

Evaluation of biological seed treatments for management of *Rotylenchulus reniformis* on cotton

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Abstract

Greenhouse experiments were conducted to assess 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 treatment 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* spp., 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 agents as seed treatments can manage plant-parasitic nematodes and limit the crop damage.

Keywords: Biological control, ILeVo, Abamectin, Plant-parasitic nematode, Root morphology, Cotton.

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 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 option for reniform nematode management in cotton, the best alternatives are the use of crop rotation, 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 with nematicides has 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 (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 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 roots were taken out from the beaker, weighed; then cut into 2-5cm lengths, placed in a flask, and vigorously shaken for 2 minutes exactly. The root fragments were thoroughly washed in a 200 mesh sieve over a 500 to remove the bleach. Eggs and vermiform nematodes were counted on a counting chamber with an Olympus H2 B071 microscope (Japan Model C35AD-4) at 40x magnification.

Extraction of eggs and juveniles: Sieving, centrifugation and sugar floatation (Jenkins, 1964) was used to extract eggs and vermiform stages from soil. Soil and water were suspended in a bucket. The contents of the bucket were poured through a 60-mesh sieve onto 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 onto 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-vermiform extraction up to 40 ml volume. Microscopic examination and counting of eggs and vermiforms 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 and 5 replicates for each treatment (Table 1).

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 in discrete 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 Win RHIZOPro™ 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 significance was assessed at 5% level. 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 the study including biological control products plus fungicide (Table 2). 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 cm³ soil (Fig. 2). All treatments, except Ile Vo, 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.

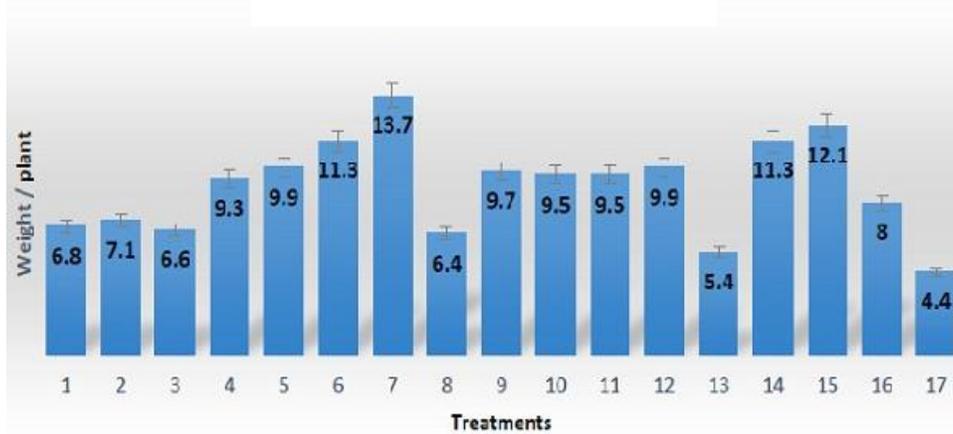


Fig. 1. The effect of biological seed treatments including *Burkholderia* sp., on management of *Rotylenchulus reniformis* Linford & Oliveira, 1940 roots weight of cotton (*Gossypium hirsutum* L.).Data are means of all replicates for each treatment after 60 days. The means compared by using Fisher`s 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.

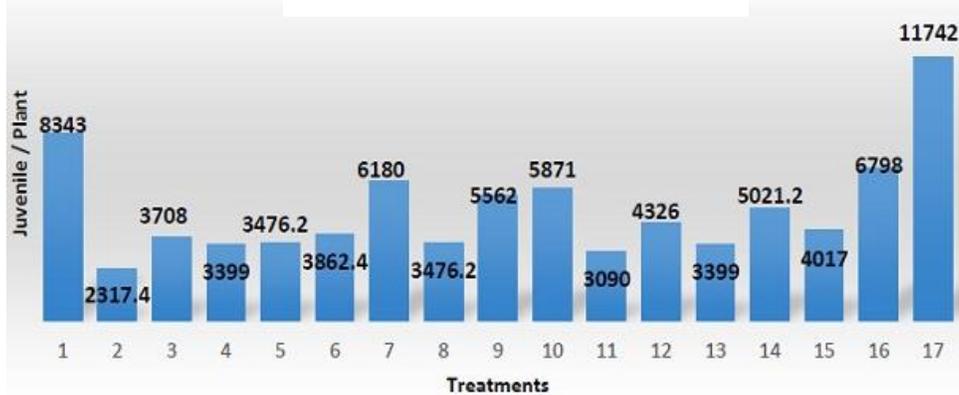


Fig. 2. Effect of biological seed treatments on cotton (*Gossypium hirsutum* L,) including *Burkholderia* sp., applied as seed application rates for management *Rotylenchulus reniformis* Linford & Oliveiera, 1940 vermiform life stages. Data are means of all replicates for each treatment after 60 days. 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.

The experimental bacteria and *B. rinojensis* treatments did not significantly affect 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* survived 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. None of the biological seed treatments had any significant effects on cotton plant growth and development by *R. reniformis*. 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 cm³ soil and eggs with the *B. rinojensis* + Harpin (SAR).

Table 1. 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 Heads up at two rates on nematodes. Use rate 0.01 floz/cwt.
4.	Heads up at two different rates with Thiabendazole at 0.64 floz/cwt on nematodes. Two modes of action - outside in protection with TBZ and inside out protection (saponin) with Heads up. Use rate of Heads up is 0.01 oz/cwt.
5.	Use rate of Heads up is 0.02 oz/cwt.
6.	Heads up at two different rates with Thiabendazole at 1.28 floz/cwt on nematodes. Use rate of Heads up is 0.01 oz/cwt.
7.	Heads up at two different rates with Thiabendazole at 0.64 floz/cwt on nematodes. Two modes of action (<i>Burkholderia rinojensis</i>)-outside in protection with TBZ and inside out protection (saponin) with Heads up. Use rate of Heads up is 0.01 oz/cwt.
8.	SAR type product associated with the harpin protein at one rate (0.25 oz/cwt) with Thiabendazole at 1.28 floz/cwt. Two modes of action - outside in protection with TBZ and inside out protection (saponin) with <i>Bacillus</i> sp.
9.	Bio-nematicide candidate (<i>Burkholderia rinojensis</i>) that was derived from a fermentation product from a bacterium. Use rate was 3 floz/cwt.
10.	Bio-nematicide candidate (<i>Burkholderia rinojensis</i>) with Thiabendazole at 0.64 floz/cwt. Two modes of action for nematode protection. Use rate of <i>Burkholderia rinojensis</i> was 3 floz/cwt.
11.	Bio-nematicide candidate (<i>Burkholderia rinojensis</i>) with Thiabendazole at 0.64 floz/cwt and the Heads up (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 <i>Burkholderia rinojensis</i> . This product's confidential rate is 3 floz/cwt.
15.	<i>Burkholderia rinojensis</i> -1 rate is 5 floz/cwt.
16.	<i>Burkholderia rinojensis</i> rate is 7 floz/cwt.
17.	<i>Burkholderia rinojensis</i> rate is 10 floz/cwt.

Table 2. Effect of biological seed treatments on root parameters of cotton infected with *Rotylenchulus reniformis* Linford & 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 $p < 0.05$

Most of these treatments (Bacteria; *B. rinojensis* + Saponin + Harpin) gave result similar to abamectin (Figs. 2, 3 and 4). All biological treatments, combination treatments and the three nematicide standards significantly reduced *R. reniformis* reproduction than the untreated controls (Figs. 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 stages as compared to control treatments. The roots image acquisition and analysis showed no negative effect on roots growth by *R. reniformis* with biological seed treatments compared to control treatments (Table 1). The effect on root development (taking image of root

scan by using Win RHIZOPro™ software) observed significant differences that improved root 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* significantly affected the number of tips, forks and crossings compared to control treatment.

Discussion

There were significant effects of all biological products used in the study that performed better than the fungicide check in suppression

of eggs and vermiforms, in addition to the overall suppression in reproduction of *R. reniformis*. Many variants and experimental bacterial products that were tested in this study, performed similar to the nematocide standard (abamectin). Biological candidates used to treat seeds, did not impact host plant development when challenged by *R. reniformis*. Avicta® (abamectin, Syngenta) and Clariva® (*Pasteuria nishizawae* Sayre *et al.*, 1992, Syngenta), and VOTiVO® (*Bacillus firmus* Bredemann & Werner 1933, Bayer Crop Science) are seed treatments that have been currently marketed to manage *R. reniformis* nematodes and have shown some control against *R. reniformis*. *Burkholderia* sp., as a biocontrol agent has shown good 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 shown some toxicity effect on the beet armyworm (Lepidoptera: Noctuidae) (*Spodoptera exigua* Hübner, 1808) and also impacted on two-different spotted spider mite species (Acari: Tetranychidae) *Tetranychusurticae* Koch, 1836 (Cordova-Kreylos *et al.*, 2013). The selected *B. rinojensis* variant 2 will be marketed by Albaugh LLC as BioST nematocide 100 contains the active ingredients by heat-killed *B. rinojensis* and its spent fermentation broth. Some nematodes including *Heterodera glycines* Ichinoe, 1952, *R. reniformis* and *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 on soybean (*Glycines max* (L.) Merr.) listed on the label of the nematocide. 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 from 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 affects respiration (Avenot & Michailides, 2010). ILeVO® (fluopyram, Bayer Crop Science Co.) was applied as a seed treatment as a new product in the 2015 planting season for the management of soybean nematodes. Plants that treated with fluopyramin, 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 fluopyramon 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 products as seed treatment 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. None of the candidates impacted host plant growth development when infected with *R. reniformis*. *B. 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. reniformis* nematode and control damage that they cause.

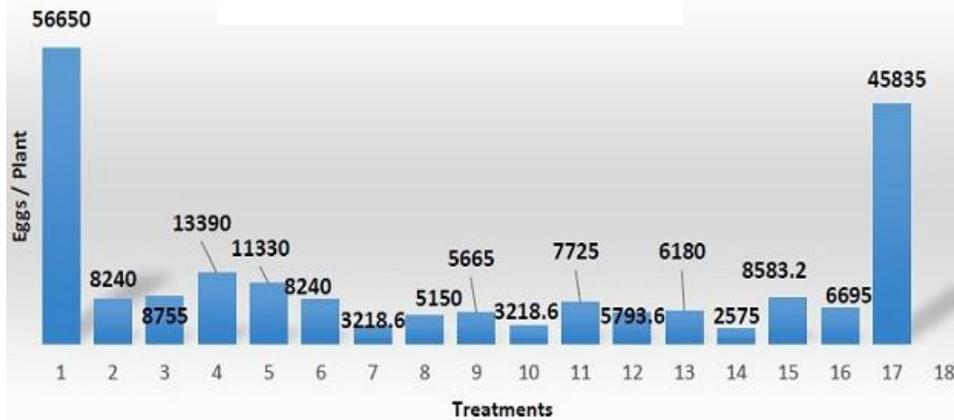


Fig. 3. Effect of biological seed treatments including *Burkholderia* sp., applied as seed application rates for management of *Rotylenchulus reniformis* Linford & Oliveira, 1940 life stages (number of eggs). Data are means of all replicates for each treatment after 60 days. The means compared by using 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.

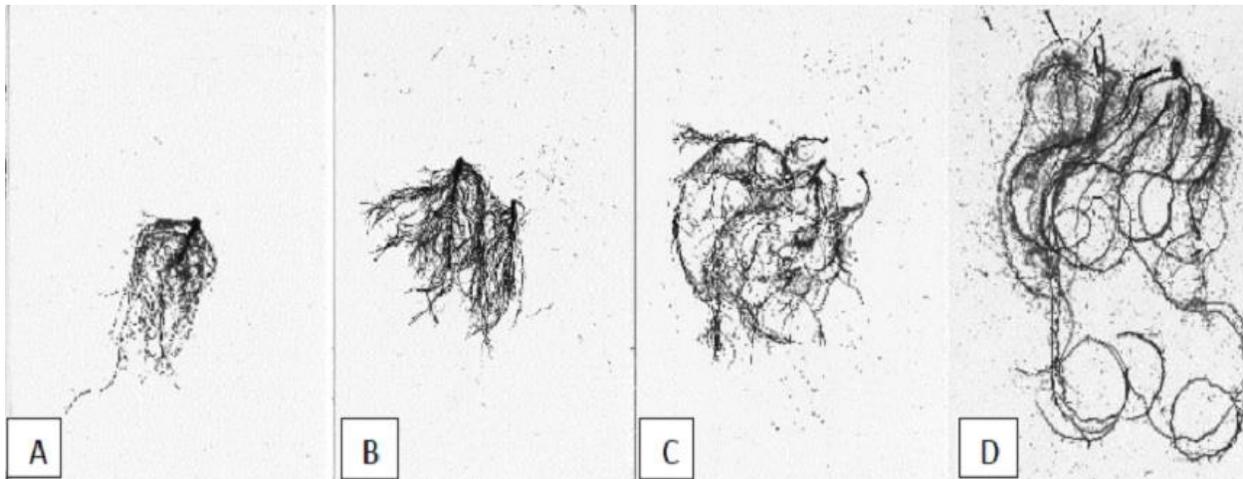


Fig. 4. (A-D). Root-scan for cotton plants (*Gossypium hirsutum* L.) with reniform nematode (*Rotylenchulus reniformis* Linford & Oliveira, 1940) 60 days of planting. A. Control; B. ILeVo; C. *Burkholderia rinojensis* (Palleroni & Holmes, 1981; Yabuuchi *et al.*, 1992), 1 rate is 5 fl. oz. /cwt; D. *Burkholderia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

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