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Association between stink bug damage and the incidence of *Phomopsis longicolla* in Mississippi soybean production

By

Joshua Lunn Jones

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Agricultural Life Sciences
in the Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

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Joshua Lunn Jones

2013

Association between stink bug damage and the incidence of *Phomopsis longicolla* in Mississippi soybean production

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Stink bugs (Hemiptera: Pentatomidae) are key pests of soybean, *Glycine max* (L.), in Mississippi. Historically, yield loss derived from direct feeding by stink bugs has been considered the greatest threat to producers. However, quality reductions resulting from seed infections caused by microorganisms including *Phomopsis longicolla* are also a concern. Experiments were conducted in 2010 and 2011 to determine if stink bugs are associated with the incidence of *P. longicolla* in Mississippi soybean production. Data from experiments suggest that stink bugs are capable of transporting *P. longicolla* between two points. Data further suggest stink bugs and *P. longicolla* have the potential to cause a yield loss of 20% when combined in soybean. Surveys of commercial fields in Mississippi determined that stink bug damaged seed was more likely to be infested with *P. longicolla* and other fungi compared to undamaged seed.

DEDICATION

I dedicate this thesis to Jehovah God, who in the beginning by the power of his Holy Spirit through his son Jesus Christ created all things according to his will.

Jonah 2:2-6

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CHAPTER I

LITERATURE REVIEW

Intoduction

Stink bugs (Hemiptera: Pentatomidae) are major pests of soybean, *Glycine max* (L.), throughout the world. Collectively, the green stink bug, *Acrosternum hilare* (Say), the southern green stink bug, *Nezara viridula* (L.), and the brown stink bug, *Euschistus servus* (Say), make up the majority of the stink bug complex in the southern United States (Turnipseed and Kogan 1976). Several other species of stink bug also feed on soybean and in some instances may become a major pest (Panizzi and Slansky 1985).

Stink bugs damage soybean by penetrating the pod hulls with their piercing-sucking mouth parts and extracting nutrients from the maturing seed. The extent of this damage can be measured in terms of both yield loss and quality reduction (Todd and Turnipseed 1974). Moreover, stink bug feeding can also affect stem and leaf development, as well as seed germination in the subsequent season, and oil and protein content (Miner 1966). However, depriving the seed of vital nutrients may not be the only risk to soybean from stink bugs. Pod hulls that are damaged may leave the seed exposed to microorganisms that can further reduce yield and quality (Russin et al. 1988).

Soybean and U. S. Agriculture

Soybean was first introduced to the U.S. in the mid-18th century (Smith 1994). Since its introduction to the Americas, soybean production has steadily increased to become a major contributor to the agricultural industry in the U.S. (Smith 1994). In 2011, the U.S. harvested approximately 29.5 million hectares of soybean worth $35.7 billion, the second greatest crop value following corn (USDA-NASS 2012). Soybean production and harvested hectares in 2009 was a record high 89.8 million metric tons and 30.9 million hectares, respectively. The total farm value of soybean in 2009 was $32.1 billion and surpassed the previous record set in 2006. Since 2006, the total production dollar amount has set a new record each year as a result of increased commodity prices (USDA-NASS 2012).

The U.S. is currently one of the world's largest producers and exporters of soybean and soybean products (USDA-NASS 2012). In 2004, U.S. production accounted for 40% of the world's soybean production with approximately 35% of domestic production being exported (FAO 2005). The top states in terms of production value are Illinois, Iowa and Minnesota (USDA-NASS, 2012). Soybean also accounts for greater than 90% of all U.S. oil seed production. Overall, soybean production continues to increase in the U.S. and is projected to continue this trend according to the USDA baseline projections until at least 2017 (USDA-NASS 2009).

Processed soybean seed is the number one source of protein and vegetable oil in the world (USDA-ERS 2010). Soybean seed is comprised of approximately 5% ash, 34% carbohydrate, 21% oil, and 40% protein (FAO 2005). Soybean was originally grown for industrial purposes and livestock feed. However, just prior to World War II, research

enabled processed soybean to replace other oils that were not available to the U.S. during this time. New oil-based products boosted soybean consumption and in turn gave rise to an increase in soybean production. Soybean rapidly became the number one oilseed crop in the edible oil industry. In the past decade, the use of soybean as an alternate energy source such as biofuel has also been pursued (Hill et al. 2006). The full potential of soybean as a biofuel is yet to be determined and is the source of extensive research and debate (Shubert 2006). United States' soybean production and world market share is expected to continue to increase in the future through increases in technology and domestic market support (USDA-NASS 2009).

Soybean Growth Stages and Maturity Groups

Soybean is generally classified as an annual legume and short day plant. However, cultivars differ significantly in critical day length requirements (Garner and Allard 1920) and temperature (Carlson and Lersten 1987) necessary for growth. There are 13 soybean maturity groups (MG) that are based on response to day length and defined numerically as 000, 00, 0, and I through X (Poehlman and Sleper 1995). Soybean cultivars are also classified as determinate and indeterminate types which are based on flowering habit, genetic variation, and growth (Poehlman and Sleper 1995). Determinate types cease vegetative growth after reproduction begins while indeterminate types continue vegetative growth after flowering (Hodges and French 1985). Determinate types will flower simultaneously on all flowering nodes and no further nodes are formed after initial flowering begins. Indeterminate types begin flowering on lower nodes and continue to progress up the plant as new nodes are formed (Hodges and French 1985). Soybean goes through eight growth stages during reproduction while the number of

vegetative stages varies depending on soybean type, maturity group and available moisture (Koger et al. 2010).

Soybean Production in the Mid-south

Over the past 20 years, the soybean production system has dramatically changed across the Mid-south. Growers in this region historically used late-maturing determinate cultivars including MG V, VI and VII (Heatherly 1999). The cultivars were typically planted in May or June and suffered yield loss due to severe drought stress throughout late summer. Drought conditions combined with insect and disease pressure, along with high production costs and low market value, provided growers with the incentive to search for alternate means of production. As an alternative to conventional practices, growers began planting earlier in the season with earlier maturing indeterminate MG IV cultivars (Heatherly 1999). This is broadly referred to as the early soybean production system (ESPS). Typically under the new system, indeterminate MG IV soybean cultivars are planted in April, generally a month before late maturing MG V and VI cultivars. This new approach to soybean production increased yields by decreasing losses from drought and specific insect pests (Baur et al. 2000).

Presently, the ESPS is widely accepted as a good agronomic practice in the Mid-south. However, producers utilizing this system have been faced with a multitude of management issues. One of the most prominent concerns is the presence of economically damaging infestations of stink bugs in late summer. Though stink bugs are not a new pest in the Mid-south, the now widespread adoption of the ESPS has given stink bugs a preferred host at a preferred phenological development period (Smith et al. 2009). If left unchecked, the ESPS may act as a nursery crop to create larger stink bug populations in

later planted soybean fields. Despite problems incurred with both systems, research suggests that the ESPS has greater yield potential when compared to conventional practices (Heatherly 1999).

Insect Pests of Soybean in the Mid-south

Across the Mid-south, numerous species of insects feed on soybean throughout the various growth stages of the plant, with the greatest economic losses generally occurring during the reproductive stages (Funderburk et al. 1999). Major insect pests utilizing soybean as a food source in the Mid-south include armyworms (*Spodoptera* spp.), bean leaf beetle (*Cerotoma trifurcata* (Forster)), corn earworm (*Helicoverpa zea* (Boddie)), soybean looper (*Chrysodeixis includens* Walker), stink bugs (Pentatomidae), threecornered alfalfa hopper (*Spissistilus festinus* (Say)), and velvetbean caterpillar (*Anticarsia gemmatalis* Hubner) (Musser et al. 2011). Other insect pests of lesser economic importance include green cloverworm (*Hypena scabra* (F.)), Mexican bean beetle (*Epilachna varivestis* Mulsant), soybean nodule fly (*Rivellia quadrifasciata* (Macquart)), and spider mites (*Tetranychus* spp.) (Funderburk et al. 1999). Although the aforementioned insects may reach damaging levels under some conditions, only those listed above as major pests have proven to be of economic importance consistently over the past decade. Together, these major pests were responsible for approximately 90% of the total cost + losses due to insect pests throughout the Mid-south during 2010 (Musser et al. 2011). Among the economically important pests of soybean in the Mid-south, the stink bug complex is generally the most important in terms of yield and quality losses (Musser et al. 2011). In 2010, stink bugs in the Mid-south region were ranked greatest

among all insect pests in soybean with respect to area treated, cost, and percent total loss (Musser et al. 2011).

Stink Bug (Hemiptera:Pentatomidae) Complex in the Mid-south

The stink bug complex is one of the most economically important pests threatening soybean production throughout the Mid-south (Funderburk et al. 1999). Collectively, the green stink bug, the southern green stink bug, and the brown stink bug make up the majority of the stink bug complex in the southern U.S. (Turnipseed and Kogan 1976). Other species of stink bug such as the redshouldered stink bug (*Thyanta custator* spp. and the redbanded stink bug (*Piezodorus guildinii* (Westwood)) have traditionally been present at low densities in isolated areas throughout the Mid-south.

Biology and Ecology of Major Stink Bug Species in the Mid-south

Green Stink Bug

The green stink bug is one of the most economically important stink bug species in the southern U.S. Even though the green stink bug is generally the predominant species in the upper regions of the southern US, the pest is not limited to this geographical region. The green stink bug is widely distributed across North America, from Canada and New England, south to Florida and Texas and reaching as far west as the Pacific Ocean (McPherson and Pitts 1982). Throughout its distribution, the species is highly polyphagous, feeding on over 30 plant species such as apple (*Malus* spp.) buckthorn (*Rhamnus* spp.) and pear (*Pyrus* spp.) (McPherson and Pitts 1982). In southern regions, the crops attacked most frequently are soybean and green beans (*Phaseolus* spp.) (Miner 1966). However, in the absence of preferred hosts, other legumes such as lima bean

(*Phaseolus lunatus* L.) (Daugherty et al. 1964) may also be damaged, as well as other cultivated crops such as cotton (*Gossypium* spp.) (McPherson and McPherson 2000).

Eggs are smooth, barrel shaped and deposited in clusters predominately on the underside of leaves. Clusters are often laid in multiples of seven and average 32 eggs, but are highly variable, ranging from 3 to 56 eggs (Miner 1966). The egg color is initially a light pastel green. After approximately 5 days, the eggs become yellowish, changing to dull pink between days 6 and 8, and finally a bright pink just prior to hatching (Miner 1966). The egg stage of the green stink bug generally requires 8 days for completion (Simmons and Yeargan 1988).

Nymphs develop though five instars prior to molting into adults (Simmons and Yeargan 1988). Developmental times for first, second, third, fourth, and fifth instars average 7.0, 8.9, 7.9, 8.9, and 12.8 days, respectively (Miner 1966). The lengths of the stages vary with respect to temperature. However, the fifth instar is reported as the longest nymphal stage, regardless of temperature.

Males and females are long lived and average 67.6 days and 59.0 days, respectively (Miner 1966). Ovipositing females live an average of 73.3 days while non-ovipositing females average 48.1 days (Miner 1966). Adults overwinter in leaf litter, field trash and within the bark of fallen or decaying trees. Adults emerge from overwintering sites in the spring and begin feeding almost immediately on wild hosts, subsequently moving into cultivated hosts as they become available. Even though appearance times vary within different regions, it is speculated that increasing temperatures signal emergence from overwintering sites (Jones and Sullivan 1981).

Southern Green Stink Bug

The southern green stink bug is one of the most important agricultural insect pests in the world today. Although the ancestral origin of this species is uncertain, the current pest range includes many tropical and subtropical regions of Africa, Asia, Australia, Europe, and the Americas, between latitudes 45° N and 45° S (Todd 1989). The southern green stink bug is a strong flier and has likely utilized wind and weather patterns to facilitate dispersal across regions (Todd 1989). Industry shipping lanes have also likely contributed to its spread to different continents. The southern green stink bug is considered one of the most economically damaging pests of soybean across the globe (Panizzi and Slansky 1985).

The southern green stink bug is polyphagous, feeding on a multitude of important food and fiber crops. This species utilizes a number of monocots and over 30 different families of dicotyledonous plants (Todd 1989). The southern green stink bug, like all known phytophagous hemipterans, penetrates plant tissues with a stylet bundle consisting of two outer mandibular and two inner maxillary stylets. In soybean, the southern green stink bug penetrates the pod wall and extracts nutrients up a canal between the appressed maxillary stylets. Fifth instar nymphs and adults prefer to feed on developing seed in soybean and cause similar amounts of damage (Todd 1989). Feeding generally occurs in the top portions of the canopy until food shortages or high population densities force them to feed on the lower portions of the plant (Miner 1966).

As temperatures rise in the early spring, southern green stink bug adults emerge from overwintering sites and begin to feed and mate almost immediately. Feeding and oviposition generally occurs first in small grains, clover (*Trifolium* spp.), and early spring

vegetables; moving into row crops such as soybean, corn (*Zea mays* L.) and tobacco (*Nicotiana* spp.) later in the summer (Todd 1989). Females primarily oviposit in the upper portion of crop canopies on the underside of the leaves. However, oviposition may also occur on the pods and stems of soybean. Females will generally leave the plant shortly after oviposition is complete. Eggs are oviposited in masses containing 30-130 eggs per mass. The eggs are deep yellow at first turning a pinkish-yellow as they mature and eventually a bright orange at emergence (Todd 1989). Eggs generally hatch simultaneously due to the emerging nymphs stimulating hatching in adjacent eggs (Harris and Todd 1980).

Immatures of the southern green stink bug develop through five nymphal stages (Harris and Todd 1980). The first stage nymphs do not feed and generally do not leave the egg mass unless they are disturbed. The second stage nymphs will feed on plant tissue but usually stay on or close to the egg mass. By the third stage, nymphs begin to move away from the egg mass but stay clustered as a group (Harris and Todd 1980). The fourth stage nymphs show an increased tendency to disperse. By the fifth stage, the nymphs are dispersed and their distribution is random (Todd 1989). Total time from egg deposition to adult takes approximately 35 days but varies due to temperature and relative humidity (Harris and Todd 1980). In the fall when available food diminishes, adults move back to winter cover crops and feed until overwintering in crop residue, litter, bark or other suitable places that offer protection (Miner 1966).

Brown Stink Bug

The brown stink bug is the most economically important member of the genus *Eushistus* in North America (Panizzi and Slansky 1985). The species is widely distributed

throughout the continental U.S. and has two generations annually throughout its range (McPherson and Mohlenbrock 1976). The brown stink bug can cause yield and quality losses to several agronomic crops including soybean, corn, cotton, alfalfa (*Medicago sativa*, L.), sorghum (*Sorghum* spp.), and tobacco (Panizzi and Slansky 1985).

Eggs are manila-colored and are deposited in loosely bound clusters generally on the underside of leaves. Eggs are often deposited in multiples of 14 and masses can vary in size. Incubation periods average 5.5 days, but vary depending on temperature (Munyaneza and McPherson 1994). Nymphs are yellow-brown with light brown spots down the middle of the abdomen. Their development through the five nymphal instars is successively longer. Developmental times average 5.0, 6.0, 6.7, 9.3, and 11.5 days for first, second, third, fourth, and fifth instars, respectively, totaling 38.5 days (Munyaneza and McPherson 1994).

The brown stink bug overwinters as an adult. Preferred overwintering sites include crop residues, forest ground litter and weedy areas (Jones and Sullivan 1982). However, low survival rates have been observed in areas with sparse ground cover or poor drainage (Jones and Sullivan 1982). Spring emergence of adults varies with temperature and generally occurs around early-March to mid-April (Rolston and Kendrick 1961).

Diseases of Soybean in the Mid-south

As the number of hectares planted to soybean throughout the Mid-south has increased over the years, so too has the incidence and severity of diseases. Over 100 organisms are known to affect soybean; however, only about 35 are considered to be economically important (Bowers and Russin 1999). Some of the more common diseases

of soybean found in Mississippi include anthracnose (*Colletotrichum truncatum* (Schwein) Andrus & W. D. Moore), Cercospora blight (*Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner), charcoal rot (*Macrophomina phaseolina* (Tassi) Goidanich), frogeye leaf spot (*Cercospera sojina* K. Hara), and pod and stem blight (*Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm.) (Wrather and Koenning 2010). Generally, one or more diseases can be found throughout the growing season in any field where soybean is grown. Moreover, the extent and severity of disease depends heavily on the compatibility between the pathogen and the host plant coupled with the influence of the environment on the association (Balducchi and McGee 1987, Rupe 1990). Because of this specificity, a disease may be economically important one season and rarely found the next (Kulik and Sinclair 1999).

Fungal Pathogens of Soybean in the Mid-south

Throughout the majority of the growing season in the Mid-south, temperatures are warm, humidity is high (especially in irrigated soybean), and dew periods are long (Heatherly 1999). Because of these environmental conditions, pathogenic fungi may infect and colonize plants rapidly and potentially initiate an epidemic. Some of the more economically problematic fungi in the Mid-south include *M. phaseolina*, *C. kikuchii*, *C. sojina*, and *Rhizoctonia solani* Kühn (Wrather and Koenning 2010). Collectively, these pathogens have the ability to cause significant economic problems for soybean production across the Mid-south.

Fungal Diseases Associated with Stink Bugs

Fungi represent the largest group of economically important plant pathogens in soybean (Kulik and Sinclair 1999). Fungal spores and mycelium are capable of being disseminated many different ways, some of the more prominent being rain splash, wind, and insects. Heteroptera have been associated with many different fungal diseases, including cankers, pod and boll rots, and leaf spots (Agrios 1980). Entry into plant tissue is commonly achieved by insect damage, primarily feeding (Agrios 1997).

In soybean, associations between stink bugs and pathogens have been described as close or loosely related (Mitchell 2004). A close relationship is one in which the pathogen is dependent upon the insect for transmission. A loose relationship is where pathogens are not dependent on the insect for transmission, but the creation of wounds or lesions by feeding provide a point of entry for the pathogen into the plant (Shortt et al. 1982). Some of the fungi in soybean associated with stink bugs include *C. truncatum*, which is described as a loose relationship and *Phomopsis* spp., for which the relationship is undetermined (Mitchell 2004, Panizzi et al. 1979, Russin et al. 1988).

Phomopsis Seed Decay and the Diaporthe-Phomopsis Complex

Phomopsis seed decay is one of the primary diseases causing poor seed quality and yield loss in many soybean producing countries (Li 2011, Sinclair 1993). Seed decay is reported to be primarily caused by the fungal pathogen *Phomopsis longicolla* Hobbs, however, other *Phomopsis* and *Diaporthe* spp. can also affect seed quality (Sinclair 1993). *Diaporthe phaseolorum* var. *sojae* causes pod and stem blight of soybean and *D. phaseolorum* var. *caulivora* Athow and Caldwell along with *D. phaseolorum* var. *meridionalis* F.A. Fernandez cause stem canker (Kulik and Sinclair 1999). All can be

associated with *P. longicolla* and together make up the *Diaporthe – Phomopsis* complex (Bowers and Russin 1999, Sinclair 1993).

Yield loss and poor seed quality resulting from infestations of the aforementioned pathogens are largely due to increases in the frequency of cracked, moldy, and/or split soybeans as well as decreases in oil content and seed viability (Kulik and Sinclair 1999, Wrather et al. 2004). The *Diaporthe – Phomopsis* complex is distributed worldwide and causes significant reductions in yield and quality during years when environmental conditions are conducive to pathogen growth and disease development (Kulik and Sinclair 1999, Sinclair 1993). Yield losses derived from this complex in the southern U.S. from 1996 to 2007 were estimated to be in excess of 410 thousand metric tons (Wrather and Koenning 2010). In the southern U.S. during 2009, Phomopsis seed decay was estimated to cause yield losses in excess of 62 thousand metric tons in 16 states; primarily due to prolonged hot and humid conditions from pod fill to harvest (Koenning 2010).

Phomopsis longicolla

Phomopsis longicolla was first described in 1985 (Hobbs et al. 1985). Most isolates used in the study were derived from pod, seed and stem tissues taken from soybean samples collected from several locations in different states throughout the U.S. Isolates were divided into two groups based on appearance on acidified potato dextrose agar (pH 4.5, 85% lactic acid) after two weeks of incubation under intermittent fluorescent light (12 hrs/day). Hobbs et al. (1985) reported that isolates from the first group matched Lehman's (1923) description of *P. sojae* and were continuously associated with the sexual form of the fungus *D. phaseolorum* var. *sojae*. However,

isolates from the second group were different from the first group and did not match Lehman's description of *D. phaseolorum* var. *sojae*. In addition, isolates in the second group did not fit previous descriptions of any of the known *Diaporthe-Phomopsis* spp. taken from soybean. Hobbs et al. (1985) reported that colonies in the second group produced floccose ropy mycelium on potato dextrose agar, were also dense and initially white with occasional greenish-yellow areas on top turning tan to brown as the culture aged. The undersides of colonies were colorless and exhibited large black stromata. Alpha-conidia were hyaline ranging from 5 to 9.5 × 1.5 to 3.5 μm in size. Beta-conidia were rare and also hyaline. Hobbs et al. (1985) suggested that isolates comparable to the second group were likely observed in past studies but were reported as *D. phaseolorum* var. *sojae*. Hobbs et al. (1985) proposed that isolates from the first group retain the name of *P. sojae* while the second group be called a new species, *P. longicolla*.

Disease Cycle and Symptoms

Phomopsis seed decay occurs throughout the soybean producing regions of the U.S. and especially in the Mid-south (Kulik and Sinclair 1999). However, the incidence and severity of the disease varies year to year, depending greatly on environmental conditions and available moisture (Kulik and Sinclair 1999, Mengistu et al. 2009, Rupe 1990, Wrather et al. 2004). In one study, Rupe (1990) reported that infection of soybean stems and petioles increased with increasing dew points at all temperatures ranging from 15 to 35°C. In another study, Mengistu et al. (2009) reported that *P. longicolla* isolated from soybean seed taken from field plots was significantly affected by available moisture. Furthermore, a study conducted from 1995 to 1997 and in 2001 exhibited significant effects on the levels of *P. longicolla* infection due to irrigation (Mengistu and

Heatherly 2006). Irrigation increased infection, suggesting that soil moisture could increase relative humidity in the crop canopy in a way that favors development of Phomopsis seed decay (Mengistu and Heatherly 2006). While environmental conditions are generally accepted as one of the most critical aspects impacting the incidence and severity of Phomopsis seed decay (Balducchi and McGee 1987), available inoculum is also important (Kmetz et al. 1979).

Fungi of the Diaporthe-Phomopsis complex are reported to be seedborne and may overwinter in soil or plant debris (Bowers and Russin 1999, Kmetz et al. 1979, Kulik and Sinclair 1999). The soybean residue left behind after harvest may contain an abundance of mycelium, pycnidia and spores; therefore, making it one of the primary sources of inoculum (Kmetz et al. 1979). This disease complex is generally among the first microorganisms to infest soybean, but may do so at any point throughout the vegetative or reproductive growth stages (Kulik and Sinclair 1999). However, seed infections are more likely to occur during later developmental stages or after physiological maturity (Sinclair 1993).

The appearance of soybean seed infected by *P. longicolla* or other *Phomopsis* spp. range from asymptomatic to shriveled, elongated or cracked, and commonly appear white or chalky (Kulik and Sinclair 1999, Mengistu et al. 2009, Li 2011). In the subsequent season, infected seed either fail to germinate or germinate more slowly than healthy seed (Kulik and Sinclair 1999; Mengistu et al. 2009). Seed that do germinate generally emerge slowly and are prone to both pre- and post-emergence damping-off (Kulik and Sinclair 1999, Mengistu et al. 2009; Sinclair 1993). The fungi initially colonize seed coats and then the cotyledons. Mycelia then invade the ovule and developing seed. The fungi

generally colonize all tissues of the cotyledons, seed coat, and eventually the radicle and plumule (Kmetz et al. 1979, Kulik and Sinclair 1999). Although *P. longicolla* may be seedborne, the pathogen may be isolated from all plant parts. Mengistu et al. (2009) reported that *P. longicolla* was recovered more frequently from all vegetative plant parts, pods, and seed compared to other *Diaporthe-Phomopsis* spp. over a 3-year period. Another study supports Mengistu et al. (2009), reporting that *P. longicolla* was the predominant species isolated from diseased plants over a 3-year study in Canada (Xue et al. 2007) and was the most frequently isolated fungal pathogen from both discolored and non-discolored mature soybean stems (Harrington et al. 2000).

Disease Management

To date, standard agronomic practices are a primary foundation for managing Phomopsis seed decay and minimizing the impact of the disease in soybean (Li 2011). Crop rotation with corn or another non-legume crop is effective because they are not hosts of the fungus (Kulik and Sinclair 1999). Fields should be plowed to accelerate decomposition of crop residues (Bowers and Russin 1999) and reduce spore dissemination (Li 2011). High quality disease free seed should be planted to assist in preventing infestations and decrease the spread of the fungus to non-infested fields. Fungicide seed treatments and foliar applications have been suggested to be effective in reducing the risk of infection; however, the fungus may infect the plant at any time and is believed to be most problematic during the mid-to late-reproductive stages. Therefore, foliar fungicide applications should be made during the reproductive stages between beginning pod (R3) through beginning seed maturity stage (R7) (Bowers and Russin 1999, Kulik and Sinclair 1999). Harvesting soybeans promptly at maturity is essential.

Delayed harvest, especially when environmental conditions are favorable for fungal growth, can result in yield and quality losses (Mengistu et al. 2010). However, these practices are preventative measures intended to reduce the risk of infection by the fungus. Once infection has occurred, management options are limited and have been reported to be largely ineffective (Kulik and Sinclair 1999). After the seed is infected, the pathogen degrades seed coat proteins and market grade can be significantly decreased.

Along with agronomic practices, the use of resistant cultivars is the most effective method for managing Phomopsis seed decay (Jackson et al. 2005, Mengistu et al. 2010, Pathan et al. 2009, Roy et al. 1994). In recent years, screening for Phomopsis seed decay resistance has brought about the identification of several resistant sources (Li 2011). Simple sequence repeat (SSR) markers have been used to identify genes linked to Phomopsis seed decay resistance in PI80837 and MO/PSD-0259. These markers should assist in selection of resistant genotypes in breeding programs (Li 2011). However, at this time, sources of Phomopsis seed decay resistance remain unclear.

The objective of this research project was to determine if there is an association between stink bugs and *P. longicolla*. In addition, a survey of commercial soybean fields in Mississippi from 2010 to 2011 was conducted to attempt to quantify the extent of stink bug feeding in soybean and the effect feeding may have had on the frequency of *P. longicolla* recovered from damaged seed.

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CHAPTER II

ASSOCIATION BETWEEN STINK BUGS AND THE INCIDENCE OF FUNGI, SPECIFICALLY *PHOMOPSIS LONGICOLLA*, IN *GLYCINE MAX*

Abstract

Experiments were conducted during 2010 and 2011 to determine if stink bugs (Hemiptera: Pentatomidae) or the damage they cause can be associated with the incidence of *Phomopsis longicolla* (seed decay) in soybean, *Glycine max* (L.). A laboratory experiment using the green stink bug, *Acrosternum hilare* (Say), brown stink bug, *Euschistus servus* (Say) and redshouldered stink bug *Thyanta custator* (McAtee), indicated that 90% of stink bugs across all three species were capable of transporting *P. longicolla* to acidified-potato dextrose agar after being exposed to the fungus. The ability to transport the fungus did not vary among tested species. Stink bugs and *P. longicolla* each reduced yield by 10 to 15% in a field cage study, but there was no interaction between the two factors. Field sleeve cage studies indicated that soybean pods and seed with openings similar to that caused by stink bug feeding were at greater risk of infestation by fungi in general when compared to undamaged seed. However, the incidence of *P. longicolla* was not impacted by stink bug feeding.

Introduction

Stink bugs (Hemiptera: Pentatomidae) are polyphagous plant feeders and major pests of various crops in the southern United States (Turnipseed and Kogan 1976). In the mid-southern U.S., stink bugs are among the most economically important insects in soybean (Musser et al. 2011). Stink bugs damage soybean by penetrating pod hulls with their piercing-sucking mouth parts and extracting nutrients from the maturing seed (Miner 1966). Yield loss from direct feeding by stink bugs is the primary concern for producers (Turnipseed and Kogan 1976). However, plant infection by pathogenic microorganisms associated with stink bugs may cause additional losses in yield and seed quality.

Soybean diseases caused by fungi play a major role in reducing yield and can also result in loss of seed quality (Wrather and Koenning 2009). Fungi in the Diaporthe-Phomopsis complex are common in fields across the Mid-south and can greatly contribute to the reduction of soybean yield. The most economically damaging impact of the Diaporthe-Phomopsis complex is the effect these fungi can have on seed quality (Bowers and Russin 1999). Infected seed may become shriveled, elongated, and moldy; a condition commonly known as seed decay (Mengistu et al. 2009). *Phomopsis longicolla* is reported to be the predominant fungus within the complex because it is most often isolated from decayed seed and is thought to cause the majority of seed decay damage (Kulik and Sinclair 1999; Mengistu et al. 2009). Previous reports indicate that stink bugs may be associated with specific fungi that can reduce seed yield and quality (Kilpatrick and Hartwig 1955; Mitchell 2004; Russin et al. 1988). The research reported here is

intended to determine if an association between stink bugs and *P. longicolla* exists and to investigate the nature of that association.

Materials and Methods

Laboratory Experiment

An experiment was conducted in 2010 to determine if stink bugs could transport *P. longicolla*. Adult green, *Acrosternum hilare* (Say), brown, *Euschistus servus* (Say), and redshouldered, *Thyanta custator*, stink bugs were collected from commercial fields in Mississippi via sweep-nets, caged according to species, and returned to the laboratory for testing. In the laboratory, 20 individuals of each species were randomly selected and transferred to surface-sterilized plastic boxes (177 mm × 381 mm) where they were held until surface disinfestation.

In a laminar flow hood, individual stink bugs were removed from the plastic box and placed in a petri dish 100 × 15 mm (Fisher Scientific, Waltham, MA) containing acidified-potato dextrose agar (A-PDA) (plate 1). A-PDA was prepared by adding 58.5 g of PDA (Difco Laboratories, Detroit, MI) to 1,500 ml of distilled water and autoclaving for 25 minutes at low pressure. After autoclaving 1 ml of lactic acid was added. Each petri dish used in this experiment contained 15 ml of A-PDA applied with a Wheaton Unispense Pump (Wheaton Instruments, Millville, NJ). After 2 minutes in the petri dish, the stink bug was removed and placed in a wire mesh cylinder (87 mm × 150 mm). The stink bug was immersed for 25 seconds in a 1,000 ml beaker containing 800 ml of a 0.06% NaOCI solution, rinsed in sterile distilled water for 10 seconds and dried on filter paper for 2 minutes. After drying, the stink bug was placed in another petri dish containing A-PDA (plate 2) for 2 minutes to confirm surface disinfestation.

The surface disinfested stink bug was then placed in a petri dish containing *P. longicolla*. The specific *P. longicolla* isolate used in this experiment originated from diseased soybean plant samples submitted to the MSU-ES Plant Diagnostic Clinic in 2010. Cultures were incubated in a growth chamber (32°C; 24 h light) and were two weeks old at the time of experimentation. The stink bug was removed after 2 minutes and placed on a fresh A-PDA petri dish (plate 3) for an additional 2 minutes and removed. The process was conducted for all bugs selected from each species.

Petri dishes were stored in a surface-sterilized clear plastic container at room temperature (22°C) under florescent lighting (12 h light: dark). After 2 weeks, plates were examined for fungi, particularly *P. longicolla*. Each petri dish was rated as positive or negative for *P. longicolla* based on colony morphology (Hobbs et al. 1985). Data were grouped by stink bug species and analyzed using the Pearson's chi-square statistic generated by PROC FREQ in SAS (Version 9.2, SAS Institute of America, Cary, NC).

Field Cage Experiment

The experiment was conducted during 2010 in a field of MG IV soybean (Pioneer 94B73) planted on 96.5 cm rows at the Rodney R. Foil Plant Science Research Center in Starkville, Mississippi. The field was conventionally tilled, uniformly managed, and weeds were controlled using Mississippi State University Extension Service suggestions (Koger et al. 2010). Insect pressure was monitored weekly using sweep net sampling through growth stage R7. When insects reached treatment thresholds (Catchot et al. 2011) prior to growth stage R5 (Fehr et al. 1971), an insecticide application was made with 117 ml/ha of lambda-cyhalothrin (Karate Z 2.08CS, Syngenta Crop Protection, Greensboro, NC) using a MudMaster Multi-Purpose Sprayer (Bowman Manufacturing, Newport, AR).

One insecticide application was made at beginning bloom (R1) and no further insecticide applications were made until the completion of the trial. At R1 a fungicide was applied to all plots using a formulated product rate of 437 ml/ha of azoxystrobin (Quadris 2.08SC, Syngenta Crop Protection, Greensboro, NC) using a MudMaster Multi-Purpose Sprayer with a spray volume of 15 gal/ha. At R5.5, field cages (1.8 m × 1.8 m × 1.8 m), made of 32-mesh Lumite screen (Lumite, Inc., Gainesville, GA), supported by an aluminum pipe frame were erected over the plots to enclose two rows of soybean (1.8 m in length). Each plot was enclosed with a single cage and the bottom edges were covered with soil to prevent insect movement in or out of the cage. Four treatments: green stink bugs, *P. longicolla* inoculum, green stink bugs + *P. longicolla* inoculum, and a non-infested control were applied at R5.5 (7 July 2010). The four treatments were replicated four times in a randomized complete block design. After 7 days the cages were removed and the plots were sprayed with 117 ml/ha of lambda-cyhalothrin.

For infestation purposes, stink bugs were collected using sweep nets from commercial soybean fields and maintained without food in an insect rearing chamber for 24 hours prior to the initiation of the experiment. Twenty-seven stink bugs were placed in each field cage that required an infestation of stink bugs by gently shaking them out of the wire containers evenly over the soybean foliage. This stink bug density was 2.25 times the recommended threshold for stink bugs in Mississippi (Catchot et al. 2011).

The *P. longicolla* isolate used originated from the above previously described culture. Based on previous research (Mengistu et al. 2009), liquid inoculum consisting of macerated mycelium and conidia was determined to be the optimum method for infesting plants. *P. longicolla* treatments were sprayed at dusk (approx. 8:15 p.m.) on 7 July 2010

with inoculum consisting of 1,000 ml of sterile distilled water, 10 ml of potato dextrose broth and four-week old cultures of *P. longicolla* grown on A-PDA . Four cultures of *P. longicolla* were placed in a blender for approximately 10 seconds on medium speed, and then combined with sterile water and PDB. *P. longicolla* treated plots were sprayed using a Solo Backpack Sprayer (Solo, Newport, VA) with the filter screens removed. The macerated inoculum was sprayed evenly onto the soybean foliage in each plot until runoff.

At physiological maturity (R8), plots were mechanically harvested and yield for each plot was measured. Two 350 g sub-samples were kept from each plot. One sub-sample was sent to Midsouth Grain Inspection Service (Stoneville, MS) for seed quality inspection including moisture, stink bug damage, and total damage. Samples from plots were allowed to standardize for moisture and are reported as dry matter. The other sub-sample was used to determine the frequency of recoverable fungi, particularly *P. longicolla.* One hundred seed were taken from each sample and separated into three damage categories: undamaged, damaged (e.g., shriveled, elongated, moldy), and stink bug damaged. Seed placed in the stink bug damaged category exhibited a definitive stylet puncture in the seed coat indicative of stink bug feeding. The damage category was determined by examining seed under a ×1.75 lighted desk top magnifier and making all quality determinations based on the Official U.S. Standards for Soybeans (USDA GIPSA 2004).

Seed were screened for the presence of *P. longicolla* by following the A-PDA method for the detection of the Phomopsis complex on soybean (ISTA 2003). Seed in each damage category from each treatment were surface disinfested separately by

submerging the seed in a 0.06% NaOCl solution for 30 seconds, triple rinsed with sterile distilled water, and dried on filter paper in a laminar flow hood. Once dried, nine randomly selected seed (or all seed when less than nine were available) were placed evenly on the surface of A-PDA in 100 × 15 mm petri plates (3 seeds per plate). Following 7 days of incubation in a growth chamber (25°C; continuous light) plates were examined in a laminar flow hood for fungal growth. Sub-cultures were made on fresh A-PDA from seed exhibiting fungal growth. Following an additional 7 days, sub-cultures were examined with the aid of a dissecting microscope. The frequency of *P. longicolla* was determined based on morphological characteristics (Hobbs et al. 1985) and reported with respect to damage category and treatment. Fungi isolated from seed samples not identified as *P. longicolla* were classified solely as fungi for the purposes of statistical analysis.

Field cage statistical analyses were conducted with mixed model ANOVA using SAS (Version 9.2, SAS Institute, Cary, NC). Initial analysis of the 2 × 2 factorial treatments suggested there was not a significant interaction between stink bugs and *P. longicolla* with regard to yield; therefore the interaction term was deleted from the final model. Yield from one untreated control plot was substantially lower than the other control plots and consequently removed from the data set after initial statistical analysis because it was an outlier with a high level of influence. Degrees of freedom were estimated using the Kenward-Roger method (Kenward and Roger 1997) and differences were determined with Fisher's protected least significance difference (LSD) test at $P \leq 0.05$.

Field Sleeve Cage Experiment

A field sleeve cage experiment was conducted in 2011 to determine if stink bugs have a role in the infestation of soybean seed by *P. longicolla* by transporting the fungus to soybean or by creating wounds during feeding. The experiment was conducted twice at the Rodney R. Foil Plant Science Research Center in Starkville, MS and once at the Delta Research and Extension Center in Stoneville, MS. MG IV soybean were planted in April on 96.5 cm wide rows at each location and all fields were managed as previously stated. Treatments consisted of surface disinfested stink bugs (two per cage), *P. longicolla* infested stink bugs (two per cage), simulated stink bug damage, and a control.

Green stink bug adults and fifth instar nymphs were collected from commercial soybean fields as previously described. The specific insect life stages were selected because they cause comparable damage (Simmons and Yeargan 1988). Stink bugs were surface disinfested as previously described and separated into two groups; one group remained surface disinfested and the other was infested with *P. longicolla*. Infestation was accomplished by placing stink bugs in two week old cultures of *P. longicolla* growing on A-PDA for three hours. The origin of the *P. longicolla* used in this experiment was described above. Each group was kept separate in sterilized clear plastic containers until initiation of the experiment.

Sleeve cages made of tuille fine mesh screen measuring 15 × 7 cm were placed over pod clusters located in the middle section of plants. This was accomplished by measuring the height of the plant and selecting the cluster closest to the middle of the plant. Each of the four treatments was replicated 10 times using a completely randomized design. Treatments were initiated at R5.5 and removed after 7 days.

Stink bug damage was simulated by piercing the developing soybean seed with a flame sterilized 18 gauge pin (1.27 mm diameter). Each seed was pierced once and the needle was flame-sterilized between piercings. This was done for all seed within the pod cluster and each cluster contained a minimum of three pods each with 2 to 3 seed. After 7 days, the sleeve cages were removed and pods were harvested by hand, placed in sterile plastic containers, and transported back to the laboratory where the seed were extracted from the pods.

Fifteen pods and seed from each of the four treatments were surface disinfested and a sub-sample of 9 pods and seed were tested for the presence of fungi, particularly *P. longicolla* (ISTA 2003). Three replicate plates per treatment, containing either three pods or three seed were plated on A-PDA. Plates were incubated for 5 days in a growth chamber as previously described. Sub-cultures were made on A-PDA from pods and seed that exhibited fungal growth. After an additional 7 days the sub-cultures were examined with a dissecting microscope. The presence of fungi and the frequency of *P. longicolla* were recorded as previously outlined. Statistical analyses of the proportion of seed infested with *P. longicolla* or other fungi were conducted with mixed model ANOVA using SAS (Version 9.2, SAS Institute, Cary, NC). Arc-sine transformation was performed to meet the assumptions of the mixed model. Degrees of freedom were estimated using the Kenward-Roger method and differences were determined with Fisher's protected least significance difference (LSD) at $P \leq 0.05$.

Results and Discussion

Laboratory Experiment

Several species of fungi were present on stink bugs collected from commercial soybean fields (data not presented). This was expected due to previous reports that have suggested associations between Pentatomidae and fungi (Foster and Daugherty 1969, Leach and Clulo 1943, Michailides et al. 1998, Russin et al. 1988). However, *P. longicolla* was not isolated from stink bugs prior to surface disinfestation. Petri dishes exposed to stink bugs after surface disinfestation were free of fungi, indicating that surface disinfestation was successful. After exposure to *P. longicolla*, 90% of the stink bugs screened transported the fungus to A-PDA. The ability to transport *P. longicolla* did not vary among species tested ($X^2 = 1.11$; df = 2; $P = 0.57$) (Table 2.1).

Previous research suggests an association between stink bugs and the *Diaporthe-Phompsis* complex (Mitchell 2004, Panizzi et al. 1979) but the authors did not elaborate on the nature of the association. Our research suggests that stink bugs are capable of transporting *P. longicolla* between two points. Internal transmission was not evaluated, but it is unlikely that the fungus resided inside the stink bugs because of the short duration between exposure to the fungus and transportation to A-PDA. This experiment further suggests that one mode of transportation is mechanical and external. However, the timeframe in which the pathogen can remain virulent while in transport was not determined.

Field Cage Experiment

Analysis of data as a 2 × 2 factorial indicated that the main factors of stink bugs and *P. longicolla* infestations caused significant yield loss (stink bugs $F = 20.15$; df = 1,

8; $P < 0.01$; *P. longicolla* $F = 35.23$; df = 1, 8; $P < 0.01$) (Figure 2.2). However, there was not an interaction between stink bugs and *P. longicolla* ($F = 4.53$; df = 1, 8; $P = 0.07$). Because the test for interaction was not significant, these data indicate that the yield loss observed when stink bugs and *P. longicolla* were combined was additive and not synergistic.

Analysis of yield data by treatment exhibited significant differences in yield ($F = 19.81$; df = 3, 9; $P < 0.01$) (Figure 2.2). While yield loss was significant, the biological basis for the observed yield loss is not apparent. Percent seed damaged by stink bugs ranged from 0.9 to 4.1% and was not significantly different with regard to treatment ($F = 1.65$; df = 3, 9; $P = 0.25$) (data not presented). Total damage was also not significantly different with regard to treatment, ranging from 1.8 to 5.4% ($F = 2.16$; df = 3, 9; $P = 0.16$) (data not presented). Also, *P. longicolla* was not recovered from any sampled seed and no significant differences were observed among treatments for seed infestations of other fungi ($F = 2.37$; df = 3, 9; $P = 0.14$). While it is possible that the yield loss was the result of the stink bug and *P. longicolla* infestations, the lack of supporting data makes that conclusion tenuous.

Field Sleeve Cage Experiment

P. longicolla was not recovered from pods collected from the caged clusters. Furthermore, the frequency of *P. longicolla* recovered from seed was not significantly different with regard to treatment ($F = 1.06$; df = 3, 6; $P = 0.43$) and less than 2% of seed were determined to be infested with the fungus. These results are consistent with Russin et al. (1988) who reported that the incidence of *P. longicolla* recovered from soybean seed was not significantly affected by stink bug damage. In contrast, 100% of plated pods

were infested with other fungi. Moreover, a significant difference was observed among treatments with regard to fungi recovered from seed ($F = 13.38$; df = 3, 6; $P < 0.01$) (Figure 2.3). Similar results have been previously reported, suggesting that the frequency of fungi may be increased in stink bug damaged seed compared to undamaged seed (Kilpatrick and Hartwig 1955, Russin et al. 1988). Surface disinfested stink bugs and *P. longicolla* infested stink bugs were not significantly different with regard to the incidence of fungi excluding *P. longicolla*. The incidence of fungi other than *P. longicolla* in the simulated stink bug damaged seeds was not significantly different than the stink bug damaged seeds despite the fact that pins (1.27 mm diameter) used to simulate stink bug damage were larger in diameter than a typical stink bug stylet (0.55 mm) (Yates et al. 1991), resulting in a larger opening.

This study illustrates the importance of stink bug damage to pods in relation to the frequency of fungal infestation of soybean seed. However, it does not determine which species of fungi are most readily recovered from stink bug damaged seed. *Phomopsis longicolla* infestations were not impacted by stink bug feeding, but the overall recovery level of *P. longicolla* was low in all treatments, so it is likely that the environmental conditions prevented this experiment from effectively testing the relationship between stink bugs and *P. longicolla*. Further research on the fungi associated with stink bugs and the damage they cause in soybean is needed to more clearly understand the factors that impact this relationship.

Table 2.1 Results for stink bug species tested for transportation of *P. longicolla* in a 2010 laboratory experiment.

	P. longicolla		
Stink bug species	Absent	Present	Total
Euschistus servus	2	18	20
Acosternum hilare	3	17	20
Thyanta custador spp.	1	19	20
Total	6	54	60

$X^2 = 1.11$; df = 2; $P = 0.57$

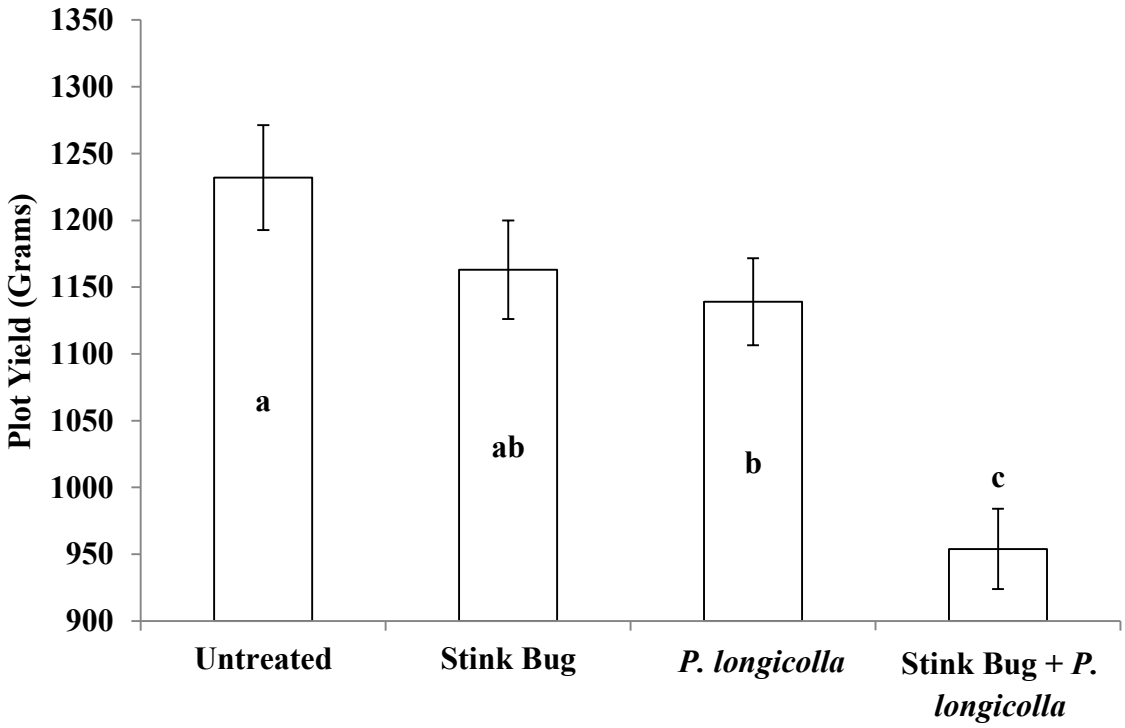

Figure 2.1 Mean yield of plots from a 2010 field cage study

Means followed by the same letter are not significantly different (α=0.05). Error bars indicate standard error of the mean.

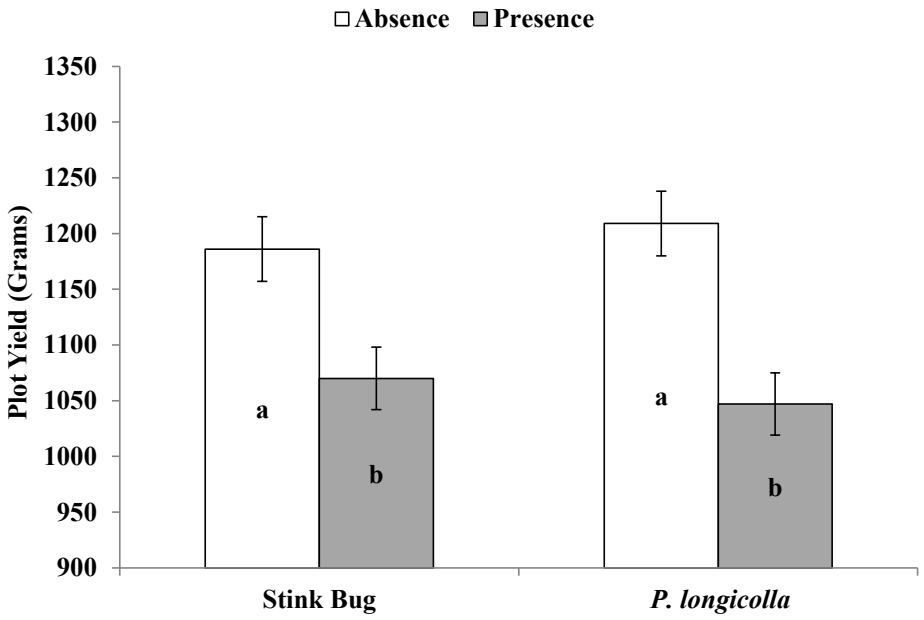

Figure 2.2 Least squared means estimates for main effects from a 2010 field cage study.

Each bar is the mean of two treatments containing the specified level of the factor. Means followed by the same letter are not significantly different (α = 0.05). Error bars indicate standard error of the mean

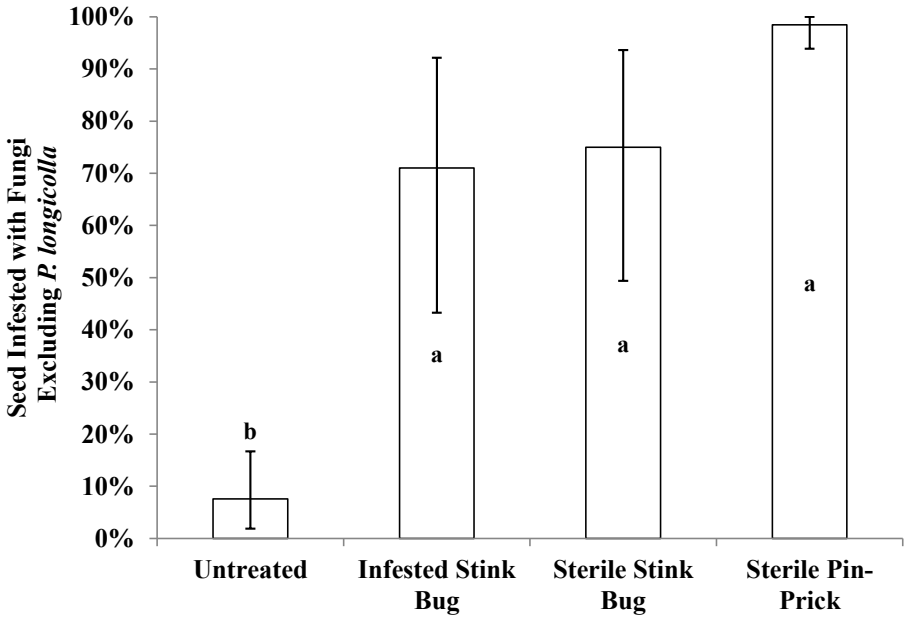

Figure 2.3 Mean percent of seed infested with fungi excluding *P. longicolla*, from a 2011 field sleeve cage study.

Means followed by the same letter are not significantly different based on an arcsine transformation of the data (α = 0.05). Error bars indicate standard error of the mean.

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CHAPTER III

SURVEY OF STINK BUG DAMAGE AND THE INCIDENCE OF *PHOMOPSIS LONGICOLLA* IN MISSISSIPPI SOYBEAN FIELDS

Abstract

A survey was conducted during 2010 and 2011 to determine if stink bug (Hemiptera: Pentatomidae) damage increased the frequency of *Phomopsis longicolla* in Mississippi soybean *Glycine max* (L.) seed. Seed were collected from the top, middle, and bottom sections of plants from 58 fields of mature soybean throughout two major agricultural regions of Mississippi. *P. longicolla* was recovered from 15% of seed damaged by stink bugs, which was significantly greater than the 5% recovery rate from undamaged seed. Similarly, other fungi were recovered significantly more frequently from stink bug damaged seed than from undamaged seed. Damaged seed or seed exhibiting blemishes by means other than stink bug feeding also had significantly greater recovery rates for *P. longicolla* and other fungi compared to undamaged seed, but not greater than stink bug damaged seed rates. Seed collected from the bottom section of plants were greater in recoverable *P. longicolla* in comparison to seed collected from the top. No significant differences were observed among plant sections with regard to the incidence of other fungi. This study suggests that minimizing stink bug feeding in soybean may reduce fungal infestations of seed and may lead to reduced disease incidence.

Introduction

Stink bug (Hemiptera: Pentatomidae) is a key pest of soybean, *Glycine max* (L.) throughout the southern United States. In the Mid-south, the three most common species found in soybean are the green stink bug, *Acrosternum hilare* (Say), the southern green stink bug, *Nezara viridula* (L.) and the brown stink bug, *Euschistus servus* (Say) (Turnipseed and Kogan 1976). Stink bugs are frequently the most economically damaging pest of soybean (Musser et al. 2011) because of reductions in yield and may cause secondary losses in quality brought about by associated pathogenic microorganisms (Russin et al. 1988).

Stink bugs damage soybean by penetrating the pod hulls with their piercing-sucking mouth parts and extracting nutrients from the maturing seeds (Miner 1966). Infestations of stink bugs generally occur during the reproductive growth stages, specifically R3 to R6 (Fehr et al. 1971) when soybean becomes preferable to other hosts (Miner 1966). Although yield loss from direct feeding is a major concern of producers, previous research has suggested that secondary losses due to associated microorganisms can further reduce yield and quality (Agrios 1980, Foster and Daugherty 1969, Russin et al. 1988).

Some of the pathogens of soybean reported to be associated with stink bugs include *Colletotrichum truncatum* (Schwein.) Andrus and W. D. Moore (Panizzi et al. 1979), *Fusarium* spp. (Russin et al. 1988), and *Phomopsis* spp. (Panizzi et al. 1979). Although all of these fungi may cause disease in soybean, some are more economically important than others (Wrather and Koenning 2009). For example, Wrather and Koenning (2009) reported that in the southern U.S. from 1996 to 2007, *C. truncatum* was

estimated to cause losses in excess of 380 thousand metric tons and *Phomopsis* spp. losses were estimated to be 410 thousand metric tons. During years when environmental conditions are suitable, fungi of the Diaporthe-Phomopsis complex can cause significant losses (Kulik and Sinclair 1999). In 2009, *Phomopsis* losses were estimated to be more than 62 thousand metric tons in 16 states (Koenning 2010). Commonly recovered members of the complex include *Diaporthe phaseolorum* var. *sojae* (pod and stem blight), and *Diaporthe phaseolorum* var. *caulivora* (stem canker). One of the most important impacts of this complex is its effect on seed quality, with *Phomopsis longicolla* being the primary pathogen causing seed decay (Bowers and Russin 1999). The objective of this study was to estimate the impact of stink bug feeding on the frequency of *P. longicolla* in soybean seed collected from commercial fields in Mississippi.

Materials and Methods

Sample Collection

During 2010, soybean seed were collected from 30 commercial fields in Mississippi (Table 3.1). Fifteen fields were sampled from the Delta region and 15 from the Northeastern Hills/Black Belt Prairie region, with five from each region collected during August, September, and October. All fields were sampled approximately at physiological maturity (R8) (Fehr et al. 1971) and each field within a month was located in a different county to create a representative sample of each region.

Within each field, soybean plants (300 per field) were arbitrarily selected along a predetermined path that included both the field interior as well as field edges. A single pod was randomly collected by hand from the top (highest node) middle or bottom (lowest node) section of each plant in rotation until 100 pods were collected from each

plant section. Sampled plants were a minimum of 5 meters apart. Pods were kept separate in freezer bags and stored in a refrigerator at 2.5°C until they could be analyzed. Samples for 2011 were collected during September and October because very few physiologically mature fields were available in August and the number of samples was reduced to a total of 14 fields per region (7 during September and October, respectively) (Table 3.1). All other aspects of the 2011 survey remained consistent with the 2010 survey.

To prepare the soybean seed for analysis, pods were hulled by hand and a random 100 seed sub-sample was taken from each plant section. Seed from each section were separated into three categories: undamaged, damaged, and stink bug damaged, by examining each seed under a ×1.75 lighted desk top magnifier and making all quality determinations based on the official U.S. standards for soybeans (USDA GIPSA 2004). Seed exhibiting no external defects or color alterations were classified as undamaged. Shriveled, discolored, or disfigured seed were classified as damaged. Only seed that displayed visual external puncture wounds typical of stink bug feeding (Miner 1966), were considered stink bug damaged. Seed in each damage category were kept separate with regard to field, total weight, and percent recoverable *P. longicolla* for both years.

Isolation of *P. longicolla* and Other Fungi

Seed samples were evaluated for the presence of *P. longicolla* as prescribed (ISTA 2003). Seed were surface disinfested and nine seed (except when nine seed were not available) from each damage category were placed on A-PDA with up to 3 seed per plate and incubated as described in Chapter 2. Sub-cultures were made seven days after initial incubation and examined using a dissecting microscope following seven days of

colony growth. The frequencies of *P. longicolla* and other fungi were recorded for each category by region, month, county, and plant section.

Morphological observations were used for initial identification of *P. longicolla* (Hobbs et al. 1985). The frequency of sub-cultures exemplifying characteristics of *P. longicolla* was recorded and the sub-cultures were kept for further confirmation via molecular techniques. All fungi not identified as *P. longicolla* were grouped together and recorded as other fungi for the purpose of statistical analysis.

DNA Extraction and Molecular Identification of *P. longicolla*

Fungal mycelium from 10 sub-cultures morphologically identified as *P. longicolla* were used for genomic DNA extraction. Mycelium was scraped from 2 week old cultures grown on A-PDA, placed in a mortar, covered with liquid nitrogen until lyophilized and placed in a 1.5 ml micro centrifuge tube. DNA was extracted from the lyophilized mycelium using DNeasy Plant Mini Kits (Qiagen, Valencia, CA) following the manufacturer's directions. Extracted genomic DNA was stored at -20 °C.

Polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region of the fungal DNA. ITS sequences were generated using ITS1 and ITS4 primers to amplify the ITS1 and ITS2 regions based on procedures described by White et al. (1990). The master mix for each aliquot of DNA included: 28 µl Millipore water, 10 µl 5x Go Taq buffer, 4 µl $MgCl_2$ (25 mM), 0.8 µl dNTPs, 3.0 µl ITS1 (5 µM), 3.0 µl ITS4 (5 µM), and 0.2 µl TAQ. The thermal cycling protocol to amplify ITS was as follows: a 95°C cycle for two minutes to denature material, followed by 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, followed by a final stage at 72°C for 10 minutes (White et al. 1990).

A 1.5% agarose gel electrophoresis was used to confirm amplification. The amplicon was purified using ExoSap-IT (USB Products, Cleveland, OH) and submitted to Eurofins MWG Operon, Huntsville, AL for nucleotide sequencing. Contigs were assembled from resultant ITS1 and ITS4 sequences and uploaded in GenBank using Basic Local Assignment Search Tool (BLAST).

Data Analysis

Analyses were conducted using the mixed model analysis of variance (ANOVA) in SAS (Version 9.2, SAS Institute, Cary, NC). The response variables measured included percent total seed weight, as well as percent recovery for *P. longicolla* and other fungi.

To analyze the differences in proportions of seed from the three damage categories, the percent of total seed weight from each plant section (bottom, middle, top) from every field was calculated for each category. This allowed comparison of rates of damage within and between each section. To prevent bias due to a lack of seed in a particular category, percent total seed weight analysis was conducted only on fields that contained all three categories in all three plant sections. This reduced the number of fields in the analysis for total seed weight to 39 while all other response variables were based on 58 fields. Region was initially evaluated as a fixed factor, but preliminary analysis failed to show any consistent influence of region (data not presented) and the impact of region was not considered central to the objectives of this survey, so region was included in the model as a random factor. Therefore, the final model for analyzing percent total seed weight included month, plant section, category, and their interactions as fixed effects with year and region as random factors. However, because no samples were

collected in August 2011, the month by category interaction was analyzed separately by year.

Fixed effects for analysis of the incidence of *P. longicolla* and other fungi included plant section and damage category. Year, region, and month were placed as random factors in the model. Month was not evaluated as a fixed factor for the incidence of fungi because fungal incidence is strongly influenced by the environment, and environmental data were not collected. The primary reason for conducting the survey in multiple months was to sample a range of fungal incidence from seed, not to predict fungal incidence by month.

Log transformations of percent total seed weight, percent recovery of *P. longicolla* and other fungi were performed to meet the assumptions of the mixed model. Degrees of freedom were estimated using the Kenward-Roger method (Kenward and Roger 1997). Differences were determined using Fisher's protected least significance difference (LSD) at $P \leq 0.05$.

Results and Discussion

Percent Total Seed Weight

In both years combined, the three-way interaction of month by plant section by damage category ($F = 0.21$; df = 8, 457; $P = 0.99$) as well as the two-way interaction for month by plant section ($F = 0.14$; df = 4, 469; $P = 0.97$) were not significant for percent total seed weight and were subsequently deleted from the final model. In 2010, there was a significant month by category interaction ($F = 11.75$; df = 4, 228; $P < 0.01$) as the proportions of undamaged and damaged seed during August were different than during September (Figure 3.1). However, in 2011, this interaction was not significant ($F = 2.13$;

df = 2, 213; $P = 0.12$) as data were only collected during September and October (Figure 3.1). In both years combined, there was a significant plant section by damage category interaction ($F = 5.75$; df = 4, 469; $P < 0.01$). The proportions of seed damaged and undamaged in the top of the canopy were different from the bottom section of the canopy (Figure 3.2).

In both years combined, stink bug damage increased slightly in later maturing soybeans, but overall, stink bug damage was minimal in the surveyed regions during both years of the survey (Figure 3.1). This is in contrast to previous years when stink bugs were a major pest of soybean in Mississippi (Musser et al. 2011). Stink bug damage was significantly more abundant in the upper and middle sections of plants compared to the bottom section (Figure 3.2), consistent with the observations of Russin et al. (1988). During the two years of this survey, stink bug densities were low and the complex was primarily composed of green and brown stink bugs. When stink bug outbreaks occur in soybean in Mississippi, the southern green stink bug is normally the dominant species. Therefore, it is possible that the results of this survey regarding stink bug damage may differ from surveys conducted under greater southern green stink bug pressure that frequently occurs in Mississippi.

One limitation of this survey was that the growth stage of the plant at the time of injury for damaged and stink bug damaged seed was not identified. It is possible that some seed recorded in the damaged category sustained injury from stink bugs during earlier reproductive stages. At physiological maturity when the seed were collected, seed damaged by stink bugs during early reproductive stages would likely be shriveled, shrunk, or infested with a microorganism, thus obscuring the stylet punctures indicative

of stink bug feeding. Therefore, the percentage of stink bug damaged seed reported is likely conservative due to our inability to accurately determine the initial cause of injury to some damaged seed.

Phomopsis longicolla

Incidence of *P. longicolla* in the survey was based on morphological identification of cultures. Nucleotide sequence BLAST results indicated ITS sequences exhibited 100% identitiy to *P. longicolla* Stam-27 (GenBank Acc. No. FJ785433) (Mengistu et al. 2009). This confirms the accuracy of *P. longicolla* identification in this survey.

There was not a significant interaction between plant section and damage category with regard to recoverable *P. longicolla* ($F = 0.50$; df = 4, 460; $P = 0.74$) (Figure 3.3). However, significant differences were observed among main effects of plant section and damage category. Incidence of recoverable *P. longicolla* was nearly doubled in seed collected from the bottom section of plants in comparison to the top section ($F = 7.31$; df = 2, 464; $P < 0.01$) (Figure 3.4). This was expected because literature reports similar findings regarding the severity of *Phomopsis* being greater in the lower sections of the plant canopy when compared to the top (Kmetz et al. 1978). One of the possible reasons for this effect is that seed lower in the canopy are closer to field trash, which is the main source of inoculum (Kmetz et al. 1979). It is also possible that the microclimate encountered in the lower plant canopy during advanced growth stages was more conducive to the needs of *P. longicolla* when compared to the temperature and relative humidity encountered at the top of the canopy, especially in furrow irrigated soybean fields that are commonly encountered in the Mississippi Delta (Mengistu et al. 2009).

A significant difference in recoverable *P. longicolla* was observed among seed damage categories ($F = 9.41$; df = 2, 464; $P < 0.01$) (Figure 3.5). More than twice as many stink bug damaged seed and damaged seed had recoverable *P. longicolla* compared to undamaged seed. These results are consistent with Kilpatrick and Hartwig (1955) who reported that the incidence of microorganisms may be increased in seed damaged by stink bugs. However, in another study, the incidence of *P. longicolla* was unaffected by stink bug feeding (Russin et al. 1988). Differences in results between our survey and Russin et al. (1988) are likely due to several factors. Russin et al. (1988) collected seed from plots of two varieties of MG V soybeans at one location and were subjected to the same environmental conditions as well as level of *P. longicolla* inoculum. In contrast, our survey was conducted over three months on multiple fields consisting of different planting dates. Therefore; it can be safely assumed that the surveyed fields were different maturity groups. Moreover, fields were in multiple locations subjected to various environmental conditions. Our survey suggests that the frequency of *P. longicolla* was greater in seed that were physically compromised by stink bug feeding. Therefore, management practices that reduced stink bug infestations could have also resulted in a reduction in the frequency of *P. longicolla* recovery.

Other Fungi Excluding *P. longicolla*

The interaction between plant section and damage category for the incidence of other fungi was not significant ($F = 0.80$; df = 4, 457; $P = 0.47$) (Figure 3.6). However, damage category was a significant factor for the incidence of other fungi ($F = 0.34$; df = 2, 461; $P < 0.01$). Stink bug damaged seed had more recoverable fungi excluding *P. longicolla* compared to damaged seed, and both damaged seed categories had more seed

infested with fungi than recovered from undamaged seed (Figure 3.7). Similar results have previously been reported (Kilpatrick and Hartwig 1955, Russin et al. 1988). In the previous studies, *Fusarium* spp. was identified as the primary fungus that was more frequent in stink bug damaged soybean seed. When seed are used for planting, the increase in fungal incidence from stink bug damaged seed may cause problems with germination due to either pre or post emergence damping-off (Jensen and Newsom 1972).

Based on this survey and previous research (Kilpatrick and Hartwig 1955, Russin et al. 1988) stink bug feeding can result in damage to the pod and seed that can increase the frequency of fungal infestations on soybean seed. When environmental conditions are suitable, these infestations lead to infections resulting in diseases that reduce soybean yield and quality (Wrather and Koenning 2009). However, the linkage between stink bug feeding and *Phomopsis* is not as consistent, suggesting that other factors may be more important in *Phomopsis* incidence than stink bugs. Good stink bug management practices can have the indirect benefit of reducing disease incidence; however this will only be evident when environmental conditions are conducive to the development of disease.

Table 3.1 Soybean seed weights by seed category collected from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Year	Sampling date	County	Region	Total weight (g)		
				Undamaged	Damaged	Stink bug damaged
2010	August	Carroll	Delta	9.79	27.3	2.76
		Chickasaw	Hills	25.0	8.21	1.86
		Grenada	Delta	7.81	20.0	2.61
		Holmes	Delta	0.00	37.6	0.00
		Lee	Hills	24.2	11.4	1.08
		Leflore	Hills	0.00	30.3	0.66
		Lowndes	Delta	6.52	24.4	0.72
		Noxubee	Hills	6.68	37.4	0.00
		Oktibbeha	Hills	12.7	28.8	0.77
		Talahatchie	Delta	17.0	12.9	1.42
	September	Bolivar	Delta	1.38	28.1	1.88
		Calhoun	Hills	14.0	12.0	0.45
		Carroll	Delta	26.9	6.22	0.97
		Chickasaw	Hills	23.3	8.57	2.22
		Leflore	Delta	42.7	1.27	2.08
		Monroe	Hills	11.5	23.0	0.19
		Oktibbeha	Hills	15.8	19.9	1.10
		Sunflower	Delta	25.9	2.63	1.88
		Washington	Delta	32.9	6.53	4.62
		Winston	Hills	26.2	6.72	2.78
	October	Bolivar	Delta	15.1	23.5	0.39
		Chickasaw	Hills	14.0	20.5	1.07
		Grenada	Delta	16.2	18.3	0.88
		Lee	Hills	20.2	23.5	2.41
		Leflore	Delta	22.6	16.5	1.39
		Monroe	Hills	12.9	22.7	1.88
		Oktibbeha	Hills	20.2	18.8	3.49
		Prentiss	Hills	21.3	12.4	5.15
		Sunflower	Delta	26.4	5.63	3.57
		Tallahatchie	Delta	23.2	4.32	3

Table 3.1 (continued)

Year	Sampling date	County	Region	Total weight (g)		
				Undamaged	Damaged	Stink bug damaged
2011	September	Bolivar	Delta	27.5	13.7	2.04
		Chickasaw	Hills	18.1	26.1	0.42
		Clay	Hills	27.9	10.9	1.26
		Holmes	Delta	20.9	23.1	1.69
		Humphreys	Delta	14.2	16.8	1.09
		Leflore	Delta	1.81	34.7	0.82
		Lowndes	Hills	10.7	17.3	0.64
		Monroe	Hills	29.9	1.93	1.23
		Noxubee	Hills	8.83	27.7	0.58
		Oktibbeha	Hills	17.6	25.3	0.22
		Pontotoc	Hills	13.2	26.7	0.43
		Sunflower	Delta	22.3	25.5	1.28
		Washington	Delta	35.2	13.4	2.44
		Yazoo	Delta	13.6	13.4	0.96
	October	Alcorn	Hills	30.2	11.5	0.32
		Coahoma	Delta	24.1	13.6	1.28
		Grenada	Delta	11.8	13.9	2.38
		Monroe	Hills	27.5	7.75	1.04
		Oktibbeha	Hills	37.3	7.44	1.39
		Panola	Delta	50.2	0.32	0.00
		Pontotoc	Hills	18.3	11.5	1.01
		Prentiss	Hills	22.6	16.1	1.92
		Quitman	Delta	30.4	4.24	1.62
		Talahatchie	Delta	20.8	7.63	6.75
		Tate	Delta	21.9	11.2	2.56
		Tishomingo	Hills	34.2	9.72	1.87
		Tunica	Delta	38.3	7.13	0.89
		Union	Hills	12.7	28.7	0.29

A total of 100 seed were evaluated from each sample.

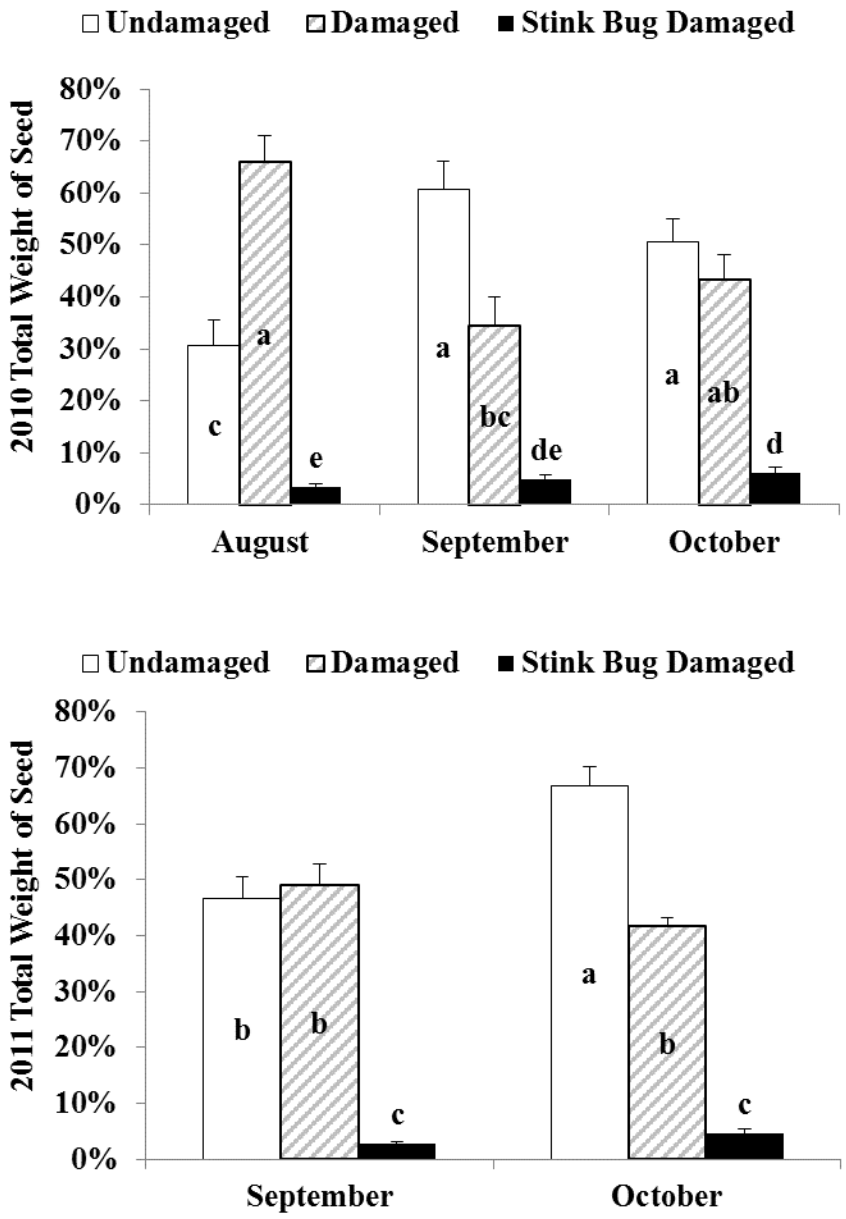

Figure 3.1 Mean percent total seed weight of undamaged, damaged, and stink bug damaged soybean seed category collected in each month from a 2010 (top) and 2011 (bottom) survey of commercial soybean fields in Mississippi.

Seed category by month interaction for 2011 was not significantly different based on analysis of log transformed data ($\alpha = 0.05$). Error bars indicate standard error of the mean.

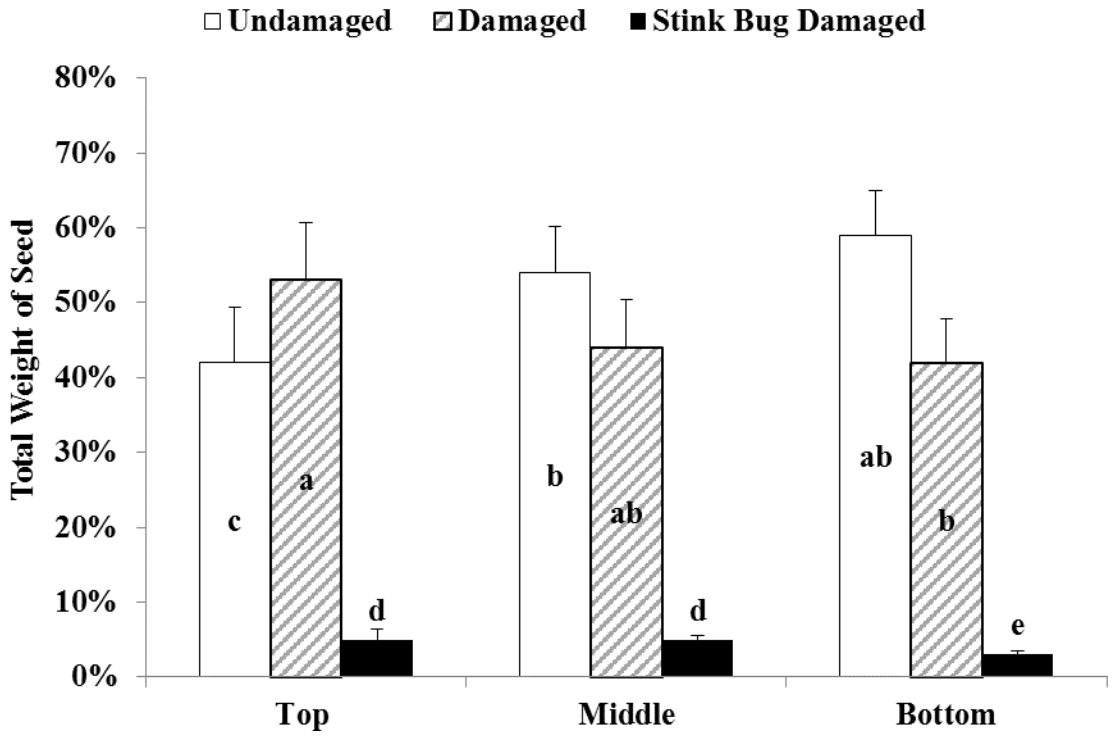

Figure 3.2 Mean percent total seed weight in each seed category by plant section from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Data includes only fields that contained all three seed categories in all three plant sections. Columns with the same letter are not significantly different based on analysis of log transformed data ($\alpha = 0.05$). Error bars indicate standard error of the mean.

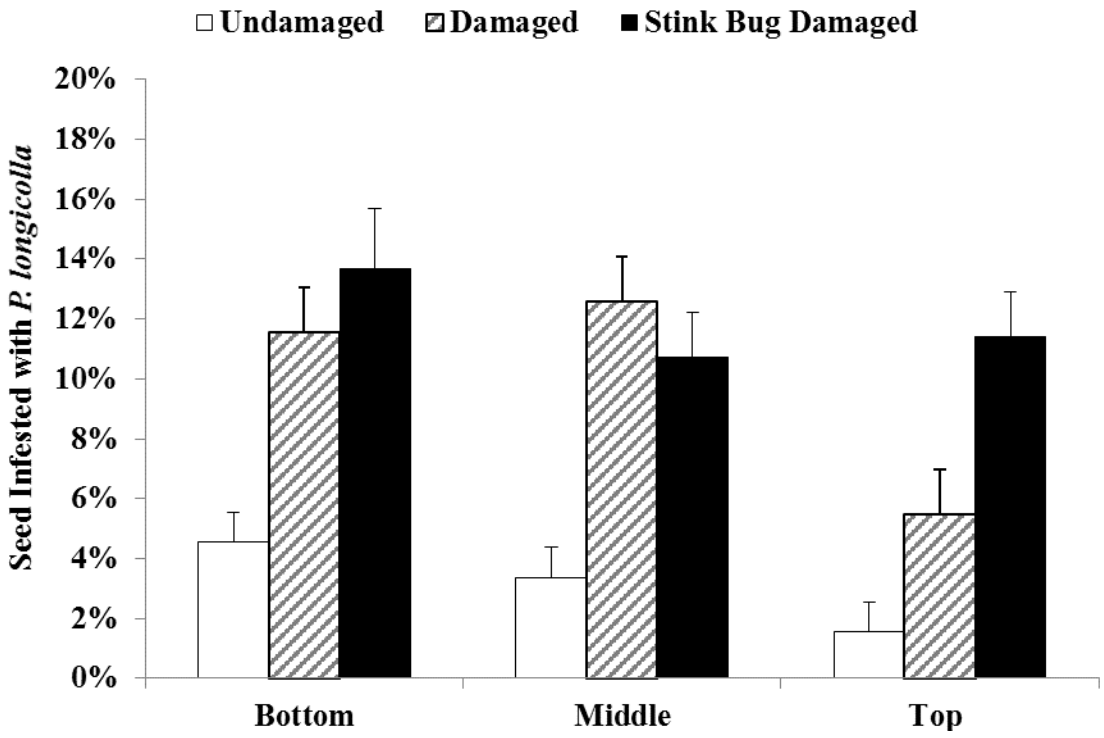

Figure 3.3 Mean percent seed infested with *P. longicolla* in each seed category by plant section from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Damage category by plant section was not significantly different ($F = 0.50$; $df = 4, 460$; $P = 0.74$) ($\alpha = 0.05$). Error bars indicate standard error of the mean.

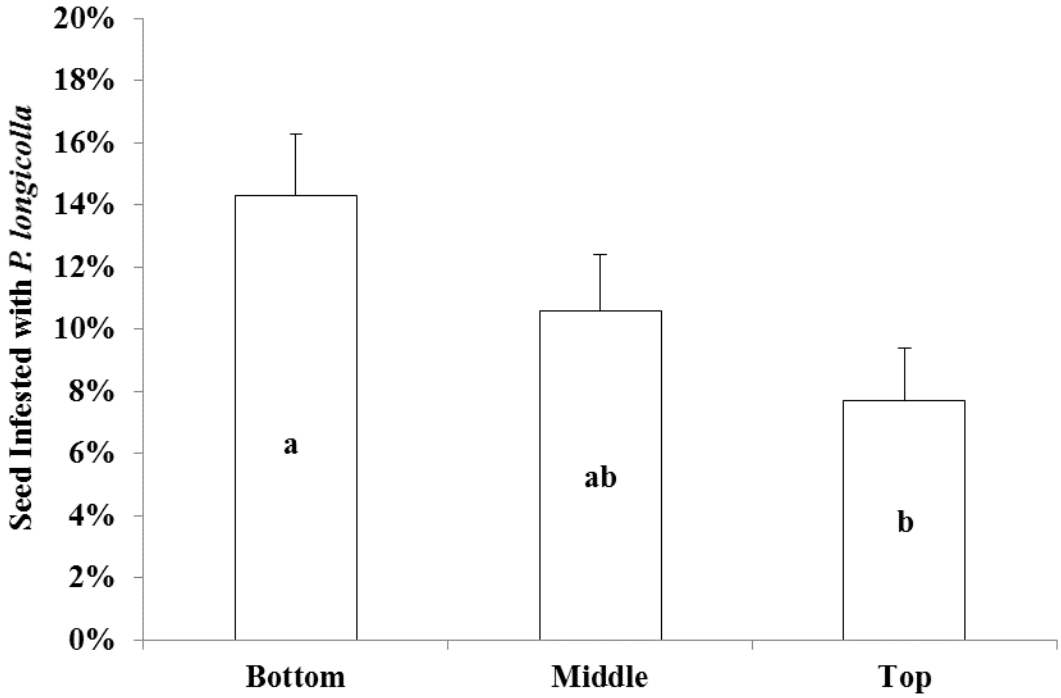

Figure 3.4 Mean percent seed infested with *P. longicolla* by plant section from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Columns with the same letter are not significantly different based on analysis of log transformed data ($\alpha = 0.05$). Error bars indicate standard error of the mean.

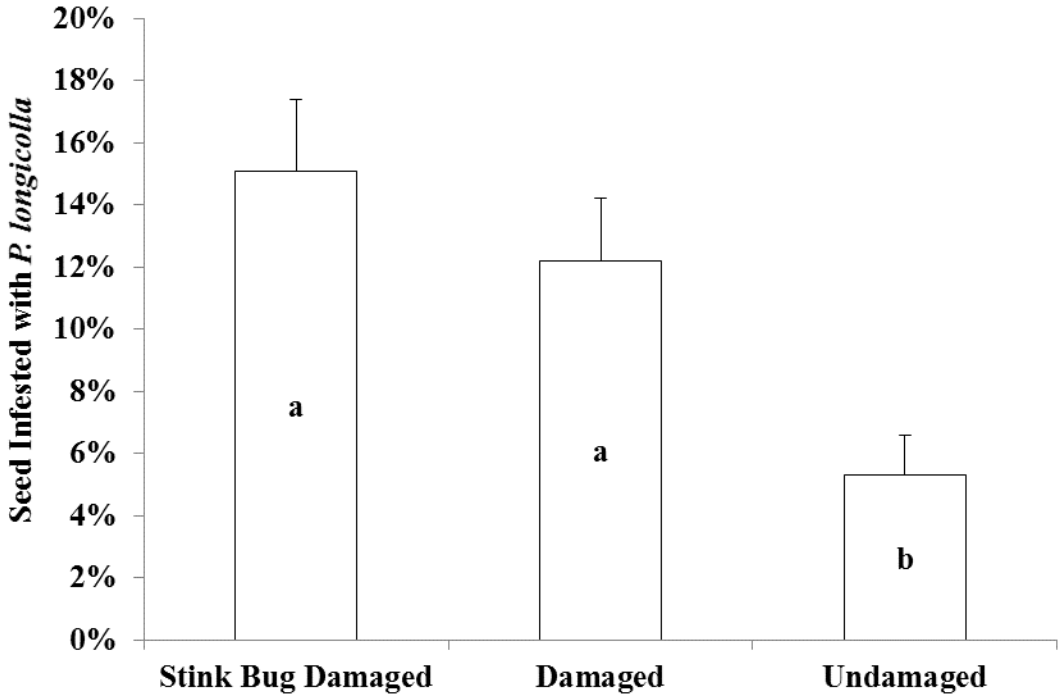

Figure 3.5 Mean percent seed infested with *P. longicolla* by seed category from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Columns with the same letter are not significantly different based on analysis of log transformed data ($\alpha = 0.05$). Error bars indicate standard error of the mean.

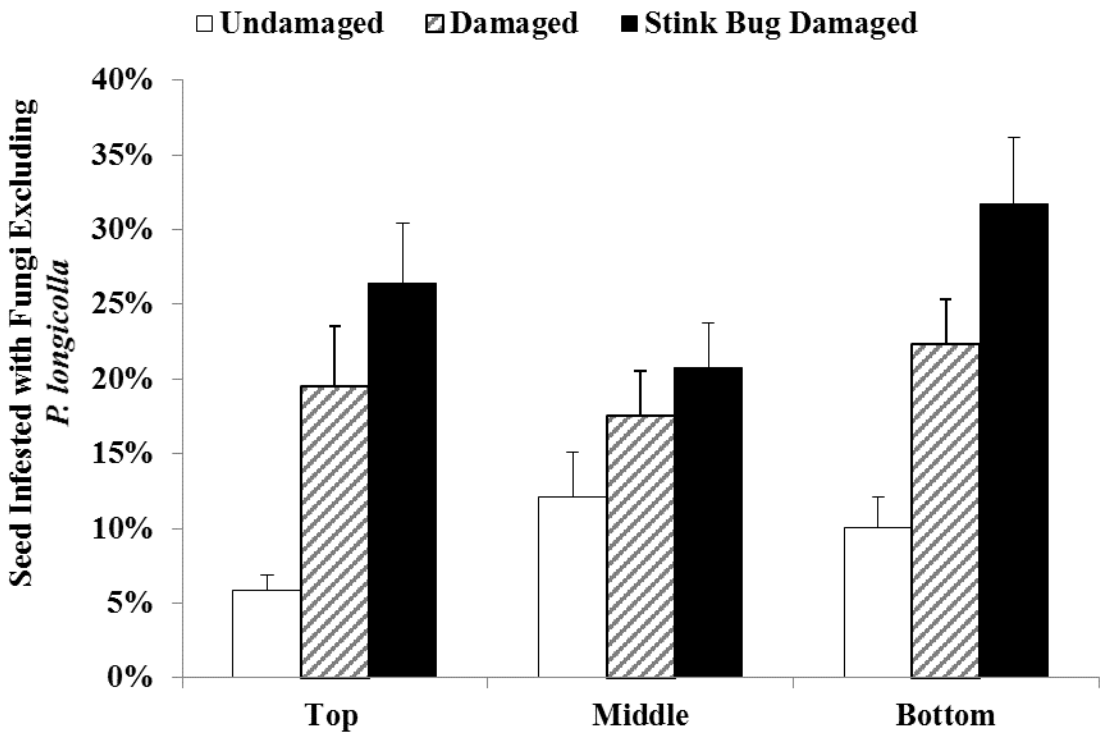

Figure 3.6 Mean percent seed infested with fungi in each seed category by plant section from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Damage category by plant section interaction was not significantly different ($F = 0.80$; $df = 4, 457$; $P = 0.47$) ($\alpha = 0.05$). Error bars indicate standard error of the mean.

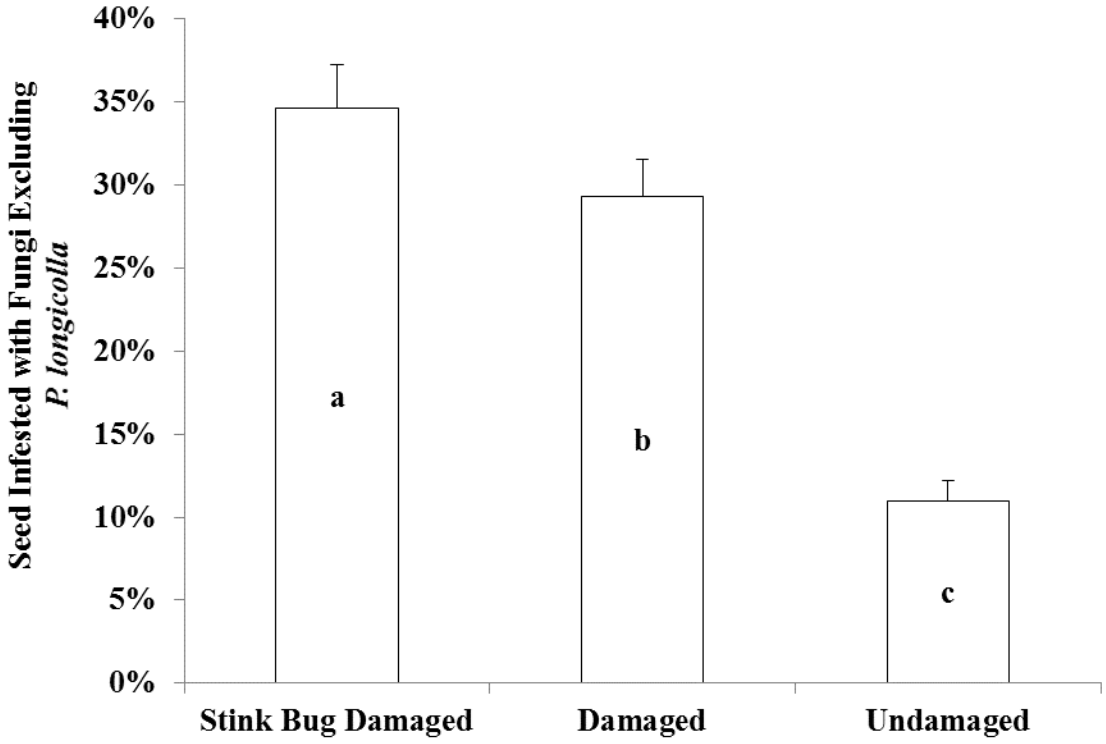

Figure 3.7 Mean percent seed infested with fungi by seed category from a 2010 and 2011 survey of commercial soybean fields in Mississippi.

Columns with the same letter are not significantly different based on analysis of log transformed data ($\alpha = 0.05$). Error bars indicate standard error of the mean.

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