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Nematicidal Activity of Some Biocontrol Agents against Root-Knot Nematodes *In-Vitro.*

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ABSTRACT

Nineteen rhizospheric bacterial isolates and three biocontrol agents (*Serratia marcescens*, *Pseudomonas fluorescens* and *Bacillus thuringiensis* BT14) were evaluated against root-knot nematodes (*Meloidogyne incognita*). The obtained data revealed that, five bacterial isolates (B38, B39, B78, B91 and B103) and *S. marcescens* strain were the most effective bacteria against the J2s of *M. incognita* whereas, these bacteria achieved the highest mortality percentages by more than 94%. Results also indicated that isolate B38 showed the highest mortality percentage of nematicidal volatile activity by 70.3%, followed by *S. marcescens* and isolate B103. Moreover, isolate B38 succeeded to record maximum hydrolysis zone values of gelatinase, protease and chitinase (4.27, 5.55 and 4.33 cm, respectively). The most potent isolates for bioagent production were chosen and these isolates were identified as *Pseudomonas fluorescens* B103 and *Bacillus subtilis* B38. In addition, poly agar medium recorded the highest values for nematicidal activities by *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens*, while Emerson's agar medium lowest one. Bacterial cells suspension showed more efficient mortality on nematode *M. incognita* compared with their cultural filtrates. The highest mortality percentages were recorded at dilutions 10^{-1} by all bacterial strains.

Keywords: biocontrol agents, root-knot nematode, nematicidal activities, *Pseudomonas fluorescens*, *Bacillus subtilis, Serratia marcescens*.

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INTRODUCTION

Plant-parasitic nematodes such as root-knot nematodes or *Meloidogyne* spp. are microscopic obligate pathogens that feed on plant roots. They cause severe damage to a wide variety of crops and lead to significant yield losses of approximately 78 billion dollar worldwide annually (Caillaud *et al*, 2008). The use of rhizobacteria that can produce nematicidal metabolites is also considered a promising tool for controlling nematodes (Gu *et al*, 2007). The control achieved by biocontrol agents with several distinct mechanisms of control may be additive or synergistic. Biocontrol agents that have different traits in the soil and rhizosphere may be used together to control plant pathogens via different mechanisms of disease suppression. Siddiqui and Mahmood (2001) observed that *Pseudomonas fluorescens* GRP3 applied with organic manure was effective for the management of the root-knot nematode *M. incognita*. *Serratia marcescens* and some other rhizobacterial strains were involved in the biocontrol of root-knot larvae *in vitro* and in soil. (Zavaleta-Mejfa and Van-Gundy, 1989).

Padgham and Sikora (2007) found that biological control promises to be such an option. Application of microorganisms antagonistic to nematodes or compounds produced by these microbes could provide additional opportunity for managing the damage caused by root-knot nematodes. Such microorganisms can produce substances that may limit the damage caused by these nematodes, e.g. by producing antibiotics, siderophores and a variety of enzymes. These microorganisms can also function as competitors of nematodes for colonization sites and nutrients. Sergeant *et al* (2006) reported that the genus *Xenorhabdus* produce a wide range of toxins and a complex set of extracellular enzymes including lipases, phospholipases, chitinases, phosphatases and proteases.

In addition, Somvanshi *et al* (2006) found that proteases represent important part of the extracellular enzymes, although their role in virulence process is yet unclear. Extracellular alkaline proteases produced by different species of *Photorhabdus*. Certain microorganisms like species of *Bacillus, Enterobacter, Pseudomonas, Serratia, Streptomyces* and *Vibrio* have been reported to produce chitinase (Bhattacharya *et al*, 2007). The bacterial strains *Paenibacillus polymyxa, Bacillus megaterium* and *Bacillus circulans* observed the highest protease, chitinase and gelatinase activities which might help to explain the way how the bacteria could act against the root-knot nematodes. It should be stated that the highest nematicidal activity exhibited by their strains against the second stage juveniles of *Meloidogyne incognita* (El-Hadad *et al*, 2010).

Volatiles have been reported to play a vital role in the recognition between entomopathogenic nematodes and their hosts. However, little is known about nematicidal volatile organic compounds (VOCs) and their potential use as biological control agents against plant parasitic nematodes (Lewis *et al*, 2006). The search for nematotoxic or antagonistic compounds in cultural filtrates has greatly intensified in recent years, due to the number of toxins, enzymes or compounds derivable from their metabolites (Liu *et al*, 2008).

The main goal of this study was concerned with evaluation of isolated bacteria and bioagent strains for controlling root-knot nematodes *in vitro* and screened to their biocontrol agents i.e. nematicidal volatiles, gelatinase, protease and chitinase activities.

MATERIALS AND METHODS

Bacterial biocontrol agent

Isolation of different bacterial isolates was carried out on different specific bacteriological media namely nutrient agar medium, colloidal chitin agar medium and King's agar medium (Atlas, 2005) from plants rhizosphere (tomato, peppers and eggplant). Three bacterial biocontrol strains namely *Serratia marcescens*, *Pseudomonas fluorescens* and *Bacillus thuringiensis* (BT14) were kindly obtained from Plant Pathology Research Institute, Agricultural Research Center, Ministry of agriculture, Giza, Egypt.

The root-knot nematode larvae

The second juvenile (J2) of *M. incognita* used throughout the study was provided from Agricultural Zoology and Nematology Department, Faculty of Agriculture, Cairo University.

Screening of biocontrol agents

Bacterial isolates and bioagent strains were screened on nutrient broth medium for their abilities to inhibit the second juvenile (J2) of *M. incognita*. Mortality percentage was calculated according to the method described by Schneider and Orelli (1947).

Determination of Biocontrol agent activity

Selected bacterial isolates and bioagent strain which showed considerable mortality percentage for *M. incognita* were investigated for their potentialities for nematicidal volatiles, gelatinase, protease and chitinase activities according to the methods of Fernando *et al* (2005), Pires-Bouças *et al* (2010), Dajanta *et al* (2009) and Roberts and Selitrennikoff (1988), respectively.

Identification of the potent isolates

The potent bacterial isolates which showed pronounced results with all examined characters of antagonistic action of nematode were identified according to Reva *et al* (2001) and Palleroni (2005).

Effect of cultivation media on production of bioagent by the potent strains

Identified bacterial strains were used to produce bioactive agents against nematode on five microbiological media namely nutrient medium as a control, tryptic soy medium (Atlas, 2005), poly medium (Bourgouin *et al*., 1984), Emerson's medium (Weiss, 1975) and acetate yeast extract medium (Sasaki *et al*., 1998).

Effect of culture filtrate on mortality percentages of *M. incognita* (J2s)

Identified bacterial strains were grown on the best medium using shaking incubator (200 rpm) for 3 days at 30˚ C. Then, the cells were harvested by centrifugation at 10000 rpm for 20 min, the culture medium was discarded. The supernatant was filtered by passing the culture broth through a sterile membrane filter 0.2 µm according to the method described by El-Boghdady (1993). Five ml sterilized porcelain cups were supplied with one mL of each dilution of either bacterial liquid cultures 10^{-1} – 10^{-3} or each dilution of culture filtrates 10^{-1} – 10^{-3} and one ml of nematode sterilized suspension (Kim and Riggs, 1991) containing 350±50 individual of J2s per cup and incubated at 30˚C for 48 hrs. Plain water in cup supplied with nematode suspension was used as a control. Cups were loosely covered which slightly permit aeration and reduce evaporation. Number of survived and died were counted after 48 hrs using one ml nematode counting slide, corrected mortality percentage was calculated (Schneider and Orelli, 1947).

RESULTS AND DISCUSSION

Isolation and screening of biocontrol agents

Data presented in Table 1 showed that among nineteen bacterial isolates and three bioagent strains, five bacterial isolates and *S. marcescens* strain showed the highest inhibition activity against the J2s of *M. incognita*.

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Table 1. Mortality percentage of *M. incognita* (J2s) as affected by the tested bacteria after 48 hrs on 30˚C

Isolates number	Number of immobile J2s	Mortality (%)	Isolates number	Number of immobile J2s	Mortality (%)
B02	220	62.9	**B78**	346	98.9
B07	240	68.6	**B91**	331	94.6
B13	210	60.0	**B92**	234	66.9
B27	271	77.4	**B97**	180	51.4
B31	235	67.1	**B99**	191	54.6
B33	198	56.6	**B100**	300	85.7
B38	348	99.4	**B101**	260	74.3
B39	345	98.6	**B103**	344	98.3
B50	216	61.7	**S**	347	99.1
B69	320	91.4	**Ps**	325	92.9
B70	280	80.0	**BT14**	328	93.7

- S: *S. marcescens* - Ps: *Ps. fluorescens* - BT14: *B. thuringiensis* BT14
- Number of juveniles in 1 ml suspension is 350 ± 50

Data also showed that these isolates (B38, B39, B78, B91 and B103) and *S. marcescens* achieved the highest mortality percentages by more than 94%.

Moreover, isolate B38 exhibited the highest mortality percentage by 99.4% followed by *S. marcescens* (99.1%). While, isolate B97 recorded the lowest mortality percentage (51.4%). In a similar study, Carneiro *et al* (1998) reported that the culture of *B. thuringiensis* and *B. laterosporus* freshly killed the emerged J2s of *M. javanica* within 24 to 48 hrs. These microorganisms can also function as competitors of nematodes for colonization sites and nutrients. The search for nematotoxic or antagonistic compounds in cultural filtrates has greatly intensified in recent years, due to the number of toxins, enzymes or compounds derivable from their metabolites (Liu *et al*, 2008).

In view of the obtained results, the bacterial isolates B38, B39, B78, B91, B103 and *S. marcescens* revealed high nematicidal effect against J2s of *M. incognita*. Therefore, it was found of interest to verify whether the effect is mainly due to the intact cells.

Biocontrol agent activity against J2s of *M. incognita*

Five potent bacterial isolates and one bioagent strain which showed the highest effect were screened for their potentialities for produce volatile compounds, gelatinase, protease and chitinase (Table 2).

Nematicidal volatiles activity (NVA)

The selected bacteria (B38, B39, B87, B91, B103 and *S. marcescens*) were tested for nematicidal activities via production of volatile compounds. It was seen from the obtained results in Table 2 that isolate B38 showed the highest mortality percentage of nematicidal volatile activity by 70.3% followed by *S. marcescens* strain (69.3%) and B103 (65.3%). These results are in harmony with those recorded by Lewis *et al*, (2006) who reported that the volatiles have been reported to play a vital role in the recognition between entomopathogenic nematodes and their hosts. However, little is known about nematicidal volatile organic compounds (VOCs) and their potential use as biological control agents against plant parasitic nematodes.

Moreover, Gu *et al* (2007) reported that the detected volatile compounds were alcohols, aldehydes, ketones, alkenes and ethers. Twenty VOCs with strong NVA (X80%), nine (phenol, 2-octanol, benzaldehyde, benzeneacetaldehyde, decanal, 2-nonanone, 2-undecanone, cyclohexene and dimethyl disulfide) displayed 100% NVA to both nematodes.

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Table 2. Values of nematicidal volatile activity, gelatinase, protease and chitinase activities produced by the selected bacteria

Isolates number	Nematicidal volatile activity (%)		Gelatinase		Protease		Chitinase	
	Number of immobile J_{2s}	NVA %	Zone of hydrolysis (cm) mean (R)	Gelatinase activity (R/r) ratio	Zone of hydrolysis (cm) mean (R)	Protease activity (R/r) ratio	Zone of hydrolysis (cm) mean (R)	Chitinase activity (R/r) ratio
B38	211	70.3	2.56	4.27	3.33	5.55	2.60	4.33
B39	162	54.0	2.33	3.88	3.13	5.22	2.41	4.01
B78	170	56.6	1.83	3.05	2.40	4.00	1.30	2.16
B91	81	27.0	2.00	3.33	2.46	4.11	1.86	3.10
B103	196	65.3	2.53	4.22	3.30	5.50	2.20	3.66
S	208	69.3	2.53	4.22	3.30	5.50	2.55	4.25

- Size of filter- paper disc (r) = 0.6 cm,
- Number of juveniles in 1 ml suspension is 300 ± 50, - S: *S. marcescens*

Table 3. Morphological and physiological characteristics of the isolate No. B103

Examined tests		Isolate No. B103	Characteristics		Isolate No. B103
KOH solubility test		Long line	**Pigmentation**		+
Gram staining		G^-	**Acid production from:**	**Xylose**	+
Cell shape		Short rods		**Glucose**	+
Spore formation		-		**Mannitol**	+
Growth on N_2-free medium		-		**Arabinose**	-
Motility		+		**Sucrose**	+
Growth at	**41-42˚C**	-	**Catalase production**		+
	4˚C	+	**Hydrolysis of:**	**Starch**	+
H_2S production		-		**Gelatin**	+
Indole production		-		**Casein**	-
V.P. test		V		**Lipids**	+
Methyl red		+	**Citrate utilization**		+
Growth on MaConkey		+			
Suggested scientific name			***Pseudomonas fluorescens* B103**		

- V: means variable

Gelatinase, protease and chitinase activity

Data in Table 2 show potentialities of the selected bacteria for excretion gelatinase, protease and chitinase. The obtained results revealed that, isolate B38 succeeded to produce high activities of gelatinase, protease and chitinase by measuring clearing zone (4.27, 5.55 and 4.33 cm, respectively), followed by *S. marcescens* and B103. It was of interest to notice that high nematicidal activity exhibited by B38 against J2s of *M. incognita* could be related to their high enzymes secretion. Nematode egg shell contains chitin fibrils embedded in a protein matrix, chitinase is a type of inducible enzymes for degrading chitin and can be produced by a wide variety of microrganisms. Their potential role in fungal infection towards nematode eggs was first suggested by Wharton (1980). Moreover, El-Hadad *et al* (2010) reported that *Paenibacillus polymyxa* NFB7, *Bacillus megaterium* PSB2 and *Bacillus circulans* KSB2 revealed high protease, chitinase and gelatinase

activities when evaluated their abilities for enzymes secretion. It should be stated that the highest nematicidal activity exhibited by these strains against the second stage juveniles of *Meloidogyne incognita*.

Finally, for *in vitro* screening, the aforementioned results indicated that, isolates B38 and B103 are promising isolates since they induced high nematicidal effect. Also, *S. marcescens* exhibited the highest nematicidal activity. Therefore these isolates B38, B103 and *S. marcescens* strain were selected for further study.

Table 4. Morphological and physiological characteristics of the isolate No. B38

Examined tests		Isolate No. B38	Examined tests		Isolate No. B38
KOH solubility test		Short line	Growth at	5˚C	-
Gram staining		G+		10˚C	-
Cell shape		Long rod		30˚C	+
Spore formation		+		40˚C	+
Growth on N_2-free medium		-		50˚C	-
Growth on MacConkey		-		55˚C	-
Motility		+	H_2S production		+
Growth at pH	6.8	+	Hydrolysis of	Starch	+
	5.7	+		Gelatin	+
Catalast production		+		Casein	+
Acid production from:	Glucose	+	Indole production		-
	Mannitol	+	Growth at NaCl 6.5%		+
	Sucrose	+	Citrate utilization		+
V.P. test		+	Anaerobic growth		-
Methyl red		-			
Suggested scientific name			*Bacillus subtilis* B38		

Effect of cultivation media on nematicidal compounds production

Data in Table 5 showed the effect of cultivation media on nematicidal volatiles, gelatinase, protease and chitinase production by *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens*. Comparing with nutrient agar medium, poly agar medium recorded the highest values for nematicidal volatiles by *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens* being 83.33%, 64.0% and 83.66%, respectively. Whereas, Emerson's agar medium showed the lowest nematicidal activity being 50.0%, 47.0% and 35.0% for *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens,* respectively.

Also, the obtained results in indicated that all the used media were suitable for enzymes production by the bacterial strains with various levels. Where, poly agar medium showed the highest activity of gelatinase, protease and chitinase (based on clearing zone measured) produced by *B. subtilis* B38 being 4.66, 5.83 and 4.25, respectively.

Concerning the *Ps. fluorescens* B103, obtained data showed the same trend proved that poly broth medium was the best medium for gelatinase, protease and chitinase activities being 4.25, 5.68 and 3.83, respectively. Moreover, poly agar medium exhibited the high activities of gelatinase, protease and chitinase produced by S. *marcescens* being 4.21, 5.83 and 4.25, respectively. High lytic activity by the tested bacteria on poly agar medium could be attributed to that medium which has a great effect for increasing of protein production. This result is in agreement with those of Rashad *et al* (2012) who reported that poly agar medium was found to be the most suitable medium since it has great effects on the growth, sporulation and biocide production by *B. sphaericus*. Also, they reported that the released proteins by local strains (*B. sphaericus* EMCC

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1931 and EMCC 1932) in poly broth medium were more toxic than those produced in the other tested growing media.

Table 5. Values of nematicidal factors produced by bioagent strains on solid cultivation media

Media	Nematicidal volatile activity (%NVA)		Gelatinase		Protease		Chitinase	
	Number of immobile J_{2s}	NVA %	Zone of hydrolysis (cm) mean (R)	Gelatinase activity (R/r) ratio	Zone of hydrolysis (cm) mean (R)	Protease activity (R/r) ratio	Zone of hydrolysis (cm) mean (R)	Chitinase activity (R/r) ratio
				***B. subtilis* B38**				
M1	205	68.33	2.61	4.35	3.00	5.00	2.55	4.25
M2	221	73.66	2.60	4.33	3.50	5.83	2.50	4.16
M3	250	83.33	2.80	4.66	3.50	5.83	2.55	4.25
M4	150	50.00	1.88	3.13	2.50	4.16	2.00	3.33
M5	189	63.00	2.11	3.51	2.82	4.70	2.13	3.55
				***Ps. fluorescens* B103**				
M1	177	59.00	2.50	4.16	3.20	5.33	2.22	3.70
M2	190	63.33	2.50	4.16	3.33	5.55	2.30	3.83
M3	192	64.00	2.55	4.25	3.41	5.68	2.30	3.83
M4	141	47.00	2.21	3.68	2.63	4.16	2.10	3.50
M5	144	48.00	2.33	3.88	2.50	4.38	2.14	3.56
				S. marcescens				
M1	205	68.33	2.44	4.06	3.30	5.50	2.50	4.16
M2	217	72.33	2.50	4.16	3.30	5.50	2.55	4.25
M3	251	83.66	2.53	4.21	3.50	5.83	2.55	4.25
M4	105	35.00	2.16	3.60	2.55	4.25	2.15	3.50
M5	113	37.66	2.20	3.66	2.81	4.68	2.10	3.58

- M1: Nutrient agar medium, **M2:** Tryptic Soy agar medium, **M3:** Poly agar medium, **M4:** Emerson's agar medium, **M5:** Acetate yeast extract agar medium.
- The number of juveniles in a ml suspension is 300 ± 50
- The size of filter- paper disc (r) = 0.6 cm.

Effect of bacterial cells suspension and cultural filtrate on mortality of *M. incognita* J_{2s}

Different dilutions of bacterial cells suspension and cultural filtrate of *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens* which grown in poly broth medium were evaluated for their effect on mortality of *M. incognita* J2s after 48 hours, these results are illustrated in Fig. (1) it could be stated that, bacterial cells suspension concentration showed more efficient mortality on nematode *M. incognita* compared with their cultural filtrates.

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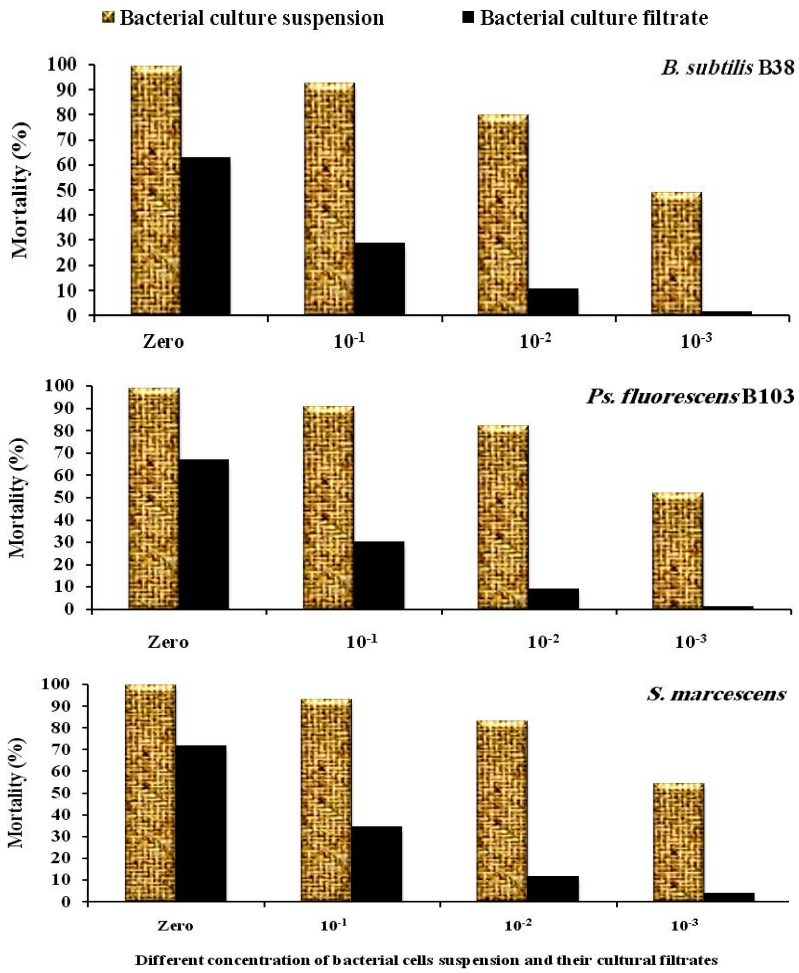

Fig.1. Mortality percentages of *M. incognita* J_{2s} affected by different concentration of bacterial cells suspension and their cultural filtrates.

This may be due to the presence of bacterial strains which produced nematicidal substances constantly in the media. The obtained results are in harmony with those of El-Hadad *et al* (2010) who found that, the filtrates of all bacterial cultures were low effective on nematodes mortality compared with their whole bacterial cultures.

Concerning the dilutions of bacterial cells suspension and their filtrates, the highest mortality percentages were recorded at dilutions 10^{-1} by all bacterial strains comparing with the other dilutions (10^{-2} and 10^{-3}). This is because, the active substances causing mortality decrease with increasing the dilution. Regarding the bacterial culture of *S. marcescens* at dilutions of 10^{-1}, 10^{-2} and 10^{-3} recorded the highest values of *M. incognita* mortality being 92.9%, 82.9% and 54.3%, respectively, compared with the other tested bacteria. While *Ps. fluorescens* B103 recorded the lowest mortality in dilution of 10^{-1} (90.6%) and 10^{-2} (82.0%), *B. subtilis* B38 culture recorded the lowest mortality at dilution 10^{-3} being 48.9%.

These results are in agreement with those of Terefe *et al* (2009) who found that an aqueous suspension of *Bacillus firmus* at 2.5 and 3% concentration caused 100% inhibition of mobility *M. incognita*, 24 hrs after treatment. In addition, the mortality percentage of *Paenibacillus polymyxa* (NFB7) at 1/100 was the highest inhibitor (99.3%) comparing with the other tested bacteria. Moreover, the mortality percentage of *Bacillus circulans* (KSB2) at dilutions of 1/100 and 1/1000 were the highest inhibitors comparing with the other tested bacteria being 97.8 and 40.3%, respectively, (El-Hadad *et al*, 2010).

Regarding the cultural filtrate, the obtained data are illustrated in Fig. (1) also explained that *S. marcescens* at dilutions 10^{-1}, 10^{-2} and 10^{-3} showed the highest mortality percentage being 34.3%, 11.4% and 3.7%, respectively. While, *B. subtilis* B38 filtrate showed the lowest mortality at dilution 10^{-1} being 28.6%, *Ps. fluorescens* B103 recorded the lowest mortality at dilution 10^{-2} (8.9%) and 10^{-3} (0.9%). The lethal effect of the cultural filtrates of these bacteria may be attributed to the production of nematicidal metabolites i.e. lytic enzymes (gelatinase, protease and chitinase) and volatile compounds in the cultural media. Similar results were reported by Ali *et al* (2002) who found that cultural filtrates of *Pseudomonas* sp. caused juvenile mortality of *M. javanica*. The exposure of *M. incognita* to various concentrations (5–100%) of cultural filtrate of *Paenibacillus polymyxa in vitro* conditions significantly reduced egg hatching and caused substantial mortality of its juveniles (Khan *et al*, 2008).

CONCLUSION

It is clear from this work that, in plant rhizosphere, region there are many bacteria have potentialities for controlling root-knot nematode. Among 19 bacterial isolates and 3 bioagent stains, two isolates and *S. marcescens* were exhibited the highest production of nematicidal activities against root-knot nematode (*M. incognita*) *in vitro* and the two isolates were identified as *B. subtilis* B38 and *Ps. fluorescens* B103. Finally, it could be recommended that using strains of *B. subtilis* B38, *Ps. fluorescens* B103 and *S. marcescens* to control of root-knot nematode.

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