*Plant Archives* Vol. 20, Supplement 2, 2020 pp. 2026-2030 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

# EVALUATION OF BIOLOGICAL SEED TREATMENTS FOR MANAGEMENT OF *ROTYLENCHULUS RENIFORMIS*

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## Abstract

Greenhouse experiments were conducted to reveal the effectiveness of potential biological control products to manage and evaluate the development of *Rotylenchulus reniformis* on cotton. Tests included seeds treated with the Abamectin, ILeVo, and a non-treated control. Results showed that seed treatments with Abamectin and *Burkholderia* sp. suppressed the numbers of *R. reniformis* eggs significantly. Seeds treated with Abamectin and bacteria had fewer vermiform adults in the soil in comparison with the non-treated seeds. The bacteria and *Burkholderia* sp. seeds treatments drastically suppressed the number of eggs isolated from cotton roots compared with the non-treated control. Abamectin also inhibited the number of vermiform life-stages found in the soil as compared to the non-treated control. Biological seed treatments produced no negative effects on plant growth. The use of different biological control as seed treatments can manage plant-parasitic nematodes and limit damage.

***Keywords*:** Biological control, ILeVo, Abamectin, plant-parasitic nematode, root morphology.

## Introduction

Cotton (*Gossypium hirsutum* L.) is primarily grown as textile fiber crop in the tropical and subtropical regions of the world (Khanal *et al*., 2018). Among several plant-parasitic nematodes that attack cotton, reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira, 1940) has become predominant and economically important (Robinson, 2007). It has been reported in at least 38 countries, suggesting wide distribution. Damage caused by this nematode includes suppression of yields and alteration of the development of the cotton plant (Agrios, 2005; Nicol *et al*., 2011).

Management options for reniform nematode include the use of crop rotation, host plant resistance, chemicals, and biologicals (Khanal *et al*., 2018). Because host plant resistance is not a currently available for reniform nematode management in cotton, the best alternatives are the use of crop rotation, and chemicals and biological nematicides (Khanal *et al*., 2018). Among the currently available management methods, seed treatment is preferred by growers over crop rotation and chemicals because it is easy to adopt and reduces the exposure of humans and off-target organisms to harmful chemicals. Biological control is a constituent of an integrated pest management program that has been used to manage soil-borne pathogens by introducing microorganisms for more than 65 years (Sikora, 1992; Baker, 1987), but has not been commercially practical. Seed treatment nematicides have been on the market since 2005 which has stimulated production practices. Management practices have changed from the standard granular in-furrow applications to seed treatments (Glare *et al*., 2012), including Avicta complete cotton, (abamectin), Aeris, (thiodicarb), and Votivo, a biological strain GB 216 of the *Bacillus firmus*. Seed treatments have simplified the farming and have reduced the producer's exposure to chemicals (Aljaafri *et al*., 2016). The aim of this present research was to examine the response of *R. reniformis* to different biological control agents and to evaluate abamectin as a seed treatment for the management of *R. reniformis* in greenhouse.

## Materials and Methods

**Extraction of nematodes from plant material:** Nematode eggs and vermiform stages were extracted from infested roots with the bleach extraction method (Hussey & Barker, 1973) as follows: roots of reniform nematode infested cotton were placed into a beaker with 10% bleach solution (5.25% NaClO). After 2 minutes, the root are cut into 2-5cm lengths, placed in a flask, and vigorously shaken for 2 minutes exactly. The root fragments are thoroughly washed in a 200 mesh sieve over a 500 to remove the bleach. Eggs and vermiform nematodes were counted on a grated counting chamber with an Olympus BH2 B071 microscope (Japan Model C35AD-4) at 40x magnification.

**Eggs and juvenile extraction:** Sieving, centrifugation and sugar floatation (Jenkins, 1964) was used to extract eggs and vermiform stages. Soil and water contents of the bucket were suspended in water in a bucket, poured through a 60-mesh sieve into a 325-mesh sieve. The 325-mesh sieve was rinsed with a gentle flow of water and about 30-40 ml was washed from the 325-mesh sieve into a 150 ml beaker. The beaker contents were allowed to settle for 2 hours and most of the water was discarded. A 1.3 M sucrose solution was added to the bottom layer contents of the beaker to increase the volume to 50 ml and was gently stirred. The sugar-nematode suspension was transferred into a 50 ml centrifuge tube and centrifuged for 1 min at 1500 rpm. The supernatant was poured into a 500-mesh sieve and rinsed with running tap water until all of the sugar was gone and collected in a 150 ml beaker. Water was added to make the egg-verminform extraction up to 40 ml volume. Microscopic examination and counting of eggs and vermiform on a grated Petri dish were made with an Olympus BH2 B071 microscope (Japan Model C35AD-4) at 40x magnification.

**Seedlings treatments with different biological control:** Cotton seedlings with 2000 eggs or vermiform nematodes were inoculated into pots with different treatments applied of 5 replicates for each treatment (Table 2).

**Root analysis:** Roots were separated from the plants and washed carefully. The cleaned root system was floated in a 0.3 × 0.2 m Plexiglas tray in 5 mm of water. Tangled and

indiscrete roots were separated with a small paint brush to free the roots from each other. The tray with roots was put on a paired Scan optical scanner (Regent Instruments, Inc., Quebec, Canada). Grey scale images of the roots were obtained at a resolution 800 by 800 dpi. They were evaluated for the accumulative number of roots, number of crossings, root volume, average root diameter, number of root lengths, length per volume, number of tips, surface area, and number of forks using WinRHIZOPro$^{TM}$ software (Version 2009c, Regent Instruments, Inc.).

**Statistical analysis:** The experiment was arranged in a randomized block design with five replications for each treatment, and repeated once. Statistical analyses were carried out with SAS version 9.4. The data were tabulated as 5 replicates +/- standard error mean (SEM), and the standard of significant was collected at 5%. Least significant difference (LSD) tests at $P = 0.05$ were employed to the differences among treatments for the parameters measured and the standard errors of the mean (SEM) was calculated as error bars in the figures.

## Results

Different seed treatments were used in the current study including biological control products plus fungicide (Table 1). The results statistically showed that no reduction in root weight was caused by *R. reniformis* with biological seed treatments compared to the control (fungicides only)(Fig.1) with significantly fewer numbers of juveniles of *R. reniformis*. Saponin reduced the numbers of juveniles and vermiform adults compared to control treatments to per 500 $cm^3$ soil (Fig. 2). All treatments, except ILeVo, significantly reduced nematode reproduction compared to the control (Table 2). The biological treatments were not significantly different from the abamectin standard, but were significantly better than the fluopyram treatment.

The experimental bacteria and *B. rinojensis* treatments were not significantly affected the plants growth parameters. Other treatments (bacteria; *B. rinojensis* + Saponin; *B. rinojensis.* + Harpin) were significantly better in comparison with control and had a higher weight of roots, especially the treatments *B. rinojensis* (Fig. 1). The number of juveniles and eggs of *R. reniformis* were reduced in most treatments with biological seed products except for *Burkholderia rinojensis* (Palleroni & Holmes, 1981; Yabuuchi *et al.,* 1992) treatment (Fig. 2). All treatments were significantly different than the fungicide check. In general, *R. reniformis* performed better at the higher application rates than the lower rate (fl. oz./cwt). In this study, all treatments were statistically similar to the abamectin standard except ILeVo as compared to the *B. rinojensis* treatment. There were no significant effects on cotton plant growth and development by *R. reniformis* from any of the biological seed treatments. The combination treatments of *B. rinojensis* Var 2+ Harpin reduced the number of juveniles and eggs compared to the control treatment for 500 juveniles per $cm^3$ soil and eggs with the *B. rinojensis* + Harpin (SAR). Most of these treatments (Bacteria; *B. rinojensis* + Saponin + Harpin) gave similar result to abamectin (Fig. 2 and 3). All biological treatments, combination treatments and the three nematicide standards were significantly reduced *R. reniformis* reproduction than the untreated controls (Fig. 2 and 3). Vermiform life stages were also reduced with all tested biological seed treatments. The effect of biological seed treatments reduced the number of vermiform and juvenile~~'s~~ stages as compared to control treatments.The roots image acquisition and analysis showed no negative effect on root~~s~~ growth by *R. reniformis* with biological seed treatments compared to control treatments (Table 1). The effect on root~~s~~ development (taking image of root scan by using WinRHIZOPro$^{TM}$ software) observed significant differences that improved roots growth with *B. rinojensis* (root length, surface area of root, average root diameter, root volume, number of tips, number of forks, and number of crossings) compared to control treatment. Also, the treatment that was combination from Saponin + *B. rinojensis* was significantly affected the number of tips, forks and crossings compared to control treatment (Fig.4).

## Discussion

There were significant effects in all biological products used that performed better than the fungicide check in regarding the suppression of eggs and vermiforms, in addition to the overall reproduction of *R. reniformis*. Many variants and experimental bacterial products that were tested in this study performed similar to the nematicide standard (abamectin). Biological candidates used to treat seeds did not impact host plant development when challenged by *R. renifomis.* Avicta® (abamectin, Syngenta) and Clariva® (*Pasteuria nishizawae* Sayre *et al.,* 1992, Syngenta), and VOTiVO® (*Bacillus firmus* Bredemann and Werner 1933, Bayer CropScience) are seed treatments that have been currently marketed to manage *R. reniformis* nematode and have been shown some control against *R. reniformis* and *Burkholderia* sp. as a biocontrol agent has been shown activity against different pathogens (Burkhead *et al*., 1994). Some *Burkholderia rinojensis* isolates that have been recovered from soil showed insecticidal activity against the new strain from Japan. Cell broth cultures of *Burkholderia rinojensis,* that reported and named as A396 strain, has been shown some toxicity effect on the beet armyworm (Lepidoptera: Noctuidae) (*Spodoptera exigua* Hübner, 1808) and also impacted on two-different spotted spider mite (Acari: Tetranychidae) *Tetranychus urticae* Koch, 1836 (Cordova-Kreylos *et al*., 2013). The selected *B. rinojensis* variant 2 will be marketed by Albaugh LLC as BioST nematicide 100 contains the active ingredients by heat-killed *B. rinojensis* and it spent fermentation broth. Some nematodes including *Heterodera glycines* Ichinoe, 1952, *R. reniformis* and *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 on soybean (*Glycines max* (L.) Merr.) listed on the label of the nematicide. The literature describes the active ingredients as being a collection of enzymes and toxins that have nematocidal properties on the above nematode via contact and ingestion.

The SAR and bacterial metabolite was statistically different than the fluopyram on *R. reniformis*. Fluopyram is a fungicide that has been shown to have activity against nematodes and as a dehydrogenase inhibitor of fungi that effects respiration (Avenot&Michailides, 2010). ILeVO® (fluopyram, Bayer CropScience Co.), and is applied as a seed treatment as a new product in the 2015 planting season for the management of soybean nematodes. Plants that treated with fluopyram in, under field conditions, reduced SDS foliar symptoms when compared to the control with just an insecticide (Mueller *et al*., 2011). Early testing has shown activity of fluopyram on plant-parasitic nematodes such as *H. glycines* (Zaworski, 2014). Harpin protein increased yields when used with cotton seeds (French, 2005). Harpin protein

plays an important role in suppressing the population of *R. reniformis* and also gave a slight yield increases when compared to the control. Also, harpin protein as a seed treatment has shown activity to suppress plant-parasitic nematodes. When applied as seed treatment Harpin stays on seeds helping them to grow long after planting (French, 2005).

Using different biological control as seed treatments to manage nematodes on cotton, *Burkholderia rinojensis* was identified as a potential organism. The two variants of *B. rinojensis* that were used in the current study suppressed the nematode numbers associated *R. reniformis*. None of the candidates impacted host plant growth development when infected with *R. renifomis. Burkholderia rinojensis* was the most consistent product in suppressing the number of eggs and vermiforms. Both *B. rinojensis* products reduced nematode reproduction and had no negative effect on plant growth. Saponin was effective at a lower rate in comparison with the bacteria. The new biological products can enhance sustainable crop production and allow growers to manage *R. renifomis* nematode and control damages that they cause.

**Acknowledgements**

Authors are grateful to the scholarship from the University of Kufa in Iraq. Technical support from the Mississippi State University Plant Growth Facilities is also gratefully acknowledged. No author has any conflict of interest to declare.

**Table 1 :** Effect of biological seed treatments on roots parameters of cotton infected with *Rotylenchulus reniformis* Linford and Oliveira, 1940.

| Treatments | Length (cm) | Surface area | Average diameter | Root volume | Tips | Froks | Crossing |
|---|---|---|---|---|---|---|---|
| 1 | 438.6533 | 116.025 | 0.45968 | 1.4982 | 1899.4 | 3656.8 | 137.2 |
| 2 | 1148.671 | 184.3048 | 0.51898 | 2.3734 | 3657.6 | 6744 | 453.6 |
| 3 | 1190.158 | 197.1138 | 0.5326 | 2.6388 | 4718 | 7253.6 | 455.2 |
| 4 | 897.3386 | 144.9562 | 0.51368 | 1.8746 | 3868.4 | 5343.8 | 300.4 |
| 5 | 891.0841 | 139.1545 | 0.50392 | 1.7474 | 3779.4 | 5134 | 296.2 |
| 6 | 596.7346 | 96.4652 | 0.52948 | 1.2544 | 2204.6 | 2994.8 | 216.6 |
| 7 | 887.4959 | 140.3145 | 0.51878 | 1.8046 | 3331.2 | 4499.8 | 321 |
| 8 | 614.586 | 99.0897 | 0.52302 | 1.2852 | 2030.4 | 2977.4 | 201.8 |
| 9 | 663.8198 | 114.8257 | 0.5719 | 1.6248 | 2617.8 | 3777.8 | 213.4 |
| 10 | 1270.279 | 203.3792 | 0.51534 | 2.6028 | 4986.6 | 7707.4 | 489.6 |
| 11 | 738.658 | 119.5016 | 0.51058 | 1.5588 | 3476 | 4053.8 | 202.2 |
| 12 | 732.1158 | 120.0763 | 0.52822 | 1.5814 | 3507 | 4085 | 229.6 |
| 13 | 639.343 | 102.8767 | 0.50946 | 1.3234 | 2184.8 | 3182.8 | 214.4 |
| 14 | 613.09 | 99.4771 | 0.518 | 1.295 | 2058.2 | 3107 | 249.8 |
| 15 | 729.7607 | 132.7626 | 0.58378 | 1.9374 | 2375.2 | 4382 | 227.6 |
| 16 | 635.3459 | 104.5256 | 0.52074 | 1.3736 | 2341.6 | 3136.8 | 340.2 |
| 17 | 867.0496 | 139.7165 | 0.5161 | 1.7956 | 2584 | 4451.6 | 245.6 |
| P-value | 0.003 | 0.066 | 0.045 | 0.067 | 0.004 | 0.0023 | 0.0012 |
| L.S.D 0.05 | 124.32 | 98.43 | 0.0026 | 1.024 | 276.34 | 344.07 | 32.34 |

Data are means of the 5 replicates for each treatment after 60 days. The means compared by using Fisher`s protected least significant difference test at $P<0.05$.

**Table 2 :** Treatments used in this study.

| S.No. | Treatments |
|---|---|
| 1 | Fungicide control - no nematicide. |
| 2 | Thiabendazole at three different rates on nematode = use rate 0.16 floz/cwt. |
| 3 | SAR product called Headsup at two rates on nematodes. Use rate 0.01 floz/cwt. |
| 4 | Headsup at two different rates with Thiabendazoleat 0.64 floz/cwt on nematodes. Two modes of action - outside in protection with TBZ and inside out protection (saponin) with Headsup. Use rate of Headsup is 0.01 oz/cwt. |
| 5 | Use rate of Headsup is 0.02 oz/cwt. |
| 6 | Headsup at two different rates with Thiabendazoleat 1.28 floz/cwt on nematodes. Use rate of Headsup is 0.01 oz/cwt. |
| 7 | Headsup at two different rates with Thiabendazoleat 0.64 floz/cwt on nematodes. Two modes of action (*Burkholderia rinojensis* )- outside in protection with TBZ and inside out protection (saponin) with Headsup. Use rate of Headsup is 0.01 oz/cwt. |
| 8 | SAR type product associated with the harpin protein at one rate (0.25 oz/cwt) with Thiabendazoleat 1.28 floz/cwt. Two modes of action - outside in protection with TBZ and inside out protection (saponin) with *Bacillus* sp. |
| 9 | Bio-nematicide candidate (*Burkholderia rinojensis*) that was derived from a fermentation product from a bacterium. Use rate was 3 floz/cwt. |
| 10 | Bio-nematicide candidate (*Burkholderia rinojensis*) with Thiabendazoleat 0.64 floz/cwt. Two modes of action for nematode protection. Use rate of *Burkholderia rinojensis*was 3 floz/cwt. |
| 11 | Bio-nematicide candidate (*Burkholderia rinojensis*) with Thiabendazoleat 0.64 floz/cwt and the Headsup (0.1 oz/cwt). |

| | |
|---|---|
| 12 | Abamectin - the active used in Avicta Complete from Syngenta on nematodes (0.15 mg ai per seed). |
| 13 | ILeVo used at the 1.14 floz / 140,000 seeds. |
| 14 | Fungicide standard with low rate TBZ and different rates of *Burkholderia rinojensis.* This product's confidential rate is 3 floz/cwt. |
| 15 | *Burkholderia rinojensis*-1 rate is 5 floz/cwt. |
| 16 | *Burkholderia rinojensis*rate is 7 floz/cwt. |
| 17 | *Burkholderia rinojensis*rate is 10 floz/cwt. |

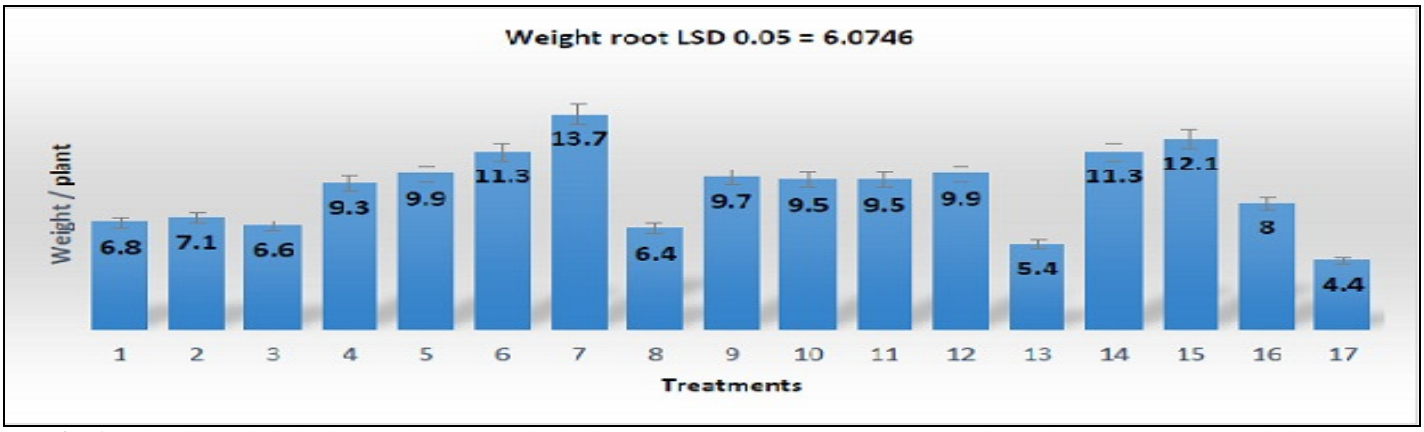


**Fig. 1 :** The effect of biological seed treatments including *Burkholderia* sp. on management of *Rotylenchulus reniformis* Linford and Oliveira, 1940 roots weight of cotton (*Gossypium hirsutum* L.).Data are means of all replicates for each treatment after 60 days. The means compared by using Fisher`s protected least significant difference test at *P*<0.05. Treatments listed from 1-17 following Table 2, the axis of this figure presents tested plants root weight.

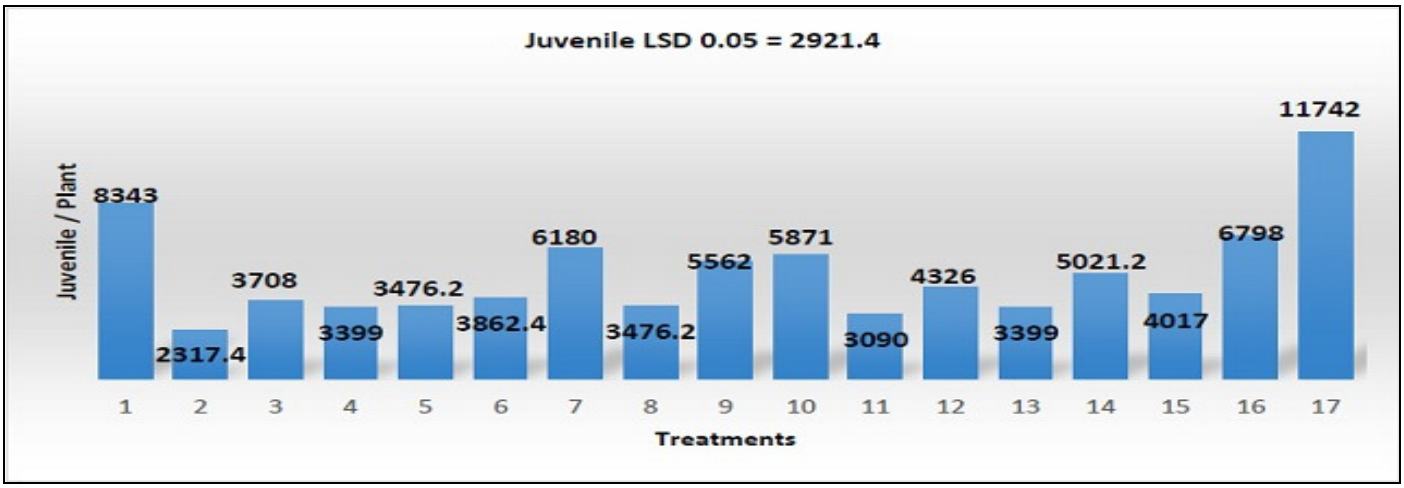


**Fig. 2 :** Effect of biological seed treatments of cotton (*Gossypium hirsutum* L,) including *Burkholderia* sp. applied as seed application rates for management *Rotylenchulus reniformis* Linford and Oliveiera, 1940 vermiform life stages. Data are means of all replicates for each treatment after 60 days. The means compared by using Fisher`s protected least significant difference test at *P*<0.05. Treatments listed from 1-17 following Table 2, the axis of this figure presents Juvenile in tested plants.

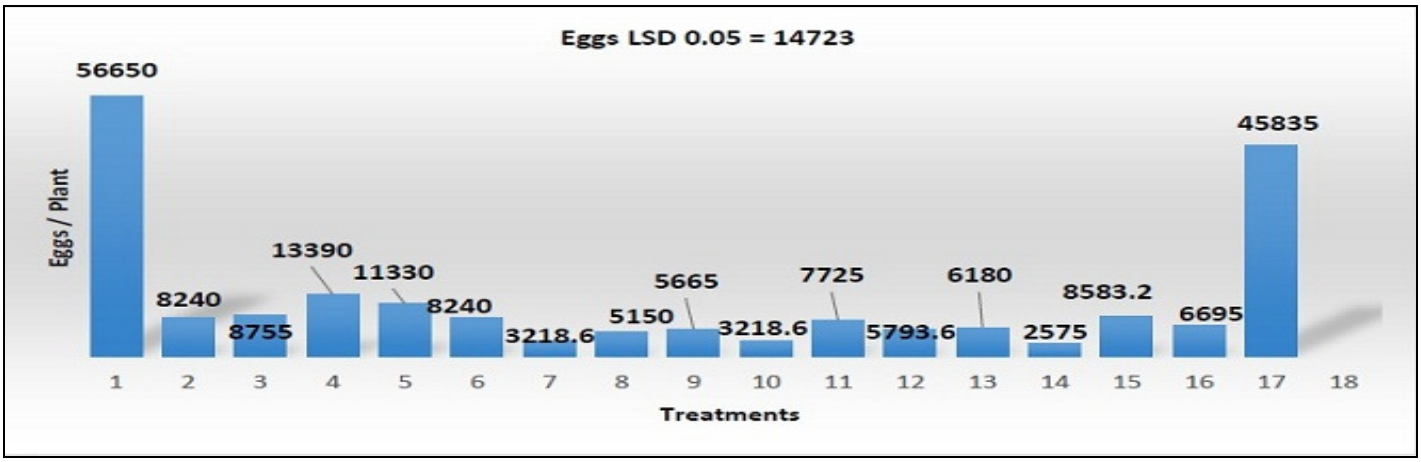


**Fig. 3 :** Effect of biological seed treatments including *Burkholderia* sp. applied as seed application rates for management of *Rotylenchulus reniformis* Linford and Olivieira, 1940 life stages (number of eggs). Data are means of all replicates for each treatment after 60 days. The means compared by using Fisher's protected least significant difference test at *P*<0.05. Treatments listed from 1-17 following Table 2, the axis of this figure presents eggs in tested plants.

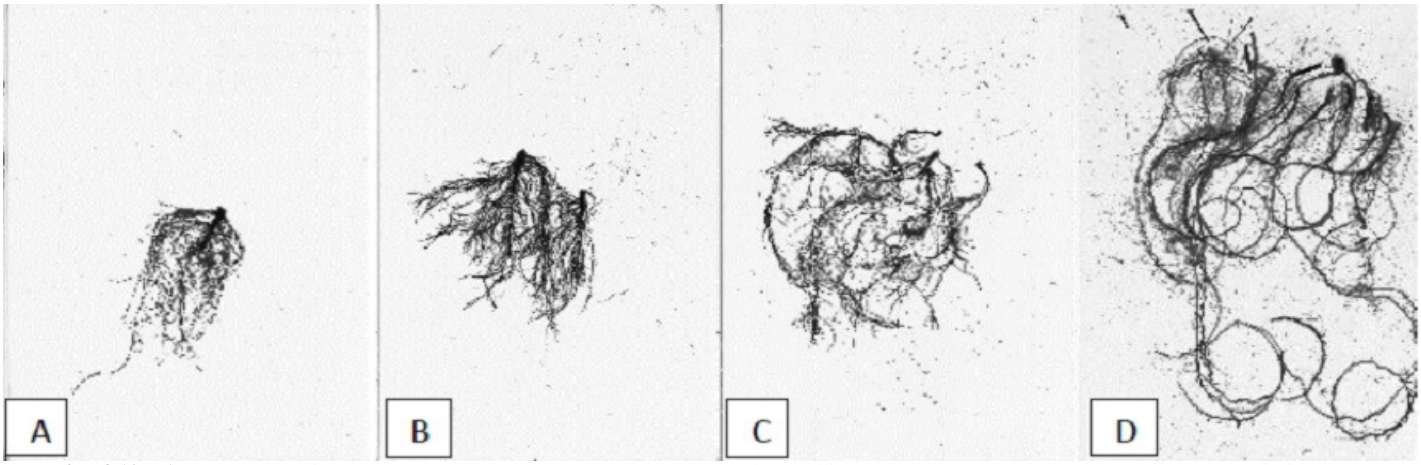


**Fig. 4 (A-D).** Root- Scan for cotton plants (*Gossypium hirsutum* L.) with reniform nematode (*Rotylenchulus reniformis* Linford and Olivieira, 1940) 60 days of planting. A. Control; B. ILeVo; C. *Burkholderia rinojensis* (Palleroni & Holmes, 1981; Yabuuchi *et al.,* 1992), 1 rate is 5 fl. oz, /cwt; D. *Burkholderia rinojensis* rate is 10 fl. oz./cwt.

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