Efficacy of some Biocontrol Agents on Reproduction and Development of *Meloidogyne incognita* Infecting Tomato

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Abstract: Three rhizobacteria and two yeasts isolates were used as biocontrol agents against Meloidogyne incognita in laboratory and greenhouse. The used biocontrol agents were identified as *Bacillus amyloliquefaciens, Brevibacterium otitidis, Sanguibacter inulinus, Candida incommunis and Wicherhamiella domercqiae*. They inhibited the egg-masses hatching *in vitro* and exhibited strong nematicidal activity by killing the second stage juveniles of *Meloidogyne incognita* to various degrees in greenhouse. The most effective treatment was the complete culture of the four biocontrol agents (propagules and filterate) suppressed galls and egg-masses formation by 100% *Br. otitidis* reduced galls and egg-masses by 43.7 and 52.19 %, respectivily compared with the untreated control. The microorganisms used in greenhouse test reduced nematode populations in the rhizosphere and promoted the growth of tomato plants over the control treatment.
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Key words: Biocontrol; *Meloidogyne incognita*; tomato; Rhizobacteria; yeast

1. Introduction:

The root-knot nematodes cause serious damage to important crops world-wide resulting in significant loss of revenue. Resistant cultivars, crop rotation, soil fumigation and chemical nematicides have been used traditionally, for management of the root-knot nematodes. Unfortunately, longevity and slow degradation rate of chemical nematicides created potential environmental and human health concerns, which have forced researchers to find other safe and efficient methods for nematode control. Several soil microbes which produce an array of biologically active compounds can serve as potential biological control agents. Plant growth-promoting rhizobacteria (PGPR) have been identified as an important biological control agent (Johnsson et al., 1998).

A group of important natural enemies of nematode pests, nematophagous bacteria exhibit diverse modes of actions including parasitizing, competing for nutrient uptake, inducing systemic resistance of plants, promoting plant health, producing toxins, antibiotics or enzymes. They act synergistically on nematodes through the direct suppression of nematodes, promoting plant growth, and facilitating the rhizosphere colonization and activity of microbial antagonists (Tian et al., 2007).

Chitinases and glucanases lyse microbial cells and these enzymes have been implicated in the reduction of deleterious and pathogenic rhizosphere microorganisms, creating an environment more favorable for root growth (Leong, 1986).

Chitin, a glucosamine polysaccharide, is a structural component of fungal cell wall, shells of insects, various crustaceans and nematode eggs. In egg shells of tylenchoid nematodes, chitin is located between the outer vitelline layer and the inner lipid layer and may occur in association with proteins (Bird and Bird, 1991). The breakdown of this polymer by chitinase can cause premature hatching which results in fewer viable juveniles (Mercer et al., 1992).

Bacteria and fungi are also capable of producing lytic enzymes such as chitinases, β(1,3) glucanases, cellulases, lipases and proteases. Some of these enzymes are involved in the breakdown of fungal cell wall by degrading its constituents, such as glucans and chitin, resulting in the destruction of pathogen structures or propagules. Biocontrol bacteria producing protease (Dunne et al., 1998) and chitinase (Rossi et al., 2000) were capable to suppress several plant diseases. The degradation products released can be used by the biocontrol agent to proliferate. Several bacterial proteases have been shown to be involved in the infection processes against nematodes (Tian et al., 2006).

A variety of nematophagous bacterial groups were isolated from soil, host-plant tissues, beside nematodes, their eggs and cysts (Meyer, 2003). They affected nematodes by variety of modes including parasitism, production of toxins, antibiotics or enzymes, hindering the nematode plant-host

recognition, competing for nutrients, inducing systemic resistance of plants and promoting their health. These bacteria had a wide range of suppressive activities on different nematode species, including free-living and predatory nematodes as well as animal and plant parasitic nematodes (Siddiqui and Mahmood, 1999). The objectives of the present study were to impact bioagents that suppress root-knot nematodes and to evaluate the potent antagonistic strain in controlling meloidogyne infesting tomato planted under greenhouse conditions.

2. Materials and Methods

2.1. Samples

Tomato seedlings (Castel rock) were provided from Horticulture Research Institute (Agric. Res. Center -ARC, Giza, Egypt). The experimental soil was collected from the ARC farm, Giza, Egypt. The soil texture was sandy clay characterized by an EC (2,32 dSm-1), pH (7.9) and available N, P & k of 57.35, 6.76 and 110 mg kg soil-1, respectively. The soil analyses were conducted by the methods described by Page et al. (1982). Five potential bio-control agents of which three bacterial strains of *Bacillus amyloliquefaciens, Brevibacterium otitidis* and *Sanguibacter inulinus* and two yeast strains of *Wickerhamiella domercqiae* and *Candida incommunis* were previously isolated by Moussa et al. (2006) and Moussa (2007). These microorganisms were tested *in vitro* and *in vivo* for their effect on controlling *Meloidogyne incognita* which causes the root-knot nematodes.

2.2. Biocontrol microorganism propagation on laboratory scale

Both bacteria and yeast strains were cultured individually in 250 ml Erlenmeyer flakes containing 100 ml king's B broth medium as described before by King et al. (1954). Incubation was in a shaker incubator at 28 ^{0}C and 150rpm min-1 for 24 hours. The obtained culture suspension contained 107cfu/ml

2.3. Nematode larvae extraction

The extraction of juveniles from the soil was accomplished using Jenkins's method (Jenkins, 1964). They were counted under the stereoscopic microscope and data were expressed as juveniles per ml of soil.

2.4. Nematode eggs extraction

Nematode Eggs were recovered from excised roots by agitation in 0.5% sodium hypochlorite solution (Jenkins, 1964). The total number of eggs was counted under a stereoscopic microscope and expressed as number of eggs per gram root.

2.5. Nematode stock culture

Nematode population of *M. incognita* was maintained on tomato plants cv. super marmand in a green house at 25 -27 oc. Plants were infested at 2-3 leaves stage by adding egg-masses to roots then covered with soil. After 60 days nematode egg-masses collected from roots by a needle, put in Petri deshes and put it in incubator for hatching at 25^{o}c. The hatched juveniles were collected daily for seven days to laboratory experiment and green house testes.

2.6. Screening of antagonisms against egg-masses and juveniles nematodes *in vitro*

Five ml from the complete culture, culture filtrate and culture suspension of strain cells of each biocontrol agent were added to five egg-masses of *M. Incognita* (hand picked) in Petri-dishes (5cm). One Petri dish containing same egg-masses number received 5 ml distilled water to serve as control. Each treatment was applied in three replicates. The nematode percentage inhibition was recorded after 3 days.

The same procedure was applied to test the effect of each biocontrol agent suspension on controlling nematode juveniles. One ml of nematode suspension containing 500 individual juveniles was placed in 8 ml glass vial and completed to 5 ml with each bioagent. Each treatment was held in three replicates. The nematode percent mortality was recorded after 48 hours under a stereoscopic microscope. Morphological changes in eggs and juveniles were observed on an inverted microscope found in Cell Manipulation Lab.

Nematode specimens were examined microscopically through phase contrast system using Olympus IX-70 inverted research microscope equipped with 100W Philips halogen lamp for maximum illumination. The objective phase contrast lens used was of 40X power (LCPlanFI40XPh) while the magnification selector knob was 1.5X power. Observation was carried out using frosted filter, color temperature conversion filter (LBD) and the green interface filter (IF 550) Magnification index: 40X (phase contrast lens) x 5X (built in lens) x 1.5X (magnification selector knob) x 2.5X (camera magnification) = 750X.

2.7. Effect of biocontrol agents against *M. incognita* using plastic cups

After 7 days of cultivation the tomato seedlings in steam sterilized sand, 500 freshly hatched juveniles of *M. incognita* poured around the roots of tomato seedlings and also, the different treatments of five biocontrol agents were added. Two

controls were maintained, one with nematode suspension in water and another in the media used for bioagents growth. All treatments replicated three times. After 60 days the plants were uprooted and the roots were washed free from the adhering sand particles. Number of galls, number of egg-masses, number of free nematodes in soil and also the plant weight were determined.

2.8. Effect of biocontrol agents against *M. incognita* under greenhouse conditions

A pot experiment was conducted to explore the effectiveness of the five biocontrol agents to reduce the population density of root-knot nematode juveniles and eggs. Three week old tomato seedlings (*Lycopersicon esculentum*) were transplanted in pots (25cm) which were previously filled with 4kg sandy clay soil. Pots were divided into thirteen groups each comprises six replicates.

The treatments included the bioagents *Bacillus amyloliquefaciens, Brevibacterium otitidis, Sanguibacter inulinus* and two yeast strains of *Wickerhamiella domercqiae* and *Candida incommunis*. They were individually incorporated into the soil at the rates of 4 ml and 8 ml per pot (107 cells/ml). This practice was repeated two times every 15 days. Pots were then, watered weekly twice. Fertilization was practiced after cultivation as Super phosphate (15% P_2O_5) at a rate 460 kg/Hectare. Nitrogen Fertilizer was added 35 kg/Hectare (Ammonium sulphate 20.5% N) and potassium Sulphate (48% K_2O) at a rate 115 kg/Hectare recommended by the Ministry of Agriculture. Prior to biocontrol agent addition, the transplanted tomato seedlings were infested with *M. incognita* by using 500 freshly hatched juveniles that were poured around the roots of tomato seedlings (7 days after tomato transplanting).

After 60 and 120 days from tomato transplantation, the developed plants in each pot were uprooted. The roots were then washed to get rid of the adhering sand particles and to determine numbers of galls, egg-masses and free nematodes in soil.

The tomato rhizosphere soil was collected to determine microbial activity by using the fluorescein diacetate hydrolysis (FDA) method as described by Schnurer and Rosswall (1982), total fungl count on potato dextrose agar (Difco, 1985), total diazotrophs bacteria (Hegazi et al. 1998), chitinase activity in soil (Rodriguez-Kabana et al., 1983), protease activity in soil (Wright and Reddy, 2001) and also fruits weight.

Throughout the pots experiment, the following treatments were statistically arranged in a completely randomized design:

Control (without root-knot nematodes and bioagents).
Control + *M. incognita*.
Soil + *M. incognita* + media used for growing bioagents.
Soil + *M. incognita* + *Bacillus amyloliquefaciens* (4 ml/ pot).
Soil + *M. incognita* + *Bacillus amyloliquefaciens* (8 ml/ pot).
Soil + *M. incognita* + *Brevibacterium otitidis* (4 ml/ pot).
Soil + *M. incognita*+ *Brevibacterium otitidis* (8 ml/ pot).
Soil + *M. incognita*+ *Sanguibacter inulinus* (4 ml/ pot).
Soil + *M. incognita* + *Sanguibacter inulinus* (8 ml/ pot).
Soil + *M. incognita* + *Wickerhamiella domercqiae* (4 ml/ pot)
Soil + *M. incognita* + *Wickerhamiella domercqiae* (8 ml/ pot).
Soil + *M. incognita* + *Candida incommunis* (4 ml/ pot).
Soil + *M. incognita* + *Candida incommunis* (8 ml/ pot).

2.9. Statistical analysis

The data were analyzed by ANOVA using SPSS version 12 statistical software (SPSS Inc. chicago, Illinois). Differences between treatments were determined by Duncan's Multiple Range Test (DMRT) at 5% significance level. Data Collected were subjected to the statistical analysis according to the standard methods recommended by Gomez and Gomez (1984) using the computer program (Costat). The differences between the mean values of various treatments were compared by Duncan's multiple range test (Duncan, 1955).

3. Results:

3.1 Effect of biocontrol agent treatments on *M. incognita* egg-masses *in vitro*

Meloidogyne incognita inhibition as affected by five biocontrol agents were studied and presented inTable1. Obviously, the inhibition of nematode egg hatching was affected by the treatment type. In general, the maximum inhibition percentage was mostly achieved by using the yeasts complete culture, as it reached 100% with both *Candida incommunis* and *Wickerhamiella domercqiae*, while by using the suspension of the cells it reached 97.79and 89.91%, respectively.

The treatment with the culture filtrates only of either yeasts suppressed hatching by 79.81 and 96.09%, respectively. On the other hand, the lowest inhibition percentage (72.97%) was acheived by *B. amyloliquefaciens* suspension of microbial cells.

The morphological change of *M. incognita* eggs and juveniles was examined using an inverted microscopy during 7 days of incubation with each microorganism. Deformation of juveniles and eggs that occurred as shown in Figure1. (A) to (E) and (F) to (J), respectively and their untreated controls are represented in Figure2. (A) and (B), respectively. Some eggs appeared to be destroyed, but no inhibition was observed with the water control. Observation through the inverted microscope demonstrated that the microorganisms widely attached to the eggs and juveniles of *M. incognita*.

3.2 Effect of biocontrol agents on development of *Meloidogyne incognita* infecting tomato roots *in vivo*

The following experiment was conducted to evaluate the five biocontrol agent treatments previously tested in suppressing root-knot nematode infection and nematode population densities under greenhouse conditions as a pre-field test.

The data presented in Table 2. revealed the highly significant response to the effect of variable treatments to tomato including *B. amyloliquefaciens, Brevibacterium otitidis, Sanguibacter inulinus, Candida incommunis* and *Wickerhamiella domercqiae* which suppressed galling compared to untreated control.

The number of juveniles extracted from roots, number of galls and also number of egg-masses were 100% reduced by the complete culture of all treatments except for *Brevibacterium otitidis* treatment which showed lower suppressive effect, whereas, the tomato plant weights were increasingly improved.

Table 1. Effect of different biocontrol agent treatments on hatching of *M. incognita* egg-masses *in vitro*.

Treatment	Bacterial sp.	% inhibition	R
Microbial cells + filtrate	*B. amyloliquefaciens*	87.95	de
	Brevibacterium otitidis	93.16	c
	Sanguibacter inulinus	93.82	c
	Candida incommunis	100	a
	Wickerhamiella domercqiae	100	ab
Filtrate	*B. amyloliquefaciens*	74.37	h
	Brevibacterium otitidis	81.76	f
	Sanguibacter inulinus	89.012	d
	Candida incommunis	79.81	g
	Wickerhamiella domercqiae	96.09	b
Microbial cell	*B. amyloliquefaciens*	72.97	i
	Brevibacterium otitidis	97.07	ab
	Sanguibacter inulinus	86.32	e
	Candida incommunis	97.79	ab
	Wickerhamiella domercqiae	89.91	d
Media		0.32	d
Control		0	J
LSD			1.99

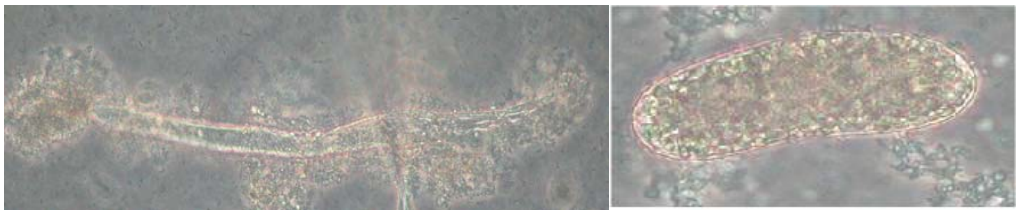

Figure1. Destroyed juvenile (A) and egg (F) of *M. incognita* as a result of using *Bacillus amyloliquefaciens*

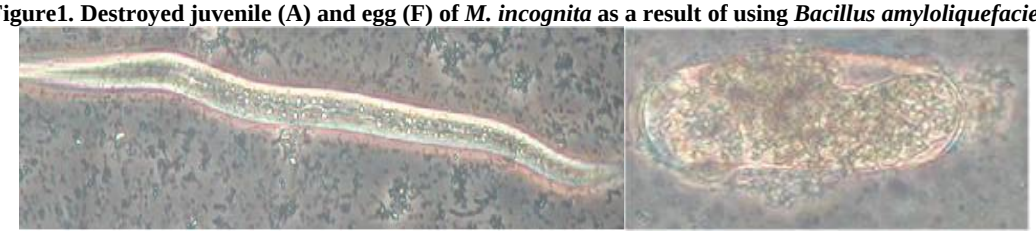

Figure1. Destroyed juvenile (B) and egg (G) of *M. incognita* as a result of using *Brevibacterium otitidis*

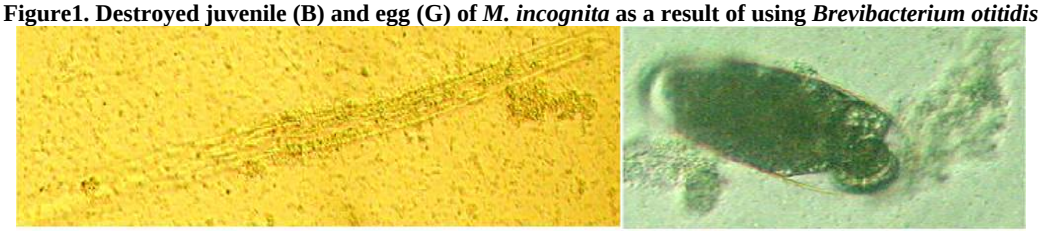

Figure1. Destroyed juvenile (C) and egg (H) of *M. incognita* as a result of using *Sanguibacter inulinus*

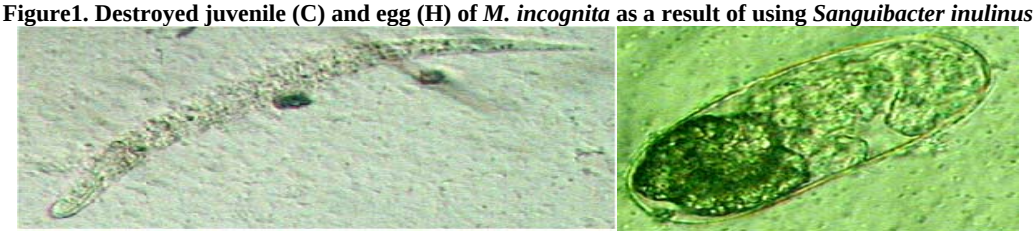

Figure 1. Destroyed juvenile (D) and egg (I) of *M. incognita* as a result of using *Wickerhamiella domercqiae*

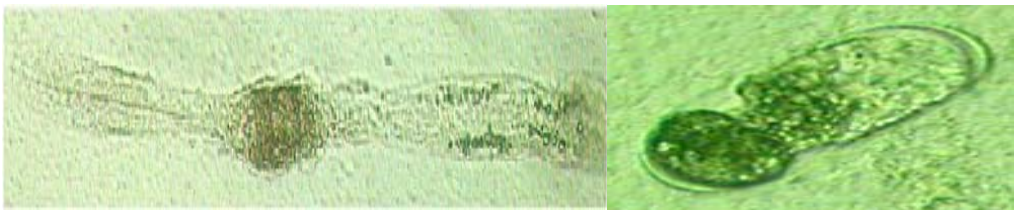

Figure1. Destroyed juvenile (E) and egg (J) of *M. incognita* as a result of using *Candida incommunis*

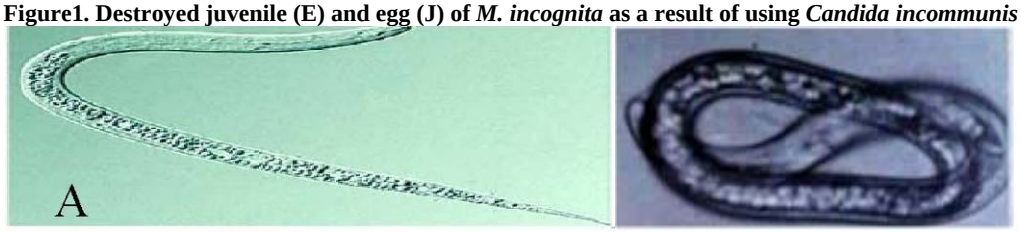

Figure 2. Untreated juvenile (A) and egg (B) of *Meloidogyne incognita*

Table 2. A Pre-field test for studying the effect of biocontrol agent on development of *M. incognita* infecting tomato roots.

Treatment	Bacterialsp.	N / 250 cm3 soil	R	Galls no.	R	egg mass no.	R	Plant wt.	R
Microbial	S1	0	f	0	e	0	d	6.7	h
cells	S2	155.7	def	118	c	126.3	c	9.9	g
+ filtrate	S3	0	f	0	e	0	d	7.6	h
	S4	0	f	0	e	0	d	12.3	cfg
	S5	0	f	0	e	0	d	14.1	def
Filtrate	S1	321.7	cd	115.3	c	145.7	c	10.9	fg
	S2	288.3	cde	87.3	d	101	c	9.2	g
	S3	129	ef	17.7	e	25	d	15.4	cd
	S4	371.3	c	116.3	c	152.66	c	12.3	ef
	S5	360	c	167.7	b	225	b	13.7	cde
Microbial	S1	66.7	f	18.3	e	31	d	15.5	c
cell	S2	66.7	f	21.7	e	33	d	12.8	ef
	S3	65.3	f	9	e	14	d	25	a
	S4	593.3	b	84	d	132.3	c	25.4	a
	S5	33.3	f	6.7	e	11.3	d	22.5	b
Control (nematodes)		2723	a	351	a	438.67	a	6.27	h
LSD 5%		171.573		21.846		53.205		2.183	

S1 (*B. amyloliquefaciens*), S2 (*Brevibacterium otitidis*), S3 (*Sanguibacter inulinus*), S4 (*Wickerhamiella domercqiae*), S5 (*Candida incommunis*).

4. Effect of biocontrol agent treatment on the numbers of nemtode, gall and egg masses after 6o days of cultivation

Among the microbial antagonists used as alternative nematicides, the biological control agents showed limitation in nematode abundance and increased tomato plant weight and height.

Data in Table 3.revealed that all treatments suppressed galling up to 100% compared to the untreated control expect those treatments with *Brevibacterium otitidis*, 8 ml/pot, and *Candida incommunis* at 4.8 ml/pot and media which reduced gall formation with 87.27%, 63.6%, 81.82% and 29.6%, respectively.

The same trend was observed when testing the parameter of egg-masses number and number of juveniles extracted from soil. In case of development in plant parameters, Figure3. shows the highest shoot length being achieved when treated with *Brevibacterium otitidis* 8ml / pot treatment, while the lowest one was with the media treatment. The highest shoot weight was achieved as revealed in Figure 4.

also with *Brevibacterium otitidis*, 8ml / pot treatment, while the lowest was with the *Wickerhamiella domercqiae* at 4 ml /pot treatment.

4.1 Effect of biocontrol agent treatment on the numbers of nemtode, gall and egg masses at harvest time

The inoculation with nematode alone resulted in extensive galling on roots of tomato at harvest. Treatments with the experimental microorganisms reduced the gall formation at harvest as shown in Table 4. Decline in gall formation was from 436 to 16 due to *Sanguibacter inulinus* at 8ml/pot in comparison with untreated control, followed by *B. amyloliqufaciens* at (8ml/pot) and *Candida incommunis* at (8ml/pot). Egg masses production was successfully inhibited due to the application of all microorganisms. The *Sanguibacter inulinus* at 8ml/pot gave the maximum decline in the egg masses, followed by *B. amyloliqufaciens* (8ml/pot) and *Candida incommunis* at 4.8ml/pot and the lowest was with media treatment.

The soil root-knot nematode populations were significantly affected by treatments with microorganisms, as the percent decrease in them was greater than that in galls or egg-masses. The highest decrease in nematode juveniles (n/250 cm3 soil) occurred with *Brevibacterium otitidis* (8ml/pot) and *Wickerhamiella domerciqae*(8ml/pot) compared to the control infected with nematode. Nevertheless, the nematode development stages inside the root system decreased descending from 7% with *Wickerhamiella domerciqae* at (8ml/pot) to 15% with either *Candida incommunis* (4ml/pot) or *B. amyloliqufaciens* (4ml/pot) compared to with control.

Table 3. Effect of biocontrol agent treatments on number of nemtodes, galls and egg masses after 60 days of cultivation.

Treatments	Nematode no. (n/250 cm3)	R	Gall no.	R	Egg mass no	R
B. amyloliquefaciens C1	0.00		0.00	E	0	
B. amyloliquefaciens C2	0.00		0.00	E	0	
Brevibacterium otitidis C1	0.00		0.00		0	
Brevibacterium otitidis C2	0.00		3.50		0	
Sanguibacter inulinus C1	0.00		0.00		0	
Sanguibacter inulinus C2	0.00		0.00		0	
Candida incommunis C1	0.00		10.00		0	
Candida incommunis C2	20.00	c	5.00	C	1	c
Wickerhamiella domercqiae C1	0.00		0.00		0	
Wickerhamiella domercqiae C2	0.00		0.00		0	
Media	50.00	b	19.00	C	3	b
Nematodes	70.00	a	27.50	A	5	a
LSD	2.1		1.63		0.47	

C1=4ml/pot., C2=8ml/pot., P1=60days after cultivation, P2=90days after cultivation, P3=120 days after cultivation.

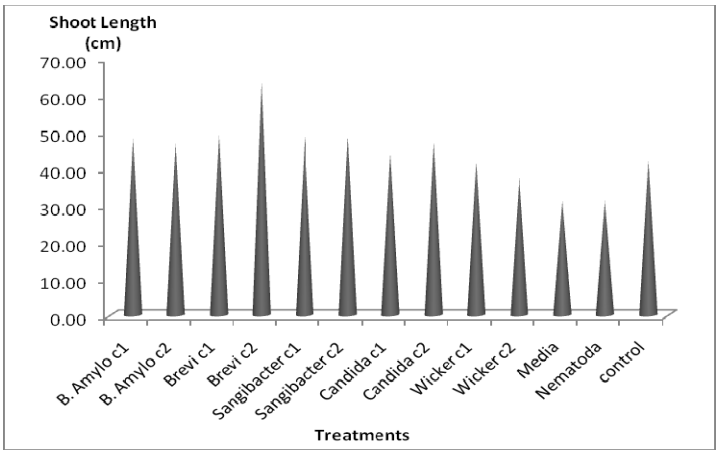

Figure 3. Effect of 12 different treatments on tomato shoot length achieved after 60 days.

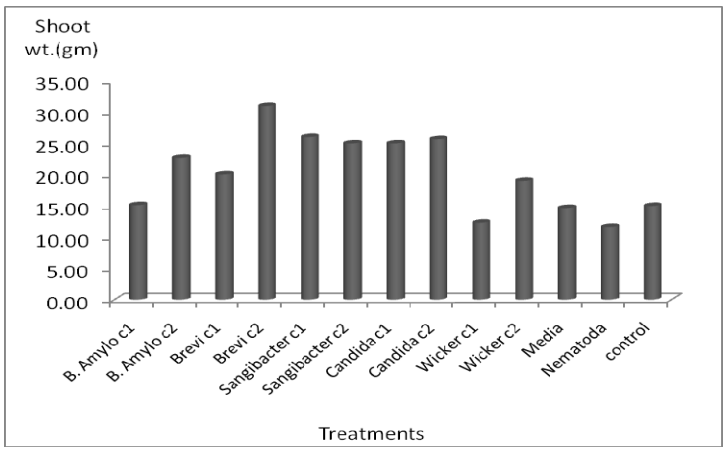

Figure4. Effect of 12 different treatments on tomato shoot weight achieved after 60 days.

Table 4.Effect of biocontrol agent treatments on the numbers of nematode, galls and egg masses after harvest.

Treatments	Nematode no. (n/250 cm3)	R	Gall no.	R	Egg mass no.	R	NDS	R
B. amyloliquefaciens C1	981	f	39	f	25	e	33	c
B. amyloliquefaciens C2	901	h	32	g	18	g	22	f
Brevibacterium otitidis C1	1200	d	60	c	31	C	19	g
Brevibacterium otitidis C2	881	i	51	d	22	F	18	h
Sanguibacter inulinus C1	1001	e	44	e	30	D	32	c
Sanguibacter inulinus C2	981	g	16	h	12	H	28	d
Candida incommunis C1	980	g	38	f	24	E	33	c
Candida incommunis C2	901	h	38	f	18	g	24	e
Wickerhamiella domercqiae C1	1301	c	51	d	32	C	23	ef
Wickerhamiella domercqiae C2	880	i	49	d	21	F	16	i
Media	2301	b	181	b	136	b	132	b
Nematodes	4101	a	436	a	305	a	218	a
LSD 5%	0.28		2.8		0.78		1.13	

C1=4ml/pot., C2=8ml/pot., P1=60days after cultivation, P2=90days after cultivation, P3=120 days after cultivation, NDS: Nematode development stage.

At harvest, both plant shoot length and weight increased with all the used microorganism treatments compared to nematode infested plant as shown in Figure 5. The highest shoot length (76 cm) being achieved when treated with *Sanguibacter inulinus* at 4ml / pot treatment, followed by (70.67cm) achieved with either *Brevibacterium otitidis* 8ml / pot treatment and *Wickerhamiella domerciqae* 4ml / pot treatment compared to the untreated control (64.67cm), while the lowest one was with the media treatment (47.67cm).

The highest shoot weights were with *Wickerhamiella domerciqae* using first concentration (58.67gm) and *B. amyloliqufaciens* (8ml/pot) recorded (52.33gm) , while *Sanguibacter inulinus* (4ml/pot), *B. amyloliquefaciens* (4ml/pot), *Brevibacterium otitidis* (4ml/pot) and *Wickerhamiella domercqiae* (8ml/pot) recorded 50.67, 46.33 and 42.67gm, respectively, as shown in Figure 6. The lowest shoot weights were 22.33gm obtained from plants infected with nematodes or 16.33 gm obtained from non- infected plants.

Fruit weights varied significantly due to the used treatments, as the highest fruit weights were obtained with *Brevibacterium otitidis* (4ml/pot), *B. amyloliqufaciens* (8ml/pot), *Sanguibacter inulinus* (4ml/pot) and *Wickerhamiella domerciqae* (8ml/pot) to be 78, 72, 68 and 66 gm respectively, while the lowest fruit weights were obtained from plants infected with nematode (14 gm) and for non-treatment with *Candida incommunis* (8ml/pot) (17 gm) as shown in Figure 7.

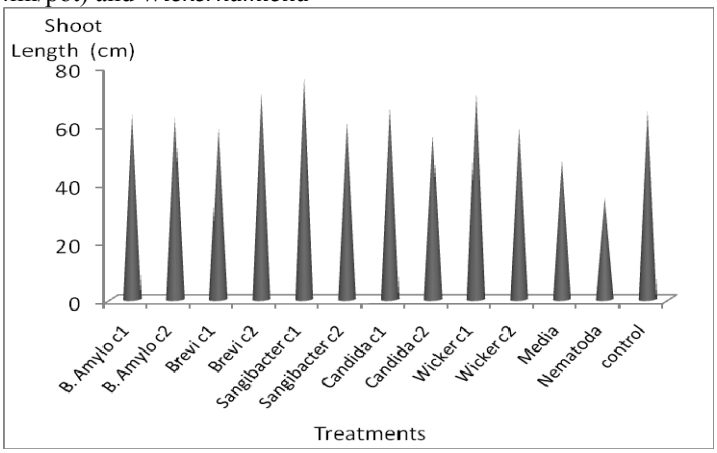

Figure 5. Effect of biocontrol agent on shoot length of tomato at harvest time.

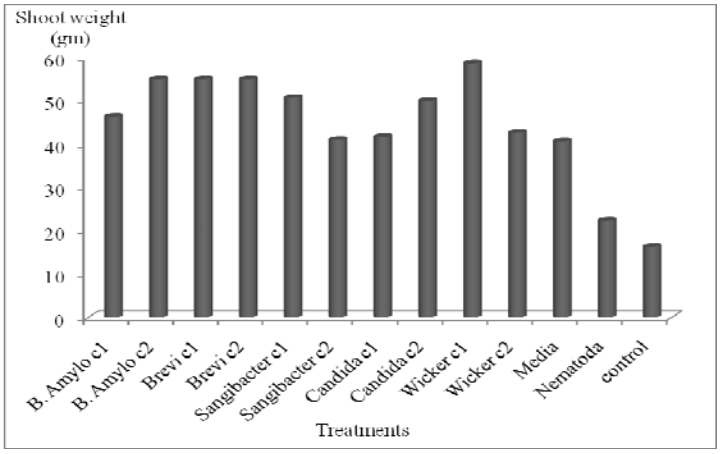

Figure 6. Effect of biocontrol agent on shoot weight of tomato at harvest time.

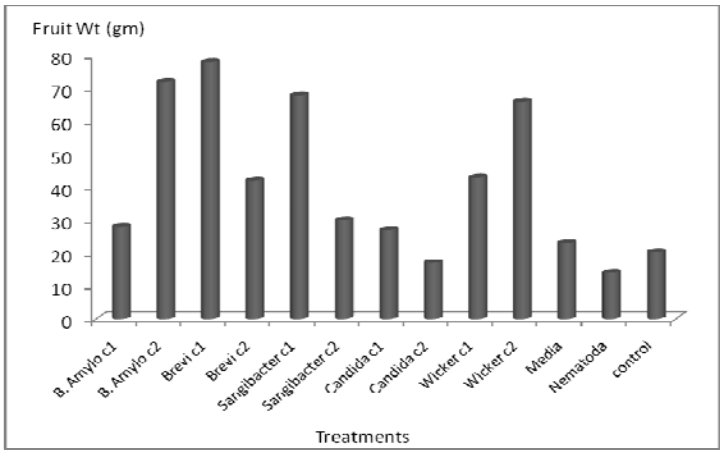

Figure 7. Effect of biocontrol agent on fruit weight of tomato.

4.2 Microbial enzymes detected in rhizosphere during plantation periods

The total microbial activity was assessed by measuring fluorescein diacetate (FDA) hydrolysis releasing fluorescein under the action of microbial enzymes such as proteases, lipases and esterases (Green et al, 2006).The effect of the biocontrol agents activities including bacteria and yeasts added to tomato rhizosphere on fluorescein diacetate compared with that of the controls 1 and 2, beside that of soil amended with media were investigated through the growth stages of tomato. Data in Table 5. indicate the frequency in the amount of fluorescein resulting according to treatment type. The significant differences indicated that not only the type of treatment affected the activity calculated but both the concentration of the biocontrol added and the intervals of sampling. The *Sanguibacter inulinus* achieved the best activity measured after 90 daysusing 8ml/pot. All soil rhizosphere treated with those biocontrol agents gave more activity than those untreated (control 1, 2 and that with media only).

Table 5. Effect of different treatments on microbial activity in soil measured as fluorescein diacetate hydrolysis.

Treatment name	Activity	Rank
Sanguibacter inulinus c2p2	14.6	A
B.amyloliquefaciens c2p1	13.3	B
Brevibacterium otitidis c2p1	13	C
Brevibacterium otitidis c1p1	12.9	C
Sanguibacter inulinus c2p1	12.9	C
Candida incommunis c1p1	12.7	D
Candida incommunis c2p1	12.7	D
Brevibacterium otitidis c1p2	12.6	De
Wickerhamiella domercqiae c2 p1	12.6	De
Brevibacterium otitidis c2p2	12.5	E
Wickerhamiella domercqiae c1 p1	12.3	F
Brevibacterium otitidis c1p3	12.2	Fg
Candida incommunis c2p3	12.1	Gh
Sanguibacter inulinus c1p1	12.07	Ghi
Wickerhamiella domercqiae c2 p2	12	Hi
B.amyloliquefaciens c2p3	12	Hi
B.amyloliquefaciens c2p2	11.9	Ij
Candida incommunis c2p2	11.9	Ij
B.amyloliquefaciens c1p1	11.8	Jk
B.amyloliquefaciens c1p3	11.8	Jk
B.amyloliquefaciens c1p2	11.7	Kl
Wickerhamiella domercqiae c1 p2	11.6	L
Sanguibacter inulinus c1p2	11.4	M
Brevibacterium otitidis c2p3	11	N
Candida incommunis c1p2	10.9	No
Wickerhamiella domercqiae c2 p3	10.8	Op

Sanguibacter inulinus c2p3	10.7	P
Sanguibacter inulinus c1p3	10.5	Q
Candida incommunis c1p3	10.3	R
Wickerhamiella domercqiae c1 p3	10	S
Medium p1	9.9	S
Control 1 (c1p2)	9.7	T
Medium p2	9.7	T
Control 1 (c1p1)	9.5	U
Medium p3	9.3	V
Control 1 (c1p3)	9.2	Vw
Control 2 (c1p2)	9.1	W
Control 2 (c1p1)	8.1	X
Control 2 (c1p3)	6.1	Y
LSD 5% =		0.16

C1=4ml/pot., C2=8ml/pot., P1=60days after cultivation, P2=90days after cultivation, P3=120 days after cultivation.

4.3 Effect of bioagents on diazotrophic bacteria in soil rhizosphere

The diazotrophic bacteria capable of utilizing atmospheric N2 as their soil nitrogen source (N-fixer) are commonly associated with plants to achieve phyto -nitrogen balance, are affected by soil rhizosphere microflora including the bioagents under study. This experiment was performed to evaluate the effect of bioagents on N-fixing population. As shown in Figure 8, the highest bacterial number among the nitrogen fixing population tested was recorded with *Sanguibacter inulinus* (8ml/pot after 45 days). Along all samples tested, the whole nitrogen fixers' numbers were the maximum after 45 days of cultivation. Obviously, five of the treatments tested (*Bacillus amyloliquifaciens* C1 and C2, *Brevibacterium otitidis* C2, *Sanguibacter inulinus* C2and *Wickerhamiella domarcqiae* C1) decreased the diazotrophic bacterial numbers throughout the whole period (120 days). On the other hand, the other five treatments tested (*Brevibacterium otitidis* C1, *Sanguibacter inulinus* C1, *Wickerhamiella domarcqiae* C2, *Candida incommunis* C1 and C2) decreased the diazotrophic bacterial numbers after 90 days but their numbers increased after 120 days. After 45 days the nitrogen fixers' numbers in the soil samples treated with the bioagents exceeded that in the control 1, 2 and that treated with medium. Concerning those untreated, the nematodal presence in the control 2 affected negatively the nitrogen fixers' numbers than those in control 1 with no nematodal investion.

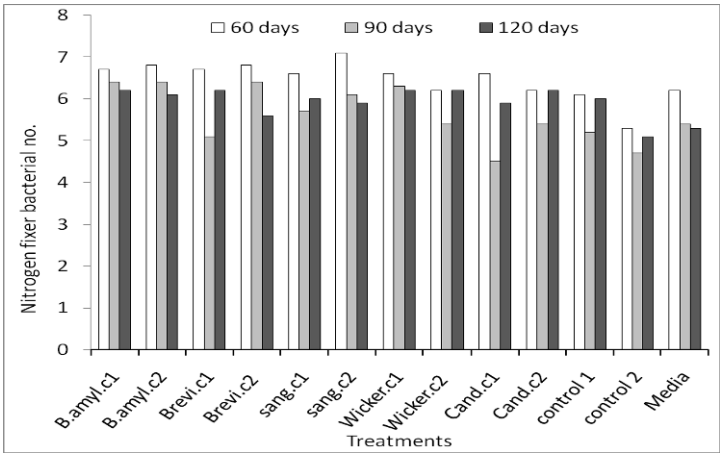

Figure 8. Effect of bioagent on Nitrogen fixer bacterial number.

4.4 Chitinolytic and proteolytic activity criteria in tomato rhizosphere during biocontrol treatments

Data presented in Table 6.And7. shows the influence of different biocontrol agents on excretion of microbial chitinase and protease, respectively in soil infested with root knot-nematode *M. incongnita*. Apparently, the most efficient bioagent in producing chitinase was *Candida incommunis* added at

concentrations of 4ml and 8ml/pot in the second period, followed by the bioagents *Brevibacterium otitidis* and *Wickerhamiella domercqiae* at concentration (4ml, 8ml/pot) in third period. Other less effective bioagents varied in accordance to their type, concentration applied and incubation period in soil. Egg masses production was successfully inhibited due to the application of all microorganisms. The *Sanguibacter inulinus* at 8ml/pot gave the maximum decline in the egg masses, followed by *B. amyloliqufaciens* (8ml/pot) and *Candida incommunis* at (4.8ml/pot) and the lowest with media treatment. The bioagent *B. amyloliquefaciens* showed maximum proteolytic activity at a concentration of 4ml/pot in the third period. Lower protease activity was detected when applying *Brevibacterium otitidis* (4ml/pot, first period) followed by *Candida incommunis* 8 and 4 ml/pot in the third period). Other bioagent actions decreased dramatically.

4.5 Total number of fungi in rhizosphere soil

Total numbers of fungi were affected by the biocontrol agents added. Their numbers were negatively pronounced with all treatments when compared to control 1 and 2 at first period, while all treatments in the second and third periods decreased fungal numbers as shown in Figure 9.Addition of these two species to the soil seeded with kidney bean and infested with the pathogen increased the percentage of control plants. This was carried out through the process of antibiosis (secretion of antifungal compounds).

Table 6.Chitinase activity in the presence biocontrol agent

Treatment name	Activity	Rank
Candida incommunis c1p2	0.6	a
Candida incommunis c2p2	0.42	b
Brevibacterium otitidis c2p3	0.18	c
Brevibacterium otitidis c1p3	0.17	cd
Wickerhamiella domercqiae c1 p3	0.17	cd
Wickerhamiella domercqiae c2 p3	0.17	cd
B. amyloliquefaciens c1p2	0.15	de
Sanguibacter inulinus c2p2	0.14	ef
Brevibacterium otitidis c2p2	0.13	efg
Sanguibacter inulinus c1p2	0.13	efg
Sanguibacter inulinus c1p3	0.13	efg
Brevibacterium otitidis c1p2	0.12	fgh
B. amyloliquefaciens c2p2	0.12	fgh
B. amyloliquefaciens c1p3	0.12	fgh
B. amyloliquefaciens c2p3	0.12	fgh
Candida incommunis c2p3	0.12	fgh
Wickerhamiella domercqiae c1 p2	0.11	gh
Candida incommunis c1p3	0.11	gh
Sanguibacter inulinus c2p3	0.10	h
Medium p1	0.07	i
Medium p2	0.07	i
Medium p3	0.06	I
Control 1 p3	0.037	J
Wickerhamiella domercqiae c2 p2	0.037	J
Wickerhamiella domercqiae c2 p1	0.014	K
Candida incommunis c2p1	0.013	K
Brevibacterium otitidis c2p1	0.013	K
Control 2 p3	0.013	K
B. amyloliquefaciens c1p1	0.012	K
Sanguibacter inulinus c2p1	0.012	K
B. amyloliquefaciens c2p1	0.012	k
Candida incommunis c1p1	0.012	K
Sanguibacter inulinus c1p1	0.012	K
Brevibacterium otitidis c1p1	0.012	K

Control 1 p2	0.011	K
Wickerhamiella domercqiae c1 p1	0.011	K
Control 2 p2	0.011	K
Control 1 p1	0.010	K
Control 2 p1	0.01	K
LSD 5% =		0.02

C1=4ml/pot., C2=8ml/pot., P1=60days after cultivation, P2=90days after cultivation, P3=120 days after cultivation

Table 7. Protease activity in the presence biocontrol agent

Treatment name	Activity	Rank
B. amyloliquefaciens c1p3	54.2	A
Brevibacterium otitidis c1p1	53	B
Candida incommunis c2p3	50	C
Candida incommunis c1p3	48	D
Sanguibacter inulinus c1p3	19.7	E
Wickerhamiella domercqiae c1 p2	19.7	E
Wickerhamiella domercqiae c2 p2	10.3	F
Brevibacterium otitidis c1p3	8.7	G
B. amyloliquefaciens c2p3	7.6	H
Brevibacterium otitidis c1p3	6.2	I
Wickerhamiella domercqiae c1 p3	6.1	I
B. amyloliquefaciens c2p2	5.8	Ij
Sanguibacter inulinus c1p1	5.3	J
Sanguibacter inulinus c2p3	4.60	K
Candida incommunis c2p1	4.21	Kl
Wickerhamiella domercqiae c2p3	3.81	Lm
Brevibacterium otitidis c1p2	3.4	M
Candida incommunis c1p1	2.4	N
Sanguibacterinulinus c1p2	2.3	N
Candida incommunis c2p2	2	No
Wickerhamiella domercqiae c1 p1	1.86	Nop
Candida incommunis c1p2	1.6	Opq
Sanguibacter inulinus c2p1	1.42	Opqr
B. amyloliquefaciens c1p1	1.3	Pqrs
B. amyloliquefaciens c2p1	1.2	Qrs
Brevibacterium otitidis c2p1	1.13	Qrs
B. amyloliquefaciens c1p2	1.03	Qrst
Sanguibacter inulinus c2p2	1	Rstu
Control 1p2	1	Rstuv
Control 1p1	0.9	Stuv
Medium p1	0.8	Tuv
Control 1p3	0.7	Tuv
Control 2p1	0.5	Tuv
Control 2p2	0.45	Tuv
Brevibacterium otitidis c2p2	0.4	Tuv
Medium p2	0.4	Tuv
Control 2p3	0.35	Uv
Medium p23	0.25	V
Wickerhamiella domercqiae c2p1	0.18	V
LSD at 5% =	0.535	

C1=4ml/pot., C2=8ml/pot., P1=60days after cultivation, P2=90days after cultivation, P3=120 days after cultivation

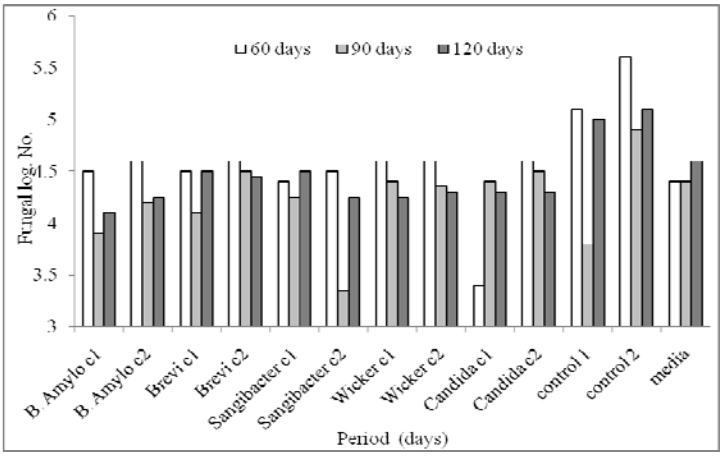

Figure 9.Effect of bioagents on fungal number log.

4. Discussion:

Meloidogyne incognita inhibition was affected by the treatment type. The maximum inhibition percentage 100% was mostly achieved by using the complete culture of both *Candida incommunis* and *Wickerhamiella domercqiae*. Similar results were obtained by Shawky et al. (2006) who reported that all the bioagent candidates *Bacillus subtilis*, *Saccharomyces uvarum* and *Saccharomyces ludwigii* proved harmful to *M. javanica* juveniles, egg masses and numbers of galls but the effect magnitude differed from one candidate to another. Jung et al. (2002) showed that *Paenibacillus illinoisensis* had caused 2.5% reduction in egg hatching on the first day and by seventh day there were no hatching eggs found from the 78 eggs/ml used. Mohamed et al. (2008) indicated that the application with the yeast isolates *Pichiagluillier mondii*, *Pachytrichospora transvaalensis* and *Candida albicans* treatments significantly reduced the number of juveniles *in vitro* after both 24h and 48h.The lethal action of toxic compounds produced by microorganisms on egg *in vitro* noted by Meadows et al. (1989) deserves further exploration.

The morphological change of *M. incognita* eggs and juveniles agreed with the study of Westcott and Kluepfel (1993). They reported that chitinases produced by PGBR was more potent in attacking the eggs rather than the cuticle of *M. incognita*. This might have resulted from the direct damage to the eggs caused by the bacterial or yeast chitinase activity.Also, Mercer et al. (1992) reported that the bacterial lytic enzymes interferes with the egg hatching of *M. hapla*that might had lysed the egg shell including various lipolytic,proteolytic and chitinolytic enzymes, causing an aberrant change in egg shape and egg rupture.

In vivo, the results of evaluating the five biocontrol agent treatments may be referred to the fact that most rhizobacteria act against plant-parasitic nematodes by means of metabolic by-products, enzymes and toxins. The effects of these toxins include the suppression of nematode reproduction, egg hatching and juvenile survival, as well as the direct killing impact on nematode itself (Siddiqui and Mahmood, 1999).

Cronin et al. (1997) reported that purified commercial chitinase inhibited the egg hatching of potato cyst nematode *Globoderarosto chiensis in vitro* up to 70%. On the other hand, Yong et al. (2004) reported that genus *Sanguibacter* produced chitinase efficiently, hindering the pathogenesis of locusts. Both protease and collagenase had adverse effects on larval motility *in vitro*, when larvae were treated with the enzymes prior to inoculation, protease-treated larvae caused a significant decrease of 40 % in galling (Galper et al. 1990). Rossi et al. (2000) reported that several bacterial proteases have been shown to be involved in the infection processes against nematode. *B. amyloliquefaciens* has been developed to control plant parasitic nematodes on tomato, bell pepper and strawberry (Meyer, 2003). Huang et al. (2005) reported that eggshells of root-knot nematode might be lysed by bacteria that produce various lipolytic, proteolytic and chitinolytic enzymes. These reports indicated that chitinase produced by soil bacteria was associated with the inhibition of egg hatching of root knot nematode. The present results showed the most efficient bioagent in producing chitinase was *Candida incommunis* added at concentrations of 4ml and 8ml/pot in the second period and the bioagent *B. amyloliquefaciens* showed

maximum proteolytic activity at a concentration of 4ml/pot in the third period this agrees with above reports.

Both plant shoot length and weight increased with all the used microorganism treatments compared to nematode infested plant. At harvest, present results showed that *Brevibacterium otitidis* affected the plant showing the highest shoot length and fruit weight, while *Wickerhamiella domerciqae* achieved to the plant the highest shoot weight at specific concentrations. Shawky et al. (2006) found that adding any of the antagonistic bacteria had increased the endophytic strains like *Sanguibacter sp.* increased biomass production of plants in the shoot part and good biomass yield. Also, *B. amyloliquefaciens* strain has been shown to induce growth promotion in tomato seedling and reduce severity of diseases caused by several pathogens and elicitation of induced systemic resistance, additionally reduced gall incidence by root-knot nematodes in tomato plants and resulted in increased yield (Kokalis-Burelle and Dickson, 2003). YU et al. (2002) found that *B. amyloliquefaciens* strains produced Iturin A2 molecules that have been used as biocontrol agents to suppress fungal plant pathogens. Also Hiradate et al. (2002) found that those molecules included seven ά- amino acids and one β-amino fatty acid and Iturin A which were produced as a mixture of up to eight isomers.

Candida incommunis was reported for its ability to produce IAA and phosphate solubilization that aid in microbial nutrition besides its ability to produce siderophores that acts as antifungal agents that aid in bacterial enrichment (Hassanin et al. 2007). These actions might affect positively the diazotrophs during the third period (120 days) in the present study.

Total numbers of fungi were affected by the biocontrol agents added. These results agree with that of Chen et al. (2006). They reported that *B. amyloliquefaciens* produced lipopeptides, surfactins, bacillomycin D and fengycins as secondary metabolites mainly of known antifungal activity. Similar results were reported by Turner and Messenger (1986), who estimated the ability of *Brevibacterium* to produce phenazine compounds acting as antibiotics. *Candida incommunis* is characterized by its ability to produce siderophores as antifungal agents and inhibitory effects on the growth of the fungal pathogen *Fusarium oxysporum*, according to Hassanin et al. (2007).

Addition of these two species to the soil seeded with kidney bean and infested with the pathogen increased the percentage of control plants. This was carried out possibly through the process of antibiosis (secretion of antifungal compounds).

5. Conclusion:

Through this study, clear evidence was presented that the most effective biocontrol treatment was the complete culture (propagules and filterate) of the four biocontrol agents mentioned *Bacillus amyloliquefaciens*, *Brevibacterium otitidis*, *Sanguibacter inulinus*, *Candida incommunis* and *Wicherhamiella domercqiae*. These biocontrol agents suppressed galls and egg-masses formation of *Meloidogyne incognita* by 100% and promoted the growth of tomato plants over the control treatment. Therefore, these biocontrol agents can substitute the use of the nematicides used in tomato fields.

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