The effect of cultural filtrates of some bio-control agents on the root-knot nematode, *Meloidogyne incognita* infecting grape seedlings.

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Abstract

Effects of cultural filtrates of six bio-control agents i.e. *Pseudomonas fluorescens*, *P. putida*, *Streptomyces aureofaciens*, *Trichoderma harzianum*, *T. viride* and *Bacillus subtilis,* as standard solution and dilutions (S/20, S/40 and S/80) on the development and reproduction of root knot nematode, *Meloidogyne incognita* and plant growth of infected grape seedlings cv. Early Sweet was evaluated under laboratory and greenhouse conditions. Also, their potential to control nematodes was compared with that achieved by using the chemical control agent, Ragby. In vitro tests, all bio-control agents were found to be highly nematicidical effects against infective juveniles of nematode. *Pseudomonas putida* at all concentrations was more effective which caused100, 95, 90 and 74 % nematode mortality, since was *Pseudomonas fluorescens*, *Trichoderma harzianum, Streptomyces aureofaciens, Bacillus subtilis* and *Trichoderma viride*. In greenhouse, all tested biofiltrates significantly controlled nematode population by reduced number of all nematode parameters compared to the control, and enhanced plant growth. Recommended dose of Ragby and standard solution of *P. putida* and *T. harzianum* has surpassed in reducing *M. incognita* population the others through successive four months. However, *T. viride*, *P. fluorescens* and *B. subtilis* was mostly effective .While, the least effective treatment was *S. aureofaciens* that gave the

minimum of the percentage efficacy in reducing of all nematode parameters.

Key Words: Biological control, *Meloidogyne incognita,* biofiltrates, *Pseudomonas fluorescens*, *P. putida*, *Streptomyces aureofaciens*, *Trichoderma harzianum*, *T. viride*, *Bacillus subtilis*, grape.

Introduction

Most of the new reclaimed areas in Egypt are planted with fruit trees especially grapes which are considered the second fruit crop in the country. The area of the vineyards has increased rapidly through the last few years and reached about 185.700 feddans (**Anonymous**,2009) with total production of 1,639,860 tons and an average of about 9.60 tons / feddan. Much effort is devoted to raising the productivity of the crop.

Unfortunately, many factors are contributing to declines in crop yield. Among these factors are root-knot nematodes, as the environmental conditions in vineyards favor their reproduction and development. *Meloidogyne incognita*, *M. arenaria* and *M. javanica* receive the greatest attention because of their widespread distribution in vineyards (**Anwar and McKenry, 2001**). In countries where no attention is given to control strategies, yield losses are likely to be in the region of 25-60 % (**Sasser and Carter, 1982**). Reports of yield reduction of grapevines caused by nematodes were recorded in different countries (**Ferris & McKenry, 1975; Eissa, 1981; Moussa *et al.*, 1983; El-Eraki *et al.*, 1984; Osman & Hendy, 1988 and Anwar & McKenry, 2001**). Growth of vines on nematode infested sites can be improved by managing the nematode populations below the damage level through application of effective chemicals.

There has also been, however, a loss of effective nematicides as some products have been withdrawn. Besides, since nematicides are very expensive and are suspected of being the cause of serious environmental pollution, the applied strategy today is directed towards replacing the use of hazardous chemical nematicides by environmentally friendly natural materials, which in the same time can maintain nematode population densities at levels that do not cause economic damage to plants.

Considerable efforts have been made by many workers for the management of different plant-parasitic nematodes using filtrates of *Pseudomonas fluorescens*, *P. putida*, *Streptomyces aureofaciens*, *Trichoderma harzianum, T. viride and Bacillus subtilis* **(Stephen & Grover, 1985; Sharma &Saxena,1992**; **Rao *et al.*, 1997**; **Faruk *et al.*, 1999**; **Hanna *et al.* ,1999;Verma *et al.*, 1999**; **Jonathan *et al.*, 2000; Khan *et al.*, 2001**; **Rajendran *et al.*, 2001; Siddiqui & Mahmood,2001**; **Abadir *et al.*, 2004; Jonathan & Umamaheswari,2006**; **Rao,2007** and **Bokhary,2009)** . **El-Nagdi and Abd-El-Khair (2008)** tested the culture filtrates of *B. subtilis, P. fluorescens, T. harzianum* and *Trichoderma viride* for managing *M. incognita* in the greenhouse. The most effective culture filtrate, applied as a soil drench, was *B. subtilis* at 10% which reduced the number of juveniles in soil, galls and egg masses of *M. incognita* on roots of eggplant cv. Pusa Purple Long by 91.9, 82 and 82.6%, respectively.

The objectives of this study were to test the efficacy of the filtrates of six Egyptian bio-control agents against the infective juveniles of root knot nematode, *Meloidogyne incognita* (laboratory test) and management of these nematodes on grape seedless cv. Early Sweet under field conditions.

Material and Methods

Propagation of *Meloidogyne incognita* in pure cultures:

A pure stock culture of the root-knot nematode *Meloidogyne incognita* was prepared from naturally infected grapes roots collected from fields. Individual egg-masses of females which were previously identified as *M. incognita* were transferred to a 25 cm diameter clay pot filled with 4 kg steam sterilized sandy loam soil and grown with a seedling of eggplant, inoculated pots were placed in a greenhouse and watered when needed. After two months of inoculation, infected roots were then chopped and used as sources of inoculation for other series of clean eggplant seedlings. By repeating this procedure, enough quantities of inocula from a single egg-mass were obtained.

Bioagents:

Six bioagents, i.e. ***Pseudomonas fluorescens***, ***Pseudomonas putida***, ***Streptomyces aureofaciens***, ***Trichoderma harzianum***, ***Trichoderma viride*** **and** ***Bacillus subtilis*** were isolated from the rhizosphere of grapevine cultivars and the bioassay tests. These isolates were identified in Plant Pathology Department, National Research Centre based on a great variety of morphological, cultural, physiological and biochemical characteristics.

The fungi (***T. harzianum*** **and** ***T. viride***) were grown on Potato Dextrose agar medium for 10 days at 25°C. The conidial suspensions were obtained by flooding the colonies with sterile water containing 0.01% tween80, then weltered and adjusted to 5×10^5 colony forming units (CFUs/ml). For long-term maintenance, strains were preserved in broth medium containing 15% (vol/vol) glycerol at 4°C. For production;

Yeast extract-Peptone-Glucose (YPG) (3 g/l yeast extract, 10 g/l peptone, 20 g/l glucose) was used.

Bacteria (***P. fluorescens, P. putida* and *Bacillus subtilis***) were grown in nutrient broth at 26°C for 24 h. Bacteria were spread onto nutrient agar plates from stocks. These plates were incubated overnight at 25°C and used to inoculate 100 ml nutrient broth (Oxoid) in 250 ml conical flasks. *Pseudomonas* was routinely cultured in King's medium B (**King *et al.* 1954**) at 28°C. For long-term maintenance, strains were preserved in broth medium containing 15% (vol/vol) glycerol at 4°C. For production; Yeast extract-malt extract medium was used (Yeast extract, 4.0; Malt extract, 10.0; Glucose, 4.0; Agar, 20.0; Distilled water 1000 ml. pH was adjusted at pH 7.2-7.4).

For ***Streptomyces aureofaciens***, Starch-nitrate medium (Soluble starch, 20.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCO_3.2H_2O$, 2.0) was used. Cells were harvested after four days, centrifuged at 3000 g for 10 min. Pellet were resuspended in 15 ml distilled water containing 0.01% Tween80 and Phosphate-buffered saline (PBS) to a density of 10^5 bacteria/ml. For production; Yeast extract-malt extract medium was used (Yeast extract, 4.0; Malt extract, 10.0; Glucose, 4.0; Agar, 20.0; Distilled water 1000 ml. The pH was adjusted at pH 7.2-7.4). For long-term maintenance, strains were preserved in broth medium containing 15% (vol/vol) glycerol at 4°C.

Biocontrol agents were cultured into broth medium and incubated on an incubator shaker (150 rpm) at 28°C for 3 and 7 for bacteria and fungi respectively. The liquid suspension of each bioagent was collected and adjusted at the concentration of 5×10^5 and 5×10^6 colony forming unit for bacteria and fungi respectively.

Bioassay test:

Culture filtrates of the six bioagents previously mentioned i.e. *P. fluorescens*, *P. putida*, *S. aureofaciens*, *T. harzianum*, *T. viride* and *B. subtilis*, were tested against the *M. incognita* survival. Each filtrates were considered to be standard solution (S). Dilutions S/20, S/40 and S/80 were made by adding distilled water. About 100 newly second stage juveniles of *M. incognita* were transferred to the different dilution of each bioagent filtrate S, S/20, S/40 and S/80 contained in sterilized Petri-dishes 5 cm diameter. Distilled water served as a control. Each treatment was replicated three times. The test was carried out at room temperature of 25±2°C. After 12,24,48,72 and 96 hrs. of exposure dead and a live larvae were counted under a stereoscopic microscope then the percentage of nematode mortality were calculated. From these counts, the percentages of nematode mortality were calculated for each treatment. Nematodes were considered alive if they moved or assumed a winding shape, and they were considered dead if they had adopted a straight shape and were immobile. To avoid incorrect classification, the nematodes in each Petri dish were then transferred to distilled water for 48 h to check whether the assumed dead nematodes regained motility or not. The corrected nematode mortality percentages were calculated according to **Abbott's Formula (1925).**

Greenhouse test:

The six bioagents which were tested against *M. incognita* development on two grapevine cultivar seedlings Early Sweet and Flame Seedless, one year old. Seedlings were grown in 20 cm diameter clay pots filled with steam sterilized loamy soil. Two months after cultivation, each pot was received about 1000 second stage juveniles (j2)

of *M. incognita* or 1000 unswollen females of *R. reniformis*. After 4 days of nematode inoculation 50 ml of each previous filtrate and dilution were separately added to each pot around the seedling roots. Each treatment was replicated three times. Equal number of untreated (control with nematode only) served as a check. All pots were arranged on a greenhouse bench at 32 ± 5°C in randomized design receiving the same horticultural treatments. Six weeks after inoculation, nematode soil population of each nematode species and their developmental stages in or on roots were counted. Also, the eggmasses per root, eggs per eggmass and rate of reproduction for each nematode species were calculated. For *M. incognita* the numbers of galls were calculated too. All plants were harvested and the root system of each plant was carefully removed from soil, length and fresh weights of both shoots and roots were estimated.

Data were then, analyzed following standard procedures for analysis of variance by **Duncan`s multiple range test (1955).**

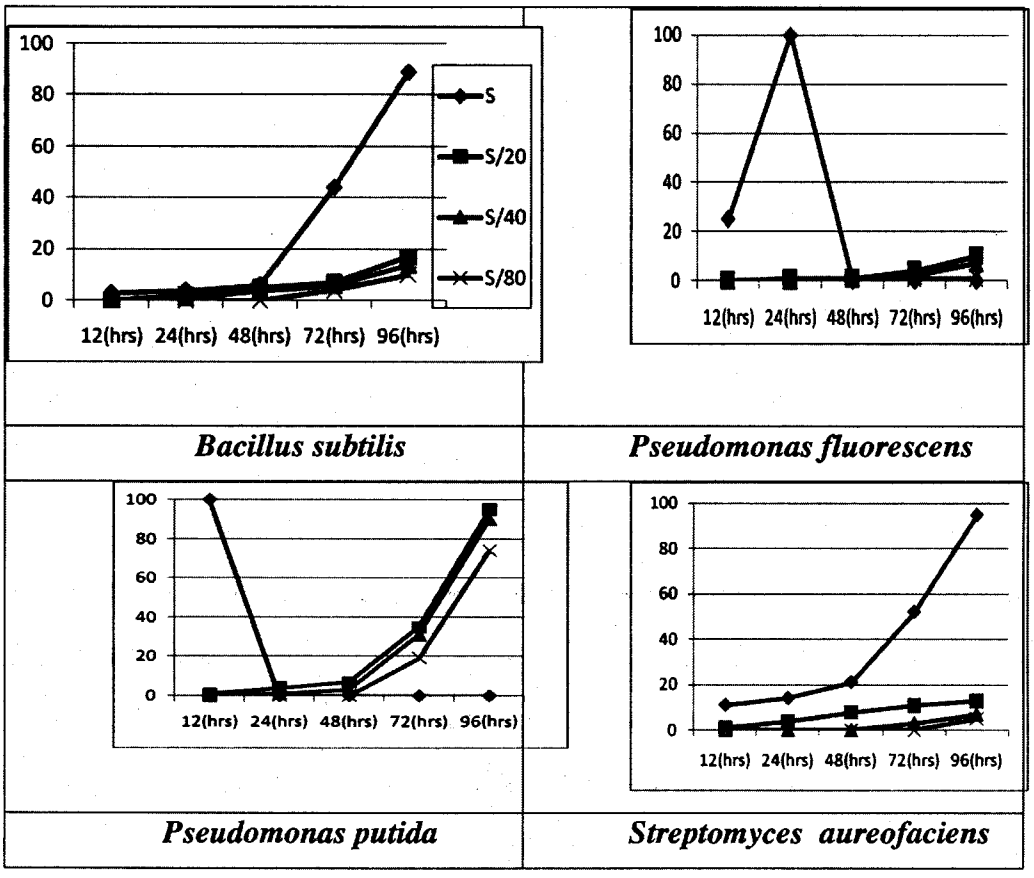
Results and Discussion

1.1): Bioassay test.

Effect of cultural filtrates of six bio-control agents of ***Pseudomonas fluorescens, P. putida, Streptomyces aureofaciens, Trichoderma harzianum, T. viride*** and ***Bacillus subtilis*** were studied at the concentrations of S, S/20, S/40 and S/80 against second stage juveniles of ***Meloidogyne incognita*** *in vitro* after 12,24,48,72 and 96 hrs. as a exposure times (Fig. , 1). These fungal and bacterial cultural filtrates had high nematicidal effects against J_2 of *M. incognita* and caused significantly higher nematode mortality when compared with control. Also, results revealed that the cultural filtrates increased the percentages

of J_2 nematode mortality with increase in the exposure time from 24 to 96 hrs.

The standard cultural filtrate concentration (S) from ***P. fluorescens, P. putida*** was the most effective against the nematode (100 %), followed by ***S. aureofaciens, T. harzianum, B. subtilis*** and ***T. viride*** (95, 95, 89 and 80%, respectively) after 96 hrs. At concentration S/20, the highest nematode mortalities was caused by ***P. putida*** (95) followed by the cultural filtrates for ***T. harzianum, B. subtilis, S. aureofaciens, T. viride*** and ***P. fluorescens*** (25, 17, 13.13 and 10%, respectively) after 96 hrs. Finally, the lowest effect was occurred by the cultural filtrate concentration (S/80) to all biofiltrates except ***P. putida*** which resulted for a noticeable increase in nematicidal effects against J_2 of ***M. incognita.***



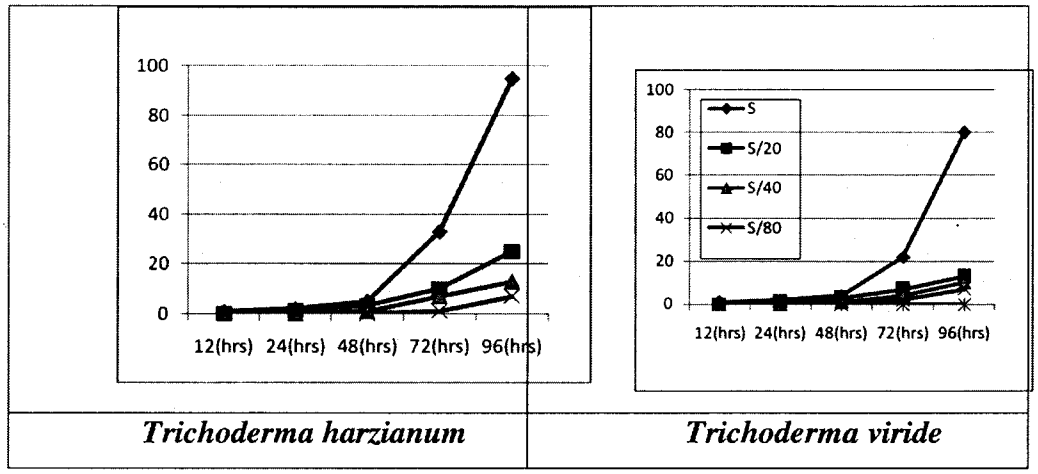


Fig. (1): Effect of biofiltrates on mortality of *Meloidogyne incognita* juveniles.

These results are in general agreement with those recorded by **El-Nagdi and Abd-Elkhair (2008), Bokhary (2009) and Abdelnabby *et al.* (2011).**

1.2): Greenhouse test.

Effects of the filtrates of ***P. fluorescens, P. putida, S. aureofaciens, T. harzianum, T. viride*** and ***B. subtilis,*** as standard solution and dilutions (S/20, S/40 and S/80) and the nematicide Ragby on the development and reproduction of ***M. incognita*** and plant growth of infected grape seedlings cv. Early Sweet was evaluated under greenhouse conditions (Table, 1). Results revealed that soil application with tested filtrates or nematicides significantly reduced number of root galls, nematode juvenile in soil per pot, nematode developmental stages, eggmasses per root, eggs per eggmass, nematode final population and the rate of reproduction of *M. incognita* compared to the inoculated control (nematode inoculation only). Also, significant differences ($P \leq 0.05$) in such nematode criteria of these filtrates were always decreased with increasing of the cultural filtrate concentration.

Recommended dose of Ragby and standard solution of ***P. putida & T. harzianum*** was the most effective treatment in galls reduction which recorded (6, 20 and 21galls numbers, respectively) followed by ***T. viride, P. fluorescens*** and ***B. subtilis*** (22, 23 and 26, respectively). While, the least effective treatment was ***S. aureofaciens*** that gave 30 galls numbers per plant root.

Table (1): The effect of biofiltrates on the development and reproduction of the root-knot nematode *Meloidogyne incognita* infecting grape seedlings.

Treatments	Dilutions	No. of galls/ root	Nematode counts				Nematode final population (P_f)	Rate of build up (P_f/P_i)
			J. in soil/ pot	Develop. stages / root	Egg- masses / root	Eggs /egg-mass		
Bacillus subtilis	S	26 j	283 fg	36 f	8 ghi	158 gh	1533	1.53
	S/20	38 h	367 de	53 cd	9 fg	173 efg	2032	2.03
	S/40	43 c-f	433 abc	54 cd	14 de	183 c-f	2984	2.98
	S/80	46 b	467 ab	54 bcd	15 cde	191 cde	3391	3.39
Pseudomonas fluorescens	S	23 jkl	333 ef	31 gh	7 hig	119 I	1158	1.16
	S/20	42 d-g	367 de	53 cd	11 f	185 c-f	2455	2.45
	S/40	43 b-e	417 bcd	54 cd	14 de	194 cd	3122	3.12
	S/80	45 bc	467 ab	55 abc	15 cde	195 cd	3442	3.44
Pseudomonas putida	S	20 l	367 de	27 i	6 j	185 c-f	1440	1.44
	S/20	39 gh	400 cd	54 cd	13 e	191 cde	2941	2.94
	S/40	42 c-g	433 abc	54 bcd	14 de	194 cd	3139	3.14
	S/80	55 a	450 abc	58 a	16 cd	219 b	3934	3.93
Streptomyces aureofaciens	S	30 i	367 de	42 e	8 gh	142 h	1592	1.59
	S/20	41 efg	433 abc	54 cd	10 fg	166 fg	2088	2.09
	S/40	42 c-g	467 ab	55 a-d	14 de	178 c-g	2959	2.96
	S/80	44 bcd	467 ab	55 abc	16 bc	180 c-f	3467	3.47
Trichoderma harzianum	S	21 l	150 h	24 j	8 ghi	112 i	1067	1.07
	S/20	24 jk	400 cd	32 gh	8 ghi	168 fg	1720	1.72
	S/40	44 b-e	433 abc	51 d	13 e	172 efg	2721	2.72
	S/80	45 bc	433 abc	54 bcd	18 g	176 d-g	3714	3.71
Trichoderma viride	S	22 kl	367 de	29 hi	6 ij	176 d-g	1510	1.51
	S/20	40 fgh	467 ab	54 cd	13 e	186 c-f	3000	3.00
	S/40	40 fgh	477 ab	55 abc	14 de	198 c	3233	3.23
	S/80	44 bcd	483 a	56 abc	14 de	223 b	3661	3.66
Ragby	Recommended	6 m	183 h	8 k	3 k	107 i	511	0.51
	Half	25 j	275 g	33 fg	9 g	222 b	2229	2.23
	Quarter	41 efg	400 cd	54 cd	10 fg	229 b	2667	2.67
Check		55 a	487 a	58 ab	18 ab	259 a	5213	5.21
LSD 0.05		2.51	52.23	3.30	1.73	17.26		

Values in a column followed by the same letter (s) are not significantly by ($p \leq 0.05$) according to Duncan`s multiple-range test.

Furthermore, the performance of the used treatments on reduction of nematode final population was also evaluated. The superior treatment was recommended dose of Ragby and standard solution of ***T. harzianum*** (511and 1067, respectively), followed by ***P. fluorescens, P. putida, T. viride*** **and** ***B. subtilis*** (1158, 1440, 1510 and 1533, consecutively). ***S. aureofaciens*** was the least effective treatment which recorded 1592.

The influence of the evaluated treatments on root-knot nematode rate of build up which considered an important indicator to the efficacy of the used compounds which recorded significant reduction when compared with control. The highest treatments of Ragby and ***T. harzianum*** with rate of build up 0.51and 1.07 at recommended dose and standard solution, respectively when compared with rate of build up of check (5.2), followed by the bio-control agents of ***P. fluorescens, P. putida, T. viride, B. subtilis*** and ***S. aureofaciens*** where the values of nematode reproduction when using the standard solution were 1.16, 1.44, 1.51, 1.53 and 1.59, respectively.

All treatments of biofiltrates significantly increased in plant growth represented by shoot, root weight and length compared with non treated control plants. The percentages of increasing of shoot and root were positively correlated by increasing the bio-filtrate concentration. Data presented in Table (2) indicated that The highest increasing in shoot and root parameters occurred by the treatment of Ragby (39.82, 42.37, 46.43 & 57.38 %) and ***T. harzianum*** (29.84, 30.61, 36.77 & 45.64 %) increasing percentage of shoot, root length and weight at recommended dose and standard solution, respectively. Followed by ***P. putida, B. subtilis, T. viride*** and ***P. fluorescens*** filtrates. While the least percentage of increasing was associated with ***S. aureofaciens*** which amounted in shoot, root length and weight (5.93, 6.42, 10.04& 23.57%, respectively). Similar effects of ***P. fluorescens*** and ***Trichoderma*** spp. on different crops against ***Meloidogyne*** **spp.** have been reported by many authors (**Hamid *et al.*, 2003; Khan *et al.*, 2005; Khan, 2007; Khan *et al.*, 2007**). The bacterium is a phosphate solubilizer (**Khan *et al.*, 2009**) but may also suppress pathogens through

antibiosis (**Nielson *et al.*, 1998**), siedorphore production (**Glick, 1995**), induced systemic resistance (**Kloepper *et al.*, 1992**), production of phytohormones (**Garcia de Salamone *et al.*, 2001**) or other compounds (**Marek-kozaczuk and Skorupska, 2001**). Application ***T. harzianum, T. hamatum*** **or** ***T. virens*** has also demonstrated potential to suppress root-knot nematodes (**Siddiqui and Shaukat, 2004**).

Table (2): Effect of biofiltrates as soil application and its dilutions on growth of grape seedlings infected with *Meloidogyne incognita.*

Treatments	Dilutions	Length(cm)		Weight(gm)	
		Shoot	Root	Shoot	Root
Bacillus subtilis	S	51 d	41 d	42.24 ef	29.15 d
	S/20	49 def	38 ef	40.57 f	27.25 def
	S/40	46 fgh	36 h-k	41.03 ef	23.87 ghi
	S/80	45 g-j	36 i-l	40.71 ef	21.69 i-l
Pseudomonas fluorescens	S	46 fgh	37 f-I	37.35 g	26.14 efg
	S/20	44 g-j	36 i-l	33.89 h	23.87 ghi
	S/40	43 hij	35 k-n	32.79 h	21.75 i-l
	S/80	43 hij	34 lmn	32.39 h	20.68 jkl
Pseudomonas putida	S	55 c	45 c	47.52 c	32.43 c
	S/20	44 g-j	35 j-m	42.42 def	22.07 ijk
	S/40	42 ij	34 mn	41.44 ef	19.22 l
	S/80	41 j	33 n	33.90 h	19.41 kl
Streptomyces aureofaciens	S	45 ghi	36 g-j	37.15 g	25.14 fgh
	S/20	44 g-j	36 i-l	35.35 gh	23.83 ghi
	S/40	44 g-j	34 lmn	33.90 h	21.75 i-l
	S/80	41 j	33 n	32.79 h	19.41 kl
Trichoderma harzianum	S	60 b	49 b	52.86 b	35.35 b
	S/20	55 c	48 b	50.81 b	33.21 bc
	S/40	49 def	37 fgh	41.78 ef	22.67 hij
	S/80	41 j	33 n	37.15 g	19.41 kl
Trichoderma viride	S	50 de	39 e	43.59 de	27.90 de
	S/20	47 efg	38 efg	42.64 def	25.99 efg
	S/40	43 hij	35 k-n	41.93 ef	20.22 jkl
	S/80	42 ij	34 mn	40.63 ef	19.19 l
Ragby	Recommended	70 a	59 a	62.39 a	45.08 a
	Half	45 ghi	38 ef	45.27 cd	23.97 ghi
	Quarter	42 ij	35 g-m	42.75 def	19.76 kl
Check		42 ij	34 mn	33.42 h	19.22 l
LSD 0.05		3.52	1.52	2.96	2.70

Values in a column followed by the same letter (s) are not significantly by ($p \leq 0.05$) according to Duncan`s multiple-range test.

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تاثير رواشح بعض الكائنات الحية على نيماتودا تعقد الجذورالتى تصيب شتلات العنب

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الملخص العربى

فى هذه الدراسة تم تقييم تاثيرروا شح ستة من الكائنات الحية وهى ثلاثة من البكتريا (*Pseudomonas fluorescens*, *P. putida*, *Bacillus subtilis*) وإثنين من الفطريات (*Trichoderma harzianum*, *T. viride*) ونوع واحد من الاكتينوميسيس (*Streptomyces aureofaciens*) وذلك على اربعة تركيزات وهى التركيز الاصلى وتخيف ٢٠ وتخفيف ٤٠ وكذلك تخفيف ٨٠ على على نيماتودا تعقد الجذوروكذلك على معدلات نمو شتلات العنب من الصنف الإيرلى سويت المصابه بها تحت ظروف المعمل والصوبة الزراعية ، ومقارنة ذلك بواحد من المبيدات النيماتودية الكيماوية وهو الراجبى.

فى الدراسة التى أجريت تحت ظروف المعمل أثبتت النتائج ان كل رواشح الكائنات المختبرة نتجت عنها تأثيرات سامه اثرت على الاطوار المعدية لنيماتودا تعقد الجذور. كانت رواشح البكتريا (*Pseudomonas putida*)هى الأكثر تأثيرا على كل التركيزات المختبرة حيث نتج عنها نسب موت ١٠٠ ، ٩٥ ، ٩٠ ، ٧٤ % .وبعد ذلك جائت نتائج رواشح باقى الكائنات المختبرة (*Pseudomonas fluorescens*, *Trichoderma harzianum, Streptomyces aureofaciens, Bacillus subtilis ,Trichoderma viride*) على الترتيب.

أما فى تجربة الصوبة الزراعية ، دلت النتائج على ان كل رواشح الكائنات المختبرة نتجت عنها مقاومه معنوية لتعداد نيماتودا تعقد الجذور ويتمثل ذلك فى خفض معنوى فى كل قياسات النيماتودا وكذلك نتج عنها درجات معنوية فى نمو شتلات العنب مقارنة بالكنترول.ودلت النتائج ايضا ان التركيز الموصى باستعماله من مبيد الراجبى وكذلك رواشح البكتريا (*P. putida*) والفطر (*T. harzianum*) اعطت اعلى النتائج فى خفض تعداد نيماتودا تعقد الجذورمقارنة بالمعاملات الاخرى . تلت هذه النتائج رواشح الفطر (*T. viride*) و رواشح كلا من البكتريا (*P. fluorescens* ,*B. subtilis*)بينما كانت اقل النتائج هى المتحصل عليها بواسطة رواشح الاكتينوميسيس (*Streptomyces aureofaciens*).