Northeastern IPM Center – IPM Partnership Grants – 2009 – Proposal
Project Description

PD: Gary C. Bergstrom
Project Title: Developing improved protocols to assess alfalfa varieties for resistance to *P. sclerotioides*.

1. Project Category: IPM Issues

2. Project Summary

Phoma sclerotioides, an economically important fungal pathogen with widespread distribution in the Northeast, causes brown root rot of alfalfa (BRR) and contributes to alfalfa spring black stem and leaf spot (SBS). Alfalfa varieties with effective resistance to *P. sclerotioides* are not available. BRR resistance of alfalfa varieties appears to differ by *P. sclerotioides* biotype, with no variety resistant to all biotypes. All varieties are at least moderately susceptible to SBS caused by *P. sclerotioides*. Current breeding protocols involve the use of a single isolate of *P. sclerotioides* to screen for resistance to BRR and do not include *P. sclerotioides* when screening for resistance for SBS. The proposed study will identify the minimum set of isolates needed to breed alfalfa for effective *P. sclerotioides* resistance and will investigate which *P. sclerotioides* biotypes contribute to SBS. Controlled studies will be conducted to test the virulence of isolates of each *P. sclerotioides* biotype to the alfalfa varieties Peace, Starbuck, and WL 347 LH. Preliminary studies suggest that the *P. sclerotioides* resistance of these varieties differs by biotype. Both foliar and root inoculations will be conducted. Field surveys will be conducted in New York and Vermont to evaluate which *P. sclerotioides* biotypes are associated with SBS in alfalfa production fields. The study will provide breeders with the tools necessary to develop alfalfa varieties with stronger, more effective resistance to both root and foliar disease caused by *P. sclerotioides*.

3. Background and Justification

Phoma sclerotioides, a fungal pathogen with widespread distribution in the Northeast, causes brown root rot of alfalfa (BRR) and contributes to alfalfa spring black stem and leaf spot (SBS). The pathogen is widespread in Maine, New Hampshire, New York, and Vermont (Wunsch et al. 2007, 2009). It is found in parts of central and northern Pennsylvania (Wunsch et al. 2009) and likely also occurs in parts of Massachusetts.

Pathogenic colonization of alfalfa roots and crowns by *P. sclerotioides* results in BRR. BRR is associated with reduced spring regrowth of alfalfa and severe stand decline of alfalfa (Berkenkamp et al. 1991, Hollingsworth et al. 2003). It causes winterkill losses that can exceed 50 percent two years after seeding (Berkenkamp et al. 1991) and 95 percent three or more years after seeding (Gray et al. 2004) and is particularly severe in regions with harsh winters. Stakeholders in the Northeast report poor alfalfa persistence where BRR incidence is high. Jay Dickson, owner of a 700-cow dairy in western New York, reports that alfalfa persistence has been reduced from six to four years in his fields, all of which have high incidence of BRR. Eric Bever, a crop consultant in northern New York, and Richard Kersbergen, an extension professor with the University of Maine, note that alfalfa stands frequently persist only to two or three

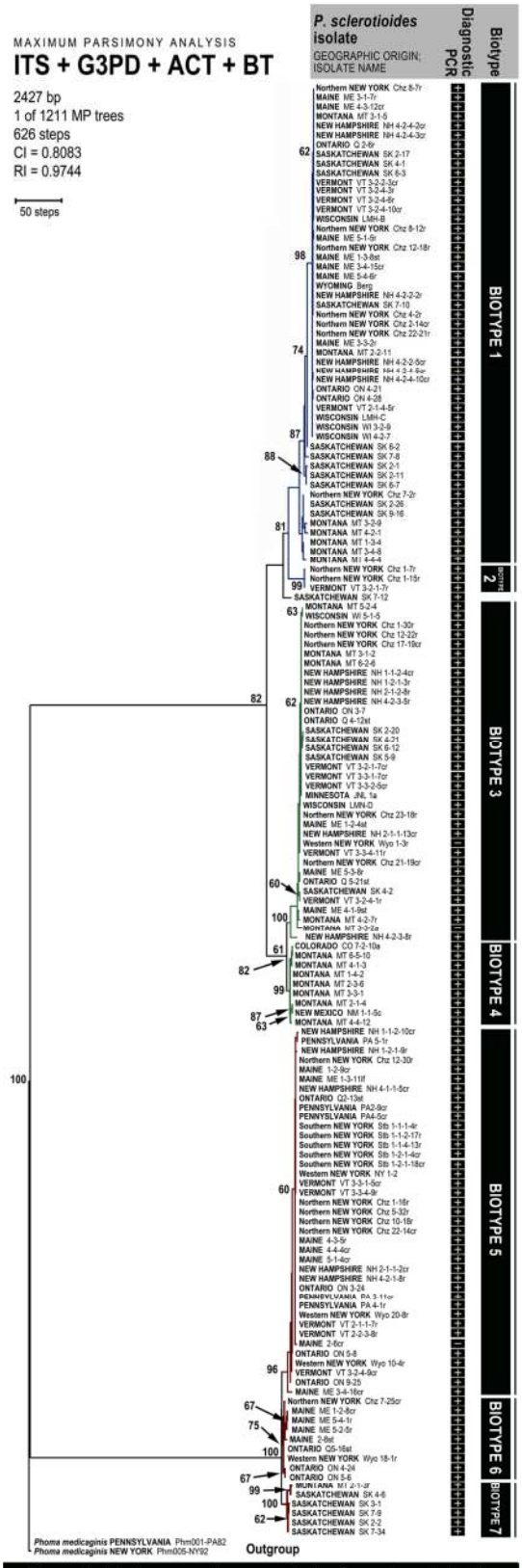

Figure 1. Maximum parsimony analysis of concatenated actin, beta-tubulin, glyceraldehyde 3-phosphate dehydrogenase, and nuclear rDNA ITS sequence data. Rooted with *P. medicaginis*. **Bootstrap values > 50% indicated at internodes. Diagnostic PCR results denoted with (+) or (-).**

production years in New York's Champlain Valley and in central Maine, respectively, regions where BRR is widespread (Wunsch et al. 2007, 2009).

Pathogenic colonization of alfalfa stems and leaves by *P. sclerotioides* results in SBS (Wang et al. 2004, Hwang et al. 2006). SBS reduces yield and sharply reduces forage quality of alfalfa, and it is one of the most important foliar diseases of alfalfa under the cool and moist conditions prevalent in the spring and fall in the Northeast. At the first and last cuttings, when SBS is most severe, alfalfa yields in the northern United States are reduced an average of 13 percent by foliar disease (Nutter et al. 2002), and in Vermont and Wyoming, SBS causes average yield losses of 10 to 15 percent at the first cutting (Gray et al. 1987, Nutter et al. 2002). Alfalfa crude protein content is reduced by an average of 22 percent in leaves affected by SBS (Hwang et al. 2006). The contribution of *P. sclerotioides* to SBS was first identified in Alberta in 1998 (Wang et al. 2004); previously, only *P. medicaginis* was believed to cause SBS (Leath 1990).

The contribution of *P. sclerotioides* to SBS can be significant. In replicated field trials conducted over multiple years and sites in Alberta, inoculation of alfalfa with *P. sclerotioides* caused an increase in SBS incidence and severity and a decrease in seed yield roughly equivalent to that caused by inoculation with *P. medicaginis* (Hwang et al. 2006). In greenhouse trials, inoculation of alfalfa with *P. sclerotioides* resulted in SBS symptoms that decreased the photosynthetic activity of leaves at a rate comparable to that caused by inoculation with *P. medicaginis* (Hwang et al. 2006). The association of *P. sclerotioides* with alfalfa SBS has also been confirmed in the northeastern U.S. In spring 2007, alfalfa with SBS was collected from two fields in New York and one field in Maine, and both *P. sclerotioides* and *P. medicaginis* were isolated from SBS lesions in all three fields (Wunsch, unpublished). The failure of previous researchers to detect *P. sclerotioides* is likely due to the difficulty of isolating the pathogen using conventional protocols.

At least seven major biotypes of *P. sclerotioides* exist in North America. Maximum parsimony analysis of multilocus sequence data places U.S. and Canadian *P. sclerotioides* isolates into seven major clades (figure 2; Wunsch and Bergstrom 2008), each of which has distinctive in-vitro culture morphology on potato dextrose agar. The isolates were obtained from symptomatic alfalfa, test positive for *P. sclerotioides* in diagnostic PCR (figure 1; Larsen et al. 2002), and produce large, beaked pycnidia and unicellular, hyaline, ovoid conidia characteristic of *P. sclerotioides* (Boerema et al. 1994). Inoculation of alfalfa roots and crowns with isolates from biotypes 1, 3, 4, and 5 results in light to dark brown lesions typical of BRR (Wunsch et al. 2008, 2009); pathogenicity to roots and crowns of isolates from other biotypes has not been evaluated. It is unclear which *P. sclerotioides* biotypes contribute to SBS. All *P. sclerotioides* isolates obtained from foliar lesions in New York and Maine were of biotype 5 (Wunsch, unpublished), but the *P. sclerotioides* isolate tested for pathogenicity to alfalfa in Alberta (Wang et al. 2004, Hwang et al. 2006) is unavailable, and its biotype is unknown.

Varieties with effective resistance to *P. sclerotioides* are not available in the Northeast. The BRR-resistant variety Peace (Berkenkamp et al. 1991, Hollingsworth et al. 2005), developed in Alberta, does not consistently show resistance to *P. sclerotioides* in New York (table 1), lacks bacterial wilt resistance, and has only moderate *Phytophthora* root rot resistance. Other management tools are inadequate. *P. sclerotioides* cannot be managed by crop rotation; it has a broad host range and persists in the soil (Davidson 1990). Fungicide control is not effective for BRR and is not employed for SBS because of cost.

Table 1. Relative susceptibililty of alfalfa cultivars to BRR.

Bath, NY (Steuben County)				Willsboro, NY (Essex County)	
SPRING 2007		SPRING 2008		SPRING 2008	
variety (seed company)	**incidence**	**variety**	**incidence**	**variety (seed company)**	**incidence**
Guardsman II (Seedway)	45 a	Starbuck	14 a	Peace (Richardson Seeds)	5 a
361 HY (Preferred Seed)	46 ab	Mariner III	25 ab	WL 347 (W-L Research)	8 ab
WL 347 LH (W-L Research)	51 ab	WL 347 LH	29 ab	ReGen (Seedway)	9 ab
54V46 (Pioneer)	51 ab	Seedway 9558	32 b	Oneida Ultra (Seedway)	12 ab
Oneida Ultra (Seedway)	52 ab	Oneida Ultra	32 b	54V46 (Pioneer)	14 ab
Starbuck (Proseed)	55 ab	54V46	33 b	Verna (Univ. of Wisconsin)	14 ab
ReGen (Seedway)	56 ab	Guardsman II	34 b	Mariner III (Allied Seed)	15 ab
Seedway 9558 (Seedway)	61 abc	361 HY	36 b	Seedway 9558 (Seedway)	18 ab
Mariner III (Allied Seed)	65 bc	Verna	36 b	Starbuck (Proseed)	21 b
Verna (Univ. of Wisconsin)	65 bc	Peace	37 b	Guardsman II (Seedway)	22 b
Peace (Richardson Seeds)	76 c	ReGen	38 b	361 HY (Preferred Seed)	22 b
LSD=16 (α=0.05)		LSD=17 (α=0.05)		LSD=13 (α=0.05)	

Incidence refers to percentage of plants from which *P. sclerotioides* was successfully isolated from the roots and/or crowns; 125 plants of each cultivar (25 plants/cultivar/rep) were sampled. Inoculations were conducted with an isolate of *P. sclerotioides* biotype 5 in Bath and biotype 1 in Willsboro. Different letters indicate significant (P<0.05) differences. **The lower *P. sclerotioides* incidence observed in Bath in 2008 reflects differences in sampling dates.** In 2007, samples were collected in mid-April, and in 2008, samples were collected in mid-May; *P. sclerotioides* is easier to recover early in the spring.

Developing varieties with effective resistance to *P. sclerotioides* will require revising the protocols used to assess resistance to the pathogen. Preliminary results suggest that resistance to *P. sclerotioides* in alfalfa differs by biotype. Peace, an alfalfa variety developed in western Canada, is resistant to the *P. sclerotioides* 'Berg' isolate (Hollingsworth et al. 2005) of biotype 1 (figure 1). In 2006 and 2007, replicated field trials were established in New York to evaluate the relative susceptibility of 11 alfalfa varieties to BRR; local *P. sclerotioides* isolates were used for inoculations. Peace showed elevated resistance to *P. sclerotioides* in Willsboro, NY (table 1), where an isolate of *P. sclerotioides* biotype 1 was used, but it was highly susceptible in Bath, NY (table 1), where an isolate of *P. sclerotioides* biotype 5 was used. Conversely, Starbuck

(Pickseed Canada; Lindsay, ON) was highly susceptible in Willsboro but showed elevated resistance in Bath (table 1). Current breeding protocols involve the use of a single isolate of *P. sclerotioides* to screen for resistance to BRR and do not include *P. sclerotioides* when screening for resistance for SBS.

The proposed study will identify the minimum set of isolates needed to breed alfalfa for effective *P. sclerotioides* resistance and will investigate which *P. sclerotioides* biotypes contribute to SBS. It will address the New York State IPM Program priority of developing tools to manage SBS and BRR of alfalfa, and it will contribute to the Northern New York Agricultural Development Program priority of improving management of BRR.

4. Objectives and Anticipated Impacts

Objectives:	*Anticipated Outcomes:*	*Anticipated Impacts:*
1. Evaluate which biotypes of *P. sclerotioides* contribute to SBS.	▪ Identification of candidate *P. sclerotioides* isolates for use in screening for SBS resistance. ▪ Improved understanding of the etiology of SBS in the Northeast.	▪ Alfalfa bred for *P. sclerotioides* resistance will exhibit more effective SBS resistance in the field.
2. Identify the minimum set of *P. sclerotioides* isolates needed to breed alfalfa for effective resistance to *P. sclerotioides*.	▪ Identification of a set of *P. sclerotioides* isolates that represents all major virulence differences found across the *P. sclerotioides* species complex. ▪ Use of this set of isolates to breed for resistance will result in alfalfa varieties with stronger, more effective resistance to SBS and BRR.	▪ Alfalfa bred for *P. sclerotioides* resistance will exhibit effective resistance regardless of the composition of the local *P. sclerotioides* population.
3. Disseminate research findings.	▪ Alfalfa breeders serving the Northeast will be informed of the results and will have access to a full set of *P. sclerotioides* isolates for breeding. ▪ Findings will be published in a refereed journal (Plant Disease or equivalent).	▪ Alfalfa breeders will modify their screening protocols to reflect the role of *P. sclerotioides* in SBS and BRR etiology.

Anticipated impacts with respect to the mission of the Northeast IPM Center:

A. *Safeguarding human health and the environment*

- The proposed study will facilitate the development of alfalfa with stronger *P. sclerotioides* resistance. The availability of alfalfa with improved *P. sclerotioides* resistance would be expected to increase the production of alfalfa relative to alternatives such as corn for silage, thereby reducing fertilizer inputs, pesticides, and erosion associated with the alfalfa alternatives. Up to 3.83 million acres in the Northeast – 2.46 million acres planted to alfalfa and 1.37 million acres planted to corn for silage (Anonymous 2002) – could be affected.

B. *Promoting economic benefits*

- The proposed study will contribute to increased profitability of alfalfa and, consequently, milk production in the Northeast. By facilitating the development of alfalfa with improved SBS and BRR resistance, it will lead to improved alfalfa yields and stand persistence.

- Alfalfa growers will benefit from this proposal. In the Northeast, the 16,800 alfalfa growers (Anonymous 2002) in the northern states of Maine, New Hampshire, New York and Vermont, where *P. sclerotioides* is most widespread, will reap the greatest benefits and be the most satisfied with research leading to varieties with improved SBS and BRR resistance.

C. *Furthering IPM implementation*

- The identification of a set of *P. sclerotioides* isolates representing all major virulence phenotypes will enable breeders to develop varieties with *P. sclerotioides* resistance that is effective across broad geographic regions. The varieties will exhibit resistance regardless of local differences in the composition of *P. sclerotioides* populations.
- Improved resistance to *P. sclerotioides* in resulting alfalfa varieties will increase the use of resistant varieties as a tool for managing plant diseases.
- The study will help address the New York State IPM Program priority of developing tools to manage SBS and BRR of alfalfa and contribute to the Northern New York Agricultural Development Program priority of improving management of BRR.

5. Approach and Procedures

Objective 1: Evaluate which biotypes of *P. sclerotioides* contribute to SBS.

In May 2009, alfalfa exhibiting symptoms of spring black stem and leafspot (SBS) will be collected from ten fields in northern New York (Clinton County) and five fields in central Vermont (Addison County). In both regions, isolates from all three *P. sclerotioides* subtypes are known to contribute to brown root rot (BRR) of alfalfa (figure 2). In each field, approximately 30 to 40 plants will be collected across multiple sampling sites. A parallel study (not funded by NE-IPM) will be conducted in central Saskatchewan (near Saskatoon).

- Pathogen isolation: Five to eight symptomatic leaflets and one to two symptomatic upper stem sections (at least 10 cm above crown) will be collected from each plant, surface sterilized, and plated onto water agar. Petri dishes will be incubated at 10˚C for 3 months under continuous light (Hollingsworth et al. 2003, Wunsch et al. 2007) and examined for the production of pycnidia characteristic of *P. sclerotioides* and *P. medicaginis*. The percentage of plants with stems and/or leaves colonized by *P. sclerotioides* and by *P. medicaginis* will be recorded.
- Identification of *P. sclerotioides* by subtype and clade: Single-conidium isolates will be established from pycnidia characteristic of *P. sclerotioides*. The subtype and clade (figure 2) to which each *P. sclerotioides* isolate pertains will be assessed by culture morphology on potato dextrose agar and by end-point PCR using clade-specific diagnostic primers developed from sequence polymorphisms at the nuclear rDNA internal transcribed spacer and intron-spanning regions of actin, alpha tubulin, beta tubulin, glyceraldehyde 3-phosphate dehydrogenase, histone, and phosphate permease loci (Wunsch, unpublished).

Objective 2: Identify the minimum set of isolates needed to breed alfalfa for effective resistance to *P. sclerotioides*.

Replicated experiments will be conducted in controlled environments to evaluate ten *P. sclerotioides* isolates and a control (uninoculated) treatment on each of three alfalfa varieties.

- Alfalfa varieties: Varieties Peace (Richardson Seeds), Starbuck (Pickseed), and WL 347 LH (W-L Research) will be evaluated. Replicated field trials (table 1) suggest that Peace is resistant to *P. sclerotioides* biotype 1 and relatively susceptible to biotype 5; that Starbuck is susceptible to *P. sclerotioides* biotype 5 and relatively resistant to biotype 1; and that WL

347 LH is relatively resistant to both biotypes. Pickseed, Richardson Seeds, and W-L Research have granted permission to use these varieties in this study.

- *P. sclerotioides* isolates: Two isolates from each of *P. sclerotioides* biotypes 1, 3, and 5 and one isolate from each of *P. sclerotioides* biotypes 2, 4, 6, and 7 will be used for inoculations. Biotypes 1, 3, and 5 are the most broadly distributed biotypes in the Northeast.
- SBS experiment: Individual plants will be established in SC10 Cone-Tainers (21 cm deep, 3.8 cm diameter; Stuewe and Sons, Inc., Corvallis, OR). Six weeks after establishment, a spore suspension of $1x10^6$ conidia/ml in 0.5% (v/v) Tween 80 or a control (0.5% Tween 80) will be applied with an airbrush until runoff from leaves (Salter and Leath 1991). Conidial viability will be assessed on water agar plates. Plants will be incubated in complete darkness and high relative humidity for 48 hours and then kept at 17˚C under 16 hours light/day. Two weeks after inoculation, disease severity of the top three and bottom three compound leaves will be assessed on a 0 to 11 scale: 0 = no disease, 1 = 1 to 10% of leaf area diseased, 2 = 11 to 20% of leaf area diseased, … , 10 = 91-100% of leaf area diseased.
- BRR experiment: Individual plants will be established in SC10 Cone-Tainers (21 cm deep, 3.8 cm diameter). Four months after plant establishment, four barley grains colonized by *P. sclerotioides* or four sterile barley grains (control) will be placed against the tap root 3 cm below the soil level. Plants will be kept at 17˚C for 1 week, at 4˚C for 2 weeks, at -2˚C for 9 weeks, at 4˚C for 4 weeks, and at 17˚C for 1 week; day length will be maintained at 16 hours light/day. At the conclusion of the experiment, root rot severity will be assessed on a 0 to 11 scale: 0 = no disease, 1 = 1 to 10% of root or crown diameter diseased, 2 = 11 to 20% of root or crown diameter diseased, … , 10 = 91-100% of root or crown diameter diseased.
- Evaluating possible cross-contamination: To confirm that cross-contamination of *P. sclerotioides* isolates has not occurred across treatments, re-isolation of *P. sclerotioides* will be attempted from leaves and stems (SBS experiment) and roots and crowns (BRR experiment) of all plants, regardless of symptoms. The biotype of recovered *P. sclerotioides* isolates will be identified by culture morphology on potato dextrose agar, and cross-contamination will be confirmed by end-point PCR of recovered isolates (see objective 1). Plants affected by cross-contamination will be excluded from the final data set.
- Experimental replicates and sample sizes: The SBS and BRR experiments will be repeated three times. In both experiments, 36 plants (12 plants in each replicate) will be tested in every alfalfa variety x treatment (*P. sclerotioides* isolate or control) combination.
- Statistical analysis: In both experiments, disease severity data will be analyzed with logistic regression implemented in PROC GENMOD (SAS, version 9.13, SAS Institute, Cary, NC). Wald Chi-square estimates will be used to contrast each inoculated treatment with the corresponding control; the Bonferroni correction will be used to control the Type I error rate.

Objective 3: Disseminate research findings.

Research findings will be communicated directly with current and recent collaborators at alfalfa seed companies, including Allied Seed, Pickseed, Pioneer, Preferred Seed, Seedway, and W-L Research, and will be published in a refereed journal. A fact sheet presenting key findings will be sent to extension personnel in Maine, New Hampshire, New York, Pennsylvania, and Vermont. Findings will also be presented at winter extension meetings in New York.

Timeline:		
Objective	Task(s)	Date
1	Collection, laboratory processing of field samples	May 2009
1	Evaluation of cultures for *P. sclerotioides, P. medicaginis* pycnidia; establishment of single-conidium isolates	Aug. 2009
1	Morphological and PCR assessment to identify *P. sclerotioides* isolates by subtype and clade	Oct. 2009
2	Plant establishment, SBS experiment replicates 1, 2, and 3	June, July, Aug 2009
2	Inoculation, SBS experiment replicates 1, 2, and 3	July, Aug, Sept 2009
2	Data collection, SBS experiment replicates 1, 2, and 3	Aug, Sept, Oct 2009
2	Plant establishment, BRR experiment replicates 1, 2, and 3	Jan, Feb, Mar 2009
2	Inoculation, BRR experiment replicates 1, 2, and 3	May, June, July 2009
2	Data collection, BRR experiment replicates 1, 2, and 3	Aug, Sept, Oct 2009
2	Data analysis	Nov 2009
3	Preparation, submission of manuscript for publication	Nov to Dec 2009
3	Dissemination of results to breeders and extension personnel	Dec 2009 - Feb 2010

6. Evaluation Plans

Objective 1: Evaluate which biotypes of *P. sclerotioides* contribute to SBS.

A successful result will be defined, as follows: All alfalfa samples are collected when temperatures are cool (10 to 20˚C; temperatures at which *P. sclerotioides* can be successfully isolated from plant tissues). Sampled plants have SBS symptoms, and *P. sclerotioides* is isolated from symptomatic leaves and stems.

Objective 2: Identify the minimum set of isolates needed to breed alfalfa for effective resistance to *P. sclerotioides*.

A successful result will be defined, as follows: The relative virulence of the ten *P. sclerotioides* isolates to leaves/stems and to roots/crowns is identified. Plants given the control treatment do not develop SBS or BRR, and *P. sclerotioides* is not recovered from stems/leaves or roots/crowns of plants in the control. Across inoculated treatments, little or no cross-contamination of isolates is detected.

Objective 3: Disseminate research findings.

A successful result will be defined, as follows: Major findings of this study are reported to alfalfa breeders in North America, and breeders modify their SBS and BRR screening protocols accordingly. Major findings are communicated to extension personnel and growers in the Northeast, and growers express increased interest in planting alfalfa varieties with elevated resistance to SBS and BRR. Results are published in a refereed journal.

Contributions to the mission of the NE-IPM Center: Safeguarding human health and the environment, promoting economic benefits, furthering IPM implementation.

The anticipated development and adoption of alfalfa varieties with improved SBS and BRR resistance cannot be evaluated within the time frame of this study. A successful result from this study will be defined, as follows: (1) The minimum set of *P. sclerotioides* isolates needed to

breed alfalfa for effective SBS and BRR resistance is identified, and (2) alfalfa breeders demonstrate interest in modifying their SBS and BRR screening protocols by inquiring about obtaining appropriate *P. sclerotioides* isolates.

7. Key Personnel

Michael Wunsch (Ph.D. candidate, Dept. of Plant Pathology and Plant-Microbe Biology, Cornell Univ.) will execute the experiments, laboratory work, and statistical analyses. He will also prepare academic and extension publications summarizing the research findings and disseminate the findings to alfalfa breeders and to extension personnel. **Gary Bergstrom** (professor, Dept. of Plant Pathology and Plant-Microbe Biology, Cornell Univ.) will advise M. Wunsch in all aspects of the work, edit academic and extension publications, and help disseminate research findings to stakeholders. **Catherine Ballard** (director of research, W.H. Miner Agricultural Research Institute, Chazy, NY) and **Heather Darby** (extension professor, University of Vermont) will assist in the field study assessing which biotypes of *P. sclerotioides* are associated with SBS (objective 1). They will help identify alfalfa production fields that M. Wunsch will sample. A parallel field study (not funded by NE-IPM) assessing which biotypes of *P. sclerotioides* are associated with SBS will be conducted in Saskatchewan, Canada by **Bruce Gossen** (research scientist, Agriculture and Agri-Food Canada, Saskatoon, SK).

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