Application of Real Time PCR for Detection And Identification of Soybean Pests in Michigan

Project Number: **GR03-004**

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Objectives

RT-PCR development takes three distinct phases:

1. Development of primers to amplify DNA in the target organism;
2. Evaluation of the primers for specificity to the target organism, and potential plant and soil interference;
3. Validation of RT-PCR with conventional diagnostic protocols

Specific Objectives

1. Develop new primers for specific soybean pathogens, and optimize conditions to diagnose soybean plants using RT-PCR.
2. Evaluate and validate RT-PCR for the soybean pathogens as a diagnostic tool in collaboration with Michigan State University Diagnostic Services.
3. Fully integrate RT-PCR into a Diagnostic Service tool as part of the MSU Diagnostic Services program.

We proposed to develop real time PCR diagnostics for Phytophthora sojae, causes Phytophthora root and stem rot (PRR), Fusarium solani, causes sudden death syndrome (SDS), and several cyst nematodes including soybean cyst nematode (SCN). Bean pod mottle virus (BPMV), and soybean mosaic virus (SMV), were added at the request of the Michigan Soybean Promotion Committee. This research was supported with a one year GREEEN grant

Phytophthora root and stem rot. A fairly common pathogen in Michigan, with a broad range of new races identified in Michigan in 1998. Dechun Wang (Crop and Soil Sciences) and I participate on a regional project on this soybean disease.

Sudden Death Syndrome (SDS). *Fusarium solani* causes sudden death syndrome and is a major soybean pathogen in Illinois, Indiana, Iowa and Ohio. It has only recently occurred in Michigan, but appears to be spreading slowly in the southern tier of Michigan counties. SDS is difficult to confirm by conventional diagnostic methods, and often occurs in association with other diseases that may mask SDS. RT-PCR will facilitate surveys for SDS in Michigan, and facilitate routine diagnosis of soybeans submitted to Diagnostic Services.

Cyst Nematodes. The soybean cyst nematode, *Heterodera glycines*, and the sugar beet cyst nematode, *H. schachtii*, are serious threats to soybeans and sugar beets respectively in Michigan, and both are common in Michigan. Currently, bioassays are utilized at MSU to distinguish between soybean and sugar beet cyst nematodes if cyst nematodes are recovered from soil samples received from growers of both soybeans and sugar beets. Bioassays consist of growing soybean or cabbage (a good host for *H. schachti*) in the soil received. If cyst nematodes develop on soybean, it is confirmation of a soybean cyst nematode infestation. If they develop on

cabbage, it confirms the presence of sugar beet cyst nematode. If they develop on both plants, then both species are present. Cyst nematodes are usually managed by growing non-host crops for a period of one to several years. Proper selection of these crops is critical to ensure the desired results. Therefore, proper identification of cyst nematodes is imperative for crop selection. Bioassays, where effective, require 30-45 days to complete. RT-PCR should expedite this process, and provide a more accurate diagnosis. The alfalfa cyst nematode (*H. trifolii*) was added because it can also occur in soil samples and cannot be distinguished microscopically from the other two cyst nematodes.

Bean pod mottle virus and soybean mosaic virus. BPMV has been increasing in the Midwest over the past five years, with significant occurrence and affect on seed quality in 2001. Although not a major cause of yield reductions by itself, infected seed has reduced vigor, and usually must be treated with a fungicide to promote good stands. This is an important problem for the growing number of organic soybean producers in Michigan who do not have seed treatment options. BPMV is seed transmitted at a rate of less than 0.1%. SMV occurs occasionally in Michigan, but can be destructive, causing significant yield losses, and it is seed transmitted. Duel infections with BPMV can cause yield reductions up to 90%.

Objective 1

Develop new primers for specific soybean pathogens, and optimize conditions to diagnose soybean plants using RT-PCR. Objective 1 has been completed for each of the pathogens, and tests completed to validate RT-PCR.

Objective 2

Evaluate and validate RT-PCR for the soybean pathogens as a diagnostic tool in collaboration with Michigan State University Diagnostic Services. Objective 2 was completed for *Phytophthora sojae, Fusarium solani*, and bean pod mottle virus. Soybean plants were inoculated in the greenhouse and laboratory with isolates of *P. sojae*, or *F. solani*. After symptoms developed the plants were harvested, and a Qiagen DNA extraction and purification kit was used to extract and purify DNA for RT-PCR. The pathogens were isolated from the plant tissue to confirm RT-PCR results. In addition, several plants with SDS or Phytophtora root and stem rot symptoms were collected from commercial soybean fields in Monroe County, and analyzed by RT-PCR, and pathogen isolations as described above. The plants with SDS symptoms were confirmed to have *F. solani*. However, the putative Phytophthora plants were all negative by RT-PCR, and *P. sojae* was not isolated. Subsequently, we isolated *Pythium* from these plants, sequenced the ITS region, and determined these plants were infected by a species of *Pythium* reported from France, but not in the U. S. We are currently running tests to confirm the pathogenicity of this new soybean disease in the U. S. These results show the power of RT-PCR as an aid in diagnosis. Usually, soybean plants with these symptoms are not tested because the symptoms are strongly associated with *P. sojae*, and conventional isolation is difficult. However, a negative RT-PCR prompted us to investigate further with the result being a putative new pathogen.

A survey was conducted in August, 2003, to collect bean leaf beetles (BPMV vector) from soybean fields to test and compare RT-PCR and a commercial ELISA (AG-DIA) for BPMV. Both tests identified BPMV in soybean seed. Forty-six fields from southwest Michigan and the thumb were surveyed. One hundred sweeps with a sweep net were made in each field, and all bean beetles collected, counted and frozen. The number of bean beetles per field ranged

from a low of 0 to a high of 193 (25 five fields had at least 10 beetles/100 sweeps, and 21 had less than 10 with 6 of these having no beetles). BPMV was detected by RT-PCR in beetles from 20 fields, and by ELISA in beetles from only 8 fields. RT-PCR detected BPMV in all beetles that were positive by ELISA. Both preliminary experiments and the field assay indicated that RT-PCR was more sensitive than the ELISA.

Diagnostic Services maintains populations of all three nematode species, and provided us with the DNA for sequencing, and blind samples of the cyst species for testing. This allowed us to run preliminary validations prior to testing of field samples in 2004. RT-PCR accurately identified all of the cyst species provided, and correctly identified mixtures.

Table 1. Primers and probes designed for detection and identification of some fungi, viruses and nematodes. Taq-probes for Heterodera species were thought to be required to separate the species using a second RT-PCR amplification, but testing indicated this was not necessary.

Phytopthora sojae	PSOJF1	GCCTGCTCTGTGTGGCTGT	rDNA-ITS
	PSOJR1	GGTTTAAAAAGTGGGCTCATGATC	
	PSOJF2	GCTGGTGAACCGTAGCTGTGT	rDNA-ITS
	PSOJR2	TCCGCAGCAAGCCGC	
Fusarium solani f.sp. *glycine*	FSG1F	GGTCGGAGCCCTCCGG	rDNA-ITS
	FSG1R	CAGCGAGACCGCCACTGA	
Bean Pod Mottle Virus	BPMV3F	TGGGAAGTTGTATGAGGGTAAGC	RNA2-COAT P
	BPMV3R	ACAGAATTTCCTCCTGTGCCAT	
Soybean Mosaic Virus	SMV2F	GCGTGTGGGTGATGATGGA	RNA
	SMV2R	CATCTCAATGTAAGCTTCTGCTGC	
Heterodera glycine	HG-1F	CCATACGTTGGAGCTGTGGTATA	rDNA-ITS
	HG-1R	CGAGCGTGCATCCCACAT	
	TAQ-PROBE	AAAGCCTGTGTATGGCTGCTGCGTG	
Heterodera trifolii	HT-1F	GTGGTAGGCACATAACACACTGACT	rDNA-ITS
	HT-1R	CAAGCACAAACACCGCTAGT	
	TAQ-PROBE	TGGTGGTTTCGTTCCCAGTCTTACGTG	
Heterodera schatii	HS-1F	CTCAGTGCTGCACACGTGAA	
	HS1R	CCCGCCACCGACACAT	
	TAQ-PROBE	CCTGTGGATGGCTGCTGTGTGGC	

Table 2. Results of RT-PCR and ELSA tests of beetle samples collected from various counties in Michigan.

Sample	Position	County	Elevation	# of BLBs	RT-PCT [1]	ELISA
AUG 15 P01	N42 42.243 W84 20.838	Ingham	887 ft	18	–	–
AUG 15 P02	N42 40.229 W84 12.289	Ingham	861 ft	11	28.84	–
AUG 15 P03	N42 38.303 W84 11.798	Ingham	890 ft	5	–	–
AUG 18 P01	N42 38.587 W84 36.758	Ingham	867 ft	9	–	–
AUG 18 P02	N42 32.296 W84 38.180	Eaton	874 ft	6	29	–
AUG 18 P03	N42 28.466 W84 39.520	Eaton	878 ft	191	–	–
AUG 18 P04	N42 24.451 W84 39.896	Jackson	905 ft	2	–	–
AUG 18 P05	N42 20.925 W84 41.633	Jackson	913 ft	0	NT	NT
AUG 18 P06	N42 16.652 W84 42.531	Jackson	1014 ft	1	–	–
AUG 18 P07	N42 12.210 W84 45.140	Calhoun	974 ft	17	–	–
AUG 18 P08	N42 09.655 W84 45.581	Calhoun	971 ft	13	–	–
AUG 18 P09	N42 07.142 W84 48.505	Calhoun	939 ft	0	NT	NT
AUG 18 P10	N42 01.739 W84 42.961	Hillsdale	999 ft	5	25.48	–
AUG 18 P11	N41 56.951 W84 39.684	Hillsdale	1048 ft	19	–	–
AUG 18 P12	N41 52.218 W84 30.319	Hillsdale	1075 ft	13	12.47	+
AUG 18 P13	N41 51.293 W84 22.977	Hillsdale	943 ft	88	16.28	+
AUG 18 P14	N41 51.378 W84 16.824	Lenawee	906 ft	173	24.37	–
AUG 18 P15	N41 52.542 W84 09.605	Lenawee	831 ft	33	13.48	+
AUG 19 P16	N41 53.318 W84 04.789	Lenawee	800 ft	21	16.02	+
AUG 19 P17	N41 59.464 W84 00.242	Lenawee	823 ft	79	17.45	+
AUG 19 P18	N42 10.492 W84 01.913	Washtenaw	981 ft	49	23.37	+
AUG 19 P19	N42 15.630 W84 02.039	Washtenaw	936 ft	54	25.66	–
AUG 19 P20	N42 24.403 W84 07.373	Washtenaw	943 ft	4	–	–
AUG 19 P21	N42 29.315 W84 11.413	Ingham	883 ft	28	–	–
AUG 19 P22	N42 33.566 W84 11.502	Ingham	909 ft	28	28.4	–
AUG 20 P01	N42 50.915 W84 13.093	Shiawassee	863 ft	9	24.94	+
AUG 20 P02	N42 56.901 W84 13.282	Shiawassee	717 ft	2	–	–
AUG 20 P03	N43 01.594 W84 10.499	Shiawassee	729 ft	0	NT	NT
AUG 20 P04	N43 07.728 W84 10.285	Shiawassee	693 ft	9	26.91	+
AUG 20 P05	N43 13.827 W84 10.002	Saginaw	604 ft	25	–	–
AUG 20 P06	N43 24.652 W83 52.584	Saginaw	589 ft	26	–	–
AUG 20 P07	N43 24.269 W83 40.770	Tuscola	638 ft	2	–	–
AUG 20 P08	N43 25.022 W83 09.371	Tuscola	789 ft	21	–	–
AUG 20 P09	N43 24.919 W82 56.941	Sanilac	785 ft	1	–	–
AUG 20 P10	N43 25.430 W82 52.164	Sanilac	779 ft	3	–	–
AUG 20 P11	N43 20.037 W82 49.290	Sanilac	749 ft	2	–	–
AUG 20 P12	N43 14.394 W82 48.361	Sanilac	774 ft	9	–	–
AUG 20 P13	N43 13.016 W82 54.644	Sanilac	781 ft	2	–	–
AUG 20 P14	N43 13.883 W83 06.997	Lapeer	813 ft	6	–	–
AUG 22 P01	N42 45.330 W84 47.775	Eaton	812 ft	13	27.35	–
AUG 22 P02	N42 45.415 W84 54.959	Eaton	851 ft	64	19.9	–
AUG 22 P03	N42 45.356 W85 03.629	Eaton	858 ft	19	28.79	–
AUG 22 P04	N42 43.505 W85 10.421	Barry	849 ft	0	NT	NT
AUG 22 P05	N42 29.222 W85 24.825	Barry	964 ft	20	27.15	–
AUG 22 P06	N42 23.458 W85 27.018	Kalamazoo	953 ft	33	26.93	+
AUG 28 P01	N42 03.854 W83 32.700	Monroe	686 ft	no sweeps	NT	NT
AUG 28 P02	N41 56.972 W83 39.656	Monroe	638 ft	no sweeps	NT	NT
AUG 28 P03	N41 56.465 W83 39.128	Monroe	682 ft	no sweeps	NT	NT
AUG 28 P04	N42 09.921 W83 21.986	Wayne	643 ft	no sweeps	NT	NT
SEP 08 P01	N41 57.318 W85 24.912	St. Joseph	854 ft	3	28.14	–
BPMV- C1[2]					14	+
BPMV-C2 [3]					13.72	+
NC					–	–
NTC					–	–

Table 3. RT-PCR detection of BPMV and SMV in soybean leaf samples.

Sample	Source	BPMV [1]	SMV [2]
AUG 18 P09	Calhoun	–	–
AUG 18 P15	Lenawee	–	–
AUG 20 P01	Shiawassee	–	–
AUG 20 P02	Shiawassee	–	–
AUG 20 P04	Shiawassee	–	–
AUG 20 P05	Saginaw	–	–
AUG 20 P06	Saginaw	–	–
AUG 20 P07	Tuscola	–	–
AUG 20 P08	Tuscola	–	–
AUG 20 P09	Sanilac	–	–
AUG 20 P10	Sanilac	–	–
AUG 20 P11	Sanilac	–	–
AUG 22 P02	Eaton	–	–
AUG 22 P03	Eaton	–	–
AUG 22 P04	Barry	–	–
AUG 22 P05	Barry	–	–
AUG 22 P06	Kalamazoo	–	–
AUG 28 P01	Monroe	–	–
AUG 28 P02	Monroe	–	–
AUG 28 P03	Monroe	–	–
S-20033785	Plant Diagnostic	–	–
S-2A	Plant Diagnostic	–	–
S-2B	Plant Diagnostic	–	–
S-4A	Plant Diagnostic	–	–
S-4B	Plant Diagnostic	–	–
S-5-SMV	Plant Diagnostic	–	–
S-6-SMV	Plant Diagnostic	–	–
BPMV-C1 [3]		13.04	–
BPMV-C2		12.81	–
SBMV-C1 [4]		–	12.35
SBMV-C2		–	12.64
NC		–	–
NTC		–	–

Summary Statement

Real time polymerase chain reaction (RT-PCR) is an excellent diagnostic resource for plant pathogens because the test is specific, sensitive, and very rapid. The test identifies specific segments of DNA that are unique to each pathogen. Because many pathogens of soybeans are difficult to accurately diagnose in a short period of time, RT-PCR will facilitate the diagnosis of many soybean pathogens. Virus diseases of soybean are especially difficult to identify and RT-PCR was developed for soybean mosaic virus (SMV) and bean pod mottle virus (BPMV). A survey in 2003 comparing ELISA with RT-PCR showed that RT-PCR was more sensitive and identified more bean beetles positive for BPMV than ELISA. RT-PCR was also developed to facilitate the detection and identification of the sudden death syndrome in soybeans caused by *Fusarium solani*. Although only identified a few times in Michigan, SDS was confirmed using RT-PCR in samples of soybeans from southeast Michigan. Our goal is provide the MSU Diagnostic Services Laboratory with appropriate RT-PCR tests for the soybean pathogens used in the GREEEN supported research project. The Michigan Agriculture community will benefit by increased capacity of Diagnostic Services, and more rapid an accurate testing for soybean pathogens.

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