Effect of Immobilized PGPRs Bacteria in Different Forms Against Root-Knot Nematodes on Tomato Plants

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Abstract: Plant growth-promoting rhizobacterium (PGPR) suppresses root–knot nematode *Meloidogyne javanica* through directly or indirectly effect. In our research four different PGPRs namely *Pseudomonas fluorescens, Azotobacter chroorcoccum, Bacillus polymyxa* and *Azospirillum brasilense* were used at three different formulated types as follows: encapsulated with sodium alginate beads, formulated with mixture of carboxymethyl-cellulose with talcum powder and suspension of culture, to investigate the potential effect against root–knot nematode *M. javanica* under greenhouse conditions. The viability of the bacterial strains studied by using two polymers; sodium alginate and carboxymethyl cellulose (CMC) *in vitro*. Viability was measured at four temperatures i.e. - 5, 10, 20 and 30°C after 10, 20, 30 and 40 days from stored (zero time). *In vivo* treatments classified into two groups; one group inoculated only one from all tested bacteria, whereas the second group inoculated twice after 15 days from the first one with the same treatments. Results showed that significantly effects on nematode and vegetative growth parameters compared to plants treated with nematode alone. All treatment reduced the nematode parameters i.e. number of galls and egg masses/root system between 25-99 % compared to control. The highest reduction percentage in galls number recorded with *P. fluorescens* at all application forms as ranged between 83-93%, the highest one 91 and 93% recorded with alginate beads types either at one or two inoculation times, respectively. The same trend of results recorded with egg masses as the reduction percentage ranged between 94 and 99%. The highest one also recorded with *P. fluorescens* alginate beads as ranged between 96 and 99 % at one and two inoculation time, respectively. The lowest effect on nematode parameters observed with *A. brasilense* at all inoculation forms and times. Moreover, results found that inoculation with encapsulated of all bacterial strains gave a significant increase in rhizosphere enzymatic microbial activities compared to other treatments and control. Also, inoculation with all bacterial strains at all forms and times significantly increased the chemical components i.e. total chlorophyll (a+b), caroteinoids contents as well as total protein content in all treatments compared to control.

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1. Introduction

Root-knot nematodes, *Meloidogyne* spp. (RKN) are plant- parasitic nematodes, which attack about more than 2000 host plants that susceptible to their infection and they cause approximately 5% of global crop losses (**Hussey and Janssen, 2002**). Root-knot nematodes are major pests of field and vegetable crops. They cause damage to many economically important horticultural crops like potato, cotton and tomato. Root-knot nematodes cause considerable losses in both commercial and subsistence tomato production systems and their control remains difficult for its wide host range. Tomato plants is highly susceptible host to RKN; *M. incognita* and various plant parasitic nematodes **(Khan *et al*., 2011).** Due to their endoparasitic style of living and feeding, they disrupt the physiology of the plants and may reduce crop yield and product quality, therefore, they are great economic importance nematodes and make control are necessary **(Adegbite, 2011).**

Due to severe human and environmental problems and high costs, achieving a sustainable agriculture will require avoidance of chemical control of plant parasitic nematodes. Recently, numerous nematologists have focused on safety alternative methods to control RKN such as biological control with some biocontrol agents i.e plant-growth promoting rhizobacteria (PGPR) such as *P.fluorescens* **(Siddiqui and Shakeel 2009**; **Ashoub and Amara 2010),** *A. brasilense* (**Shamseldin *et al*., 2010)** and *A.chroorcoccum* (**Siddiqui, and Futai, 2009)** to overcome the nematode damage. PGPR seem to promote growth through suppression of causal organisms **(Veerubommu and Kanoujia 2011);** competition for space, nutrients and ecological niches, induce systemic resistance; production of

antimicrobial substances; phytohormones and peptides acting as bio- stimulants without negative effects on the user, consumer or the environment **(Jimenez-Delgadillo, 2004)**.

Beneficial microorganisms such as diazotrophs bacteria, bio-control agents (BCAs) and PGPRs, can play an important role in this major challenge, as they fulfill important ecosystem functions for plants and soil **(Rosa Hermosa *et al*., 2011)**. Some rhizosphere microorganisms, including *P. fluorescens* CHA0, can also act as antagonists of plant-pathogenic nematodes **Siddiqui and Shaukat (2003).** Alkaline protease Prb1 from *T. harzianum* IMI 206040 has been demonstrated to play a major role in biological control **Benítez *et al.,* (1998).**

Carriers are classified as inorganic material i.e. zeolite, clay, anthracite, porous glass, activated charcoal, and ceramics and organic polymers i.e. alginate, carrageenan, polyacrylamide and talcum powder. Inorganic carriers were selected to immobilize microorganisms because they can resist microbial degradation and are thermostable (**Verma *et al*., 2006).** The organic polymeric carriers are more abundant than inorganic carriers and can be natural and synthetic polymeric carriers **(Cassidy *et al*., 1996).** Other formulations useful to the application of beneficial microorganisms to seeds or plants make use of cross-linking organic polymers. These materials have been used extensively to experimentally immobilize plant, animal or microbial cells and even isolated enzymes (**Stormo and Crawford, 1992).** This bacterium was used as a model for this work and it must be underlined that the new carrier could be applied to various nitrogen fixing bacteria that were isolated from various soils in Europe **(Elena *et al*., 2003).** Bioformulation of plant-growth promoting rhizobacteria should be composed of a superior carrier material such as high water holding capacity, high water retention capacity, no heat production from wetting, nearly sterile, chemically uniform, physically uniform, nontoxic in nature, easily biodegradable, nonpolluting, nearly neutral pH (or easily adjustable pH), and supports bacterial growth and survival. Apart from these materials, many other synthetic and inert materials, such as vermiculite, ground rock phosphate, calcium sulfate, polyacrylamide gels, and alginate have also been evaluated (**Domenech *et al*., 2006).** This carrier was **s**elected after optimization of the encapsulation procedure which consists in assessing the maximum bacterial surviving with respect to the polymer characteristics (low cost, biocompatible, biodegradable, with release properties that are temperature and water dependent). Therefore, the aim of this study was to develop a new form of PGPRs on their survival and application as a bio-control agent against root-knot nematode infecting tomato plants under green house conditions.

2. Materials and Methods

Bacterial strains:

P.fluorescens, A. chroorcoccum, B.polymyxa (JQU15993) and *A. brasilense* (HQ678675) were obtained from Agric. Microbiology Department, Soils, Water and Environment Res. Inst., Agric. Research Center, Giza, Egypt**.** All strains enriched on nutrient broth medium **(Difco Manual, 1985)** for 48 hours at 28°C to reach the maximum growth (10^{7}cfu /ml).

Protease production

This test was carried out using King's B medium **(King *et al*., 1954)** containing 0.01% (w/v^{-1}) sodium caseinate (NBC). An alginate bead of *P. fluorescens* was inoculated on casein agar medium and the plates were incubated at 27°C for 48 hours. The clear zones around the colonies were considered a positive reaction for protease production (**Olajuyigbe and Ajele, 2005).**

Inoculums formulation

1- Sodium Alginate (encapsulation process)

Four bacterial strains were grown in 100 ml of nutrient broth medium for 72 hrs at 30°C. The cells were harvested at log phase (10^{8} cfu^{-1}) by centrifugation at 4°C for 5000 g. Cells pellet of each Bacterial strain was mixed with 2 ml of 1% alkaline humic acid (extracted from peat as described in **Young *et al*., (2004)** and 8 ml of 2% aqueous solution (w/v) reagent grade sodium alginate (KISHIDA Chemical Reagents, Japan) and vortexed for uniform dispersion of the cells in the polymer mixture. This mixture was extruded from a sterile syringe (26G) into 1.5% $CaCl_2$ solution with mild stirring. Instantaneously formed beads (Fig. 1) were maintained under room temperature condition for 6 hrs and washed three times with sterile tap water then stored in 0.85% NaCl till used Each seedling needs 100 mg beads **(Rekha *et al*., 2007).**

2- Talcum powder

A mixture of 10 g carboxymethyl cellulose (CMC) and 1 kg of talcum powder was used to prepare the powder formulation; calcium carbonate was added to the mixture to adjust the pH7. The mixture was autoclaved at 121°C for 20 min. as described by **Vidhyasekaran and Muthamilan (1995).** Both four bacterial homogenized biomass were added to the carrier (1kg talc powder for 100 ml of each used strain) and mixed well under aseptic conditions to form pasta. The pasta was air dried under laminar flow hood for 24 hrs. The dried product was powdered using a blender sieved and packed in sterilized polyethylene bags. One gram of each microbial type was taken to count the colony forming

unit (10^7cfu/ml) by using dilution plate technique on particular media for all bacterial strains.

To study the viability of beads and formula, ten beads and 1g talc powder formula were solubilized for cell counts in 0.2M phosphate buffered saline (PBS) pH7 after 10, 20, 30 and 40 days under gentle shaking for 30 min. Serial dilutions of each bacterial strain was prepared and total count was determined on nutrient agar medium as described by **Rekha *et al*., (2007).** Immobilized cells and formula of each strain was also maintained at 5°C, 10°C, 20°C and 30°C during 40 days, to estimate bacterial survival (shelf life) as described by **Daniel Rojas *et al*., (2015).**

Fig.1: Shape of *P. fluorescens* carried on alginate sodium (encapsulation).

Bacterial suspension

In this method a bacterial suspension of each strain was prepared on nutrient broth medium for 48 hrs at 28°C to reach the maximum growth (10^7cfu /ml) **Difco Manual, 1985).** Each bacterial strain was applied at a rate of 10ml/plant (10^7cfu/ml) around the root zone.

Seeds

Tomato seeds (*Lycopersicon esculentum* L. cv Castlerock II PVP) were obtained from Agric. Research Center (ARC), Giza, Egypt. Seeds of tomato were surface sterilized for 1 min with 70% ethanol, then rinsed five times with sterile distilled water. The seeds were germinated as described by **Asaka and Shoda (1996)**. After four weeks the seedlings were utilized for greenhouse experiment.

Greenhouse experiment:

A pot experiment was conducted at the experimental greenhouse of Fac. of Agric., Menoufia Univ., during season 2015/2016. A pot with 15 cm in diameter was filled with 2 Kg sandy-clay mixture soil (2:1, v/v). The chemical and physical analysis of soil have: 1.12% organic matter, soil pH7.85 and electric conductivity (EC) of 1.7 ds m^{-1} **(Jakson, 1973).** Pots were arranged in a randomized complete block design with three replicates. Pots were daily watered and fertilized weekly with 5 ml of 2g/L of NPK (20:20:20), international for agricultural and industrial development to maintain field capacity.

Nematode inoculation:

Three days after bacteria inoculation, nematode was inoculated by pipetting 3000 eggs of *M. javanica*/plant in three holes around the root zone. The treatments were applied as follows:

T_1. *P. fluorescens* en-capsulated with sodium alginate.
T_2. *P. fluorescens* formulated with talcum powder.
T_3. *P. fluorescens* carried on free suspension (10^7 cells/ml).
T_4. *A. chroorcoccum* en-capsulated with sodium alginate.
T_5. *A. chroorcoccum* formulated with talcum powder.
T_6. *A. chroorcoccum* carried on free suspension (10^7 cells/ml).
T_7. *B. polymyxa* (JQU15993) en-capsulated with sodium alginate.
T_8. *B. polymyxa* (JQU15993) formulated with talcum powder.
T_9. *B. polymyxa* (JQU15993) carried on free suspension (10^7 cells/ml).
T_{10}. *A. brasilense* (HQ678675) en-capsulated with sodium alginate.
T_{11}. *A. brasilense* (HQ678675) formulated with talcum powder.
T_{12}. *A.brasilense* (HQ678675) carried on free suspension (10^7 cells/ml).
T_{13}. Nematode only.
T_{14}. Control.

The experiment was divided into two groups. (1) Adding microbial formula for one time only, (2) adding microbial formula twice after inoculation. Plants were harvested after 60 days of nematode inoculation; samples were taken to obtain growth parameters i.e. plant height (cm), shoot and root weights (g) and length (cm). Number of galls; egg mass/root system; eggs/egg mass (EM) were measured. Egg masses, were stained prior to count by dipping the infected roots in 0.05 % phloxine- B solution for 20 minutes as described by **Daykin and Hussey, (1985).** Percentage of nematode reduction was measured according to the following equal:

$$\text{Reduction (\%)} = \frac{\text{Control-Treatment}}{\text{control}} \times 100$$

Nitrogenase and Dehydrogenase activities were determined according to **Somasegran and Hoben, (1994)** and **Skujins and Burns (1976),** respectively**.** Chemical analysis was conducted to determine total chlorophylls (a+b) and carotenoids content in fresh

leaves using the method described by **Bradford (1976).**

Statistical analysis:

Data were statistically analyzed as described by **Duncan (1955)**.

3. Results and Discussion

In Vitro Experiment

I- Protease test

In our research paper the best active strain used and estimated to produce the protease enzyme by clear zone around the colonies. Cassase is an exoenzyme which produced by some bacteria to degrade casein. *P. fluorescens* showed high ability to produce proteases as showed a largest clear zone after 48 hrs, protease activities were measured based on the diameter of clear zone which ranges from 10 to 60 mm indicating high level of protease production (Fig. 2). This enzyme likely plays an important role in bacteria-nematode-plant-environment interactions and it may serve as an important in balancing nematode populations in the soil. Protease production is an effective mechanism for controlling nematodes. Extracellular enzymes, including subtilisin-like serine protease corresponding to the main chemical constituents of nematode cuticle and eggshell, have been reported to be involved in the infection as virulence factors **Huang *et al.*, (2004).** In the interaction between pathogen and hosts, several experimental evidence supported that serine protease can destroy the integrity of cuticle to help penetration of pathogen **Qiuhong *et al.*, (2006)** and initiate or trap nematophagous fungi **Ahman *et al.*, (2002).** The activity of protease was increased as there was increase in the enzyme concentration **Shanti Naidu (2011).** Some of the antagonistic fungi which synthesize protease enzymes such as *T. harzianum* IMI 206040 has been demonstrated to play an important role in biological control **Benítez., *et al* (1998)** and **Ait-Lahsen *et al.*, (2001).**

II- Viability of microorganisms

The viability of the tested biocontrol agents was determined at different intervals times i.e. 10, 20, 30 and 40 days under room temperature. Results revealed that incubation times had greatest effect on bacterial survival regardless of the protective agent used. All bacterial cell viability was progressively decreased and consequently the lowest bacterial counts observed after 30 days of storage, except *B. polymyxa* as showed an increase in cell viability after 40 days on the model of alginate beads and C.M.C agent as recorded 5.3 and 4.5×10^8 cells/ml, respectively. *P. fluorescens* recorded 6.6 and 6.4 $\times10^8$ cells/ml, respectively after 40 days with alginate beads and C.M.C. On the contrary, alginate beads and C.M.C agent used with *A. brasilense* recorded decreased in cell viability after 40 days it recorded 52 and 50 $\times10^7$ cells/ml respectively with alginate beads and C.M.C. Extended time resulted in a decrease in bacterial viability, this results agreement with **Daniel Rojas *et al.*, (2015)** and **Trivedi and Pandey (2008)** as they showed that encapsulation of *Bacillus subtilis* and *Pseudomonas corrugate*, both of them in alginate beads maintained bacterial count 10^7 cfu/ml after three years of storage. Similarly, **Hanaa and Maie (2016)** found that beads of *Paenibacillus polymxa* revealed maximum growth up to $1\text{x}10^9$ cfu/ml compared to *Azospirillum lipoferum* as recorded 4 $\times10^8$ cells/ml. After 40 days we observed a difference associated to the protective agent used. Results confirmed that, alginate beads proved to be the best polymer to maintain bacterial viability compared to C.M.C. These results were agreement with those obtained by **Bashan and Gonzalez (1999)** as they reported that dry alginate is suitable for preservation of *A. brasilense* and *P. fluorescens* under room temperature.

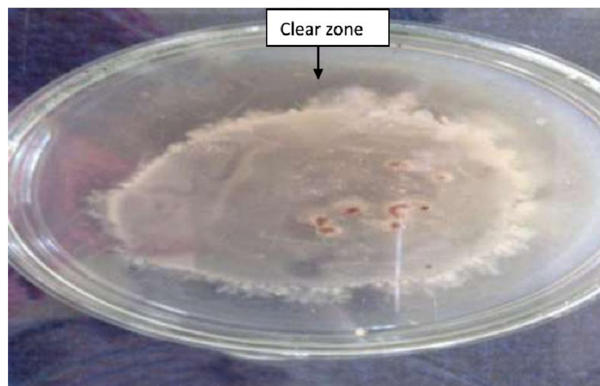


Fig. 2: Production of extracellular proteases in King's B medium containing casine as a substrate of en-capsulated *P. fluorescens.*

In vivo Experiment

Nematode parameters as affected by antagonistic microorganisms in different formulation carriers

Results showed that all bacterial strains on different formula decreased the number of galls and egg masses/ root system compared to plants treated with *M. javanica* alone (Fig. 3&4) as well as the percentage of galls and egg masses reduction was increased (Fig. 5&6). T_1 (*P. fluorescens* en-capsulated with sodium alginate) recorded the highest reduction of galls and egg masses either in once or twice as it recorded 93 and 99 % at one time, whereas 91 and 96% at twice, respectively compared to other treatments as illustrated in Fig (5 and 6), followed by T_2 (*P. fluorescens* formulated with talcum powder), by 89 and 95% at one time and 88 and 95% at twice. T_{12} (*A.brasilense* carried on free suspension) recorded the lowest effective one on nematode parameters. The

reduction % recorded 26 and 49% at one time, whereas 25 and 59% at. These results are agreement with those obtained by **Omar *et al*., (2013)** they found that the lowest number of galls and egg masses/root system observed in the root-knot nematode *M. javanica* infected plants inoculated with *P. fluorescens*. The plant growth promoting rhizobacteria significantly reduced galling and egg masses on tomato roots infected by root–knot nematodes and resulted in increasing in tomato yield **Kokalis-Burella and Dickson (2003). Mahdy *et al*., (2004)** found that mixing the biocontrol agent formula as talc powder thoroughly with soil pots reduced the number of galls and egg masses of *M. javanica*/root system compared to plants treated with nematode only. The reduction in the number of egg masses may refer to the harmful effect of the biocontrol agents on *M. javanica* after penetration. **Siddiqui *et al.*, (2009) Siddiqui** and **Shaukat (2005)** cleared that *P.aeruginosa* suppress root–knot nematode indirectly via enhanced plants defense mechanism (induce systemic resistance), which is independent of salicylic acid accumulation in the host.

Table (1): Viability of microorganisms in sodium alginate and carboxymethyl cellulose (C.M.C) at intervals days under room temperature.

Strains	days	Sodium alginate cfu/ml	C.M.C (cfu/ml)
P. fluorescences	10	42	40
	20	52	51
	30	60	56
	40	66	64
A.chroochoccum	10	42	39
	20	52	50
	30	65	56
	40	66	58
B. Polymyxa	10	44	41
	20	60	54
	30	62	60
	40	71	58
A. barislense	10	41	38
	20	51	50
	30	56	56
	40	52	50

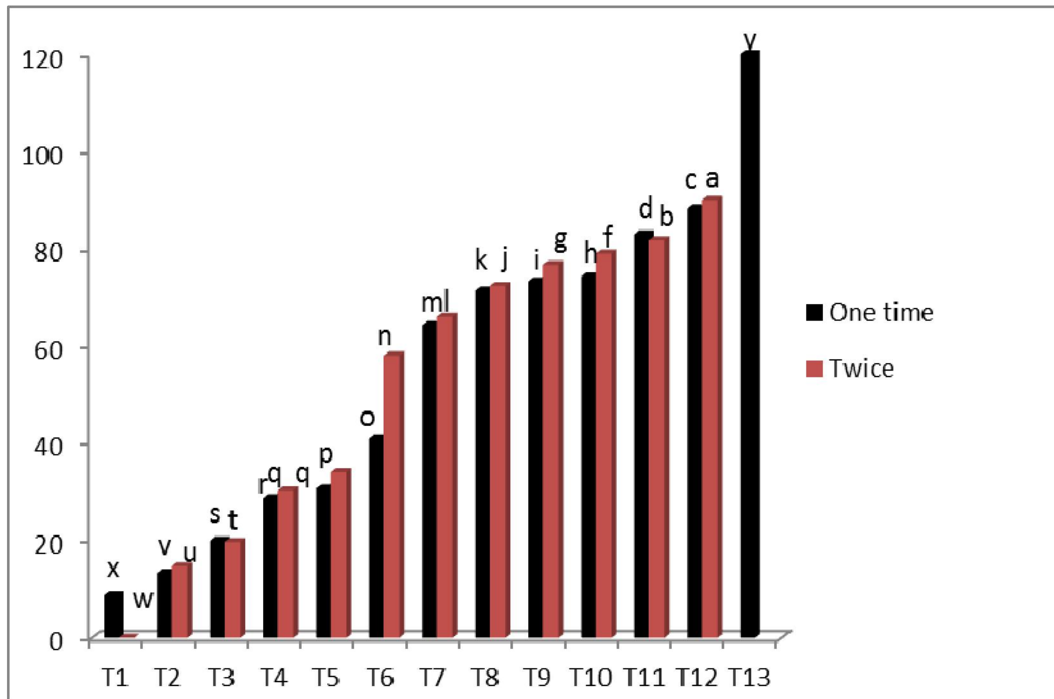


Fig. (3): Impact of microorganisms in different formulation carriers applied one and two times on the number of galls of *M. javanica* infected tomato plants.

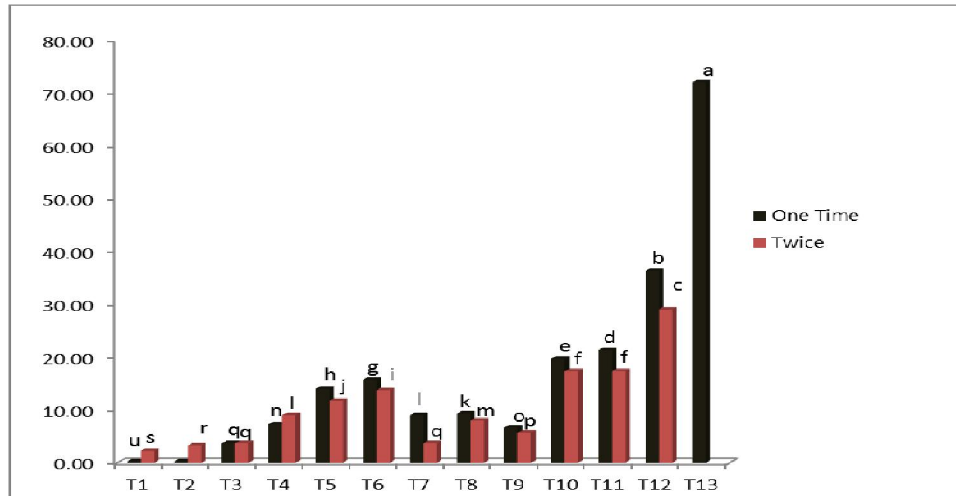


Fig. (4): Impact of microorganisms on different formulation carriers applied one and two times on the number of egg masses of *M.javanica* infected tomato plants.

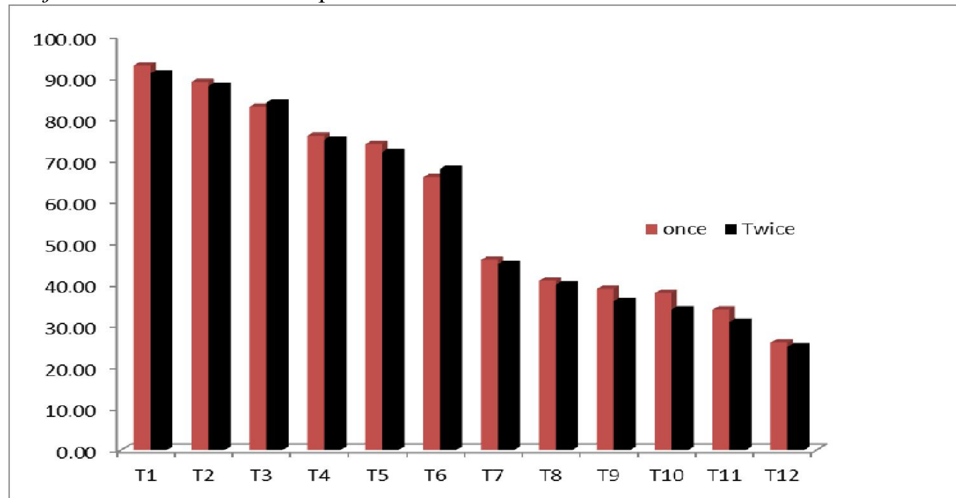


Fig (5): Impact of microorganisms in different formulation carriers on the reduction% of galls of *M.javanica* infected tomato plants

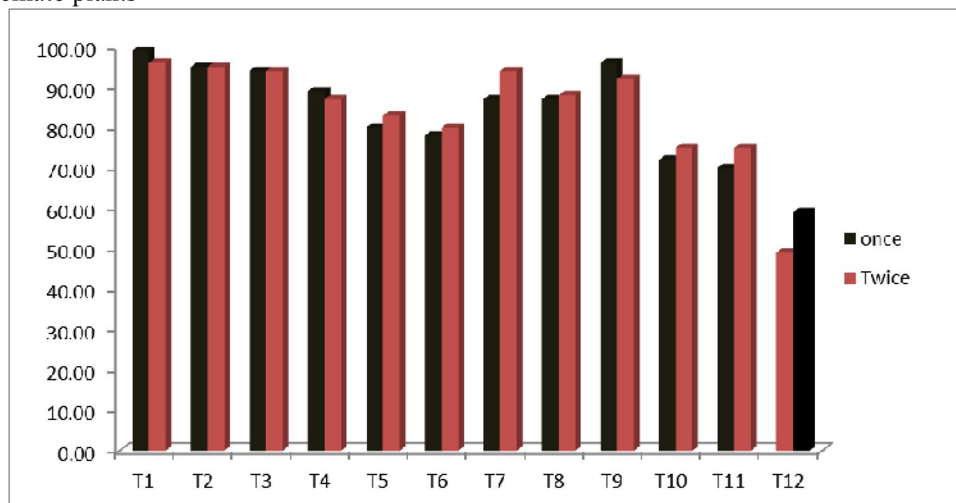


Fig.(6): Impact of microorganisms in different formulation carriers on the reduction% of egg masses of *M.javanica* infected tomato plants.

Growth parameters

In concern, data shown in Table (2) revealed that, all bacterial strains on different formula showed stimulatory effect on vegetative growth in terms of plant height, fresh shoot & root weights and lengths of root. The highest increase for majority of the growth parameters was shown in T_1, T_2 and T_3 recorded 66 cm, 17 g, 1.6g and 15 cm on plant height, fresh shoot & root weights and length of root, respectively in comparison to uninoculated plants and plants infected with nematode alone. T_{13} (nematode alone) recorded the lowest growth parameters 40 cm, 10.7 g, 0.9 g and 9.0 cm, on plant height, fresh shoot & root weights and length of root, respectively, followed by T9 (*B. polymyxa* carried on free suspension) recorded 35.0 cm, 8.0 g, 0.4 g and 8.0 cm on plant height, fresh shoot & root weights and lengths of root, respectively when adding bacterial strains one time, whereas it recorded 40.7 cm, 9.0 g, 0.6 g and 10.0 cm on plant height, fresh shoot & root weights and lengths of root, respectively when adding bacterial twice. The results showed that there is no difference between adding all formula of bacterial strains either one time or twice for tomato plants. These results are agreement with **Omar *et al.*, (2013)** as they found that inoculation with PGPR on tomato plants infected with *M. javanica* increased growth parameters, through a variety of mechanisms such as: production of phytohormones suppression of deleterious organisms, activation of phosphate solubilization and usually believed to be involved in plant growth promotion. PGPR have been reported to improve plant growth either through direct stimulation by synthesis of phytohormones **(Xie *et al.*, 1996)** or by decreasing the effect of pathogens **(Weller *et al.*, 2002)**. The beneficial effects on seedling growth given by the rhizobacteria would mainly be due to two reasons: firstly, plant growth was enhanced due to reduction in root-knot disease intensity; secondly, the nutritional status of the growing seedlings was directly enhanced by providing nitrogen **(Dobbelaere *et al.*, 2003). Siddiqui and Akhtar, (2007**) found that PGPR strains usually increase the root length and biomass and this better developed root system may increase the mineral uptake in plants. Bio-yield, a product that contains spores of *B. subtilis* and amyloliquefacies on a chitosan carrier induced growth promotion in tomato seedlings and reduce severity of diseases cause by several pathogens **Kloepper and Ryu (2006).** Also **EL Sayed and Nada (2014)** found that PGPR inoculated with tomato plants significant increase on root length, shoot fresh weight compared to plants infected with *M.incognita.*

Table (2): Impact of microorganisms in different formulation carriers applied once or twice on the growth parameters of tomato plants infected with *M. javanica*.

Treatments	Plant height (cm)		Shoot fresh weight (g)		Root fresh weight (g)		Root length (cm)	
	Once	Twice	Once	Twice	Once	Twice	Once	Twice
T1	66.0 **a**	65.3 **b**	17.0 **b**	18.0 **a**	1.6 **a**	1.5 **ab**	15.0 **d**	15.0 **c**
T2	65.3 **b**	65.7 **b**	16.7 **c**	15.0 **e**	1.6 **a**	1.1 **e**	14.3 **e**	12.0 **h**
T3	56.3 **b**	56.3 **i**	15.7 **d**	14.3 **f**	1.2 **d**	0.8 **gh**	11.3 **i**	11.3 **i**
T4	60.7 **e**	58.7 **g**	15.0 **e**	15.3 **de**	1.4 **bc**	1.0 **ef**	10.3 **k**	12.0 **h**
T5	59.3 **f**	58.0 **h**	14.0 **f**	16.3 **c**	1.3 **c**	1.4 **bc**	9.7 **l**	15.0 **c**
T6	50.0 **m**	54.7 **k**	10.0 **l**	10.0 **n**	0.9 **g**	0.6 **fg**	10.0 **k**	10.0 **k**
T7	43.7 **n**	43.0 s	12.3 **h**	13.3 **g**	0.8 **gh**	0.7 **ij**	10.0 **k**	10.7 **j**
T8	40.7 **r**	42.7 **o**	10.3 **l**	12.0 **h**	0.6 **jk**	1.0 **ef**	11.3 **i**	10.7 **j**
T9	35.0 **t**	40.7 **p**	8.0 **l**	9.0 **m**	0.4 **l**	0.6 **gh**	8.0 **n**	10.0 **k**
T10	37.3 s	55.0 **j**	10.0 **l**	11.7 **i**	0.7 **hi**	0.9 **g**	10.3 **k**	17.3 **a**
T11	61.0 **d**	42.3 **p**	16.3 **c**	11.0 **j**	1.0 **ef**	0.9 **k**	12.3 **g**	10.0 **k**
T12	41.0 **q**	50.7 **l**	10.7 **k**	11.0 **j**	1.4 **bc**	1.1 **e**	12.0 **h**	13.7 **f**
T13	40.0 **r**	-	10.7 **k**	-	0.9 **g**	-	9.0 **m**	-
T14	62.3 **c**	-	18.0 **a**	-	1.6 **a**	-	16.0 **b**	-

Enzymatic activities:

Dehydrogenase enzyme (DHA) is very important, it gives us large amount of information about biological characteristic of the soil, while nitrogenase enzyme catalyze the reduction of N_2 into NH_3 an evolution of H_2. Data in (Table3) revealed that the impact of three types from polymers of microorganisms on enzymes activity of microflora in soil rhizosphere. Results showed that inoculation with alginate beads, talc powder formula and suspension of

bacterial strains significantly increased the enzymes activities in soil compared to control. Inoculation with alginate beads of *P. fluorescens* recorded higher dehydrogenase effect compared to others. Also, nitrogenase activity increased with inoculation with PGPR in alginate beads compared to other treatment and control (nematode only). Inoculation with *P. fluorescens* at all formula carriers T_1, T_2 and T_3 recoded highest dehydrogenase and nitrogenase activities recorded 93.01, 85.71 and 84.71 (µg TPF/g dry soil /day) for dehydrogenase and 23.51, 23.36 and 24.31 (µ mole C_2H_4/g soil / h) for nitrogenase activity respectively. While adding formula of *P. fluorescens* twice, dehydrogenase and nitrogenase activities recorded 90.21, 85.66 and 84.66 (µg TPF/g dry soil /day) for dehydrogenase and 25.01, 23.26 and 24.11 (µ mole C_2H_4/g soil / h) for nitrogenase activity, respectively. Soil enzyme activities have been suggested as suitable indicators of soil quality because of their intimate relationship with soil biology. Aside from soil enzyme activity, there is interest in the soil nematode community, especially free-living species, as bio-indicators of soil quality (**Yeates and Bongers, 1999).** Results indicated that there is no difference between adding the inoculums once or twice for tomato plants. T_{13} recorded the lowest enzyme activity compared to other treatment. The increase of dehydrogenase activity in alginate beads more than other forms relied on the viability of these microorganisms and the existence in high population that colonized the rhizosphere and these increased the bio-control agent against *M. incognita* and increased the plant growth **Omer and Ismail (2002).**

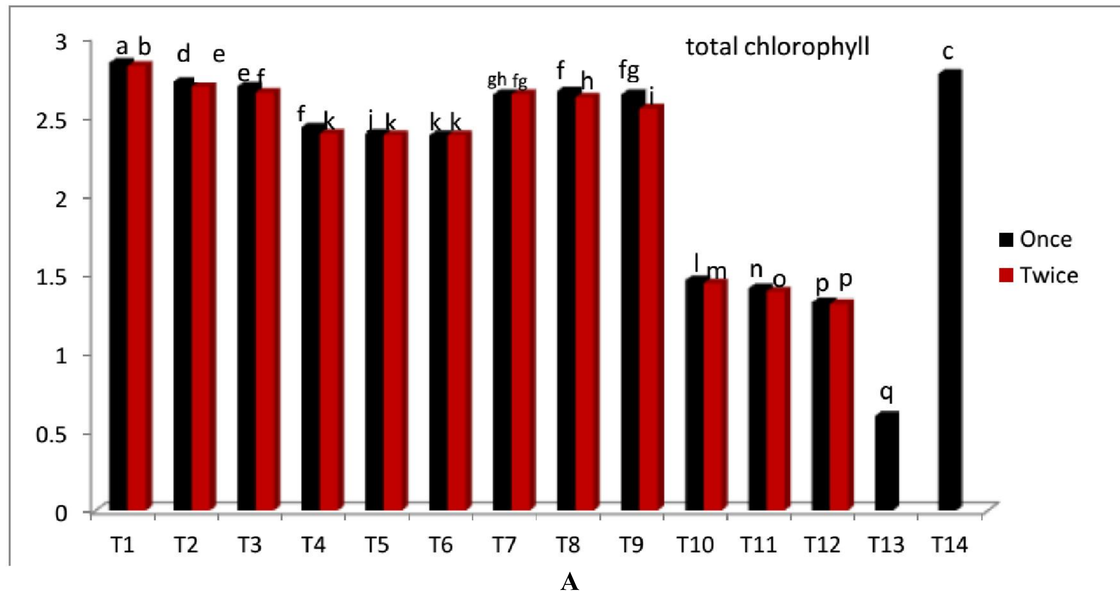
Table (3): Impact of microorganisms in different formulation carriers applied once and twice on enzymes activity of soil rhizosphere.

Treatments	Dehydrogenase(µg TPF/g soil/day)		Nitrogenase (µ mole C_2H_4/g soil/h)	
	Once	Twice	Once	Twice
T1	93.01 **a**	90.21 **b**	23.51 **d**	25.01 **d**
T2	85.71 **d**	85.66 **d**	23.36 **c**	23.26 **c**
T3	84.71 **e**	84.66 **e**	24.31 **b**	24.11 b
T4	61.26 **i**	61.21 **i**	19.41 **g**	19.06 **g**
T5	60.16 **j**	60.11 **j**	16.81 **h**	16.8 **l**
T6	60.00 **j**	60.01 **j**	15.91 **i**	15.06 **i**
T7	77.21 **f**	77.11 **f**	23.69 **bc**	21.07 **d**
T8	66.31 **g**	66.16 **g**	22.11 **b**	20.86 **f**
T9	65.06 **h**	65.00 **h**	21.00 **ef**	14.36 **ki**
T10	58.21 **k**	58.11 **k**	14.56 **jk**	13.96 **ki**
T11	57.26 **l**	57.16 **l**	14.01 **ki**	13.86 **ki**
T12	56.16 **m**	56.11 **m**	14.00 **ki**	27.32 **l**
T13	18.36 **n**	-	11.31 **m**	-
T14	91.11 **c**	-	21.96 **a**	-

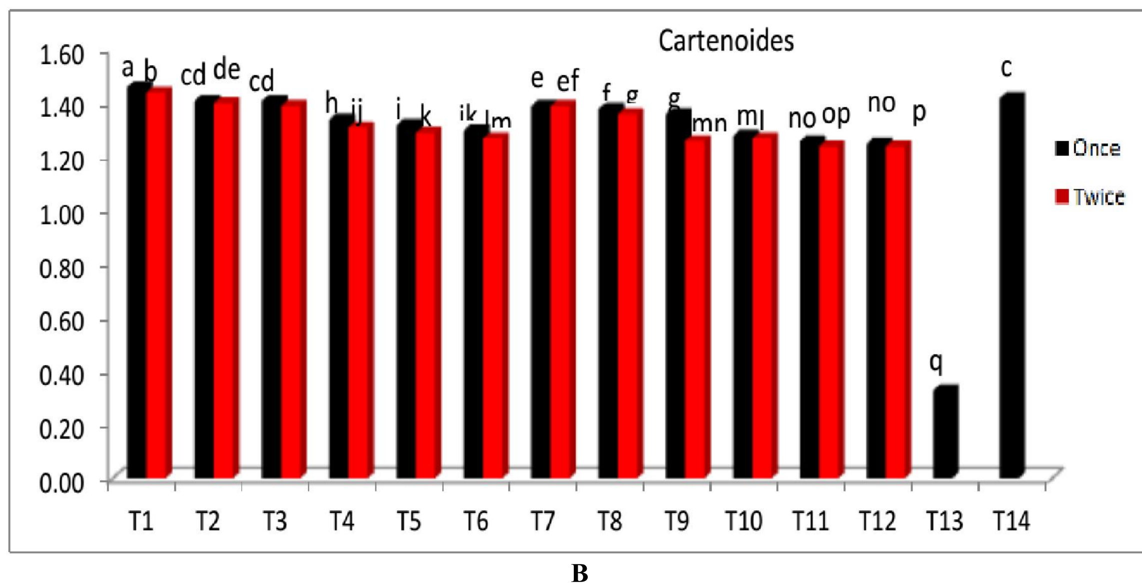
Total chlorophyll and carotenoids:

Data illustrated in (Fig. 7 a + b) showed the effect of three forms of PGPRs inoculation on total chlorophyll and carotenoids of tomato plants infected with *M. javanica*. All PGPRs forms increased the tomato growth and that reflected on the amount of pigments represented in total chlorophyll (a+b) as well as carotenoids. T_1 recorded the highest total chlorophyll (a+b) and carotinoids recoded 2.84 and 1.46 (mg/g.d.w), respectively at one time, while it recorded 2.82 and 1.44 (mg/g d.w), respectively at twice. Nematode alone (T_{13}) recorded the lowest total chlorophyll (a+b) and carotenoids being 0.60 and 0.33 (mg/g d.w), respectively at one time. *P. fluorescens, A. chroorcoccum* and *A. brasilense* may also improve plant growth, by the production of biologically active substances of growth hormones and their reflect of pigment amounts of plant **(Morsy *et al.*, 2009). Sharma *et al.*, (2003)** assessed the role of the siderophores-producing by *Pseudomonas* strain GRP3 on iron nutrition of *Vigna radiate*, and they found a decline in chlorite and iron symptoms. Chlorophyll a and b contents increased when plants inoculated with strain GRP3 compared to control and plants infected by nematode alone. **Saharan and Nehra (2011)** found that inoculating tomato with *Pseudomonas aeraginosa* infected with *Meloidogyne incognita*, enhanced chlorophyll and carotenoids content in tomato plants. The increasing of chlorophyll in all treatments probably resulted in higher photosynthetic rates and thus improved plants biomass **(Farinaz *et al.*, 2013). Amir *et al.*, (2001)** reported that the highest total

chlorophyll observed in plants inoculated with *A. brasilense*, the increasing lead to higher rates of photosynthesis is dependent on one factor, by improved nutrition of the host plants.



A



B

Fig (7a+b): Impact of microorganisms in different formulation carriers applied once and twice on total chlorophyll and carotinoids (mg/g.d.w) of tomato plants infected with *M. javanica*.

Total protein:

Data illustrated in (Fig. 8) showed the highest total protein content with treatment of T_1, T_2 and T_{14} being 70.21, 63.11 and 67.16 (µg/ml), respectively, whereas the lowest contents were recorded with T_{13}, T_6 and T_4 being 23.86, 34.01 and 39.26 (µg/ml) respectively. These results agreement with **Akbari *et al.*, (2011)** as they reported that the protein content was improved by the inoculation of PGPR as compared to the control. **El Sayed and Nada (2014)** found significant increase in total protein content of PGBR inoculated plants at 60 days plant-old compared to uninoculated plants and plants infected with nematodes alone. Also, **Stefan *et al.*, (2010)** found that inoculation plants with *Bacillus pumilus* Rs3 increased the total amount of seed soluble protein by 66%, probably due to stimulation of protein biosynthesis processes in soybean plants, providing in this way soybean seeds with higher nutritional value.

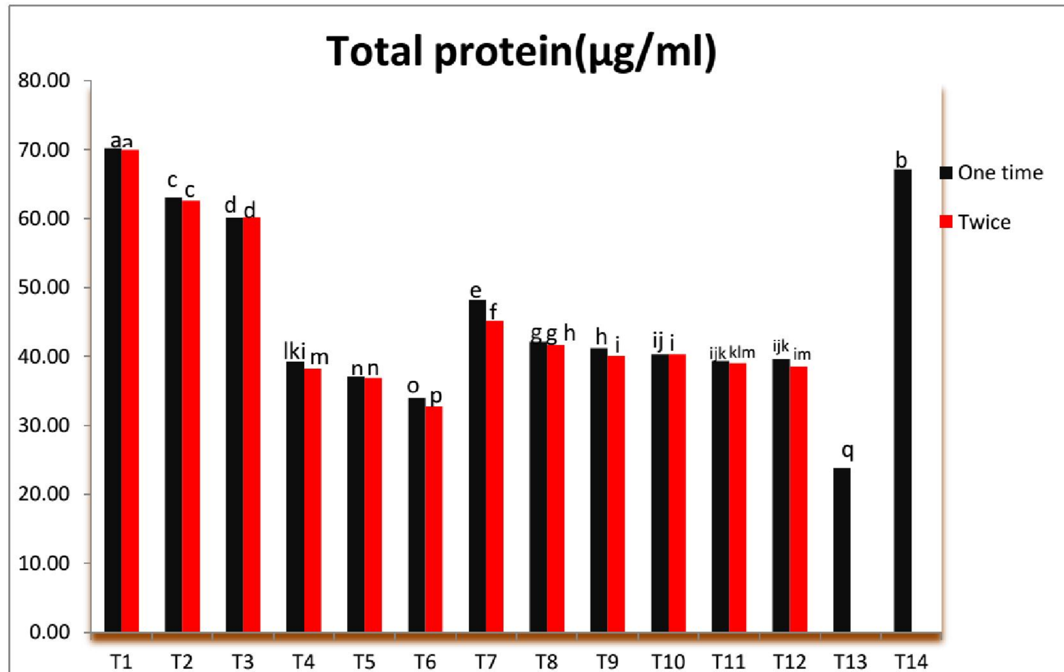


Fig. (8): Impact of microorganisms in different formulation carriers applied once and twice on total protein content (µg/ ml) of tomato plants infected with *M. javanica*.

Conclusion

P. fluorescens has the ability to produce protease enzymes, this enzymes play an important role for controlling root-knot nematode. Root-knot nematode, *M. javanica* can be managed by inoculation infected tomato plants with PGPR at different formulation carriers as reflected by improving the plant vigor, growth parameters and chemical components. Also, results suggested that alginate beads formula was the best polymer one to maintain the viability of bacterial cells for a long time at room temperature.

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