IN VITRO SCREENING OF BIOLOGICAL CONTROL AGENTS ON *MELOIDOGYNE INCOGNITA*

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Abstract

Meloidogyne incognita (root-knot nematode, RK) is the most damaging pathogen on cotton in the U.S. and causes economic yield losses. Due to its extremely wide range of hosts, crop diseases caused by root-knot nematode are usually very difficult to manage. The objective of this research project is to select potential biocontrol agents that paralyze the infective stage juveniles (J2s) of the root-knot nematode. The commercial products (Aeris and Avicta) and 19 bacterial strains were evaluated. An *in vitro* assay was established in 100 µl 96-well plate with 30 ~ 40 J2s in each well at 25℃ to evaluate the effect of paralysis of the J2s. Numbers of live J2s were counted and recorded before exposure and 48 hours after treatment. Eleven *Bacillus* strains paralyzed 75% or more of the root-knot J2s. The eleven strains were isolates of *Bacillus amyloliquefaciens, B. mojavensis, B. safensis, and B. subtilis.* Aeris and Avicta paralyzed 92.4% of the root-knot J2s. The selected *Bacillus* strains from the *in vitro* screening will be evaluated in the greenhouse for further selections. Field trial will be established in 2014 and may provide more options for growers to manage the nematode disease on cotton and prevent yield loss.

Introduction

The withdrawal of aldicarb (Temik 15G), has driven an increase in examinations of possible new control agents including biologicals in nematode management. The root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood is a serious pathogen of most crops and causes an average of a 3% or $216 million dollar loss in cotton annually. *Bacillus* species are one the most important biological control agents that has been reported to inhibit plant parasitic nematodes and promote plant growth. According to Kloepper (2004), protection resulting from induced systemic resistance (ISR) elicited by *Bacillus* spp. (such as specific strains of the species *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus*) has been reported against root-knot nematode, many fungal and bacterial plant pathogens, and viruses. In addition, *B. thuringiensis* (Bt) has been proved to express a nematicidal Cry protein Cry5B which could inhibit the establishment or maintenance of root-knot nematode sites reducing nearly 3-fold the root-knot nematode progeny produced (Li, et al., 2008). The commercial formulation of *B. firmus*, (BioNem WP) applied at 200 and 400 kg ha^{-1} was effective in reducing the number of galls (75–84%) and increasing shoot height (29–31%) and weight (20–24%) on tomato plants (Terefe, et al., 2009). *B. megaterium* volatile organic compounds (VOCs) also have been found to cause death (100%) of root-knot nematode J2s and egg-hatching inhibition (Huang, et al., 2010). *Bacillus* spp. appear to be promising biological control agents against the root-knot nematode.

In this study, the commercial products (Aeris and Avicta) and 18 *Bacillus* strains (*B. amyloliquefaciens* subsp. *plantarum*, *B. megaterium*, *B. subtilis* subsp. *subtilis*, *B. amyloliquefaciens*, *B. safensis*, *B. mojavensis*, *B. pumilus*, and *B. solisalsi*), and a strain of *Lysinibacillus xylanilyticus* were evaluated in 96-well plate *in vitro* to select the strains that have the potential to be used as a biological control agent of root-knot nematode (*M. incognita*). The objective of this experiment is to screen *Bacillus* strains and identify those that paralyze RK J2s *in vitro*.

Materials and Methods

Preparation of second stage juveniles (J2s) of root-knot nematode

Root-knot eggs were extracted from the roots of corn plants, which were previously inoculated with root-knot nematodes and planted in the greenhouse. Root-knot eggs were extracted by shaking the roots in a 6.0% NaOCl solutions for 4 minutes and collecting the eggs on a 25 µm sieve. Root-knot eggs were poured in a modified Baermann funnel and incubated on the warmer at 31 ˚C for about 5 days to hatch the eggs. The hatched J2s were collected from the bottom of the funnel and used in the *in vitro* assay.

***In vitro* screening of *Bacillus* strains on root-knot nematode**

Bacterial strains were grown on TSA (Tryptic Soy Agar) medium plates at pH 7 and at 35˚C in the incubator for 48 hours. Inoculated colonies of the strains from the plates into 10 ml of 1× M9 medium (Dissolved 200 ml sterilized 5× M9 salts which made by 56.4 g 5× M9 Minimal medium stock in 1,000 ml distilled water, 2 ml 1M $MgSO_4$, 100 µl 1M $CaCl_2$, 20 ml carbon source, and some distilled water to a total of 1,000 ml) in tubes and incubated for 96 hours on a rotary shaker at 150 rpm. Thirty to forty RK J2s were added in the wells of a 100 µl 96-well plate. 90 µl of each bacterial culture was transferred into 4 wells (4 reps) of the 96-well plates. The industry standards of Aeris and Avicta were added at 1.5 µl into their respective 4 wells of the 96-well plate. Water was used as control. The plate was sealed with Para film and incubated at room temperature for 48 hours. Numbers of live J2s were counted and recorded before exposure and 48 hours after exposure to the bacteria and the commercial products.

Experimental Design

The experimental design for in *vitro* assay was randomized complete block design with four replications. The trial was repeated twice. Data collected from the experiment were analyzed with JMP Pro 10 (SAS Institute Inc.) using Student's t-test and Dunnett's Method to separate the means at a $P \leq 0.05$ level.

Results and Discussion

The average percentage paralyzed RK J2s for Aeris and Avicta were 92.4%, with a range from 80.6% to 104.2% and 81.1% and 103.8%, respectively (Table 1). The water control had an average of 6.4% paralyzed RK J2s, with a range of 0% to 15.8%. All of the *Bacillus* strains and the *Lysinibacillus xylanilyticus* isolate caused paralysis of the RK J2's compared to the water control. Percentages of paralyzed RK J2s were statistically similar to the Aeris and Avicta, the industry standards, in the treatments AP-301, AP-279, AP-136, AP-278, AP-52, AP-153, AP-188, AP-209, AP-280, AP-3, and AP-218 with an average over 75% paralysis. Interestingly these treatments represent multiple species of *Bacillus* that caused paralysis of the RK J2's. *B. amyloliquefaciens, B. mojavensis, B. safensis, and B. subtilis* all caused paralysis. Five different isolates of *B. amyloliquefaciens* (AP-301, AP-136, AP-188, AP-218, and AP-219) were evaluated and between isolates there were significant differences in paralysis of the RK J2's. Three separate isolates of *B. subtilis* subsp. *subtilis* (AP-279, AP-278, and AP-52) all preformed very similarly. Thus multiple species of *Bacillus* may have very different pathogenicity potentials to the RK J2's.

Table 1: Least squares estimate percentage of paralyzed RK J2s and 19 bacterial strains at 48 hours after exposure, confidence intervals, and *P*-values based on Dunnett's versus the checks Water and the commercial products (Aeris and Avicta) ($P \leq 0.05$).

		95% Confidence limit			Dunnett's *P* vs.		
Treatment	Scientific name	Avg.	Lower	Upper	Water	Aeris	Avicta
AP-301	*Bacillus amyloliquefaciens* subsp. *plantarum*	88.3	78.6	97.9	<.0001	1.000	1.000
AP-279	*Bacillus subtilis* subsp. *Subtilis*	84.8	75.1	94.4	<.0001	0.9734	0.9715
AP-136	*Bacillus amyloliquefaciens*	84.5	74.8	94.1	<.0001	0.9631	0.9607
AP-278	*Bacillus subtilis* subsp. *Subtilis*	84.3	74.7	94.0	<.0001	0.9589	0.9563
AP-52	*Bacillus subtilis* subsp. *Subtilis*	83.0	73.4	92.6	<.0001	0.8804	0.8755
AP-153	*Bacillus megaterium*	83.0	73.3	92.6	<.0001	0.8785	0.8736
AP-188	*Bacillus amyloliquefaciens*	82.5	72.9	92.2	<.0001	0.8446	0.839
AP-209	*Bacillus mojavensis*	79.4	69.7	89.0	<.0001	0.5359	0.529
AP-280	*Bacillus safensis*	78.3	68.7	88.0	<.0001	0.4391	0.4327
AP-3	*Bacillus safensis*	77.6	68.0	87.2	<.0001	0.3784	0.3724
AP-218	*Bacillus amyloliquefaciens*	75.1	65.5	84.7	<.0001	0.2083	0.2041
AP-217	*Bacillus solisalsi*	68.0	58.4	77.6	<.0001	0.0216	0.0209
AP-283	*Bacillus safensis*	67.6	58.0	77.3	<.0001	0.0188	0.0182
AP-219	*Bacillus amyloliquefaciens*	67.4	57.8	77.0	<.0001	0.017	0.0164
AP-7	*Bacillus safensis*	66.4	56.7	76.0	<.0001	0.0113	0.0109
AP-18	*Bacillus pumilus*	64.5	54.9	74.1	<.0001	0.0052	0.005
AP-281	*Bacillus safensis*	59.7	50.1	69.3	<.0001	0.0005	0.0005
AP-282	*Lysinibacillus xylanilyticus*	54.9	45.3	64.5	<.0001	<.0001	<.0001
AP-305	*Bacillus amyloliquefaciens* subsp. *plantarum*	52.6	43.0	62.2	<.0001	<.0001	<.0001
Aeris		92.4	80.6	104.2		1.000	
Avicta		92.4	81.1	100.0			1.000
Water		6.4	0.0	15.8	1.000		

Conclusions

In this study, the percentages of paralyzed RK J2s were significantly increased when the commercial products (Aeris and Avicta) and 19 bacterial strains were applied to RK J2s *in vitro*. Aeris, Avicta, bacterial strains AP-301, AP-279, AP-136, AP-278, AP-52, AP-153, AP-188, AP-209, AP-280, AP-3, and AP-218 had similar and high effects of paralyzed RK J2s. The eleven bacterial strains of the species *B. amyloliquefaciens, B. mojavensis, B. safensis, and B. subtilis* could be promising candidates to manage root-knot nematode and further work needs to be done to determine their further potential.

References

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