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The morphological and molecular variability of *Sporisorium scitamineum* isolates from Eswatini

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Abstract

Sugarcane smut is a disease of economic importance in the sugarcane industry, occasioning losses of up to 50%. Current management practices have been ineffective in controlling the fungal disease, and hence the understanding of the pathogen and the development of appropriate control measures are required. This study investigates the morphology, virulence and molecular variability of *Sporisorium scitamineum* isolates from Eswatini to understand its pathogenicity for effective control. Fungal isolates were collected along the Sugarbelt in the Lowveld of Eswatini. The isolates were verified by polymerase chain reaction using the *bE*4 and *bE*8 specific primers with amplification of a 459bp fragment. The identity was further verified by DNA sequencing. The teliospores from the isolates were uniform in brown colour, spiny texture and circular shape. The teliospore sizes were significantly (*P*=0.05) different among the isolates. The isolate from Big-Bend had a mean spore diameter of 5.55µm, while Simunye, Nsoko and Mhlume had average diameters of 4.69µm, 4.98µm and 4.87µm, respectively. The documentation of the developmental stages revealed that the samples were of variable virulence with significantly (*P*=0.05) different rates of promycelium development. The variable virulence of the isolates was further confirmed by the *in-vivo* pathogen biomass quantification by RT-qPCR. Genetic distance matrix analysis and cluster analysis showed high homology (99-100%) among the local isolates which indicate that there is a low variability among the strains that are found in the selected sugarcane growing areas of Eswatini.

Keywords: *Sporisorium scitamineum*; sugarcane smut; molecular analysis; morphology analysis; fungi.

Introduction

Sugarcane**,** a perennial grass belonging to the *Poaceae* family, is primarily cultivated for its juice from which sugar is processed with other products and by-products such as biofuel, ethyl alcohol (ethanol), molasses, rum, straw and bagasse. The plant itself can be used as thatching material and as livestock fodder (Yamane, 2018). Sugarcane production is faced with several insect pests and diseases, and among these is the sugarcane smut, which is caused by the fungus *Sporisorium scitamineum* (Syd) M. Piepenbr., M. Stoll & Oberw (Comstock, 2000; Nalayeni et al., 2021).

Sugarcane smut is a disease of economic importance in the sugarcane industry as it can cause losses that range between 10% and 50% (Schenck et al., 2013; Rajput, 2021). The disease is considered a major constraint to sugarcane production because of its potential to spread quickly and cause considerable losses, as each sorus can release about a 100million teliospores a day (Cui et al., 2020; Jacques-Edouard et al., 2020, Bhuiyan et al., 2021). The fungus affects the stalk yield as well as the sucrose content of the sugarcane. *Sporisorium scitamineum* produces two types of haploid teliospores which are complementary to each other (Croft et al., 2006). Teliospores that are deposited on wet surfaces of healthy cane will germinate and fuse to form a dikaryon mycelium, which then develops to grow systematically in the plant to infect the meristem tissue (Liu et al., 2017, Vincente, 2021).

A few months after infection of the meristem tissue, the sugarcane develops a long sorus, which is called a whip. The sori appear on the growing points, and this is one of the recognizable symptoms of sugarcane smut. Apart from the sorus, other recognizable symptoms are the grassy, weak and lanky appearance of the stalks (Que et al., 2014, Vincente, 2021).

Sugarcane smut is primarily managed by the use of resistant varieties. However, this is sometimes difficult to achieve because the *S. scitamineum* sometimes produces new physiological strains that are not inhibited by the host resistance mechanism, consequently rendering breeding programmes expensive and time-consuming (Jacques-Edouard et al., 2020). There is also the possibility of a resistant variety being susceptible in another region because of the existence of different strains of the fungus. The reaction of different varieties of sugarcane is therefore expected to vary from country to country and from region to

region (Singh et al., 2005). Hitherto, the variability of *S. scitamineum* has been documented based on morphological traits (Jacques-Edouard et al., 2020). To optimise breeding strategies, it is important to understand the genetic variation of the pathogen populations in the target area (Bhuiyan et al., 2021).

The level of disease severity can be dependent on the resistance or susceptibility of the host plant and the virulence of the pathogen. The difference in the virulence of the pathogen can be observed through the rate of germination and colonization of the fungus in the plant (Su et al., 2016; Deng, 2020). The rate of teliospore germination and colonization of a virulent isolate is significantly higher than that of a less virulent isolate. The low virulent strains possess dormancy in the initial growth stages. This difference can often be seen during the comparison of the developmental stages of the pathogen isolates *in-vitro* (Nalayeni et al., 2021; Su et al., 2016).

The fungal Internal Transcribed Spacer (ITS) region has the highest likelihood of identification for a wide range of fungal pathogens and is an important locus in phylogenetic studies (Martin, 2002; Thomas and Richard, 2004; Benevenuto, 2016). The use of the nuclear ribosomal large unit in identification is less effective than using the ITS, while the nuclear ribosomal small unit has a poor species-level resolution (Schoch et al., 2011). The ITS region is relatively easy to amplify from virtually all fungi mainly due to its multicopy arrangement and highly conserved priming sites (Hibbertt, 1992; Zambino et al., 1993).

The existence of the pathogenic races of *S. scitamineum* in different environments and locations presents challenges for uniform control of the pathogen either through breeding or other biological means (Luzaran et al., 2012). The emergence of new physiological strains of the pathogen also causes challenges to control this pathogen (Jacques-Edouard et al., 2020). Therefore, the identification and knowledge of the local pathogens are essential in the management of *S. scitamineum.*

This study aims to determine the morphology, development patterns and molecular variability of the *S. scitamineum* isolates in Eswatini to understand the pathogenicity of the fungus. Hitherto, the variability of the fungal isolates in Eswatini was not investigated, hence the challenges in managing the disease. This study will bring about insight on the basis of prospective management of the pathogen using biological agents or resistant varieties.

Results

Identification of the fungus

To confirm the identity of the fungus, the collected isolates were screened by conventional PCR using the *S. scitamineum*-specific primers *bE*4 and *bE*8. The four isolates had a positive amplification of a 459bp fragment (Figure 1), which was consistent with the findings by (Izadi and Moosawi-jorf, 2007;. The identification of the pathogen was also verified by the sequencing of the internal transcribed spacer (ITS) region which produced a sequence of 725bp.

Morphology analysis

The *S. scitamineum* teliospores were circular with a spiny membrane and were brown for all the isolates. Although the surface texture of the teliospores was not visible under a light microscope, it was possible to see the spiny nature of the spore membrane under the SEM at a magnification = 7000x. The isolates had different teliospore sizes. The isolate from Big-Bend had a mean diameter of 5.55µm, while Simunye, Nsoko and Mhlume had an average diameter of 4.69µm, 4.98µm and 4.87µm, respectively (Figure 2), which was consistent with the findings of Bhuiyan et al. (2021). The size analysis revealed that only the isolate from Big-Bend was significantly (at $P < 0.05$) larger when compared to the other isolates.

Documentation of the in-vitro development of the isolates

The means of the four samples of *S. scitamineum* obtained from the development rate of the promycelium length measured at 3-, 6-, 12- and 18hours post culturing (Figure 3). At three hours, there was a significant difference (at $P<0.05$) in the growth of the promycelium as the sample from Mhlume recorded a mean growth of 14.65µm, while Nsoko had a mean of 15.60µm, Simunye had 5.55µm and Big-Bend had no growth. At six hours the isolate from Nsoko has a significantly ($P<0.05$) higher mycelium development, while Big-Bend had a significantly low development. At 12 hours the isolate from Mhlume had a significantly ($P<0.05$) high mycelium length when compared to the other isolates. At 18 hours post culturing, there was no significant difference between the isolates from Mhlume, Nsoko and Simunye, while the isolate from Big-Bend was significantly lower ($P<0.05$), the highest mycelial growth was observed in the isolate from Mhlume which had a mean of 46.97µm, followed by the isolate from Nsoko with a mean of 36.35µm, while the isolates from Simunye and Big-Bend had means of 34.75µm and 5.15µm respectively.

Absolute quantification of pathogen biomass

The *bE* primer was used for the RT-qPCR amplification. This primer was first amplified using conventional PCR to confirm the presence and size of a 179bp amplicon (Figure 4). This confirmation provided confidence for using the primer for the RT-qPCR assays.

The rate at which the different isolates colonize the host plant was assessed by absolute RT-qPCR quantification five days after inoculation. The isolates from Nsoko and Mhlume had significantly ($P < 0.05$) high concentrations of 11.63 and 11.35, respectively when compared to the isolates from Big-Bend and Simunye, which had concentrations of 5.02 and 4.67, respectively (Figure 5).

Sequence analysis of the isolates

The genetic distance matrix and cluster analysis of the ITS sequences of the *S. scitamineum* samples analysed using the MEGA X software (Tamura *et al*., 2004; Tamura *et al*., 2021) are shown in Table 1 and Figure 6. This analysis had a total of 13 nucleotide sequences which were the four smut isolates from Eswatini and nine other ITS sequences that were found on the NCBI database and an out-group of *Sporisorium sorghi* (JX183795). The genetic distance matrix showed the sequences to be having a high homology of 99% - 100%. The high homology was also observed when comparing the local isolates in this study with the other isolates from South Africa (DQ004830 and MH865951), China (MZ470432, MZ470433, EF185066, EF185072 and EF185076) and India (KP 893340) that were obtained from the NCBI database (Table 1).

Table 1. Genetic distance matrix of the ITS sequences of *S. scitamineum* isolates from Eswatini analysed alongside other isolates from China, India and South Africa.

	Mhlume	Nsoko	Simunye	Big-Bend	DQ004830	EF185066	EF185072	MH865951	MZ470432	MZ470433	EF185076	KP893340
Mhlume												
Nsoko	0.001											
Simunye	0.001	0.000										
Big-Bend	0.000	0.001	0.001									
DQ004830	0.000	0.005	0.001	0.000								
EF185066	0.004	0.004	0.006	0.004	0.004							
EF185072	0.003	0.013	0.004	0.003	0.003	0.001						
MH865951	0.000	0.001	0.001	0.000	0.000	0.004	0.003					
MZ470432	0.000	0.001	0.001	0.000	0.000	0.004	0.003	0.003				
MZ470433	0.013	0.004	0.004	0.003	0.003	0.001	0.000	0.003	0.003			
EF185076	0.000	0.001	0.001	0.000	0.000	0.004	0.003	0.000	0.000	0.003		
KP893340	0.014	0.006	0.006	0.004	0.004	0.002	0.001	0.004	0.004	0.001	0.004	
MW692228	1.685	1.679	1.682	1.707	1.681	1.751	1.747	1.705	1.685	1.712	1.718	1.715

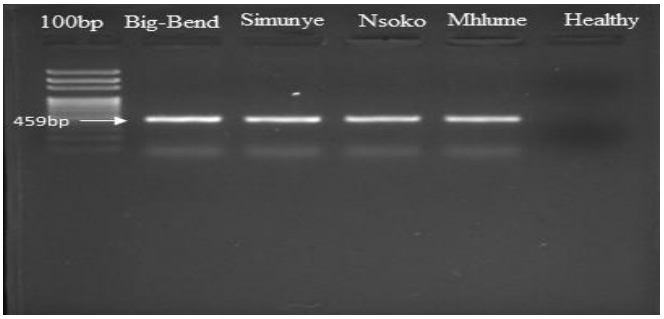

Figure 1. Gel documentation indicates the amplification of the fungal genomic DNA using the *S. scitamineum* specific primers, *bE4* and *bE8*, that were used to confirm the identity of the pathogen. The primers had a positive amplification with a 459bp amplicon size.

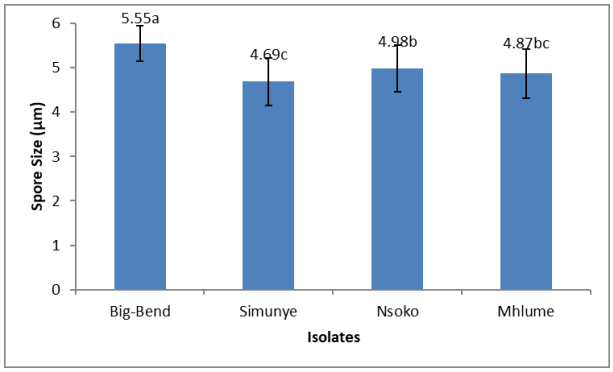

Figure 2. The teliospore size of different *S. scitamineum* isolates from Eswatini. The diameter was determined for 40 teliospores per isolate and there was a significant difference (P<0.05) among the spore diameters.

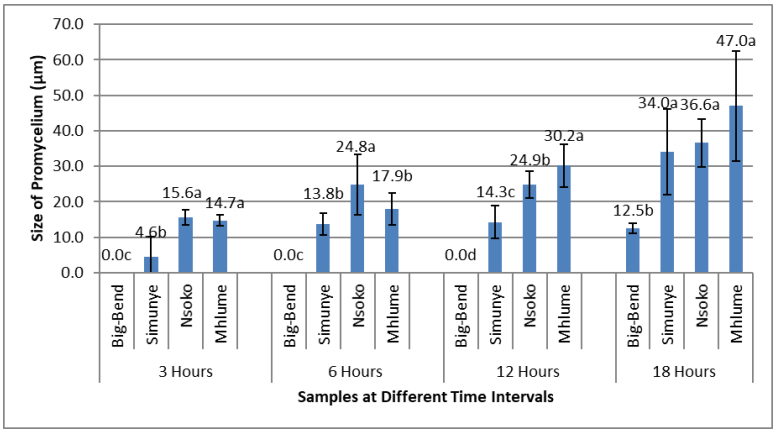

Figure 3. *In-vitro* development rates of the promycelium (mean length (µm)) of the four *S. scitamineum* isolates measured 3, 6, 12 and 18 hours after culturing. The standard deviations from the means are represented by the error bars. There was a significant difference (P<0.05) in the development of the promycelium at all the time intervals.

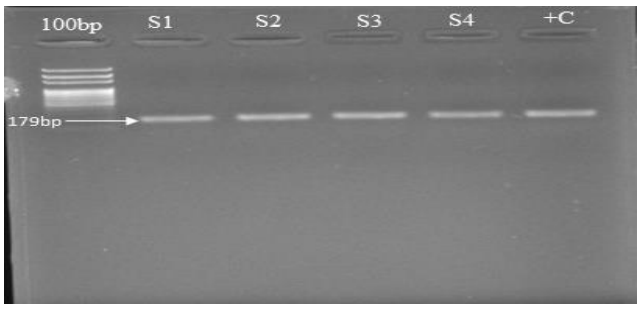

Figure 4. Confirmation of amplification of the *bE* primers by yielding a 179bp fragment on conventional PCR before using them for RT-qPCR amplification. The amplification was done on the four isolates (S1 – S4) and pure fungal DNA as the positive control.

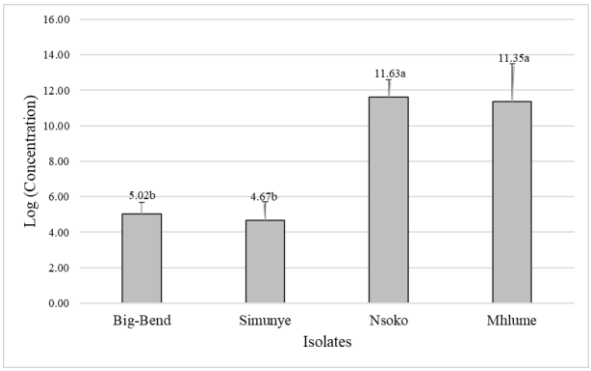

Figure 5. The absolute quantification of the in-planta pathogen biomass of four *S. scitamineum* isolates five days after inoculation. There was a significant difference (P<0.05) in the pathogen biomass of the different isolates in the inoculated sugarcane.

Figure 6. Cluster analysis of the ITS sequences for the *S. scitamineum* isolates from Eswatini analysed along with other isolates from China (MZ470432, MZ470433, EF185066, EF185072 and EF185076), India KP893340) and South Africa (DQ004830 and MH865951) and an out-group of *Sporisorium sorghi* (JX183795).

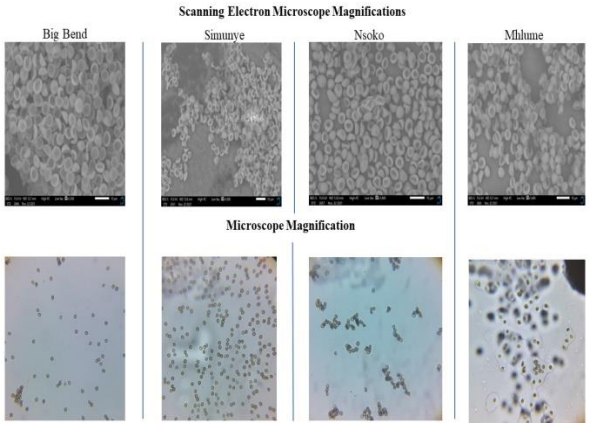

Figure 7. Morphology analysis of the teliospore isolates from the different areas using a Scanning Electron Microscope and a light microscope.

Discussions

Identification of the fungus

DNA fingerprinting is a molecular technique that has been reliably used to identify organisms by recognizing precise sections of their genome. It can be further used to determine the genus, species and strain of that particular organism. The b-East (*bE)* mating-type gene has been previously used to determine the identity of *S. scitamineum* isolates. In this study, the detection of this gene in the isolates by the specific primers *bE4* and *bE8* produced PCR amplicons that are 459bp in length (Izadi and Moosawi-jorf, 2007; Zhang et al., 2015).

Morphology analysis

The morphological analysis of the teliospores using a light microscope enabled the determination of the shape and colour of the spores. The size and texture of the teliospores were determined using the SEM. Morphological characteristics of the teliospores can be used to determine if the isolated pathogen from the different areas or regions is composed of different strains of *S. scitamineum* (Singh *et al*., 2005). Challenges to controlling sugarcane smut have been attributed to the production of new physiological strains of the pathogen (Jacques-Edouard *et al*., 2020). The isolates that were found in Eswatini had a similar appearance in colour. There are studies where isolates with different colours (white and yellow) have been found in the same country (Jacques-Edouard et al., 2020).

Documentation of the in-vitro development of the isolates

The time intervals provided information to determine the virulence patterns of the collected isolates. *Sporisorium scitamineum* teliospores are known to start to germinate after 20 minutes upon culturing *in-vitro* (Izadi and Moosawi-jorf, 2007). The *in-vitro* developmental assay was able to identify the virulent isolates by observing the rapid germination and growth. The less virulent isolates were dormant at the early stages of the assay and they had a slower development rate when compared to the virulent isolates. Su et al. (2016) and Nalayeni et al. (2021) observed that the rate of teliospores' germination and colonization of a highly virulent isolate is significantly higher than that of a less virulent isolate. The low virulent strains possess dormancy in the initial growth stages. This difference can often be seen during the comparison of the developmental stages of the pathogen *in-vitro.* The isolates from Nsoko and Mhlume were observed to be highly virulent when compared to the isolates from Big-Bend and Simunye, although their morphology was suggestive of belonging to the same strain.

Absolute quantification of pathogen biomass

The high pathogen biomass that was observed in sugarcane plants that were inoculated with the isolates from Nsoko and Mhlume, when compared to the plants that were inoculated with the isolates from Big-Bend and Simunye shows that the isolates from Nsoko and Mhlume were more virulent than the isolates from Big Bend and Simunye. The ability of the fungus to rapidly colonize the host is attributed to the virulence factors of the fungus. The fungus secretes protein molecules that overcome the plant's defence system, protect the fungus against the plant's immune system and signals for the fungus to build up a critical mass to put up resistance to the plant's defence system (Vincente et al., 2021). These results confirm the findings of the *in-vitro* development assays that were done in this study, as well as the findings of Nalayeni et al. (2021). This shows that there is a relationship between the rate of mycelial development *in-vitro* and the virulence of the pathogen *in-vivo*. Su et al. (2016) also observed a positive correlation between the time after inoculation and the amount of smut pathogen in sugarcane. The biomass quantification of the smut pathogen can be effectively evaluated from four to seven days after inoculation, especially in susceptible genotypes.

Sequence analysis of the isolates

The high homology (99% - 100%) of the isolates could be an indication that the *S. scitamineum* isolates have not evolved that much, globally. These results also suggest that the ITS region of the *S. scitamineum* is highly conserved in the pathogen. The sequences were deposited to the GenBank and they were assigned the following accession numbers: Big-Bend – ON366376, Mhlume – ON366377, Nsoko – ON366378 and Simunye – ON366379. The close relationship of the isolates is also observed in the phylogenetic relationships. A close relationship between the isolates ON3663760 (Big-Bend) and ON366377 (Mhlume) was observed as they formed their clade, while the isolate ON366378 (Nsoko) formed the same clade with the isolate ON366379 (Simunye). The isolates from Mhlume and Big-Bend were also observed to have evolved more than the isolates from Nsoko and Simunye. The bootstrap values, at 1000 replications, showed strong support for the isolates from Mhlume and Big-Bend with a bootstrap figure of 96, while the isolates from Nsoko and Simunye had a weakly supported confidence value of 56 (Tamura et al., 2004; Tamura et al., 2021).

Materials and methods

Sample collection and culturing

This study was done in Eswatini during the 2021/2022 sugarcane-growing season. The smut-infected samples were obtained from the main sugarcane growing areas, namely Big-Bend, Simunye, Nsoko and Mhlume where the smut-infected plants were identified. The fields with the smut-susceptible sugarcane variety N25 were scouted for collection. Visible sori were cut from the infected sugarcane plants and bagged to prevent any spread to healthy plants. A total of 16 isolates were collected from all the locations. The teliospores were maintained at the molecular biology laboratory in the Faculty of Agriculture, University of Eswatini. These spores were then rinsed three times with distilled water and cultured in potato dextrose agar (PDA). The plates were incubated in darkness at 28^{o}C (Singh *et al*., 2005; Cui *et al*., 2020). To purify the cultures, single colonies were transferred onto new plates and incubated in darkness at 28^{o}C (Que et al., 2014).

DNA extraction, amplification and sequence analysis

DNA extraction for pathogen verification

The fungal genomic deoxyribonucleic acid (DNA) was extracted from mycelia using a Zymo Fungal/Bacterial Genomic DNA Extraction Kit (Inqaba Biotech, South Africa), following the instructions of the manufacturer. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and a nanodrop spectrophotometer. To verify the identity of the fungus, the DNA that was extracted

from the samples was amplified on conventional PCR using the *bE*4 (5'- CGCTCTGGTTCATCAACG - 3') and *bE*8 (5'- TGCTGTCGATGGAAGGTGT - 3') primers that are specific for *S. scitamineum* (Izadi and Moosawi-jorf, 2007; Zhang et al., 2015).

Conventional PCR amplification was carried out in a 25 μL volume containing 1µl of 0.1ng/µl gDNA, 12.5μL of 2x OneTaq master mix, 0.5μL of each of the upstream and downstream primers and 10.5μL of water. The PCR amplification was performed following a thermal cycling programme of 95^{o}C for 5min; 35 cycles of 95^{o}C for 30s, 52^{o}C for 30s, and 68^{o}C for 40s; and a final extension at 72^{o}C for 5min. The PCR amplicons were checked for quality in a 1% agarose gel electrophoresis and then documented.

DNA extraction for sequencing

Fungal genomic DNA was extracted using a using Quick-DNA^{TM} Zymo Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005, from Inqaba Biotech, South Africa) following the instructions of the manufacturer. The quality and concentration of DNA were analysed using 1% gel electrophoresis and a nanodrop spectrophotometer. The DNA was sent for sequencing at Inqaba Biotech, South Africa. Sequencing was based on the ITS region of the fungal genomic DNA using the ITS1 (5'- TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5' - TCCTCCGCTTATTGATATGC - 3') primer pair (White *et al*., 1990; Zhang *et al*., 2015). The ITS target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486).

The PCR products were run on a 1.2% gel and extracted from the gel using the Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye^{TM} Terminator Cycle Sequencing Kit V3.1 and BRD3-100/1000) and purified (Zymo Research, ZR96 DNA Sequencing Clean-up Kit^{TM}, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction of every sample. The CLC Bio Main Workbench v7.6 was used to analyse the .ab1 files generated by the ABI 3500XL Genetic Analyser and the results were then converted to FASTA format using the SnapGene® Viewer V5.3.2 (Altschul et al., 1996; White et al., 1990).

Multiple sequence alignment (MSA) for the four sequences from Eswatini was done using the Molecular Evolutionary Genetic Analysis (MEGA) Software V11.0.10 (Tamura et al., 2021). The alignment also included nine sequences from other countries that were obtained from the NCBI database, including an out-group of *Sporisorium sorghi*. The combined sequences were used to construct a phylogenetic tree of the ITS regions using the neighbour-joining method with a bootstrap of 1000 replications (Tamura et al., 2004).

Morphological analysis

The morphological description of the isolates was done by measuring the teliospore sizes, as well as by observing the surface texture and colour of the teliospores (Jacques-Edouard et al., 2020). To determine the colour of the teliospores, the samples were mounted on a glass slide, stained in lactophenol blue and then observed on a light microscope at a magnification of 400X. The JCM-7000 Benchtop Scanning Electron Microscope (SEM) was used to observe the surface texture and to measure the sizes of the teliospores at 7000X and 850X, respectively. For morphology analysis, a total of 40 teliospores were observed for each sample. For sample preparation, the teliospores were dried to ensure that the particles are well separated. Then a black double-sided tape was fixed onto the stage, with the dried teliospores deposited onto the showing side of the tape. The stage was then placed in the sample chamber for real-time automated analysis of each sample. The low-vacuum mode was used for the analysis, owing to its suitability for biological and non-conductive samples.

Determination of in-vitro development rates

This assay was done to ascertain the development rate of the teliospores from the different *S. scitamineum* isolates. The teliospores were cultured on PDA media and they were kept at 28^{o}C. The different promycelium development rates were observed under a light microscope at 3, 6, 12 and 18 hours post culturing at 400X magnification. Each sample was mounted on a glass slide and stained in Lactophenol blue to observe the teliospore germination and promycelium development rates at these time intervals (Keterly et al., 2018). A total of 15 promycelium, from each isolate, were measured for the analysis. The length of the fungal developmental structures (promycelium) was measured using the ImageJ software v1.48. The scaling was done with the help of a photo of a microscope graticule slide with a micrometre calibrator that was taken at the same magnification (400X) as the images that were analysed.

In-vivo pathogen biomass quantification of the isolates

The teliospores that were isolated from the different locations were cultured and used to inoculate healthy sugarcane seedlings of the variety N25, which is susceptible to smut. Inoculation was done using the injure and paste method. The inoculum suspension was calibrated using phosphate-buffered saline (PBS) solution to match the 0.5 McFarland turbidity standard which is equivalent to 1.5x10^{8} colony forming units (CFU). This standard was made by mixing 1% Barium Chloride (0.05ml) and 1% Sulphuric acid (9.95ml). The fungal cells were then harvested by centrifuging at 4000rpm for 5 minutes. The cells were rinsed twice with distilled water before resuspending them in 1ml distilled water.

The total genomic DNA was extracted using a Zymo Plant Genomic DNA Extraction Kit (Inqaba Biotech, South Africa) following the manufacturer's instructions five days after inoculation. The quality and concentration of DNA were analysed by 1% agarose gel electrophoresis and by using a nanodrop spectrophotometer.

For the absolute qPCR quantification (SYBR Green), pure fungal DNA was diluted in tenfold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) with a known starting DNA concentration to generate the standard regression line that was used to interpolate the Ct values of the DNA from the treatments (Nalayeni et al., 2021).

The four treatments were composed of the DNA from the plants that were inoculated with the isolates from Big Bend, Simunye, Nsoko and Mhlume. The qPCR amplification was done using the *bE* primers (F- TGGATCAGATATGGCGTCAA and R- GCTCTCTGCTTAGCCCTCCT).

Data analysis

The data collected for the morphology studies were analysed by both ANOVA and T-Test. The plant pathogen biomass concentrations were analysed by ANOVA and the homology of the sequences was analysed using the genetic

distance matrix between the different sequences. A phylogenetic tree was built using the Neighbour-Joining method (Saitou and Nei, 1987; Dereeper et al., 2008; Zhang et al., 2015).

Conclusion

This research revealed the morphological differences observed between the virulent isolates as well as in isolates which have a low virulence in Eswatini does have no genetic basis. In addition, the study has demonstrated that there is very low variability in the strains that are found in the selected sugarcane growing regions in Eswatini. The *S. scitamineum* showed similar virulence patterns when growing *in-vitro and in-vivo.* The sequence analysis further verifies the close relatedness of the *S. scitamineum* isolates that are found in these areas when analysed using the ITS region. This relatedness could suggest that there would be no requirement for a variable management approach for *Sporisorium scitamineum* in Eswatini.

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