Identification and characterization of soybean sudden death causal species in South Africa

By

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Contents

Abstract

Soybean sudden death syndrome (SDS) was first reported in South Africa by a series of pathogen surveys conducted in 2013. SDS is caused by different *Fusarium* species within the *Fusarium solani* species complex, *F. virguliforme* is a species in that complex and the most prevalent SDS causal species worldwide. The primary objective of this study was to identify and characterise the causal species of soybean sudden death in South Africa to assist in the breeding for resistance against the disease.

Higher frequencies of *F. virguliforme* and *F. graminearum* over lower frequencies of *F. oxysporum*, *F. sporotrichioides* and *F. equiseti* were morphologically identified from trials that were situated in Petrusburg (Free State) and Delmas (Mpumalanga) in South Africa. Isolates identified as *F. graminearum* and *F. virguliforme* was confirmed by species-specific PCR assays using Fg16F and Fg16R primer pair combination amplifying a 410 bp fragment and also Fsg1/Fsg2 and FsgEF1/ FsgEF2 primer pair combinations to amplify a 438 bp and 237 bp fragment, respectively. This is not the report of *F.virguliforme* causing soybean SDS in South Africa. However, we cannot ignore the fact that *F. graminearum* is in such high frequencies in the field and thus could be associated with SDS. Results of the mycotoxin and pathogenicity experiment revealed that mycotoxins are correlated with the pathogenicity of the disease, however, conversely the growth rates of the pathogen aren't correlated pathogenicity of the disease. The identification and characterisation of SDS causal species will enable the improvement of management and control strategies with the emphasis on disease resistance breeding.

Introduction

Soybean, (*Glycine max*), is an annual legume crop of the *Fabaceae* family (pea). Soybean is also an edible crop mainly produced in South Africa for the life stock industry and oil production. The most economically important bean species in the world is soybean, which provides essential proteins for millions of people and is used as the main ingredients for hundreds of chemical products (Aoki et al., 2003).

In South Africa's agricultural economy soybean is a small but very important component and its steadily increasing (O'Donnell et al., 1995). The importance of soybean budding in South Africa is demonstrated by the area was brought into production in recent years (2013). Rising yields is mainly supported by environment agricultural policy that is focusing on the commercialisation and use of agricultural biotechnologies (Al-Kaisi et al., 2007). Thus, it has facilitated a smooth transition of commercial farmers from the production of traditional grains to soybean production and to be able to rotate soybeans with other grain crops to maximise profits (Al-Kaisi et al., 2007).

For example, (Van der Merwe et al.,2013) stated that the increase in production is recognised by commercial farmers becoming cognisant of the benefits of soybean in crop rotation systems with maize. The government of South Africa recognises the importance of soybeans in the economy. The Industrial Policy and Action Plan (IPAP)

2012 – 2015 recognises soybeans as having the potential for creating opportunities for new investments and job creation (DTI, 2010).

Soybean receives the status “economical important” due to its increasing domestication in countries across the globe, but it also means that the impact and management of diseases is even more important. Such important diseases include, sudden death syndrome (SDS), it’s a relatively new disease for soybean caused by blue-pigmented strains of *Fusarium solani (Mart.) Sacc.* that have been designated *F. solani f. sp. glycines* (Hirrel, 1987).

Sudden death syndrome (SDS), is caused by numerous of fusarium species in various parts of the globe, however, *Fusarium virguliforme (Fv)*, is the most common occurring pathogen of SDS on soybean *(Glycine max)* (Aoki et al., 2003).

Sudden death syndrome is a disease that targets the roots of a plant resulting in severe foliar symptoms during the reproductive stage (R1-R8) of the plant which is approximately 20 days after emergence of seedlings. Occasionally SDS is unnoticed because its symptoms are comparable to those of brown stem rot (BSR), also a common soybean disease. However, they do differ from BSR due to SDS not causing internal stem browning, which is a crucial symptom of brown stem rot (Smith et al., 2017).

A startling discovery is that soybean cyst nematodes (SCN) has been associated with SDS occurrences (Rupe et al., 1991; Gibson et al., 1994; Rupe et al., 1995). The chlamydospores of *F. virguliforme* were discovered in within SCN cysts nematodes. It is being understood that the chlamydospores of *F. virguliforme* is capable of overwintering in the SCN and can consequently cause severe SDS yield losses in the next growing season (Roy et al., 1997). Apart from *F. virguliforme* which is accountable for causing SDS in North America and South Africa, *Fusarium tucumaniae*, has revealed to also cause SDS of soybean in South America (Aoki et al., 2003). Despite the fact that *F. tucumaniae* comprises of a sexual reproductive stage and has two mating types in its life cycle, samples of *F. virguliforme* are of the related mating type and for this reason it is determined that *F. virguliforme* species does not reproduce sexually (Covert et al., 2007).

SDS of soybean (Glycine max L. Merr.) is an economically important disease, caused by the semi-biotrophic fungus *Fusarium solani f. sp. glycines* (Roy et al., 1997), that was recently renamed *Fusarium virguliforme*. This is due to the length and complexity of the *Fusarium*-soybean interaction, however the molecular mechanisms fundamental for plant susceptibility and resistance to the pathogen are not entirely understood (Yuan, 2008).

SDS reduces yield dramatically when symptoms are very severe in the early reproductive development of the soybean (Melgar et al., 1992). Yield losses due to SDS can range from 20-80% or more, depending greatly on the growth stage of the host at the initiating stages of symptom development and whether the SDS disease progresses rapidly and becomes severe (Smith, 2017).

A high throughput multi locus assay was established based on the nucleotide polymorphism within two anonymous intergenic regions (locus 51 & 96) and the nuclear ribosomal intergenic spacer region rDNA by O'Donnell et al. (2010). This high throughput multi locus assay was capable of successfully differentiate between *F. virguliforme* and *F. tucumaniae* on an allelic basis (O'Donnell et al., 2010). Hence, it was discovered that four phylogenetically distinct Fusarium species, *F. tucumaniae, F. cuneirostrum sp. nov, F. brasiliense sp. nov., and F. virguliforme,* has the ability to cause SDS disease in soybean (Aoki et al., 2003).

However, *F. graminearum* is also primarily regarded as an economically important pathogen on corn *(Zea mays L.)* and small grains. But recently, *F. graminearum* was discovered to be more pathogenic on soybean (Pioli et al. 2004). In the meantime, *F. graminearum* has been shown to cause seed and root rot, pod blight, and pre- and post-emergence damping-off on soybean (Ellis et al. 2011). As already known *F. virguliforme* is the causal agent of soybean sudden death syndrome (SDS) on soybean in the U.S.A. and other parts of the world (Aoki et al. 2003). However, both *Fusarium* species can be found in most of the major crop growing regions.

The fungi *Fusarium solani* is one of the many species complexes and *F. virguliforme* is one of the species within this complex (Aoki et al., 2003). However, in South America*, F. tucumaniae, F. cuneirostrum, F. brasiliense* and *F. virguliforme* cause SDS symptoms in plants as already discussed.A fungus of this taxonomic complexity develops rather slowly in culture and it's challenging to isolate from diseased plants. As soon as a pure culture is isolated, the iconic blue spores of the pathogen and other cultural characteristics single out the SDS pathogens from other *Fusarium* species that can also infect soybean roots.

According to (Covert et al., 2007), *F. virguliforme* is contemplated to be clonal and has been considered asexual, but recent crossing experiments revealed the existence of a teleomorph in North America. In South American one of the SDS pathogens, *F. tucumaniae*, produced sexual reproductive structures (fertile perithecia) in crosses under specific laboratory conditions. As stipulated in the Vienna Code of the International Code of Botanical Nomenclature, Article 59.7 (Rijckevorsel, 2006) the anamorph title has been retained for this fungus (Covert et al., 2007). *F. virguliforme*, the North America and South Africa SDS-pathogen, failed to produce fertile crosses with *F. tucumaniae* or with itself; only infertile sexual structures were developed in a few latter crosses (Covert et al., 2007).

These discoveries substantiate the hypothesis that the North American SDS-pathogen is in fact clonal and that distinct species do cause SDS in North America, South America and South Africa, respectively (Westphal, 2018).

SDS pathogen survives growing seasons as chlamydospores in crop residue, freely in the soil or in cyst nematodes. The thick-walled chlamydospores develop on soybean roots during disease development and thereafter in the soil. Chlamydospores is capable to endure and survive the wide range of fluctuations in soil temperature this

also includes resist desiccation and freezing. In the spring as soil temperature increases, chlamydospores neighbouring the soybean roots are stimulated to germinate, and infect soybean roots (Hashmi, 2004).

F. virguliforme, also produces macroconidia on the taproot's surface during the summer, these spores are only dispersed a short distance within a growing season. However, over a period of years, cultivation practices and flowing water that cause soil move can disperse spores over longer distances between or within fields. Symptoms of SDS initially appears after hefty rains during reproductive stages of the soybean, disease severity increases with high soil moisture (Xing et al., 2006).

When incubated in a soil extract solution the macroconidia of *F. virguliforme* converts to chlamydospores, and these chlamydospores transpire in the cortical tissue of the roots in the field (Melgar et al., 1994). The fungus survives in the root debris and in soil primarily as chlamydospores, which make up the primary inoculum of the pathogen.

The method by which *F. virguliforme* penetrates the roots is mysterious, however, some evidence does suggest that it is a direct infection. Roots of 2- to 3-week-old seedlings are colonized and infected by the pathogen (Njiti et al., 1997), and it appears that the colonization is limited to cortical tissue of the lower stem and root (Melgar et al., 1994). Hyphae that's being developed grow inter- and intracellularly, but mostly intracellularly.

Secreted molecular weight proteins or toxins of pathogenic fungi have been confirmed to be the basis of some diseases in plants (Rep, 2005). However, there are host selective toxins (HSTs) due to their 7 capabilities to develop disease only in specific host plants.

The most known virulent fungi species that produces toxin is *Fusarium* (Creppy, 2002). Toxins produced by *Fusarium* species affect either plants (fusaric acid) or animals such as *F. graminearum* and, in some rare cases, both plants and animals like enniatins, fumonisins and moniliformin that is produced by *F. verticillioides* (Desjardins et al.,1997).

Compared with other fungus species the *fusarium* is amongst the most virulent toxin-producing fungi of today (Creppy, 2002). Up to the present time, twenty fungal pathogens have been discovered to produce HSTs. The majority of the HSTs are proteins or low-molecular-weight metabolites (Wolpert et al., 2002).

Toxins produced by *F. virguliforme* in the roots of the soybean that are translocated to the leaves causing foliar symptoms; the fungus itself does not invade the stems more than a few centimetres above the soil line (Roy et al., 1997). It is well known by now that *F. virguliforme* is the causal agent of Sudden death syndrome disease. However, there are two components that plays a role, i.e., foliar SDS and root necrosis. Interveinal chlorosis and necrosis of leaves is characterized at the initial stage of foliar SDS.

F. virguliforme excretes toxins into the culture media that give rise to foliar SDS-like symptoms in 3-week-old soybean seedlings (Li et al., 2009). However, it is contemplated that one or more toxins released by *F. virguliforme* into the soybean roots causes foliar SDS (Li et al., 2009).

Bhattacharyya (2011) reported the discovery of the FvTox1 gene, which is responsible for the encoding of a 13.5-kDa acidic protein. The expressed cell, FvTox1, of the cell-free *F. virguliforme* culture filtrates produced necrosis and chlorosis in susceptible SDS soybean cultivars. The SDS-susceptible soybean lines are highly sensitive to the expressed FvTox1 protein (Friesen et al., 2008). These discoveries indicate that the FvTox1 plays a major part in the pathogenicity factor for the development of SDS foliar symptoms in soybean.

When FvTox1 is expressed in an insect cell line, it will cause necrosis as well as loss of chlorophylls in soybean leaf discs. The loss of chlorophyll is, thus, a very usual foliar SDS symptom (Ji et al., 2006). However, only in the presence of light can the cell-free *Fv* culture filtrates that contains the phytotoxin will cause SDS foliar symptoms (Ji et al., 2006).

Role of FvTox1 in foliar SDS symptom development is quite unique where one of the mechanisms utilised by fungal pathogens to incapacitate plant defences is the induction of host cell death machinery. It has been shown that phytotoxins can trigger programmed cell death (PCD) that will lead to large scale necrosis and chlorosis in the host plant (Rep, 2005).

Furthermore, certain *Fusarium* species possess the ability produce trichothecenes deoxynivalenol (DON) and nivalenol (NIV), that are harmful toxins to the health of both animals and humans through association with certain pathologies—e.g., feed refusal and emesis or loss of appetite, ens (Schöneberg, et al., 2016).

F. poae and *F. graminearum* are typical type-B-trichothecene producers, the former being the greater NIV producer and the latter the greater DON producer (Schöneberg, et al., 2016, Osborne, et al., 2007, Stenglein, et al., 2014, Yli-Mattila, et al., 2008). DON-producing strains such as *F. graminearum* were found to inhibit the thickening of the host cell walls which is a defence mechanism of plants; therefore, it has been determined that the DON toxin facilitates the spreading of infection (Maier, et al., 2006).

Accordingly, the production of DON, NIV and other trichothecenes is controlled by a cluster of TRI genes, namely TRI10 (transcription factor regulating TRI6), TRI13 the most prevalent gene (cytochrome P450 oxygenase) which is used in our study, TRI4 (multifunctional cytochrome P450 monooxygenase catalysing the transition of trichodiene to isotrichodermin and trichothecene), TRI5 (encoding trichodiene synthase), TRI6 (zinc finger protease transcription factor that regulates the biosynthetic pathway) (Yli-Mattila, et al., 2008). *Fusarium* species and toxins associated with them contributes to the future development of viable management strategies, such as plant resistance, crop rotations, sanitation and the control of toxins in crop production.

However, attention has been focused on researching soybean in depth since the early 2000s, using a team approach by combining breeding and molecular methods for the domestication of plants and animals and development and needs of human society (Barzegar et al., 2019).

Soybean breeders worldwide are attempting to breed for resistance to SDS as part of a control strategy to contain this disease. However, it's challenging, because each breeder's objective is to create a resistant cultivar adapted to their region with high and stable yield and quality. Resistance is inherited quantitatively and is affected by environmental conditions, which makes it difficult to score disease severity reliably. It's still unknown if the stability or durability of improved commercial crops over a widespread range of environmental factors are associated with climate change despite important advances in plant breeding (Sinclair, 2011). Thus, its crucial to keep in mind that genetic variation in resistance to SDS is recognised in most parts of the world.

Breeding for yield, morphological traits and resistance to abiotic stress, are conceptually different from breeding for resistance to biotic stresses. Breeding for abiotic stress entails the manipulation of only one genetic system the plant. Breeding for resistance to biotic stress, is a different ballgame, it involves the manipulation of two genetic systems the plant (host) and organism (pathogen). Breeders shouldn't regard the two genetic systems as independent but to the understand the interaction and relationships between the two systems.

Resistance components include resistance to penetration and colonisation and the reduction of mycotoxin content. Many sources of partial SDS resistance have been identified, but no sources with complete resistance are known. Most measures, which have successfully worked with most fungal diseases, such as crop rotation, fungicides, deep ploughing, manipulation of plant density etc., have either completely failed or have been insufficiently effective with soybean SDS.

Disease resistance, therefore, is the only viable option. Though complex, disease resistance is described as an economic and durable control option for controlling most diseases (Butzen, 2006) and would be the most effective strategy for SDS management (Giammaria *et al.*, 2014).

The aim of this study is to identify and characterise the causal species of soybean sudden death in South Africa to assist in the breeding for resistance against the disease.

Material and Methods

Plant material and locations

Plant material used in this study was obtained from two soybean field trials that were planted for yield evaluations by the division of Plant Breeding at the University of the Free State during the 2016/2017 growing season. These trials were situated in Petrusburg (Free State) and Delmas (Mpumalanga), in South Africa with similar environments but different climates. The trials had 18 cultivars (Table 1) that comprised nine edamame and nine commercial soybean cultivars, and were planted in a randomized complete block design (RCBD), with three replications.

Table 1: List of 18 soybean cultivars in the field trials and their sources: EDP (Edamame Development Project), ARC (Agriculture Research Council).

Entry number	Cultivar	Description	Source
1	E429	Edamame	EDP
2	LS6161	Commercial	ARC
3	PAN1614	Commercial	ARC
4	PHB95Y20	Commercial	ARC
5	DM62i	Commercial	ARC
6	PAN1454	Commercial	ARC
7	PAN1521	Commercial	ARC
8	LS6248	Commercial	ARC
9	LS6146	Commercial	ARC
10	DM5953	Commercial	ARC
11	E353	Edamame	EDP
12	E335	Edamame	EDP
13	E292	Edamame	EDP
14	E418	Edamame	EDP
15	E382	Edamame	EDP
16	E432	Edamame	EDP
17	Tanba	Edamame	EDP
18	E457	Edamame	EDP

Fungal isolates:

Infected and healthy plants were sampled from SDS infected fields. Six whole seeds, six stem pieces and six pod pieces were excised from each plant.

Plant parts were surface-sterilised by immersing it into a 1.25% (v/v) sodium hypochloride solution for 3 min and then rinsing three times for 30 s with sterilised distilled water. Surface-sterilised plant parts were then plated onto Van Wyk's agar a semi-selective medium for *Fusarium* (Van Wyk et al., 1986) and incubated under a 12 h light/dark cycle for 4-7 days at 25°C. One *Fusarium* isolate was obtained per infected plant part.

After 4-7 days fungal hyphae were transferred to potato dextrose agar (PDA) and incubated in a sterile growth chamber with a 12 h light/dark cycle at 25°C for an

additional 4-7 days. The fungal isolates were also cultured on Spezieller Nährstoffarmer agar (SNA) (Nirenberg, 1976) plates until conidia formed. Cultures originating from SNA plates were purified by subculturing single spores for identification purposes following dilution plating onto fresh SNA (Leslie and Summerell, 2006).

Morphological identification

Purified *Fusarium* cultures were sub-cultured on PDA, SNA and carnation leaf agar (Fisher et al., 1982) for identification. Morphological characterisation followed the protocol of Summerell et al. (2003). All macroconidia used for identification purposes were obtained from sporodochia.

Table 2: Morphological characterisation followed the protocol of Summerell et al. (2003)

Colony description	Microconidia	Macroconidia	Phialides	Chlamydospores
Colour Sporodochia colour	Presence Shape Number of septa Formation	Presence Shape Number of septa Shape of apical cell Shape of basal cell	Mono Poly	Presence Position Shape Formation

Molecular validation

DNA extraction

All isolates morphologically identified as *Fusarium* species were sub-cultured into a liquid growth medium, potato dextrose broth, to grow adequate mycelia for DNA isolation. Cultures used were incubated in a 12 h light/dark cycle for 7-10 days at 25°C for optimal growth. On day seven, the mycelia were harvested over a Buchner funnel and filter paper. Mycelia were freeze-dried for three days at -60°C. Freeze-dried samples were stored at -80°C until further use.

Before isolation of total genomic DNA, the freeze-dried fungal material was homogenised using Qiagen's TissueLyser. A piece of the material was transferred to a 2 ml microcentrifuge tube with two 5 mm stainless steel ball bearings and homogenised for 30 s at 30 r/s. Isolation of total genomic DNA was done by using a CTAB (hexadecyltrimethylammonium bromide) DNA isolation method (Saghai-Maroof et al., 1984).

DNA quantity and quality were estimated from a 0.8% (w/v) agarose gel with electrophoresis at 80 V in UNTAN (40 mM Tris-Cl; 2 mM EDTA, pH adjusted to pH 7.4 with acetic acid) buffer. DNA was visualised with ethidium bromide staining under UV light. The concentration of the DNA samples was determined by using a UV spectrophotometer and measuring absorbance at A_{260} and A_{280}. Samples were diluted to a working concentration of 200 ng/µl for subsequent experiments.

Species-specific PCR

The identity of these 53 isolates as *F. graminearum* and *F.virguliforme* was confirmed by using species-specific PCR assays. The Fg16F and Fg16R primer pair combination was used to amplify a 410 bp fragment associated with *F. graminearum* and the Fsg1/Fsg2 and FsgEF1/ FsgEF2 primer pair combinations was used to amplify a 438 bp and 237 bp fragment, respectively. These primer combinations are associated with *F. virguliforme*.

The reaction mixtures were prepared to a total volume of 20 ul which was made up from 4 µl Promega Buffer, 1.25 mM of each dNTP's and 25 mM MgCl2. For each reaction 5U/µl Taq polymerase, 50 pmol/ µl of each primer and approximately 50 ng/ µl of fungal template DNA were used. Reactions were performed in a GeneAmp® PCR system 9700 thermal cycler (Applied Biosystems, USA) using the following PCR conditions: denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 45 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min, followed by cooling at 10°C until recovery of the samples. PCR amplification products were separated by electrophoresis through a 2% (w/v) agarose gels stained with ethidium bromide (Mule etal., 2004).

Mycotoxin PCR

The mycotoxin genotype of the morphologically identified *F. virguliforme* and *F. graminearum* isolates were determined following PCR amplification with specific PCR primer pairs. For *F. graminearum* the primer pair combination Tri13/Tri13R that targets the Tri13 gene was used to identify both genotypes in a single reaction. By amplifying a DNA fragment of 1075 bp associated with the NIV allele and/or a 799 bp fragment associated with the DON allele. With *F. virguliforme* primer pair combination FvTox1F/FvTox1R targets the FvTox1gene and was used to amplify a 483 bp fragment associated with the FvTox1 allele.

Same reaction mixtures were prepared as in with species specific PCR for mycotoxin PCR. However, the PCR conditions do differ: denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 min, followed by cooling at 10°C until recovery of the samples. PCR amplification products were also separated by electrophoresis through a 2% (w/v) agarose gels stained with ethidium bromide (Mule etal., 2004).

Pathogenicity profile

Plant material

Fifteen test cultivars (Table 1), based on resistance of cultivars were used to test the pathogenicity of the pre-dominant Fusarium isolates associated with SDS.

Pathogenicity test

Not all the Fusarium isolates were subjected to pathogenicity tests. Only selected isolates from the pre-dominant Fusarium species associated with SDS such as *F.*

virguliforme and *F. graminearum* (must still be confirmed) were selected based on pathogen growth rate (Fast; intermediate; slow) together with 2 reference isolates (1 *F. virguliforme* and 1 *F. graminearum*)

Seedling inoculation assay

Method described by Tewoldemedhin et al., (2017) was used. Isolates were cultured and incubated under a 12 h light/dark cycle for 4-7 days at 25°C on PDA in a sterile growth chamber. Selected *Fusarium* cultures were grown in potato sucrose broth to obtain a spore suspension.

Ten seeds per cultivar (15 cultivars) were germinated and only seeds with 10-15 mm sprouts were inoculated for evaluation. The phenotypic evaluation was done in a greenhouse cubicle with temperatures set at 18/24°C minimum/maximum. Selected isolates and the control seedlings were submerged in a spore suspension ($1x10^5$) and incubated at 22-25°C for 4-5 h, before transplanting. A non-inoculation was included as a negative control. Three seeds were planted per pot. SDS symptoms were visually evaluated on 7, 14 and 21 days after inoculation on a scale of 1-5, where 1 = 10%, low infection; 2 = 10 – 20% moderate low infection; 3 = 20 – 50% moderate infection; 4 = 50 – 80% high infection and 5 = 80 – 100% total infection (Tewoldemedhin et al., 2017).

Results

Morphological identification

Morphological characterisation followed the protocol of Summerell et al. (2003) was conducted on 53 purified *Fusarium* isolates, out of these: 27 *F. virguliforme*, 17 *F. graminearum*, 5 *F. oxysporum*, 3 *F. sporotrichioides* and only one *F. equiseti* were identified. A total of 14 isolates originated from Delmas. Only *F. virguliforme* and *F.graminearum Fusarium* species were identified. Out of the 14 isolates 8 *F. graminearum* were identified which were in higher frequency than *F. virguliforme* where only 6 were identified.

In Petrusburg there were more variation of *Fusarium* species in the field where a total of 39 isolates out of the 53 purified *Fusarium* isolates were identiefied as: 21 *F. virguliforme*; 9 *F. graminearum*; 5 *F. oxysporum*; 3 *F. sporotrichioides* and1 *F. equiseti*.

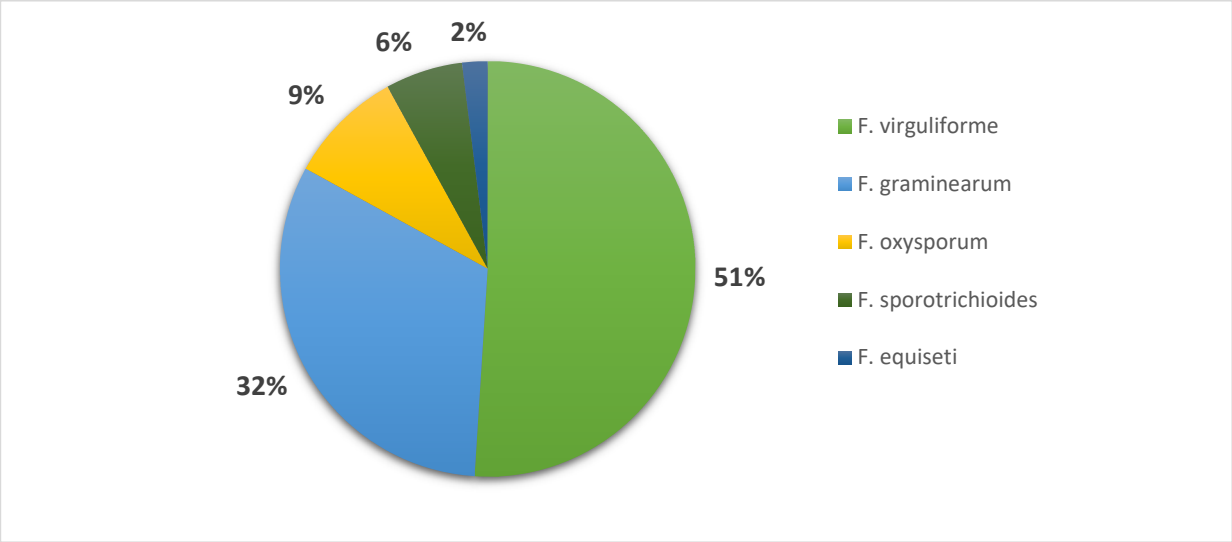

Figure 1: 5 Different Fusarium species identified in the field.

From the SDS infected fields seeds, stem and pod pieces were excised from healthy and infected plants were 53 purified *Fusarium* isolates were identified based on morphological characteristics described by Summerell et al. (2003). Out of 53 purified *Fusarium* isolates 28 isolates derived from the lower stems, 21 from pods and 4 from seeds. The highest frequencies of *Fusarium* species present were *F. virguliforme* and *F. graminearum. Fusarium* species *F. graminearum* and *F.virguliforme* was confirmed by using species-specific PCR assays. *F. virguliforme* is a known associate with SDS of soybean, however, we cannot ignore the fact that *F. graminearum* is in such high frequencies thus only they were focused on.

Mycotoxin profile

Morphologically identified *F. virguliforme* and *F. graminearum* isolates mycotoxin genotypes were determined following PCR amplification with specific PCR primer pairs. From the 27 *F. virguliforme* isolates only 22 amplified fragments for the FvToxI mycotoxin, five isolates did not amplify any fragments. However, 13 isolates of the 17 *F. graminearum* isolates amplified fragments for DON accumulation and four isolates amplified fragments for NIV accumulation. This shows that DON accumulation is much more dominant in the field.

Pathogenicity profile

Seedling inoculation assay described by Tewoldemedhin et al., (2017) was employed on 27 *F. virguliforme* and 17 *F. graminearum* isolates confirmed by using species-specific PCR assays.

For the infection on the five isolates of the 27 *F. virguliforme* isolates that did not amplify any fragments with the mycotoxin tests, showed the lowest infection with a disease rating of 1 (10%). Not only did these five isolates obtained the lowest disease rating it was also observed that these isolates were all fast growers for the pathogen. Seven isolates showed moderate infection with a disease rating of 3 (20 – 50%), these isolates were included intermediate and fast growers for the pathogen. High infection

was observed by 15 isolates with a disease rating of 4 (50 – 80%). Included in these isolates slow, intermediate and fast growers of the pathogen was observed.

Of the 17 *F. graminearum* isolates 13 was identified for the accumulation of DON. These isolates showed high infection with a disease rating of 4 (50 – 80%), these isolates were observed to be intermediate and fast growers for the pathogen. However, the 4 NIV producing isolates were all slow growers of the pathogen and showed a low infection with a 1 (10%) disease rating. DON producing isolates are more virulent than NIV producing isolates. A total of 56% high infection; 25% moderate infection and 19% low infection was observed for both fungal populations.

Discussion

Morphological Profile

Distinguishing species within the *Fusarium solani* species complex using morphological characters is difficult even for specialists (Summerell et al, 2003), so DNA sequence-based identifications and species-specific PCR assays are usually needed to accurately identify species within the complex. Thus, we used species-specific PCR and sequence analyses in some cases to confirm our morphological identifications.

Five Fusarium species were morphologically identified based on morphological characteristics described by Summerell et al. (2003). Three *Fusarium* species *F. oxysporum*, *F. sporotrichioides* and *F. equiseti* were in very low frequencies whereas *F. virguliforme* and *F. graminearum* were in higher frequencies among the isolates thus also in the field. Both *F. virguliforme* and *F. graminearum* are soilborne diseases, affecting roots and lower stems of its host plat. Thus, it correlates with the highest number of isolates identified from the lower stems of the infected plants. Disease that are not seedborne correlates with lowest number of isolates. *F. virguliforme* produces macroconidia on the taproot's surface during the summer, these iconic blue spores are only dispersed a short distance within a growing season.

SDS is caused by different *Fusarium* species within the *Fusarium solani* species complex (Aoki et al., 2003). Known *Fusarium* species that causes SDS of soybean are *F. basiliense* (United States and Brazil), *F. cuneirostrum* (United States, Brazil, Canada and Japan), *F. tucumaniae* (Brazil and Argentina), *F. phaseoli* (United States), however, *F. virguliforme* is the most prevalent SDS causal species worldwide.

F. graminearum causes several diseases, such as Gibberella stalk rot, ear rot, and kernel rot in maize, and head blight of wheat. Wheat and maize rotation are a popular dryland cropping system, and increases both wheat and maize diseases caused by *F. graminearum*. However, in South Africa it is also common to implement soybean and maize rotation. *F. graminearum* also survives in the soil on crop residue and infect the roots of its host plant. *F. graminearum* has never before been associated with SDS, *F. graminearum* is indeed a cosmopolitan, however, we cannot ignore the fact that it is in such high frequencies in the field and be part of the Fusarium complex associated with SDS of soybean.

Over all *F. virguliforme* was in higher frequency than *F. graminearum* in the fields situated at Delmas and Petrusburg. Environmental conditions are more or less the same there is not much difference between the average temperature & rainfall in Delmas and Petrusburg (Table 3). The climates however do differ with higher levels of humidity from Delmas and less humid from Petrusburg. Scherm and Yang (65) indicate that high humidity, soil moisture and low temperatures during the early part of the growing season, but warmer temperatures during soybean reproduction, are optimal for SDS symptom expression. Thus, it might suggest the higher frequency of *F. virguliforme* over *F. graminearum* in Petrusburg vice versa.

Table 3: Climate characteristics of trial sites.

	Petrusburg	**Delmas**
GPS coordinates	29°29'30.5"S 25°31'12.1"E	25°59'11.0"S 28°31'28.5"E
Climate		
Annual summer rainfall	450 mm	540 mm
Maximum summer temperature	31.5ºC	27.8ºC
Minimum summer temperature	13.7ºC	14.8ºC
Relative humidity (summer)	82.64%	97%
Soil characteristics		
Ca (mg/kg)	946	309
Mg (mg/kg)	306	61
K (mg/kg)	258	239
Na (mg/kg)	44	6.0
Zn (mg/kg)	3.9	-
P (mg/kg)	24.66	103
pH (H_2O)	7.52	5.44

On the other hand, as already discussed soybean cyst nematodes (SCN) have been associated with *F. virguliforme* occurrences (Rupe et al., 1991; Gibson et al., 1994; Rupe et al., 1994). The chlamydospores of *F. virguliforme* is capable of overwintering in the SCN and can consequently cause severe SDS yield losses in the next growing season (Roy et al., 1997). SCN have not been associated with *F. graminearum.* Weather conditions which favour maximum soybean yields are those which favour maximum SCN reproduction. In years with dryer conditions, especially in sandy soils, yield losses are higher.

In a regional survey, higher SCN populations have been associated with sandier, well drained soils such in Petrusburg. Fields with high clay content soils such as Delmas tend to have lower SCN populations. Petrusburg soils have a high soil pH this also associated with high SCN populations (Table 3). Thus, SCN can also be one of the factors to the higher frequencies of *F. virguliforme* over *F. graminearum* in Petrusburg.

Mycotoxin Profile

Once established in the xylem, the pathogen produces toxins which are systemically transferred throughout the plant leading to the foliar symptoms. Toxin production and subsequent transport depends on the presence of light and is increased under wet and humid conditions. Other diseases associated with said conditions as well as invading *F. graminearum* may increase the infection potential and consequently disease severity. PCR amplification for *F. virguliforme* with primer pair combination FvTox1F/FvTox1R that targets the FvTox1gene was used to amplify a 483 bp fragment associated with the FvTox1 allele.

One of our objectives was to see if mycotoxin contribute to pathogenicity of the disease. The five isolates that did not amplify any fragments also showed low disease infection. No amplification of the five isolates meant that no mycotoxin accumulation in the host plant, hence the low disease rating. However, 22 isolates that did indeed amplify fragments that corresponds with the accumulation of mycotoxins showed moderate (7 isolates) and high infection ratings (15 isolates). Therefore, it suggests that mycotoxins contribute to pathogenicity of a disease.

The most common species of toxin-producing *Fusarium* contaminating crops are *Fusarium graminearum*; with both elaborating diverse toxins, especially deoxynivalenol (DON) and nivalenol (NIV), respectively are hazardous to the health of both humans and animals through association with certain pathologies. Severity of infection by *F. graminearum* relays completely upon the production of the DON. Reports suggest that factors, such as water activity, temperature, and growth time, has a direct influence on DON and NIV production in *F. culmorum, F. graminearum*, and *Fusarium meridionale* (Llorens, et al., 2004, Hope, et al., 2005, Rybecky, et al., 2018).

Llorens et al. (2004) reported that the most favourable temperature for the growth of *F. graminearum* ranged from 20 °C to 32 °C, and fungal growth was reduced at 15 °C. The expression studies of the *Tri* cluster showed that it required an optimal water activity for the expression of all the genes (Hope, et al., 2005). The optimal temperature for the production of DON is 28 °C, whereas NIV production depends on the species, strain, and also on the temperature (Llorens, et al., 2004). Hence, NIV accumulation is in lower frequencies than DON accumulation in the field.

Pathogenicity Profile

Mycotoxin can be associated with pathogenicity as already mentioned. Hence, the growth rate of the pathogen might also be correlated with pathogenicity. Our other objective was to test whether pathogenicity is positively correlated with parasite growth rate. The five isolates that showed low infection rate also displayed slow growth of the pathogen. However, isolates that demonstrated high infection rates, 25%-56% of the *F. virguliforme* isolates obtained slow, intermediate and fast growers for the pathogen. In contrast with the standard assumption that increased pathogen growth leads to greater host mortality, we found the opposite that there was no correlation between growth rate and pathogenicity.

This does not demonstrate that the trade-off theory for pathogenicity is inaccurate or does not exist. The no correlation between growth rate and pathogenicity could occur because other life-history details that determine how pathogens infect and exploit their hosts change the predicted trade-off between growth, transmission and pathogenicity.

On the contrary, with the *F. graminearum* isolates only four amplified any fragments for NIV accumulation which demonstrated low infection rate and slow growers of the pathogen. Where 13 DON accumulation isolates presented high infection rates which included intermediate and fast growers for the pathogen. This observation might suggest that DON accumulation is more virulent than NIV accumulation under the certain environmental conditions.

Conclusions

Five species of *Fusarium* were identified from two soybean field trials that were planted for yield evaluations by the division of Plant Breeding at the University of the Free State during the 2016/2017 growing season. In previous studies *F. virguliforme* is confirmed to be the causal pathogen responsible for SDS foliar symptom development of soybean worldwide. However, there have been speculations that *F. basiliense* could be the causal pathogen for SDS of soybean in South Africa.

Conversely, the morphological identification and characterisation of together with species specific PCR confirms that *F. virguliforme* is present at both locations which is situated in different provinces. Thus, *F. virguliforme* can be the only *Fusarium* species associated with SDS on soybean in South Africa. However, the fact still remains that *F. graminearum* was in such high frequencies at both locations that it could also have associations with SDS of soybean. To confirm *F. graminearum* is associated with SDS of soybean further studies are recommended.

Mycotoxin correlations with pathogenicity of the disease, have been discussed. There was no indication of mycotoxin potential associated with low infection rate isolates. Though, all isolates indicating mycotoxin potential demonstrated either moderate or high infection rates. Also, high infection rate isolates displaying slow, intermediate and fast growers of the disease suggest that growth rate does not correlate with pathogenicity. Our ability to confirm some relationships with pathogenicity such as the growth rate of the disease while questioning the effect of other variables for instance our lack of plant material. Highlights the value of the objective in further understanding the evolution of pathogenicity by conducting further studies with adequate plant material.

The identification and characterisation of SDS causal species will enable the improvement of management and control strategies with the emphasis on disease resistance breeding.

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