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Epidemiological studies on the infection process and symptom expression of soybean sudden death syndrome

Carlos Cecilio Gongora-canul

Iowa State University

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Epidemiological studies on the infection process and symptom expression of soybean sudden death syndrome

by

Carlos Cecilio Góngora-Canul

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Pathology

Program of Study Committee:
Leonor Leandro, Major Professor
Gary Munkvold
Greg Tylka
X. B Yang
Dan Nordman

Iowa State University
Ames, Iowa

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DEDICATION

To my Lord, for giving me the blessing and the adventure to live. To my mother Elsa and my father Elias for their endless love and to all my brothers and sisters (Javier, Roberto, Martha, Manuel, Enrique and Nicte-Há) for all their great love affection. To my wife Tania for grabbing my hand and walking together during the journey of the PhD and to my God’s gift, my sons Carlos and Pablo.
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ABSTRACT

Sudden death syndrome (SDS) caused by *Fusarium virguliforme* (Aoki, O’Donnel, Homma & Lattanzi) is one of the most important soybean (*Glycine max* (L.) Merr.) diseases in the US. Management strategies currently available for this disease are not always effective, partly due to the high variability in symptom expression that occurs in field environments. To clarify the relationship between progress of root rot and foliar symptoms, soybean seedlings were inoculated at five inoculum densities and were destructively sampled over a 50 day period. Disease severity and area under disease progress curve (AUDPC) increased in response to increasing inoculum density (*P* < 0.01), particularly for foliar symptoms. Root rot severity evaluated 15 to 30 days after inoculation (DAI) was most highly correlated (*r* >0.8, *P* < 0.01) with foliar severity at 40 and 50 DAI, while weak correlations were found when roots and leaves were assessed simultaneously. Rate of disease progress generally increased as inoculum densities increased for both root and foliar symptoms. Root biomass was reduced by up to 80% at the three highest inoculum densities. To study the effect of plant age on symptom expression, plants were sown at intervals over a five week period to obtain plants at different stages of development at the time of inoculation. Plants were incubated in growth chambers to 17°C for 7 days and then to 24°C, and assessed for root rot and foliar severity at 18 and 38 DAI. Root rot developed on plants inoculated at all ages, but plants inoculated 0 days after planting (DAP) had the highest (*P* < 0.01) root rot severity compared to plants inoculated at older ages. Foliar symptoms were severe on plants inoculated 0 DAP, but never developed on plants inoculated at all other ages. Fungal colonization of the xylem was more frequent (56%) in plants inoculated 0 DAP than on plants inoculated at later stages (2-14%). These findings show that soybean roots are
susceptible to infection at different vegetative growth stages but that plants become less susceptible to xylem colonization and development of foliar symptoms as they mature. The interaction between plant age at inoculation and soil temperature was studied in greenhouse conditions. Soybeans were grown at 17, 23 and 29°C and inoculated at 0, 3, 7 and 13 DAP. Root rot developed in all inoculated plants, but severity decreased with increasing temperature and root age at inoculation. Foliar symptoms also became less severe at warmer soil temperatures, but severity was greatly dependent on plant age at inoculation. At 17°C, plants inoculated at all ages except 13 DAP developed foliar symptoms, while at 29°C only plants inoculated 0 DAP showed foliar symptoms for the duration of the experiment. Plants inoculated 0 DAP showed severe root rot and foliar symptoms at all temperatures. Root growth rate and root length were negatively correlated with root rot AUDPC and root rot rate, and positively correlated with root dry weight. This suggests that accelerated root growth in warm soils restricts xylem colonization and reduces the window for infections conducive to foliar symptoms. The fact that temperature did not affect disease severity on plants inoculated 0 DAP, may explain why delayed planting may not prevent severe epidemics if infection occurs shortly after planting.
CHAPTER 1.
GENERAL INTRODUCTION

Dissertation organization
This thesis is divided into five chapters. The first chapter includes a literature review of the history, importance, symptomatology, and management of soybean sudden death syndrome (SDS), followed by a justification for the research conducted. The second chapter is a study on the temporal dynamics of root and foliar symptom of SDS at different inoculum densities. The third chapter describes research on the effect of plant age at time of inoculation on root and foliar symptoms, and the fourth chapter focuses on the interaction of soil temperature and plant age at inoculation on the expression of SDS symptoms. The last chapter is a summary and general conclusion of this thesis.

Literature review

History and distribution of soybean sudden death syndrome
Sudden death syndrome (SDS) of soybean (Glycine max (Merr.) L.) caused by Fusarium virguliforme is an economically important disease with worldwide distribution. In the US, the disease was first observed by H. J. Walters in Arkansas in 1971 (64), but it was first named in 1982 when Hirrel (19) called the disease sudden death syndrome because of the fast onset of foliar symptoms. Since its first detection, the disease spread to different regions of the US, including most Midwestern states (6, 32, 75, 88, 95). Sudden death syndrome was first reported in Tennessee, Missouri, and Mississippi in 1984, Illinois, Kentucky, Kansas, and Indiana in 1985 (70), Iowa in 1993 (96), Minnesota in 2002 (32), and
in Wisconsin (6) and Nebraska (99) in 2006. According to Scherm and Yang (75) SDS distribution in the US is restricted by low moisture west of the Missouri River and by cold stress north of 43-44 degrees latitude. Outside the US, the disease has been reported in Canada (2), Argentina (73, 74), Brazil (45), Paraguay (97), Bolivia (98), and Uruguay (59).

**Economic importance**

According to Wrather and Koenning (88, 90), SDS is among the top ten diseases that suppress yield in soybean, ranking between second and fifth place during the period of 1996-2007 in soybean-producing states including Iowa, Illinois, Indiana, Arkansas, Missouri, and Tennessee. From 2000 to 2007, yield suppression ranged from an estimated 12.4 to 75.7 millions of bushels in 28 US states (88), representing losses of hundreds of millions of US dollars annually (3). Sudden death syndrome generally occurs in fields with high yield potential (usually in southern US) and can result in severe to total yield loss in affected areas (20, 65). Estimates of incremental yield reductions due to SDS have ranged from 7 to 34 kg/ha per unit increase in SDS incidence (12), and 12 to 22% of total yield per unit increase in foliar disease severity (54). In addition, Lou et al. (39) reported that per unit increases in foliar disease index caused yield losses of 18 to 29 kg/ha.

**Causal agents of SDS**

**Species distribution.** Sudden death syndrome is caused by four *Fusarium* species worldwide (3, 4), namely *F. virguliforme*, *F. brasiliense* *F. cuneirostrum*, and *F. tucumaniae* sp. nov. These four species are causal agents of SDS in South America, but in North America only *F. virguliforme* has been associated with the disease on soybeans. *Fusarium brasiliense*
is responsible for causing SDS in Brazil, *F. tucummaniae* causes SDS in Argentina and Brazil and *F. virguliforme* causes SDS in US and Argentina. In addition, *F. cuneirostrum* causes SDS in Brazil, and causes root rot of dry bean and mung bean in Japan, US and Canada. *Fusarium phaseoli* causes root rot in dry bean in US (3) and may cause SDS-like symptoms on soybean plants although incidence is low (68).

**Host range.** Although *F. tucummaniae* and *F. brasiliense* are only known to be pathogenic to soybean (*Glycine max* (Merr.) L.) *F. cuneirostrum* is pathogenic to multiple fabaceous hosts (i.e., soybean, dry bean, and mung bean). However, a recent study done by Kolander et al. (30), *F. virguliforme* caused symptoms on mung bean (*Vigna radiata*) and green bean (*Phaseolus vulgaris*) when inoculated without wounding and on lima bean (*P. lunatus*) and cowpea (*V. unguiculata*) when plants were wounded. According to the same authors, other plant species, including alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*), and pinto bean (*P. vulgaris*) can develop symptoms when infected with *F. virguliforme*. Five species, including corn (*Zea mays*) and the lambsquarter (*Chenopodium album*), appear to serve as asymptomatic hosts capable of increasing or sustaining *F. virguliforme*.

The species *Fusarium virguliforme*  

**Classification and identification.** *Fusarium virguliforme* (3, 4) belongs to section Martiella of the *Fusarium* genus (48, 55, 63, 65). The taxonomical classification of *F. virguliforme* (29, 48, 81) is as follows:
Fusarium virguliforme was formerly named *F. solani* f. sp. *glycines* and was recently renamed (3, 4) because all isolates of *F. virguliforme* belong to a single mtDNA RFLP haplotype, which distinguished it from other isolates of *F. solani*. However, *F. virguliforme* shares some similarities with some isolates of *F. solani* f.sp. *phaseoli* because it belongs to a single mtDNA RFLP haplotype and they may be very similar in culture (68). There is phylogenetic evidence indicating that *F. virguliforme* and *F. tucumaniae* may have evolved convergently, since they do not share a most recent common ancestor. *Fusarium virguliforme*, *F. tucumaniae*, and *F. phaseoli* appear to have evolutionary origins in the southern hemisphere (4).
**Morphological description.** *Fusarium virguliforme* produces macroconidia, microconidia (although rarely), and chlamydospores (43, 65). The macroconidia are formed by monophialides on simple or branched conidiophores (4, 61, 63), and are mostly two, three or four-septate (63, 65). Macroconidia are cylindrical to falcate, with a foot cell on tall conidiophores. The average size of a macroconidia on synthetic low nutrient agar (SNA) is 50-60 µm long and 5-5.5 µm wide, the apical cell is acutate, and the basal foot cell is indistinct. Microconidia are minute, oblong-ellipsoidal, short-clavate, mostly as septate or 1 septate on short conidiophores (3). *Fusarium virguliforme* can be grown on different media, including potato dextrose agar (PDA), modified Nash and Snyder’s medium (MNSM), modified Bilay’s medium, potato sucrose agar, and V8 juice agar. These media have been used to isolate the pathogen from rotted roots and from soil (8, 40, 64, 65, 71, 72). Colony morphology and pigmentation vary depending on the media where the fungus is grown and on the number of repeated transfers, but a blue pigmentation is most often associated with the pathogen in culture (61, 62). After repeated transfers onto PDA, the fungus can change from a blue to a white color, reduce the sporulation and increase the nonsporulating aerial mycelium (64). Although sexual stages have been found for *Fusarium tucumaniae* (9), the *F. virguliforme* population in the US. is believed to be exclusively asexual due to lack of a compatible mating type or special requirements of environment conditions that stimulate sexual reproduction.
SDS symptomatology

Like many other Fusarium species, *F. virguliforme* is a root pathogen capable of causing root rot and loss of root mass. However, this specie also produces a toxin that is responsible for foliar symptoms. The symptoms on roots and leaves are described below.

**Root symptoms.** Root symptoms are characterized by reddish to brown discoloration of the tap root and lateral roots. The discoloration may extend up in the stem several nodes, but the pith remains white. In severely attacked plants with high levels of foliar severity, the root discoloration is most pronounced and root volume and dry weight are reduced, causing the plants to be easily pulled out of the soil (35). In field conditions, it is also possible to observe a bluish sporulation on the tap root and lower stem caused by masses of macroconidia on the root surface (62). In controlled environment studies, root infection was shown to occur as soon as 2.5 h after initial exposure of seedling roots to *F. virguliforme* (46), while discoloration of the taproot was observed 3 days after planting in another study (22). In field conditions, Gao et al. (10) detected root infection as early as 18 days after planting. The progress of root rot may follow a monomolecular curve model which implies a rapid progress at the beginning of the epidemic and a slowdown at the end (7, 44). According to Li et al. (35) the ability of the fungus to colonize the root may not always be related to toxin production.

**Foliar symptoms.** Once root infection has occurred, foliar symptoms may be visible within 2 or 3 weeks (47). Foliar symptoms start with circular scattered mottling on leaves, rugosity, and marginal cupping (34). The spots then enlarge and become chlorotic, and later coalesce to form large regions of interveinal chlorosis and necrosis. Severely attacked leaflets may drop off leaving the petioles attached. Foliar symptoms progress faster in the upper
leaves than lower leaves, resulting in upper canopy defoliation, finally complete defoliation may occur under high disease severity (64). Foliar symptoms typically appear at reproductive stages in field conditions (40, 64), but foliar symptom during vegetative stages has also been observed both in naturally infested fields and in artificial inoculation studies (13, 15, 38-40, 47, 67). In contrast, under controlled environments, foliar symptoms typically appear 2 or 3 weeks after inoculation (15, 47).

**Disease cycle and epidemiology**

**Infection process.** Macroconidia converted into chlamydospores in sloughed root tissue (43) are considered the primary source of inoculum of *F. virguliforme* (64). The chlamydospores germinate to produce hyphae that penetrate the root (41). Microscopic observation revealed that the higher penetration frequency of the fungus occurred near the root-cap zone where few or no root hairs were observed, but also occurred in the base of the root hairs. Germ tubes, appressoria, and infection pegs were also observed on radicles (47). Once penetration occurs, hyphae grow mainly intracellularly or sometimes intercellularly (43). The fungus then starts to cause necrosis of the root tissue, and plants start to show mottling and mosaic symptoms on leaves within 10 days after the pathogen-root contact (22). The hyphae need to reach the xylem to induce foliar symptoms, but the mode of entry of the germ tube into the xylem is unknown (47). Cool soils (15-17°C) and wet soils (10, 76) are most favorable to root infection, while expression of foliar symptoms is favored by 22-24°C (76). However, severe levels of root rot can also occur in warm soil.

**Pathogen toxin.** *Fusarium virguliforme* produces toxins that are responsible for causing foliar symptoms in soybean (28, 35). Jin et al. (27) determined that one toxin
involved in the foliar symptoms had a molecular weight of 17kDa. This protein was identified in soybean stem exudates of plants inoculated with *F. viguliforme* and was designated as FISP 17 (28, 34). The mechanism by which the toxin produced foliar symptoms involves the disappearance of the protein ribulose 1,5 bisphosphate carboxylase/oxygenase (Rubisco), which is involved in carbon assimilation and photorespiration from leaves; and light appears to be essential for its toxic activity (26).

Typical SDS symptoms can be produced by immersing of soybean seedling with cut stems in cell-free culture filtrates of *F. virguliforme*, but symptoms do not develop on plants exposed to filtrates of other *Fusarium* species (36). Not much is known about the ability of different isolates to produce toxins, but it is likely that some isolates may be good toxin producers but poor root colonizers, while others may be poor toxin producers but good root colonizers (35).

**Pathogen survival.** Root and foliar symptoms progress throughout the growing season until complete defoliation occurs (64). Once the soybean plant dies, the pathogen overwinters in crop debris, soil particles and maybe also in soybean cyst nematodes (SNC) (41, 64). *Fusarium viguliforme* survival is thought to occur in the form of chlamydospores in plant debris and soil, since the pathogen has been isolated from sloughed cortical roots tissue (43, 62). In addition, there is evidence that sporulation on plants may be greater in a no-tillage field due to decayed roots that persist after harvest to the next growing season (62). *Fusarium virguliforme* has also been isolated from SCN cysts (it’s the egg-filled body of death female) (43), and it is thought that survival of the fungus is greater inside the cysts than in free soil due to the lack of competition in the cyst (41, 42). In a study using artificial inoculation, *F. virguliforme* was shown to survive as chlamydospores or mycelia externally
adhering to a soybean seed coat for six months (5), but the study was not extended to the field. The inoculum density in soil and roots is dynamic. In a study by Lou et al. (38), pathogen populations reached the highest density on roots (about 1,500 CFU/g of root) at the end of the growing season, the pathogen maintained a moderate density from October through December, and then decreased, with a lowest density throughout the winter. Then in early spring, pathogen populations increased again. Rupe and Becton (66) also found that inoculum density in the soil increased at the time of harvest of the soybean, probably due to highest sporulation in sloughed root tissues. According to Rupe et al. (72), vertical distribution of inoculum was highest in the top 15 cm of soil layer, reaching densities of $1.6 \times 10^3$ and $2.8 \times 10^3$ CFU per cm$^3$ of soil at planting and harvest, respectively. Under a controlled environment, inoculum densities of $10^3$ to $10^4$ per cm$^3$ of plant growth media (52) have been reported to cause high levels of disease severity.

**Pathogen dispersal.** *Fusarium virguliforme* can be dispersed by different mechanisms. Macroconidia can be rain-splashed, dispersed by water runoff, or dispersed by air during soybean harvesting (62), potentially resulting in the external infestation of seeds that can contribute to dissemination (5, 43). In addition, the pathogen can be dispersed in the SCN cysts by soil movement with water or air (41, 42). Luo et al. (38) determined that short-distance dispersal may also occur from root to root on neighboring plants or by growth of mycelia in the soil bulk.

**Interactions with soybean cyst nematode (SCN)**

The relationship between *Heterodera glycines* and *F. virguliforme* has been investigated for more than two decades, but the interaction is still not clearly understood (11,
Foliar symptoms of SDS were shown to appear earlier and be more severe when plants were inoculated with *F. virguliforme* and *H. glycines* than with the fungus alone (42). In addition, Xing and Westphal (92) determined that the presence of both of the SCN and *F. virguliforme* increased the root rot and foliar severity. In contrast, Gao et al. (11) concluded that the interaction between *F. virguliforme* and *H. glycines* was seldom significant and did not increase the severity of SDS, even though both pathogens reduced plant growth. Correlations between SCN and *F. virguliforme* have been inconsistent, with some studies showing no correlation between SCN densities and SDS foliar AUDPC (18) and others indicating a consistent association between *F. virguliforme* and *H. glycines* population (77). *Fusarium virguliforme* can affect population density and life-stage development of *H. glycines* (41) and has been isolated from cysts as well as colonized epidermal and cortical cells adjacent to developing syncytia.

**SDS Management**

There are several practices recommended for SDS management but their effectiveness is limited and dependent on factors, such as environment, soil type, cultural practices, and the region where the crop is planted. Information released in some extensions programs (82, 83, 94) agree that while there is no fully effective management practice, some practices are more effective than others.

**Resistant cultivars.** The use of resistant cultivars is the best approach to manage SDS. Thus, good variety selection is critical, since a high yielding variety could compensate for the impact of SDS (58). However, this management strategy faces some challenges, since resistance to SDS is polygenic (21, 37) and there are no immune genotypes to SDS.
Examples of field resistant cultivars have included Forrest, Jack, and Ripley, (53), but more plant introductions need to be explored as sources of resistance (15, 22, 25, 69) since commercial options available to growers are limited (87), especially in the northern states. In addition, results from cultivar screenings done in greenhouse conditions are not always consistent with field screenings (52), leading to difficulties in developing resistant cultivars.

The use of molecular techniques for cultivar screening is promising. Iqbal et al. (25), suggested that QTLs for SDS resistance serve to delay symptoms or confer resistance by maintaining or increasing the expression of specific genes after infection. They also determined that the responses of known plant defense genes, signal recognition and transduction, and metabolic processes, were different in partially resistant and partially susceptible soybean roots, and proposed that these responses could potentially be used to assay resistance to the disease (24). In addition, Njiti et al. (51) determined that the soybean plants have two separate QTLs that confer resistance to foliar severity and root severity, and this may suggest that cultivar screening for resistance not only should rely on the foliar phase, but also on the root phase. So far, twelve resistance loci for SDS have been found on 8 chromosomes (37).

**Planting date.** Recommendations for delayed planting have been used for SDS management based on evidence that cool and wet soil favors SDS (76), and that early planting can increase SDS severity. For example, studies in Kentucky (18) and Missouri (89), showed that mid-May plantings resulted in higher SDS than plantings in mid-June through early July. Reports support that cool temperatures (15°C) and wet soils (76, 77) at the beginning of the growing season that may favor infection and increase root rot caused by *F. virguliforme*. However, although delayed planting can reduce levels of SDS, it does not
always prevent its occurrence later in the season (67), and late planting may reduce yield as result of the shorter growing season, especially in early maturity zones of the US. For these reasons, delayed planting is not usually recommended.

**SCN management.** Soybean cyst nematode management is important to manage SDS because the interaction of the two pathogens may potentially increase SDS severity, and because SCN is widespread and can be responsible alone for reduced yields (88). Proper monitoring of soil infestation levels is a first step to manage SCN (23). Current extension recommendations for managing SCN involve the use of resistant varieties as well as crop rotation with non-host crops. Other cultural practices may include maintaining adequate soil fertility, breaking hardpans, irrigating, and controlling weeds, diseases, and insects to improve soybean plant health (17, 49). Due to the interaction with SDS, dual management has been proposed mainly based on the selection of resistant cultivars. For example, one study found that a total of 93 out of 535 lines had genomic regions that underlie resistance to both SDS and SCN in Essex x Forrest soybean populations, and alleles conferring resistance to SDS and SCN in Essex x Forrest are transferable to other populations (60).

**Crop rotation.** Although crop rotation is recommended for management of several soilborne pathogens, it does not consistently appear to be an effective measure to manage SDS. Rupe et al. (71) showed that rotation with crops other than soybean, such as sorghum (*Sorghum bicolor*), fescue (*Festuca arundinacea*), and wheat (*Triticum aestivum*), significantly reduced the *F. virguliforme* and *H. glycines* population densities, especially rotation with sorghum and wheat. However, other researchers reported that even many years of rotation with corn does not reduce the risk of SDS (93). Interestingly, Xing and Westphal (91) found that year-to-year rotation of corn and soybean was not considered effective in
reducing SDS, but monoculture of soybean resulted in some disease suppression possibly because of soil microbial suppressiveness. A strong argument against crop rotation as a feasible method to control SDS, is that *F. virguliforme* has been reported to have several plant hosts (30) that do not necessary show foliar symptoms, but whose root colonization can cause deleterious effects on the plant, and serve to maintain or even increase the population of the fungus.

**Tillage.** Tillage practices also impact SDS development. Wrather et al. (89) found that the percentage of leaves with SDS symptoms in some cultivars were greater for no-tillage than for either disked-tillage or ridge-tillage in mid-May plantings. Vick et al. (84) found that subsoil tillage dramatically reduced SDS foliar symptoms and hypothesized that the decrease in soil compaction would increase soil porosity and provide a more aerated root zone that hinders root infection and decreases SDS symptoms and probably improve traits such as plant vigor and yield (16). Soil compaction also increases loss of carbon by the living root system, reduces growth rate, affects the ability of the roots to obtain water and nutrients, and causes a lack of oxygen supply to the roots (1, 31).

**Seed treatment.** Since *F. virguliforme* is not a seed-borne pathogen, seed treatments have not been used as a control measure for the disease (78). However, several studies on seed treatment are being conducted at several research institutes and industry. In a controlled environment study in 2009 (57), seed treatment consistently reduced SDS severity by 0.50 (on a scale of 1 to 9), and all seed treatments reduced the root health index compared to the control. Testen and Malvick (80) also showed that soybean seed treatment reduced SDS severity by using a commercial fungicide treatment.
Research justification

Sudden death syndrome (SDS) has been among the top ten (ranking between second and fifth place) diseases that causes yield losses on soybean in the US, and has spread to different parts of the American continent (3, 4, 88). Current management of the SDS mainly relies on the use of resistant cultivars (15, 22, 69), but availability of these cultivars is limited in the northern regions of the US, including Iowa. Although, alternative disease management approached exist, such as crop rotation (71, 91), tillage, planting time (84, 89), and SCN management (60), those practices have not been highly effective in the control of the disease.

One of the major challenges that has been faced for about four decades with the development of effective management of SDS and with studies on the biology and epidemiology of this disease (64) has been the lack of understanding of the factors that control symptom expression. In particular, the relationship between root rot and foliar severity phase of the disease is poorly understood, since severity of root rot is not always related with severity of foliar severity (14, 35, 39, 40, 56, 76). In addition, the disease is highly dependent on environmental conditions making the disease very variable and unpredictable. This creates a challenge with the interpretation and repeatability of resistance screening assays and tests on the effectiveness of disease management practices. Thus, the study of symptom expression, the pathogen, host and environmental factors that influence the variability of the disease, may reveal important epidemiological insights to clarify the relationship between root and foliar symptoms and improve development of effective management tools for SDS.

Most of the research of SDS has focused on the foliar phase of the disease. A better understanding of the relationship between the root and foliar phases may explain some of the
causes of variability in SDS development and clarify the reason for the lack of relationship between foliar and root symptom severity in previous research. We hypothesize that a predictive relationship between foliar and root disease assessments would be found if epidemiological disease variables such as incubation period, disease progress rates, and area under disease progress curve (AUDPC), were compared on plants exposed to different conditions, such as different levels of inoculum.

Most of the literature concerning the time of appearance of foliar symptoms of the disease agrees that under field conditions foliar symptoms typically occur at flowering or after flowering (64, 67, 69). However, foliar symptoms can also appear at vegetative stages in both field and controlled environments (47, 65, 76). It is also known that roots can get infected at young stages of growth, and it is not known why there is a lag between the development of root rot and expression of foliar symptoms, or if infections can occur throughout the growing season. In addition there is a lack of knowledge about the period of susceptibility of the soybean plant to root infection and foliar symptoms. The fact that greenhouse screening protocols rely either on the first foliar disease phase at vegetative stage, (65) while field screenings are based on the second phase of the disease at reproductive stage (69), raises important questions about the meaning of results from each type of screening. Perhaps both periods of symptom expression should be taken into account for cultivar screening for resistance. Accurate information on the period of root susceptibility that can leads to foliar symptoms and on the factors that influence that period is vital not only in evaluating resistant cultivar screening, but also for understanding the epidemiology of the disease.
Some studies have focused on the effect of some environmental factors on SDS (75-77). So far, those studies agree that cool and wet soil favor the disease. In fact, delaying planting has been recommended as a management option based on avoiding cool and wet soils at the beginning of the growing season (89). The period of susceptibility of roots to infection conducive to foliar symptoms is not well understood and undoubtedly can be influenced by environmental factors (67). It is clear that soil temperature can influence soybean root traits such as root growth (79), and there are reports on the interaction of root growth and disease severity, such as the cotton-\textit{Thielaviopsis basicola} pathosystem, where decreasing temperature resulted in decreasing root growth and thus increasing disease severity (85, 86). Currently there is no information on the effect of soybean plant age on the progress of root and foliar symptoms and how the period of susceptibility to root infection conducive to foliar symptoms can be dynamically changed by different soil temperatures.

**Research objectives**

Based on the above knowledge gaps, the objectives of this research were:

1. To characterize the temporal dynamics of root and foliar symptoms of soybean sudden death syndrome.

2. To determine if plant age at time of inoculation affects the ability of \textit{F. virguliforme} to infect roots and cause SDS foliar symptoms.

3. To determine the effect of soil temperature and plant age at inoculation on progress SDS root rot and foliar severity.
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CHAPTER 2.
TEMPORAL DYNAMICS OF ROOT AND FOLIAR SYMPTOMS OF SOYBEAN SUDDEN DEATH SYNDROME

ABSTRACT
Temporal dynamics of soybean sudden death syndrome (SDS) root and foliar symptoms were studied in growth chamber experiments on susceptible plants exposed to different inoculum densities (0, 1, $10^1$, $10^2$, and $10^3$ conidia/g soil) of *Fusarium virguliforme*. The monomolecular model provided the best fit to describe the rate of foliar and root disease development over time. Disease severity and area under disease progress curve (AUDPC) increased in response to increasing inoculum density ($P < 0.01$), particularly for foliar symptoms. Root rot severity evaluated 15 to 30 days after inoculation (DAI) were most highly correlated ($r > 0.8$, $P < 0.01$) with foliar disease at 40 and 50 DAI. Rate of disease progress generally increased as inoculum densities increased for both root and foliar symptoms. The incubation period for root and foliar symptoms ranged from 9 to 20 and 15 to 30 days, respectively. In our study, differences in root rot severity were more easily detected in early stages of infection, while root rot and foliar symptoms were weakly correlated when both were assessed simultaneously. Root biomass was reduced by up to 80% at the three highest inoculum densities, suggesting that the root rot phase of the disease may contribute to yield losses caused by SDS.

INTRODUCTION
Sudden death syndrome (SDS) of soybean [*Glycine max* (L) Merr.] is a root rot and leaf scorch disease caused by *Fusarium virguliforme* (Aoki, O'Donnell, Homma & Lattanzi)
(formerly *Fusarium solani* f. sp. *glycines*) (1, 2). Currently, SDS ranks from second to fifth among the top ten diseases that suppress soybean yield in the US Midwestern states, including Iowa (39). Toxins produced by the pathogen in the roots are translocated to the above-ground plant parts causing the typical SDS leaf scorch symptoms of the disease (32, 33). The relationship between severity of foliar symptoms and root rot severity, however, is not well understood (11, 12, 17, 21, 22, 30). Most studies and screening protocols have focused primarily on assessing the foliar phase of the disease (12, 27, 34), whereas relatively little is known about the temporal dynamic of the root rot phase (11). Several researchers (10-12, 33) have reported that plants may have severe root rot but mild or no symptoms on leaves, suggesting that foliar symptoms alone are a poor indicator of root colonization by the pathogen and cannot be used to predict yield losses caused by the root rot phase.

Within-field variability in SDS hampers the development of resistant soybean cultivars because it is difficult to achieve consistent disease development in screening trials (11, 12, 38). One factor that may be responsible for the high degree of variability in SDS symptom expression is the variation in inoculum density within and among field plots (23, 25, 27). Generally, increasing inoculum density in soil results in an increase in SDS foliar severity, but the effect on root rot severity has not been clearly defined (9, 11, 27). For example, in Iowa, a positive correlation was reported between foliar disease severity and population density of *F. virguliforme* in field soil (36). In contrast, Killebrew et al. (15) found no relationship between inoculum density of *F. virguliforme* and disease severity in the field in Mississippi. No attempt was made in either of the two studies to quantify association between root rot and foliar severities over time. Additionally, limited information is available on the effect of SDS on root growth.
There is a need to investigate the causes of the variability in SDS symptom severity in roots in order to clarify the epidemiology of this disease, improve screening protocols for disease resistance, and evaluate the effectiveness of disease management practices (11, 13, 22, 35). The objectives of this study were to: i) characterize and compare the dynamics of root and foliar disease development under different inoculum densities, ii) determine the optimum sampling time to assess root rot for severity and to relate with final foliar severity, and iii) assess the impact of root rot severity on root biomass.

MATERIAL AND METHODS

Plant material and inoculation. Soybean cultivar AG2403 (Monsanto Co., St. Louis, MO), which is susceptible to SDS, was used in all experiments. Seeds were surface disinfested in 0.5% sodium hypochlorite for 2 minutes, followed by rinsing twice in sterile deionized water (SDW). The seeds were then placed on moist sterile germination paper in a tray and incubated at approximately 24°C for 5 days in the dark.

Inoculum preparation. A single-spore isolate of *F. virguliforme* (LL0009) was used in all experiments. This isolate was obtained in Nevada, IA in 2006, from roots of plants exhibiting SDS symptoms. The isolate was cultured on carnation leaf agar and stored periodically on potato dextrose agar (PDA) (Difco Industries, Detroit, MI). Before inoculation, cultures of the isolate were grown on PDA for 11 to12 days at room temperature (24 ± 2°C) in darkness. Spore suspensions for inoculation were prepared by flooding the cultures with SDW dislodging conidia with a rubber policeman, filtering through three layers of sterile cheese cloth, and then adding SDW to obtain a final volume of 250 ml. The spore
concentrations were counted using a hematocytometer and adjusted to four inoculum concentrations: $10^1$, $10^2$, $10^3$ and $10^4$ conidia/ ml. Sterile water was used as a control.

**Growth chamber experiment.** A two-way factorial, randomized complete block design was used, with five blocks, five inoculum density treatments, and six (destructive) sampling times. Pots assigned to each inoculum density and sampling time combination were completely randomized within each block. An experimental unit consisted of a pot containing three plants, and there were five replicate pots (one per block) per inoculum density treatment for each sampling time. The experiment was repeated once. Germinated seedlings were grown in a mixture of pasteurized soil and sand (1:1 vol:vol) amended with 1.5% (wt/wt) sterilized cornmeal as a source of organic matter to mimic corn residue in the field (5). The soil mixture plus cornmeal was infested with the *F. virguliforme* conidial suspensions (100 ml/kg soil) or water in order to obtain the following five initial inoculum densities: 0, $10^0$, $10^1$, $10^2$, and $10^3$ conidia/g soil. The total volume of soil for each inoculum density treatment was thoroughly mixed by hand and immediately used to fill 12-cm-diameter plastic pots. Three 5-day-old pre-germinated seedlings were then transplanted into each pot at a depth of 1 cm. Potted plants were incubated in a growth chamber at 17°C for 7 days to provide optimum conditions for root infection, followed by 24°C for 43 days in the same growth chamber to promote foliar symptoms (35). The duration of the experiment was 50 days after inoculation (DAI), at which time the plants were at the V6 growth stage (8). The photoperiod was 14 h light/10 h dark using a combination of incandescent and fluorescent lights. Each pot was watered daily with approximately 80 ml of tap water.

**Disease assessments.** Severity of foliar and root rot symptoms were assessed visually at the following six destructive sampling times: 9, 15, 20, 30, 40 and 50 DAI. The severity of
foliar symptoms was estimated visually as the percentage of the total leaf area exhibiting chlorosis and necrosis relative to the size of the leaf area assessed. After roots were washed under running tap water, root rot severity was assessed visually as the percentage of root area showing brown or black discoloration relative to the total root area. Roots sampled at 20, 30, 40, and 50 DAI were dried at 89°C for 24 h and weighed to determine dry weight.

**Pathogen isolation and identification.** To quantify the isolation frequency of *F. virguliforme* from roots, two 1-cm-long root pieces were arbitrarily selected from each of the three soybean plants per pot, one piece from necrotic tissue and one from apparently healthy root tissue at each of the five sampling time. The root pieces were then plated onto 9-cm-diam petri dishes containing modified Nash and Snyder’s medium, which is selective for *F. virguliforme* (7). Cultures were incubated in the dark at 24 ± 2°C for 7 to 10 days. The number of root pieces with characteristic blue, purple or white colonies of *F. virguliforme* was counted 5 to 10 days after incubation. Fungal cultures were observed under the microscope for confirmation of *F. virguliforme* identity based on the presence and morphology of the macroconidia.

Polymerase chain reaction (PCR) was also used to confirm the presence of the pathogen in the roots at 30 DAI using specific primers Fsg-1 and Fsg-2 (16, 18). One colony arbitrary sampled from each of five replicate plates per inoculum density was tested. DNA was extracted from cultures using PrepmanUltra (Applied Biosystem, Foster City, CA) buffer, following the manufacturer’s instructions. PCR mixes were carried out in a total volume of 25µl containing 2 µl of genomic DNA, 0.04 µM of each primer, 2.5 mM of dNTP, 2.5 mM of MgCl₂, 1X of DMSO, and 1.25 U of DNA *Taq* Polymerase (Econotaq, Lucigen Corp., Middleton, WI). Cycling parameters were at 94 °C for 5 min for denaturation. Cycles
of 30 s at 95 °C for 5 min, 65 °C for 1 min, and 72 °C for 1.5 min, with a final extension period of 10 min at 72 °C were performed with a Techne thermal cycler (Techne Inc., Burlington, NJ). PCR amplicons were resolved in 1 % agarose gels and visualized after staining with ethidium bromide.

**Data analysis.** Due to an interaction between experiment and inoculum density ($P<0.01$), the two experiments were analyzed separately. The area under disease progress curve (AUDPC) was calculated for each inoculum density by using the trapezoidal integration method (6). Analysis of variance was performed using the PROC GLM procedure of SAS version 9.1 (SAS Institute, Cary, NC) on the AUDPC for root rot and foliar severity. The Tukey-Kramer test was used (alpha=0.05) to detect differences in means for root rot and foliar severity AUDPC. Pearson correlation coefficients were calculated to test the strength of the association between rot root and foliar severity within and among sampling times. These associations were presented in interpolation plots (37) constructed using Surfer 6.4 software (Golden Software Inc., Golden, CO).

To characterize and compare the dynamics of root and foliar disease development as affected by different inoculum densities, the monomolecular population growth curve model (\(\ln[1/(1 – y)] = \ln[1/(1 – y_0)] + rMt\) (6) was fitted to the temporal progress curves of root and foliar severity, since this model provided the best fit compared to other population growth models (29) for this monocyclic disease. Temporal data curves were modeled from the time disease symptoms were first detected until 40 DAI for all inoculum levels except the highest, where disease progress was modeled until 30 DAI since disease severity decreased at 40 DAI due to the production of new apparently-healthy root and leaf tissue. Model fit was evaluated according to the following criteria: the significance statistic ($P$ value) of the overall model,
the coefficient of determination ($R^2$), standard error for the estimate for Y ($\text{SEE}_y$), and the normality of the residuals (assessed with the Shapiro-Wilk test). Regression analysis was conducted on linear transformations of the data using the monomolecular model. Mean comparison of linear monomolecular rates of each inoculum density treatment were conducted using the Tukey-Kramer test (alpha=0.05). For root dry weight, linear regressions were performed to quantify the relationships between root dry weight and sampling time (from 20 to 50 DAI) to obtain rates of dry weight loss of each inoculum density treatment. Root biomass loss was estimated as a percentage of root dry weight relative to non-inoculated plants.

**RESULTS**

**Root rot and foliar disease severity.** Root rot and foliar disease became more severe with increasing inoculum density, but the effect of inoculum density on disease severity was more pronounced on leaves than on roots (Fig. 1). The greatest differences in disease severity among inoculum densities were observed at 15 DAI on roots and 40 DAI for leaves. A decrease in severity of both root rot and foliar symptoms was observed between 30 and 40 DAI for the highest inoculum density, due to the production of new, apparently healthy leaves and roots (Fig. 1). Root rot severity and isolation frequency of *F. virguliforme* from roots had similar progress curves (Fig. 1). No disease developed on non-inoculated control plants (Fig. 4).

**Relationship among root rot and foliar disease severity.** Correlation among root rot severity and foliar disease severity varied greatly depending upon the time of assessment (Fig. 2). Correlation coefficient ($r$) and corresponding $P$ values are presented in 2D plots (37), where
interpolation was used to estimate correlation statistics for unsampled times. Root rot assessments conducted at 15, 20 and 30 DAI were significantly ($P < 0.05$) correlated ($r \geq 0.8$) with final foliar severity at 40 and 50 DAI in both experiments (Fig. 2), with the strongest correlations ($P < 0.01$) observed when roots were assessed between 20 and 30 DAI as shown in the darkest areas of the plots.

**Analysis of disease progress curves.** The coefficient of determination ($R^2$) for the fit of the monomolecular model to the root rot progress curves ranged from 79.0 to 98.9% and from 81.4 and 95.1%, for experiments one and two, respectively. For progress of foliar severity, the $R^2$ ranged from 81.1 to 99.7% and 98.2 to 99.5%, for experiments one and two, respectively (Table 1).

Analysis of residuals based on the Shapiro-Wilk test indicated normality, supporting the validity of the model except for three of the curves in experiment two. As inoculum density increased, disease progress rates increased for root and foliar symptoms up to 40 DAI (Table 1). The monomolecular rate for root rot severity ranged from 0.06 to 0.11 ln(1/(1-y))/day units (5.8 to 10.4% severity /day) and from 0.04 and 0.11ln(1/(1-y))/day units (3.9 to 10.4% severity/day) for experiments one and two, respectively. The rate of root rot progress was lowest ($P < 0.05$) for the lowest inoculum density ($10^6$ conidia/g soil), but did not differ significantly among the other inoculum density treatments. In contrast, the rates for foliar severity tended to increase ($P < 0.05$) with an increase in inoculum density. For foliar severity, the rate of disease progress ranged from 0.03 to 0.16 ln(1/(1-y))/day units (2.9 to 14.7% severity/day) and from 0.009 to 0.19 ln(1/(1-y))/day units (0.9 to 17.3% severity/day) for experiments one and two, respectively
The area under the disease progress curve (AUDPC) increased as inoculum density increased and was generally greater for root rot than for foliar disease severity (Table 2). Root rot AUDPC did not differ among the three highest inoculum density treatments, while foliar severity AUDPC significantly increased with inoculum density. Incubation periods were shorter for root rot severity (9 to 20 days) than for foliar severity (15 to 30 days), and were shortest at highest inoculum densities (Table 2).

**Root biomass.** Root dry weight (g) decreased over time in proportion to inoculum density. Linear regression between time and dry weight showed that rate of dry weight accumulation in non-inoculated plants was 0.033 g/day and 0.026 g/day, for experiments one and two respectively, while the rates of root dry weight accumulation over time in inoculated plants were from 46 to 94% and from 24 to 69% lower, respectively (Table 3). At the final assessment time, root biomass of all inoculated plants, was lower than the control. For the lowest inoculum density, root weight was 40% and 23% lower than controls in experiment 1 and 2, respectively, while at the three highest inoculum densities, losses in root weight ranged from 40 to 80% (Fig. 3).

**DISCUSSION**

In this study, early assessments of root rot severity caused by *F. virguliforme* were more highly correlated with assessments of SDS foliar severity than those performed in later phases of root rot development. Specifically, we demonstrated that root rot severity assessed from 15 to 30 days after inoculation is strongly correlated with foliar severity evaluated four to six weeks later. However, root rot and foliar severity were not strongly correlated if disease assessments were conducted simultaneously at the end of the experimental period.
This finding suggests that physiological processes related to the infection of young roots may be associated with severity of foliar symptoms that develop later, possibly due to the ability of the fungus to colonize root vascular tissues (22).

Our results agree with earlier studies that have reported weak correlations between root and foliar severity when both were assessed simultaneously at later stages of disease development (11, 17, 21, 22, 26, 35). For example, Luo et al. (22) found that assessments using a foliar disease index was not significantly correlated with the rate of root rot disease parameters. In addition, Njiti et al. (28) found a poor association between foliar disease index and infection frequency of the pathogen in two environments and reported that resistant soybean cultivars had reduced foliar severity, even though infection frequency and severity on roots were high. Gray and Achenbach (11) did not find clear differences in root rot severity between resistant and susceptible soybean cultivars under different inoculum densities. Since we used a single susceptible variety in our study, we cannot conclude if soybean genotypes with different levels of root resistance would have shown different correlations between root and foliar symptoms. However, our results suggest that the inconsistent association between root and foliar disease severity reported previously may have resulted from using evaluation methods that relied on single assessments of root rot severity at the end of experiments (11, 35) when differences in root rot may not have been as evident. In contrast, in other diseases, such as root rot of chickpea (caused by F. solani f. sp pisi), increasing inoculum density caused a linear increase in root rot severity, resulting in detectable and significant differences in root rot severity when assessed 30 days after emergence (4). In our study, the incubation period for root rot and foliar severity became shorter as inoculum density increased, causing an increase in AUDPC values for both root rot
and foliar disease severity. Similarly, in the cotton-*Phymatotrichum* pathosystem, a delay in the sloughing process of the root cortex was shown to reduce and delay foliar symptoms (14).

The fact that in our study plants with similar levels of severe root rot exhibited a range of foliar severity levels may be a consequence of the etiological processes of *F. virguliforme*. Sudden death syndrome foliar symptoms are caused by pathogen toxin(s) that are translocated in the vascular system from the roots to leaves (31, 32), but effective movement of the toxin to the leaves requires colonization of the xylem. Navi and Yang (26) found that roots of resistant cultivars were colonized in the cortex only, while roots of susceptible cultivars were colonized by the fungus in both the cortex and xylem. It is therefore possible that roots may show severe root rot, but that pathogen colonization is limited mostly to the cortex, thereby preventing or restricting toxin movement to the leaves via the xylem. The fact that the frequency of isolation of *F. virguliforme* from roots was similar among inoculum density treatments, at assessment times when foliar symptoms differed among treatments, is an indication that overall colonization of the roots may not be indicative of the amount of fungus in the xylem. Therefore, care must be taken when interpreting the significance of root rot severity or isolation frequency of *F. virguliforme* evaluations as a measure of soybean resistance to SDS (11, 26).

The best fit of the monomolecular model to the root and foliar disease progress curves in our study is consistent with monocyclic diseases where rate of disease progress is fastest during the early stages of the epidemic and then progressively slows due to a decrease in the amount of apparently healthy or disease-free plant tissue (6, 29). This model has been successfully applied to describe rate of disease intensity of chick pea wilt caused by *F. oxysporum* f. sp. *ciceris* (23, 24). In our study, root and foliar symptoms decreased between
30 and 50 DAI at the highest inoculum density (10^3 conidia/g of soil), due to the production of new apparently healthy tissues.

Infection by *F. virguliforme* caused up to 80% loss in root biomass in our study, indicating that this fungus is an aggressive root pathogen, as has been reported for many other *Fusarium* species (3, 19). Other studies (9, 10, 17) have also reported that high densities of *F. virguliforme* can reduce root and shoot dry weight on soybeans, but this is the first study to measure the impact of *F. virguliforme* on root growth over time. We observed that roots almost stopped growing in plants exposed to an initial density of 10 or more conidia per gram of soil, but continued to grow at a constant rate in plants exposed to an initial density of one conidium per gram of soil.

In summary, we used inoculum density as a tool to study the epidemiology of the temporal dynamics of SDS root and foliar severity during vegetative plant development. Inoculum density was shown to affect the monomolecular rate of root rot severity and foliar disease severity and the final level of disease severity, but the impact on root rot severity was only detectable in early stages of the epidemic. Therefore, we suggest that in greenhouse assays in which plants are evaluated during the vegetative growth stages, root rot assessments performed earlier than foliar disease assessment may be more informative than those conducted when the pathogen has already extensively colonized the roots. The fact that root biomass was greatly reduced by *F. virguliforme* in our experiments may suggests that there is a major potential for yield loss to occur due to the root rot phase of the SDS, even if foliar symptoms are absent. However, our data does not support the relationship of root rot and yield loss. However, additional work with other soybean cultivars and in field trials is needed to verify the presence of similar associations between early season root rot assessments and foliar assessments performed.
later in the growing season. However, due to the aggregated spatial pattern of *F. virguliforme* in field soils (20), such association may best be studied in greenhouse or growth chamber environments, in which inoculum densities can be more reliably regulated.

**ACKNOWLEDGEMENTS**

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TABLE 1. Summary of regression analysis parameters and statistics for linear transformations of data according to the monomolecular population growth curve model ([ln (1/1-y)] fitted to describe the progress of root rot severity (%) and foliar disease severity (%) of sudden death syndrome caused by *Fusarium virguliforme* on soybean (cv. AG2403).

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5 $P$ values and LSD correspond to mean comparison of the slopes of the model by using Tukey-Kramer test (alpha=0.05).

7 Statistical significance of the analysis of normality of the residuals based on the Shapiro-Wilk analysis. Each experiment was based on 15 individual plants per sampling time and inoculum density treatment. For the $10^3$ inoculum density treatment, the temporal analysis was done up to 30 DAI.
TABLE 2. Area under disease progress curve and incubation period for root and foliar severity of soybean plants (cv. AG2403) inoculated with five inoculum densities of *Fusarium virguliforme*, causal agent of sudden death syndrome.

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<td>Incubation Period (days)</td>
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<td>9</td>
<td>2650.3 a</td>
<td>15</td>
</tr>
<tr>
<td>LSD</td>
<td>762.4 ab</td>
<td>-</td>
<td>741.5</td>
<td>-</td>
</tr>
<tr>
<td>$P$ value</td>
<td>&lt;0.0001</td>
<td>-</td>
<td>&lt;0.0001</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 3. Parameter estimates and statistics of the linear regression model accounting for the effect of five inoculums densities of *Fusarium virguliforme* on root dry weight (g) over time of soybean (cv. AG2403).

<table>
<thead>
<tr>
<th>Inoculum density (Conidia/g soil)</th>
<th>Intercept</th>
<th>Slope</th>
<th>$R^2$</th>
<th>P value</th>
<th>$SEy^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.005</td>
<td>0.017</td>
<td>0.88</td>
<td>0.059</td>
<td>0.004</td>
</tr>
<tr>
<td>$10^0$</td>
<td>0.185</td>
<td>0.003</td>
<td>0.31</td>
<td>0.442</td>
<td>0.003</td>
</tr>
<tr>
<td>$10^1$</td>
<td>0.449</td>
<td>0.001</td>
<td>0.13</td>
<td>0.634</td>
<td>0.003</td>
</tr>
<tr>
<td>$10^2$</td>
<td>0.335</td>
<td>0.002</td>
<td>0.15</td>
<td>0.602</td>
<td>0.004</td>
</tr>
<tr>
<td>$10^3$</td>
<td>-0.075</td>
<td>0.033</td>
<td>0.99</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

$^z$Standard error of the estimate of Y.
**Fig. 1.** Disease progress curves for sudden death syndrome root rot severity, foliar severity and isolation frequency (%) of *Fusarium virguliforme* from soybean roots (cv. AG2403) inoculated with five different conidial densities, for experiment one (A, B and C) and experiment two (D, E and F). Plants were incubated in a growth chamber at 17 °C for 7 days, followed by 42 days at 25°C. (n=15).

**Fig. 2.** Pearson correlation coefficients ($r$) and statistical significance ($P$) for the associations between root rot severity and foliar disease severity of sudden death syndrome on soybeans (cv. AG2403) assessed at six sampling times (9, 15, 20, 30, 40, and 50 days after inoculation); (A and B) for experiment one and (C and D) experiment two. Darker shades of gray correspond to higher $r$ and $P$ value in the top and bottom graphs, respectively.

**Fig. 3.** Effect of five conidial densities of *Fusarium virguliforme* on root dry weight (g) over time of soybean plants (cv. AG2403) for (A) experiment one and (B) experiment two. (n=15).

**Fig. 4.** Soybean plants (cv. AG2403) showing root rot and foliar symptoms 30 days after inoculation with five conidial densities of *Fusarium virguliforme*. Plants show similar levels of root rot severity and different levels of foliar severity.
Fig. 1.
Fig. 3.

(A) Root dry weight (g) against days after inoculation with different levels of conidia/g of soil. The levels are indicated by different symbols: 0, 10^0, 10^1, 10^2, and 10^3.

(B) Root dry weight (g) against days after inoculation with different levels of conidia/g of soil. The levels are indicated by different symbols: 0, 10^0, 10^1, 10^2, and 10^3.
Fig. 4.
CHAPTER 3.

PLANT AGE AFFECTS ROOT INFECTION AND DEVELOPMENT OF FOLIAR SYMPTOMS OF SOYBEAN SUDDEN DEATH SYNDROME

ABSTRACT

Soybean sudden death syndrome is characterized by root rot, followed by the development of foliar symptoms caused by pathogen toxins. The timing of root infections conducive to foliar symptoms is unclear. Soybeans grown in pots were inoculated at plant ages ranging from 0 to 35 days after planting (DAP) by drenching soil with conidia of *Fusarium virguliforme*. Plants were incubated in growth chambers at 17°C for 7 days, followed by 24°C for 31 days. Root rot and foliar severity were assessed at 18 and 38 days after inoculation (DAI). Root rot developed on plants inoculated at all ages, but plants inoculated 0 DAP had the highest ($P < 0.01$) root rot severity compared to older plants. Foliar symptoms developed on plants inoculated 0 DAP but never developed on plants inoculated at all other ages. Fungal colonization of the xylem was more frequent in plants inoculated 0 DAP than on plants inoculated at later stages. This study revealed that soybean roots are susceptible to root infection at different vegetative growth stages but that plants become less susceptible to xylem colonization and development of foliar symptoms as they mature. Management practices aimed at protecting seedling roots from infection should be investigated.
INTRODUCCION

Sudden death syndrome (SDS) is an economically important soybean (*Glycine Max* (L.) Merr.) disease caused by *Fusarium virguliforme* (Aoki, O’Donnel, Homma & Lattanzi) (1, 2, 35). The disease can cause yield losses of up to 90% (26). Although SDS is characterized by root rot and foliar intervenial chlorosis and necrosis (26), the foliar symptoms are caused by pathogen toxins that are translocated from the roots to the leaves (13, 14, 17, 26, 27) when the pathogen colonizes the root vascular system (20). Under field conditions, the appearance of SDS foliar symptoms typically occurs at or after flowering (26), but root infections can occur in early stages of root development (25). For example, Gao et al. (7) demonstrated that roots of young soybean seedlings were colonized by *F. virguliforme* within two weeks after emergence in field soil, and Huang and Hartman (11) isolated the fungus within 3 to 10 days after inoculation in controlled environment. The fact that early planting of soybean increases the risk of high severity of SDS (10, 36) suggests that root infections occurring in early growth stages are important to foliar disease severity development later in the season. However, it is not known whether susceptibility varies with growth stages nor how the timing of infection affects disease severity.

The age at which plant roots have contact with inoculum can affect severity of other diseases (8, 16, 31). Kuruppu et al. (16) found that 1-week old plants were more susceptible to red crown rot of soybean caused by *Calonectria illicola*, than older plants. Similarly, soybeans became less susceptible to diaporthe stem canker caused by *Diaporthe phaseolorum* f. sp. *meridionalis* as plant age increased (31), but the opposite was observed with brown stem rot on soybean caused by *Phialophora gregata* (22). For SDS the period of susceptibility for root infections and subsequent foliar symptoms have not been defined, but
such knowledge may provide useful in understanding the etiology of this disease, and contribute to more effective management. Our hypothesis is that soybeans become less susceptible to root infection conducive to SDS foliar symptoms with increasing plant age at time of infection. A clarification of how susceptibility to SDS varies with plant age may have implications for disease management practices such as application of seedling protectants, decisions about planting time, and development of criteria for resistance breeding.

The objective of this work was to determine if plant age at time of inoculation affects the ability of *F. virguliforme* to infect roots and cause SDS foliar symptoms.

**MATERIAL AND METHODS**

**Plant material and inoculum.** Susceptible soybean cultivar AG2403 (Monsanto Co., St. Louis, MO) was used in all experiments. This is a glyphosate tolerant, maturity group II cultivar, with an indeterminate growth habit. An inoculum source was a single-spore isolate of *Fusarium virguliforme* (LL0009) obtained in 2006 from roots of plants with typical SDS foliar symptoms from a field in Nevada, IA. Colonies of *F. virguliforme* were grown on carnation leaf agar and stored by transferring periodically to new potato dextrose agar (PDA) plates. Before inoculation, cultures of the isolate were grown on PDA for 12 days at room temperature (24 ± 2 °C), in darkness. Conidial suspensions were prepared by flooding the cultures with sterile deionized water (SDW) dislodging the conidia with a rubber policeman, then filtering through three layers of sterile cheese cloths, and SDW was used to adjust the density to $6 \times 10^3$ conidia/ml.

**Growth chamber experiment.** The experiment was established as two-way factorial randomized complete block design, with five replications, seven plant age treatments, and
two sampling times. The experimental unit was a pot with three plants, and there were five replicate pots (one per block) for each plant age and sampling time combination. In order to obtain plants of different ages at inoculation, soybean seeds were planted over five week period. Before planting, soybean seeds were surface sterilized by immersion in 0.5% sodium hypochlorite for 2 minutes, and rinsed twice in SDW. Three seeds were planted in 12-cm diameter pots containing 1.1 kg of a pasteurized soil and sand mix (1:1 vol:vol) amended with 1.5% (wt/wt) sterilized cornmeal. The cornmeal was used to serve as a source of organic matter and to facilitate homogenous colonization of the soil by *F. virguliforme*. After planting, the plants were maintained in a growth chamber at a continuous temperature of 24°C and with a 14 h photoperiod using incandescent and fluorescent lights. Plants were watered daily using 150-200 ml water per pot.

Plants were inoculated 0, 4, 7, 14, 21, 28 and 35 days after planting (DAP), corresponding to growth stages ranging from seed to V5, respectively (6) (Table 1). Soybeans were inoculated with *F. virguliforme* by drenching each pot with 130 ml of conidial suspension, resulting in an initial concentration of 780 conidia/g of soil. After inoculation, plants were exposed to 17±2°C for 7 days to induce root infection, followed by 24±2°C for 30 days to induce foliar symptoms. The experiment was repeated once. The 4 DAP plant age treatment was only included in the second repetition of the experiment.

Root rot severity and foliar severity were evaluated at 18 and 38 days after inoculation (DAI). Plant roots were destructively sampled and gently washed in running tap water. Root rot severity was visually rated as the percentage of root area showing brown or black discoloration. Severity of foliar symptoms was visually rated as the percentage of the total leaf area of each plant exhibiting chlorosis and necrosis typical of SDS. Frequency of
root colonization by *F. virguliforme* was calculated by culturing 1-cm-long root pieces on modified Nash and Snyder’s medium (MSNM) (4). Two root pieces were arbitrarily excised from diseased and healthy areas of the tap and lateral roots from each of the three plants per pot. The plates were incubated at 24 ± 2°C in the dark for 7-10 days, and the percentage of root pieces showing blue or purple colonies characteristic of *F. virguliforme* was counted 5 to 10 days after plating (27).

Analysis of variance was performed with the PROC GLM procedure of the SAS software version 9.1 (SAS Institute, Cary, NC). Data of the two experiments were analyzed separately due to a significant interaction between experiment and plant age, and the fact that the 4 DAP plant age treatment that was not included in experiment one. Interaction between evaluation time and plant age at inoculation were evaluated using the “slice” option in SAS. The Tukey-Kramer procedure was used (alpha = 0.05) to test differences in root and foliar severity, as well frequency of root colonization by *F. virguliforme*.

**Microscopy experiment.** An experiment was conducted to compare the infection process of *F. virguliforme* on plants inoculated 0, 4, 7 and 14 DAP. Plants were grown in growth chambers and inoculated as described above. For each plant age and sampling time, six plants were inoculated and three plants were used a non-inoculated controls. Root rot severity and foliar disease severity were evaluated 17 and 27 DAI as described above, and the roots were washed and prepared for microscopic observation. Three 1-cm-long root pieces were arbitrarily excised from healthy and diseased portions from the top, middle and bottom of the tap root of each plant. The 18 root pieces were pooled for each plant age and sampling time combination, and fixed in formalin-acetic acid-alcohol (FAA) from two to four days at 4°C. Ten arbitrarily selected root pieces were then dehydrated in a graded
ethanol series (1 hour each step at 50, 70, 8, 9, and 100%), cleared with xylene (100%), and infiltrated and embedded using paraplast paraffin (Fisher Scientific, Pittsburgh, PA). Sections were cut at 8 µm thickness using an A/O 820 rotary microtome (Fisher Scientific, Pittsburgh, PA). The sections were then mounted onto glass slides, deparaffinized, stained with 1% toluidine blue in 1% borax, dehydrated in graded ethanol solutions and cleared with xylene. Five sections of each of the ten root pieces were observed using a Zeiss AxioPlan II Imaging compound microscope (Carl Zeiss Inc, Thornwood, NY) at a 400X magnification. Presence or absence of *F. virguliforme* in xylem and cortical root tissues was recorded on a binary scale, and the frequency of xylem and cortex colonization was estimated as the proportion of root pieces showing fungal hyphae in each tissue. Digital images of roots were collected using a bright field Zeiss Axiocam MRC microscope camera on a Zeiss AxioPlan II (Carl Zeiss Inc, Thornwood, NY) compound microscope.

Analysis of variance was performed using the PROC GLM procedure of SAS version 9.1 (SAS Institute, Cary, NC). Interaction between sampling time and plant age at inoculation were evaluated using the “slice” option in SAS. The Tukey-Kramer test was used (alpha =0.05) to compare means for root rot and foliar severity, and frequency of xylem and cortex colonization by *F. virguliforme*.

**Field experiment.** A microplot trial was established in 2008 and 2009 at the Iowa State University Hinds Research Farm near Ames, Iowa, to determine if the effect of plant age observed in growth chambers trials also occurred under field conditions. The field plot had been cropped with continuous soybeans for more than five years, and had a history of SDS due to natural and artificial infestations. A completely randomized design was used with four plant age treatments and ten replicate plants per treatment.
Soybean seeds were planted in 8 cm-diameter styrofoam cups containing 280 g of the soil:sand:cornmeal mix described above that have been maintained in growth chamber conditions at 24±2°C. Planting was staggered over a two week period in order to obtain plants 0, 4, 7 and 14 days old at the time of inoculation. On May 24, 2008, and May 4, 2009, plants were removed from the styrofoam cups and transplanted in the field into 10-cm-deep holes, spaced 25 cm between and within rows. Inoculation was done immediately after transplanting by drenching 100 ml of a 5 x 10^4 conidia/ml suspension around the root zone of each plant to achieve an approximate inoculum concentration of 1.8 x 10^4 conidia/g of soil. Plants were watered with a hose as needed to maintain adequate soil moisture throughout the experiment. Severity and incidence of SDS foliar symptoms were evaluated weekly until senescence.

The area under disease progress curve (AUDPC) for foliar severity was calculated for each plant age treatment by using the trapezoidal integration method (3). Analysis of variance was performed using the PROC GLM procedure of SAS version 9.1 (SAS Institute, Cary, NC). To detect differences in foliar severity AUDPC, the Tukey-Kramer procedure was used (alpha = 0.05). Only data from the 2008 trial was analyzed due to lack of disease development in 2009.

**RESULTS**

**Growth chamber experiment.** Plants inoculated at all ages developed root rot, but differences (P < 0.01) in root rot severity were detected among plant age treatments in both experiments (Fig. 1). For the first sampling time (18 DAI), root rot severity was greatest in plants inoculated 0 DAP (>75%), older plant ages ranged from 7.6% to 24.6% in experiment...
1 and from 15.5 % to 40.3% in experiment 2. For the second sampling time (38 DAI), plants inoculated 0 DAP showed severe root rot (>94%), while root rot severity on plants inoculated at 4 DAP or older ranged between 25 to 57 %, and did not differ significantly among plant age treatments (Fig. 1A and D).

Differences in SDS foliar symptoms were significant \( (P < 0.01) \) among plant ages. At both sampling times, only plants inoculated 0 DAP developed foliar symptoms, while plants inoculated 4 DAP or later never developed foliar symptoms throughout the duration of the experiment (Fig. 1B and E). The growth stage of plants evaluated 38 DAI ranged from V2 to R2 (Table 1).

Frequency of \( F. \) virguliforme isolation from soybean roots differed among plant ages at both sampling times \( (P < 0.01) \), except at sampling time of 18 DAI in the first experiment. In both experiments, the pathogen was isolated more frequently from plants inoculated 0 DAP than those inoculated at later ages in the 18 DAI assessment time. At 38 DAI, frequency of pathogen isolation was greater (75 and 77%) from plants inoculated at 0 DAP, than from older plants (15 to 67%) (Fig. 1C and F).

**Microscopy experiment.** The effect of plant age on severity of root rot and foliar symptoms was consistent with the results from the growth chamber experiment described above. Root rot developed on plants of all ages, but was greater \( (P < 0.01) \) on plants inoculated 0 DAP than those inoculated at older ages at 27 DAI sampling time (Fig. 2A and B). In contrast, foliar symptoms were only observed on plants inoculated 0 DAP (Fig. 2C and D).

Hyphae of \( F. \) virguliforme was observed in roots of all inoculated plants, but hyphae were never observed in non-inoculated plants. At 17 DAI, hyphae were observed in the root
cortex of plants inoculated at all ages, with the frequency of hyphae colonization ranging from 70 to 94% (Fig. 3A). Colonization of the xylem was <5% in roots of plants in plants inoculated 4 and 7 DAP (Fig. 3A and 4A), but these values did not differ significantly from zero ($P = 0.53$). At 27 DAI, hyphae formed dense networks in the cortical tissue of plants inoculated at all ages. Frequency of cortex colonization ranged from 82 to 98% and did not differ among plant ages (Fig. 3B and 4B). Hyphae were also observed forming dense and long intercellular and extracellular networks in the xylem of 56.5% of roots pieces from plants inoculated 0 DAP (Fig. 3B and 4C), and on 14% of plants inoculated 14 DAP. However, the frequency of xylem colonization on the 14 DAP treatment did not differ significantly from the other plant ages, where no xylem colonization was observed.

**Field experiment.** In the 2008 trial, progress of SDS foliar severity differed significantly among plants inoculated at different ages. Two phases of foliar symptoms were observed on these plants (Fig. 5). The first phase occurred at vegetative stages V2-V4 (26 to 58 DAI). A remission of foliar symptoms was then observed due to defoliation of symptomatic leaves and production of new leaves. A second phase occurred at reproductive stages R5-R7, starting at 79 DAI. For the first phase of foliar symptoms, plants inoculated 0 DAP showed significantly greater foliar severity AUDPC than all other plant age treatments ($P < 0.0004$), whereas plants inoculated 4 DAP showed low foliar severity AUDPC and those inoculated 7 and 14 DAP did not show foliar symptoms (Table 2). In the second phase, however, plants inoculated at all ages developed foliar symptoms, and no significant differences in foliar severity AUDPC were observed among plant age treatments ($P = 0.14$).

Total foliar severity AUDPC calculated for the complete duration of the experiment was
greatest \((P =0.0004)\) for the 0 DAP plant age treatment and did not differ significantly among the older plant ages (Table 2).

**DISCUSSION**

This is the first study to document that plant age at time of inoculation influences susceptibility to soybean SDS. In both growth chamber and field trials, we found that severity of root rot and foliar symptoms was reduced by increasing plant age at the time of exposure to inoculum. Although root rot developed on plants inoculated at all plant ages, foliar symptoms only developed on plants inoculated at the seed stage (0 DAP) during the 38 day duration of the experiment. These findings indicate that *F. virguliforme* is able to infect and colonize roots of plants at different ages, but that infection may not always result in the development of foliar symptoms.

Plant age is known to affect disease development in other pathosystems. Kuruppu et al. (16) determined that soybean roots were most susceptible to red crown rot caused by *Calonectria ilicicola* during the first week after seedling emergence, and then disease severity dropped by half when plants were inoculated two or more weeks after emergence. Younger celery plants also developed more severe disease when infected by *Fusarium oxysporum* f. sp. *apii* than older plants (8). In contrast, root rot of cereals caused by *Cochliobolus sativus* (33) and brown stem rot of soybean caused *Phialophora gregata* (22), older plants are more susceptible to the disease than plants are earlier growth stages.

In our study, the fact that root rot severity and frequency of *F. virguliforme* isolation from roots was greatest in plants inoculated at seed stage and tended to decrease on progressively older plants at inoculation suggests that soybean roots are most susceptible to
infection at early seedling stages. Several mechanisms could be involved in the decreased susceptibility of older roots. For example, higher suberin content in older sections of roots of soybean has been associated with increased resistance to infection by *Phytophthora sojae*, and this is thought to play a role in partial resistance to this pathogen (23, 32). Suberin can be synthesized within 2 to 6 days after soybean inoculation with *P. sojae* (23), so it is possible that suberin accumulation within this timeframe contribute in the dramatic differences in foliar symptoms that we observed between plants inoculated 0 and 4 days after planting. Flavones and phytoalexins such as glyceollin may also be involved in root resistance to *F. virguliforme* in soybean (12, 18), and their concentration is known to increase within days after inoculation with other pathogens (5).

In our study, plants inoculated 0 DAP showed foliar symptoms, showed the highest frequency of colonization in the xylem compared to plants inoculated at older ages. Although xylem colonization was also sometimes observed in low frequency in plants inoculated at older ages, hyphal colonization was primarily limited to the cortical tissue. The fact that we observed a higher frequency of *F. virguliforme* in the xylem of plants inoculated 0 DAP, compared to plants inoculated at older ages, suggests that the mechanisms involved may restrict colonization of the plant vascular system, thereby limiting toxin movement to the leaves (20). It is also possible that enough fungal biomass must build up in the xylem until the toxin concentration is sufficient to affect the leaves. In addition, the higher susceptibility of plants inoculated 0 DAP may be related to the fact that the root cell wall of seedlings is less lignified than root cell walls of older plants (18). Since *F. virguliforme* is considered a very efficient lignin degrader (19, 24), further studies to clarify the role of lignin on the infection process of this fungus would be valuable.
In field conditions, SDS foliar symptoms commonly develop during plant reproductive stages (26). In our growth chamber studies, however, plants inoculated 28 DAP and 35 DAP did not develop SDS foliar symptoms even though they were at the R1-R2 growth stage by the end of the experiments. Since we did not monitor disease progress into later reproductive stages, we cannot conclude that plants would have remained asymptomatic if they were maintained for a longer period of time. In fact, results of our 2008 field experiment suggests that there are two phases of development of SDS foliar symptoms, one during vegetative and one during reproductive stages. This dual phase of disease has been observed in other SDS field trials (29) (Leandro, unpublished data). In our field study, only plants inoculated at planting developed severe foliar symptoms during the vegetative stage, but plants inoculated at all ages developed foliar symptoms at the reproductive stage. The decrease in foliar symptoms on plants inoculated 0 DAP was caused by defoliation of diseased leaves, and the production of new, leaves. Remission of SDS foliar symptoms has been attributed to dry soil conditions (9), but drought stress was not a limiting factor in our trials since plots were irrigated. Furthermore, we have observed a similar remission in foliar symptoms in greenhouse studies where plants were watered daily (Gongora-Canul, unpublished data).

Although development of SDS symptoms in vegetative stages have been previously reported in field trails (28), rapid increases in disease progress mostly occur in reproductive stages of the plant. Rupe and Gbur (28) suggested that this rapid increase in foliar symptoms may be caused by physiological changes that occur in plants during the shift from vegetative to reproductive stages. For example, re-allocation of photosynthates from vegetative to reproductive plant organs may reduce plant defenses (34), while a reduction in root growth
after R2 stage (15) may shift the ratio between relative growth rates of the roots and pathogen, possibly giving the pathogen a competitive advantage. In contrast, in another study (29), expression of foliar symptoms appeared to be independent of the reproductive stage of the plant and mostly dependent on the chronological age of the plants.

We did not investigate if the second period of foliar symptom expression resulted from new root infections during plant reproductive stages or from infections that occurred during vegetative growth stages and remained latent until the second phase of the disease. However, it is possible that plants that were asymptomatic during vegetative stages were already infected, and that the development of foliar symptoms at reproductive stages resulted from breakdown of plant defenses that allows fungal penetration of the xylem, or from an increase in pathogen biomass in the root system. It is also possible that the buildup of soybean cyst nematode (SCN) naturally present in most Iowa fields increased susceptibility of the plants during reproductive stages, since an interaction between SDS and SCN is known to exist (21, 30).

In this study, we found that soybean roots were susceptible to infection at all plant ages tested, but that root infection at seedling stages was most conducive to the development of SDS foliar symptoms. However, we cannot exclude the possibility that foliar symptoms may have developed in plants inoculated at older ages if exposed to different environmental conditions, inoculum density or with plants of different genotypes. The use of cornmeal as a source of organic matter is not believed to have affected the development of SDS in our study, since root rot was rarely observed, and the pathogen was not isolated from non-inoculated control plants.
Since soybeans were most susceptible to SDS when inoculated at seed stage, the effectiveness of management practices aimed at protecting seedlings should be investigated as a possible disease management option. In addition, studies on the mechanisms involved in SDS symptom development during vegetative and reproductive stages could clarify the mechanisms of root resistance to the disease.

ACKNOWLEDGEMENTS

This study was funded by the Iowa Soybean Association and the North Central Soybean Research Program. We thank Dr. Tom Kaspar, USDA-ARS National Soil Tilth Laboratory, Ames, IA, for advising on root growth and physiology and Dr. Harry Horner and Tracey Pepper, of the Iowa State University Microscopy and Nanoimaging Facility, for their assistance with the microscopy.


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79:139-143.


composition, and relationship to partial resistance to *Phytophthora sojae* Plant
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TABLE 1. Soybean growth stage (GS) at time of inoculation, and at 18 and 38 days after inoculation (DAI), for soybean plants (cv. AG2403) inoculated with a conidia suspension of *Fusarium virguliforme* at seven different ages and grown at 24°C in growth chamber conditions for the two repetitions of the experiment.

<table>
<thead>
<tr>
<th>Plant age at inoculation (days)</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>14</th>
<th>21</th>
<th>28</th>
<th>35</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS at inoculation</td>
<td>Seed</td>
<td>VE</td>
<td>VC</td>
<td>V1/V2</td>
<td>V2</td>
<td>V3</td>
<td>V4/V5</td>
</tr>
<tr>
<td>GS at first sampling date (18DAI)</td>
<td>V1</td>
<td>V2</td>
<td>V2/V3</td>
<td>V3/V4</td>
<td>V4/V5</td>
<td>V5</td>
<td>V6/V7</td>
</tr>
<tr>
<td>GS at second sampling date (38 DAI)</td>
<td>V2/V3</td>
<td>V4</td>
<td>V5</td>
<td>V6</td>
<td>V7/V8</td>
<td>V8/R1</td>
<td>R1/R2</td>
</tr>
</tbody>
</table>
TABLE 2. Area under disease progress curve (AUDPC) for foliar severity of sudden death syndrome on susceptible soybean plants (cv. AG2403) inoculated with *Fusarium virguliforme* at four different ages after transplanting into field conditions in 2008.

<table>
<thead>
<tr>
<th>Plant age at inoculation (DAP)(^x)</th>
<th>First phase (0-63 DAI(^y))</th>
<th>Second phase (63-93 DAI)</th>
<th>Total phase(^z) (0-93 DAI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>780.2 a</td>
<td>112.2 a</td>
<td>892.4 a</td>
</tr>
<tr>
<td>4</td>
<td>69.0 b</td>
<td>238.3 a</td>
<td>307.3 b</td>
</tr>
<tr>
<td>7</td>
<td>0.0 b</td>
<td>275.1 a</td>
<td>275.2 b</td>
</tr>
<tr>
<td>14</td>
<td>0.0 b</td>
<td>96.1 a</td>
<td>96.1 b</td>
</tr>
</tbody>
</table>

\(^x\) DAP is the number of days after planting when plants were inoculated. Plants were grown in pots in the greenhouse and inoculated on the day of transplanting into a field plot.

\(^y\) DAI is the number of days after inoculation.

\(^z\) Phase stands for period of foliar symptoms expression.

\(Pr > F\) \(P = 0.14\) \(P < 0.0004\)
**Fig. 1** Root rot, foliar severity of sudden death syndrome and frequency of isolation of *Fusarium virguliforme* on soybean plants inoculated in growth chamber experiment at different ages with *F. virguliforme* and evaluated 18 and 38 days after inoculation (DAI). Data represent repetition one (A, B and C) and two (D, E and F) of the experiment (n=15 plants/age/sampling time).

**Fig. 2.** Root rot and foliar severity of sudden death syndrome in soybean plants inoculated at four different ages. Plants were assessed at 17 (A, C) and 27 (B, D) days after inoculation (DAI) (n=6).

**Fig. 3.** Frequency of colonization (%) by *Fusarium virguliforme* hyphae of cortex and xylem root tissues of soybean plants inoculated at four different ages sampled A, at 17 and, B, at 27 days after inoculation. Five longitudinal sections of each of 10 root pieces per plant age treatment were observed at each sample time (n=50).

**Fig. 4.** Colonization by *Fusarium virguliforme* hyphae on soybean roots inoculated at four plant ages and sampled 27 days after inoculation. A, healthy cortical and vascular tissue of a non-inoculated plant; B, intra- and intercellular colonization of the cortical tissue cells in a plant inoculated 4 DAP; and C, hyphae in the cortex and xylem tissues of a plant inoculated at planting (0 DAP). Roots were observed at a magnification of 400x using an optical microscope.
**Fig. 5.** Progress of sudden death syndrome foliar severity on soybean plants inoculated at four different ages on the day of transplanting into a field plot in Iowa in 2008 (n=10).
Fig. 1.
Fig. 2.
Fig. 3.

Hyphal colonization (%) as a function of plant age at inoculation (days). A: Xylem vs. Cortex colonization, Xylem: $P = 0.5318$, Cortex: $P = 0.0049$. B: Xylem vs. Cortex colonization, Xylem: $P < 0.0001$, Cortex: $P = 0.1188$. Plant age at inoculation (days): 0, 4, 7, 14.
Days after inoculation

SDS foliar severity (%)

0 2 4 6 8 10

0 DAP

4 DAP

7 DAP

14 DAP

Fig. 5.
CHAPTER 4.
EFFECT OF TEMPERATURE AND PLANT AGE AT TIME OF INOCULATION ON PROGRESS OF ROOT ROT AND FOLIAR SYMPTOMS OF SOYBEAN SUDDEN DEATH SYNDROME

ABSTRACT
Sudden death syndrome (SDS) is favored by planting in cool soil, but epidemics can be severe even when planting occurs later in the season into warmer soil. The objective of this study was to determine how soil temperature affects susceptibility of plants to SDS when exposed to *Fusarium virguliforme* at different ages. Soybeans were grown in rhizotrons in water baths at 17, 23 and 29°C. Subsets of plants were inoculated at 0, 3, 7 and 13 days after planting (DAP) by drenching the soil with a conidial suspension. Root growth, root rot severity and foliar severity were evaluated over 36 days after inoculation. Root rot developed in all inoculated plants, but severity of rotting decreased with increasing temperature and plant age at inoculation. When foliar symptoms occurred, severity decreased with the increasing plant age at inoculation. However, while plants inoculated 0 DAP developed severe symptoms at all temperatures, plants inoculated 3 and 7 DAP only developed symptoms at 17 and 23°C, and plants inoculated 13 DAP did not develop foliar symptoms at any temperature. Root growth rate was negatively correlated with root rot AUDPC and root rot rate and positively correlated with root dry weight. While root length at inoculation was negative correlated with root rot and foliar severity variables and positive correlated with root dry weight. These findings suggest that accelerated root growth in warm soils reduces the window of susceptibility to root infection conducive to SDS foliar symptoms. These
results may explain why delayed planting reduces risk of SDS, but may not prevent severe epidemics if infection occurs shortly after planting.

**INTRODUCTION**

Sudden death syndrome (SDS), caused by the fungus *Fusarium virguliforme* (Aoki, O'Donnell, Homma & Lattanzi) (1, 2, 42), is an economically important disease in soybean (*Glycine max* (L.) Merr.) (42). *Fusarium virguliforme* is a soilborne pathogen that causes root rot and produces a toxin that is translocated to the leaves causing foliar chlorosis and necrosis (13, 14). Colonization of the xylem is thought to be needed for development of foliar symptoms, and it is possible for plants to show root rot but not foliar symptoms if colonization is restricted to the cortical tissue (9, 24). In addition, SDS is highly dependent on environmental conditions, especially temperature and moisture (31-33, 44). There is abundant evidence that the disease is favored by cool and wet soil, and early season planting increases risk of SDS (11, 30, 32, 43). Adequate season-long moisture also seems to favor foliar symptom development (19). Scherm and Yang (32) reported that a low temperature of 15°C is optimum for root rot, whereas temperatures of 22-24°C are optimum for foliar symptoms.

In previous studies (7) we found that plant age at time of inoculation had a major impact on SDS severity. In that study, soybean plants were inoculated at several growth stages ranging from ungerminated seed to V5 (6) (fifth trifoliate leaf), and incubated first at 17°C to favor infection, then at 24°C to induce foliar symptoms. We observed that plants inoculated at all ages developed root rot, but foliar symptoms only developed on those inoculated at seed stage under the conditions of our experiment. We also found that the lack
of foliar symptoms was associated with limited colonization of the xylem in older plants. This suggested that roots become less susceptible to SDS as they mature, probably due to defense mechanisms that prevent xylem colonization and subsequent movement of pathogen toxins to the leaves (24). However, we did not report on how plant age affected susceptibility to SDS under different environmental conditions.

Soil temperature influences the rate of root growth and development (37) as well as root rot severity (4). In addition, plant roots can develop resistance to pathogen invasion as they mature (10) as a result of processes such as accumulation of suberin and lignin in the root tissues (27, 28, 38). Therefore, growing conditions that favor root development are likely to reduce susceptibility to disease. For example, in black root rot of cotton, caused by *Thielaviopsis basicola*, plant growth in cool temperatures resulted in increased disease severity as a result of the slower plant growth (29, 40, 41). With SDS, planting early in the growing season in cool and wet soil increases the risk of disease, suggesting that young roots growing in cool soils are most susceptible to infection. However, severe SDS can sometimes develop in fields where planting was delayed. Studies on the interaction between plant age and temperature have not been conducted on the SDS pathosystem. We hypothesized that soybeans grown in warmer soil temperatures would be less susceptible to root infection and conducive to foliar symptoms than plants grown in cool soil due the faster root growth rate. The objective of the study was to determine the effect of soil temperature and plant age at inoculation on progress of SDS root rot and foliar severity.
MATERIAL AND METHODS

Inoculum preparation. A single-spore isolate of *Fusarium virguliforme* (LL0009) was used in all experiments. This isolate was obtained in 2006 from roots of a soybean plant with typical SDS symptoms from a field in Nevada, IA. The isolate was grown on carnation leaf agar and stored by transferring to potato dextrose agar (PDA; Difco Laboratories, Detroit MI) plates every two months. To prepare inoculum, cultures of the isolate were grown on PDA for 17 to 19 days at 24 ± 2°C in the dark. A conidial suspension was obtained by flooding the cultures on PDA with sterilized deionized water (SDW) and dislodging the conidia with a rubber policeman. The conidia suspension was then filtered through three layers of cheese cloth, diluted with SDW, and adjusted to 4.0 x 10⁴ conidia/ml using a hemacytometer.

Plant source and growth medium. Soybean cultivar AG2403 (Monsanto Co., St. Louis, MO) was used in all experiments. This cultivar belongs to maturity group II and is susceptible to SDS. Soybean seeds were surface sterilized by immersion in 0.5% sodium hypochlorite for 2 min, followed by rinsing twice in SDW. The plant growth media consisted on a mixture of pasteurized soil and sand (1:1 w/w ratio) amended with 1.5% sterilized cornmeal as a source of organic matter.

Experimental design. The experiment consisted of a split-plot design, with soil temperature as the whole plot factor and plant age at inoculation as the sub-plot factor. Experiment repetition was used as the block factor and there were six replicate plants of each plant age within each main plot. Soybeans were grown in rhizotrons to allow repeated observations of the same plants over the course of the experiment. Rhizotrons consisted of transparent boxes (24 cm high × 30 cm long × 2.5 cm wide) made 0.5 cm thick plexiglass
sheets. Each rhizotron was divided vertically into two cells, each holding approximately 1 kg of soil mixture (Fig. 1). The rhizotrons were incubated at three different temperatures in waterbaths made from rectangular plywood boxes (152 cm long x 91 cm wide x 30 cm high) lined with rubber pond liner to avoid leaking. Water temperature in the waterbaths was controlled by a fully submergible aquarium heater (18.8 to 32.2°C/300 W; Aquarium Pharmaceutical Inc., Chalfont, PA), a thermostat (model A19ABC-24; Johnson controls Inc, Milwaukee, WI) and an asco solenoid valve (model 8210G015; Asco, Florham Park, N.J). To ensure homogenous temperature, a pondmaster 1.9 pump (EG Danner MGF Inc. Islandia, NY) was used to circulate the water within the waterbaths. For the experiments, waterbaths were set at 17, 23 and 29°C (± 1°C), and temperature was monitored daily using manual submergible digital thermometers.

Soybean seeds for all treatments were planted on the same day at a depth of 1 cm in each rhizotron cell by approaching the seed to one wall of the rhizotron. Rhizotrons were then wrapped with aluminum foil to maintain roots in the dark and submerged into the waterbaths at a 30° angle to promote root growth against the plexiglass surface and facilitate their observation. A total of 16 rhizotrons were placed in each waterbath on the day of planting, and with three rhizotrons randomly assigned to each of the four plant age treatments. For each plant age treatment, three replicate rhizotrons were inoculated and one was used as a non-inoculated control. Inoculation was conducted 0, 3, 7 and 13 days after planting (DAP) on subsets of plants assigned to the corresponding plant age treatments by drenching the soil in each rhizotron cell with 70 ml of conidial suspension prepared as described above. This resulted in an initial concentration of approximately 2.9 x 10^3 conidia/g of soil. Control plants were drenched with 70 ml of sterile deionized water. The
volume used for inoculation was chosen because it resulted in uniform wetting of the soil profile without accumulation of free water at the bottom of the rhizotron. Throughout the experiment, plants were exposed to a 14 h photoperiod using overhead incandescent light (high pressure sodium, 400 w), and were fertilized 20 and 26 days after planting with a 20 % N, 10% P, 20% K general purpose fertilizer. The experiment was repeated once. Watering of the plant was done as needed.

**Assessments.** Root and foliar disease severity were evaluated every 2 or 4 days after inoculation for 50 days. Root rot severity was observed directly through the rhizotron wall and assessed as the percentage of root area showing brown or black discoloration. Foliar severity was estimated visually as the percentage of total leaf area exhibiting chlorosis and/or necrosis typical of SDS. To determine the root growth rate, tap root length was measured every 2 days until the roots reached the bottom of the rhizotron. Root dry weight was estimated 50 DAP after drying at 89°C for 24 h.

**Data analysis.** Data were analyzed as the progress of disease severity versus time after inoculation. Since planting occurred on the same day for all treatments, and inoculation was staggered, the day of the inoculation of each plant age treatment was considered day zero after inoculation and only assessments conducted up to 36 days after inoculation (DAI) were considered for each plant age treatment. Area under disease progress curves (AUDPC) for root and foliar severity were calculated for each plant age and temperature treatment by using the trapezoidal integration method (5). A linearized monomolecular model \( \ln\left[1/(1 - y)\right] = \ln\left[1/(1 - y_0)\right] + rMt \) was used to estimate root and foliar severity progress rates (5, 20, 25), since in previous studies this model provided the best fit to SDS progress curves (8). A simple linear regression was done for the relationship between time and root length up to 8 days.
after inoculation in all plant age treatments (inoculated and no inoculated plants) exposed to the three temperatures to estimate root growth rate (cm/day) due to by 8 DAI roots reach the bottom of the rhizotron.

Analysis of variance was performed according to a split plot design using the PROC GLM procedure of SAS version 9.1 (SAS Institute, Cary, NC) on AUDPC, monomolecular rate of disease progress $\ln[1/(1 - y_0)]$ units/day, root growth rate (cm/day) and root dry weight. The two experiments were pooled since there was no interaction between experiment and temperature for the root rot and foliar severity AUDPC and monomolecular rate. Main effects for soil temperature and plant age at inoculation were evaluated using the “slice” option is SAS. The Tukey-Kramer test (LSD) was used (alpha < 0.05) to test differences in means for AUDPC, disease progress rate, root dry weight and root growth rate. Pearson correlation coefficient and statistical significance were calculated to determine the association among root traits, root rot and foliar severity variables by using the SAS software.

**RESULTS**

Soil temperature and plant age at inoculation affected ($P < 0.05$) the development of root rot and foliar symptoms (Table 1 and 2). In general, root rot and foliar symptoms became less severe with increasing soil temperature and with increasing in plant age at inoculation. However, foliar symptoms only developed in plants inoculated at 0, 3, and 7 DAP when exposed at soil temperature of 17°C, in 0, 3 DAP when exposed at 23°C, and only 0 DAP when exposed at 29°C (Fig. 2). By the time of inoculation root growth and growth
stage of the soybean plants of the same age was accelerated as soil temperature increased (Fig. 3).

Analysis of variance showed a significant interaction between plant age and temperature (Table 2), so means for treatment effects will be presented separately for each plant age and temperature combination. Temperature did not affect root rota AUDPC in plants inoculated at 0 and 13 DAP, but plants of 3 and 7 DAP showed a significant decrease in root rot AUDPC as temperature increased from 17 to 29°C (Table 1). For foliar severity AUDPC, soil temperature had an effect on plants inoculated at 0 and 3 DAP, but, differences were not observed in plants inoculated at 7 and 13 DAP. Root rot AUDPC was greatest on plants inoculated 0 DAP at 17°C, while foliar severity AUDPC was greatest on plants inoculated 0 DAP at 23°C (Table 1). Plant age at inoculation significantly affected severity of root and foliar symptoms at all temperatures (Table 1 and 2). Plants inoculated 0 DAP had the greatest root rot and foliar AUDPC at all soil temperatures, and AUDPC decreased with increasing plant age at inoculation.

The monomolecular model showed a significant fit to root and foliar severity progress curves for all plant ages at inoculation and all soil temperatures, except for foliar severity on plants inoculated 7 DAP and incubated at 23°C (Table 3). The monomolecular rate of root and foliar disease progress was generally not affected by soil temperature, but root rot rate on plants inoculated at 3 and 7 DAP was significantly lower ($P < 0.01$) at 29°C (Table 3). In contrast, plant age at inoculation significantly affected ($P < 0.01$) the rate of root rot and foliar symptoms at all soil temperatures. Plants inoculated 0 DAP showed the greatest rates of root rot and foliar severity in all three soil temperature, and the rates decreased with increasing plant age at inoculation (Table 3). Rates for foliar severity could not be calculated
for plants inoculated 14 DAP and for plants inoculated 3 and 7 DAP at 29°C due to the lack of foliar symptoms.

Soil temperature and plant age at inoculation had an effect on root growth rate (cm/day) on inoculated and non-inoculated plants (Fig. 3). Root growth rate was approximately two-fold faster at 29°C than 17°C (Table 4). Since root growth was measured only for the first 8 days after planting, there were no differences in growth rates between for plants inoculated 0, 3, 7 and 13 DAP and non-inoculated plants at 17°C. However, there was a clear difference in root growth rate between plants inoculated 0 and 3 DAP and those inoculated 7 and non-inoculated at 13 DAP and control plants when incubated at 23°C and 29°C (Table 4). Root dry weight (g) increased significantly with increasing soil temperature in all plants ages except 0 DAP, and was significantly lower on plants inoculated 0 DAP than plants inoculated at older ages (Table 4).

Root growth rate was negatively correlated with root rot AUDPC and root rot rate, and positively correlated with root dry weight (Table 5). Root length at inoculation was positively correlated with dry weight and negatively correlated with root rot and foliar severity variables. Rate of root rot and foliar severity and AUDPC were positively correlated with each other, and negatively correlated with root dry weight (Table 5).

**DISCUSSION**

In this study we found that temperature and plant age at inoculation greatly affect severity of root rot and foliar symptoms of soybean SDS. Generally, we found that disease became more severe as soil temperature and plant age at inoculation decreased, but there was a significant interaction between these factors.
Increasing soil temperature significantly reduced root rot severity when inoculation occurred at 3 and 7 days after planting (DAP), but significant differences were not observed in plants inoculated 0 and 13 DAP due to high levels of severity and lack of symptoms respectively. In agreement with this study, Miller and Burke (22) showed no significant differences in Fusarium root rot in bean plants exposed at 18 and 27°C when inoculation occurred at seed stage (0 DAP). Foliar symptoms also tended to be less severe at warmer soil temperatures, but severity was greatly dependent on plant age at inoculation. Our study also confirmed that root rot and foliar symptoms are favored by different temperatures since the maximum AUDPC for plants inoculated 0 DAP was observed at 23 °C for leaves, and at 17 °C for root rot. This is consistent with findings by Scherm and Yang (32) that 17 °C is optimum for root rot and 22-24 °C is the optimum for foliar severity of SDS.

At all temperatures, plants inoculated 0 DAP showed severe foliar symptoms, while plants inoculated at older ages showed a dramatic decrease in severity with increasing temperature. However, while at 17°C plants inoculated at all ages except 14 DAP developed foliar symptoms, at 29°C only plants inoculated 0 DAP showed foliar symptoms for the duration of the experiment. This is consistent with our previous findings that plants inoculated at seed stage (0 DAP) were more susceptible to SDS than plants inoculated 3 or more days after planting. In this study, we have additionally shown that the period of susceptibility to infection conducive to foliar symptoms is shorter at warmer temperatures than at cool temperatures. Since root growth was accelerated at warmer temperatures, our findings suggest that susceptibility is affected by the developmental stage of the roots when they are exposed to the pathogen.
The importance of root growth to susceptibility to SDS was further indicated by the significant correlations found between root weight and length and the severity of root and foliar symptoms. The negative correlation between root growth rate, root dry weight and root length, and disease variables (AUDPC and rate), suggesting that accelerated root growth in warm soils may be a factor influencing root rot severity (23, 29). Other studies also showed that low soil temperature constrained root growth on beans and potentially increased Fusarium root rot (3, 4, 21, 22).

Differences in disease severity observed among temperatures on plants inoculated more than 3 DAP may be related to the longer roots and different growth stage induced by different soil temperatures when infection occurred (15, 37). There is evidence that plants may be more resistant to disease as they mature (16, 34, 38). Ranathunge et al. (27) determined that induced suberin can be synthesized after 2 to 4 days inoculation in a cultivar with strong partial resistance, probably rapidly enough to influence the initial phases of infection, while in a susceptible cultivar suberin was synthesized only 6 days after inoculation. Suberin is known to be important as a physical defense against pathogen invasion (38). It is therefore possible that suberin deposition or other defense mechanisms are activated within the three days of growth. Other possible mechanism involved in resistant on older plats is the high production of phytoalexin such as glyceolllyn as soybean stem-root tissues mature (17, 26). These defense mechanisms may have caused a delay in colonization and penetration of *F. virguliforme* into the xylem and subsequently delayed the movement of the toxin to the leaves (13, 14), resulting in less severe foliar symptoms on plants inoculated later than 0 DAP. In other studies, low soil temperature and wet soil extended the spermosphere influence in space and time allowing to produce more spores and increase
infection points causing more rot as showed in the pathosystem *F. solani* f. sp. *pisi*-pea (*Pisum sativum*) (35, 36). Such a phenomenon also could have played a factor in this study.

On plants inoculated 0 DAP, soil temperature did not affect root rot AUDPC and disease progress rates because the seeds had not been exposed to different temperatures, and therefore did not differ in maturity or development at the time of inoculation.

Our study also confirmed that root rot and foliar symptoms are favored by different temperatures since the maximum AUDPC for plants inoculated 0 DAP was observed at 23 °C for leaves and at 17 °C for root rot. This consistent with findings by Scherm and Yang (32). The fit of the monomolecular model to disease progress curves provided additional information about the effect of temperature and plant age on SDS. For example, the rate of root rot and foliar disease progress tended to be slower at warmer temperatures and faster at the coolest temperature, although differences in rate were only statistically significant for root rot on plants inoculated 3 and 7 DAP. It is possible that the fastest severity rates were observed at the low temperature because at this temperature the expansion rate of the root rot lesions was faster than the rate of root growth, giving a competitive advantage to the pathogen in relation to the plant (12).

The rate of disease progress on both roots and leaves was highly affected by plant age at inoculation; with rates decreasing by 4 to 14-fold between plants inoculated 0 and 14 DAP. The slower rates on older plants may be explained by a greater length of the root area that is resistant to infection (17, 26) compared to younger roots. Since plant were inoculated by application of a spore drench to soil mixed with cornmeal, it is unlikely that the differences in rate were related to different inoculum pressure in roots of different ages. Further evidence
for homogenous inoculum pressure was obtained by observations through the rhizotrons that allowed visualization of root rot developing simultaneously in young and older root areas.

Like other soil physical factors, such as compaction and oxygen content, that affect root development (3, 4, 22), root growth rate and dry weight were greatly reduced by cool temperatures in plants inoculated at all ages in our study. However, such reductions in root growth may result from a direct effect of soil temperature on root growth and an indirect effect on pathogen growth and ability to cause root rot. Soil temperature alone can affect root growth and development (15, 37) and can therefore affect root dry weight and tap root extension. Alternatively, root dry weight can be a useful measure of pathogen aggressiveness (18), e.g. a reduction in the number of roots in clover was attributed to infection by F. crokwellence (39). In our study, root weight increased as plant age at inoculation increased, particularly at the cooler temperatures. Since all plants were assessed for root weight 50 days after planting, the smaller reduction in weight observed in plant inoculated at older plants resulted from the shorter period of exposure to the pathogen.

In this study, we found that plants inoculated at planting developed severe symptoms at all temperatures but that disease severity decreased with increasing temperature when infection occurs on older plants. This may explain why late plantings into warmer soil temperature may reduce SDS severity (11) but not prevent from severe epidemic especially if infection occurred at seed stage. These findings also highlight the need to clarify the role of root growth parameters on susceptibility to SDS and the mechanisms involved in root resistance to infection conducive to foliar symptoms. This knowledge may assist breeders in determining root traits that should be taken into account when developing soybean cultivars with resistance to SDS.
Finally, this paper also provides the first detailed description of SDS root rot progress over time based on non-destructive root evaluations conducted using rhizotrons. This methodology facilitated the analysis of disease progress curves, calculation of disease parameters such as AUDPC and disease progress rates and measurements of root growth, that were valuable in assessing the impact of temperature and plant age on SDS root and foliar symptoms. The use of the rhizotrons was therefore shown to be a reliable and easy method to non destructively study root rot development over time, which reduces variability and can provide valuable information for studies on the epidemiology and infection of *F. virguliforme*.

**ACKNOWLEDGEMENTS**

This study was funded by the Iowa Soybean Association and the North Central Soybean Research Program. We thank Dr. Tom Kaspar, USDA-ARS National Soil Tilth Laboratory, Ames, IA, for advice on root growth and physiology.
LITERATURE CITED


TABLE 1. Mean values and standard error of root rot and foliar severity AUDPC of soybean plants (cv. AG2403) of four ages at inoculation exposed at three soil temperatures.

<table>
<thead>
<tr>
<th>Plant Age</th>
<th>Root rot AUDPC</th>
<th>Foliar severity AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17°C</td>
<td>23°C</td>
</tr>
<tr>
<td>0</td>
<td>2098 ± 57</td>
<td>2022 ± 84</td>
</tr>
<tr>
<td>3</td>
<td>1471 ± 172</td>
<td>1339 ± 248</td>
</tr>
<tr>
<td>7</td>
<td>1760 ± 118</td>
<td>929 ± 190</td>
</tr>
<tr>
<td>13</td>
<td>635 ± 121</td>
<td>767 ± 146</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Statistical significance of mean comparison of AUDPC among plant age at inoculation at a single soil temperature.
*Statistical significance of mean comparison of AUDPC of a single plant age at inoculation across the three soil temperature.
TABLE 2. ANOVA on the effects of soil temperature and plant age at inoculation on the area under disease progress curve (AUDPC) for root rot and foliar severity of sudden death syndrome of soybean (cv. AG2403).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MSE</th>
<th>F</th>
<th>P &gt; F</th>
<th>MSE</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replication (Block)</td>
<td>1</td>
<td>759306.1</td>
<td>7.4</td>
<td>0.008</td>
<td>244015.1</td>
<td>2.93</td>
<td>0.0925</td>
</tr>
<tr>
<td>Soil temperature (Main plot)</td>
<td>2</td>
<td>232047.83</td>
<td>8.1</td>
<td>0.109</td>
<td>104110.8</td>
<td>2.5</td>
<td>0.284</td>
</tr>
<tr>
<td>Main plot Error</td>
<td>2</td>
<td>283879.22</td>
<td>2.8</td>
<td>0.069</td>
<td>41285.6</td>
<td>0.50</td>
<td>0.611</td>
</tr>
<tr>
<td>Plant age (subplot)</td>
<td>3</td>
<td>6455510.8</td>
<td>63.6</td>
<td>&lt;0.001</td>
<td>7221000.1</td>
<td>86.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Soil temperature* Plant age</td>
<td>6</td>
<td>540130.4</td>
<td>5.32</td>
<td>0.0002</td>
<td>240392.0</td>
<td>2.8</td>
<td>0.0159</td>
</tr>
<tr>
<td>Subplot error</td>
<td>57</td>
<td>101457.6</td>
<td>...</td>
<td>...</td>
<td>83322.8</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*Analysis was done by pooling data of the two experiments.
TABLE 3. Parameters of the linear regression analysis of the monomolecular growth curve model used to describe root rot and foliar severity progress curve of sudden death syndrome on soybeans (cv. AG2403) inoculated at four ages and exposed to three soil temperatures.

| Soil temperature (°C) | Plant age (Days) | Root rot severity (%) | | | Foliar severity (%) | | |
|---|---|---|---|---|---|---|
| | | Intercept | Slope | $R^2$ | $P$ value | $SEE_y$ | Intercept | Slope | $R^2$ | $P$ value | $SEE_y$ |
| 17 | 0 | -0.540 | 0.104 | 98.6 | $<0.0001$ | 0.004 | 2.04 | 0.122 | 95.7 | 0.003 | 0.014 |
| | 3 | -0.226 | 0.048 | 99.3 | $<0.0001$ | 0.001 | -0.71 | 0.044 | 91.1 | 0.0008 | 0.006 |
| | 7 | -0.252 | 0.056 | 95.6 | $<0.0001$ | 0.003 | -0.32 | 0.019 | 76.6 | 0.009 | 0.004 |
| | 13 | -0.047 | 0.015 | 82.9 | 0.0006 | 0.002 | ... | ... | ... | ... | ... |
| 23 | 0 | -0.312 | 0.081 | 93.9 | $<0.0001$ | 0.007 | -0.97 | 0.11 | 88.9 | 0.001 | 0.017 |
| | 3 | -0.174 | 0.047 | 97.8 | $<0.0001$ | 0.002 | -0.34 | 0.02 | 88.6 | 0.001 | 0.003 |
| | 7 | -0.024 | 0.018 | 84.9 | 0.0002 | 0.002 | 0.001 | 0.001 | 47.3 | 0.059 | 0.576 |
| | 13 | -0.070 | 0.018 | 84.8 | 0.0004 | 0.002 | ... | ... | ... | ... | ... |
| | | $P<0.0001$ | | | | | $P=0.01$ | | | | | |
| 29 | 0 | -0.398 | 0.085 | 90.7 | $<0.0001$ | 0.010 | -0.403 | 0.055 | 90.4 | 0.001 | 0.007 |
| | 3 | 0.009 | 0.009 | 60.5 | 0.0135 | 0.002 | ... | ... | ... | ... | ... |
| | 7 | 0.006 | 0.008 | 85.0 | 0.0001 | 0.001 | ... | ... | ... | ... | ... |
| | 13 | 0.011 | 0.006 | 91.9 | $<0.0001$ | 0.0007 | ... | ... | ... | ... | ... |
| | | $P<0.0001$ | | | | | $P=0.01$ | | | | | |

*Statistical significance of mean comparisons for rate of disease progress (slope) among plant age treatments within each soil temperature.
*The rate of root rot severity (slope) only differed significantly ($P<0.01$) among temperatures for plants inoculated 3 and 7 DAP.
*The rate of foliar severity (slope) did not differ significantly ($P<0.01$) among temperatures for plant age.
*Standard error of the estimate of Y.
TABLE 4. Mean and standard error of root growth rate and root dry weight of soybean plants (cv. AG2403) inoculated with *Fusarium virguliforme* at four different ages and incubated at three soil temperatures.

<table>
<thead>
<tr>
<th>Soil temperature (°C)</th>
<th>Plant age (days)</th>
<th>Root growth rate (cm/day)</th>
<th>(^{*})Non inoculated plants</th>
<th>(^{y})P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>0</td>
<td>1.1 ± 0.11</td>
<td>1.3 ± 0.04</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.1 ± 0.11</td>
<td>1.3 ± 0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>23</td>
<td>2.5 ± 0.04</td>
<td>2.5 ± 0.04</td>
<td>0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>29</td>
<td>2.3 ± 0.11</td>
<td>2.3 ± 0.11</td>
<td>2.8 ± 0.03</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

\(^{x}\)P value <0.0001 <0.0001 <0.0001 <0.0001

<table>
<thead>
<tr>
<th>Soil temperature (°C)</th>
<th>Root dry weight (g)</th>
<th>(^{w})P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.04 ± 0.005</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>0.15 ± 0.025</td>
<td>0.006</td>
</tr>
<tr>
<td>7</td>
<td>0.18 ± 0.049</td>
<td>0.006</td>
</tr>
<tr>
<td>13</td>
<td>0.35 ± 0.062</td>
<td>0.006</td>
</tr>
<tr>
<td>Control</td>
<td>0.28 ± 0.029</td>
<td>0.006</td>
</tr>
</tbody>
</table>

\(^{w}\)Dry weight estimation was done by destructively sampling by 50 DAP.

\(^{y}\)Statistical significance for mean comparison among plant ages within each soil temperature.

\(^{x}\)Measurements were done up to 8 DAP since root reached the bottom of the rhizotron.

\(^{*}\)Statistical significance for mean comparison among temperatures within each plant age.

\(^{z}\)Is the mean of the average of plant ages at inoculation of 7, 13 DAP and control plants.
TABLE 5. Pearson correlation coefficients between disease variables and root variables of soybean plants (cv. AG2403) inoculated with *Fusarium virguliforme* at four ages and incubated at three soil temperatures.

<table>
<thead>
<tr>
<th>Disease associated variables</th>
<th>Root growth rate (cm/day)</th>
<th>Root length at inoc. (cm)</th>
<th>Root dry weight (g)</th>
<th>Root rot AUDPC</th>
<th>Root rot rate</th>
<th>Foliar severity AUDPC</th>
<th>Foliar severity rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root growth rate (cm/day)</td>
<td>...</td>
<td>0.58*</td>
<td>0.77**</td>
<td>-0.61*</td>
<td>-0.58*</td>
<td>-0.35ns</td>
<td>-0.55ns**</td>
</tr>
<tr>
<td>Root length at inoc. (cm)</td>
<td>...</td>
<td>...</td>
<td>0.76**</td>
<td>-0.85**</td>
<td>-0.84**</td>
<td>-0.79**</td>
<td>-0.76**</td>
</tr>
<tr>
<td>Root dry weight (g)</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>-0.90**</td>
<td>-0.86**</td>
<td>-0.73**</td>
<td>-0.74**</td>
</tr>
<tr>
<td>Root rot AUDPC</td>
<td></td>
<td></td>
<td>0.97**</td>
<td>0.86**</td>
<td>0.85**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root rot rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.89**</td>
<td>0.91**</td>
</tr>
<tr>
<td>Foliar severity AUDPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>...</td>
<td>0.90**</td>
</tr>
<tr>
<td>Foliar severity rate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>...</td>
<td></td>
</tr>
</tbody>
</table>

xThe correlation was done by considering the effect of the three soil temperature on the root traits and disease variables only in inoculated plants of all four ages.

yCorrelation between growth rate and foliar severity variables was done up to 7 DAP.

zStatistical significance of ** was $P < 0.01$, and for * was $P < 0.05$. 
**Fig. 1.** Soybean plants (cv. AG2403) growing in rhizotrons and incubated at 23 °C for 49 days after planting. Left: non-inoculated plant, right: plant drench-inoculated, both at planting (0 DAP) with a conidial suspension of *Fusarium virguliforme* showing root rot and foliar symptoms. White dashed lines shows the rhizotron dimension.

**Fig. 2.** Progress of root rot and foliar symptoms of soybean sudden death syndrome severity on plants (cv. AG2403) inoculated with *Fusarium virguliforme* at 0, 3, 7 and 13 days after planting (DAP) and incubated at three soil temperatures: A-B 17°C, C-D, 23°C, and E-F 29°C. Soybean growth stages 36 days after inoculation are shown for each plant age treatment in the foliar severity graphs (values from top to bottom are for plants inoculated 0, 3, 7 and 13 DAP, respectively.

**Fig. 3.** Root length and growth stage of soybean plants (cv. AG2403) at time of inoculation with *Fusarium virguliforme*. Plants were grown in rhizotrons incubated at three soil temperatures and inoculated at four different ages after planting.
Fig. 1
Fig. 2.
Fig 3.
CHAPTER 5.
GENERAL CONCLUSIONS

The work contained in this thesis investigated epidemiological aspects of the infection process and symptom expression of sudden death syndrome of soybean (*Glycine max* (Merr.) L.) caused by *Fusarium virguliforme*, specifically the temporal dynamic of root rot and foliar symptoms at different inoculum densities, the effect of plant age at inoculation on root rot and foliar symptoms, the mechanisms of fungal colonization of the vascular and cortical root tissue; and finally the interaction between soil temperature and plant age at inoculation.

Our results confirmed that early assessments of root rot 15-30 days after inoculation (DAI) show a stronger correlation with final foliar severity (40-50 DAI) than when roots and leaves are assessed simultaneously. It was also shown for the first time that the monomolecular growth model had the best fit to describe progress of SDS root rot and foliar severity due to the fast progress of the disease early in the epidemic, and a decrease in rate towards the end of the epidemic, resulting from the production of new apparently healthy tissue by the plants. We also found that infection by *F. virguliforme* can significantly reduce root mass by 30 to 80%, even at low inoculum levels. The use of a range of inoculum densities of *F. virguliforme* was found to be a useful tool to compare disease parameters and evaluate the relationship between root rot and foliar severity.

Our work also provided evidence that plant age at inoculation (days after planting-DAP) greatly impacts root rot and foliar severity in both controlled and field environments. Inoculation at seed stage (0 DAP) resulted in the most severe root rot and foliar symptoms, with severity above 80%. In contrast, plants inoculated at ages ranging from 3 to 35 DAP
also showed high levels of root rot severity (from 25 to 60%), but foliar symptoms never
developed under growth chamber conditions. In the field, two phases of foliar symptoms
expression could be observed, one at vegetative stage (V2-V4) when the effect of plant age
was similar to the effect observed in growth chambers, and the other phase at reproductive
stages (R5-R7) when plants inoculated at all ages showed foliar symptoms. We also
demonstrated that plants that showed root rot and foliar symptoms had a high frequency of
xylem colonization by the pathogen, while in plants that showed severe root rot severity but
no foliar symptoms, colonization was mostly limited to the cortical tissue. Plants inoculated
4, 7 and 14 DAP sometimes showed xylem colonization but this occurred at a very low
frequency that probably did not provide enough toxin to induce foliar symptoms.
Finally, we found that the period of susceptibility for root rot infection conducive to foliar
symptoms was different at soil temperatures. Low soil temperature of 17°C resulted in a root
growth rate (1.2 cm/day) that was two-fold slower than the rate at 29°C (2.5 cm/day). Plant
roots inoculated 0, 3, 7 and 13 DAP and exposed to 17, 23 and 29°C developed different
levels of root rot and foliar severity over time. Disease parameters such as root rot and foliar
severity area under disease progress curve (AUDPC), and monomolecular rates of disease
progress, decreased as temperature and plant age at inoculation increased. However, while
plants inoculated at 0, 3 and 7 DAP developed foliar symptoms at 17°C, foliar severity only
developed on the 0, 3 and 7 DAP treatments at 23°C, and only on plants inoculated at seed
stage at 29 °C. When inoculation occurred at 0 DAP, differences in root rot and foliar
severity were not evident among temperatures. However, increases in soil temperature
resulted in reduced severity on plants inoculated at ages older than seed stage. These results
may explain why delayed planting reduces risk of SDS, but may not prevent severe epidemics if infection occurs shortly after planting.
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To God for his omnipotence over everything. “You helped me when I less thought”