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Final Report – Production Research

Title of Research Project:	Seed health of grey leaf spot diseased maize
Name and Trading Name of the Institution/Employer:	University of Pretoria
Name of Lead Researcher:	Prof Dave Berger
Contact details of Lead Researcher ***E-mail address:*** ***Phone:***	 Dave.berger@fabi.up.ac.za 072 2444 382
Duration of Study:	Four years
Are there changes to the original project proposal (yes/no)	No.

1. Summary of Progress

Please provide the main findings of the project during the funding year and the remaining challenges. Please do so at hand of the Gantt Chart submitted during the application process.

Field visits and industry engagement.

Field sampling of maize grain for this project had been carried out in 2021 (season 1) in KwaZulu-Natal, 2022 (season 2) in KwaZulu-Natal and Eastern Cape, and 2023 (season 3) in KwaZulu-Natal. This sampling had been carried out with the co-operation of Syngenta South Africa Technical Field Scientists in KwaZulu-Natal, and with the FABI team of the National Grain Research Programme in the Eastern Cape.

Further industry and stakeholder engagements during the reporting period were that the project leader Prof Dave Berger was (i) an invited speaker at the 15th Southern African Plant Breeders Symposium in Bloemfontein in March 2024, (ii) was a guest at the 35th SANSOR Congress in April 2024, (iii) a guest of GrainSA at NAMPO Harvest week in May 2024, (iv) hosted the International Day of Plant Health event at the University of Pretoria in May 2024, and (v) participated in the AgBiz Workshop on Gene Editing in February 2024 and subsequent industry engagements. In addition, the co-PI Prof Cobus Visagie presented the maize research at the 2nd Symposium of the National Grain Research Programme in Bloemfontein in April 2024. Student N.Ndaba attended the 3rd Annual National Grain Research Day, Potchefstroom in March 2025, and presented at the 1st FABI-GRP Student Research Day, University of Pretoria in 2025.

Prof Dave Berger has served as member of the AgBiz working group on Gene Editing during 2024 and 2025, where there will several engagements with the Department of Agriculture (Genetic Resources and GMO registrar), including a meeting in November 2024 that the Minister of Agriculture attended.

WP1 Fungal culture and identification
WP1 was completed in year 3. The deliverable was to make 150 cultures from maize grain from the three field seasons. This target was exceeded with 170 fungi cultured and identified using phylogenetic methods, which were deposited in the culture collection at FABI. Depending on the fungal genus, appropriate phylogenetic genes were sequenced to obtain species identities, namely Translation Elongation Factor 1 (TEF1) for *Fusarium, Beauveria* and *Trichoderma* species, Beta-Tubulin (BenA) for *Pencillium*, *Cladosporium* and *Talaromyces* species, and internal transcribed spacer (ITS) region for other species. A total of 13 fungal genera were identified, with *Fusarium* species present in grain from all eight sites in all seasons. *Cladosporium*, *Penicillium*, and *Talaromyces* were the next abundant - isolated from five, four and three of the sites, respectively.

WP2 Fungal microbiome, long read sequencing.
WP2 completed in Year 4. The aim of WP2 was to investigate the fungal mycobiome in maize grains with long-read DNA sequencing (using the PacBio platform). The approach, where organisms (fungi in this case) are identified and quantified at a large scale in a sample based on the count of their DNA barcodes, is called **metabarcoding**. Maize grain from all three seasons were sampled for DNA extraction. Metabarcoding targeting the ITS ribosomal DNA gene region was carried out. The main findings were that (i) *Fusarium* spp. were the most abundant accounting for >90% of fungi identified; (ii) *Penicillium* and *Talaromyces* were identified from all fields; (iii) metabarcoding detected a greater diversity of fungi than culture-based methods; (iv) grain from maize with GLS-diseased leaves did not show significant differences in fungal metabarcodes compared to grain from fungicide-sprayed maize in the same field. However, fungal microbiomes in the grain of the same maize hybrid did differ between the field locations, with a greater number of fungal metabarcodes in the Cedara site than in the Bergville site. This indicates that the geographic location is an important factor to maize seed fungal microbiome structure. Based on observations of the sites during sampling, it may be that environmental conditions or the soil bacteria could be involved in the fungal community structure but this is yet to be verified. Finally, the metabarcoding results of the maize grain did not contain any DNA barcodes for the causal agent of grey leaf spot disease, *Cercospora zeina*. These grain samples were from maize sampled in all three seasons, and included plots in each field where fungicides had not been sprayed, and thus there had been high GLS disease pressure on the leaves.

Validation experiments were carried out to confirm the absence of *C. zeina* in the maize grain that had been metabarcoded. The quantitative PCR method developed in the Berger lab is currently the most sensitive method to detect *C. zeina* in maize. The qPCR method is based on quantifying a *C. zeina* fungal gene relative to a maize gene. Standard curves showed that the detection limit of the qPCR assay was 156 pg of *C. zeina* in 10 ng of maize gDNA extracted from maize grains. The qPCR assay validated the metabarcoding results of the samples from both season 2 and season 3 since *C. zeina* could not be detected with either assay.

Maize grain samples from commercial farms in KwaZulu-Natal and the small-holder farms in the Eastern Cape were screened for the presence of *C. zeina* using the qPCR assay. The results confirmed the conclusion from the metabarcoding that *C. zeina* is not a seed-borne pathogen in different maize lines and from different regions and farm types.

WP3 Seed transmission experiment

WP3 completed in year 4. The aim of WP3 was to artificially infect maize plants with *C. zeina* to evaluate whether *C. zeina* can be transmitted during the plant development stages into the seeds/kernels. Maize plants were inoculated with a virulent strain of *C. zeina* from Cedara-KZN on both leaves and ear husks, and GLS disease developed well over the trial. The maize ears were harvested and the grain stored for two experiments. First, the qPCR assay to detect *C. zeina* was carried out on replicate samples of DNA extracted from the maize grain. There was no *C. zeina* detected. Second, kernels of the maize grain from these plants were planted in a follow-up glasshouse trial in the 2024 season. These plants were grown in the glasshouse under conditions that are optimal for GLS disease, namely high humidity and temperatures that simulate the conditions in GLS hotspots such as Cedara-KZN. The plants were closely monitored for symptoms of GLS disease. Seedlings of some of the plants developed chlorotic spots. Therefore, leaf samples were collected for the qPCR assay in the next year. No *C. zeina* was detected in these leaf samples, indicating that the fungus was not present. The chlorotic spots were likely from an unknown external factor (abiotic or biotic). This supports the final conclusion that *C. zeina* is not seed transmitted.

Capacity building
PhD student N. Ndaba attended three technical training workshops in laboratory methods and data analysis during 2023 to gain skills required for this research project, namely (i) a Quantitative PCR

workshop by Thermo-Fisher, (ii) Analysing amplicon sequence data with QIIME2 (Bite-sized Biology workshop) by the AgriMicrobiome Group, UP, (iii) Introduction to Metabarcoding at FABI, UP, and (iv) a bioinformatics training workshop at the ISME conference. He was able to apply this training to produce the results reported in WP2. xx

2. Additional information

(*e.g. Tables; Figures; Contributions by co-workers; Brief discussion of accomplishments*)

WP1: Fungal culture and identification

WP1 aimed to isolate fungi that were present inside the maize kernels (grain) and avoid any fungi that were on the outer surface of the kernels.

The maize kernels (30 kernels per ear, three ears per field site) were removed from the ears aseptically. The maize kernels were surface sterilized as follows; firstly, the maize kernels were washed with tap water with 0.01% of Tween20. This was followed by soaking the maize kernels in 1% sodium hypochlorite for 5 minutes and the sodium hypochlorite residues were removed with two washes of distilled water. Then, the maize kernels were immersed in 70% ethanol for 2 minutes and this was followed by three washes using distilled water. The maize kernels were then dried in a laminar flow until further processing.

Surface sterilized maize kernels were milled to a fine powder using an electric grinder. The electric grinder was washed with 1% sodium hypochlorite in between samples. The powdered maize kernels (flour) were sprinkled onto ¼ × Potato Dextrose Agar (PDA), and Water Agar (WA) media in petri dishes and incubated at room temperature for 5 days. Fungal growth was observed under a stereomicroscope, and samples positive for growth were cultured onto fresh ¼ × PDA plates. This was then followed by subculturing of the fungi until pure cultures were obtained. The pure cultures were grouped based on their macro-morphological characters before phylogenetic identification.

A total of 170 fungal cultures were obtained from the maize grain from the same three main field sites that were analysed with metabarcoding (Howick 2021, Cedara 2022, Bergville 2023), plus additional sites in KwaZulu-Natal and Eastern Cape. Genomic DNA was extracted from all 170 fungal cultures. Figure 1 shows that the dominant genus was *Fusarium*, followed by *Talaromyces*, *Cladosporium*, and *Penicillium*. A total of 13 different fungal genera were present.

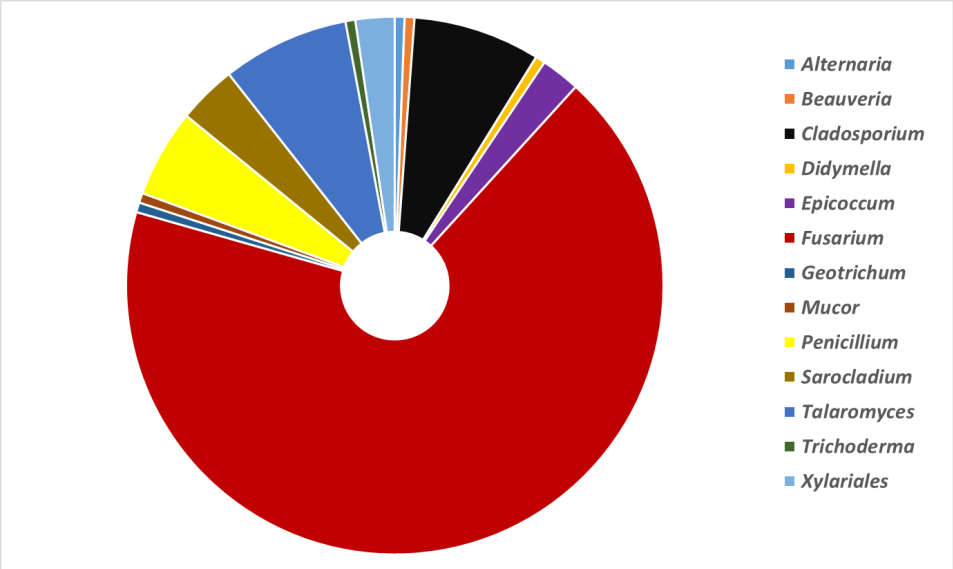


Figure 1 Fusarium was the most abundant genus out of the 170 cultures identified from the maize grain.

Phylogenetics was used to identify the cultures to species level where possible (Table 1). *Fusarium, Trichoderma, and Beauveria* spp. were identified by sequencing the TEF1 gene, *Penicillium, Cladosporium* and *Talaromyces* spp. with the BenA gene and other species with the ITS region. Table 1 lists the fungal species that were identified, showing that three *Fusarium* spp. were the most dominant. Interestingly, two fungi that are known as biological control agents were also isolated, namely the entomopathogenic fungus *Beauveria bassiana* and *Trichoderma gamsii*. The causal agent of GLS, *Cercospora zeina*, was not isolated by culture-based methods from any of the maize grain samples.

Table 1 Fungal species cultured from the maize grain.

Species	Number of isolates	Species	Number of isolates
Fusarium temperatum	50	*Sarocladium strictum*	3
Fusarium meridionale	24	*Sarocladium zeae*	3
Fusarium boothii	12	*Fusarium annulatum*	2
Cladosporium cucumerinum	9	*Penicillium sp.*	2
Fusarium graminearum	8	*Alternaria alternata*	1
Talaromyces stollii	8	*Beauveria bassiana*	1
Fusarium verticilliodes	6	*Cladosporium angulosum*	1
Penicillium cairnsense	6	*Didymella dimorpha*	1
Epicoccum sorghinum	4	*Fusarium cortaderiae*	1
Talaromyces amestolkiae	4	*Fusarium sylviaearle*	1
Xylariales sp.	4	*Geotrichum candidum*	1
Cladosporium oxysporum	3	*Mucor orantomantidis*	1
Fusarium caucasicum	3	*Penicillium steckii*	1
Fusarium poae	3	*Talaromyces wortmannii*	1
Fusarium udum	3	*Trichoderma gamsii*	1
Fusarium acacia-mearnsii	2		

The fungal cultures were isolated from maize grain from eight field sites in KwaZulu-Natal and the Eastern Cape. Figure 2 shows the distribution of genera per site, which illustrates again that *Fusarium* was the most common (light brown). A common pattern is seen between the commercial maize production fields of Baynesfield and Howick with *Penicillium* (light green) and *Talaromyces* (dark brown) also well represented.

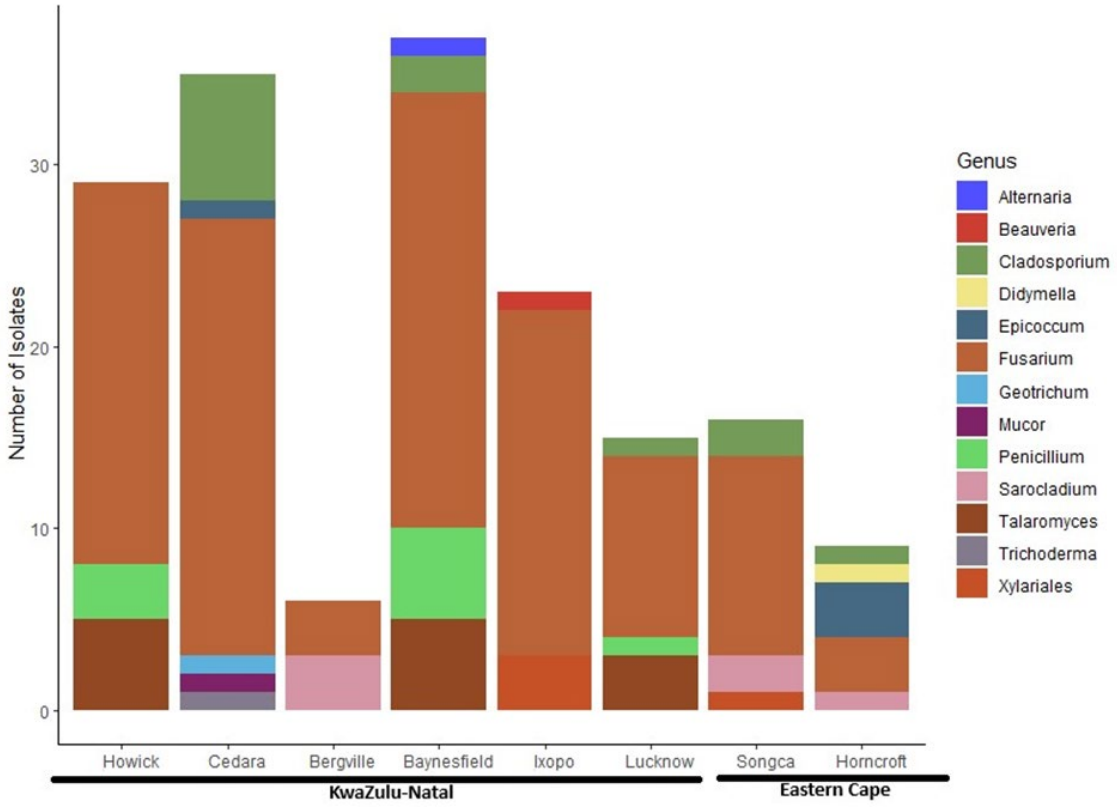


Figure 2 Fungal genera isolated from each field site showing the number of fungal isolates per genus per field site. There were six sites in KwaZulu-Natal, with Lucknow at the border with Eastern Cape. There were two field sites in Eastern Cape. The Howick and Baynesfield sites were commercial fields, with Cedara a commercial research site, and the other sites were small-holder farms.

WP2: Fungal microbiome, long reads sequencing

This work package aimed to analyse the fungal microbiome of maize grains from GLS infected maize in farmers' fields using DNA metabarcoding of the ITS gene region over three seasons. We applied several innovations in the DNA metabarcoding process. First, PCR primers V9G and LS266 were used to amplify a 800 – 1200 bp fragment of the fungal ITS gene region (compared to the standard ITS fragment of ~600 bp). Second, we used the PacBio DNA sequencing platform to obtain the full-length ITS sequences. This is in contrast to previous work on maize that used Illumina DNA sequencing which can only sequence short (100-150 bp) reads, which can't resolve repeats or complex regions such as those typical to the ITS regio which increases the error rate.

Furthermore, Dr. J. Gokul joined the team as a co-supervisor of PhD student N. Ndaba. She is an expert in DNA metabarcoding data analysis and a Lecturer in the Department of Plant and Soil Sciences at UP. The season 1 samples were used to optimize the lab protocols and data analysis methods. Excellent progress was made by Dr Gokul and Mr Ndaba since they developed an in-house maize grain DNA barcoding analysis protocol and applied this to all three seasons of data. The output of the metabarcoding is a dataset of all the unique ITS DNA barcodes for a sample, which are called "Amplicon Sequence Variants" (ASVs), which were used as proxies to represent different fungal species. For example, a single grain sample might generate a total of ~10,000 ASVs and 5,000 of them can match the ITS sequence for *Fusarium temperatum*, resulting in a 50% relative abundance for this fungal species, indicating it is the predominant fungus.

The pipeline output was used to evaluate the reproducibility of the metabarcoding data from season 1 maize grains, looking at both biological and technical replication. The biological replication was that gDNA was extracted and used for ITS PCR from grain from three different maize plants of each treatment. The technical replication was that two independent PacBio libraries were made from each maize grain sample. This meant that there were six replicates from each treatment. Figure 3 shows the different fungal ASVs found in each sample presented as a Non-metric Multi Dimensional Scaling (NMDS) cluster plot. It shows that the biological replicates for treatment S1, S3 and S4 cluster within each treatment, indicating that the fungal microbiome was similar for each of the three independent maize plants per treatment. The treatment S2, which were the maize plants sprayed with the effective fungicide, and thus exhibiting low GLS disease symptoms, were also clustered, however with a wider spread, indicating some differences in fungal microbiome. Importantly, the pairs of technical replicates showed high reproducibility when different PacBio libraries were made from the same maize grain gDNA source, for example samples A13 & A16 of the S4 treatment, and samples A7 and A10 of the S2 treatment (Figure 3).

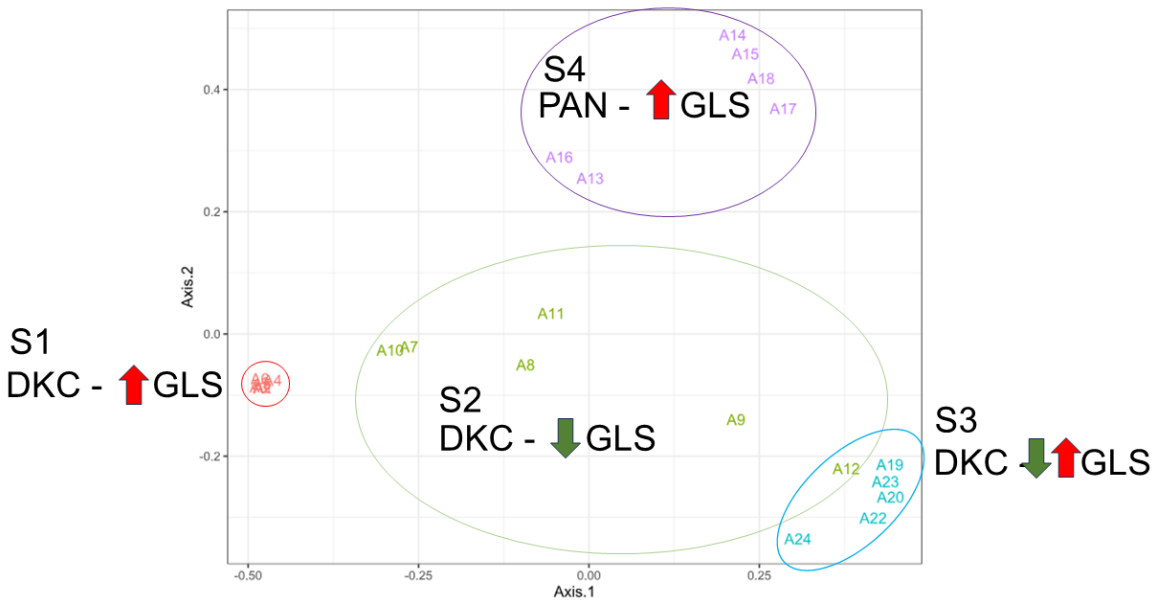


Figure 3. NMDS plot illustrating the fungal microbiome relatedness between the 23 maize grain samples based on the ASVs in each sample. The plot shows that there were differences in the ASVs between each treatment. The plots also show that the biological and technical replicates for each treatment (S1-S4) were reproducible between biological and technical replicates. Treatment S1 (DKC hybrid with high GLS infection) and treatment S2 (DKC hybrid with low GLS infection) had the lowest and highest variation between replicates. The season 2 and 3 data will be used to determine if this result is related to the level of GLS infection.

Metabarcoding of Season 2 and Season 3 maize grain with a maize ITS PCR blocker – PNA

One limitation that we identified from season 1 maize grain data was that the ITS metabarcoding data included reads of the maize ITS region because the primer binding sites are highly conserved in fungi and plants. This meant that the method would be less sensitive in picking up DNA barcodes of rare fungi that may be present in small amounts, such as *C. zeina*. This is because a proportion of the DNA sequencing reads on the PacBio instrument would be "wasted" on maize ITS barcodes. We therefore applied the innovation of using a maize ITS PCR blocker called Peptide Nucleic Acid (PNA), recently developed in leading labs in the USA for this purpose.

The expected sizes of the fungal ITS product are ~ 900 bp. Figure 4 shows that the PNA is able to block PCR amplification of the maize ITS region (lane D) but does not block the PCR of fungal ITS when fungal gDNA is mixed with maize gDNA (lanes B & C) or gDNA from five different fungi (lanes E to J).

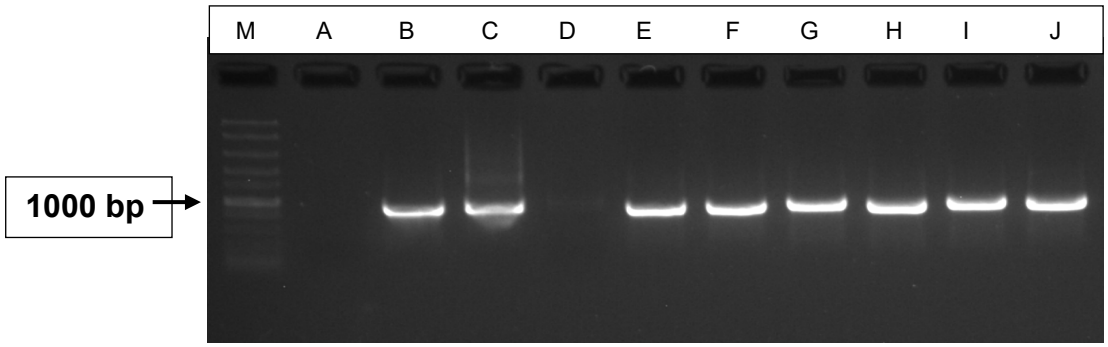


Figure 4. ITS PCR of maize and different fungi with the maize ITS PNA blocker. Lanes: **M** DNA size marker, **A** (non-template control), **B** (Maize + *C. zeina*), **C** (Maize + 6 fungi), **D** (Maize only), **E** (*C. zeina* only), **F** (*Fusarium* sp.), **G** (*Penicillium* sp.), **H** (*Epicoccum* sp.), **I** (*Talaromyces* sp.), **J** (*Cladosporium* sp.).

Metabarcoding of grain from the maize hybrid DKC from high and low GLS plots in season 2 (Cedara) and season 3 (Bergville) was carried out using all samples treated with PNA. The number of ITS reads from each sample ranged from 79,056 to 145,992 with an average of 99,082. Figure 5 shows that the ITS PNA blocker worked effectively to block PCR of maize ITS sequences since only two Bergville samples had maize (family *Viridiplantae*) reads at below 15% relative abundance.

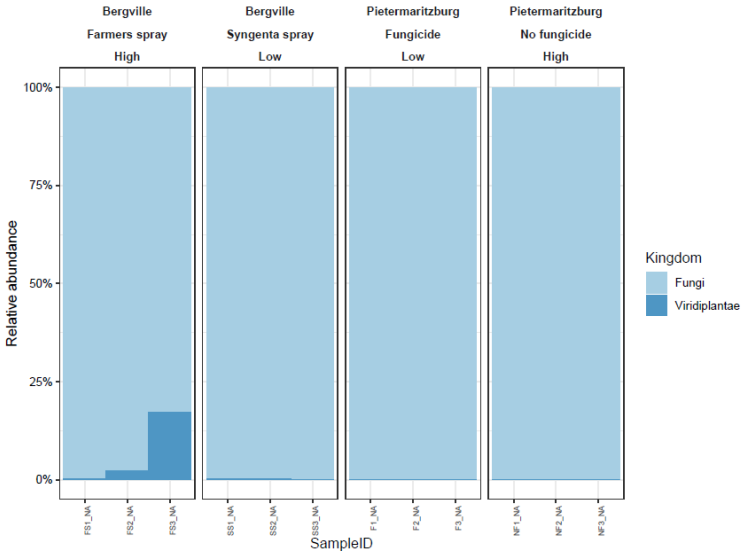


Figure 5 Metabarcoding reads matching maize (Viridiplantae) or fungi ITS sequences. This shows that the PNA ITS PCR blocker was effective in blocking PCR of maize ITS from the season 2 (Cedara) and season 3 (Bergville) grain. Only two samples (Bergville) showed negligible maize reads. The PNA blocker therefore resulted in targeted enrichment of fungal reads.

First, the fungal community composition (based on ITS metabarcodes) was compared between plots with low GLS and those with high GLS. The data from both seasons was combined. Figure 6 shows that biological replicates from each of four treatments cluster together, indicating consistency between

the treatments. It also shows that grain from maize plants with high GLS (red) has overlapping fungal composition to grain from maize with low GLS infection (blue). This would indicate that leaf GLS infection does not significantly affect the fungal mycobiome in the grain of the same plants (PERMANOVA, p = 0.46).

However, Figure 7 shows a comparison of the abundance of fungi at genus level, and it can be seen that the high GLS infection (i.e. treatments where fungicide spray was not effective) resulted in a higher proportion of *Penicillium* and lower proportion of *Fusarium* compared to the low GLS infection.

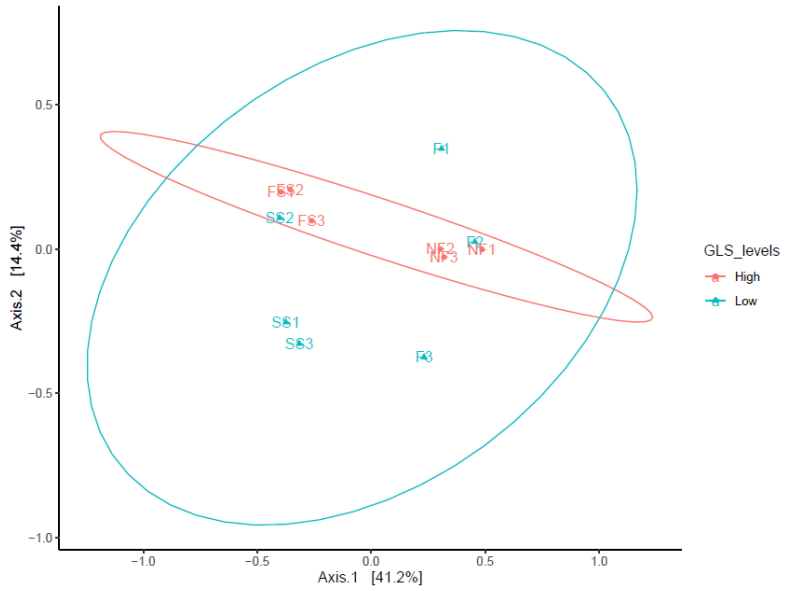


Figure 6 PCoA ordination plot of fungal communities in grain from maize with high or low GLS infection. There is overlap indicating that grain fungi diversity are not significantly different between grains of maize from high (red) or low (blue) GLS infection plots. The two axes combined explain that a total of 56% of the total variance is due to GLS infection.

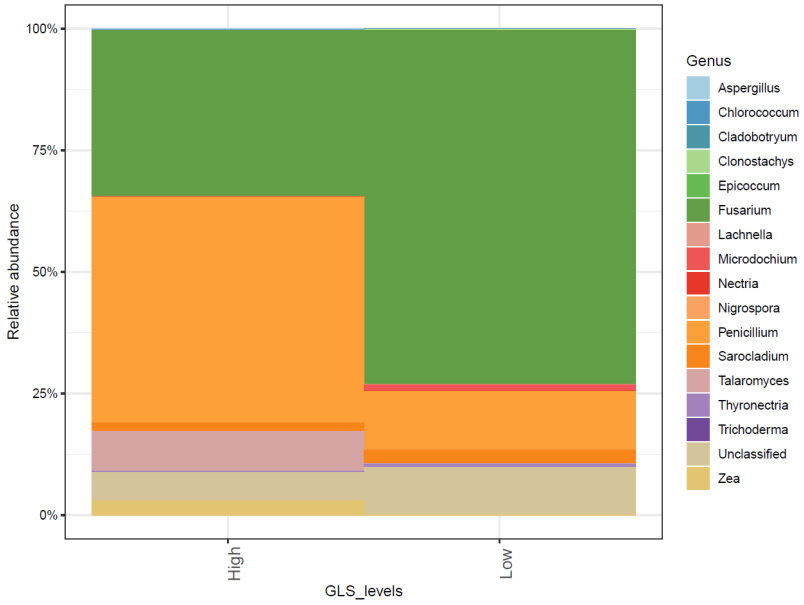


Figure 7 Comparison of fungal genera from maize grain from plots with high and low GLS. ITS Metabarcoding data was combined from both seasons. The data indicates that *Penicillium* was relatively more abundant in grain from maize with high GLS infection compared to low GLS infection.

When comparing the maize grain microbiome between the two different sites, Cedara and Bergville, there is a clear separation indicating that site location is an important factor affecting the fungal variation (Figure 8 and Figure 9).

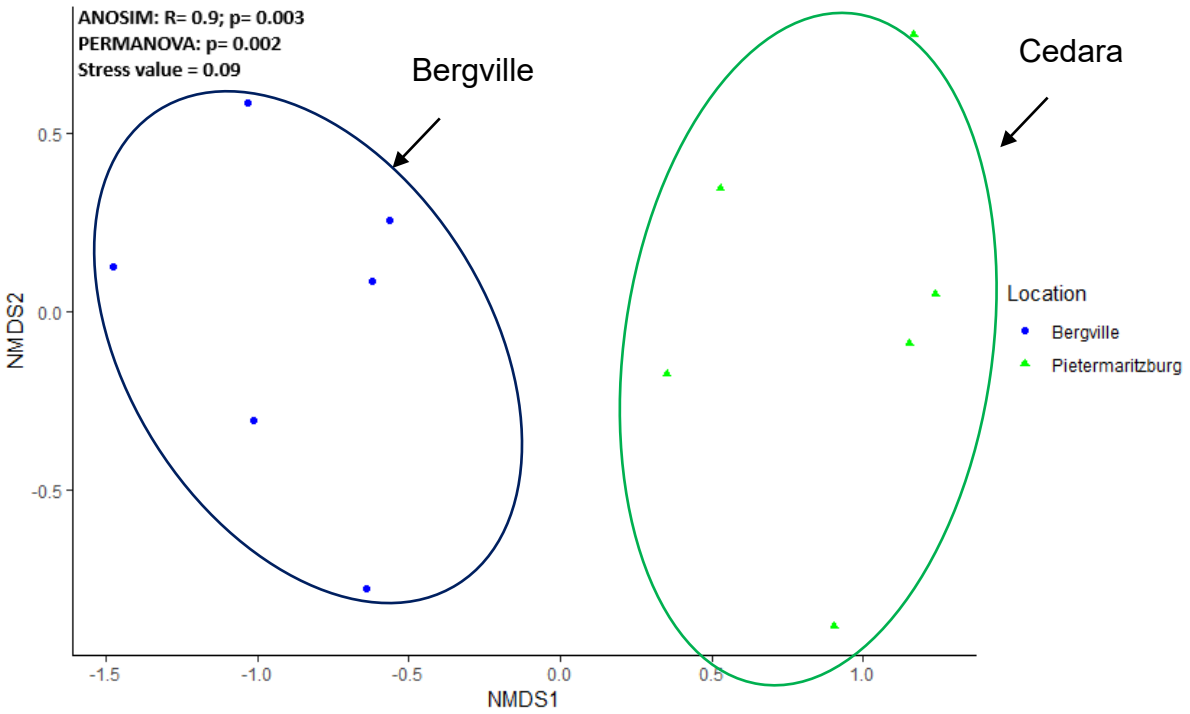


Figure 8: Non-metric multidimensional scaling (NMDS) plot showing the effect of geographical locations on maize grain mycobiome for all treatments. Fungal metabarcodes of samples at Bergville clustered separately from samples at Cedara. The fungicide treatments/GLS levels at each site have less effect on the composition than the site location.

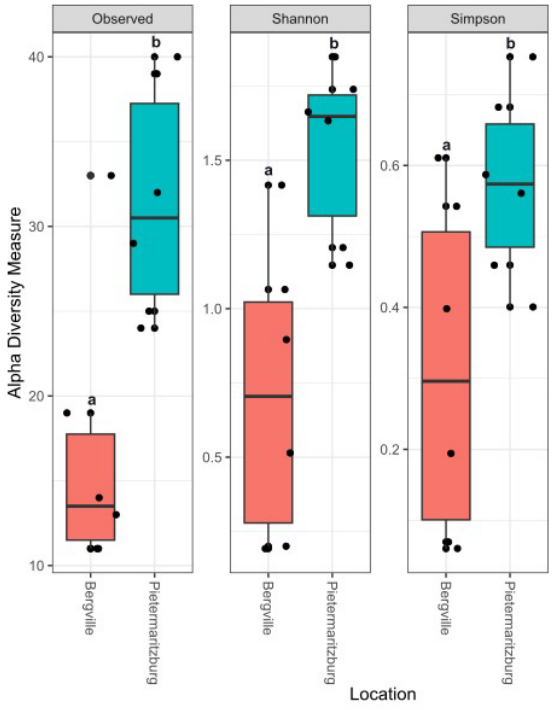


Figure 9 Alpha diversity indices for observed richness, and overall diversity (Shannon and Simpson) differed significantly between the Bergville and Cedara (Pietermaritzburg) sites. Alpha diversity is a measure of how many different fungal metabarcodes (genotypes) were identified within a sample. This shows that there was greater sample richness and diversity at the Cedara site.

Quantitative PCR assay to test for *C. zeina* in maize grain samples.

The qPCR assay for detection and quantification of *C. zeina* in maize samples developed in the Berger lab (Korsman et al 2012, EJPP, 133, 461-471) had only previously been used for maize leaf tissue, and therefore it was tested in maize grain samples to ensure specificity. Figure 10 shows that the *C. zeina* CPR1 qPCR was specific for *C. zeina* only (lane 2) and did not amplify a product from maize or the five common fungi from maize grains (lanes 4-8). Figure 10 also shows the maize GST3 qPCR is specific for maize (lane 11) and does not amplify fungi (lanes 10 and 12-16).

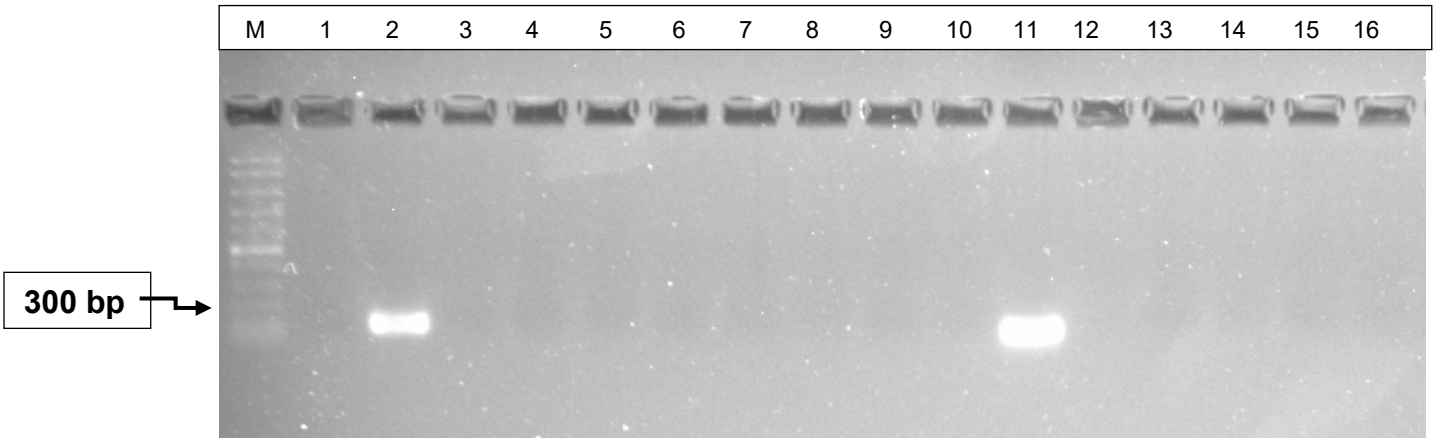


Figure 10 Specificity of the qPCR assays for *C. zeina CPR1* (164 bp) and maize *GST3* (106 bp). **M** = DNA ladder. **Lanes 1 – 8** represent PCR with the *CPR1* primers. **Lanes 9 – 16** represent PCR with the *GST3* primers. Lanes **1 & 9** (non-template controls), **2 & 10** (*C. zeina*), **3 & 11** (Maize), **4 & 12** (*Fusarium* sp.), **5 & 13** (*Penicillium* sp.), **6 & 14** (*Epicoccum* sp.), **7 & 15** (*Talaromyces* sp.), **8 & 16** (*Cladosporium* sp.).

A standard curve for detection of *C. zeina* in maize grain was constructed by making a dilution series of *C. zeina* gDNA in 10 ng/ul of maize gDNA and conducting the CPR1 qPCR for each dilution. In addition, a standard curve was made for quantifying the amount of maize gDNA in a sample by conducting the maize GST3 qPCR with a dilution series of maize gDNA. The standard curves showed that the detection limit of the qPCR assay was 156 pg of *C. zeina* gDNA in 10 ng of maize gDNA extracted from maize grains. Figure 11 shows the successful CPR1 qPCR with the *C. zeina* dilution series where a single product with a melting temperature of 84 °C is visible as a peak. The four horizontal lines with no peak were the expected result for the water control reactions indicating the absence of any *C. zeina* gDNA.

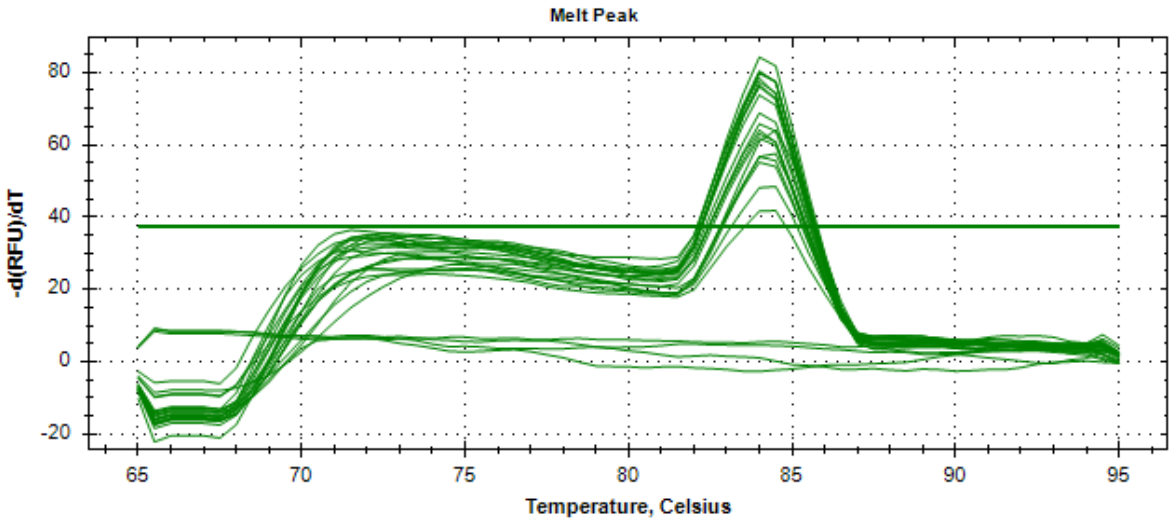


Figure 11 *C. zeina* genomic DNA dilutions produce the expected CPR1 qPCR product with a Tm = 84 °C. Each line in the graph represents the melt curves of the CPR1 qPCR products of the dilution series of *C. zeina* gDNA in 10 ng/ul of maize grain gDNA. The four flat lines are the water controls of the CPR1 qPCR in which no gDNA was added.

The DNA from the maize grain samples that had been metabarcoded from season 2 (Cedara) and season 3 (Bergville) were screened for the presence of *C. zeina* using the qPCR assay described above. Figure 12 shows the result of the CPR1 qPCR melt curves which lack the *C. zeina* CPR1 peak at Tm = 84 °C shown in the previous figure. All of the lines are below the threshold and thus the conclusion is that *C. zeina* could not be detected with this assay in these six replicate samples per field trial in season 2 and season 3. These results are consistent with the metabarcoding results for the same samples, which were unable to detect the *C. zeina* fungus in grain of GLS-infected maize as well as maize that had been treated with fungicides and thus lacked GLS.

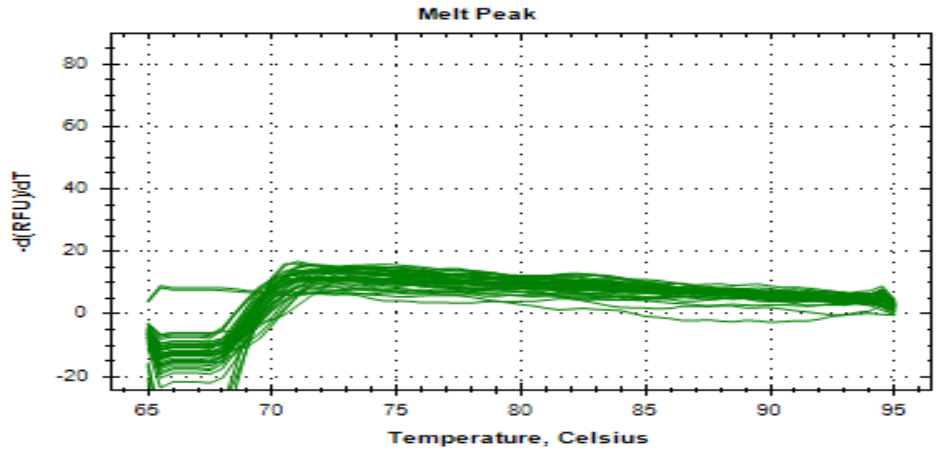


Figure 12 Grain from GLS infected maize in Season 2 (Cedara) and Season 3 (Bergville) does not contain *C. zeina* with the CPR1 qPCR assay. Each line in the graph represents the melt curves of the CPR1 qPCR products of gDNA from maize grain. No samples had peaks at 84 °C which would be diagnostic of *C. zeina*. There were six biological replicates each from Season 2 (Cedara) and Season 3 (Bergville) and non-template / water as negative controls. There were three technical replicates of each biological replicate.

***C.zeina* is not present in maize grain from small-holder farms in KwaZulu-Natal and Eastern Cape.**

The highly sensitive qPCR assay for detection of the fungus *C. zeina* was applied to maize grain samples from commercial scale farms (Cedara, Baynesfield) and small-holder farms (Ixopo, Lucknow) in KwaZulu-Natal, as well as the Eastern Cape (Songca, Thorncroft). This grain had been collected from fields where GLS was prevalent in leaf samples due to lack of fungicide control. All samples were negative for this fungus (Figure 13 and 14, Table 2), indicating that the pathogen is not seed-borne on maize

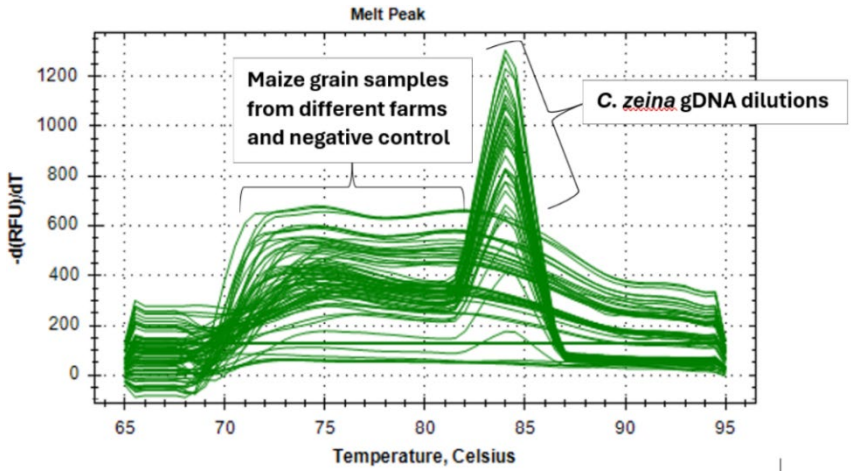


Figure 13: *C. zeina* genomic DNA dilutions produce the expected CPR1 qPCR product with a Tm = 84 °C. Each line in the graph represents the melt curves of the CPR1 qPCR products of the dilution series of *C. zeina* gDNA in 10 ng/ul of maize grain gDNA. The lines with no peak at 84 °C are the maize grain samples and water controls of the CPR1 qPCR in which no gDNA was added. There were four biological replicates each from the farms (**Cedara 2023, Songca 2022, and Horncroft 2022**). There were three technical replicates of each biological replicate.

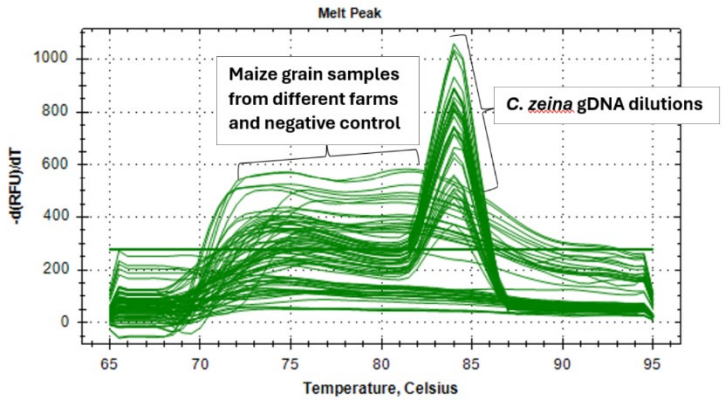


Figure 14: *C. zeina* genomic DNA dilutions produce the expected CPR1 qPCR product with a Tm = 84 °C. This graph is the same analysis as Figure 13 with the same positive controls but with different samples from the farms **Baynesfield 2022, Lucknow 2022, and Ixopo 2022**).

Table 2. qPCR assay results of different farms in KZN and EC

Sample names	**Farm type**	***C. zeina* detected in maize grain**
Positive Control	*C. zeina* genomic DNA dilutions	*C. zeina* qPCR positive
Negative Control	No DNA	*C. zeina* qPCR negative
KZN FARMS		
CEDNF2-2023	Commercial research farm	NO
CEDNF3-2023	Commercial research farm	NO
CEDNF5-2023	Commercial research farm	NO
CEDNF9-2023	Commercial research farm	NO
BAYNF1-2022	Commercial farm	NO
BAYNF2-2022	Commercial farm	NO
BAYNF3-2022	Commercial farm	NO
BAYNF4-2022	Commercial farm	NO
IXONF1-2022	Small-scale farm	NO
IXONF2-2022	Small-scale farm	NO
IXONF3-2022	Small-scale farm	NO
IXONF4-2022	Small-scale farm	NO
LUCK1-2022	Small-scale farm	NO
LUCK2-2022	Small-scale farm	NO
LUCK3-2022	Small-scale farm	NO
LUCK4-2022	Small-scale farm	NO
EC FARMS		
SONGCA1-2022	Small-scale farm	NO
SONGCA2-2022	Small-scale farm	NO
SONGCA3-2022	Small-scale farm	NO
SONGCA4-2022	Small-scale farm	NO
HORN1-2022	Small-scale farm	NO
HORN2-2022	Small-scale farm	NO
HORN3-2022	Small-scale farm	NO
HORN4-2022	Small-scale farm	NO

WP 3 Glasshouse experiment for seed transmission

Seed transmission experimental trial at UP glasshouse

In the previous year, maize plants of the DKC and PAN hybrids (susceptible to GLS) were planted in the glasshouse at the University of Pretoria. Eight plants of each hybrid were leaf inoculated with *Cercospora zeina* strain from Cedara and eight control plants of each hybrid were not inoculated. Inoculation was carried out at the V7-V8 stage of development (51 dap).

In addition, four of the leaf-inoculated plants were re-inoculated on the ear husk green tissue at an early stage of ear development. This experiment was done to test whether *C. zeina* can infect maize grain directly from the ear husk tissue. Stomatal peels showed that green maize ear husks do have stomata on their surface (Fig. 15), which indicates the potential for *C. zeina* to infect and cause GLS disease in this tissue directly above the developing maize grain.

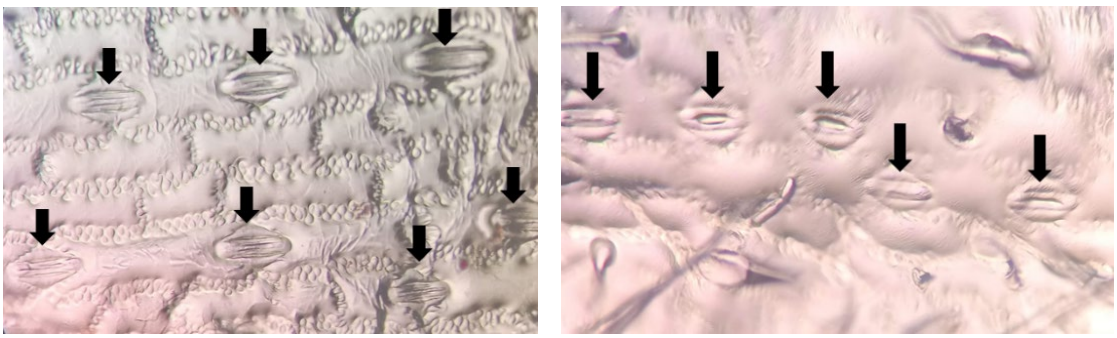


Figure 15. Epidermal tissue peels of DKC hybrid maize leaf (A) and ear husk (B) to show stomata (arrows).

Figure 16A shows the maize glasshouse set up. Good GLS disease symptoms developed on the DKC leaves (Figure 16B) and PAN leaves (not shown). In addition, a few GLS-like lesions were observed on the husks of PAN ear-husk inoculated plants (Figure 16C). Uninoculated plants were not infected with GLS. The maize plants were allowed to self-pollinate so that maize grain could develop. At the end of the trial, the maize grain was harvested to screen for the presence of *C. zeina* using the qPCR assay.

Figure 16 Glasshouse trial where maize plants were inoculated on leaves with *C. zeina* to test for transmission into grain. **A.** Glasshouse setup. **B.** high level of GLS disease symptoms that developed on the DKC hybrid leaves indicating that the inoculation was successful. **C.** GLS lesion on inoculated earhusk of PAN hybrid (white arrow). Images taken 69 dpi. The grain from this trial was harvested and used to test for *C. zeina* by qPCR.

Quantitative PCR assay of maize grain.

Quantitative PCR was done to test for the presence of *C. zeina* in the grain of maize plants that had been inoculated with the pathogen. The grain was harvested from the glasshouse trial and gDNA was extracted from pools of 30 kernels per ear per treatment.

Grain from five treatments was tested by qPCR:
GH1 = DKC-leaf + husk inoculated
GH2 = DKC-uninoculated
GH3 = DKC-leaf inoculated
GH4 = PAN-uninoculated
GH5 = PAN-leaf + husk inoculated

Quantitative PCR was carried out on the five types of grain samples with DNA extracts at 10 ng per reaction. No melt curve peaks at 84 °C were observed in any of the samples, indicating that *C. zeina* was not present in the maize grain (Figure 17). The results therefore indicate that *C. zeina* is not seed borne.

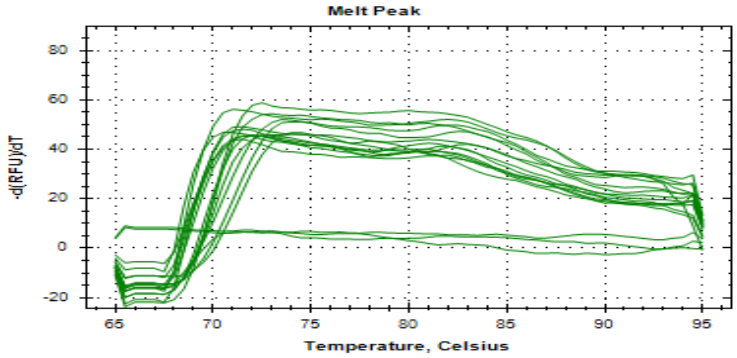


Figure 17 Grain from GLS inoculated maize in the glasshouse does not contain *C. zeina* with the CPR1 qPCR assay. Each line in the graph represents the melt curves of the CPR1 qPCR products of gDNA from maize grain. No samples had peaks at 84 °C which would be diagnostic of *C. zeina*. The grain samples were DKC hybrid - leaf and ear husk inoculated, DKC – un-inoculated, DKC – leaf inoculated, PAN hybrid – un-inoculated, PAN hybrid – leaf and ear husk inoculated, non-template negative control. There were three technical replicates of each sample.

WP3 – continued Seed transmission experiment

The DKC maize grain/seed harvested from inoculated plants from the previous glasshouse trial was next planted in a follow-up UP glasshouse trial 2 (Feb-May 2024) to determine if any GLS disease would develop in the plants. This was an additional test to find out if there was any evidence that *C. zeina* could be seed-transmitted. The seed that was planted was obtained from the same batches that had been tested for the fungus using qPCR (Figure 17 above), and which did not show any presence of the pathogen. However, it was important to carry out this additional test since it was still possible that *C. zeina* had been present in the seed at very low amounts, below the detection threshold of the qPCR assay.

The following maize seed batches were planted:
(1) Leaf + Husk inoculated-DKC (GH1), (2) Leaf only inoculated-DKC (GH3), and (3) Mock-DKC (GH2). Ten plants were grown per treatment. In this trial, the 24 plants that survived were monitored for GLS symptoms development. Seed germination percentage was reduced when compared to standard seed batches, since the seed was not conditioned for planting as occurs for commercial batches. The maize plants were grown under the same glasshouse conditions used for inoculation, which are conducive for GLS disease development. Plants were inspected for symptoms on a daily basis and photos taken from the day that chlorotic spots formed. Out of 10 plants for Leaf + Husk inoculated-DKC only 4 plants developed chlorotic spots (Fig.18). The Leaf only inoculated-DKC plants show no chlorotic spots (Fig.19), and likewise for the Mock-DKC (Fig.20).

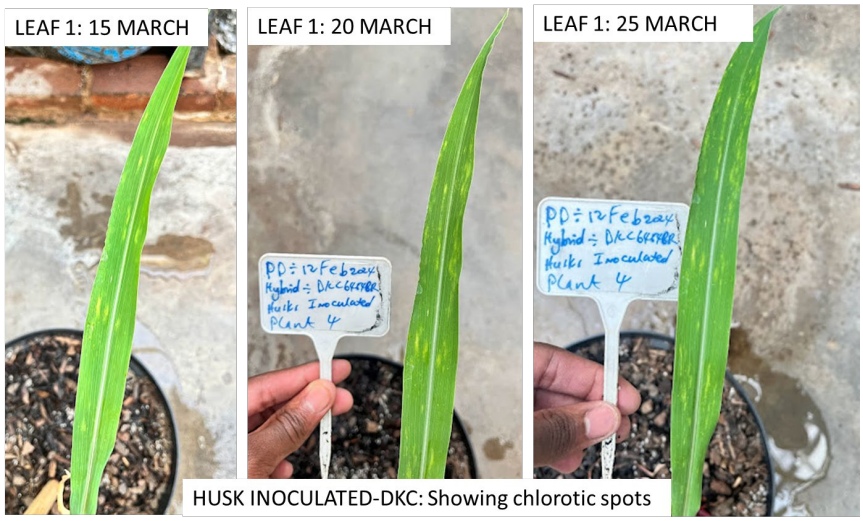


Figure 18. Maize seedlings from seed planted from DKC hybrid plants that were inoculated with *C. zeina* on leaves and ear husks (GH1 treatment). In this plant, chlorotic spots can be seen on the leaves at 32 days after planting (DAP). The chlorotic spots did not develop further over the next 10 days or later in the trial. The leaves were sampled for DNA analysis before they became senescent, since they were the earliest leaves. This leaf is representative of four different plants from seed of the Leaf + Husk inoculated-DKC (GH1) treatment. There were six other plants from this treatment that did not develop chlorotic spots.

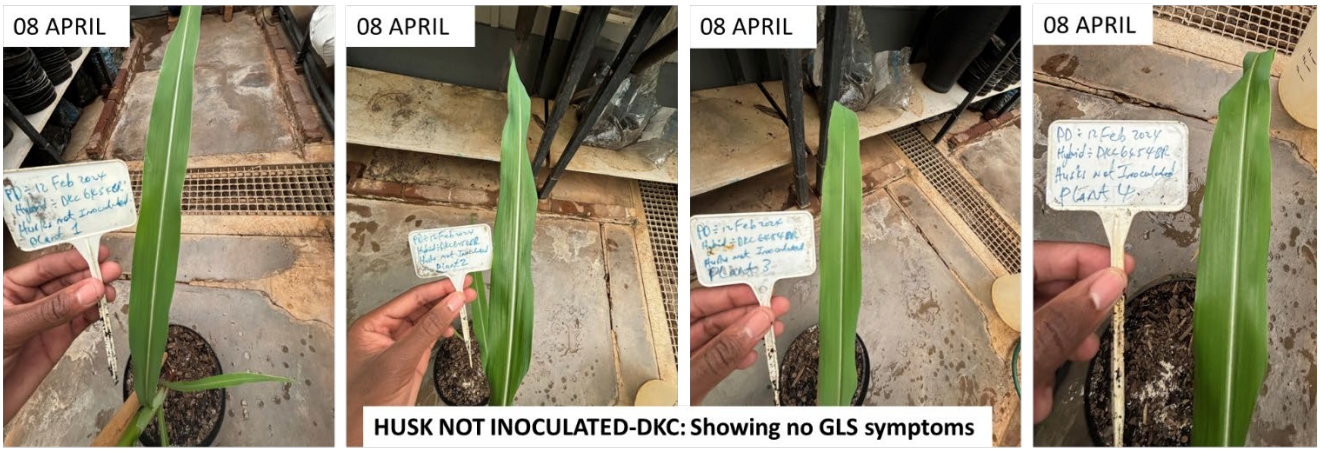


Figure 19. Maize seedlings from seed planted from DKC hybrid plants that were inoculated with *C. zeina* on leaves only (GH3 treatment). No disease symptoms such as chlorotic spots or GLS-like lesions are evident on leaves of all 4 plants at approximately 55 DAP.

Figure 20. Maize seedlings from seed planted from DKC hybrid plants that were NOT inoculated with *C. zeina* on leaves or ear husks (GH2 treatment). No disease symptoms such as chlorotic spots were evident.

Seed-transmission deliverable (continued WP3)

Maize leaf samples (DKC 64-54BR) from the Glasshouse trial 2 were tested for *Cercospora zeina* using the qPCR assay. These plants had grown from seed of plants that had been inoculated with *C. zeina* in Glasshouse trial 1 with the three treatments (GH1-GH3 treatment shown in Fig 18-20). The results show that the qPCR assay to detect *C. zeina* was again effective for the positive control samples which had been prepared by mixing maize leaf gDNA with *C. zeina* gDNA, since the peak at Tm = 84 °C was obtained (Figure 21A). In contrast, *C. zeina* was not detected in any of the leaf samples from glasshouse trial 2, which were derived from plant seeds inoculated with *C. zeina* (Figure 21B, Table 3). This confirms that the chlorotic spots seen in treatment GH1 (Fig. 18) were caused by an unknown external factor and not *C. zeina*. This is consistent with the fact that these chlorotic spots did not develop into GLS-like lesions as the plants matured. Importantly, these results confirm that *C. zeina* is not seed borne or seed transmissible.

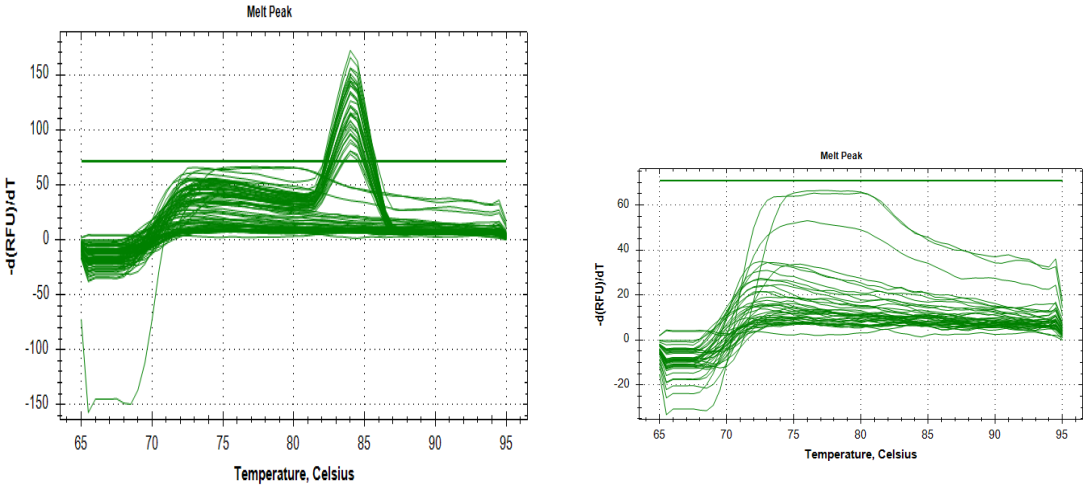


Figure 21: *C. zeina* qPCR assay of positive controls and maize leaf samples from Glasshouse Trial 2. **A**. Positive control graph showing the diagnostic *C. zeina* peak at Tm = 84 °C. *C. zeina* genomic DNA dilutions produce the expected CPR1 qPCR product with a Tm = 84 °C. Each line in the graph represents the melt curves of the CPR1 qPCR products of the dilution series of *C. zeina* gDNA in 10 ng/ul of maize leaf gDNA. The flat lines are the water controls of the CPR1 qPCR in which no gDNA was added. **B**: Leaves from glasshouse trial 2 do not contain *C. zeina* with the CPR1 qPCR assay. Each line in the graph represents the melt curves of the CPR1 qPCR products of gDNA from maize leaves/. No samples had peaks at 84 °C which would be diagnostic of *C. zeina*. There were four biological replicates each from mock (GH2), leaf + husk inoculated (GH1), and leaf inoculated (GH3), and non-template / water as negative controls. There were three technical replicates of each biological replicate.

Table 3. qPCR results of leaf samples from the Glasshouse trial 2

Treatment	**Sample replicates**	***C. zeina* detected in maize leaves**
Positive Controls (Figure 21A)	*C. zeina* genomic DNA dilutions in maize leaf gDNA	*C. zeina* qPCR positive
Negative Control (Figure 21A, B)	No DNA	*C. zeina* qPCR negative
Figure 21B results		
GH2 (seed from plants not inoculated)	Mock1	NO
GH2 (seed from plants not inoculated)	Mock2	NO
GH2 (seed from plants not inoculated)	Mock3	NO
GH2 (seed from plants not inoculated)	Mock4	NO
GH1 (seed from plants inoculated on leaves & husks)	Leaf + Husk inoculated1	NO
GH1 (seed from plants inoculated on leaves & husks)	Leaf + Husk inoculated2	NO
GH1 (seed from plants inoculated on leaves & husks)	Leaf + Husk inoculated3	NO
GH1 (seed from plants inoculated on leaves & husks)	Leaf + Husk inoculated4	NO
GH3 (seed from plants inoculated on leaves)	Leaf inoculated1	NO
GH3 (seed from plants inoculated on leaves)	Leaf inoculated2	NO
GH3 (seed from plants inoculated on leaves)	Leaf inoculated3	NO
GH3 (seed from plants inoculated on leaves)	Leaf inoculated4	NO

3. Please report on the Deliverables and milestones:

(As presented in section 6 of Application)

Deliverables

Deliverable	Progress on deliverables
Question answered scientifically – is grey leaf spot disease of maize seed-borne?	Completed. The following evidence was obtained that GLS is not seed borne: *Cercospora zeina*, the causal agent of GLS disease was not found to be present in maize grains of GLS-infected maize from three seasons using two methods – fungal culturing and metabarcoding (mycobiome analysis). qPCR analysis also did not detect *C. zeina* in these field samples. *C. zeina* was not found in grain of maize plants that had been artificially inoculated with the pathogen, and that had developed GLS leaf symptoms. No *C. zeina* was detected in maize grain from GLS-infected maize of small-holder farms in KwaZulu-Natal and Eastern Cape that were screened for seed-borne *C. zeina* using the qPCR assay.
Question answered scientifically – is grey leaf spot disease of maize seed-transmitted?	Completed: Glasshouse trial 1 completed (leaf and husk inoculation of maize with *C. zeina*) to produce high GLS disease pressure. Maize seed/grain was harvested. Glasshouse trial 2 completed – maize grain from GLS diseased maize plants was grown in the glasshouse and monitored for symptoms. No *C. zeina* was detected in leaf samples from glasshouse trial 2 tested for *C. zeina* using the qPCR assay.
150 fungal cultures from maize grain of GLS-infected maize identified, preserved, and accessioned.	Completed: 170 fungal cultures obtained from maize grain from the three seasons and field sites were identified with phylogenetic methods.
Fungal microbiomes from GLS-infected maize determined from commercial, smallholder and home consumption farmers	Completed: Maize grain metabarcoding (microbiome analysis) method optimized using Season 1 samples from the KZN field infected with GLS. An in-house metabarcoding PacBio data analysis pipeline was established and applied to Season 2 and Season 3 samples. Preliminary analyses were completed and reported in this report. The results show that *C. zeina* was not detected in these field samples using metabarcoding. The most abundant genus was *Fusarium* which was present in all fields. The level of GLS infection in plots did not significantly affect the maize grain microbiomes. However, there was a distinct "site location" effect, where fungal microbiomes differed significantly between the Season 2 and Season 3 sites. This indicates that, although the same hybrid seed was planted at each site, specific factors at each geographic location or planting time potentially influence on the resultant grain microbiome. Metabarcoding analyses from Season 2 and Season 3 have been completed, confirming absence of *C. zeina* and that the major determinant of fungal diversity in maize grain of the same hybrid is the field location which is more important than whether the plants were sprayed with fungicides or not.

Milestones

Date	Milestone	Progress in Year 3
Month 12	Maize grain fungal microbiome analysis optimized	Completed in Year 1: Grain samples from KZN maize field infected with GLS collected, gDNA extracted, ITS PCR done, and PacBio sequencing done on 24 samples, producing 18000 – 44000 DNA sequence reads per sample to be used for fungal identification.
	Developing the data analysis pipeline for fungal microbiome species identification from	Completed. In-house data analysis pipeline for fungal microbiome species identification (metabarcoding) was developed using Season 1 data, and this was applied to

	PacBio data PNA optimization	Season 2 and 3 data. Completed. PNA blocker reagent arrived from USA supplier. PNA blocker of maize ITS shown to block maize ITS region amplification but it does not block fungal ITS PCR. PNA blocker added to metabarcoding reactions for maize grain from season 2 and season 3. Metabarcoding with PacBio sequencing completed, and maize ITS sequences were removed almost completely as expected.
Month 18	150 fungal isolates from maize grain cultured and identified	Completed. 170 fungal isolates cultured and identified. *Cercospora zeina* was not isolated from any of the grain samples.
Month 24	Seed transmission experiment completed	Completed: Both glasshouse trials have been completed. The first glasshouse trial was to inoculate maize with *C. zeina* for high GLS disease pressure. This was achieved. The kernels/seed from these plants were (i) screened with qPCR to test for *C. zeina*; (ii) planted in the second glasshouse trial under conditions for GLS disease development and screened for GLS disease symptoms. The results were that *C. zeina* was not detected with qPCR in the grain of Trial 1, but the early growth leaves of the maize in the second trial showed some chlorotic lesions that did not develop further. Leaf samples were screened with the qPCR assay and *C. zeina* was absent.
Month 30	Maize grain fungal microbiome screened for *C. zeina* in samples from two seasons. Complete set of 150 fungal cultures identified.	Completed: The maize grain fungal microbiomes (metabarcoding) were completed for Season 2 and Season 3. An average of 99,082 ITS reads per sample were obtained from the metabarcoding but no barcodes corresponding to *C. zeina* were identified. In addition, the qPCR diagnostic assay for *C. zeina* was applied to the same samples without any detection of this fungus. The completed set of 170 fungal cultures from maize grains in this project were identified using DNA-based phylogenetics. The Season 2 and 3 metabarcoding data was only obtained in April 2024, so the final analyses to obtain species-level identifications, and comparison with the culture-based method results are still to be done.
Month 36	Seed dissection experiment completed	Revised and Completed. *C. zeina* was not detected in maize grain samples so in Year 4 this milestone was replaced by qPCR screening of maize grain from small-holder maize farms in KwaZulu-Natal and Eastern Cape. The results confirmed results from commercial farms that *C. zeina* is not present in grain from small-holder farms.

4. Changes to project

Describe changes to the project, provide justification for the changes and impact on outcomes.

- Not applicable – final report.

5. Scientific Outputs

(*Give full references and indicate poster or oral presentation for conference contributions*)

Scientific papers:	Ndaba NS, CM Visagie, JK Gokul, and DK Berger, Species resolution metabarcoding of endophytic fungi in maize grain – ITS long read sequencing plus phylogenetics, Journal of Plant Pathology, in preparation Nsibo, D.L., Barnes, I., and Berger, D.K. (2024). Recent advances in the population biology and management of maize foliar fungal pathogens *Exserohilum turcicum*, *Cercospora zina* and *Bipolaris maydis* in Africa. *Frontiers in Plant Science* 15**,** 1-23. doi: 10.3389/fpls.2024.1404483

	Omondi DO, Dida MM, Berger DK, Beyene Y, Nsibo DL, Juma C, Mahabaleswara SL, Gowda M (2023) Combination of linkage and association mapping with genomic prediction to infer QTL regions associated with gray leaf spot and northern corn leaf blight resistance in tropical maize. Frontiers in Genetics 14:1-16. doi:10.3389/fgene.2023.1282673 Welgemoed T, Duong TA, Barnes I, Stukenbrock EH, Berger DK (2023) Population genomic analyses suggest recent dispersal events of the pathogen *Cercospora zeina* into East and Southern African maize cropping systems. G3 Genes|Genomes|Genetics 13 (11):1-16. doi:10.1093/g3journal/jkad214 Abkallo HM, Arbuthnot P, Auer TO, Berger DK, et al (2024) Making genome editing a success story in Africa. Nature Biotechnology. 42, 551-554. doi:10.1038/s41587-024-02187-2
Technical reports:	Prof DK Berger was a co-author on Policy Brief by the African Union Development Agency for the Pan-African Parliament "Harnessing the benefits of Gene Editing for Africans" (2024)
Articles in industry magazines:	
Conference contributions:	Ndaba NS, Visagie CM, Gokul JK, and Berger DK. (2026) Unravelling the endophytic maize grain mycobiome of grey leaf spot diseased plants: Assessing the seed-borne potential of *Cercospora zeina,* Southern African Society for Plant Pathology Biennial Congress, 18-21 January 2026, accepted as oral presentation. Speed talk: Ndaba NS, Visagie CM, Gokul JK, and Berger DK. Seed health of grey leaf spot diseased maize. 1st FABI-GRP Student Research Day, University of Pretoria, South Africa, 29 July 2025. Meeting attendee: Ndaba NS. 3rd Annual National Grain Research Day, Potchefstroom, South Africa, 18 – 20 March 2025. Poster presentation: Ndaba NS, Visagie CM, Gokul JK, and Berger DK (2024). Maize grain mycobiome of grey leaf spot diseased plants. 19th International Symposium on Microbial Ecology (ISME19), Cape Town, South Africa, 18 – 23 August 2024. Oral presentation: Ndaba NS, Visagie CM, Gokul JK, and Berger DK. Unravelling the endophytic mycobiome of the maize grain from grey leaf spot diseased plants. FABI Seminar, University of Pretoria, South Africa, 08 May 2025. Invited talk: Berger DK (28 September 2023) SA Academic perspective: Genome Editing Innovation, 4th Sustainable Bio-Innovation Symposium, Diep in die Berg, Pretoria. Invited talk: Berger DK (11 March 2024) Regulation of New Breeding Technologies in plants – should we focus on the process or the product? 15th Southern African Plant Breeding Symposium, Bloemfontein, 11-13 March 2024. Berger DK (2024). Invited guest at 35th SANSOR Congress (Connect, collaborate, create change), Gordons Bay, 24-25 April 2024. Berger DK (2024). NAMPO Harvest week, Bothaville,15 May 2024 Visagie CM (2024) 3rd Annual National Grain Research Programme, University of the Free State (3-5 April 2024) Berger DK and Ndaba N (2024). Plant Sciences Department display, Open Day for school learners, Faculty of Natural and Agricultural Sciences, University of Pretoria (16 March 2024). Berger DK and Ndaba N (2024). International Day of Plant Health Workshop and outreach to school learners, Department of Plant and Soil Sciences, University of Pretoria, 10 May 2024. Oral: Ndaba NN (2023) Fungal diversity of grains and leaves from grey leaf spot diseased maize, Department of Plant and Soil Sciences Postgraduate Seminar series (21 July 2023). Oral: Berger DK (2023) Population genomics of *Cercospora zeina*, the maize grey leaf spot pathogen in Africa, Genetics of Maize-Microbe Interactions Seminar series (online), co-ordinated by Prof. P. Balint-Kurti, North Carolina State University, USA (November 2023)
Human capacity development:	PhD student, Mr Nkosinathi Ndaba attended two training workshops: 1. **Name of the workshop**: Bite-sized Biology: Analysing amplicon sequence data with QIIME2 (Working with Illumina data) Date of the workshop: 24 to 25 August 2023 Venue of the workshop: Centre for Microbial Ecology and Genomics, Natural Sciences Building II, University of Pretoria Learning outcomes: Importing and Exporting Data using QIIME2 Sequence analysis and feature table construction in QIIME2 Taxonomic analysis in QIIME2 Diversity analysis using RStudio

	Differential abundance analysis in RStudio Integrative analysis **2. Name of the workshop:** Introduction to Metabarcoding (Working with Illumina data) Date of the workshop: 04 to 05 September 2023 Venue of the workshop: University of Pretoria, FABI2 Learning outcomes: Introduction to metabarcoding library preparation and analysis Basics of Linux bash terminal and basic linux commands Raw data review and quality check Reads pre-processing (primer removal, pair merging, length filtering) ASV generation and quality filtering Mapping reads to ASVs Taxonomic identification of ASVs **3.** Bioinformatics workshop at ISME19 Congress (August 2024)
Technology transfer:	
Other outputs (Procedures, Methods, Databases, etc):	

6. Personnel / Management / Risk factors that influenced progress and lessons learned (*if applicable*)

Not applicable

7. Distribution of the project results to parties in the maize industry.

The results of the project have been presented during the course of the project at events of the National Grain Research Programme.

The results have also been accepted for presentation at the Southern African Society for Plant Pathology 2026 Biennial Congress to be held in January 2026 in Durban. The dissemination plan is for the results to be published in scientific papers, which once published will then be followed up by a popular article, which will then be able to refer to the journal article to confirm that the conclusions are from an “evidence-based report”.

Prof. Berger will continue as a member of the AgBiz working group on Genome Editing Technology to advocate to the Department of Agriculture for regulations that are in line with international best practices. This is an important issue for maize seed companies, SANSOR, and technology developers in the public and private sectors.

8. The possible utilisation of the project results within the South African maize industry.

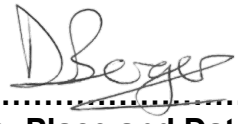
The results from the project have not been able to provide evidence that the fungus *Cercospora zeina* that causes grey leaf spot disease on maize in South Africa is seed borne or seed transmitted. This upholds the current disease cycle of GLS disease, and is good news for the maize seed industry and maize farmers in South Africa since it means that this pathogen is not a phytosanitary concern for import or export of maize seed.

Some technical innovations from this project will benefit future maize production research, namely:

1. Peptide Nucleic Acid (PNA) for enriching fungal PCR and blocking maize PCR in maize grain
2. Metabarcoding analysis pipeline for fungal identification of long read sequencing data.
3. qPCR assay to screen for *C. zeina* in maize grain (previously it had only been shown to work in maize leaves)

With respect to points (1) and (2) this will be implemented immediately: the Grain Research Team at FABI (Prof Cobus Visagie) have been approached by the Maize Grain Millers and Grain SA to research an issue related to mycotoxigenic fungi in certain grain types. This new project will be carried out by FABI-GRP and the Southern African Grain Laboratory (SAGL) using the Peptide Nucleic Acid (PNA) technology that Prof Berger brought into this project for metabarcoding maize grain. This will optimise the identification of fungi by blocking PCR of maize DNA. In addition, the data analysis pipelines for metabarcoding from our project will also be used in this new project.

9. Signature of Project Leader

Prof DK Berger  Pretoria, 28 August 2025

……

Name, Signature, Place and Date

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

FACULTY OF NATURAL AND AGRICULTURAL SCIENCES

FABI Maize Trust Grain Research Programme - Seed Health of Grey Leaf
INCOME AND EXPENDITURE STATEMENT FOR THE PERIOD 1 JUNE 2021 TO 31 MAY 2025

Our ref: N1D389DB01

	Notes	ZAR
Income		**614 808**
Donation - Maize Trust		598 768
Interest received		16 040
Total expenditure		**564 182**
Research activities		***558 668***
Bank charges		427
Laboratory expenses		452 253
Entertainment and refreshments		25 139
Postage and courier fees		6 267
Conference		8 368
Stationery		979
Office consumables		164
Printing expenses		742
Marketing expenses		724
IT Licences + Peripherals		30 000
Assets not Cap		373
Cellphone expenses		955
Travelling expenses		9 312
Subsistence and accommodation		22 964
Capital expenditure		**5 514**
Computer Equipment		5 514
Surplus / (deficit) for the period		**50 626**
Surplus / (deficit) from the previous period		**-**
Surplus / (deficit) at 31 May 2025		**50 626**

This income and expenditure statement reflects the financial position of this fund according to the records of the University of Pretoria.

Deputy Director: Finance

20/08/2025
Date