

Study on Black Gram (*Vigna mungo* L.) Infected with *Meloidogyne incognita* under the Influence of *Pseudomonas fluorescens*, *Bacillus subtilis* and Urea

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Abstract

Pot studies were carried out to assess the growth and biochemical components of *Vigna mungo* L. cultivar 'Azad-2' with the root-knot nematode and treated with two biofertilizers viz. *Pseudomonas fluorescens* strain CHA0, and *Bacillus subtilis* strain Bs-5. The plants were grown in soil at N50 and N100 doses of urea. Uninoculated untreated plants served as control. From the results, it was evident that *V. mungo* plants exhibited profused vegetative growth at N100 dose of urea as was observed in the treatment 5. The plants of treatment 5 were inoculated with *P. fluorescens* CHA0 (20 ml). The number of nodules per plant was found increased to 14.33 per plant, total chlorophyll content by 2.91 mg/g of leaf tissue, leghaemoglobin by 3.81 mg/g, and protein content in leaves and seeds by 0.83 and 0.76 mg/g, respectively, when compared with the control and the plants that received different doses of *B. subtilis* Bs-5 in the absence of the root-knot nematode. Significant reduction in gall number (41.66) and number of nematodes per gram of root (4.66) was recorded in the treatment 5.

Keywords: *Bacillus subtilis*; Inorganic fertilizer; *Meloidogyne incognita*; *Pseudomonas fluorescens*; *Vigna mungo*

Introduction

Root-knot nematodes, the obligate sedentary endoparasites, reproduce parthenogenetically by mitotic divisions [1] and complete greater part of their life cycle inside the host plant. These are more than 2,000 plants [2-5] which comprise of all major crop plants are attached by *Meloidogyne* spp. The plant parasitic nematodes are responsible in causing agriculture losses amounting to \$157 billions annually [6]. Pulses contain 17 to 30% protein and upto 60% carbohydrates. They also contain reasonable amounts of niacin and thiamine but only small amounts of carotene, riboflavin and ascorbic acid [7]. The leguminous plants have the ability to fix the atmospheric nitrogen that helps in increasing the soil fertility. *Vigna mungo*, a nitrogen fixing leguminous plant also seems as the host of the root-knot nematodes, *Meloidogyne* spp. In addition to the formation of root galls, the nematodes interfere in the process of symbiotic nitrogen fixation.

Among the various pests, *M. incognita* poses a potential threat to the cultivation of pulse crops by inflicting upon severe yield losses [8-10]. Physiological and biochemical activities of the host plant are drastically affected on nematode infection [11]. *Pseudomonas fluorescens* strain CHA0 and *Bacillus subtilis* strain Bs-5, the biocontrolling agents, have been reported to have direct antagonistic effects as a result of physical contact and pathogenic selectivity [12]. *Pseudomonas* induce host defense mechanism by synthesizing an antibiotic 2, 4-diacetylphloroglucinol (DAPG) [13].

For the growth of plants, unavailability of nitrogen acts as critical limiting factor [14]. In beans, reserved food material is stored in the form of proteins, therefore, these require more nitrogen than any other nutrient [15]. Nitrogen is a constituent of proteins, enzymes, chlorophyll and growth regulators. Deficiency of nitrogen results in stunting, chlorosis, reduced branching and surface area of leaves [15].

The experiment was carried out to investigate the effects of PGPR, antagonistic to *M. incognita*, at different doses of nitrogen fertilizer on black gram cv. Azad-2.

Materials and Methods

The experiment was performed in glass house. *Vigna mungo* cv. Azad-2 was selected as test plant; the root-knot nematode, *Meloidogyne incognita* was selected as the test pathogen; *Pseudomonas fluorescens* and *Bacillus subtilis* as the test biocontrol agents, which were added into the soil together with the nitrogen fertilizer, urea.

Plant culture

The seeds of black gram (*Vigna mungo* cv. Azad-2) procured from IARI, New Delhi were surface sterilized with NaOCl [16] for 10 minutes and then rinsed three times with sterile water. The seeds were sown in steam sterilized soil mixture of 3:1:1, soil: sand: organic manure, in 30 cm clay pots. One week old seedlings were transplanted as one seedling per pot and after three days the seedlings were treated with different doses (C=Control, 1=*P. fluorescens* (10 ml)+N50, 2=*P. fluorescens* (10 ml)+N100, 3=*P. fluorescens* (20 ml)+N50, 4=*P. fluorescens* (20 ml)+N100, 5=*B. subtilis* (10 ml)+N50, 6=*B. subtilis* (10 ml)+N100, 7=*B. subtilis* (20 ml)+N50 and 8=*B. subtilis* (20 ml)+N100).

Uninoculated plants served as control. The pots were arranged in a randomized block design and all the treatments were replicated thrice. The plants were lightly watered after inoculation and thereafter, whenever, required. The experiment was terminated 60 days after inoculation and different parameters were determined.

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Culturing of nematodes

In order to obtain sufficient number of second-stage juveniles of *Meloidogyne incognita* for the experiment, the population of the test nematode was developed from a single egg mass on brinjal plants grown in microplots, well ahead of the beginning of the experiment. The required numbers of freshly hatched second-stage juveniles were collected from this pure culture as per the requirement.

Nematode inoculum: In order to procure second stage juveniles of *Meloidogyne incognita*, egg masses were handpicked, with the help of sterilized forceps from heavily infested egg plant roots on which pure culture of the nematode was maintained. The brinjal roots with egg masses were rinsed with distilled water and placed in 10 cm diameter 15 mesh coarse sieves which covered with crossed layers of tissue paper. The sieves were placed on petridishes containing sufficient water so that their lower part remained partially submerged in water. To avoid evaporation of water the petridishes were kept covered with their lids and kept in an incubator running at 25°C. After 24 h onwards second-stage juveniles were collected in the form of suspension and stored for later use, and fresh water was added. The number of juveniles was counted using counting dish. The amount of water in the suspension was so adjusted that 10 ml of this suspension contained 1000 freshly hatched juveniles. The plants were inoculated by adding 20 ml of suspension per pot [17].

Bacterial inoculum (*Pseudomonas fluorescens* and *Bacillus subtilis*)

The pure cultures of *Pseudomonas fluorescens* strain CHA0 and *Bacillus subtilis* strain Bs-5 obtained from IARI, New Delhi, were maintained on King's B agar [18] and nutrient agar [19] media, respectively. Both the cultures (*P. fluorescens* and *B. subtilis*) were prepared on the described media by standard procedures. The suspension of bacterial culture comprised of 109 CFU ml⁻¹ for *B. subtilis* and 10⁶ CFU ml⁻¹ for *P. fluorescens* sp. Two to three day old broth of both the bacterial cultures were used to give root dip treatment to the seedlings in the planting hole.

Urea [Co(NH₂)₂] was used as fertilizer; where one gram urea was equivalent to 460 mg of nitrogen [20]. N50 and N100 values were evaluated as 107.29 and 215.51 mg urea/kg soil.

Leghaemoglobin content

The leghaemoglobin content, in fresh nodules was estimated following the method described by Sadasivam and Mannickam [21]. The reading was taken at 539 and 556 nm on spectrophotometer, against a reagent blank.

$$\text{Lb concentration (mM)} = \frac{A_{556} - A_{539}}{23.4} \times 2D$$

D= Initial dilution

Protein content

The seed and leaf protein content was estimated by adopting the methodology of Lowry et al. [22]. 50 mg of oven dried black gram seeds and leaves were ground with the addition of 1 ml of 5% trichloroacetic acid. The absorbance was read at 660 nm using spectrophotometer. The total protein content was calculated by comparing the absorbance of each sample with a calibration curve plotted by taking known graded concentration of bovin serum albumin.

Total chlorophyll content

Total chlorophyll content in fresh leaves was estimated by using the methodology of MacKinney [23]. 1 gm finely cut fresh leaves were ground to a fine paste using mortar and pestle after pouring 20 ml of 80% acetone. The mixture was centrifuged at 5,000 rpm for 5 minutes. The supernatant was collected and the absorbance was read at 645 and 663 nm against the blank on spectrophotometer. The total chlorophyll content present in the extract was calculated using the following equation:

$$22.9 (A_{645}) + 8.02 (A_{663}) \times \frac{V}{1000 \times W}$$

A=Absorbance at specific wavelength

V=Final volume of chlorophyll extract in 80% acetone

W=Fresh weight of tissue, used for extraction

Nitrogenase activity

The nitrogenase activity was assayed adopting the procedure of Hardy et al. [24]. The results were expressed in terms of μ moles ethylene formed/plant/hour.

Statistical analysis

The results were analysed statistically by the analysis of variance followed by least significant difference (LSD) at p=0.05 [25]. Duncan's multiple test were applied for testing significant differences [26].

1=Control

2=*Pseudomonas fluorescens* (10 ml)+N50

3=*P. fluorescens* (10 ml)+N100

4=*P. fluorescens* (20 ml)+N50

5=*P. fluorescens* (20 ml)+N100

6=*Bacillus subtilis* (10 ml)+N50

7=*B. subtilis* (10 ml)+N100

8=*B. subtilis* (20 ml)+N50

9=*B. subtilis* (20 ml)+N100

Result and Discussion

The above experiment revealed the beneficial effects of different doses of plant growth promoting rhizobacteria on the growth of black gram and antagonistic to nematode. Nitrogenous fertilizer and bio-fertilizers had influenced significantly over the growth attributes, nodulation, leghaemoglobin content, total chlorophyll content, nitrogenase activity and protein content of leaves and seeds.

It was a three factor experiment analysis comprising of bio-fertilizers (*Pseudomonas fluorescens*, *Bacillus subtilis*), inorganic fertilizer (Urea) and nematode (*M. incognita*). The effect of both the bio-fertilizers and the nitrogenous fertilizer showed significant increase in different growth and biochemical parameters with and without nematode infestation at P = 0.05 level. The above experiment showed that the given optimal doses of bio and chemical fertilizer were most effective in the plant growth and suppression of root galls caused by *M. incognita* infection.

Shoot and root length

The data presented in table 1 revealed that the combined inoculation of *P. fluorescens* and N100 significantly increased shoot and root length

over the control as well as other treatments. The shoot length of the treated plants with bio and chemical fertilizers increased significantly except in the treatments 2, 6 and 7. In nematode inoculated treatments, the values showed significant differences except in 3 and 8. Highest reduction was found in treatment 6 on comparing with control by 15.98% in the presence of 1000 J₂. Maximum increase in shoot length was recorded in the treatment 5 (36.76 cm). Same finding was found by Shankar et al. [27] who reported that there was a significant increase in plant height, shoot and root weight and reduction in gall index due to the inoculation of *P. aeruginosa* in tomato plants. There was a significant increase in the root length but the treatment 6 showed non-significant result on inoculating the nematode by 10.51% over control due to lower dose of bioagents, *B. subtilis*.

Liu et al. [28] found suppression of the root-knot nematode, *M. incognita* on tomato by dual inoculation with arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria. The plant height due to nematode infection were significantly increased in the presence of *P. fluorescens* [29]. Nitrogen fertilizer was helpful in increasing the biomass attributes of plant and reduced nematode population in root [30]. Tariq et al. [31] conducted in vitro test, and recorded that cell free culture filtrate of some strains showed nematicidal activity against *M. javanica* root-knot nematode by killing the second stage juveniles and by retarding the egg hatch some *Pseudomonas aeruginosa* strains also showed positive impact on plant growth by increasing the plant height and fresh shoot weight and were found to produce indole-acetic acid at varying degree in chilli.

Number of nodules and nodular dry weight

Maximum number of nodules was observed in the treatment 5 (14.33) due to the higher doses of bio and chemical fertilizers. Increase in nodule number and nodule dry weight was higher in the presence of *P. fluorescens* than *B. subtilis*. All the treatments showed significant increase except in the treatment 6 (without nematode inoculating plant). Roopa et al. [32] evaluated the effectiveness of PGPR and *Rhizobium* on nodulation and growth productivity in chickpea. Among nematode inoculated plants non-significant difference was noticed in the treatment 2 when compared with control (Table 1). In all the treatments significant increase in nodule weight was noticed. Maximum and minimum increased values were recorded in the treatments 5 and 6, respectively. *P. fluorescens* and *B. subtilis*, the biocontrolling agents controlled the nematode by killing second stage juveniles activity [33]. Jaizme-Vega et al. [34] concluded the beneficial effect of dual inoculation of AMF and *Bacillus* sp. on papaya and improved results in terms of nematode infection. Infection by root-knot nematodes decreased the number of functional nodules either through inhibition in overall

nodulation or premature conversion of functional nodules into non-functional ones due to nematode invasion [35]. Some rhizobacteria also released volatiles for promoting plant growth as reported by Phillips et al. [36]. Stirling and Sharma [37] reported that biocontrol agents not only reduced infectivity of nematodes, but also increased the number of rhizobial nodules on the root system. Inoculation of nematodes which ultimately led to the reduced production and supply of carbohydrates to the nodules to fix atmospheric nitrogen [38,39].

Leghaemoglobin (Lb) content and nitrogenase activity

From the above experiment it was revealed that variations occurred in the leghaemoglobin content and the nitrogenase activity. All the treatments showed significant increase on comparing with the control except in treatment 2 and 3, where the increment was non-significant. Treatment 5 (3.81 mg/gm) showed significant highest value of Lb content on inoculating maximum dose of *Pseudomonas fluorescens* and nitrogen and lowest value recorded in treatment 6 (3.14 mg/g) which increased non significantly (Figure 1).

Bacillus subtilis treatment with nitrogenous fertilizer was significantly less effective than *P. fluorescens* treatments with urea. On nematode infestation, treatment 4, 8 and 9 showed non-significant increment result. Nitrogenase activity was highly affected and significantly increased by the inoculation of bio and chemical fertilizer. The highest reduction was recorded in treatment 6 on nematode infestation (Figure 5). Chahal et al. [40] showed the maximum number of nodules and nitrogenase activity on applying *Rhizobium* with or without nematode infestation at various levels of iron. Bhat et al. [41] concluded that *M. incognita* caused adverse effect on the growth of black gram, nodule development, nitrogen content of shoot and root, leghaemoglobin, bacteroids and nitrogenase activity. Plant growth promoting rhizobacteria showed its beneficial effect on leghaemoglobin content.

Chlorophyll content

Chlorophyll content of black gram leaves was significantly higher on applying various doses of biofertilizers and inorganic fertilizer with and without nematode infestation. The highest and lowest increased values were recorded in the treatment 5 and 6, respectively. On inoculating nematode, the chlorophyll content values decreased significantly, whereas increased significantly on applying the maximum doses of bio and chemical fertilizer. The chlorophyll content in the treatment 8 increased non-significantly on comparing with control in nematode inoculated plant (Figure 2). Karnwal [42] with the help of experiment showed beneficial and best plant growth promoting activity

Parameters/Treatments	Shoot Length (cm)		Root Length (cm)		No. of Nodules per plant		Nodule Dry Weight (g) per plant	
		Nematode infested (1,000J ₂)		Nematode infested (1,000J ₂)		Nematode infested (1,000J ₂)		Nematode infested (1,000J ₂)
1 (Without nematode)	27.53 ± 0.38	27.53 ± 0.38	6.66 ± 0.14	6.66 ± 0.14	4.33 ± 0.33	4.33 ± 0.33	0.02 ± 0.003	0.02 ± 0.003
2	*28.40 ± 0.60	24.36 ± 0.24	8.73 ± 0.27	8.20 ± 0.05	6.66 ± 0.33	*4.66 ± 0.33	0.043 ± 0.003	0.03 ± 0.003
3	29.46 ± 0.31	*26.93 ± 0.27	10.46 ± 0.32	9.16 ± 0.08	9.33 ± 0.33	6.66 ± 0.33	0.070 ± 0.017	0.05 ± 0.01
4	33.10 ± 0.60	31.33 ± 0.27	12.13 ± 0.14	10.86 ± 0.33	9.66 ± 0.33	7.66 ± 0.33	0.110 ± 0.005	0.10 ± 0.005
5	36.76 ± 0.33	34.36 ± 0.28	14.40 ± 0.25	13.13 ± 0.14	13.66 ± 0.33	10.33 ± 0.33	0.140 ± 0.003	0.12 ± 0.01
6	*27.86 ± 0.33	23.13 ± 0.26	8.50 ± 0.15	*7.36 ± 0.12	*5.00 ± 0.57	4.33 ± 0.33	0.030 ± 0.01	0.02 ± 0.01
7	*28.36 ± 0.58	23.83 ± 0.18	9.46 ± 0.27	8.26 ± 0.16	7.66 ± 0.33	6.33 ± 0.33	0.046 ± 0.005	0.013 ± 0.003
8	31.60 ± 0.26	*28.73 ± 0.20	10.20 ± 0.08	9.60 ± 0.05	9.33 ± 0.33	7.33 ± 0.33	0.093 ± 0.006	0.07 ± 0.005
9	32.61 ± 0.16	32.30 ± 0.17	12.20 ± 0.37	12.20 ± 0.05	12.33 ± 0.33	9.33 ± 0.33	0.133 ± 0.003	0.130 ± 0.01
S.E.	± 0.74	± 0.73	± 0.43	± 0.40	± 0.58	± 0.40	± 0.008	± 0.009
LSD (0.05)	1.56	1.54	0.91	0.84	1.22	0.84	0.01	0.01

*Non-significant

Table 1: Effect of *P. fluorescens*, *B. subtilis* and urea on the growth attributes of *Vigna mungo* L. infested by *M. incognita*.

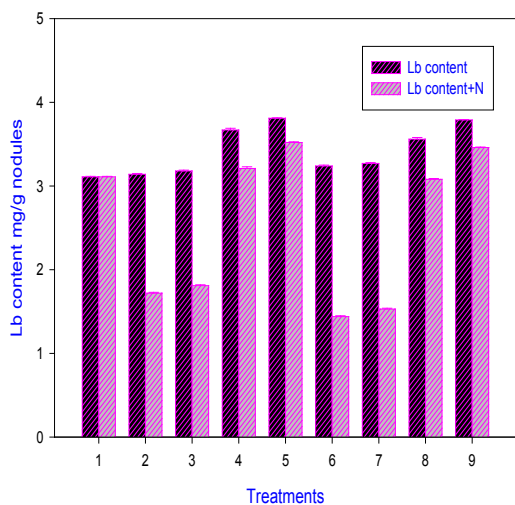


Figure 1: Variations occurred in the leghaemoglobin content and the nitrogenase activity.

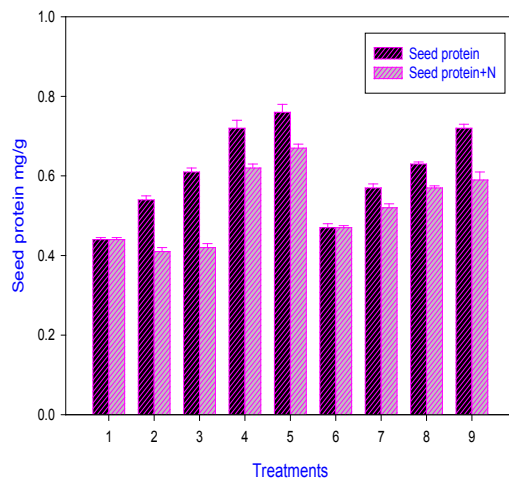


Figure 4: Data pertaining to seed protein contents.

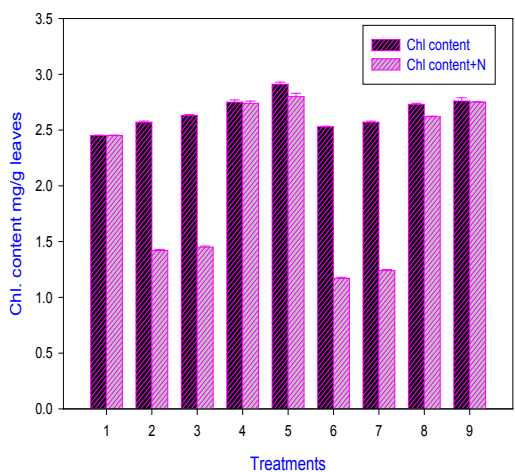


Figure 2: Chlorophyll content of black gram leaves.

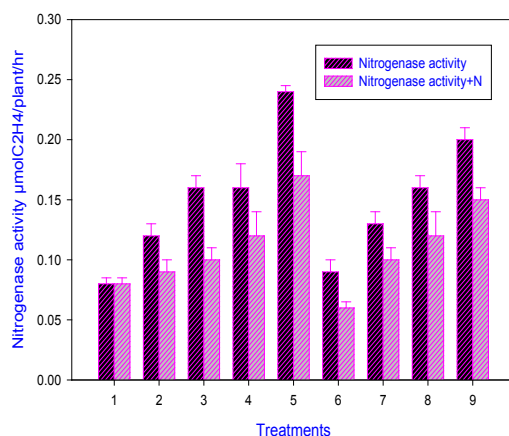


Figure 5: Nematode infestation on nitrogenous activity.

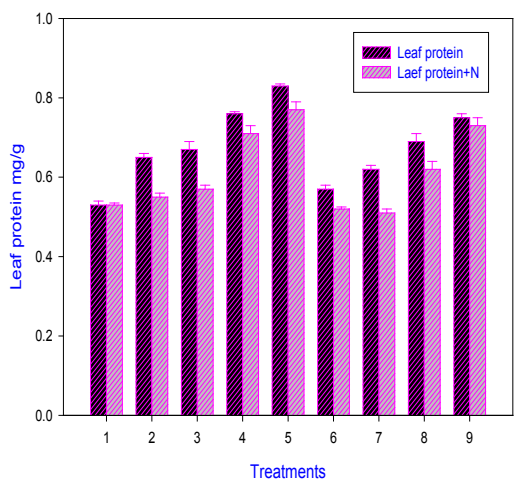


Figure 3: Data pertaining to leaves protein contents.

of *P. fluorescens* and *P. aeruginosa* due to the production of IAA. Plant growth promoting rhizobacteria significantly influence the chlorophyll content [43].

Protein content in leaves and seeds

Data pertaining to leaves and seed protein contents depicted that the treatments showed significant increase in protein content of leaf. The highest and lowest values were recorded in the treatment 5 and 6, but on nematode inoculation the treatment 6 recorded as non significant, and the treatment 7 showed significant decrease in leaf protein content. *B. subtilis* showed less significant results as compared to *P. fluorescens* with or without nematode inoculation (Figure 3). *P. fluorescens* and *B. subtilis* also helpful in solubilizing the insoluble phosphorus into solubilized form and make it available to the growth host plant [44,45].

In seeds, the protein content, in all the treatments, significantly increased except in the treatment 6 where the increment was non-significant. The higher and lower values were recorded in treatment 5 (0.67 mg/g) and 2 (0.41 mg/g) (Figure 4). PGPRs reduced pathogenic activity through induced systemic resistance. Some rhizobacteria (*Bacillus* spp.) have been found to produce lipopeptides, surfactants, bacillomycin D, and fengycins which are secondary metabolites mainly

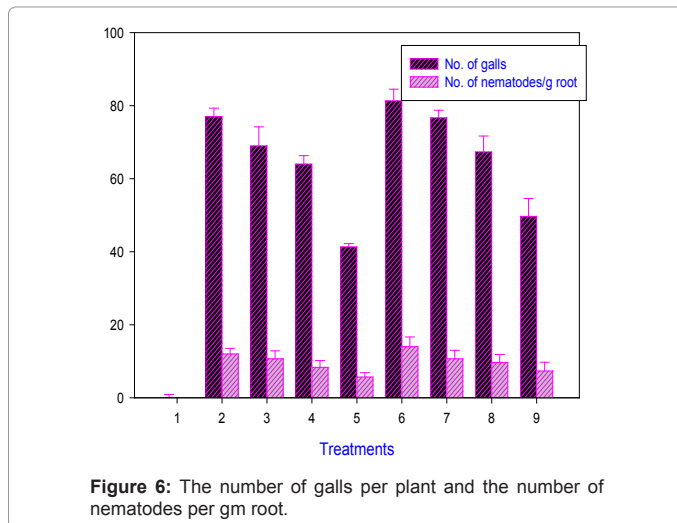


Figure 6: The number of galls per plant and the number of nematodes per gm root.

with inhabitant pathogen activity [46]. In addition to some species of *Pseudomonas*, *Bacillus* reported to induce systemic resistance in plants against invading pathogens and antagonists to root-knot nematodes of meloidogyne spp. [47-51].

Root galling and number of nematodes per gm root

The number of galls per plant and the number of nematodes per gm root were decreased on increasing the doses of biocontrol agents. Maximum number of galls per plant, and nematodes per gm root was recorded in treatment 6, and maximum reduction was recorded in the treatment 5 (Figure 6). Aino et al. [52] recorded protection of tomato from wilting disease by *P. fluorescens* colonization. *Pseudomonas fluorescens* produced an antibiotic 2, 4 diacetylphloroglucinol (DAPG) which decreased *M. incognita* egg hatching rate and increased crop yield reported by Meyer et al. (2009). Olowe [53] recorded that nitrogen resulted in better performance in plant yield and other biomass parameters and also reduced the *M. incognita* infection and galling. Combined treatment of NPK was significantly improved all the parameters. Sharoar et al. [54] found the significant effect of nitrogen fertilizer. Egunjobi and Olaitan [55] also revealed the reduction in galling by inorganic fertilizer application. Some authors also reported the great reduction in galling in other crops by NPK application [56-60]. Rodriguez-Kabana (1986) [61] showed that on applying nitrogen fertilizer, the soil became more effective to suppress nematode populations due to excessive metabolizaion of N causing toxicity. On applying nitrogen to the soil, the phenol level increased as nematode declined [62].

B. subtilis and *B. thuringiensis* were considered the most used bacteria against plant parasitic nematodes [63], also recorded the reduced hatching of *M. incognita* in vitro by *B. subtilis*, *B. thuringiensis* and *B. cereus* on cowpea and mashbean. Various workers have reported about the suppressive effect of *P. fluorescens* and *B. subtilis* on *M. incognita* [64,65]. It was observed that in the presence of *P. fluorescens* and *B. subtilis* there was significant reduction in the severity of root-knot disease [29]. The mechanism of biocontrol activated due to production of antibiotic, siderophores and hydrogen cyanide [66].

From the results of above experiment it can be deduced that biofertilizers (*P. fluorescens* and *B. subtilis*) with nitrogenous fertilizer (Urea) could be utilized for improving crop yield under field conditions.

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