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# Simplicillium lanosoniveum, a mycoparasite of Phakopsora pachyrhizi and its use as a biological control agent

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*SIMPLICILLIUM LANOSONIVEUM*, A MYCOPARASITE OF *PHAKOPSORA PACHYRHIZI*  
AND ITS USE AS A BIOLOGICAL CONTROL AGENT

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
In partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

In

The Department of Plant Pathology and Crop Physiology

By  
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B.S., Louisiana State University, 2007  
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## ABSTRACT

In 2007, a filamentous fungus was recovered from sori of soybean rust (SBR), caused by *Phakopsora pachyrhizi*, collected from Louisiana and Florida. This fungus was identified as *Simplicillium lanosoniveum* on the basis of ITS sequence data and morphological traits. *Simplicillium lanosoniveum* was found coiling within sori and around urediniospores and showed a trophic attraction to rust sori, extending from sorus to sorus. In co-inoculated soybean leaves, the fungus did not grow or establish on leaf surfaces until sori erupted. Similarly, *S. lanosoniveum* colonized within 3 days and sporulated within 4 days on leaves showing disease symptoms. In field studies, when soybean leaves were inoculated with *S. lanosoniveum* during the latent stages of infection of SBR, disease progression was significantly limited. Additionally, sori became increasingly red-brown, which appeared to represent accelerated aging of sori. In the presence of *S. lanosoniveum*, urediniospores turned brown and failed to germinate. To examine the mode of action by which *S. lanosoniveum* antagonized urediniospores, we used scanning and transmission electron microscopy as well as confocal microscopy to characterize the interaction. Putative penetration points were observed over germ pores, and hyphae penetrated urediniospores through germ pores within the first 24 hours. By the third day, hyphae exited urediniospores and sporulated on the surface of colonized urediniospores. These studies provide evidence of a mycoparasitic interaction between *S. lanosoniveum* and *P. pachyrhizi*. Implications of this mycoparasitic relationship include potential use of *S. lanosoniveum* as a component of an integrated pest management program or as a biological control agent in organic soybean production.

## CHAPTER 1. INTRODUCTION

Soybean, *Glycine max* (and its wild ancestor *Glycine soja*) has been used in China for over 5000 years [30]. The seed was used as a food source and as a component of medicinal remedies, while the plant was used in crop rotations as a source of nitrogen in soil. Widespread commercial production in the US did not begin until the 1970s, and it has become increasingly important over the last 40 years. Today, soybean is used primarily for its oil and lecithin, which are found in food products such as chocolate, peanut butter, ice cream, margarines and dressings, and frozen and canned foods. Industrial uses include paints, plastics, and even diesel fuel. The seed meal that remains after oil and lecithin extraction is used as a protein source for humans and livestock [31].

Soybean represents 53% of world oilseed production. The US is the largest producer of soybean, producing 38% of the world's supply [2]. In 2010, 77.5 million acres of soybean were planted in the US, and the United States Department of Agriculture projects increases in 2011. Soybean acreage is second only to corn in the US [1]. Worldwide, soybean is the world's fourth largest crop by planting area, with an estimated 95 million hectares planted in 2007 [32].

Soybean is subject to a variety of fungal and bacterial diseases, viruses, and nematodes. In fact, soybean disease loss reports are over 10% worldwide and over 15% in the US [102]. In recent years, one of the most severe diseases in the southern US was soybean rust (SBR). Soybean rust, also known as Asian soybean rust, is caused by the fungus *Phakopsora pachyrhizi* Syd. & P. Syd., which was first reported in Japan in 1902. Within 30 years, the pathogen spread as far north as Siberia and North Korea and throughout much of Asia and Australia. By 2001, SBR

was prevalent throughout Africa and was discovered in South America. Hurricane Ivan is believed to have carried spores of *P. pachyrhizi* from South America into the United States in 2004 at which time it was discovered at the Louisiana State University Agricultural Center's Ben Hur Research Farm near Baton Rouge, LA, the first report of the disease in the continental US [46, 84]. SBR has become endemic in tropical and subtropical soybean-growing regions around the world [73].

The obligate basidiomycete fungus produces both urediniospores and teliospores, but the aecial stage has never been observed. Teliospores are not often observed in nature, and sexual reproduction has never been reported. However, teliospore germination and basidiospore formation have been induced under laboratory conditions [81]. It is not known whether *P. pachyrhizi* is autoecious or heteroecious because these basidiospores are not known to infect, and the alternate host is not known. Thus, only urediniospores are known to be functional, and continued production of urediniospores on a suitable host is required for continuation of the disease cycle.

Soybean rust is a polycyclic disease that is maintained by asexual urediniospores. These urediniospores germinate 1-4 hours after deposition at optimal temperatures of 17°C to 27°C, and infection requires temperatures of 10°C to 27°C and at least 6 hours of free moisture from dew or rainfall [18, 40, 62]. Appresoria form penetration pegs, which penetrate leaf epidermal cells directly, unlike most rusts, which utilize stomates [51]. Seven to 10 days after infection, urediniospores erupt from uredinial sori under ideal conditions, 18°C to 24°C [18]. Extended periods of leaf wetness (18h) increase disease severity [49]. New lesions, often referred to as pustules, include sori and surrounding chlorotic or necrotic leaf tissue approximately 1 mm in diameter. These sori are volcano-shaped and mostly on the lower leaf surface. Severe infections

cause intense chlorosis and premature defoliation of soybean [52]. Soybean can be infected by *P. pachyrhizi* at all growth stages. In the field, the rate of symptom development is related to the physiological age of the plant [92, 103]. Typically, sori do not develop until the onset of flowering; however, it is more common for sori to develop at seed set.

Soybean rust occurs in tropical, subtropical, and temperate climates in the major soybean producing countries on six continents. *Phakopsora pachyrhizi* infects over 175 plant species, including *Glycine max*, other soybean relatives, as well as wild and edible legumes [22, 64, 70]. The primary host range of *P. pachyrhizi* includes members of the Fabaceae family, which serve as alternative hosts. One such legume is kudzu (*Pueraria lobata*), an invasive, weedy vine in the southeastern United States, and based on reports, kudzu is the primary source of inoculum in the US each spring [16, 22, 49, 62, 70-71].

Since its discovery in North America in 2004, *P. pachyrhizi* has been documented to overwinter throughout the Gulf South. Reports of SBR increased annually between 2005 and 2009, with disease spreading to 10 to 20 states each year [96]. This cycle was broken in 2010 when unseasonably cold winter temperatures destroyed overwintering inoculum sources throughout the Gulf South region. In early January 2010, temperatures were approximately 12° C below average. This greatly reduced the amount of green kudzu in the southeastern US, and SBR was not reported in kudzu from January through April 2010. Consequently, the first report of SBR on kudzu was in May, and soybeans were only affected in 37 counties across 7 states in 2010 [96]. Disease pressure was greatly reduced, and reported yield losses in the Gulf South were less than 10% [102].

Despite the spread of disease into major soybean-producing states between 2005 and 2009, disease losses never reached epidemic levels in the major soybean-producing regions of the US. Soybean rust does not overwinter in northern or Midwestern states, and inoculum, so far, reached these areas too late in the growing season for disease epidemics to establish [28, 42].

Worldwide crop losses caused by SBR have been reported to range from 10-90%, though there are reported losses as high as 100 percent in Taiwan and Africa [40, 50, 73, 90, 106-107]. Yield loss reports in the southeastern US are as high as 35 to 40% in Louisiana and as high as 82% in Florida on susceptible varieties that were not sprayed with fungicides (Schneider and Walker, personal communication). Overall, however, yield losses never reached levels indicated in predictive models, which anticipated losses greater than 10% in nearly all the U.S. soybean-growing areas and up to 50% in the Mississippi Delta and the Gulf Coast [74, 105].

Ideally, disease management would be more efficient with resistant cultivars. However, commercial cultivars resistant to *P. pachyrhizi* are not yet available to growers [41]. Partial resistance to *P. pachyrhizi* has been identified, but there are no soybean cultivars or breeding lines with complete resistance to all SBR isolates [41]. Five accessions have been identified, each of which show resistance to one or more isolates of *P. pachyrhizi*. Results confirmed the existence of five major resistance genes (*R* genes), *Rpp1*, *Rpp2*, *Rpp3*, *Rpp4* and *Rpp5* [17, 21, 65, 98]. Breeders also are selecting for tolerance, which may lead to reduced yield losses [41]. Furthermore, assessment of sources for resistance to *P. pachyrhizi* has been expanded to close relatives of soybean, such as perennial *Glycine* spp. [41].

Until resistant cultivars are available to protect commercial soybeans, growers use sentinel plots as early predictive systems. Preventative fungicides are the primary method of protection against

SBR in the southern US, but timing of these preventative applications depends heavily on early disease detection [96]. Despite these applications of preventative fungicides, SBR continued to affect soybean in the South [67, 85]. Furthermore, inoculum buildup in the South increases the possibility of disease establishment in the Midwestern soybean belt. These concerns led to increased studies of aerial dispersal of urediniospores and disease forecasting models as well as sophisticated electrostatic spore traps [47, 86]. Researchers and growers continue to utilize sentinel plots to track disease progress.

Researchers have evaluated combinations of preventative fungicide treatments for SBR and other diseases. Before the introduction of SBR, late-season diseases such as *Cercospora* leaf blight (CLB), frogeye leaf spot, and stem and pod diseases were routinely treated with strobilurins and benzimidazole fungicides. However, thiophanate-methyl is ineffective against SBR, and strobilurins do not have curative properties against the disease. Triazole fungicides, on the other hand, have been reported to be effective for the management of SBR but ineffective against most late season diseases. Studies continue to investigate more efficient ways to concurrently manage this spectrum of late-season diseases. For example, in 2010, field studies in Baton Rouge, LA concluded that early applications of triazole fungicides reduced the severity of CLB (C. L. Robertson, unpublished). These results indicated that SBR and CLB may have similar requirements for effective control. Management of the two most important soybean diseases with one fungicide regime would be a breakthrough for growers in the southeast.

As previously described, preventative fungicide applications are critical for the management of SBR in the southern US. Because of a probable extended latent infection stage and the need for early fungicide applications, growers in the South rely heavily on preventative fungicide applications for control of SBR. Thus, fungicide resistance management is a critical

consideration. Rotation of fungicides with different modes of action is the most common defense against fungicide-resistant pathogens. Overall, pathogens have a greater tendency to develop resistance to strobilurins than to triazoles, but baseline sensitivity studies for triazoles are ongoing (R. W. Schneider, unpublished).

### **Dissertation Research**

There is still much to be learned about the biology and epidemiology of SBR. Disease symptoms were observed in August 2007 in soybean research plots at the Louisiana State University Agricultural Center's Ben Hur Research Farm. Diseased leaves were collected on a weekly basis for use in various research projects. Detached leaves were stored at room temperature in plastic boxes lined with moist paper towels to promote sporulation. Additionally, diseased leaves were collected from the University of Florida's North Florida Research and Education Center in Quincy, Florida for other studies.

On August 2007, upon examining 4-day-old sporulating sori under 25x magnification with a dissecting microscope, fungal hyphae were observed colonizing sori of SBR. These hyphae were confined to sori that did not have surrounding necrotic leaf tissue. Approximately 10-15% of the non-necrotic sori had such fungal growth. Sori with necrotic margins also had fungi growing within and around them. However, these fungi were clearly different from the aforementioned in that they had large branching conidiophores, and hyphae were suspended above sori as well as around the outer margins of SBR lesions. Hyphae of both types were plated onto potato dextrose agar for characterization.

Within 3 days, fungi from the two types of lesions had distinctive morphological characteristics. The fungus confined to sori grew into a whitish cream-colored culture with small conidia borne



on single phialides. The other fungus, which encompassed both sori and surrounding tissue, grew rapidly and developed into a dark culture with large conidia borne on branched conidiophores.

Meanwhile, sampling of soybean leaves from several soybean fields continued from commercial fields in Louisiana, and from research farms in Louisiana and Florida. Sori from SBR on kudzu also were sampled. The resulting collection contained over 80 isolates from research farms in Louisiana and Florida. Commercial soybeans, which are routinely sprayed with fungicides to prevent SBR and other late-season diseases, yielded no mycophilic fungal isolates.

Preliminary studies included inoculation of diseased soybean leaves with two isolates each of the white and black fungi at different concentrations of conidia. After 10 days, only the white isolates colonized sori. Scanning electron microscope observations showed this fungus, unknown at that time, colonized sori and did not establish on disease-free leaf surfaces or within disease lesions of other fungi. Hyphae intertwined within sori and coiled around individual urediniospores. There was apparently a trophic response, and this fungus appeared to be mycotrophic. As described in the following chapters, this fungus, identified as *Simplicillium lanosoniveum*, is mycoparasitic and has potential to be used as a biological control agent [109]. The black cultures that did not colonize sori were identified as *Cladosporium* spp., and we concluded that they were secondary invaders of the necrotic tissue.

Following these observations, questions were posed as to whether the mycophilic fungus, *S. lanosoniveum*, was mycoparasitic and whether it affected SBR disease development.

The objectives of this doctoral research project include:

1. Determine the identity of fungus described above.
  - This is a first report of the association of *Simplicillium lanosoniveum* with sori of SBR and the ecological relationship between the two fungi.
2. Determine whether *S. lanosoniveum* affects disease development.
  - To determine whether this fungus affected disease, sorus production, or urediniospore production, detached leaves were inoculated with *S. lanosoniveum* and *P. pachyrhizi*.
3. Determine whether *S. lanosoniveum* has antagonistic properties under field conditions.
  - After successfully reducing numbers of sori in detached leaf assays in the laboratory, the study was expanded to include three field tests in a two-year period.
4. Explore mode of action of *S. lanosoniveum* through various microscopic techniques.
  - Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy were utilized to determine the mode of action by this putative mycoparasitic fungus.
5. Examine the host range of *S. lanosoniveum* through a survey other rust species.
6. Determine whether the brown urediniospores that developed in sori have increased resistance to ultraviolet light.
7. Determine the extent of the latent infection period of SBR.

## CHAPTER 2. COLONIZATION OF SOYBEAN RUST SORI BY *SIMPLICILLIUM LANOSONIVEUM*

### 2.1 Introduction

Soybean rust (SBR), caused by *Phakopsora pachyrhizi* Syd. & P. Syd., was first reported in Japan in 1902. The disease reached Africa in 1997, South America in 2001, and the US in 2004 [63, 84]. Before becoming established in the Gulf South of the southeastern US, researchers speculated that the disease would spread into the Midwest and cause devastating yield losses as seen in Africa, South America, and Southeast Asia [56, 73-74, 104]. Recent reports describe yield losses exceeding 80% on highly susceptible varieties in Florida and as much as 100% in Asia and South America (D.R. Walker, personal communication) [30, 76]. The disease is endemic in the Gulf South, but it has not yet become widespread in the Midwest or other major soybean growing areas of the US [96]. Yield losses are caused by reduced rates of photosynthesis and defoliation [52].

The only known reproductive stage of *P. pachyrhizi* is the uredinial stage. Telia have been reported, but an alternate host is not known [22, 39]. Uredinial sori develop within 7 to 10 days of inoculation at temperatures between 18°C and 27°C and high humidity. Although soybean is the most common uredinial host, over 50 other leguminous hosts have been documented, including kudzu (*Pueraria lobata*) on which the pathogen overwinters in the southeastern US [87]. Each year, SBR survives in Florida and Alabama on kudzu, thus providing early sources of inoculum for soybean in southern states [49, 96]. In the US, symptoms of SBR on soybean are

first observed during host mid to late reproductive stages, although infection occurs much earlier [107].

In 2007, while examining rust-infected soybean leaves that had been maintained in a moist chamber, hyphae were repeatedly observed within sori of SBR and intertwined around urediniospores. Fungal growth was clearly associated with sori but absent on healthy leaf surfaces, and the fungus seemed to exhibit a trophic response to SBR. The objectives of this study were to identify this mycophilic fungus and to determine whether SBR sori are a preferred habitat. Herein, a previously unknown association between *P. pachyrhizi* and the mycophilic filamentous fungus *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001 are described.

## **2.2 Methods**

### **2.2.1 Identification of Colonists**

Field-grown soybean leaves infected with *P. pachyrhizi* were collected from Louisiana and Florida in 2007, 2008, and 2009 for isolation of filamentous mycophilic fungi. In 2007, fungal inhabitants were recovered from infected soybean leaves from four fields in mid- to late-August from the Ben Hur Research Farm of the LSU Agricultural Center near Baton Rouge, LA and from three samplings in August from the University of Florida, North Florida Research and Education Center near Quincy, FL. None of these plants was treated with fungicides. Isolation attempts were also made from five commercial soybean fields near Baton Rouge, Louisiana in 2007. In 2008 and 2009, isolations were attempted from soybean leaves collected from approximately 20 commercial fields from five parishes (counties) in central and south Louisiana. Disease severity ranged from zero to 10% diseased leaf area in commercial fields. In 2009, we

also attempted a total of 50 isolations from diseased soybeans collected from Alabama, Florida, Mississippi, and Oklahoma. Disease severity ranged from 25% to 50% affected leaf area. After incubating leaves in moist chambers (plastic boxes lined with moist paper towels) at room temperature (26°C to 28°C) for 1 to 3 days, hyphae were transferred from within sori of *P. pachyrhizi* onto 1.5% potato dextrose agar (PDA; DIFCO; Beckton, Dickinson, and Company; Franklin Lakes, NJ ) using a needle and a dissecting microscope.

After 7 days, each culture was single-spored by streaking conidial suspensions onto 1.5% water agar and selecting single colonies after 24 hours with the aid of a dissecting microscope. One isolate from each locale from each sampling date was selected for further experimentation. In order to determine whether inhabitants within sori differed from those in proximity to sori, the same procedures were used to recover filamentous fungi from the edges of SBR sori, symptomless soybean leaf surfaces, lesions of frog-eye leaf spot (*Cercospora sojina*) and brown spot (*Septoria glycines*), as well as from senescing soybean leaves.

All seven isolates of the mycophilic fungus, four from Louisiana and three from Florida, were selected for DNA analyses. Genomic DNA was extracted from mycelia from each 14-day old culture using the Promega Wizard Genomic DNA Purification Kit (Promega Corp.; Madison, WI) following a modified protocol (Aime, unpublished). DNA was diluted up to 1:100 based on band strength after electrophoresis on 1% agarose gel. Promega PCR Master Mix (Promega Corp.; Madison, WI), and internal transcribed spacer primer pairs ITS1-F and ITS4 were used for DNA amplification [36]. PCR product was adjusted to 10ng / $\mu$ l and purified with Millipore Montage PCR Centrifugal Filters (Millipore; Billerica, MA). PCR products from each isolate were sequenced at the DNA Sequencing Core/ ICBR (University of Florida; Gainesville, FL) with the same primers used for amplification. Sequences were analyzed by BLASTn

(<http://www.ncbi.nlm.nih.gov>) and sorted by maximum identity [6, 69]. Three isolates were resequenced for confirmation.

### **2.2.2 Verification of Colonization**

To verify colonization of sori, one isolate each from Louisiana and Florida was selected for co-inoculations. Because *P. pachyrhizi* is an obligate parasite, assays were performed *in vivo* with detached leaves as described below. Soybean cultivar Asgrow AG5903 (Monsanto; St. Louis, MO) was grown outdoors in 22-cm diameter pots with supplemental irrigation and fertilized with slow release 13-13-13 fertilizer (Osmocote; Scotts Miracle-Gro; Marysville, OH). When plants began to flower, leaflets were collected and transported in plastic bags (Ziploc®) to the laboratory. Samples were maintained at 20°C to 25°C during collection and transport.

Urediniospores of *P. pachyrhizi* were collected one week prior to co-inoculations from infected field-grown soybean plants at the Ben Hur Research Farm with a spore collector (G-R Manufacturing; Manhattan, KS) and stored dry at -30°C. To assess germination rate, urediniospores were rehydrated in humidity chambers for 12 hours, plated onto 1.5% water agar, and rated after incubation for 6 hours at 25°C in the dark [20]. They were considered germinated if the germ tube was at least as long as the longest dimension of the spore. A minimum of 100 urediniospores were counted, and spore collections with germination rates above 75% were used for co-inoculations.

For each treatment, each of four symptomless leaflets was marked with three 4 cm<sup>2</sup> sampling circles using a felt tip pen. Individual sampling circles were inoculated with spore suspensions of each of the two test isolates. Spore suspensions were prepared by flooding Petri dish cultures with sterile phosphate buffer (0.5 mM, pH 7.1), gently rubbing the colony surface with a glass

rod, then filtering the suspension through three layers of cheese cloth to remove mycelial fragments. Spore suspensions were adjusted to 90,000 spores per ml with a hemacytometer in 0.01% Tween 20<sup>®</sup>. Approximately 1,800 spores of each of the test fungi were applied to each sampling area in 20 µl droplets with a micropipettor, which was equivalent to 450 spores/cm<sup>2</sup>. After inoculation with the test isolates, leaves were allowed to dry for one hour. Next, suspensions of urediniospores of *P. pachyrhizi* were introduced by pipetting 20 µl of a urediniospore suspension into each sample area to give a final inoculum density of 450 urediniospores/cm<sup>2</sup>. Leaves were allowed to dry for 1 hour and then incubated in moist chambers at 23°C to 26°C with a 12-hour photoperiod under cool white fluorescent lights (800-1000 lux) for 14 days. Relative humidity remained at or above 98% for the duration of the experiment. Other treatments included sterile buffer only, buffer with 0.01% Tween 20<sup>®</sup>, individual test isolates in buffer and Tween 20<sup>®</sup> but without *P. pachyrhizi*, and urediniospores in 0.01% Tween 20<sup>®</sup> only. A nontreated control also was included. Fungi were re-isolated, and morphological characteristics were examined for verification. Experiments were replicated three times.

After 14 days, newly developed sori were prepared for examination using scanning electron microscopy (SEM). Leaf tissue was fixed in formalin acetic acid overnight then dehydrated in an ethanol series culminating in 100% ethanol [77, 82]. After critical point drying, each sample was mounted onto stubs and sputter coated with gold:palladium (60:40). Mounted samples were viewed and photographed with a JEOL JSM-6610LV SEM (JEOL, Ltd; Tokyo, Japan) at the Louisiana State University Socolofsky Microscopy Center.

### **2.2.3 Morphological Characterization**

Each of the test isolates was grown on cornmeal agar (BBL; Beckton, Dickinson, and Company; Franklin Lakes, NJ) for 10 days at 25°C in darkness for assessment of conidial size and shape, as well as length of phialides. Arrangement of phialides would separate *Simplicillium* from *Lecanicillium* [109]. Length and width of twenty-five spores were measured from each of five plates using Image Pro Express 6.0 (Media Cybernetics; Bethesda, MD) under 1000x magnification with a Leica (Wetzlar, Germany) compound microscope. The average size of conidia from each isolate was calculated. Twenty-five phialides from each of five plates also were measured and averaged.

Fungal growth rates were assessed following Zare and Gams [109]. Agar plugs (7 mm diameter) from 14-day-old cultures from each of the test isolates were placed at the center of Petri dishes containing 1.5% potato dextrose agar in order to measure radial growth as a function of temperature. Four replications of each isolate were incubated at temperatures from 15°C to 39°C at three-degree increments in the dark. After 7 and 14 days, measurements were recorded from two radii in each plate with a ruler and averaged.

## **2.3 Results**

### **2.3.1 Identification of Colonists**

Each of the seven isolates that was recovered from within sori of *P. pachyrhizi* was identified as *Simplicillium lanosoniveum* [97] Zare & W. Gams by BLASTn sequence analyses of the ITS region. All sequences were identical. Results revealed 99% identity with GenBank accessions EU284715.1 and FJ861375.1 of *S. lanosoniveum*. Sequences of two of these test isolates, one



each from Louisiana and Florida, were submitted to GenBank: isolate BH081707-1A from Louisiana (accession number HQ270477) and D082307-2A from Florida (accession number HQ270476).

Morphological examination revealed long, narrow, single phialides arising from aerial hyphae and oval or ellipsoid conidia adhered in globose heads. Cultures were high and white with a creamy yellow reverse (Fig 2.1). Conidia were oval, 1.5-3.0  $\mu\text{m}$  long and 0.75-1.5  $\mu\text{m}$  wide, appearing in globose heads on solitary phialides. Conidia of isolate BH081707-1A averaged 2.22  $\mu\text{m}$  long and 1.23  $\mu\text{m}$  wide, while conidia of D082307-2A averaged 2.43  $\mu\text{m}$  long and 1.30  $\mu\text{m}$  wide. There were no conidiophores present, and phialides ranged from 10 to 30  $\mu\text{m}$  in length.

After 14 days, *S. lanosoniveum* grew between 18°C and 33°C, with an optimum temperature range from 27°C to 30°C (Fig 2.2). After 14 days at 27°C, isolate BH081707-1A grew 6.7 cm in diameter, while isolate D082307-2A grew 6.0 cm. Neither isolate grew at 15°C or 36°C.

*Simplicillium lanosoniveum* was not recovered from commercial fields, nor was it recovered from sori of SBR in 2008 or 2009 at any location. When compared to fungi from elsewhere on leaf surfaces, we found that *S. lanosoniveum* mycelia were limited to SBR sori.

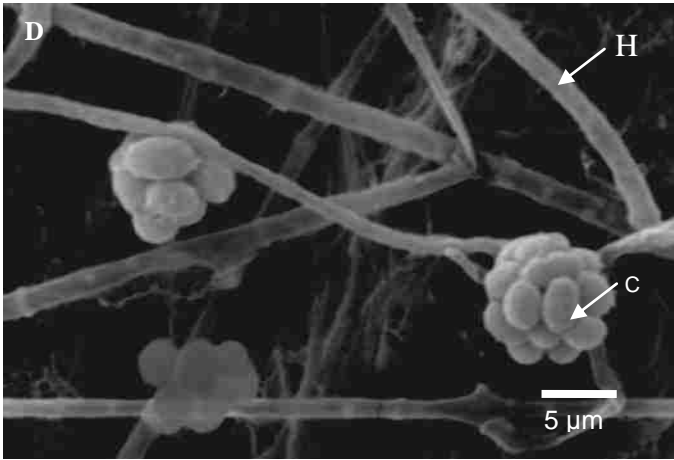
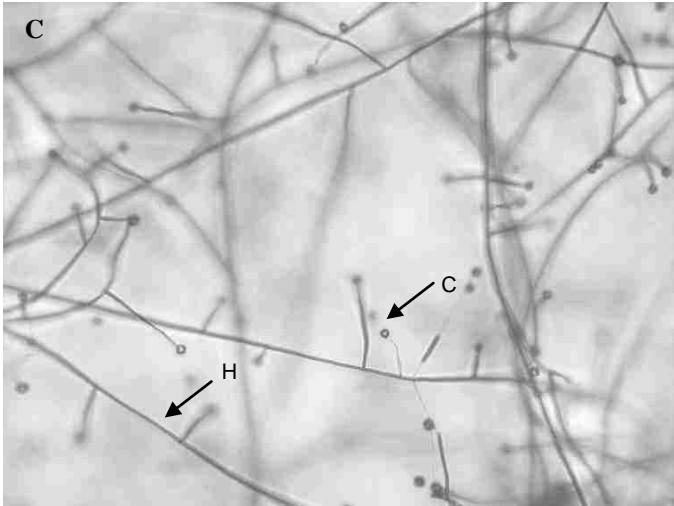
### **2.3.2 Verification of Colonization.**

After co-inoculating *S. lanosoniveum* and *P. pachyrhizi* onto symptomless soybean leaves, sori of SBR developed within 14 days of inoculation. Colonization by *S. lanosoniveum* was readily observed immediately upon sorus eruption and recovered in culture. Growth or colonization by *S. lanosoniveum* was not detected until sorus eruption 14 days after inoculation.



Fig 2.1. Upper (A) and lower (B) surfaces of a colony of *Simplicillium lanosoniveum* grown on potato dextrose agar. C Light microscope views of solitary phialides and conidial heads of *S. lanosoniveum* grown on for 7 days on cornmeal agar. D Scanning electron microscope view of conidial heads on 7-day old cultures. (C) conidia, (H) hypha

Fig 2.1 continued



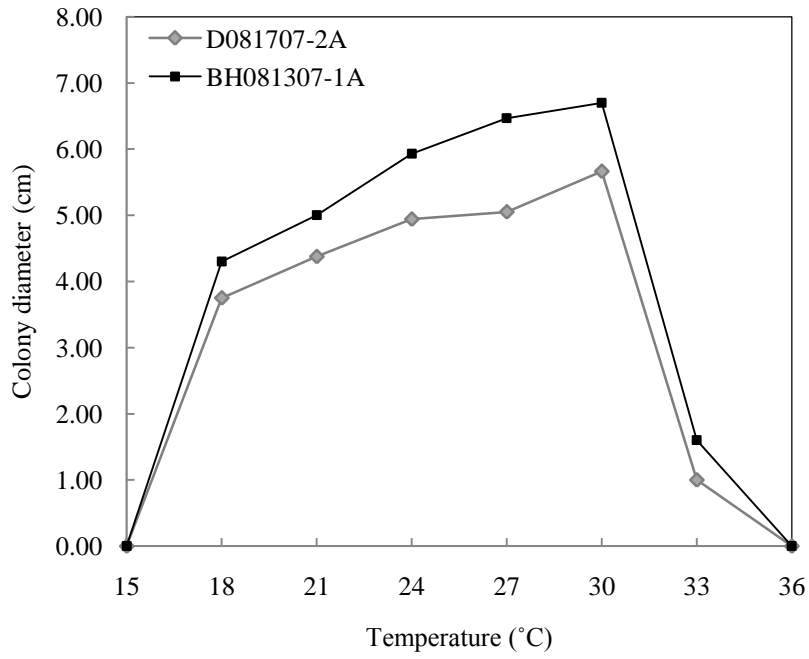


Fig 2.2. Growth responses of two isolates of *Simplicillium lanosoniveum* as affected by temperature. Colonies were grown on potato dextrose agar in the dark.

Light and SEM micrographs revealed heavy colonization and conidial formation by *S. lanosoniveum* (Fig 2.3). Furthermore, these examinations revealed numerous hyphae wrapping around urediniospores and colonizing sori, but, except for hyphal strands traversing the leaf surface, hyphae were not observed apart from sori. Hyphae seemed to exhibit a trophic response to urediniospores in that they preferentially colonized sori as compared to disease-free leaf surfaces (Fig 2.4). Microscopic observations of at least 300 sori revealed that about 90% of sori were colonized by the fungus 3-4 days after sorus eruption. Repeated experiments and observations yielded similar results.

## **2.4 Discussion**

When SBR was prevalent during the latter part of the 2007 growing season, *S. lanosoniveum* was readily recovered from sori in Louisiana and Florida. The fungus was not recovered from at least 100 examined leaves infected with SBR from either of the research farms or from commercial fields in 2008 or 2009, even though disease severity was above 10% and weather conditions were similar to that of 2007. One possible explanation is that *S. lanosoniveum* is not a normal component of the soybean phylloplane population and that it must be introduced.

Although *S. lanosoniveum* was easily recovered from sori of SBR in 2007 from nontreated soybeans from the two research farms, the fungus was not recovered from commercial fields in 2007, 2008, or 2009. This lack of recovery from commercial fields may be attributed to the widespread use of fungicides applied to protect soybeans from late-season diseases such as *Cercospora* leaf blight, frogeye leaf spot, aerial blight, pod and stem blight, and anthracnose [85].

Although not reported as an entomopathogen or mycoparasite, *S. lanosoniveum* has been recovered from the coffee rust pathogen, *Hemileia vastatrix*, and from scale insects on coffee (Centraalbureau voor Schimmelcultures; Zare and Gams 2001). Teleomorphs of *Simplicillium* are *Torrubiella* spp. (Cordyciptaceae) that are pathogens of spiders and scale insects [15, 25]. There are no documented cases in which *S. lanosoniveum* has been associated with *P. pachyrhizi*. On the other hand, *S. lanosoniveum* was recently reported to be the causal agent of brown spot on the aquatic fern *Salvinia auriculata* and *S. molesta* in Taiwan [27]. In our studies, *S. lanosoniveum* did not cause lesions or necrosis on soybean in either co-inoculated treatments or *Simplicillium*-only controls [100]. Our SEM observations revealed that the fungus was visible only on leaf surfaces when urediniospores were present. Otherwise, it did not establish or colonize leaf tissue, and lesions did not develop.

Questions arise about the origin of *S. lanosoniveum*. The fungus was not found in association with other soybean diseases or any of 26 other rust species in the US (Ward, unpublished). Accessions from CBS were isolated from coffee rust, other fungi, and scale insects from Venezuela, Sri Lanka, Puerto Rico, and Iran [25]. With the absence of *S. lanosoniveum* on other rusts and soybean diseases in south Louisiana, one possibility is that *S. lanosoniveum* co-disseminated with urediniospores of *P. pachyrhizi*. Furthermore, while we sampled various other rusts in 2008 and 2009, the preferred habitat of *S. lanosoniveum* in 2007 may have been rusts that were not present in 2008 and 2009. Likewise, because *Simplicillium* belongs to a group of fungi that often parasitize insects, a population of insects may have been the source in 2007, and again, not present or widespread in 2008 and 2009.

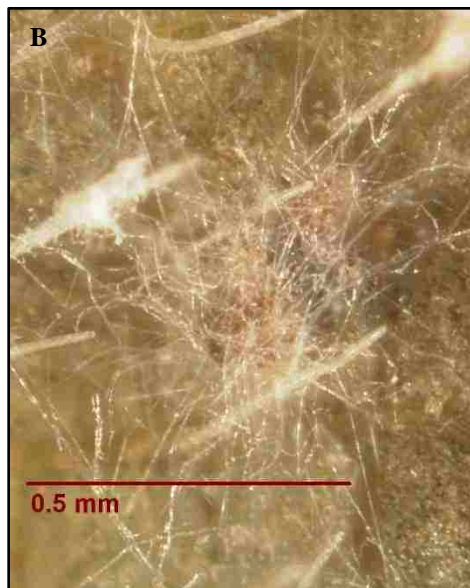
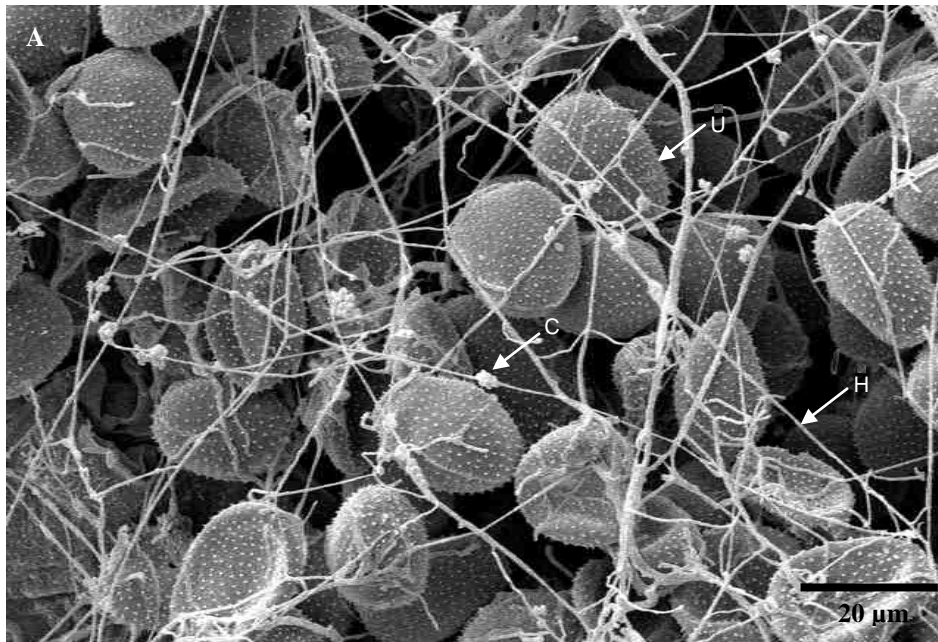


Fig 2.3. Microscopic views of colonization of sori of soybean rust by *Simplicillium lanosoniveum*. Scanning electron microscope (A) and light microscope (B) views of sporulating sori with hyphae of *S. lanosoniveum* four days after inoculation. (C) conidia, (H) hyphae, (U) urediniospore.

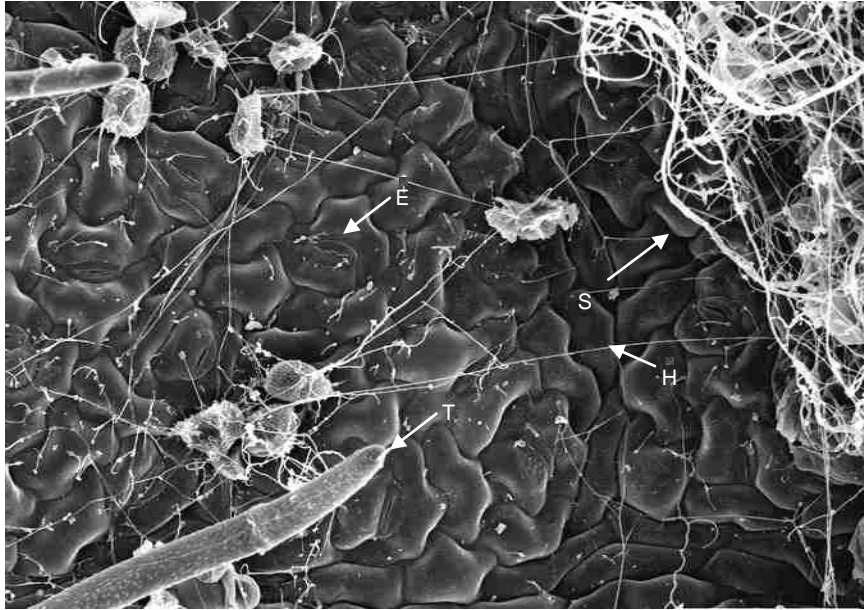


Fig 2.4. Scanning electron microscope view of soybean leaf surface with urediniospores of *Phakopsora pachyrhizi* and a sorus colonized by *Simplicillium lanosoniveum*. A portion of the sorus is visible in the upper right corner of the figure. Thickened hyphae often result from multiple hyphal strands. (E) epidermal cell, (H) hyphae, (S) sorus, (T) trichome.



SEM was used to verify colonization in SBR sori after each of the inoculations. These micrographs revealed not only colonization and trophic growth within sori, but also the absence of hyphae of *S. lanosoniveum* elsewhere on the phylloplane. Hyphae of *S. lanosoniveum* were found coiled tightly around urediniospores, but were not observed in association with fungal structures of other phylloplane fungi such as *Cercospora sojina* and *Cladosporium* spp., which are common inhabitants of the soybean phylloplane. It is also notable that *S. lanosoniveum* did not grow or establish on leaf surfaces until rust sori erupted on co-inoculated leaves. In the presence of urediniospores, *S. lanosoniveum* colonized over 90% of sori within 3 days and sporulated within 4 days. Based on these observations and experimental results, we conclude that sorus development is necessary for establishment of *S. lanosoniveum* on soybean leaf surfaces. We did not determine whether conidia germinated immediately upon inoculation onto leaf surfaces or after sorus eruption; however, *S. lanosoniveum* survived for 14 days following inoculation on leaf surfaces until sori erupted.

The nature of the interaction (i.e. mycoparasitism, cohabitation, and necrotrophic growth) between *S. lanosoniveum* and *P. pachyrhizi* is currently unknown. Mycoparasitism of *P. pachyrhizi* by *Verticillium psalliotae* was described by Saksirirt and Hoppe [78]. Additionally, similar mycoparasitic interactions were described on other rusts by *Verticillium*, *Cladosporium*, and other *Simplicillium* species [8, 38, 78]. The micrographs in these studies have striking similarities to the associations between the two fungi described in the present work.

In 2001, following the revision of the genus *Verticillium*, Zare and Gams (2001) created the genera *Lecanicillium* and *Simplicillium* and included them in the family Cordyciptaceae. This family, which also includes other anamorphic genera such as *Beauveria* and *Isaria*, contains primarily entomopathogenic ascomycetes [43, 89]. Although *S. lanosoniveum* is not reported as

an entomopathogen, *S. lamellicola* (F.E.V. Sm.) Zare & W. Gams was evaluated as a candidate entomopathogenic fungus for biological control of ticks and scale insects [75, 109]. Species of *Lecanicillium*, the sister taxon to *Simplicillium*, are often reported as entomopathogenic fungi associated with scale insects, ticks, whiteflies, and aphids [15, 29, 72]. Several species of *Verticillium*, which once included species now placed in *Simplicillium*, have been reported as parasites of cultivated mushrooms, pathogens of nematode eggs, and as parasites of rusts on bean, oat, coffee, and peanut, as well as *P. pachyrhizi* on soybean [37, 53, 55, 78, 80].

Surveys of other rusts and insects will continue. Future work includes development of a selective medium to quickly sample insects and rust-infected leaves. Furthermore, we will examine the effects of *S. lanosoniveum* on disease development in soybean. We will introduce the fungus to field-grown soybeans infected with SBR and monitor colonization throughout the season.

# CHAPTER 3. SUPPRESSION OF *PHAKOPSORA PACHYRHIZI*, CAUSAL AGENT OF SOYBEAN RUST, BY THE MYCOPHILIC FUNGUS *SIMPLICILLIUM LANOSONIVEUM*

## 3.1 Introduction

Soybean rust (SBR), caused by *Phakopsora pachyrhizi* Syd. & P. Syd., was discovered in the United States in 2004 [84, 96]. Since becoming established in the Gulf South, it was speculated that the disease would spread into the Midwest and cause devastating yield losses as seen in Africa, Asia, and South America [56, 104]. Recent reports documented yield losses of 35 to 40% in Louisiana and as much as 82% in Florida on soybeans that were not protected with a fungicide (R. W. Schneider, D. R. Walker – personal communication).

Research efforts in management of SBR have focused on applied methods such as developing fungicide application protocols. In spite of these efforts, control of SBR is not always feasible because of specific application requirements. Preventative applications of protectant fungicides must be accurately timed because they may not be effective if disease is already present at very low levels [85]. Furthermore, breeding efforts have yet to produce resistant cultivars [30].

Although there has been interest during the past 30 years in identifying microorganisms that are antagonistic to rust fungi, there are relatively few reports of such associations with *P. pachyrhizi* [78, 88, 108]. However, mycoparasitic interactions between *Verticillium psalliotae* (current name *Lecanicillium psalliotae* (Treschew) Zare & W. Gams), and *P. pachyrhizi* were reported in which *V. psalliotae* formed appresoria-like structures at possible infection sites on urediniospores [78, 109]. The primary mode of parasitism was reported to be degradation of urediniospores by

$\beta$ -glucanase, chitinase, and protease [79-80]. Other *Lecanicillium* spp. were reported as pathogens of aphids, scale insects, ticks, and whiteflies [5, 29, 57, 72]. *Simplicillium* spp. were reported in association with ticks, nematodes, and scale insects, as well as other rusts such as *Hemileia vastatrix* (coffee rust) and *Uromyces pencanus*. *Lecanicillium* and *Simplicillium* (both formerly *Verticillium* spp.) are included in the family Cordyciptaceae [89, 109], which also includes the anamorphic genera *Beauveria* and *Isaria*. This family consists of entomopathogenic and mycoparasitic ascomycetes.

In 2007, we observed the mycotrophic fungus *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001 intertwined within, around, and suspended above uredinial sori of *P. pachyrhizi*. Fungal growth was clearly associated with sori but absent on healthy leaf surfaces [101]. *Simplicillium lanosoniveum* has not been reported as either entomopathogenic or mycoparasitic, however, *S. lamellicola* (F.E.V. Sm.) Zare and Gams was studied for its entomopathogenic properties [75].

The objectives of this study were to evaluate the effects of *S. lanosoniveum* on SBR disease development, including effects on colonization of sori, sorus development, and viability of urediniospores.

## **3.2 Methods**

### **3.2.1 Co-inoculations of Disease-free Soybean Leaves**

Detached soybean leaves were used to investigate the effects of *S. lanosoniveum* on SBR sorus development. A susceptible soybean cultivar that is commonly planted for commercial production in Louisiana, Asgrow AG6202 (Monsanto; St. Louis, MO), was maintained at the

Ben Hur Research Farm of the Louisiana State University Agricultural Center near Baton Rouge, Louisiana in 2007 according to standard practices. Insecticides were applied as needed, but no fungicides were applied. Leaves from plants during early reproductive stages of growth (R2 growth stage) from a field that showed no SBR symptoms were selected for these tests [34].

In a previous study, sori of *P. pachyrhizi* were determined to be a unique habitat of *S. lanosoniveum* isolates BH081707-1A (GenBank accession HQ270477) and D082307-2A (GenBank accession HQ270476) collected from research stations in Louisiana and Florida, respectively [101]. Preliminary observations determined that isolate D082307-2A coiled around more urediniospores per sorus than isolate BH081707-1A, but colonization appeared to be similar among the isolates. Detached soybean leaves were examined with a dissecting microscope at 20x magnification to select symptomless leaves, then leaflets were co-inoculated with either of the two isolates above and urediniospores of *P. pachyrhizi* collected from nearby fields [101].

Conidia were washed from 14-day-old cultures of *S. lanosoniveum* grown on potato dextrose agar (PDA, Difco, Sparks, MD) with sterile phosphate buffer (0.5mM, pH 7.1) plus 0.01% Tween 20® by gently rubbing with a glass rod. The resulting conidial suspension was adjusted to  $10^6$  conidia/ml with the aid of a hemacytometer.

Urediniospores of *P. pachyrhizi* were collected less than one week before inoculations from infected field-grown soybean plants at the Louisiana State University Agricultural Ben Hur Research Farm in Baton Rouge, Louisiana with a spore collector (G-R Manufacturing; Manhattan, KS) and stored in 20ml vials at  $-30^{\circ}\text{C}$ . Urediniospores were rehydrated in a moist chamber for 24 hr before use [20]. Urediniospore germination was determined by plating

urediniospores onto 1.5% water agar (Bacto, Becton, Dickinson, and Company, Sparks, MD) and incubating for 6 hours at 25°C in the dark. A urediniospore was considered germinated if the germ tube was at least as long as the urediniospore. Urediniospores with a germination rate of at least 90% were used for inoculations.

Three 4cm<sup>2</sup> subsampling areas were marked on the abaxial side of the apical leaflet of each of eight symptomless soybean leaves for each of the following treatments: co-inoculation with *S. lanosoniveum* and *P. pachyrhizi*; sterile buffer only, 0.01% Tween 20<sup>®</sup> only, individual test isolate only, urediniospores in 0.01% Tween 20<sup>®</sup> only, and a nontreated control. Co-inoculations were prepared by applying approximately 1,800 conidia of *S. lanosoniveum* to each marked area by applying 20µl spore suspension with a micropipettor and spreading with a glass rod. After air drying for one hour, 1,800 urediniospores of *P. pachyrhizi* were similarly applied to the same subsampling area [101]. Sterile buffer (0.5mM, pH 7.1) was used to prepare all solutions and spore suspensions. Leaves were incubated in moist chambers made from clear polystyrene boxes lined with moist paper towels. These moist chambers were incubated under cool white fluorescent lights (850-1000 lux) with a 12 hr photoperiod. Numbers of sori were determined on day 1 and day 7 after initiation of these experiments in order to quantify latent infection that may have occurred in the field, although this check probably does not account for all latent infection that may have occurred in the field. Final soral counts were taken on day 14 because SBR sori typically develop 7 to 10 days after inoculation.

Samples were processed for SEM in order to visualize colonization by *S. lanosoniveum* [77, 82]. Leaf tissue was fixed in formalin acetic acid overnight and then dehydrated in an ethanol series as follows (20 min in each solution): 50%, 70%, 95% and culminating in 100%. Samples were critical point dried, plated with gold:palladium (60:40) and viewed with a JEOL JSM-6610LV

SEM (JEOL, Ltd; Tokyo, Japan) at the Louisiana State University Socolofsky Microscopy Center.

### **3.2.2 Inoculation of Infected Soybeans Leaves**

To further investigate the effects of *S. lanosoniveum* on SBR, two isolates of *S. lanosoniveum*, D082307-2A and BH081707-1A, were applied individually to field-grown detached soybean leaves that showed symptoms of SBR. Symptoms included sori of various ages and stages of development as they occurred under field conditions. Soybean plants (cv AG5903; Monsanto) were grown in fields as described above, and rust-infected trifoliolate leaves with a SBR severity of 5 to 10% (Bayer rating cards, percent diseased leaf area) were picked from mid-canopy from plants with fully developed pods and early seed development (R5 growth stage) [33]. After rinsing in distilled water (pH 7) for 10 seconds, two opposite leaflets of each trifoliolate were assigned to inoculated or noninoculated groups. Ten such leaflets per treatment were selected from the apical leaflet from 10 leaves and placed adaxial side down in moist chambers as described above. Conidia were washed from 14-day-old cultures of each isolate of *S. lanosoniveum* grown on PDA and adjusted to  $10^6$  conidia/ml. One ml of conidial suspension, prepared as described above, was spread evenly across the abaxial surface with a glass rod. After inoculation, leaves were allowed to dry for 1 hour before closing the moist chamber. In contrast to co-inoculations, this experiment did not use marked sample areas. Instead, entire leaf surfaces were inoculated with conidial suspensions of *S. lanosoniveum*. Because soybean leaves were already infected, we did not inoculate with *P. pachyrhizi*. Additional treatments included sterile buffer (pH 7) only, 0.01% Tween 20<sup>®</sup> only, and a nontreated control. This experiment was repeated with infected soybean leaves (cv Asgrow AG6202; Monsanto; St. Louis, MO) from a different field at the research farm.

After 14 days, leaves were rated for sorus development, color of sori, and color of urediniospores in randomly selected fields of vision (5cm<sup>2</sup>) using a dissecting microscope at 25x magnification. Red-brown sori, often associated with hypersensitive reactions, were characterized by dark halos or necrotic tissue surrounding the sorus [21, 60]. Tan sori had little or no dark surrounding tissue. These tan sori varied in urediniospore production; therefore tan sori were subdivided into minimal, moderate, and maximum sporulation (Fig 3.1). Maximum sporulation indicated that urediniospores were abundant enough that uredinia were not visible. Minimal sporulation described sori for which entire uredinia were visible because of the small number of urediniospores, and moderate sporulation included all those sori in which a portion of the uredinia were visible. Three randomly selected subsamples with at least 25 nonoverlapping sori were selected for each leaflet for categorization.

While sorus color was apparent without the aid of a microscope, urediniospore color differed when examined with a dissecting microscope (25x). Hyaline urediniospores appeared opaque or white, while other urediniospores appeared crimson-brown or dark brown. Three fields of vision (5cm<sup>2</sup>) were randomly selected for rating urediniospore color according to the following rating scale: 1 = 75-100% hyaline urediniospores; 2 = 50-75% hyaline urediniospores; 3 = 25-50% hyaline urediniospores; 4 = 1-25% hyaline urediniospores; 5 = no hyaline urediniospores.

### **3.2.3 Urediniospore Production and Germination**

The experiments described above were designed to determine whether *S. lanosoniveum* affected sorus development. To determine whether these inoculations affected urediniospore production, leaves from the above experiment were sampled 14 days after inoculation with isolate D082307-2A to quantify the number of urediniospores per sorus. Three randomly selected leaf discs (5



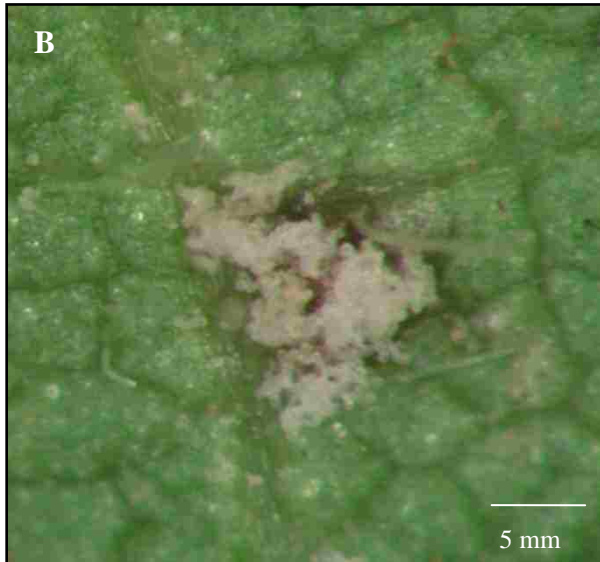
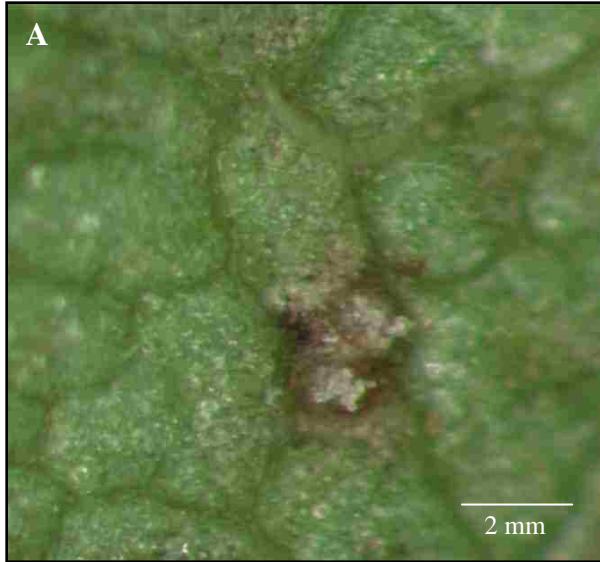
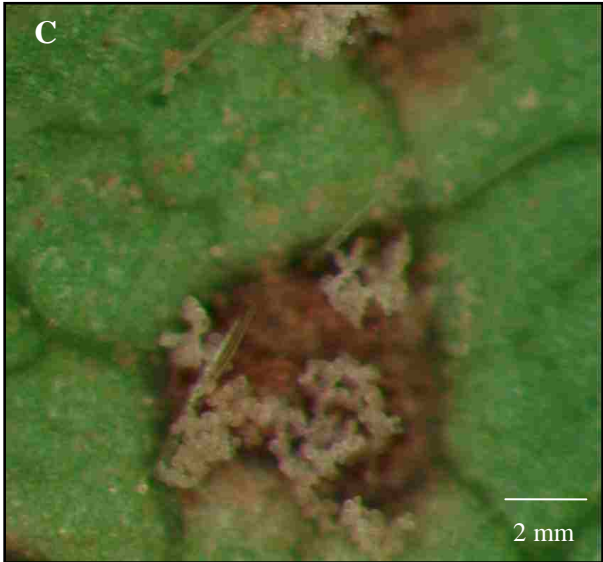


Fig 3.1. Examples of minimal (A) and maximum sporulation of tan soybean rust sori. Brown soybean rust sorus (C) showing necrotic tissue at 14 days after inoculation.

Fig 3.1 continued



cm<sup>2</sup>) were cut with a cork borer from each leaflet in the inoculated treatment and its matched-pair control. Numbers of sori on each disc were determined with a dissecting microscope at 25x magnification. Discs were placed in 10 ml buffer with 0.01% Tween 20<sup>®</sup> and vortexed for 30 seconds to suspend urediniospores. Five samples were drawn from each suspension, and urediniospores were counted with the aid of a hemacytometer (Hausser Scientific, Horsham, PA), and numbers of urediniospores per sorus were calculated.

Sori that contained a majority of either hyaline or brown urediniospores were used for tests of germination. Urediniospores were lifted with a needle from 10 sori of each urediniospore classification described above and suspended in one ml 0.01% Tween 20<sup>®</sup> in phosphate buffer (0.5mM, 7.1 pH). Three such suspensions were made for each urediniospore type from inoculated and noninoculated treatments, and three subsamples (30 µl each) were drawn from each suspension for germination tests. Subsamples were spread individually across petri dishes containing 1.5% water agar. Fifty urediniospores were assessed for germination on each dish after incubation for 6 hours at 25° C in the dark. A urediniospore was considered germinated if the germ tube was at least as long as the longest dimension of the urediniospore.

### **3.2.4 Sorus Age Study**

Previous observations indicated that urediniospores turned brown with age. The following experiment was conducted in order to document photographically the effects of sorus age on urediniospore color. Naturally infected field-grown soybeans (cv. Asgrow 6202; Monsanto; St. Louis, MO) were assessed for disease severity by estimating the percent diseased leaf area using a visual key developed by Bayer Crop Sciences (Kansas City, MO). Fifty leaflets were examined using a dissecting microscope at 25x magnification, and immature sori that had not yet

erupted were marked. At least five sori were selected from each leaflet. These sori were numbered for future reference.

Urediniospore production and color development were monitored during daily increments and cumulatively during the 14 day duration of the experiment. For daily assessments, urediniospores were brushed away with an artist's paintbrush each day in order to examine newly produced urediniospores from the same uredinium. For cumulative assessments, urediniospores were allowed to accumulate in sori throughout the course of the experiment. Sori in both treatments were photographed daily.

This study was repeated with soybean cultivar Pioneer 95Y20 (Pioneer Hi-Bred; Johnston, IA).

### **3.2.5 Statistical Analyses**

Detached leaf assays were arranged in randomized complete block designs within moist chambers. Percentage data were transformed with the arcsine function, and then pairwise t-tests and ANOVAs were calculated [3]. Outliers were removed if they fell outside of the interquartile range [94]. Standard deviations (s.d.) are presented where appropriate. All statistical calculations were performed with JMP version 9 (SAS Institute, Cary, NC).

## **3.3 Results**

### **3.3.1 Co-inoculations of Disease-free Soybean Leaves**

No rust symptoms were observed on days 1 and 7 following inoculations indicating that subsequent SBR symptom development could be attributed to experimental inoculations rather than symptoms arising from latent infections that may have occurred in the field. Symptoms

began to appear on day 10, and final uredinial counts were made on day 14. The mean number of sori/4 cm<sup>2</sup> sample area was 61.0 (s.d.= 3.46) on leaves inoculated with only *P. pachyrhizi*. The numbers of sori/sampling area for leaves coinoculated with *S. lanosoniveum* isolates BH081707-1A or D082307-2A were 13.0 (s.d. = 2.3) or 16.0 (s.d. = 1.6), respectively. This was about a 4-fold reduction in sorus development as compared to the leaves inoculated with only *P. pachyrhizi* (Fig 3.2). By day 14, distinct mycelial masses were clearly visible within sori in the co-inoculated treatment under low magnification.

In SEM micrographs, *S. lanosoniveum* did not colonize leaves in the absence of *P. pachyrhizi*. SEM micrographs showed that the fungus colonized sori and wrapped around urediniospores and hyphae radiating into a centralized, putative penetration site (Fig 3.3). Soybean rust symptoms were not apparent in other treatments (buffer, buffer with Tween 20<sup>®</sup>, *S. lanosoniveum* isolates only, and nontreated control). Repeated experiments yielded similar results.

### **3.3.2 Inoculation of Infected Soybean Leaves**

Field grown soybean infected with *P. pachyrhizi* were inoculated with *S. lanosoniveum*, and sorus counts were categorized by lesion color. By day 14, the treatment with isolate BH081707-1A had 69.4% (s.d. = 0.3) red-brown lesions and 33.0% tan lesions. The noninoculated control yielded 33.0% (s.d.= 0.2) red-brown lesions and 67% tan lesions. Isolate D082307-2A of *S. lanosoniveum* yielded 53.0% (s.d.= 0.3) red-brown lesions and 47.0% tan lesions. The nontreated control yielded 19.2% (s.d.= 0.2) red-brown lesions and 80.8% tan lesions. Thus, in the presence of *S. lanosoniveum*, soybean rust symptoms produced 2 to 2.5 times more red-brown lesions (Fig 3.4).

In addition to sorus color, urediniospore color also was determined in these experiments. After 14 days, isolate BH081707-1A induced 79.0% (s.d.= 26.0) brown urediniospores compared to 30.0% (s.d.= 22.0) brown urediniospores in the noninoculated control. Isolate D082307-2A induced 83.9% (s.d.= 12.6) brown urediniospores compared to 22.2% (s.d.= 15.8) brown urediniospores in the nontreated matched-pair control (Fig 3.5). Repeated experiments yielded similar results.

### **3.3.3 Urediniospore Production**

To determine whether *S. lanosoniveum* affected numbers of urediniospores produced per sorus, three subsamples were evaluated from each of three replications in field-collected infected leaves. Leaves inoculated with isolate D082307-2A of *S. lanosoniveum* yielded 407 (s.d. =152.9) spores per sorus, while nontreated sori averaged 362 (s.d. = 240). Likewise, leaves inoculated with isolate BH081707-1A yielded 470 (s.d. = 144) urediniospores per sorus as compared to 500 (s.d. = 109) urediniospores per sorus in the noninoculated control. Thus, while urediniospore color was affected by *S. lanosoniveum*, the number of urediniospores per sorus was not statistically different between the two isolates of *S. lanosoniveum*.

Hyaline urediniospores collected from inoculated sori germinated at a rate of 69% (s.d. = 6.8), while only 3% (s.d.=2.8) of brown urediniospores germinated. Hyaline urediniospores recovered from noninoculated sori showed a significantly higher germination rate than brown urediniospores recovered from noninoculated sori, with hyaline and brown spores germinating at 59% (s.d. = 27.8) and 28% (s.d. = 11.0), respectively. There was no significant difference in germination rates for hyaline spores from inoculated as compared to noninoculated treatments.

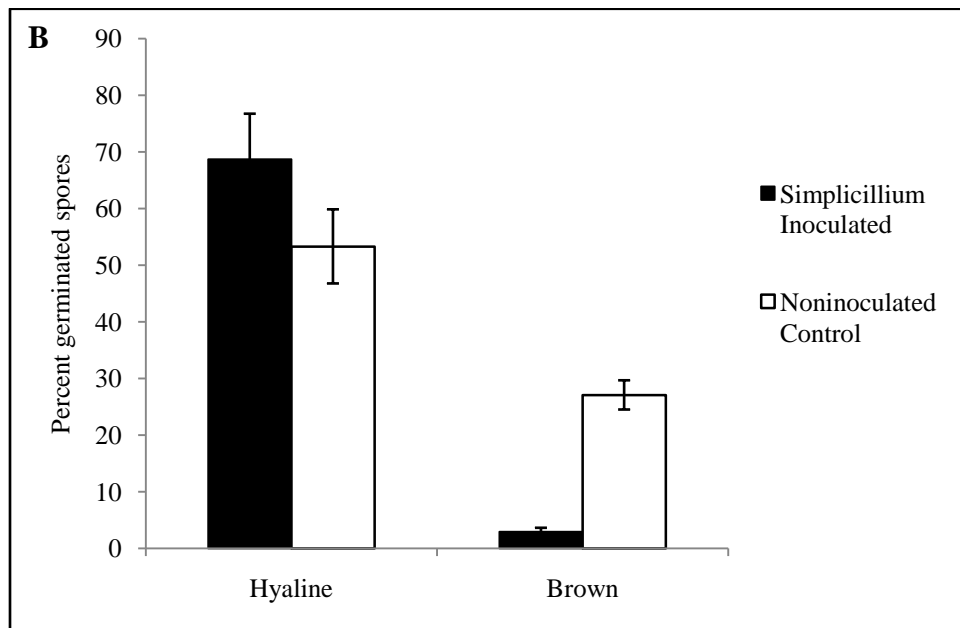
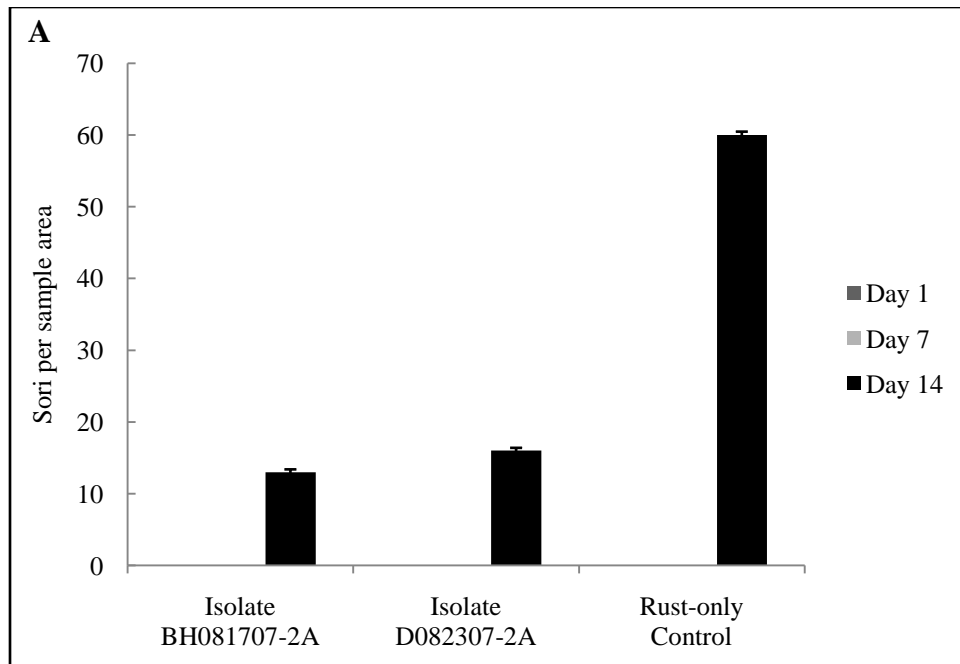


Fig 3.2. Effect of co-inoculation of soybean leaves with two isolates of *S. lanosoniveum* and *Phakopsora pachyrhizi* on development of rust sori. Numbers of sori were determined after 1, 7, and 14 days. Bars represent standard error.

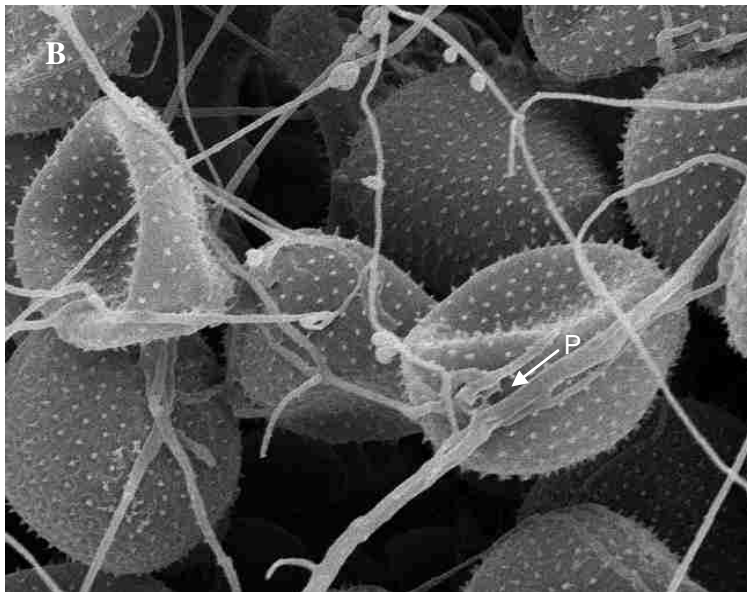
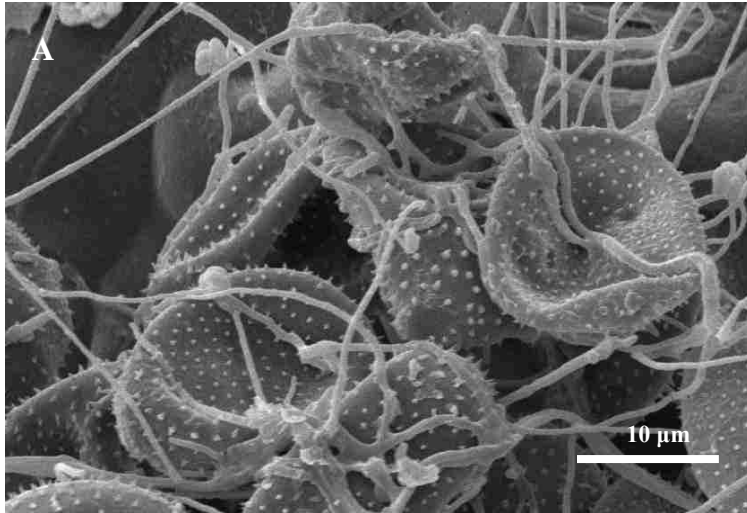


Fig 3.3. (A) Example of mycophilic attraction towards and colonization of *Phakopsora pachyrhizi* by *Simplicillium lanosoniveum*. (B) Putative penetration points by hyphae of *S. lanosoniveum* on colonized urediniospores of *P. pachyrhizi*. (P) Putative penetration site.



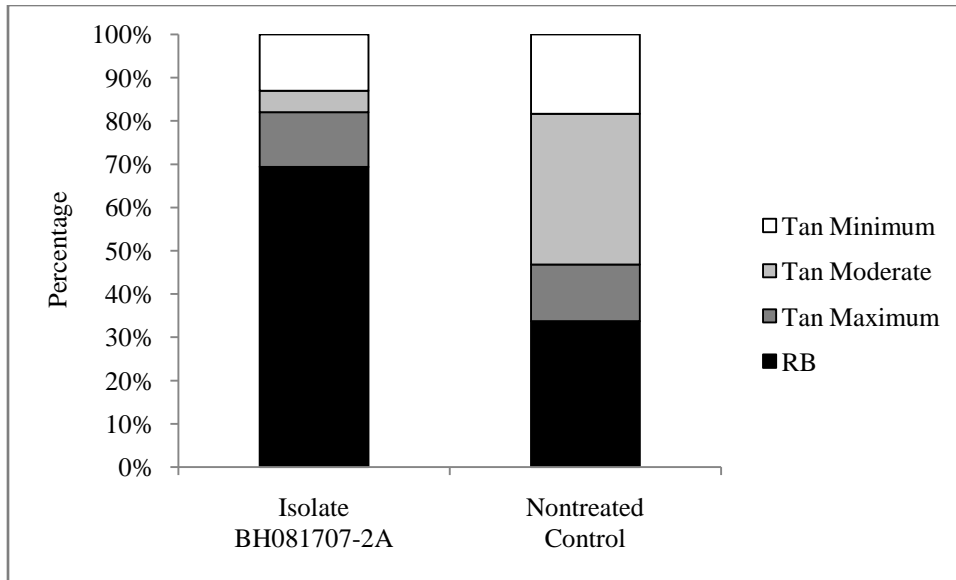


Fig 3.4. Effects on soybean rust sori type following inoculation of field-grown symptomatic soybean leaves with two isolates of *Simplicillium lanosoniveum*. Lesion type was classified at 14 days after inoculation. Tan sori were rated as minimum, moderate, or maximum sporulation.

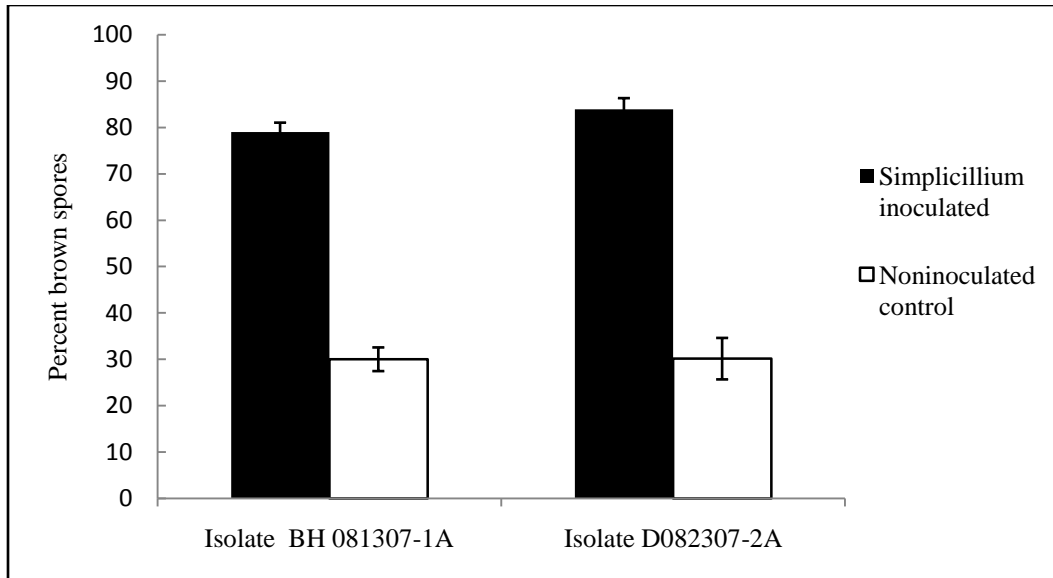


Fig 3.5. Effects on color of soybean rust urediniospores following inoculation of symptomatic, field-grown soybean leaves with two isolates of *Simplicillium lanosoniveum* at 14 days after inoculation. Urediniospore color was classified as either brown or hyaline, and data are presented as percent brown spores. Bars represent standard error.

However, germination rates for brown spores recovered from sori inoculated with *S. lanosoniveum* and those collected from noninoculated sori were significantly different (Fig 3.6).

### 3.3.4 Sorus Age Study

Urediniospore color as a function of sorus age was monitored and documented photographically. When urediniospores were brushed from sori daily, newly formed urediniospores were hyaline regardless of the age of the sorus. In sori in which urediniospores were allowed to accumulate, urediniospores became brown after 5 to 7 days (Fig 3.7). Primary sori sporulated for 7 to 10 days, and secondary sori developed 5 to 7 days after primary sori developed. By day 14, sporulation of uredinia ceased. Urediniospores from older sori turned brown at the same age after eruption as urediniospores from younger sori. Likewise, urediniospores produced in secondary sori browned at the same age as those from primary sori. By day 10 to 14, leaf tissue around sori became necrotic, and as sporulation declined, sori appeared red-brown. Results were similar in both soybean cultivars.

## 3.4 Discussion

We previously documented that *S. lanosoniveum* is a colonist unique to sori of *P. pachyrhizi* [101]. SEM micrographs and attempts at recovering *S. lanosoniveum* revealed that *S. lanosoniveum* colonized only sori and was not found in association with lesions of other diseases of soybean or healthy leaf surfaces. When *S. lanosoniveum* was introduced to sori, structures were observed that resembled penetration sites on the surface of urediniospores. These observations suggest that *S. lanosoniveum* may be a mycoparasite of *P. pachyrhizi*, and we are addressing this possibility in further studies. Mycophilic refers to the attraction of *S.*

*lanosoniveum* to sori of another fungus *P. pachyrhizi*. Until further evidence of mycoparasitism is obtained, we refer to these interactions as mycophilic.

In previous studies and in these experiments, *S. lanosoniveum* did not grow or establish on leaf surfaces until rust sori erupted on co-inoculated leaves. However, in the presence of urediniospores *P. pachyrhizi*, *S. lanosoniveum* colonized sori within 3 days and sporulated within 4 days. Sorus development was repressed in both leaf inoculation protocols in comparison to the nontreated controls. Based on these observations and from previous studies, we conclude that sorus development is necessary for establishment of *S. lanosoniveum* on soybean leaf surfaces.

In addition to a reduction in sorus development, rust-infected detached soybean leaves inoculated with *S. lanosoniveum* developed 2 to 2.5-fold increases in red-brown sori, which have been associated with hypersensitive resistance reactions [19, 60-61]. McLean developed this classification system in which tan lesions had two to five uredinia and sporulation was abundant as compared to red-brown sori in which only one or two uredinia were present and sporulation was sparse. Since then, breeders have adopted these color characteristics to differentiate between resistant and susceptible reactions [21, 41]. We observed similar lesion coloration, and we used a similar rating scale. However, we do not believe that these red-brown lesions are a hypersensitive response because they occurred on susceptible cultivars and because SBR symptoms occurred in the absence of *S. lanosoniveum*. The red-brown sori may be caused by necrosis resulting from accelerated aging of uredinia that were colonized by *S. lanosoniveum*. The sorus age study also showed that leaf tissue surrounding sori on the same cultivar became necrotic with age, and sporulation decreased dramatically by day 10. Thus, the significant increase in red-brown lesions in the presence of *S. lanosoniveum* mimicked an accelerated soral

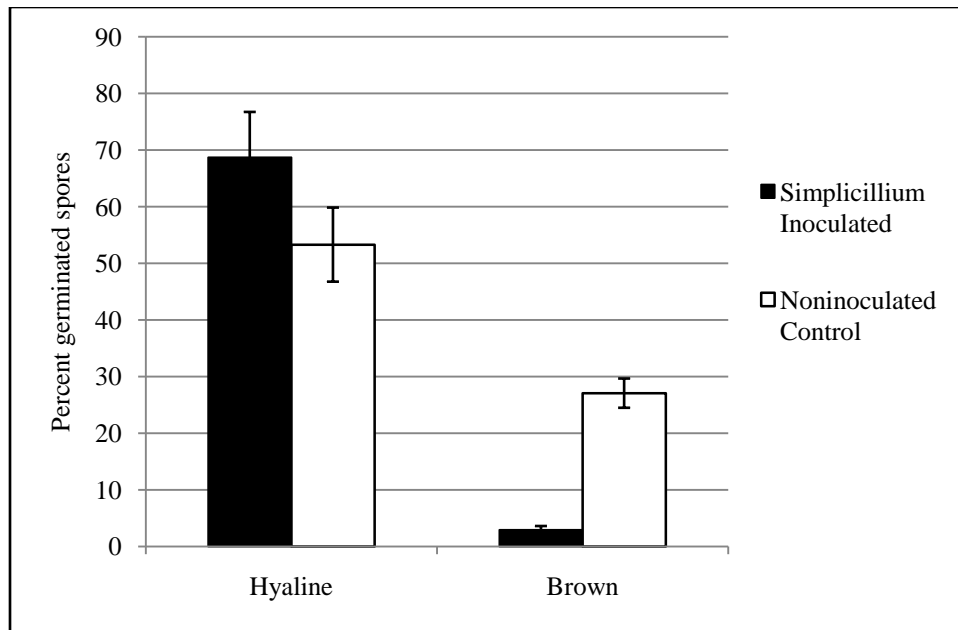


Fig 3.6. Effects on germination rate of brown and hyaline urediniospores of *Phakopsora pachyrhizi* following inoculation with *Simplicillium lanosoniveum* isolate D082307-2A. Germination rates were assessed at 14 days after inoculation. Bars indicate standard error.

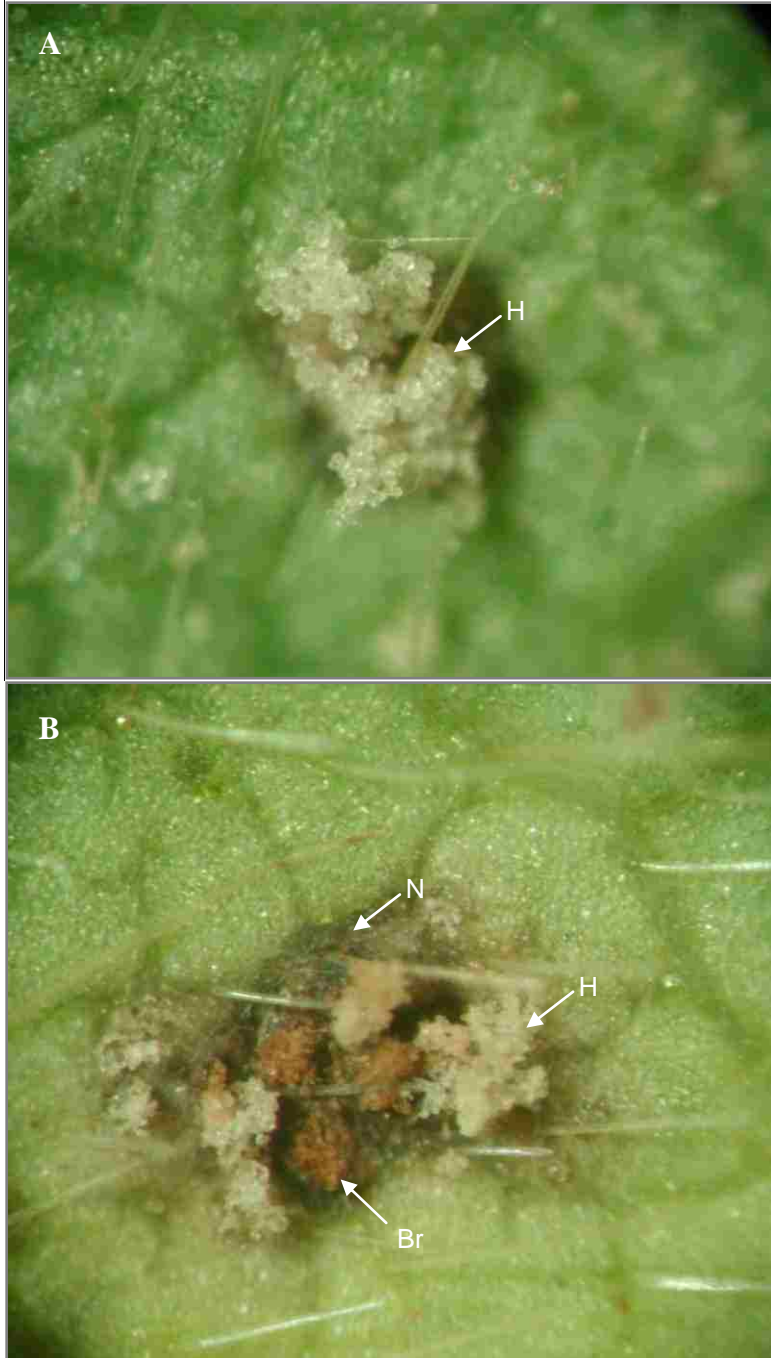


Fig 3.7. Sorus age study in which changes in urediniospore color were documented as they aged. Three (A) and 10 (B) days after sporulation began. (Br) Brown urediniospores, (H) Hyaline urediniospores, and (N) Necrotic leaf tissue.

aging process that may be unrelated to hypersensitivity. Breeders and others should be aware that, in addition to a resistance reaction, there are other factors that cause RB sori.

In addition to a change in lesion color, *S. lanosoniveum* also caused a change in urediniospore color in which they became increasingly brown within 3 to 5 days. The sorus age study indicated that urediniospores darkened with time, but when inoculated with *S. lanosoniveum*, they darkened more rapidly. Previous work indicated that SBR sori sporulated for 3 weeks [59]. Brown urediniospores from sori inoculated with the antagonist had a significantly lower germination rate. It is not clear whether this darkening of urediniospores is the result of melanization or cell decompartmentalization resulting from parasitism by the putative mycoparasite. It was apparent that brown urediniospores from inoculated sori failed to germinate. Studies are in progress to address the cause of brown urediniospore development and their failure to germinate.

Clearly, *S. lanosoniveum* reduced sorus development and inhibited spore germination. Its ability to inhibit urediniospore production in this polycyclic disease may confer this organism with disease-suppressing capability by reducing inoculum load, thereby limiting reinfection and delaying disease development. This organism may have potential as a biological control agent.

## **CHAPTER 4. EFFECTS OF FIELD INOCULATIONS OF SOYBEANS WITH THE MYCOPHILIC FUNGUS *SIMPLICILLIUM LANOSONIVEUM* ON *PHAKOPSORA PACHYRHIZI* AND SOYBEAN RUST**

### **4.1 Introduction**

Soybean rust (SBR), caused by *Phakopsora pachyrhizi* Syd. & P. Syd. was first reported in Japan in 1904 and has since spread throughout Asia, Africa, and the Americas [64, 107].

Disease losses ranged from 10 to 90 %, though there are reports as high as 100% [64]. SBR was first discovered in the US in 2004, where it quickly became established in the southeastern states [84-85, 96]. Yield losses have been reported between 35 and 40% in Louisiana and as high as 82% in Florida on susceptible varieties that were not sprayed with fungicides (R. W. Schneider, D. R. Walker, personal communications).

Breeding efforts have yet to produce resistant cultivars [41]. Therefore, disease management studies have focused mainly on fungicide applications. These studies showed that preventative applications of protectant fungicides must be accurately timed and applied very early in the infection process for effective control of the disease [85]. This may lead to unnecessary fungicide applications, especially throughout the southern US, because growers fear rapidly escalating epidemics such as those seen in Africa and Asia.

In 2007, a mycoparasitic fungus, *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001, which colonized sori of SBR and penetrated urediniospores was discovered [101]. Using detached leaf assays, a reduction in production of sori in the presence of *S. lanosoniveum* was observed, as well as a significant reduction in viability of urediniospores. The objective of this



study was to evaluate the effects of *S. lanosoniveum* under field conditions. Development of *S. lanosoniveum* and the rust pathogen was monitored using quantitative real-time PCR (qPCR).

## 4.2 Methods

### 4.2.1 Primer and Probe Development

*Simplicillium lanosoniveum*. To develop highly specific primers and probe, the internal transcribed spacer region (ITS) of the nuclear ribosomal RNA gene repeat was sequenced from five isolates of *S. lanosoniveum* that had been recovered from soybean leaves collected in Louisiana and Florida, as well as two additional isolates (CBS101895 and CBS70486) obtained from Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands)[101]. Two other species of *Simplicillium*, *S. obclavatum* (CBS 51082) and *S. lamellicola* (CBS 13837), and other phylloplane inhabitants (*Fusarium* spp. and *Cladosporium* spp.) were evaluated to eliminate overlapping sequences and to prevent false positives. Genomic DNA was extracted from 14-day old mycelia using Promega Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI). Internal transcribed spacer primer pairs ITS1-F and ITS4 were used for DNA amplification [36]. PCR product was purified with Millipore Montage PCR Centrifugal Filters (Millipore, Billerica, MA) and adjusted to 10 ng/μl. PCR products from each isolate were sequenced at the DNA Sequencing Core/ ICBR (University of Florida, Gainesville, FL) with the same primers used for amplification. All sequences were aligned with Clustal 2.0.11 (Conway Institute UCD, Dublin, Ireland). Primers and probe were designed to include all isolates of *S. lanosoniveum* and to exclude other species of *Simplicillium* and phylloplane inhabitants commonly isolated from soybean. Probe was labeled with 5' 6-FAM (fluorescent reporter dye 6-carboxy-fluorescein) and

with 3' TAMRA (quencher dye 6-carboxytetramethyl-rhodamine). Primers and probe were purchased from Integrated DNA Technologies (Coralville, IA).

Upon alignment of ITS sequences of *S. lanosoniveum*, *Simplicillium* spp., and other soybean phylloplane inhabitants, the following sequences for primers and probe were selected (Fig 4.1). Forward primer SimpF-NW (5'-TTTATCCA ACTCCCAACCC-3') was specific to *S. lanosoniveum*. Reverse primer SimpR-NW (5'-ACGCGTAGTCCCGGGAG-3') was specific to the Louisiana and Florida isolates but excluded the two CBS isolates of *S. lanosoniveum* by one or two bases. Probe SimpPR-NW (5'-FAM- CCGGGAGCCCCCTAG-TAMRA-3') was specific to *S. lanosoniveum*, and the last two bases of the 3' end were highly specific to *S. lanosoniveum* (Fig 4.1). Optimization of primers and probe for the ABI 7000 yielded the following dilutions: 900 nM each forward and reverse primers and 200nM probe. Primers and probe developed for *S. lanosoniveum* were very specific and had no cross reactivity when tested against *S. lamilicolla*, *S. obclavatum*, *Fusarium* spp. and *Cladosporium* spp. Sensitivity of the primers and probe was as low as 1.0 pg of DNA of *S. lanosoniveum* per 10 ng total genomic DNA as calculated with the standard curve at a mean Ct of 36.3.

Validity of the primers and probe was tested with both pure dilutions of *S. lanosoniveum* mycelial DNA spiked with soybean leaf DNA to identify possible inhibitors. Next, various primers and probe concentration combinations were evaluated to test for sensitivity and to test the ability to detect lowest of *S. lanosoniveum* DNA. Specificity among *S. lanosoniveum* isolates was tested against pure dilutions of mycelial DNA of *Fusarium* and *Cladosporium* and soybean

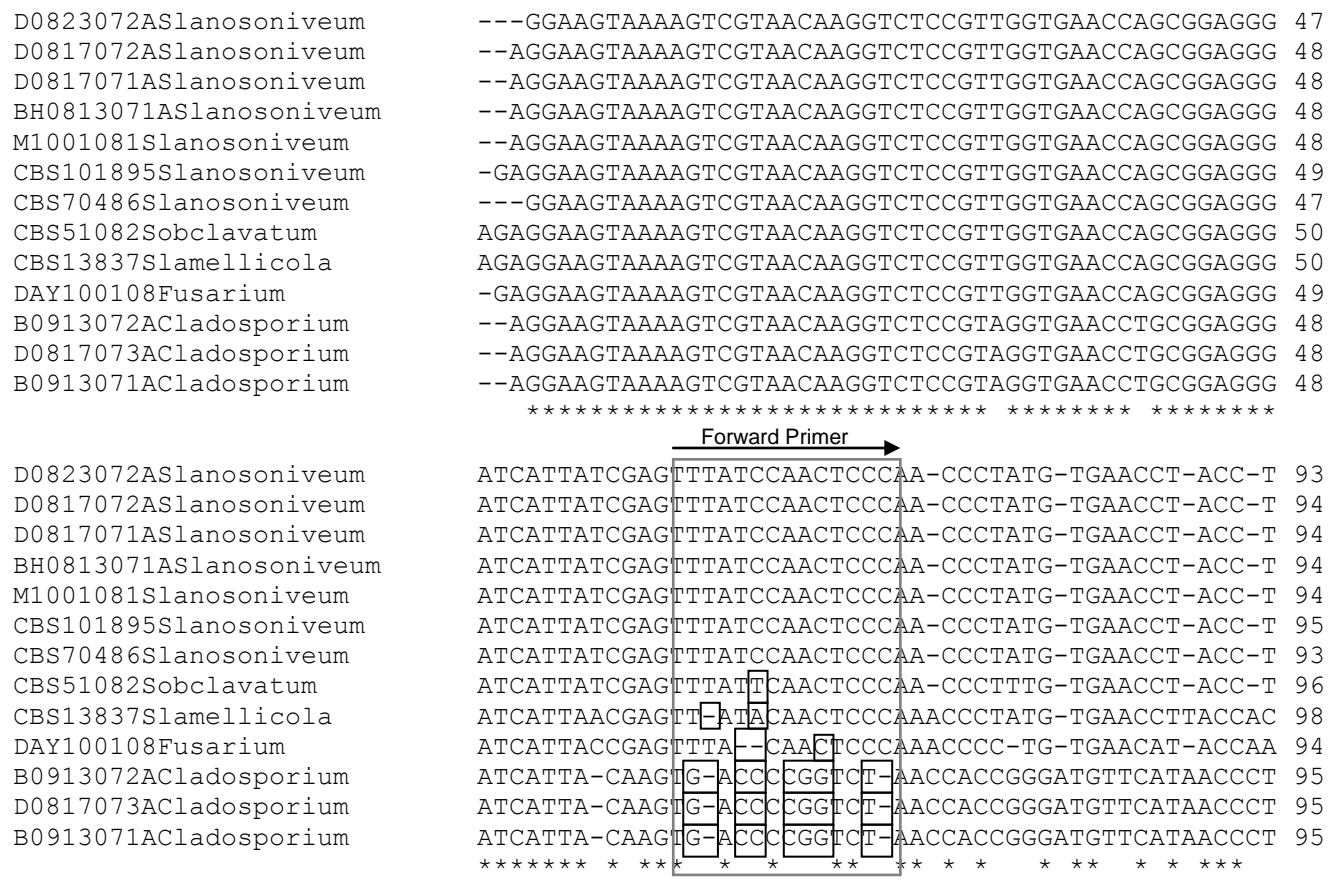
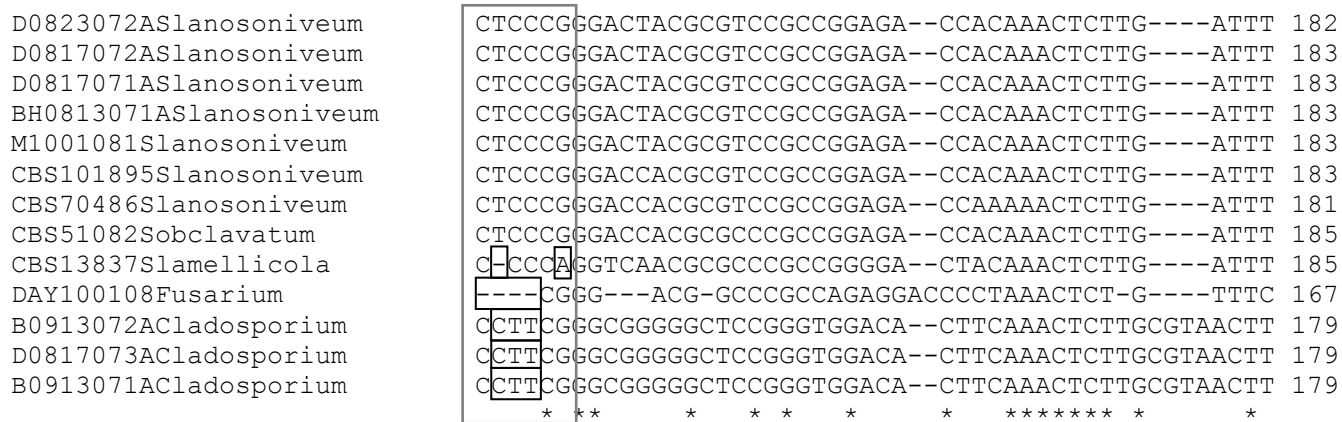
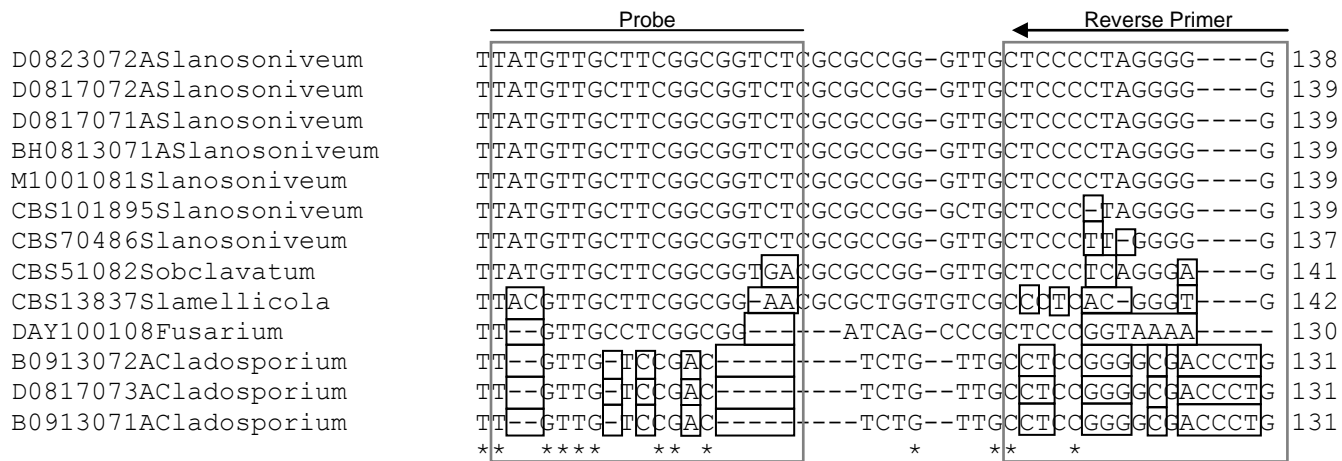


Fig 4.1. Sequence alignment of the internal transcribed spacer (ITS) region from *Simplicillium* spp., *Fusarium* sp., and *Cladosporium* sp. Nucleotide differences that occur between *S. lanosoniveum* and other species are highlighted with open boxes. Primer and probe sequences are shown by arrows.

Fig 4.1 continued



leaves infected with other pathogens such as *Cercospora kikuchii* and *Septoria glycines*. A standard curve was generated by running six replications of 10-fold dilutions of 10 ng genomic DNA from *S. lanosoniveum* plus 10 ng of DNA from soybean. Assays were replicated twice.

***Phakopsora pachyrhizi***. Primers and probe were selected from previous work [35]. Primers Ppm1 (5'-GCAGAATTCAGTGAATCATCAAG-3') and Ppa2 (5'-GCAACACTCAAAATCCAACAAT-3') were reported to be specific to *P. pachyrhizi*. Specific probe (5'-FAM-CCAAAAGGTACACCTGTTTGAGTGTC-TAMRA-3') was labeled at the 5' end with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA). Primers and probe were purchased from Integrated DNA Technologies (Coralville, IA).

#### **4.2.2 Field Studies**

Field experiments were conducted in three soybean fields in Louisiana and one field in Florida in 2009 and 2010. Conidia and/or mycelial fragments of *S. lanosoniveum* were introduced to soybean leaves at various times as described below. SBR epidemics were initiated from naturally occurring inoculum; plants were not inoculated with the SBR pathogen. Leaf samples were collected weekly and DNA of both *S. lanosoniveum* and *P. pachyrhizi* was quantified by qPCR as described below.

**Preparation of conidial inoculum.** Conidial suspensions of *S. lanosoniveum* were produced by flooding petri dishes of 2-week old cultures with 20 ml sterile phosphate buffer (0.5 mM, pH 7.1) and rubbing the colony surfaces with a glass rod [101]. Two ml of this suspension were used to inoculate 100 ml of potato dextrose agar that had been poured in the bottoms of 2 L flasks. Flasks were shaken gently to spread inoculum across the agar surface. Cultures were

incubated at 25°C in the dark for 7 days. At the time of inoculation in the field, flasks were filled with 2 L distilled water, shaken vigorously, and agar pieces were removed by straining the suspension through a wire mesh sieve with 500 µm openings. Conidial suspensions were adjusted to pH 7.0 with phosphate buffer (final concentration 0.5 mM) and amended with 100µl Tween 20® per liter. The final inoculum concentration was approximately 10<sup>6</sup> conidia/ml. Plants were inoculated during late afternoon when temperatures dropped below 27°C and immediately after inoculum was prepared.

**Preparation of mycelial inoculum.** Cultures of *S. lanosoniveum* were flooded as described above. Two ml of conidial suspension were added to 250 ml potato dextrose broth and shaken at 200 rpm on an orbital shaker for 7 days at 25°C in the dark. Cultures were strained through a mesh sieve (500 µm openings), rinsed twice with deionized water, and blended in 500 ml sterile phosphate buffer (0.5 mM, pH 7.1). Two hundred fifty ml of this suspension were added to each liter of conidial suspension to produce inoculum of 10<sup>4</sup> colony forming units (CFU) per ml of mycelial fragments.

### 4.2.3 Field Experiments

To monitor colonization of *S. lanosoniveum* and *P. pachyrhizi* on soybean leaves, 10 trifoliolate leaves were sampled weekly from each plot beginning in the late vegetative stages or early reproductive stages of growth. Leaves were stored in freezer bags at -20°C until they were processed for DNA extraction. The soybean crops were maintained according to recommended protocols with regard to insect and weed control and fertilization.

**Field 1.** The purpose of this experiment was to compare isolates of *S. lanosoniveum* and to determine whether they colonized sori of SBR under field conditions. Soybean cultivars Asgrow

6202 (Monsanto Corp.), Deltagrow 4770 (Deltagrow Corp.), Deltagrow 4771 (Deltagrow Corp.), and Delta King GP-533 (Armor Seed Co.) were grown at the Louisiana State University Agricultural Center's Ben Hur Research Farm near Baton Rouge, LA. Plants were sampled in October and November 2009 while they were at the R5 to R6 stage of reproductive growth [33]. Plots were four rows wide by 9 m long and arranged in a randomized complete block with four replicates per cultivar. When SBR severity in all plots reached at least 15% (85 DAP, conidial suspensions of  $10^6$  spores/ml of isolates BH081707-1A (GenBank accession number HQ270477) or D082307-2A (GenBank accession number HQ270476) were applied to each of the center two rows with a 3.8 liter hand-held garden sprayer at approximately 18 to 20 ml/m<sup>2</sup> after temperatures dropped below 27°C. The nontreated control received no treatment. Seven days after inoculation, 10 trifoliolate leaves were sampled from each plot for disease assessment and quantification of DNA by qPCR. Plots were inoculated again, as previously described, immediately after sampling (92 DAP) and sampled again on day 14 (99 DAP).

**Field 2.** SBR was detected within 44 km of the research farm on June 5, 2009, 46 days before soybeans were planted. To determine when *S. lanosoniveum* affected SBR infection, we inoculated soybeans before and after disease symptoms occurred. Soybean cultivar Asgrow 6202 was planted in July 2009, at the Ben Hur Research Farm, near Baton Rouge, LA. Plots were 8 rows wide by 9 m long. The following four treatments were included: 1) inoculation with *S. lanosoniveum* at first flower (R1/R2); 2) inoculation with *S. lanosoniveum* at first occurrence of rust (<2.5% severity) (R5); 3) application of pyraclostrobin fungicide (Headline, BASF Corp; 876 ml product in 187 liters of water per ha) at R1/R2; and 4) nontreated control. There were four replications per treatment, and the experiment was arranged in a randomized complete block design. The center 4 rows of each plot were sprayed until leaves were wet

(approximately 18 to 20 ml/m<sup>2</sup>) with conidial suspensions (10<sup>6</sup> spores/ml) of *S. lanosoniveum* isolate D082307-2A with a hand-held garden sprayer. Ten trifoliolate leaves were collected each week beginning during mid-vegetative stages (V4) and continuing through senescence (R7). One hour after each inoculation, three trifoliolate leaves were sampled from each plot to quantify initial inoculum.

Corn was included as a nonhost control in this experiment in order to examine colonization of *S. lanosoniveum* in the absence of SBR. Corn leaves were inoculated with conidial suspensions of 10<sup>6</sup> conidia/ml as described above. Three rows, each 9 m long, were inoculated with 10<sup>6</sup> spores/ml of *S. lanosoniveum*. Three leaves from each plot were sampled 1, 7, 14, and 21 days after inoculation. The experiment was conducted twice.

**Field 3.** In July 2010, soybean cultivar Pioneer 95Y20 was planted at the University of Florida North Florida Research and Education Center in Quincy, Florida. There were four treatments in this experiment: 1) inoculation with *S. lanosoniveum* at first flower (R1); 2) inoculation with *S. lanosoniveum* at beginning seed development (R3); 3) application of pyraclostrobin fungicide (Headline, BASF Corp; 876 ml product in 187 liters of water per ha) as described above at V6/R1; and 4) nontreated control. Plots were four rows wide by 9 m long, and six replications per treatment were arranged in a randomized complete block design. The center two rows were treated with suspensions of 10<sup>6</sup> spores/ml plus 10<sup>4</sup> CFU/ml of *S. lanosoniveum* isolate D082307-2A. Plants were sprayed until visibly wet (approximately 18-20 ml/m<sup>2</sup>) during the late afternoon when temperatures dropped below 27°C. Ten trifoliolate leaves were sampled weekly from each plot beginning during vegetative stages (V6) of growth and ending at the onset of senescence (R7) (80 DAP). SBR was detected in kudzu approximately 180 m from the plots at 8 DAP.



However, SBR was not detected in this field until 68 DAP when plants were in the R5 growth stage.

#### 4.2.4 DNA Quantification

**Numbers of Sori.** Immediately after sampling, sori were counted on each apical leaflet from each of the 10 trifoliates collected from each plot. Numbers of sori were counted within three fields of vision ( $5 \text{ cm}^2$ ) per leaf with a dissecting microscope at 25x magnification, and sori per/ $\text{cm}^2$  were calculated for each treatment. Outliers were removed if they fell outside the inner quartile range, and one-way ANOVA was used to compare means of sori/ $\text{cm}^2$  [94].

**DNA Extractions.** Leaves stored at  $-20^\circ\text{C}$  until the leaves were ground in liquid nitrogen with mortar and pestle. Subsamples of 50 mg of ground leaf material were transferred to 1.5 ml microcentrifuge tubes and ground again with plastic pestle grinders for 30 sec in extraction buffer (Qiagen, DNeasy Plant Mini Kit). Samples were incubated on a heat block at  $55^\circ\text{C}$  for 30 minutes during which time they were vortexed twice. Genomic DNA was extracted from the ground leaf samples using Qiagen's DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Final DNA concentrations were determined as described above and diluted to  $10 \text{ ng}/\mu\text{l}$  for use in qPCR assays.

**qPCR Assay.** qPCR was used to quantify the amount of DNA of *S. lanosoniveum* and *P. pachyrhizi*. Triplicate samples were tested in a total volume of  $25 \mu\text{l}$  per reaction. Each test for *S. lanosoniveum* included 10 ng template DNA,  $12.5 \mu\text{l}$  TaqMan® Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), 900 nM each forward and reverse primer and 200 nM probe. Reactions for *P. pachyrhizi* included 10 ng template DNA,  $12.5 \mu\text{l}$  TaqMan Universal PCR Master Mix, 15 mM primers Ppm1 and Ppa2, and 10mM FAM probe [35] (Z.Y. Chen and

S. Park, unpublished). The qPCR instrument (ABI 7000, Applied Biosystems, Carlsbad, CA) was run on the following protocol: initial denaturation at 95°C for 10 min followed by 40 cycles of 15°C for 15 sec and 60°C for 1 min. The instrument software package (Applied Biosystems, Carlsbad, CA) automatically analyzed the critical threshold values (Ct) for each reaction [54]. Using absolute quantification, Ct values were converted to picograms (pg) DNA based on standard curves, and outliers were removed if they fell outside of the inner quartile range [94].

#### 4.4 Results

**Field 1.** In the late-planted field, rust severity was at least 25% at the time of inoculation with *S. lanosoniveum*. One hour after inoculation with isolate D082307-2A, there were 4.0 pg (s. d. = 1.2) DNA of *S. lanosoniveum* per 10 ng soybean DNA. At 14 days after inoculation, this value increased to 7.2 pg (s. d. = 1.2). DNA concentration of *S. lanosoniveum* isolate BH081707-1A decreased from 4.0 pg (s. d. = 1.2) to 1.3 pg (s. d. = 1.7) by day 14. Amounts of DNA of *P. pachyrhizi* were used as a measure of disease potential. On day 14, experimental units inoculated with isolate D082307-2A had a mean of 11.0 ng (s. d. = 3.5) DNA of *P. pachyrhizi*, which was significantly lower than 16.5 ng (s. d. = 6.6) detected in the nontreated control. In plots inoculated with isolate BH081707-1A, a mean of 21.7 ng (s. d. = 17.7) DNA of *P. pachyrhizi* was detected, which was not significantly different from the nontreated control. DNA of *P. pachyrhizi* increased between days 7 and 14 in all treatments, but *S. lanosoniveum* isolate D082307-2A resulted in significantly less DNA of *P. pachyrhizi* than isolate BH081707 or the nontreated control by the end of the study (Fig 4.2). Numbers of sori/cm<sup>2</sup> did not differ between inoculated treatments and the nontreated control.

**Field 2.** SBR symptoms were observed on soybeans at 68 DAP when plants reached the R5 growth stage. DNA of *P. pachyrhizi* was detected at 30 days after planting, which resulted in a latent infection period of 38 days. By physiological maturity (R7, 79 DAP), early inoculations with *S. lanosoniveum* (R1) resulted in 7.5 ng (s. d. = 3.8) of DNA of *P. pachyrhizi*, which was not statistically different from 8.5 ng (s. d. = 5.1) detected in fungicide treated leaves. Inoculations that were made after rust symptom development resulted in 12.6 ng (s. d. = 2.4) of rust DNA, which was not significantly different from 11.7 ng (s. d. = 1.5) detected in the nontreated control. By physiological maturity (R7), early treatments with *S. lanosoniveum* (R1) and treatments with pyraclostrobin (R1) both resulted in lower amounts of rust severity and reduced numbers of sori. Disease severity did not differ between these two treatments by the time plants reached physiological maturity (79 DAP) (Fig 4.3).

By the time soybean plants reached physiological maturity (79 DAP), the nontreated control had a mean of 196.8 sori per cm<sup>2</sup> (s. d. = 14.9), while the mean number of sori in the R1 treatment was 143.4 sori per cm<sup>2</sup> (s. d. = 27.7) ( $P = 0.001$ ). The fungicide treatment also had a significantly lower number of sori by the end of the study, 137.8 (s. d. = 31.2), as compared to the nontreated control (Fig 4.4).

*Simplicillium lanosoniveum* increased in all inoculated treatments, with sharp increases in DNA 7 days after application. At 73 DAP (R6), rust severity increased to 25%, and DNA of *S. lanosoniveum* reached 0.3 pg (s. d. = 0.1) in the R1 treatment. In the treatment that was inoculated at >2.5% disease severity, DNA of *S. lanosoniveum* spiked to 0.6 pg (s. d. = 0.1) when disease severity was 50%. By R7, DNA of *S. lanosoniveum* decreased sharply in all treatments.

*Simplicillium lanosoniveum* was quantified in each treatment one hour after inoculation. In the three inoculation treatments, 0.04 pg (s. d. = 0) and 0.06 pg (s. d. = 0.1) were detected in the R1 and <2.5% rust treatments, respectively.

The corn treatments yielded a mean of 0.06 pg (s. d. = 0.1) of *S. lanosoniveum* one hour after inoculation, which was similar to the amount detected in soybean 1-hour after inoculation. In one experiment, corn samples yielded 0.5 pg (s. d. = 0) of *S. lanosoniveum* after 7 days, but on day 14 DNA of *S. lanosoniveum* was not detected. There was no rainfall during this period. In another experiment, 2.5 cm of rain was recorded 3 days after inoculation. DNA of *S. lanosoniveum* was not detected upon sampling at 7 days after inoculation.

**Field 3.** In fields that were planted before soybean rust was detected in the area, SBR symptoms developed at R6 (66 DAP). By the end of the experiment, one sorus was detected in each of two plots. A qPCR assay value of 0.27 pg (s. d. = 0.03) DNA of *P. pachyrhizi* indicated that there was latent infection in some plots as early as 48 DAP (R3). There was a sharp increase in DNA of *P. pachyrhizi* one week before symptoms appeared. However, amounts of DNA were low, and there were no statistical differences in the amounts of DNA of *P. pachyrhizi* among treatments (Fig 4.5).

Initial inoculum levels of *S. lanosoniveum* (one hour after inoculation) were 4.0 pg (s. d. = 0.2) and 4.5 pg (s. d. = 0.1) per 10 ng of total DNA in the R1 and R3 treatments, respectively. By the end of the study (R7), DNA of *S. lanosoniveum* decreased to 2.4 pg (s. d. = 3.4) in early inoculations (R1 treatment). Late inoculations (R3 treatment) resulted in a significant increase to 75.9 pg (s. d. = 56.6) (Fig 4.5).

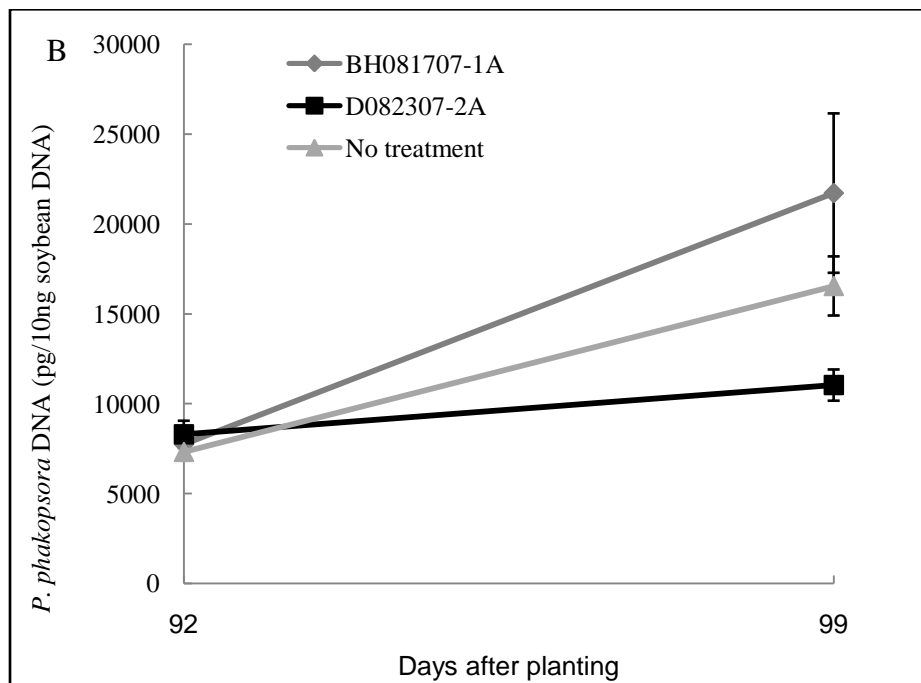
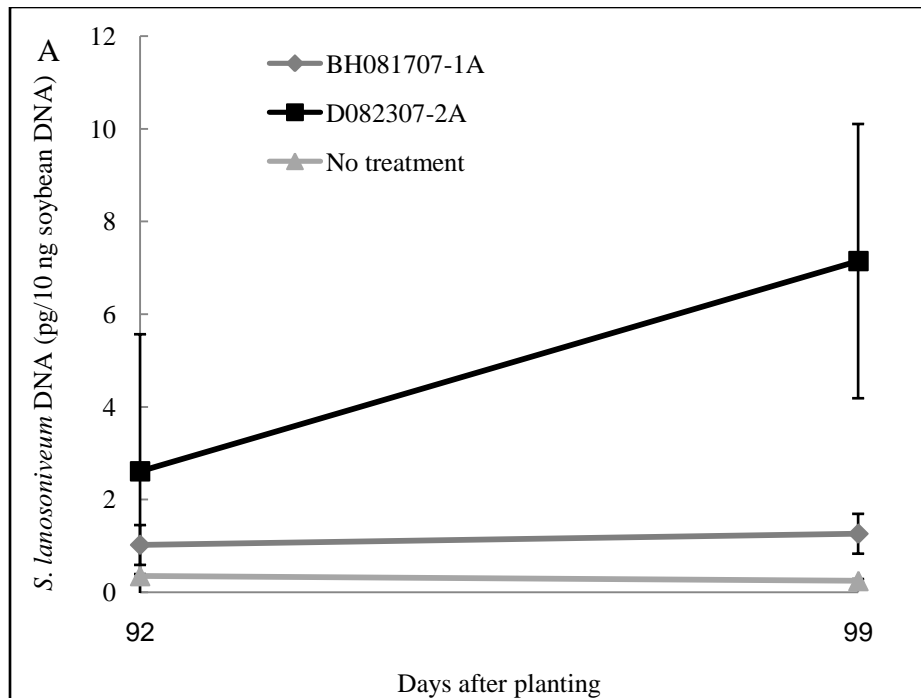


Fig 4.2. Population dynamics of (A) two isolates of *Simplicillium lanosoniveum* and (B) *Phakopsora pachyrhizi* as assessed by monitoring DNA concentrations of these organisms in soybean leaves using quantitative PCR. Assays were conducted following inoculation of field-grown soybean plants with the antagonist, *S. lanosoniveum*. Inoculum was applied 85 and 92 days after planting (DAP), and leaves were sampled 7 days after each inoculation (92 and 99 DAP). Bars represent standard error of the mean.

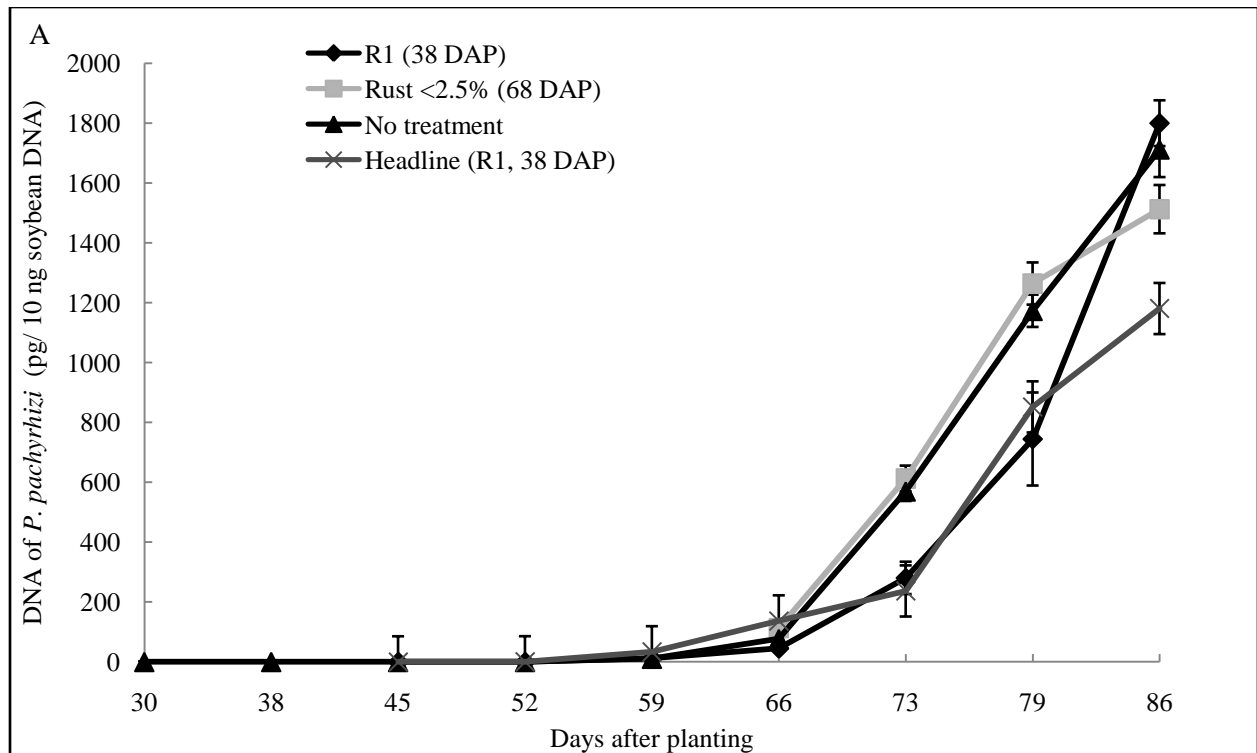
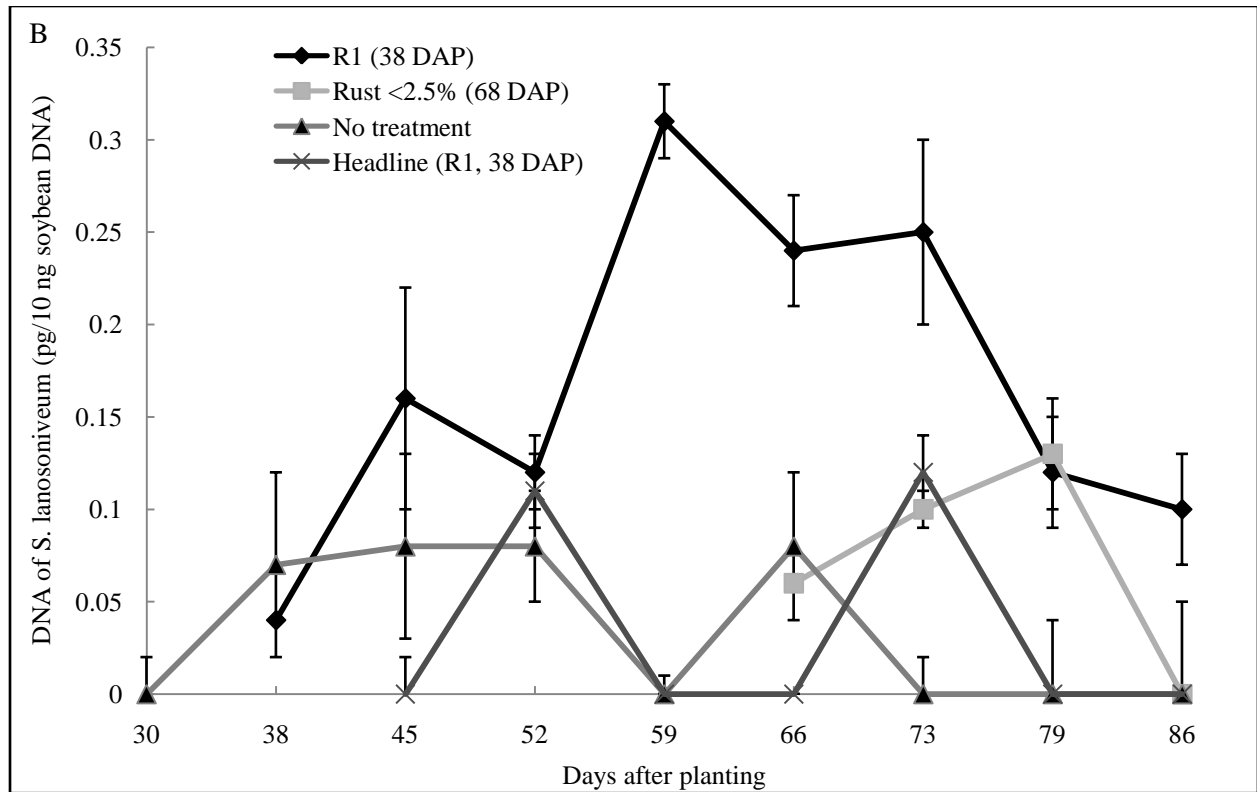


Fig 4.3. Population dynamics of (A) *Phakopsora pachyrhizi* and (B) *Simplicillium lanosoniveum* as assessed by monitoring DNA concentrations of these organisms in soybean leaves using quantitative PCR. Assays were conducted on soybean leaves collected from field plots that had been subjected to the following four treatments: Plants were inoculated with *S. lanosoniveum* either at R1 when there were no symptoms of soybean rust (R1) or when rust severity was no more than 2.5% (Rust <2.5%). Plants were sampled one hour after inoculation and once per week thereafter. Other treatments included the fungicide pyraclostrobin (Headline) applied at R1 or a nontreated control (No treatment). Bars represent standard error of the mean.

Fig 4.3 continued



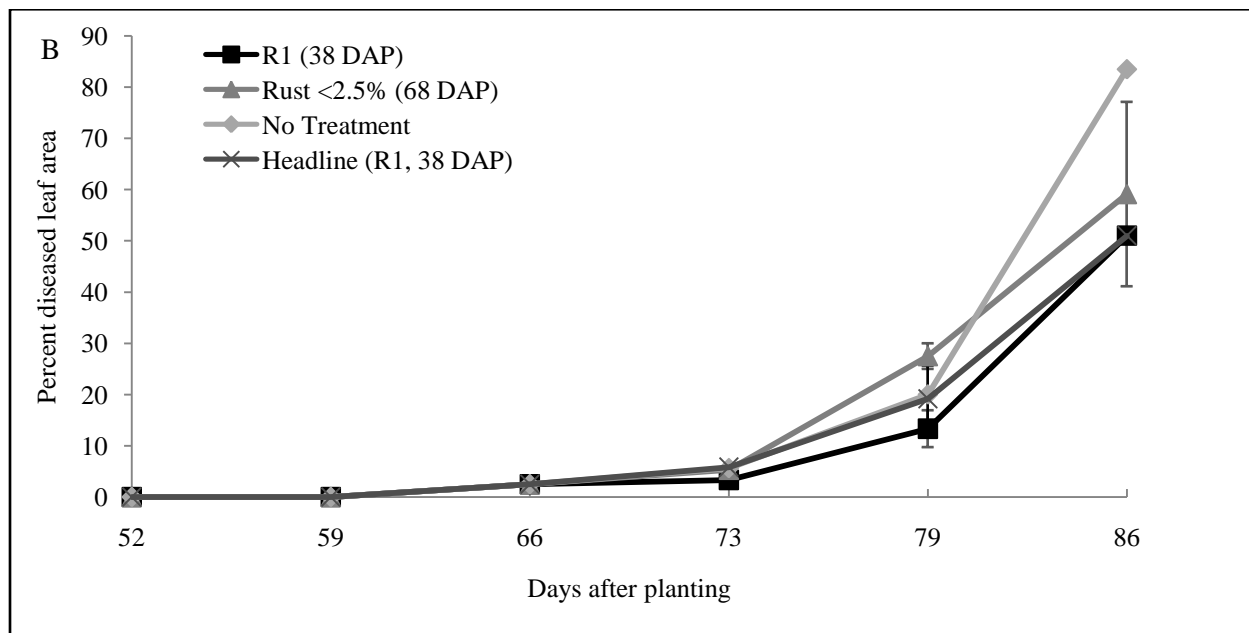
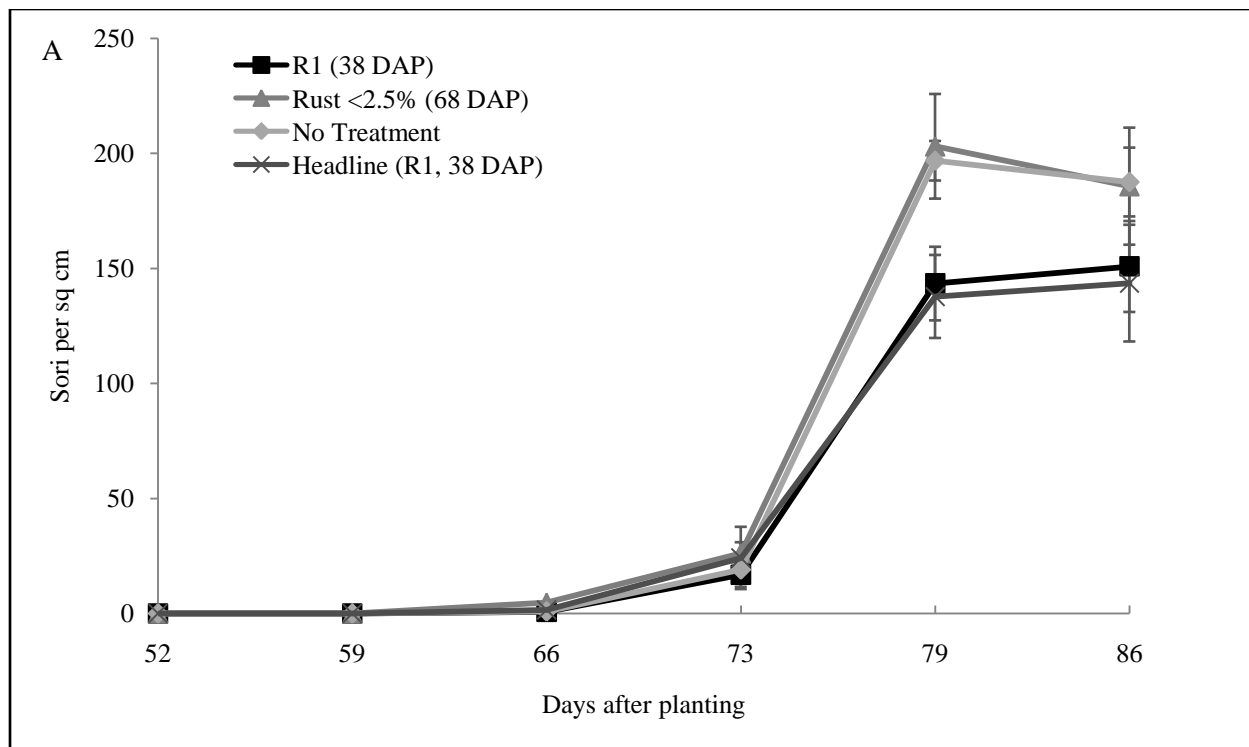


Fig 4.4. Effects of inoculation of soybean leaves with *Simplicillium lanosoniveum* on soybean rust as assessed by (A) numbers of sori per cm<sup>2</sup> leaf area and (B) percent diseased leaf area as a function of days after planting. Plants were inoculated with *S. lanosoniveum* either at R1 when there were no symptoms of soybean rust (R1) or when rust severity was no more than 2.5% (Rust <2.5%). Plants also were sprayed with the fungicide pyraclostrobin (Headline) at R1, or they were not treated (No treatment). Bars represent standard error of the mean.



## 4.4 Discussion

In earlier studies, hyphae of *S. lanosoniveum* were documented through scanning electron microscopy wrap around urediniospores of *P. pachyrhizi* and colonized sori [101]. Furthermore, detached leaf assays were used to evaluate the effects of *S. lanosoniveum* on SBR. When inoculated with *S. lanosoniveum*, soybean leaves contained significantly fewer sori and viability of urediniospores was significantly reduced [100]. To evaluate the effects of *S. lanosoniveum* under field conditions, field trials were conducted in Louisiana and Florida in 2009 and 2010. qPCR was used to quantify the establishment of *S. lanosoniveum* and its effects on SBR on both diseased soybeans and disease-free plants under field conditions.

In Field 1, two isolates of *S. lanosoniveum* used in previous experiments were tested [101]. This field study utilized several commercial soybean cultivars, all with similar degrees of SBR severity (25%). Isolate D082307-2A colonized and established in sori in all cultivars. Mycelia were often visible under low magnification with a dissecting microscope. Isolate BH081707-1A, on the other hand, did not readily colonize sori. Mycelia were visible in less than 1% of sori, and amounts of DNA were not significantly different from that of the nontreated control. In Field 2 and Field 3, only isolate D082307-2A was used for inoculations.

Weather appeared to have an effect on colonization of *S. lanosoniveum* in soybean fields. The Field 1 study was conducted in October and November 2009. It was suspected that there were more *S. lanosoniveum* in sori, as assessed with qPCR and visibly observed mycelia and conidia in sori, in this late-planted field than in the other studies because conditions were favorable for SBR development. Average high temperature was 23°C, and there was 9.2 cm rainfall during the month of the study. In Field 2 in 2009, mycelia were occasionally observed in sori. This field

study was conducted from August through October and temperatures were lower than normal because of frequent rainstorms and cloudy skies. Maximum daytime temperatures averaged 30° to 33°C, and precipitation ranged from 5.5 to 9.5 cm per month ([www.lsuagcenter.com](http://www.lsuagcenter.com)). Field 3 study was conducted in August and September 2010. Rainfall at this site was similar to Field 2 (7 to 11 cm per month), however, maximum daytime temperatures averaged 36°C ([www.fawn.ifas.ulf.edu](http://www.fawn.ifas.ulf.edu)).

Whether temperatures were favorable or not for SBR development, *S. lanosoniveum* failed to colonize field-grown leaves unless SBR was present in some form. *Simplicillium lanosoniveum* was an aggressive colonist when sori were present.

Additionally, *S. lanosoniveum* colonized leaves when SBR was in a latent stage, even in the absence of sori. It is likely that urediniospores on leaf surfaces may have provided a sufficient nutrient source to sustain *S. lanosoniveum* until sori developed. This phenomenon was observed in Field 2 in the earliest (R1) treatment. In this instance, latent infection was detected, but SBR symptoms did not occur until 30 days later. *Simplicillium lanosoniveum* colonized soybean leaves early in the disease cycle. In Field 3, on the other hand, *S. lanosoniveum* did not readily colonize leaves following the early (R1) inoculation. There was no latent infection at this stage, and we suspect that this was because inoculum of *P. pachyrhizi* was extremely low or nonexistent. The later inoculation (R3) was applied 2 weeks before disease symptoms developed. Latent infection was detected and we suspect that inoculum of *P. pachyrhizi* was present in large enough quantities to sustain the antagonist. At this point, *S. lanosoniveum* began to colonize soybean leaves.

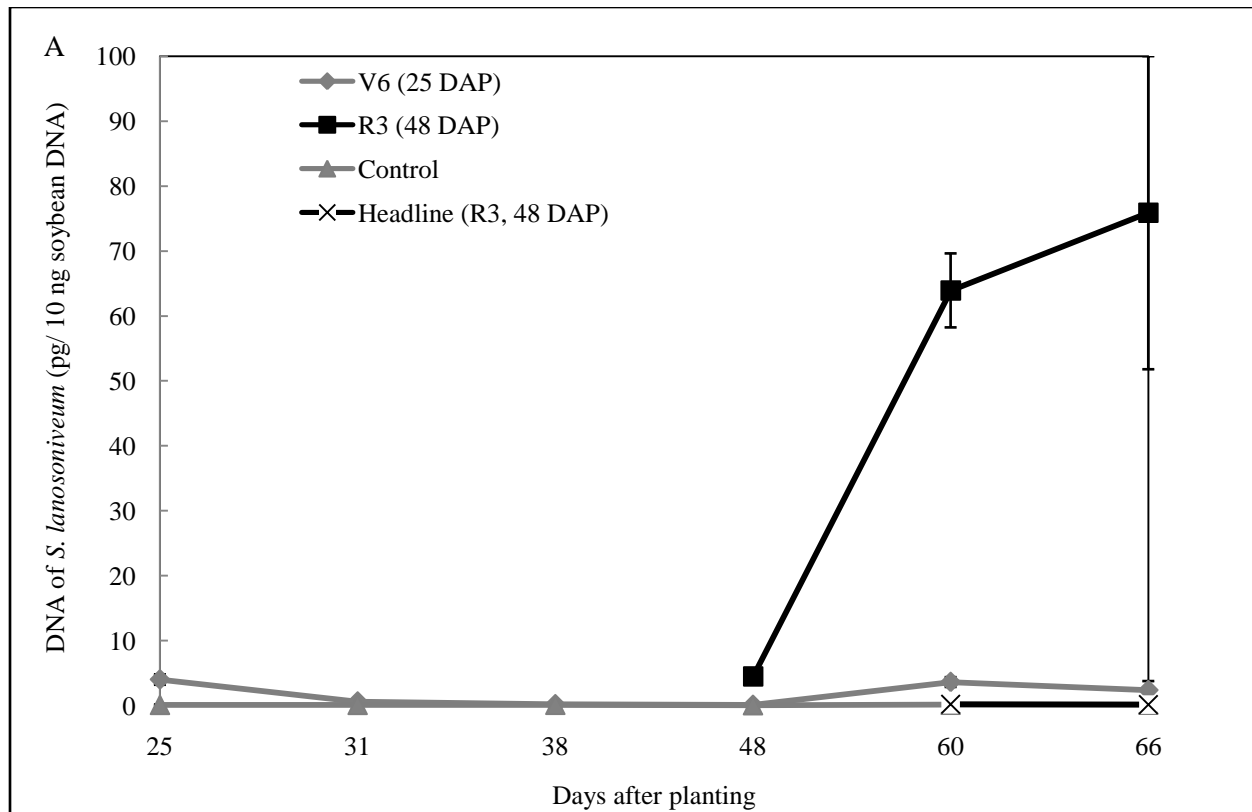
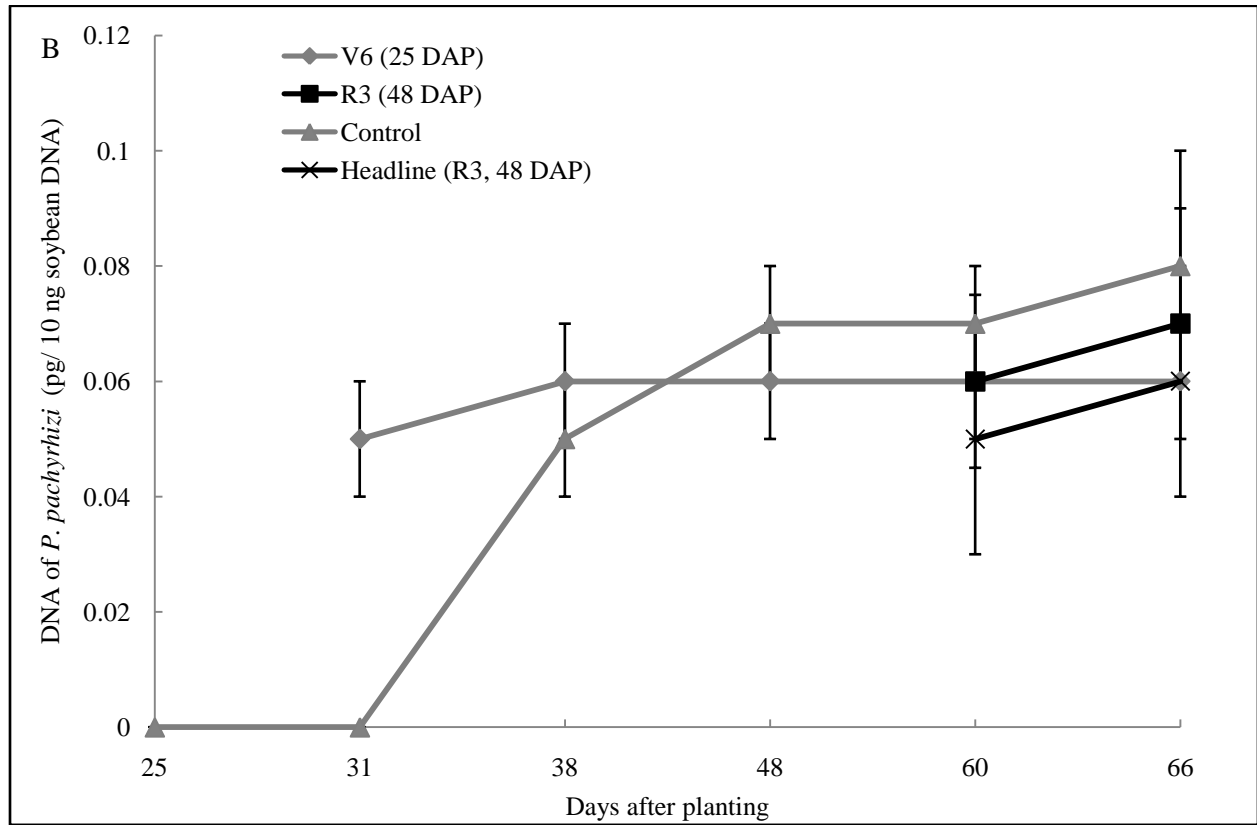


Fig 4.5. Population dynamics of (A) *Simplicillium lanosoniveum* and (B) *Phakopsora pachyrhizi* as assessed by monitoring DNA concentrations of these organisms in soybean leaves using quantitative PCR. Assays were conducted following inoculation of field-grown soybean plants with the antagonist, *S. lanosoniveum*. Samples were collected from field plots that had been subjected to the following four treatments: Plants were inoculated with *S. lanosoniveum* at either the V6 or R3 growth stages when there were no symptoms of soybean rust. Sampling began one hour after inoculations and continued every 7 to 10 days thereafter. Other treatments included the fungicide pyraclostrobin at R3 (Headline R3) and a nontreated control (Control). Bars represent standard error of the mean.

Fig 4.5 continued



In early inoculations in Field 2, amounts of *S. lanosoniveum* increased significantly for the first 4 weeks after inoculation, although it fluctuated from week to week. However, at 73 DAP (R7), there were sharp decreases in amounts of *S. lanosoniveum* in the two inoculated treatments. This decrease was not associated with weather extremes such as high temperatures or dry weather. Sori were heavily sporulating at this point, and many phylloplane inhabitants were observed as leaves senesced. Soybean leaves became increasingly chlorotic and sori developed necrotic margins. Most likely, competition for nutrients or exudates from uredinial inhabitants may have affected growth of *S. lanosoniveum*. This phenomenon was not observed in Field 3 because SBR did not develop until late in the study, SBR severity was extremely low, and amounts of DNA of *P. pachyrhizi* were too small to distinguish differences between treatments.

Initial amounts of *S. lanosoniveum* inoculum applied to soybeans in these field studies were quantified. Approximately 0.06 pg of DNA per 10 ng soybean DNA was detected when conidia alone were used as inoculum in Field 2. This was similar to results obtained with corn, which served as a nonhost control. In corn, there was no increase in amounts of DNA 7 days after inoculation, and then there was a sharp decrease in amounts of *S. lanosoniveum*. When rain occurred after inoculation of corn leaves, *S. lanosoniveum* was not detected on the day 7 sampling. In Field 3, 4.0 to 4.5 pg DNA of *S. lanosoniveum* was detected one hour after inoculation. In this field study, both conidia and mycelia were used as inoculum. In both Field 2 and Field 3, *S. lanosoniveum* was not detected 14 days after application unless rust (latent infection or visible sori) was present. In all field studies, *S. lanosoniveum* colonized diseased soybean leaves and failed to colonize disease-free leaves.

In Field 2, there were significant differences in numbers of sori per cm<sup>2</sup> between the treated and the nontreated control. Additionally, this field contained more red-brown sori, which contained

fewer secondary sori, and more necrotic tissue surrounding primary sori [61, 100]. Overall, disease was less severe in treatments inoculated with *S. lanosoniveum*. In Field 1, on the other hand, numbers of sori were extremely high when we began the experiment, and by day 14, SBR severity reached a plateau, and defoliation had already begun, which is a reasonable explanation for the lack of significant differences in sorus production. However, differences were observed between treatments in sorus size and apparent age as observed in Field 2. In Field 3, only two experimental units showed disease symptoms, but only one sorus was detected from each one.

*Simplicillium lanosoniveum* was effective in slowing the rate of disease development, and this was reflected in the DNA assays in two of the three field experiments. In the third experiment conducted in 2010, following hard freezes and significant reductions in alternative hosts such as kudzu, SBR was not detected on soybean in Florida until July. Moreover, SBR was not detected within 22 km of this field until 11 days before symptoms were discovered. Once inoculum of *P. pachyrhizi* was sufficient or sori developed, *S. lanosoniveum* colonized sori.

Based on the aforementioned results, *S. lanosoniveum* is an ideal candidate for biological control because it readily colonized soybean leaves and was effective in reducing SBR severity. Results from these and other studies warrant the use of this antagonistic fungus as part of an IPM program in combination with other cultural practices or as a biological control agent in organic soybean production systems [100-101]. Additional research should include the effects of sunlight on survival and colonization of leaf surfaces by *S. lanosoniveum* and the development of formulations with extended shelf life.

## CHAPTER 5. MYCOPARASITISM OF *PHAKOPSORA PACHYRHIZI* BY *SIMPLICILLIUM LANOSONIVEUM*: A MICROSCOPY STUDY

### 5.1 Introduction

Soybean rust (SBR), caused by *Phakopsora pachyrhizi* Syd. & P. Syd., was first reported in Japan in 1902 [22, 73]. Since then, it has spread throughout soybean-growing regions of Asia, Africa, and the Americas. The disease was reported in the continental United States in 2004 [84]. Yield losses in the southern US, where the disease has become endemic, range between 30% and 80% in soybean not protected with fungicides (D. R. Walker and R. W. Schneider, personal communication) [30, 76]. Thus, preventative fungicides are critical for effective disease management. SBR has been shown to overwinter on kudzu in the lower Gulf South, where the invasive vine may remain green during the winter months.

Our previous studies showed that *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001 (family Cordyciptaceae) colonized sori of SBR. This fungus is discussed in taxonomical references (Zare and Gams) and collections but has never been examined as a mycoparasite [25, 109]. *Simplicillium lanosoniveum* belongs to the family Cordyciptaceae, which also includes mycoparasitic and entomopathogenic fungi.

Our previous studies included detached leaf assays, which documented significant reductions in SBR disease severity was documented, and increases in brown urediniospores that failed to germinate were observed. Additionally, field studies indicated that *S. lanosoniveum* colonized soybean leaves infected by the pathogen. In all experiments, *S. lanosoniveum* failed to colonize leaves that were not infected by *P. pachyrhizi* [100].

Early observations using scanning electron microscopy indicated that *S. lanosoniveum* colonized sori of SBR, and hyphae coiled through sori and wrapped around urediniospores. These microscopic studies also confirmed that the fungus failed to establish on healthy leaf surfaces. Hyphae traversed leaf surfaces until reaching sori, at which point they rapidly colonized sori.

Examination of these hyphae of *S. lanosoniveum* stained with Calcofluor white® and viewed with a fluorescence microscope revealed hyphae of the antagonist coiled inside urediniospores after 3 to 5 days [99]. This preliminary study indicated that *S. lanosoniveum* may be mycoparasitic, but we could not confirm whether observed hyphae were from *S. lanosoniveum* or another inhabitant from field-collected urediniospores. Using this method, mode of action of this mycoparasitic interaction could not be verified.

In this work, microscopy studies were conducted, including scanning electron microscopy (SEM), transmission electron microscopy (TEM), and confocal microscopy to examine interactions between *S. lanosoniveum* and *P. pachyrhizi*. Our objectives were to determine the mode of action of *S. lanosoniveum* by examining surface interactions between the two fungi, observing entire sori, the inner and outer interactions, and determining the effect of *S. lanosoniveum* on urediniospore membranes in order to determine if they were enzymatically degraded.

## **5.2 Methods**

### **5.2.1 Urediniospore Collection**

Field-grown soybeans were infected with naturally occurring inoculum of the SBR pathogen in 2009. Urediniospores were collected with a spore collector (G&R Mfg, Manhattan, KS)



retrofitted for a hand-held battery powered vacuum (Craftsman, Sears, Hoffman Estates, IL) in October and November 2009. Urediniospores were collected into 20 ml scintillation vials and stored dry at -80°C until use. Upon removal from storage, approximately 5 mg urediniospores were rehydrated by placing them in an uncovered petri dish lid in a 100 mm glass desiccator with 2 cm distilled water in the bottom [17, 20]. Urediniospores were rehydrated for 24 hours, and germination was assessed by placing them on 1.5% water agar for 4 hours in the dark at 25°C [95]. Urediniospores were considered germinated when germ tubes were at least as long as the length of the urediniospore. Urediniospore collections with germination rates above 80% were used in all experiments.

### **5.2.2 Scanning Electron Microscopy**

SEM was used to examine the topography of colonized sori, to view possible adhesion of hyphae to urediniospores, to determine whether the integrity of urediniospores remained intact, and to determine whether hyphae were confined to sori.

Inoculum was prepared as follows: Urediniospore suspensions of *P. pachyrhizi* were prepared by suspending rehydrated spores in 0.01% Tween 20<sup>®</sup> and adjusting to 10<sup>6</sup> spores/ml using a hemacytometer. Inoculum of *S. lanosoniveum* (isolate D082307-2A [GenBank accession number HQ270476]) was prepared by growing the fungus on potato dextrose agar for 14 days. Dishes were flooded with sterile phosphate buffer (0.5 mM, pH 7.1) and rubbed with a glass rod to dislodge conidia. Conidial suspensions were diluted in 0.01% Tween 20<sup>®</sup> and adjusted to 10<sup>6</sup> spores/ml using a hemacytometer [101].

Soybean cultivar Asgrow 6202 (Monsanto) was grown outdoors in 22 cm containers in sterile potting mix (Scotts, Marysville, OH). Leaves were harvested after flowering and placed in moist

chambers made with 10cm x 23cm x 25 cm polystyrene boxes lined with moist paper towels. Suspensions of urediniospores were spread on the abaxial side of leaves at 1 ml per leaf with a glass rod. Leaves were allowed to dry (approximately one hour) and then moist chambers were closed and incubated at 25°C with a 12-hour photoperiod under fluorescent lights (850-1000 lux) [101]. Sori developed within 7 to 10 days. On day 14, conidial suspensions of *S. lanosoniveum* were introduced at one ml per leaf and spread with a glass rod.

On days 5, 7, 10, and 14 after inoculation with the mycoparasite, colonized sori were cut from leaves and fixed in FAA overnight [77, 101]. After dehydration in an ethanol series culminating in 100% EtOH, samples were critical point dried and affixed to stubs [82]. Stubs were sputter coated with 60:40 gold:palladium. Samples were viewed and photographed with a SEM (JEOL JSM-6610).

### **5.2.3 Confocal Microscopy**

Because insides of colonized urediniospores could not be visualized using SEM, optical sectioning with a confocal microscope was used to view both inner and outer interactions of colonized urediniospores. Use of fluorescent probes with this microscope allowed the determination of the proportion of urediniospores that became colonized during cohabitation and to construct a time line for this process.

For visualization with the confocal microscope, *S. lanosoniveum* isolate D082307-2A (Genbank accession number HQ270476) was transformed with green fluorescent protein (GFP). The fungus was grown on potato dextrose agar at 25°C. It was initially tested for sensitivity to the antibiotic geneticin at different concentrations (up to 15,000 µg/ml). Transformants were generated using *A. tumefaciens* strain EHA105 harboring the pSK2265 T-DNA binary vector

(Maruthachalam et al, unpublished). Conidia of the individual transformants were harvested, resuspended in sterile distilled water, and plated on water agar to obtain monoconidial cultures. A single conidium from each of the transformants was picked and transferred to a Petri dish with potato dextrose agar. The fluorescence of ZsGreen from all the transformants was confirmed with a compound microscope (Olympus BX60) equipped with a GFP filter (450-490 nm excitation, 500 nm emission). Transformants were stored in glycerol stocks at -80°C and regrown on potato dextrose agar for routine laboratory work. Transformants were tested for their ability to colonize sori, and one isolate, D082307-2A-GFP15, was selected for further studies.

Upon reactivation, the test isolate was grown on 1% cornmeal agar topped with sterile Watman #10 filter paper by spreading one ml of a  $10^6$  conidial suspension over the filter paper for ease of sampling. Urediniospores were removed from storage and rehydrated as previously described, and urediniospores with germination rates of at least 80% were used in these studies.

Approximately one mg of dry urediniospores was spread across a 5 cm<sup>2</sup> area on 14-day old cultures of *S. lanosoniveum*. After 2 to 5 days, urediniospores were picked from the filter paper and placed on microscopic slides.

Confocal images were generated using a Leica TCS SP2 scanning laser confocal microscope with a 63x 1.4 NA Apo lens. A 488nm Krypton/Argon laser was used to excite both the GFP in the hyphae of *S. lanosoniveum* and the autofluorescent compounds in the urediniospores of *P. pachyrhizi*. Hyphal GFP images were obtained by collecting the 510-535 nm emission spectra, and the images of the red autofluorescence from the urediniospores were made using the 550-650 nm spectra. Images were processed using the Leica TCS software.

#### 5.2.4 Transmission Electron Microscopy

To examine the subcellular interactions of *S. lanosoniveum* on urediniospores of *P. pachyrhizi*, TEM was used to examine whether the fungus directly penetrated the urediniospore walls, entered through germ pores, or degraded outer membranes of urediniospores before infecting. Furthermore, effects of *S. lanosoniveum* was examined on subcellular membranes upon colonization of urediniospores.

Filter paper cultures were prepared as described above. After 1 to 5 days, filter paper sections with urediniospores were cut into 1 mm squares and prepared as follows. Controls consisted of urediniospores spread onto filter paper cultures as described above except that the cultures did not include *S. lanosoniveum*.

Samples were fixed in formalin acetic acid alcohol for 4 hrs, washed 4x in cacodylate buffer, and rinsed overnight. Fixation in OsO<sub>4</sub> followed for 2 hrs, and samples were washed 2x in cacodylate buffer and 2x in distilled water. Samples were stained with uranyl acetate blocking stain for 1 hour, and then washed 2x with distilled water. Dehydration in an EtOH series followed ending in 100% EtOH. Infiltration with LR White™ resin culminated with 100% resin, and then samples were embedded in 100% LR White™ resin and polymerized overnight at 70°C.

Embedded samples were sectioned at 1.5µm thick using an ultramicrotome equipped with a diamond knife. The contrast stain was uranyl acetate. Ultra-thin sections were viewed with a JEOL 100-CX TEM at an acceleration voltage of 80 KV.

## 5.3 Results

### 5.3.1 Scanning Electron Microscopy

SEM revealed a mycophilic attraction of *S. lanosoniveum* to urediniospores of *P. pachyrhizi*. Hyphae were observed traversing leaf surfaces, colonizing sori but failing to colonize leaf surfaces that were free of pustules. Within 3 days, hyphae coiled through sori and wrapped tightly around urediniospores (Fig 5.1). Urediniospores often collapsed within 5 days. Possible penetration points made of numerous hyphae growing together in a radial pattern were observed (Fig 5.2). The mycoparasite produced fibrous adhesive material between hyphae and urediniospores, and hyphae appeared to adhere to urediniospores. Five days after inoculation, sunken germ pores were observed, and after 14 days, there were numerous holes in urediniospores (Fig 5.3). The fungus produced conidia from hyphae as early as 3 days after inoculation, and after 7-10 days, conidia were produced on long single phialides exiting collapsed urediniospores (Fig 5.4).

### 5.3.2 Confocal Microscopy

Isolate D082307-2A-GFP15 was observed colonizing urediniospores three days after co-culture at which time it had ramified throughout uredinia (Fig 5.5). While hyphae were observed wrapping around urediniospores, they did not appear to constrict urediniospores with mechanical pressure. Urediniospores autofluoresced red at a wavelength of 514 nm, allowing a visible observation that a spore coat had thinned over germ pores by 3 days after inoculation. Hyphae of *S. lanosoniveum* were frequently observed entering or exiting germ pores (Fig 5.6).

Hyphae branched extensively upon entering urediniospores where they formed tight coils. Three-day-old coinoculations revealed heavy infection, with over 60% of urediniospores colonized by the antagonist. By day five, over 90% of urediniospores were colonized with GFP-transformed *S. lanosoniveum*. Colonization was evenly distributed within urediniospores, and hyphae coiled around the inner spore coat and throughout the cytoplasm.

### **5.3.3 Transmission Electron Microscopy**

Ultrathin sections of infected urediniospores revealed sites of penetration and degradation of urediniospores. One day after inoculation, hyphae were observed in about 10% of urediniospores. Urediniospore organelles were not recognizable, appearing aggregated (Fig 5.7). Germ pores were often visible as a thinning spore coat. Throughout this entire process, the integrity of the urediniospore wall was preserved, and there was no evidence of thigmotrophic pressure exerted by the fungus. In the noninoculated control

On day two, urediniospore cytoplasm appeared to be aggregated and restricted to undefined masses of granulated material. By this time, urediniospores began to appear to be evacuated, and remaining cytoplasm was disorganized. Hyphae of *S. lanosoniveum* were observed in about 25% of urediniospores.

After three days, coinoculated urediniospores were heavily colonized with the antagonist.

Ultrathin sections revealed that hyphae of *S. lanosoniveum* entered and exited germ pores.

Approximately 60% of urediniospores were heavily colonized with hyphae, and organelles were completely absent (Fig 5.8).

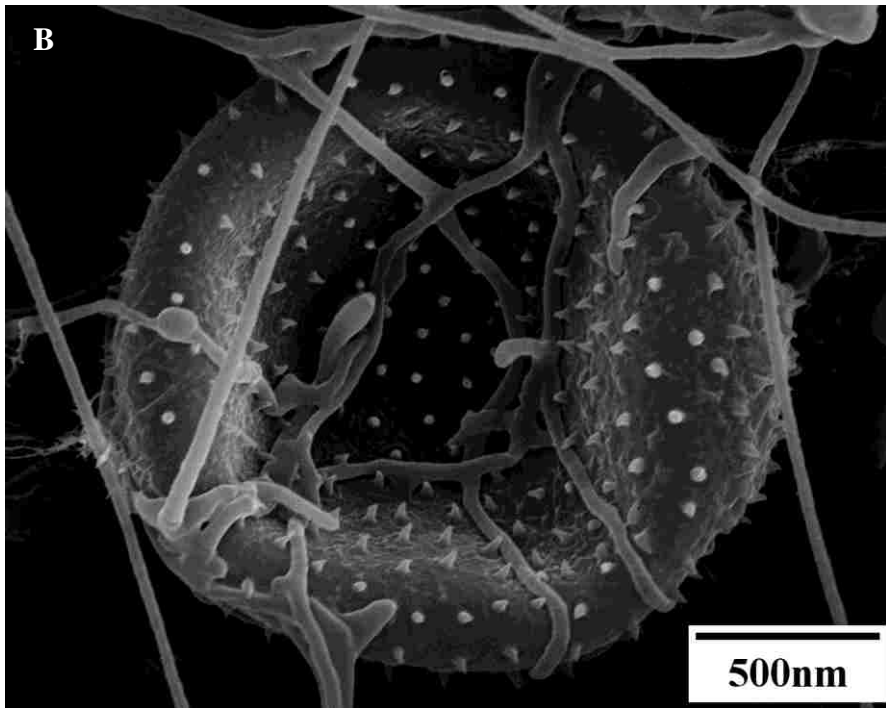
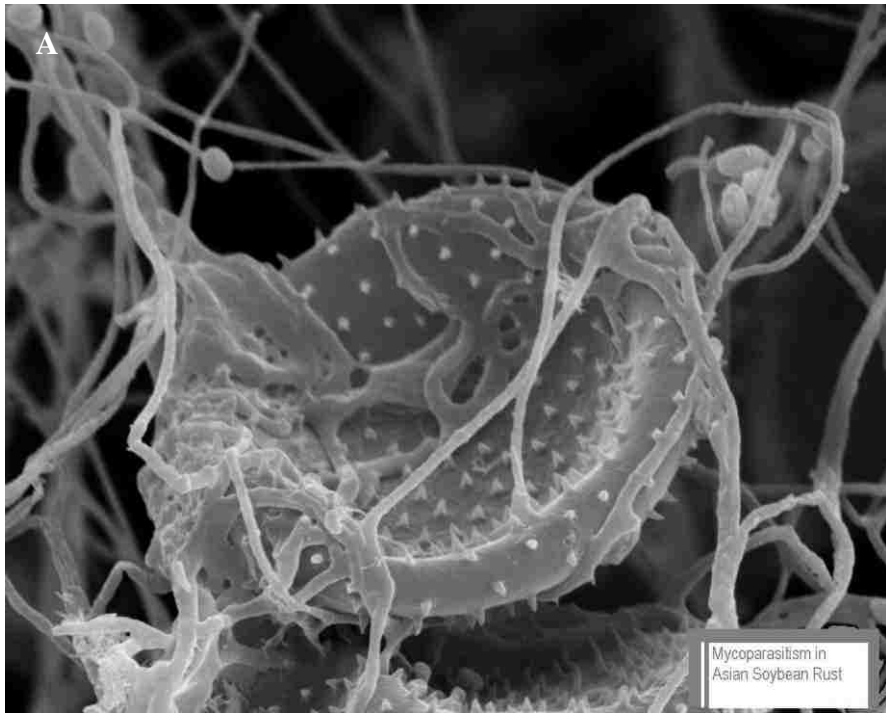


Fig 5.1. Scanning electron microscope view of hyphae of *Simplicillium lanosoniveum* coiled around urediniospores of *Phakopsora pachyrhizi*.

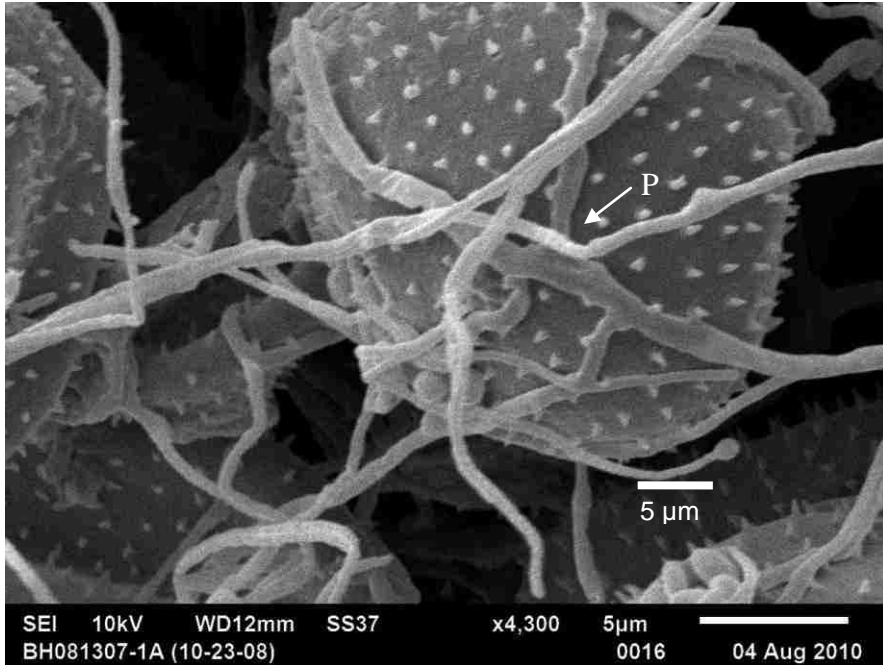


Fig 5.2. Scanning electron microscope view of putative penetration points produced by *Simplicillium lanosoniveum* after 7 days.

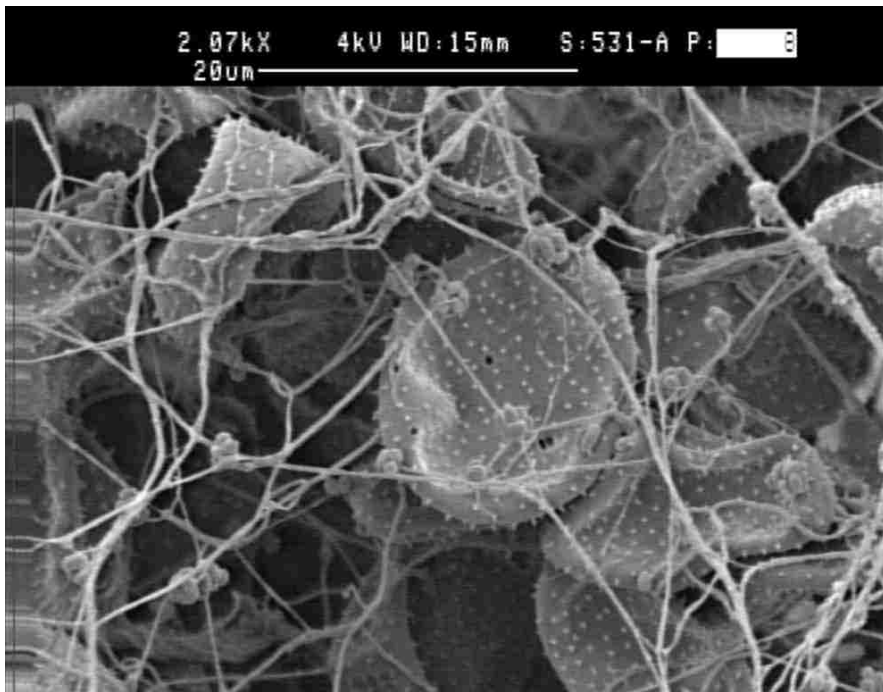


Fig 5.3. Scanning electron microscope view of urediniospores of *Phakopsora pachyrhizi* 14 days after being colonized by *Simplicillium lanosoniveum*.



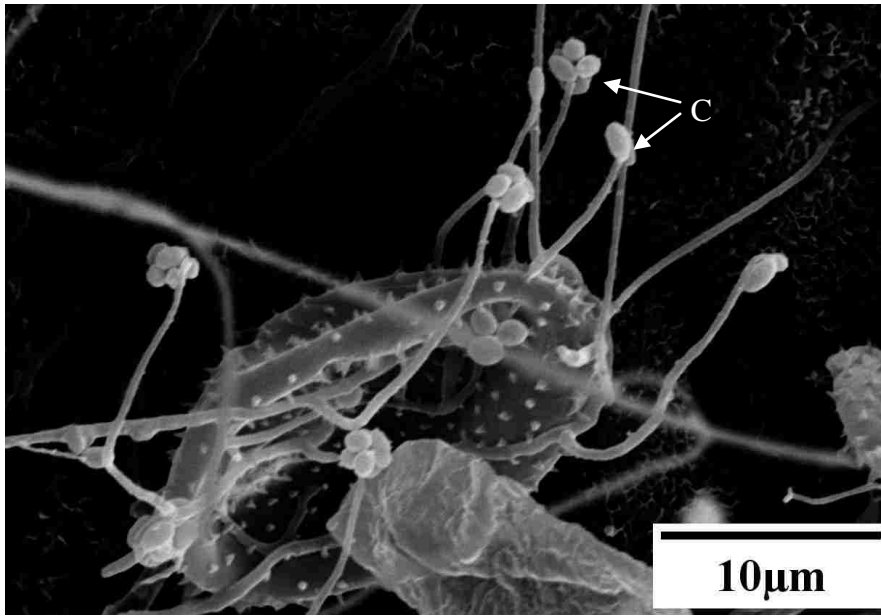


Fig 5.4. Scanning electron microscope view of conidia of *Simplicillium lanosoniveum* produced on long phialides extending from colonized urediniospores.

On day 10, all urediniospores were empty of their contents, no organelles were observed, and there was a near absence of hyphae of *S. lanosoniveum*. Most germ pores were degraded.

Urediniospores were misshapen and appeared collapsed (Fig 5.9).

Urediniospores not exposed to *S. lanosoniveum* were normally shaped and were surrounded by a thick spore coat. All organelles appeared intact, and germ pores were not sunken and their walls were relatively thick (Fig 5.10).

## 5.4 Discussion

Mycoparasitism, defined fungi that feed on other fungi, was clearly documented in this microscopic study, and our results are similar to interactions observed in other systems [4]. For example, putative penetration points were observed in *Cladosporium* when it parasitized bean rust (*Uromyces appendiculatus*) and western gall rust (*Endocronartium harknessii*) [8, 93]. In these interactions, adhesive material allowed close contact and provided the physical means by which lytic enzymes or toxic metabolites attacked the host at entry sites [7, 14, 66]. Assante determined that this enzymatic degradation of the cell wall polysaccharides and breakage of their secondary linkages with polymeric chitin microfibrils must occur to supply *C. tenuissimum* with nutrients during the first steps of this interaction [8].

Mycoparasites and entomopathogens are common in the Cordyciptaceae family, which includes *S. lanosoniveum*. *Verticillium lecanii*, now classified as *Lecanicillium lecanii*, also is a member of this family, and this entomopathogen penetrated insects within 72 hours [57]. Askary indicated that *L. lecanii* also was hyperparasitic on powdery mildew [7]. In this interaction, there were indications that cell-wall degrading enzymes supplemented active penetration by the pathogen. In addition, *L. psalliotae* is parasitic on root knot nematode eggs [5].

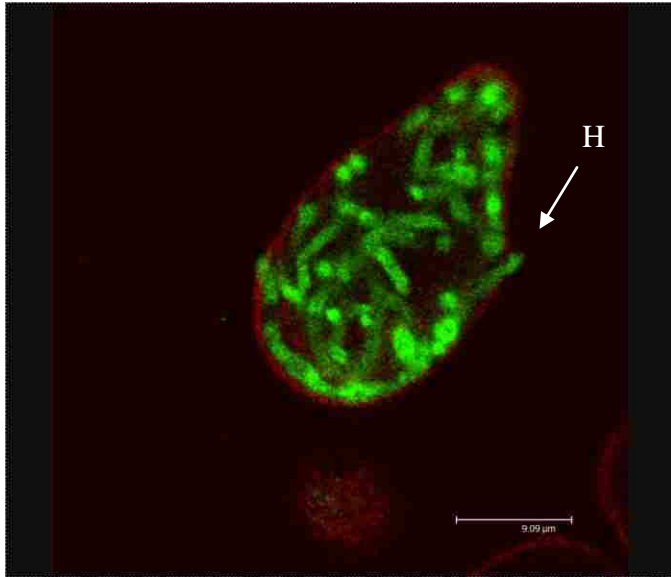


Fig 5.5. Confocal microscope view of a urediniospore of *Phakopsora pachyrhizi* colonized by *Simplicillium lanosoniveum* transformed with green fluorescent protein three days after coculture. (H) Hypha of *S. lanosoniveum* penetrating spore wall through a germ pore.

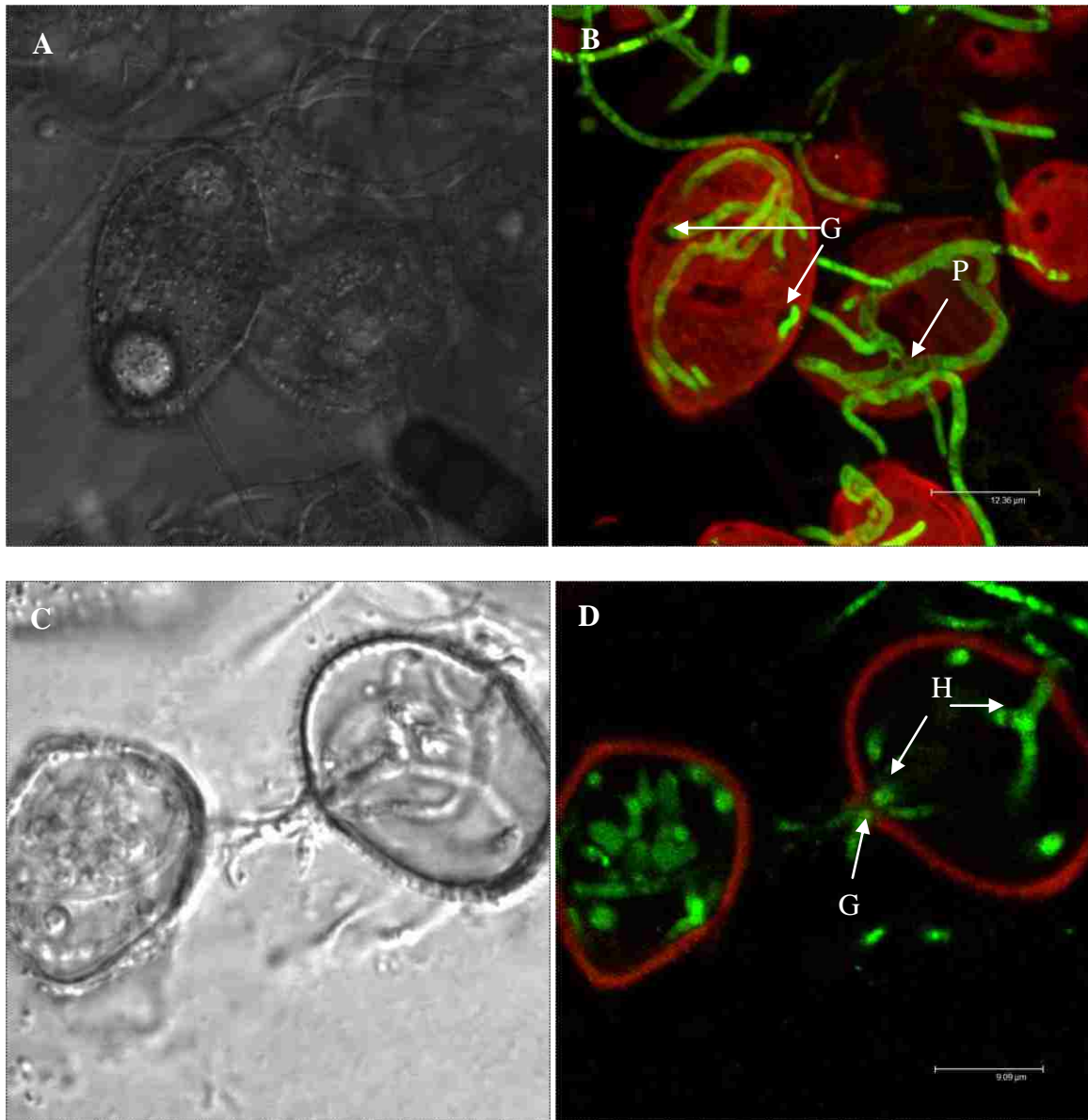


Fig 5.6. Confocal microscope (B & D) and brightfield images (A & C) of urediniospores of *Phakopsora pachyrhizi* colonized by *Simplicillium lanosoniveum* three days after co-culture. Confocal images consist of 50-60 focal planes through a section approximately 20  $\mu\text{m}$ . After 3 days, (H) hyphae of *Simplicillium lanosoniveum* were observed entering and exiting germ pores (G) of *Phakopsora pachyrhizi*. (P) Putative penetration point

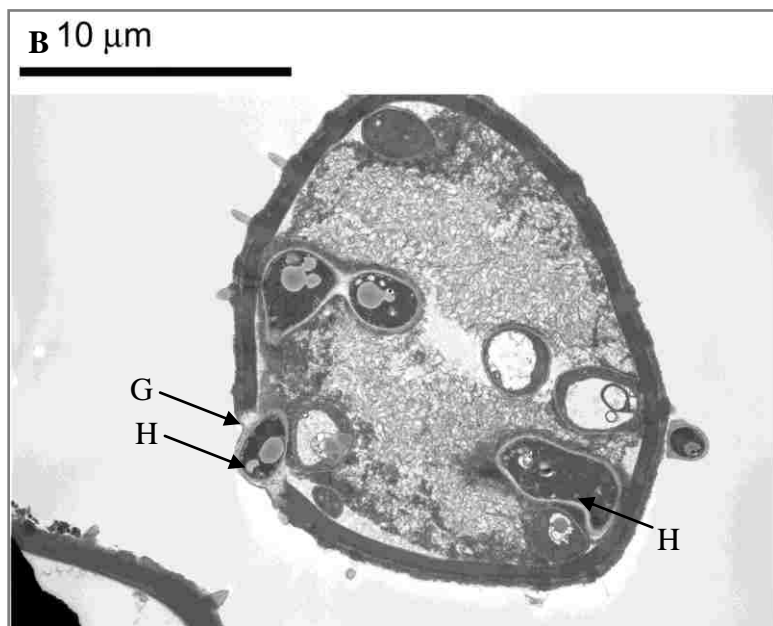
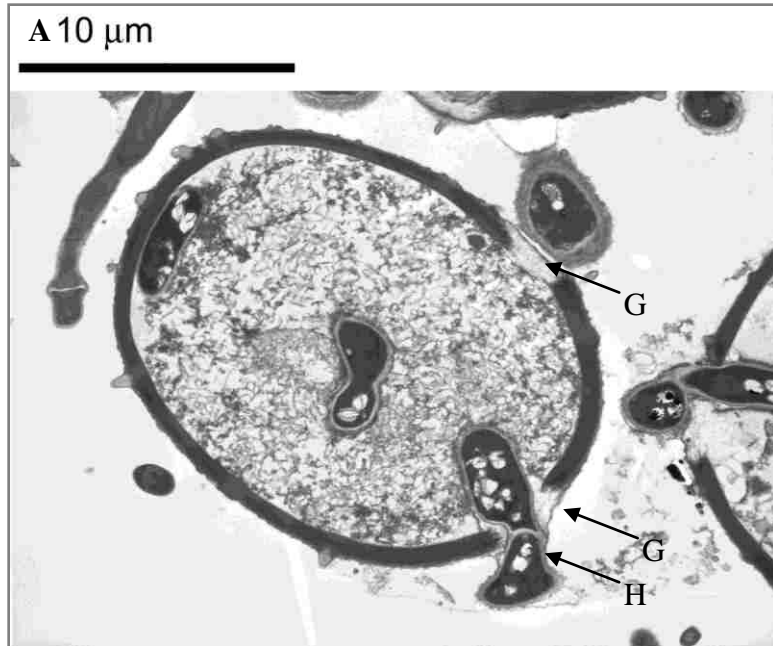


Fig 5.7. Transmission electron microscope views of urediniospores of *Phakopsora pachyrhizi* one day after co-culturing with *Simplicillium lanosoniveum*. Cytoplasm was often granulated and hyphae of *S. lanosoniveum* were often present (A & B).

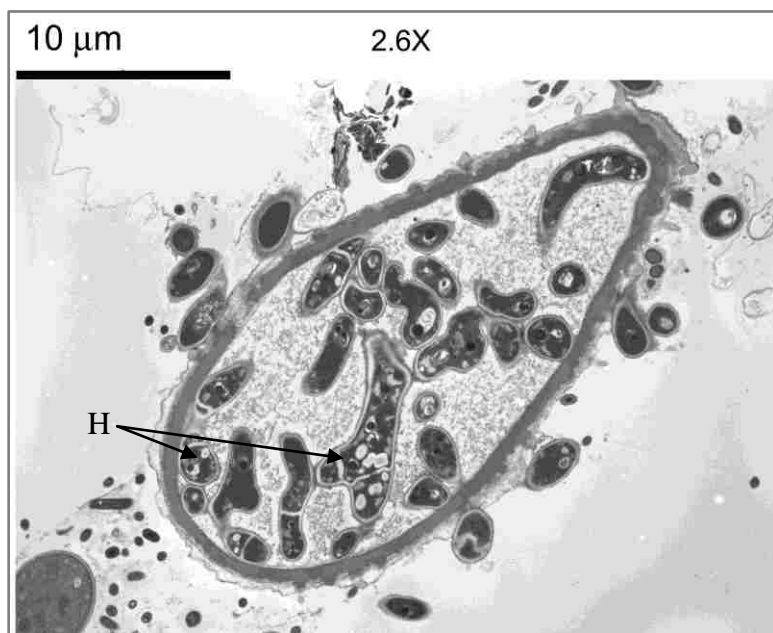
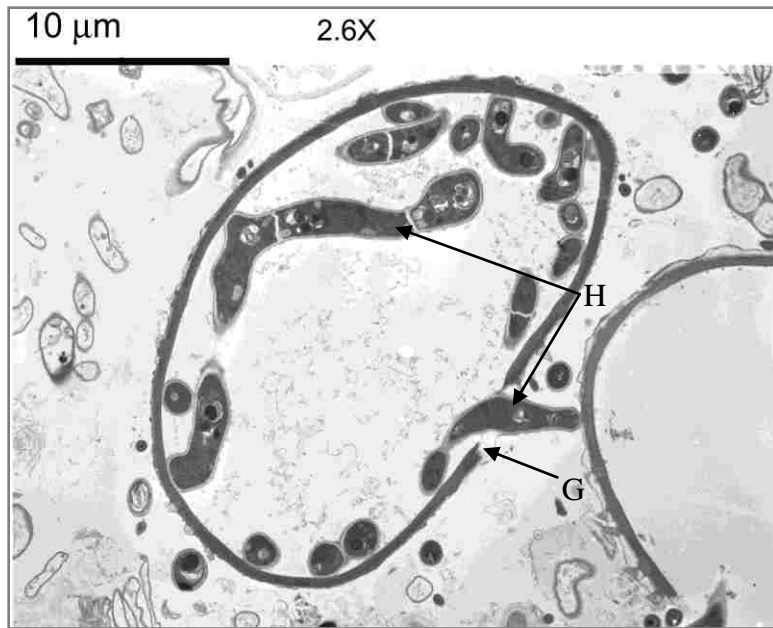


Fig 5.8. Transmission electron microscope view of a urediniospore of *Phakopsora pachyrhizi* three days after co-culturing with *Simplicillium lanosoniveum*. Urediniospores were colonized with hyphae of *S. lanosoniveum*, while organelles were degraded and cytoplasm was aggregated or often nonexistent.

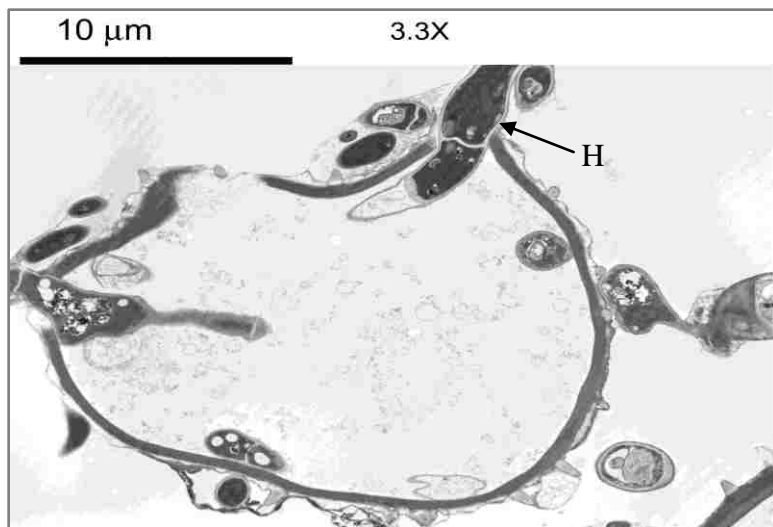


Fig 5.9. Transmission electron microscope view of a urediniospore of *Phakopsora pachyrhizi* 10 days after co-culturing with *Simplicillium lanosoniveum*. Urediniospores were misshapen and a reduced amount of hyphae of the antagonist was observed.

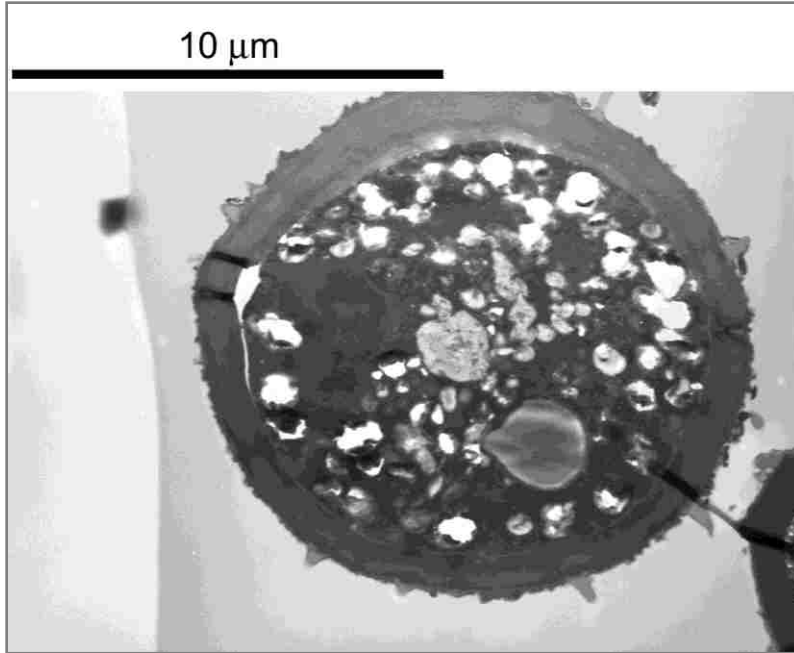


Fig 5.10. Urediniospore incubated for 24 hours on moist filter paper but not exposed to *S. lanosoniveum*.



Rust fungi are known to be parasitized by various fungi. *Cladosporium* is the most commonly described mycoparasite of rusts with *C. uredinicola* on *Puccinia* spp. being the prime example [11]. Other interactions include *Eudarluca caricis* on species of *Puccinia* and *Melampsora* and *Verticillium psalliotae* on *P. pachyrhizi* [68, 78, 108].

*Simplicillium lanosoniveum* has not been previously documented as a mycoparasite of fungi. It has been described as the causal agent of brown spot disease in the aquatic weed salvinia (*Salvinia molesta*) in Taiwan [27]. In our studies, *S. lanosoniveum* did not cause disease in salvinia (Ward, unpublished). We documented that *S. lanosoniveum* parasitized several other rusts, including *P. graminis* var. *tritici* (stem rust of wheat), *P. striiformis* (stripe rust of wheat), *Physopella fici* (fig rust), and *Phragmidium tuberculatum* (rust on rose). The mycoparasite rapidly colonized sori of these and other rusts, as was observed in *P. pachyrhizi* (Ward, unpublished).

In summary, these results clearly demonstrated that *P. pachyrhizi* is susceptible to attack by *S. lanosoniveum*. This study provided the first evidence that *S. lanosoniveum* is a mycoparasite. Furthermore, we demonstrated that the fungus parasitizes urediniospores within 24 hours, and these findings support our contention that *S. lanosoniveum* may be an effective biological control agent.

## CHAPTER 6. SURVEY OF ADDITIONAL RUSTS FOR *SIMPLICILLIUM LANOSONIVEUM*

### 6.1 Introduction

In 2007, *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001 was isolated from sori of soybean rust, a disease that is caused by *Phakopsora pachyrhizi* Syd. & P. Syd. The fungus was recovered from infected soybean leaves collected on four occasions from Baton Rouge, LA and three samplings from Quincy, Florida in August 2007. After incubation in moist chambers for 1 to 3 days, 10 to 15% of sori had visible hyphae of *S. lanosoniveum* when viewed under low magnification. Approximately 80 isolates were recovered from these two locations. Attempts to recover *S. lanosoniveum* from commercial soybean fields were unsuccessful and may be caused by the widespread use of fungicides to combat late season soybean diseases.

Using isolates collected in 2007, the relationship between *S. lanosoniveum* and urediniospores of *P. pachyrhizi* was characterized. Previous studies determined that urediniospores of *P. pachyrhizi* were colonized and parasitized by *S. lanosoniveum* within one day of co-culture (Ward, unpublished). These urediniospores became brown and failed to germinate [100]. Under field conditions, *S. lanosoniveum* colonized infected soybean leaves regardless of whether they were showing symptoms of SBR or they were in the latent stage of the infection process (Ward, unpublished).

In 2008 through 2010, our survey was expanded to include other rust species. The objective of this survey was to determine whether *S. lanosoniveum* colonized other rust species.

## 6.2 Methods

Leaves were collected from other plant species infected with rust diseases for examination and isolation of *S. lanosoniveum*. Leaves were incubated in polystyrene boxes lined with moist paper towels at approximately 25°C with a 12-hour photoperiod under cool fluorescent lights (850-1000 lux) to promote fungal growth. After 1 to 3 days, leaves were examined with a dissecting microscope at 25x to 50x to identify mycelia that colonized sori. Hyphae were isolated with a sterile needle (31 gauge) and placed onto potato dextrose agar or a semi-selective medium. After 5 to 7 days, cultures were single-spored and uniformly growing cultures were added to the isolate collection.

A semi-selective medium for *S. lanosoniveum* was developed by modifying an existing protocol for isolation of *Lecanicillium* [26]. *Simplicillium lanosoniveum* was found to exhibit a tolerance for Hygromycin B (K. Maruthachalam and N. A. Ward, unpublished). Various concentrations of this antibiotic were combined in a modified malt agar medium and tested the medium by pressing rust-infected leaves directly onto plates (Ward, unpublished). Thus, the following selective medium was used for isolation of *S. lanosoniveum* from rust sori: Malt extract 20 g, streptomycin 300 mg, tetracycline 300 mg, Hygromycin B 500 ppm (0.5 ml), agar 15 g, water 1 liter. Using this medium, rust-infected leaves were pressed onto agar surfaces for isolations.

To determine whether *S. lanosoniveum* colonized other rusts, *S. lanosoniveum* was introduced to rust-infected leaves to determine whether the fungus exhibited a mycophilic attraction to colonized sori. Conidial suspensions were produced by flooding 14-day old cultures with 0.01% Tween 20® and rubbing gently with a glass rod [101]. Conidial suspensions were diluted to 10<sup>6</sup> conidia per ml using a hemacytometer. One ml of conidial suspension was introduced to rust-

infected leaves and spread with a glass rod. Leaves were incubated in moist chambers as previously described, and then viewed with a dissecting microscope 3 to 5 days later to determine whether the fungus colonized sori [101].

### 6.3 Results

During the course of this study, one isolate each of *S. lanosoniveum* was recovered from morning glory (*Ipomea hederacea*) in 2008, from salvinia (*Salvinia molesta*) in 2009, and from elephant's foot (*Elephantopus carolinianus*) in 2009. The following table summarizes the rust-infected plants from which isolations were attempted (Table 6.1). Additionally, in inoculation studies, several rusts were determined to be susceptible to parasitism by *S. lanosoniveum* (Table 6.2).

### 6.4 Discussion

After our initial discovery, we recovered *S. lanosoniveum* on three occasions during a three year survey period. In 2008, morning glory rust, caused by *Puccinia cassipes*, was collected from a soybean field in Baton Rouge, LA. Pustules were colonized by the mycoparasite. These plants were growing adjacent to soybean research plots that were showing symptoms of SBR. In 2010, large amounts of mycelia of *S. lanosoniveum* were visible in rust pustules (*Coleosporium vernoniae*) on elephant's foot without the aid of a microscope. These plants were located in a wooded area, and SBR was not reported nearby. The third isolate of *S. lanosoniveum* was recovered from the aquatic weed giant salvinia (*Salvinia molesta*) collected from Louisiana. These plants showed no rust symptoms, but plants were sampled because of a previous report by a group in Taiwan [27]. In this report, *S. auriculata* and *S. molesta* were reported to have a newly described leaf spot caused by *S. lanosoniveum*. Although we determined that *S.*

*lanosoniveum* did not cause disease symptoms in our samples, we isolated the fungus from healthy leaf surfaces.

According to collections by Centralbureau voor Schimmelcultures (the Netherlands) and genomic sequences reported by the National Center for Biotechnology Information (Bethesda, MD), *S. lanosoniveum* has been isolated from coffee rust, other fungi, and scale insects from Venezuela, Sri Lanka, Puerto Rico, and Iran. Although our survey suggested that the fungus is not a common inhabitant of rusts in the US, *S. lanosoniveum* exhibited a mycotrophic attraction to a variety of other rusts when artificially introduced. The mycoparasite colonized a variety of rusts within five days of inoculation and was visible under low magnification. Since 2007, only 10 isolates of *S. lanosoniveum* were recovered from the southern US.

Table 6.1. Recovery of *Simplicillium lanosoniveum* from various rust species collected over a 3-year period from several states. Isolates were recovered either by pressing infected leaves onto a selective medium or by extracting hyphae from sori with a needle.

Host	Rust	Location	Date	Isolation of <i>Simplicillium lanosoniveum</i>	Isolate number	% sori infected
Soybean	<i>Phakopsora pachyrhizi</i>	Baton Rouge, LA	8/17/2007	+	BH081307	10%
Soybean	<i>Phakopsora pachyrhizi</i>	Quincy, FL	8/23/2007	+	D082307	15%
Soybean	<i>Phakopsora pachyrhizi</i>	Baton Rouge, LA	10/6/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Macon Co., GA	10/6/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Jeff Davis Co., GA	10/6/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Lanier Co., GA	10/10/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Berrin Co., GA	10/10/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Tallahassee, FL	10/11/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Byran Co., OK	10/13/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Baton Rouge, LA	10/13/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	TN	10/15/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Baton Rouge, LA	11/12/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Baton Rouge, LA	11/17/2009	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Quincy, FL	8-1-2010	-	-	-
Soybean	<i>Phakopsora pachyrhizi</i>	Quincy, FL	8-1-2010	-	-	-
Bamboo	unidentified	Baton Rouge, LA	3/9/2009	-	-	-
Bamboo	unidentified	Baton Rouge, LA	4/10/2010	-	-	-
Beachgrass	unidentified	Newport, OR	8/20/2010	-	-	-
Blackberry	unidentified	Baton Rouge, LA	5/9/2009	-	-	-
Blackberry	unidentified	Baton Rouge, LA	5/7/2010	-	-	-
Canna Lily	unidentified	New Orleans, LA	5/9/2009	-	-	-
Corn	unidentified	Lettsworth, LA	5/8/2008	-	-	-

Table 6.1 continued

Corn	<i>Puccinia polysora</i>	Baton Rouge, LA	5/9/2009	-	-	-
Corn	<i>Puccinia polysora</i>	Concordia Parish, LA	7/6/2010	-	-	-
Corn	<i>Puccinia polysora</i>	Bossier Parish, LA	7/6/2010	-	-	-
Corn	<i>Puccinia polysora</i>	Quincy, FL	8/25/2010	-	-	-
Elephant's foot	<i>Coleosporium vernoniae</i>	Pointe Coupee Parish, LA	10/21/2010	+	E101210	90%
Fig	unidentified	Livingston Parish, LA	5/8/2008	-	-	-
Fig	unidentified	Baton Rouge, LA	9/20/2010	-	-	-
Ground ivy	unidentified	Baton Rouge, LA	3/9/2009	-	-	-
Ground ivy	unidentified	Baton Rouge, LA	3/1/2010	-	-	-
Gaura	unidentified	Baton Rouge, LA	5-12-2010	-	-	-
Hollyhock	unidentified	Baton Rouge, LA	4-29-2010	-	-	-
Hollyhock	unidentified	Baton Rouge, LA	5-12-2010	-	-	-
Hollyhock	unidentified	Salinas, CA	5-18-2010			
Kudzu	<i>Phakopsora pachyrhizi</i>	Quincy, FL	7/22/2010	-	-	-
Kudzu	<i>Phakopsora pachyrhizi</i>	Quincy, FL	8/3/2010	-	-	-
Kudzu	<i>Phakopsora pachyrhizi</i>	Quincy, FL	8/25/2010	-	-	-
Morning glory	<i>Puccinia cassipae</i>	Baton Rouge, LA	10/8/2008	+	M101008	10%
Morning glory	<i>Coleosporium ipomoeae</i>	West Carroll Parish, LA	7/22/2010	-	-	-
Morning glory	<i>Coleosporium ipomoeae</i>	Quincy, FL	8/25/2010	-	-	-
Morning glory	<i>Coleosporium ipomoeae</i>	Quincy, FL	9/20/2010	-	-	-
Morning glory	<i>Coleosporium ipomoeae</i>	Baton Rouge, LA	10/1/2010	-	-	-
Morning glory, Brazilian	<i>Coleosporium ipomoeae</i>	Baton Rouge, LA	10/9/2009	-	-	-
Morning Glory, ivyleaf and bigleaf	<i>Coleosporium ipomoeae</i>	Baton Rouge, LA	10/9/2009	-	-	-
Morning Glory	<i>Coleosporium ipomoeae</i>	West Carroll Parish, LA	7-22-2010	-	-	-

Table 6.1 continued

Morning Glory, ivyleaf and bigleaf	<i>Coleosporium ipomoeae</i>	Baton Rouge, LA	9-19-2010	-	-	-
Oxalis	unidentified	Baton Rouge, LA	4/8/2008	-	-	-
Oxalis	unidentified	Cottonport, LA	3/9/2009	-	-	-
Peanut	unidentified	Quincy, FL	8/25/2010	-	-	-
Plumeria	unidentified	Baton Rouge, LA	9/9/2009	-	-	-
Raspberry	unidentified	Baton Rouge, LA	5-7-2010	-	-	-
Rose	unidentified	Corvallis, OR	8/15/2010	-	-	-
Salvinia	no rust	Golden Meadow, LA	6/2009	+	S091709	50%
Snapdragon	unidentified	Salinas, CA	5-18-2010	-	-	-
St. John's Wort, Hypericum	unidentified	Corvallis, OR	8/15/2010	-	-	-
Sugarcane	<i>Puccinia melanocephala</i>	St. Gabriel, LA	4/9/2009	-	-	-
Sugarcane	<i>Puccinia melanocephala</i>	St. Gabriel, LA	7/9/2009	-	-	-
Sugarcane	<i>Puccinia melanocephala</i>	St. Gabriel, LA	8/1/2010	-	-	-
Wheat	<i>Puccinia striiformis</i>	Baton Rouge, LA	5/9/2009	-	-	-
Wheat	<i>Puccinia striiformis</i>	Baton Rouge, LA	4/10/2010	-	-	-
Willow	unidentified	Lettsworth, LA	3/9/2009	-	-	-



Table 6.2. Colonization of various rusts by *Simplicillium lanosoniveum* upon inoculation with conidial suspensions. To determine whether sori were colonized, they were examined under a dissecting microscope. Hyphae were transferred onto potato dextrose agar or semi-selective medium, and morphological characteristics were analyzed.

<b>Rust</b>	<b>Host</b>	<b>Colonization by S. lanosoniveum</b>
<i>Phakopsora pachyrhizi</i>	Soybean	+
unidentified	Rose	+
unidentified	Blackberry	+
unidentified	Fig	+
unidentified	Snapdragon	+
<i>Coleosporium ipomoeae</i>	Morning glory	+
unidentified	Ground ivy	-
<i>Puccinia graminis tritici</i>	Wheat	+
<i>Puccinia ploysora</i>	Corn	-
<i>Puccinia sorghii</i>	Wheat	+
<i>Puccinia striiformis</i>	Wheat	+

## CHAPTER 7. EFFECT OF EXPOSURE OF HYALINE AND BROWN UREDINIOSPORES OF *PHAKOPSORA PACHYRHIZI* TO SOLAR RADIATION

### 7.1 Introduction

Soybean rust (SBR), caused by the fungus *Phakopsora pachyrhizi* Syd., has been documented to overwinter on kudzu and other leguminous hosts in the southern US [19]. Urediniospores are produced in pustules (sori) on the abaxial sides of leaves. These urediniospores, which are disseminated by wind, are reported to travel up to thousands of miles in the upper atmosphere [47]. Models predict that SBR came to the continental US from South America in 2004 during Hurricane Ivan [44-45]. These urediniospores may have survived for up to two weeks in the upper atmosphere during transcontinental transport. Studies indicate that the limiting factor in atmospheric transport of fungal propagules is ultraviolet (UV) light [9-10, 47, 58, 83].

In many fungal species, melanin is reported to serve as a protectant against UV light and other adverse environmental conditions such as dehydration [12, 23]. Melanin is believed to be the primary cause for the darkening of urediniospores of *P. pachyrhizi* during stressful events such as aging and parasitism. Previous work has documented that as urediniospores aged for 7 to 10 days, they became progressively more brown and that after 10 to 14 days, necrotic halos formed on leaf tissue surrounding pustules (Ward, unpublished). These brown urediniospores had a lower germination rate than the younger hyaline urediniospores [100].

The objective of this study was to determine whether dark urediniospores became melanized as they aged, and whether they were more resistant to solar radiation than those that were hyaline.

## 7.2 Methods

Soybean cultivar AG6202 (Monsanto Corp.) was planted at Louisiana State University Agricultural Center's Ben Hur Research Farm near Baton Rouge, LA on July 21, 2009. SBR epidemics were initiated from naturally occurring inoculum, and disease symptoms were observed 66 days after planting when plants were in the seed filling (R5) growth stage [34]. Four weeks after the first disease symptoms were documented, as disease severity reached >50% leaf area, soybean leaves were collected for experiments.

In the laboratory, urediniospores were brushed from all pustules on all leaves. One hundred leaves each were separated into two groups for production of hyaline and brown urediniospores. Mature pustules were marked to indicate which ones were present at the initiation of the study. Mature pustules were approximately 1 mm across and were not surrounded by necrotic tissue. This process allowed us to select pustules which were all approximately the same age. Previous work indicated that these pustules were approximately 5 days old (Ward, unpublished). For hyaline urediniospore induction, urediniospores were brushed from leaves daily, so they were less than 24 hours old at the time of collection. Removal of urediniospores ceased 24 hours before the end of the study. To induce brown urediniospore production, urediniospores were not brushed during the course of the study. By day seven, urediniospores from this treatment were brown.

After incubating detached leaves for seven days, as described above, urediniospores were collected from approximately 200 marked pustules from each treatment with a needle or blade and dissecting microscope. Urediniospores were placed in dry Petri dishes and exposed to varying amounts of solar radiation on the same day they were collected. Exposure to sunlight

began approximately 2 hours after sunrise (8:30 am) and ended approximately one hour before sunset (4:30 pm). Ten dishes were prepared from each treatment. One half of the dishes were exposed to full sunlight, while the other half were shaded with a knitted polyethylene greenhouse shade cloth (80% shade) (Grower Supply, Forest Hill, LA). Dishes were floated in 5 cm of water in a 20 cm deep plastic container to stabilize the temperature of urediniospores. One dish of urediniospores was collected every 2 hrs (2 hr, 10:30 am; 4 hr, 12:30 pm; 6 hr, 2:30 pm; 8 hr, 4:30 pm) from each treatment.

To measure solar radiation, an Eppley radiometer (Eppley Laboratory Inc., Newport, RI) was utilized, equipped with a CR3000 datalogger, temperature and relative humidity sensors, and a wind speed sensor. Solar radiation was recorded as  $\text{kJ/m}^2$ , which is a measure of the energy across the entire spectrum of sunlight. Sensors scanned environmental conditions every second and values were averaged in 1-minute intervals. Water temperature was recorded in the sampling baths during each sampling as an indication of urediniospore temperature.

Experiments were repeated one week later.

After exposure to varying amounts of solar radiation, germination rates for urediniospores were evaluated as an indication of viability. Urediniospores were suspended in small amounts of 0.01% Tween® 20 to generate spore suspensions of  $10^6$  urediniospores per ml. Suspensions were sonicated for 30 seconds to eliminate clumps. To establish the germinability of each treatment, one ml of the spore suspensions was placed onto five Petri dishes containing 1% water agar. Petri dishes were incubated at  $25^\circ\text{C}$  in the dark for 4 hrs. Germination rate was determined for 100 urediniospores per plate with a compound microscope at 200x magnification.

Urediniospores were considered germinated if germ tubes were at least as long as the urediniospore's longest dimension.

Additionally, we tested urediniospores from each treatment to determine whether melanin was a component of urediniospore browning. We used copper sulfide silver in which copper was bound to fungal melanin and amplified with a silver enhancement step according to published protocols [23].

### **7.3 Results**

Total solar radiation increased as the day proceeded. There were significant increases in solar radiation between 12:30 pm (4 hr treatment) and 4:30 pm (8 hr treatment) (Fig 7.1).

Germination rate was lower in urediniospores that were exposed to sunlight as opposed to urediniospores protected by shade. After 4 hrs, there was no significant difference in germination rates between hyaline and brown urediniospores that were exposed to solar radiation. However, after 6 hrs of exposure, hyaline urediniospores had a germination rate of 39.2% while brown urediniospores had a germination rate of 12.3%. After 8 hrs, all urediniospores that were exposed to solar radiation had germination rates lower than 20%, with no significant difference in germination rates between brown and hyaline urediniospores.

However, under both sun and shaded conditions, brown urediniospores had lower germination rates than their hyaline counterparts (Fig 7.2). Repeated experiments yielded similar results.

Our melanization test revealed that brown urediniospores did not contain melanin.

### **7.4 Discussion**

Previous studies revealed that as urediniospores aged, they became increasingly brown [100]. In many fungi, this brown characteristic is caused by melanization [12]. Melanized spores are

reported to be more tolerant of UV light, and melanized spores and other fungal structures are known to serve as long term survival structures [12]. However, in our previous work with the SBR pathogen, we documented that brown urediniospores germinated at lower rates than hyaline urediniospores. Thus, we hypothesized that brown urediniospores were less viable than hyaline urediniospores.

This study was conducted in October 2009 when soybean plants were heavily diseased and urediniospores were abundant. Temperatures reached a high of 26°C at 2:00 pm and remained above 24°C between 12:30 and 4:45 pm. This correlated with the 4 hr, 6hr, and 8hr sampling periods.

Results indicated that germination rates were reduced in urediniospores that were exposed to solar radiation, but brown urediniospores were less tolerant of exposure than hyaline urediniospores. The most noticeable reduction in germination came after 6 hrs of exposure.

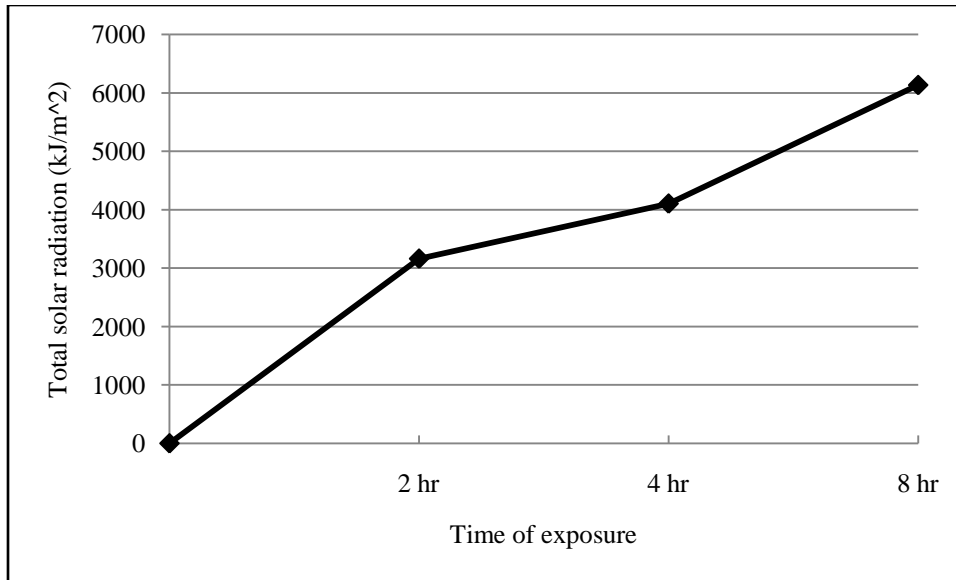


Fig 7.1. Accumulated solar radiation per minute during the course of exposure of brown and hyaline urediniospores of *Phakopsora pachyrhizi* to sunlight.

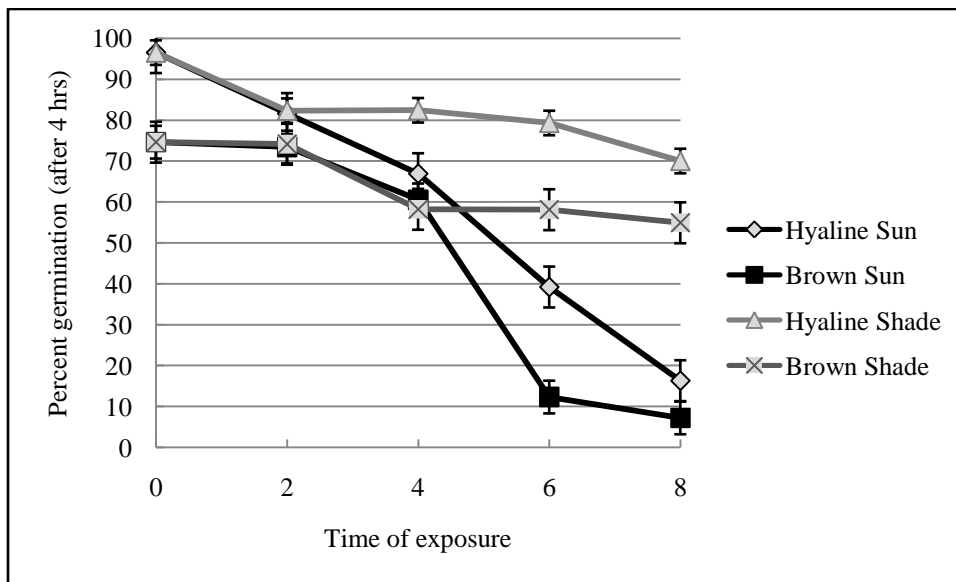


Fig 7.2. Percent germination of brown and hyaline urediniospores of *Phakopsora pachyrhizi* after exposure to either full sunlight or 80% shade.

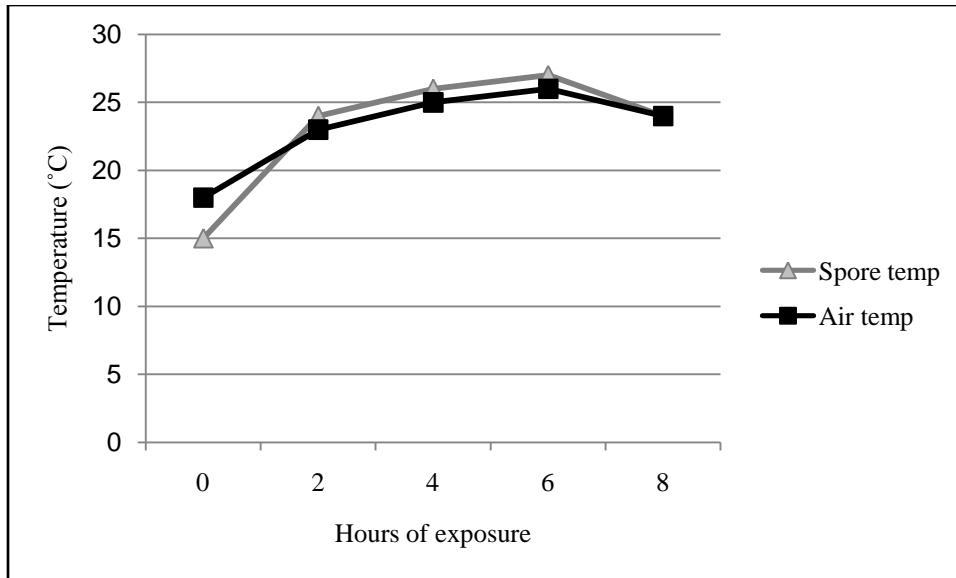


Fig 7.3. Temperature of urediniospores as indicated by water temperature of sun-exposed dishes, and temperature of the air at each time of sampling.



Brown urediniospores that were exposed to sunlight for more than 4 hours germinated at extremely low rates. We attempted to further induce germination of these brown urediniospores by employing techniques such as rehydrating in humidity chambers before germinating and extending the time exposed to water agar. However, these attempts did not increase germination rates of brown urediniospores. Urediniospores exposed to shade and those incubated in the laboratory confirmed that brown urediniospores were less viable than hyaline spores. By the end of the day during which these experiments were conducted, exposure to solar radiation further decreased germinability of all urediniospores, especially brown urediniospores.

We determined that browning of urediniospores of the SBR pathogen was not caused by the accumulation of melanin, an indicator of UV tolerance and increased survival of fungal propagules. Furthermore, we concluded that brown urediniospores were more susceptible to solar radiation than hyaline urediniospores, which may be indicative of poor vigor of urediniospores due to aging or other environmental stress factors.

This study indicated that freshly sporulating pustules are necessary for production of effective inoculum. We suspect that if urediniospores do not dislodge from pustules and disperse immediately, viability may be lost and the relationships between inoculum load and disease severity will be affected. These findings have important implications for spatial infectivity models, which assume that source strength is affected by the numbers of urediniospores released per unit area [48]. If these spores have been exposed to solar radiation for less than one day, their viability will be greatly reduced, and their ability to cause disease at distant locations will be compromised. Further studies should address this important variable in order to increase the reliability of spore transport models.

These findings also indicate that browning of urediniospores, especially those found in sori of SBR containing *S. lanosoniveum*, are not viable.

## CHAPTER 8. DOCUMENTATION OF AN EXTENDED LATENT INFECTION PERIOD BY *PHAKOPSORA PACHYRHIZI*, THE SOYBEAN RUST PATHOGEN

### 8.1 Introduction

Soybean rust (SBR), caused by *Phakopsora pachyrhizi*, was first reported in Asia in 1904 and has since spread to Africa and the Americas [63, 73, 107]. Disease losses caused by SBR have ranged from 10 to 90%, though there are reported losses as high as 100%. SBR was first discovered in the US in 2004, and yield losses between 35 and 40% were reported in Louisiana and as high as 82% in Florida on susceptible cultivars that were not sprayed with fungicides [84] (R. W. Schneider and D. R. Walker, personal communication).

Breeding efforts have yet to produce resistant cultivars [41]. Therefore, disease management studies have focused mainly on fungicide applications. These investigations demonstrated that preventative applications of fungicides must be accurately timed and applied very early in the infection process for effective control of the disease [85]. Such practices may lead to unnecessary fungicide applications, especially throughout the southern US, as growers fear rapidly escalating epidemics such as those seen in Africa, Asia, and South America [73-74, 104].

SBR has become endemic in the southern US as it overwinters on kudzu (*Pueraria lobata*) in the lower Gulf South [49, 71]. Infected kudzu is thought to be an alternative source of urediniospores that spread to soybean and cause SBR each season. Despite this source of inoculum each spring, the disease usually is not reported on soybean until the summer months. In our experience, SBR is not visibly observed until the mid-reproductive stages (R5) of plant growth when seeds begin to develop [34]. However, in laboratory and greenhouse studies, SBR

symptoms typically appear 7 to 14 days after inoculation. The objective of this study was to determine whether infection of soybean occurred early in the growing season and whether there was an extended latent infection period. Development of the rust pathogen was monitored using quantitative real-time PCR (qPCR), a technique used to quantify DNA and hence provide a quantitative assessment of the pathogen in leaf tissue.

## **8.2 Methods**

### **8.2.1 Field Studies**

Three soybean fields were monitored for SBR in Louisiana and one in Florida in 2009 and 2010. Soybean plants were not inoculated with the pathogen; epidemics were initiated from naturally occurring inoculum. To monitor the SBR pathogen in soybean, 10 trifoliolate leaves (30 leaflets) were sampled weekly from each field. Immediately after sampling, each apical leaflet was assessed to determine whether symptoms had developed. Three fields of vision (5 cm<sup>2</sup>) were examined per leaflet with a dissecting microscope at 25x magnification. Leaves were then stored in freezer bags at -20°C until DNA was extracted. DNA of *P. pachyrhizi* was quantified using qPCR as described below.

**Louisiana Field Studies 2009.** SBR overwintered in Baton Rouge on kudzu during the winter of 2008/2009. Symptomatic kudzu was reported less than 10 km from the Louisiana State University Agricultural Center's Ben Hur Research Farm where field tests were located. Field 1: Soybean cultivar Asgrow 3905 (Monsanto Corp., Creve Coeur, MO) was planted on April 17, 2009. Ten trifoliolates were collected weekly beginning at flowering (R1) and continuing through senescence (R7). Field 2: Soybean cultivar Asgrow 5802 (Monsanto Corp.) was

planted on May 20, 2009 in a field adjacent to Field 1. Sampling began at flowering (R1) and continued through senescence (R7). Field 3: Soybean cultivar Asgrow 6202 (Monsanto Corp.) was planted July 21, 2009 adjacent to Field 1 and Field 2. Leaf collections began during mid-vegetative stages (V4) and continued through senescence (R7). Nonhost controls: Corn and morning glory vine were included as nonhost controls in order to quantify urediniospores found on leaves. Three corn leaves were sampled from the outer edge of the field on a weekly basis between June 4 and July 29, 2009. Morning glory was used as a nonhost control between August 5 and September 30, 2009. Ten leaves were collected weekly from wild vines on the outer edge of soybean plots.

**Florida Field Study 2010.** Field 4: In July 2010, soybean cultivar Pioneer 95Y20 (Pioneer Hi-Bred) was planted at the University of Florida North Florida Research and Education Center in Quincy, Florida. SBR was reported in kudzu approximately 180 m from soybean plots about one week after soybeans were planted. However, SBR was not visibly detected in this field until 68 days after planting, when plants were in the R5 growth stage. Sampling began during the vegetative stages (V6) of growth and ended at the onset of senescence (R7). Nonhost control: Morning glory was included as a nonhost control in order to urediniospores found on leaf surfaces as described above.

All soybean plots were maintained according to common agricultural practices with regard to insect and weed control and fertilization. None of the plants in this study was sprayed with fungicides.

### 8.2.2 DNA Quantification

**Primers and probe.** Primers and probe were based upon previous work by Frederick et al. [35] in which primers Ppm1 and Ppa2 were bound specifically to DNA of *P. pachyrhizi*. FAM-probe was labeled at the 5' end with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) and at the 3' end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA) for quantification of fluorescence in qPCR assays.

**DNA Extractions.** Freezer bags containing leaves from each plot were stored at -20°C until they were ground in liquid nitrogen with mortar and pestle. Genomic DNA was extracted from 50 mg of ground leaf tissue using Qiagen's DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Final DNA concentrations were diluted to 10 ng/μl for use in qPCR assays.

**qPCR Assay:** qPCR was used to quantify the amount of DNA of *P. pachyrhizi* per 10 ng soybean DNA. Triplicate samples were tested in a total volume of 25 μl per reaction as previously described. The qPCR instrument software package (Applied Biosystems, Carlsbad, CA) automatically calculated the critical threshold values (Ct) for each reaction [54]. Ct values were converted to picograms (pg) DNA according to standard curves. Less than 1.0 pg DNA of *P. pachyrhizi* was detected with this protocol.

## 8.3 Results

**Field 1.** Latent infection is expressed as pg of DNA of *P. pachyrhizi* per 10 ng of soybean DNA. Latent infection was detected in April-planted soybean on the first sampling (R1), in which we

quantified 1.4 pg of DNA of *P. pachyrhizi* (Figs 8.1 and 8.2). However, at the R6.5 growth stage, DNA of *P. pachyrhizi* increased significantly to 2.6 pg. One week later (R7), 10.2 pg of DNA was detected. Symptoms of SBR were not observed, but necrotic tissue and other disease lesions made leaf examinations difficult. Surface inoculum was not detected using corn as a nonhost control.

**Field 2.** At first flowering (R1), 2.5 pg DNA of the SBR pathogen was detected, which indicated that soybean plants were infected (Fig 8.1). Amounts of DNA of *P. pachyrhizi* increased from 7.5 pg of DNA from asymptomatic leaves at the R5 growth stage to 53.5 pg when the first signs and symptoms of SBR were observed one week later (R5.5) (Fig 8.2). There was a 43-day latent infection period. Surface inoculum was not detected on corn during this study.

**Field 3.** Latent infection was detected at the V4 stage, with 2.0 pg of *P. pachyrhizi*. Symptoms developed after a 33-day latent infection period at R5. One week before symptoms developed, 2.6 pg of DNA of the SBR pathogen was detected, and after symptoms were first observed, DNA of *P. pachyrhizi* increased to 60.5 pg. Surface inoculum was detected on morning glory at the edge of Fields 2 and 3 on September 4, 2009.

**Field 4.** SBR was detected on kudzu, which was approximately 1.5 km from the soybean plots, at the Florida research farm one week after soybeans were planted, but only a limited amount of disease developed (less than 1% incidence). Latent infection was confirmed at 0.7 pg DNA 18 days before symptoms developed at R5. Epidemics of SBR were delayed throughout the Gulf

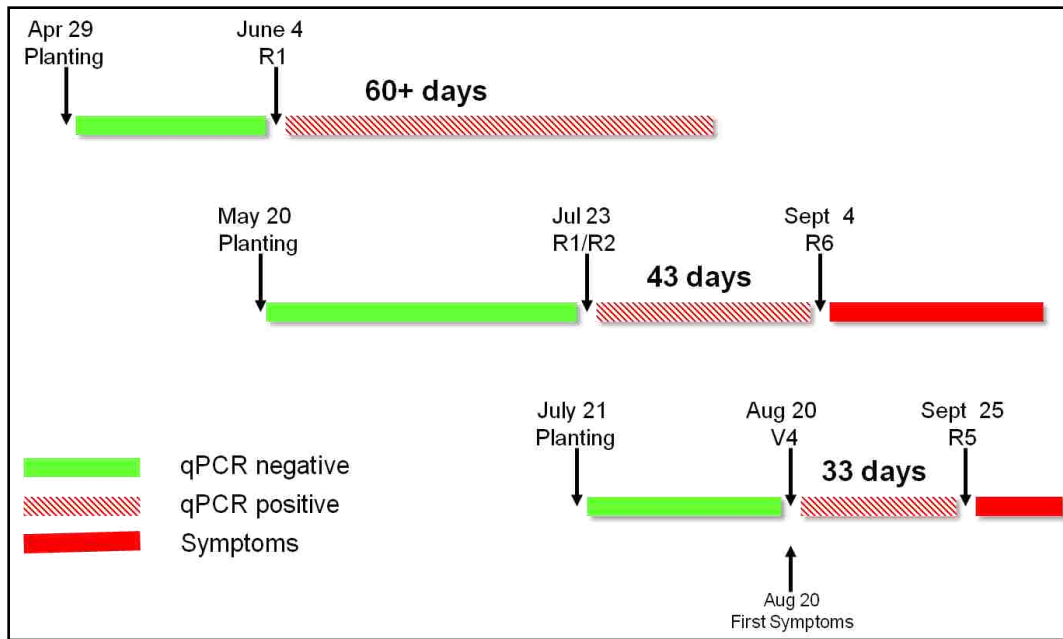


Fig 8.1. Time lines for latent infection of soybeans by *Phakopsora pachyrhizi* and the development of symptoms of soybean rust (SBR) in three field experiments near Baton Rouge, Louisiana in 2009. Fields were adjacent to each other and within 10 km of kudzu that was infected with *P. pachyrhizi*.



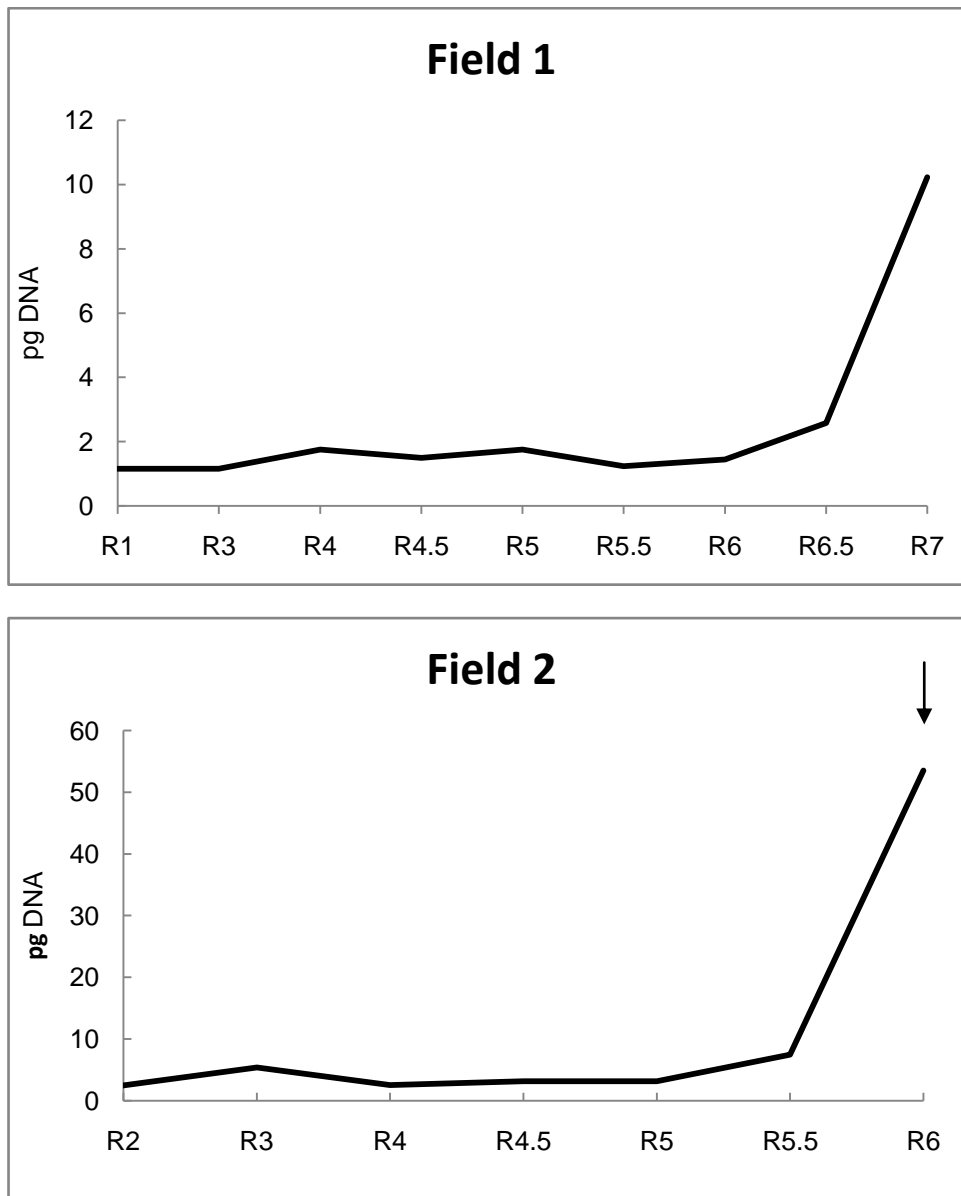
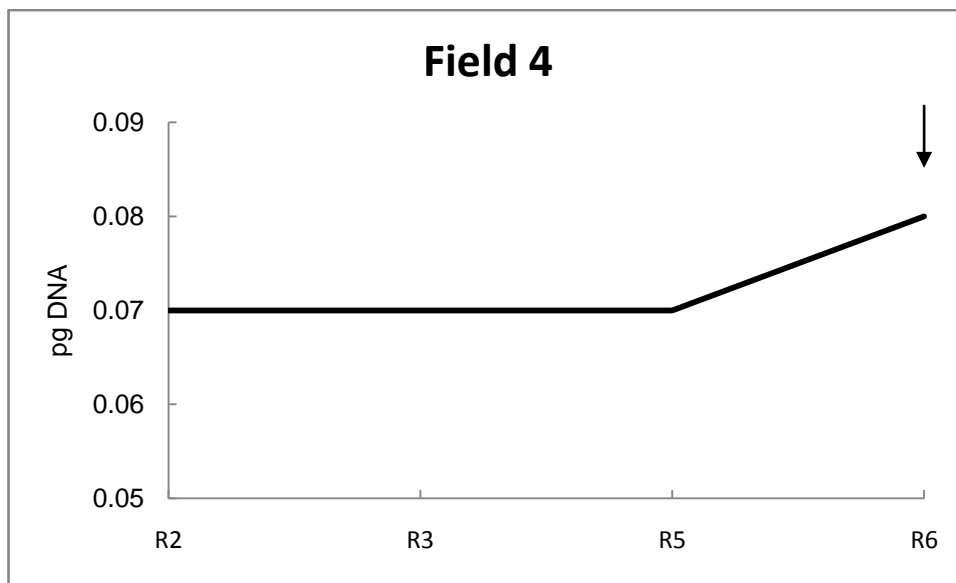
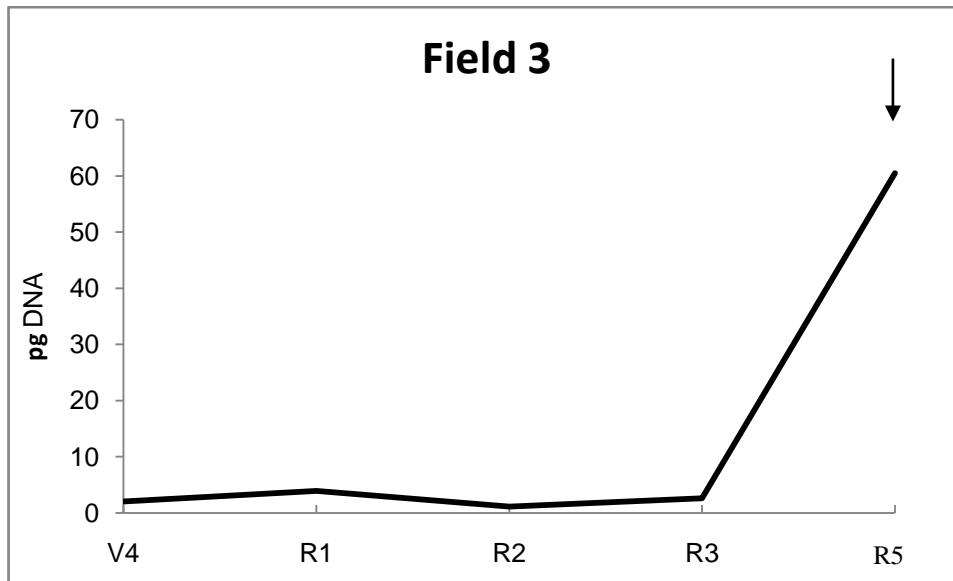


Fig 8.2. Relationships between soybean crop developmental stages and concentrations of DNA of *Phakopsora pachyrhizi*, in soybean leaves from four fields. Concentrations are expressed as pg of pathogen DNA per 10 ng of soybean DNA. Arrows indicate when symptoms were first observed. Symptoms were not observed in Field 1.

Fig 8.2 continued



South in 2010 because of subfreezing winter temperatures [96]. Surface inoculum was not detected on morning glory during the course of this experiment.

## 8.4 Discussion

We documented that visible SBR symptoms first appeared during mid-reproductive stages (R5 and later) of plant growth when seeds began to develop in pods. This contrasts with previous laboratory and greenhouse studies in which symptoms typically developed 7 to 14 days after inoculation. Furthermore, in Brazil, SBR has been reported on cotyledons and unifoliolate leaves of young soybean plants [22]. In the southern US, however, there have been no reports to date of symptom development on field-grown plants at these early growth stages.

In 2009, field plots in Baton Rouge were established less than 10 km from infected kudzu, and sporulating pustules were reported in early February on kudzu leaves that had been protected from frost. April-planted soybean never developed symptoms, but latent infection was detected in the early reproductive stages (R1). Collection of leaf samples did not begin in this field until R1 when we detected a high concentration of DNA of the *P. pachyrhizi*. Although we never observed signs or symptoms in Field 1, there was an increase in DNA of *P. pachyrhizi* at the R7 growth stage. We attempted to find SBR pustules at the R7 growth stage, but Cercospora leaf blight and other late-season diseases hindered accurate assessment. It is possible that we overlooked signs and symptoms that may have developed late in the season. In Field 2, we detected infection in the May-planted field during early reproductive stages of plant growth. Latent infection persisted for 33 days until symptoms appeared during the R5 growth stage. We suspected that small amounts of SBR inoculum were present during the early vegetative stages of

growth in Field 2 because we detected DNA of *P. pachyrhizi* in an adjacent field (Field 1) just as seedlings emerged in Field 2. Additionally, surface inoculum was not detected when Field 3 was planted or infected. We suspect that the amount of inoculum was lower than the detection limits of our qPCR assay, which was about 1.0 pg DNA of *P. pachyrhizi*. Field 3 was planted as soybean plants from Field 1 reached their highest concentration of the SBR pathogen and as latent infection was first detected in Field 2. July-planted soybeans in Field 3 became infected during vegetative stages of plant growth, but symptoms were not observed until R5. Thus, we documented latent infection periods of more than 30 days in all fields in this study.

Besides East Baton Rouge Parish, SBR typically overwintered in south-central Louisiana in Acadia, Iberia, and St. Martin Parishes. The first reported instances of SBR in 2009 in Louisiana were from soybean sentinel plots in these parishes. These soybeans did not develop symptoms until the R4 to R6 stages of growth despite nearby sources of SBR inoculum on kudzu. These observations support our conclusion that SBR symptoms usually do not develop until plants enter seed development stages even though they may be infected several weeks before signs or symptoms are observed.

In 2010, we evaluated soybean plants in North Florida, where SBR frequently overwinters on kudzu, and some of the first symptoms are observed on soybean in the US each year.

Temperatures reached record lows throughout the Gulf South during the winter of 2009/2010, and SBR was reported on kudzu and soybean later than normal. In the Florida field (Field 4), *P. pachyrhizi* was detected by qPCR during early reproductive stages of growth, but symptoms did not develop until the R6 growth stage at which time very few pustules were observed (disease incidence was less than 1%). Nevertheless, there was an extended latent infection period.

Sporulation was not as prolific on kudzu as observed in soybean. In fact, we did not detect surface inoculum on our nonhost control plants until two weeks after symptoms appeared on soybean. Because our qPCR assay was able to detect as little as 1.0 pg of DNA of *P. pachyrhizi*, we concluded that inoculum load was extremely low when kudzu served as the primary source of inoculum in the area.

In these studies, we determined that even after *P. pachyrhizi* infected soybean, DNA of the pathogen increased only minimally in leaves. Approximately one week before symptom development, there were significant increases in amounts of DNA of *P. pachyrhizi*.

Additionally, as disease severity increased, we documented a concomitant increase in DNA of the pathogen. Given the extended time span that plants were exposed to inoculum and the protracted latent infection period, it seems likely that physiological changes in soybean leaves are required for rampant colonization by the pathogen followed by the production of uredinia within about one week.

In field studies, fungicides applied during the latent infection period, as compared to applications at first symptom development, were most effective in disease suppression (Schneider, unpublished). Current practice is to spray at 5% incidence, but we show that by the time symptoms occur, the pathogen entered a logarithmic increase in biomass within leaf tissue, and fungicide efficacy may be compromised during this phase of the infection process. Fungicide applications during the latent period may either delay or modulate this geometric increase in fungal biomass. Therefore, as a practical disease management tactic, monitoring latent infection may be crucial in determining optimal times for fungicide application.

During the past few years, sentinel plots have been planted in advance of the commercial soybean crop. These plots were then monitored on a weekly basis, and fungicide spray advisories were issued when pustules were first observed in these plots. Our findings suggest that widespread sampling of leaves in commercial fields for qPCR analyses beginning during late vegetative stages of plant growth may provide a more reliable and quantitative indicator of imminent disease development.

These findings also suggest that early applications of *S. lanosoniveum* are most effective just before rust symptom development due to this latent infection period.

## CHAPTER 9. CONCLUSION

Upon examining sori of soybean rust (SBR), caused by *Phakopsora pachyrhizi*, from Louisiana and Florida in August 2007, hyphae of the mycophilic fungus *Simplicillium lanosoniveum* were observed coiling through uredinia, but these hyphae failed to colonize healthy leaf surfaces. This was the first description of colonization of sori of SBR by *S. lanosoniveum*. Further examinations determined that this fungus reduced numbers of sori when coinoculated with urediniospores of the pathogen. These sori appeared red-brown, resembling hypersensitive reactions reported in resistant soybean cultivars. Urediniospores in these sori turned brown at an accelerated rate, an indication of accelerated aging. Hyphae from *S. lanosoniveum* penetrated urediniospores of SBR within 24 hours of co-culture and concurrently digested organelles within urediniospores. Within five days, 90% of urediniospores were colonized by the mycoparasite, and subcellular contents were absent. Under field conditions, *S. lanosoniveum* reduced disease when applied to soybean between the time of initial infection and symptom development. Delaying application of the mycoparasite until sori erupted was too late for disease control. However, *S. lanosoniveum* colonized sori of SBR regardless of time of application.

*Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams 2001 is an anamorph of *Torrubiella* spp. Homotypic synonyms of *S. lanosoniveum* include *Cephalosporium lanosoniveum* J.F.H. Beyma 1942 and *Verticillium lanosoniveum* (J.F.H. Beyma) W. Gams [24, 109]. Current classification of *S. lanosoniveum* is as follows [24, 43, 89]:  
Fungi>Ascomycota>Pezizomycotina>Sordariomycetes>Hypocreomycetidae>Hypocreales>Cordycipitaceae

Fungi belonging to the family Cordycepsaceae include numerous mycoparasites and entomopathogens such as *Beauveria*, *Cordyceps*, and *Isaria* [43, 89]. While many members of this taxon have been documented to possess mycoparasitic properties, *S. lanosoniveum* had been reported as neither an inhabitant nor a parasite of rust fungi or insects. However, *S. lamellicola* (F.E.V. Sm.) Zare & W. Gams was evaluated as a candidate entomopathogenic fungus for biological control of ticks and scale insects [75, 109]. Species of *Lecanicillium*, sister taxon to *Simplicillium*, are often reported as entomopathogenic fungi associated with scale insects, ticks, whiteflies, and aphids [15, 29, 72]. Several species of *Verticillium*, which once included species now placed in *Simplicillium*, have been reported as parasites of cultivated mushrooms, pathogens of nematode eggs, and as parasites of rusts on bean, coffee, oat, and peanut [37, 53, 55, 78, 80].

The nature of the interaction (i.e. mycoparasitism, cohabitation, and necrotrophic growth) between *S. lanosoniveum* and *P. pachyrhizi* also was examined in this study. Direct penetration and quick digestion of subcellular membranes were observed to occur simultaneously upon co-culture of the two fungi. Similar mycoparasitic interactions were described with other rusts by species of *Verticillium*, *Cladosporium*, and other *Simplicillium* [8, 38, 78]. The micrographs depicting these interactions have striking similarities to the associations between the two fungi described in the present work. Direct penetration by mycoparasites was described in powdery mildew, *Trichoderma*, and cultivated mushrooms [7, 13, 91]. Additionally, direct penetration was documented to occur through penetration pegs within appressorium-like bodies by *Cladosporium* [8, 11]. On the other hand, enzymatic degradation of urediniospores was described by Saksirirt and Hoppe [78] during parasitism of *P. pachyrhizi* by *Verticillium psalliotae*. This mycoparasite secreted  $\beta$ -1,3-glucanase, chitinase, and protease enzymes in the presence of urediniospores of the SBR pathogen [79-80].



In order to evaluate the potential of *S. lanosoniveum* for biological control of SBR, conidial and mycelial suspensions of the antagonist were applied. Under field conditions, the fungus colonized sori and reduced the rate of disease increase. Early applications, introduced during latent infection stages, provided effective control of the disease. Later inoculations, those applied after disease symptoms occurred, had no effect on disease development. However, *S. lanosoniveum* colonized sori in all treatments.

We conclude from this study that *S. lanosoniveum* readily colonizes sori of SBR and parasitizes urediniospores of *P. pachyrhizi*. Implications of this research include development of the mycoparasite for biological control of SBR in organic soybean production. Furthermore, use of the biological control agent may serve as an effective rotation in integrated pest management (IPM) systems or other rotation regimes. With increasing use of preventative fungicides for SBR management, it is very likely that fungicide resistant strains of the pathogen will be selected. Rotation of these fungicides with *S. lanosoniveum* may be effective when applied during the early stages of infection or even before initial deposition of SBR inoculum. While we reported on the suppressive effects of *S. lanosoniveum* on *P. pachyrhizi* and SBR, it is also important to note that the fungus colonized sori of other rusts in laboratory studies. We confirmed similar mycophilic interactions between *S. lanosoniveum* and fig rust, rust on rose, and stripe and stem rust of wheat. Thus, we hypothesize that mycoparasitic interactions are taking place in these sori as well.

Future work may include development of *S. lanosoniveum* as a commercial biological control product. Studies of conidial survival and formulations may lead to extended shelf life of a viable product. Additionally, stability under commercial field conditions may be aided by selection of heat tolerant or fungicide resistant isolates. Survival under adverse environmental

conditions and extended survivability in commercial production may include use of sunscreens, antidessicants, and UV protectants. Development of *S. lanosoniveum* as a commercial product should be conducted with this naturally occurring, noninvasive fungus.

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## **VITA**

Nicole Ann Ward was born in 1973 and was raised in a small community outside of Cottonport, Louisiana. After graduation from Avoyelles High School in 1991, she started her own landscape contracting business. During those twelve years as a business owner, Nicole attended Louisiana State University in pursuit of her Bachelor's degree in Horticulture Science. In 2007, she joined the soybean pathology lab group of Dr. Raymond Schneider in the Department of Plant Pathology and Crop Physiology at Louisiana State University. Nicole currently resides in Lettsworth, Louisiana, with her husband James Ward and son Adam Ward.