



Molecular Identification and Phylogenetic Analysis of Maize Ear Rot Fungi in Rwanda

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Abbreviations

Abbreviation	Description
BS	Bootstrap
CTAB	Cetyltrimethylammonium bromide
DON	Deoxynivalenol
FGSC	<i>Fusarium graminearum</i> species complex
FFSC	<i>Fusarium fujikuroi</i> species complex
FOSC	<i>Fusarium oxysporum</i> species complex
FIESC	<i>Fusarium incarnatum-equiseti</i> species complex (FIESC)
<i>rpb2</i>	<i>RNA polymerase II</i> second largest subunit gene
<i>tef1-α</i>	<i>Translation elongation factor 1-alpha</i> gene
ZON	Zearalenone

Table of contents

1. Introduction	10
1.1 Study aim and objectives	13
2. Literature review	14
2.1 Maize ear rot and mycotoxin	14
2.2 <i>Fusarium</i> species associated with maize	16
2.3 Other fungal pathogens associated with maize	16
2.3.1 <i>Aspergillus</i>	16
2.3.2 <i>Penicillium</i>	17
2.3.3 <i>Trichoderma</i>	17
2.3.4 <i>Stenocarpella</i>	17
3. Material and methodology	19
3.1 DNA sequence processing & phylogenetic analysis	19
3.1.1 Sequence quality assessment	19
3.1.2 Consensus sequence generation	19
3.1.3 Species identification using BLAST	19
3.1.4 Sequence alignment	20
3.1.5 Phylogenetic analysis	20
3.2 Co-culture assay	21
3.2.1 Selection of fungal isolates	21
3.2.2 Preparation of culture media	21
3.2.3 Potato dextrose broth	21
3.2.4 Carboxymethyl cellulose broth	21
3.2.5 Mung bean extract broth	21
3.2.6 Musli oat meal agar media	22
3.2.7 Preparation of spore suspension	22
3.2.8 Inoculation of maize seeds	22
3.2.9 DNA extraction	23
3.2.10 Polymerase chain reaction	24
3.3 Statistical analysis	26
4. Results	27
4.1 <i>rpb2</i> and <i>tef1</i> -based phylogeny of <i>Fusarium</i> isolates	27
4.2 Concatenated <i>rpb2</i> and <i>tef1</i> phylogeny	28
4.3 <i>tef1</i> and <i>rpb2</i> -based phylogeny of <i>Trichoderma</i>	29
4.4 Concatenated <i>rpb2</i> + <i>tef1</i> tree for <i>Trichoderma</i>	31
4.5 β -tubulin phylogeny of <i>Penicillium</i>	31

4.6	Distribution of maize ear rot pathogens is determined by geography and maize variety.....	32
4.7	<i>In-vitro</i> assays.....	34
4.7.1	PCR (Polymerase chain reaction)	34
5.	Discussion	35
6.	References.....	38

List of tables

Table 1: Major fungal pathogens associated with maize ear rot, their secondary metabolites, including mycotoxins and potential effects on human and animal health.	15
Table 2: Individual and co-inoculation of maize kernels for <i>in-vitro</i> co-culture assay	23
Table 3: List of Primers that were used in this study	24
Table 4: Association between fungal species distribution and explanatory variables	33
Table 5: Distribution of fungal isolates across maize variety	33

List of figures

Figure 1: Phylogenetic tree based on <i>rpb2</i> sequences of <i>Fusarium</i> isolates. Major clades are shown in different colors with Bootstrap (BS) values (Created by Author) ..	9
Figure 2. Phylogenetic tree of <i>Fusarium</i> isolates based on <i>tef1</i> . Major clades are shown in different colors (Created by Author).....	28
Figure 3: Collapsed phylogenetic tree based on concatenated <i>rpb2</i> and <i>tef1</i> sequences of <i>Fusarium</i> isolates. Major clades are shown in different colors (Created by Author)	29
Figure 4: Maximum likelihood tree based on <i>rpb2</i> sequences of <i>Trichoderma</i> isolates. Major clades are shown in different colors with bootstrap (BS) value (Created by Author)	30
Figure 5: Maximum likelihood tree based on <i>tef1</i> sequences of <i>Trichoderma</i> isolates (Created by Author)	30
Figure 6: Collapsed maximum likelihood tree based on concatenated <i>tef1</i> and <i>rpb2</i> sequences of <i>Trichoderma</i> isolates. Major clades are shown in different colors with bootstrap support (Created by Author).....	31
Figure 7: Maximum likelihood phylogenetic tree based on β -tubulin sequences of <i>Penicillium</i> from this study (Created by Author)	32
Figure 8: Testing PCR primers specificity for fungal isolates	34

Abstract

Maize is a key staple crop globally and plays a critical role in food security in Rwanda. However, its production is threatened by ear rot diseases caused by various fungal pathogens, many of which are known to produce harmful mycotoxins. This study aimed to determine the fungal isolates associated with ear rot in many maize-growing regions of Rwanda by multilocus phylogenetic analysis, and to investigate some of the selected fungal interactions using *in vitro* assays. The *tef1*- α , *rpb2* and *tub2* genes were sequenced to characterise the fungal isolates, and phylogenetic relationships were inferred via maximum likelihood analysis. Additionally, pathogenicity assays and PCR-based detection of species and toxin markers were performed to evaluate the selected isolates. Phylogenetic analysis revealed considerable fungal diversity associated with maize in Rwanda. *Fusarium* isolates were grouped within the following species complexes: *Fusarium graminearum* species complex (FGSC), *Fusarium fujikuroi* species complex (FFSC), *Fusarium incarnatum-equiseti* species complex (FIESC), and *Fusarium oxysporum* species complex (FOSC). β -tubulin analysis identified several *Penicillium* species, with *Penicillium crustosum* forming the predominant cluster. Phylogenetic analysis identified seven *Trichoderma* species that formed distinct groups.

Chi-square analyses showed that the distribution of fungal species differed significantly between provinces, districts, and maize varieties, suggesting that geographic location and host genotype affected the occurrence and distribution of maize ear rot pathogens. PCR amplification using Tri13DON and AFL primers produced inconsistent or non-specific results, whereas VER1 and Fg16N primers produced clear, specific amplicons. Successful amplification in all inoculated treatments confirmed fungal establishment in both single- and co-inoculation assays. Overall, this study highlights the diverse fungal community associated with maize ear rot in Rwanda, emphasising the effectiveness of multilocus phylogenetic analysis in fungal identification and characterization. These findings serve as a foundation for future research into fungal ecology, pathogen interactions, and mycotoxin challenges in maize production systems.

Keywords: Maize, ear rot diseases, *Fusarium*, *Trichoderma*, *Penicillium*, Molecular Markers (*tef1* & *rpb2*), Phylogenetic analysis, Molecular identification, *in vitro* assays

1. Introduction

Maize is one of the world's leading cereals, cultivated on around 200 million hectares, and global production exceeds one billion metric tons (Erenstein et al., 2022). It originated in Mesoamerica, where indigenous people domesticated it over 10,000 years ago. It played a fundamental role in the diet and culture of ancient civilisations such as the Aztecs and the Maya (Logrieco et al., 2021; Pérez et al., 2011). Following European exploration the crop was introduced to Europe, Africa, and Asia, eventually becoming a globally significant agricultural commodity. Beyond its economic value, maize makes a significant contribution to global food security by serving as a major source of carbohydrates, proteins, and micronutrients for millions of people. Its ability to adapt to diverse agro-ecological conditions has enabled its widespread cultivation across more than 160 countries (Smale et al., 2012). Maize production has increased markedly in recent decades globally, due to rising demand and a combination of technological advances, area expansion and increased yield. Maize is the leading cereal in terms of production output and is set to become the most commonly cultivated and export crop in the coming decade (Erenstein et al., 2022). It is a versatile multipurpose crop, but is also important as a food crop, particularly in sub-Saharan Africa and Latin America, as well as for non-food uses (Erenstein et al., 2022).

Maize is a major part of subsistence and commercial agriculture systems and is widely grown by smallholder farmers in Rwanda and elsewhere. It contributes to food security, household income generation, and the economic stability of the country. Maize is an important cereal crop for household nutrition, livestock feed systems and national food security strategies in Rwanda, with an annual consumption of more than 130,000 tonnes (MINAGRI, 2021). Studies have shown that maize accounts for a significant proportion of cereal consumption in East Africa, where it is used in various forms such as flour, porridge, and processed foods (Smale et al., 2012.). Therefore, maize remains essential not only as a staple food but also as a driver of economic development and rural livelihoods across Africa.

Maize ear rot is caused by a complex of fungal pathogens that infect maize ears during the growing season. This results in mold growth and the deterioration of grain quality and kernel discolouration. Multiple fungal genera cause this disease, including *Aspergillus*, *Penicillium*, *Fusarium*, *Gibberella*, *Diplodia* and *Trichoderma* in some regions (Parsons & Munkvold, 2012; Santiago et al., 2020). Ear rot infections typically appear under warm, humid conditions or when the ears are damaged by insects or environmental stress. This allows fungal pathogens to colonise the kernels. The economic importance of ear rot is considerable, as the disease can cause yield losses ranging from 10% to upto 50% depending on

environmental conditions, cultivar susceptibility and pathogen pressure (Logrieco et al., 2003; Munkvold, 2003). The grain quality is severely affected because many ear rot pathogens produce toxic secondary metabolites, including fumonisins, deoxynivalenol (DON), zearalenone and aflatoxins, which contribute to serious health risks to humans and livestock (Logrieco et al. 2021). Contaminated grain often becomes unfit for human consumption, livestock feed, or export markets, resulting in major economic losses. Moreover, infected kernels have reduced nutritional value, poor shelf life, and increased susceptibility to secondary microbial contaminants, further lowering grain quality and marketability (Logrieco et al., 2021; Munkvold, 2003). Consequently, maize ear rot reflects both an agronomic and food safety challenge with worldwide implications. Furthermore, the severity of ear rot symptoms and fungal colonization patterns can influence microbial interactions. The presence of various fungal species within the same maize ear can alter secondary metabolite production, infection dynamics and pathogen fitness (Logrieco et al. 2021). However, these interactions are not yet fully understood, particularly under diverse agro ecological conditions and poor post-harvest environments, where mixed infections may be more prevalent.

DNA-based molecular approaches have proven extremely useful in mycological research for species identification, taxonomic classification, and phylogenetic analyses. The nuclear ribosomal regions, including the internal transcribed spacer (ITS), large-subunit ribosomal RNA gene (LSU) and small-subunit ribosomal RNA gene (SSU), together with other gene markers such as the RNA polymerase II largest-subunit gene (*rpb1*), RNA polymerase II second-largest-subunit gene (*rpb2*), translation elongation factor 1-alpha gene (*tef1- α*), calmodulin gene (*cal*), and β -tubulin gene (*tub2*), have been widely used for fungal species-level identification and phylogenetic analysis (Mehta et al., 2022). Two of the most commonly used protein-coding genes for fungal classification and species-level identification are the translation elongation factor 1 alpha (*tef1- α*) and the RNA polymerase II second largest subunit (*rpb2*). *tef1- α* is very informative due to its moderate rate of evolution, strong resolution capacity among closely related species, and robust amplification across major fungal groups, making it a most suitable secondary barcode for genera such as *Fusarium*, *Penicillium*, and *Aspergillus* (Tekpinar & Kalmer, 2019). *rpb2*, on the other hand, diverges more rapidly than *tef1- α* and provides excellent phylogenetic resolution at both shallow and deep taxonomic levels, retrieving well-supported clades across diverse fungal lineages (Brandon Matheny et al., 2007). For instance, a recent study on *Fusarium* showed that combined *tef1- α* and *rpb2* sequencing enables clear segregation of species within the *F. solani* and *F. fujikuroi* complexes, which are otherwise difficult to differentiate using single-gene approaches (Yassin et al., 2022). *tef1- α* and *rpb2* are frequently employed in

combination in multilocus phylogenetic frameworks, providing excellent species-level identification, phylogenetic accuracy, and greater confidence in taxonomic decisions within Ascomycota and Basidiomycota. These molecular tools are particularly important for bridging specific fungal species to their pathogenic potential and mycotoxin-producing capability in the context of maize ear rot.

1.1 Study aim and objectives

The aim of this study was to examine multilocus sequence data from fungal isolates associated with maize ear rot gathered throughout Rwanda, to identify them and establish their phylogenetic relationships. Additionally, the study sought to investigate whether the distribution of fungal species varies across provinces and districts, and whether the occurrence of these species differs among maize varieties, using statistical analyses. The study also aimed to assess the effect of *in vitro* co-cultivation of selected pathogens on their biomass and mycotoxin production.

Objectives:

1. To examine *tefl* α , *rpb2*, and β tubulin (*tub2*) gene sequences obtained from fungal isolates collected across various maize-growing regions in Rwanda and to generate phylogenetic trees for *Fusarium*, *Trichoderma*, and *Penicillium* isolates for their species-level identification.
2. To determine whether fungal species distribution differs among provinces and districts and whether fungal species occurrence is associated with maize variety using statistical analysis.
3. To conduct an *in vitro* co-culture assay to evaluate the effects of the co-occurrence of selected maize ear rot pathogens on their establishment and mycotoxigenic potential.

2. Literature review

2.1 Maize ear rot and mycotoxin

Maize ear rots are found globally wherever maize is grown, minimizing yield and quality (Munkvold, 2003). Approximately 19 genera of fungal species can infect maize ears, among them *Fusarium* spp., *Penicillium* spp., *Aspergillus* spp., and *Stenocarpella* spp. are mycotoxigenic and severely affect maize yield and quality through the production of mycotoxins (Mukanga et al., 2010). Maize ear rot is increasingly recognised as a field-to-storage disease complex rather than a single event associated with harvest. Infection often begins in the field during the flowering or grain-filling stage, but the effects continue after harvest when the kernels are dried, shelled, stored and transported under conditions that favour fungal growth (Hunde, 2025). Empirical evidence from post-harvest maize handling studies demonstrates that inadequate drying and suboptimal storage conditions markedly increase fungal colonization and mycotoxin accumulation (Chen et al., 2023). The occurrence and distribution of maize ear rot vary mostly depending on environmental conditions and geographic location, maize variety, forecrop, lack of residue management, and insect damage (Atnafu et al., 2024).

Examples of the mycotoxins, that are obtained by the ear rot fungi, are fumonisins (B1, B2 and B3) by *F. verticillioides*, *F. proliferatum*, and *F. nygamai*; aflatoxin by the *A. flavus* and *A. parasiticus*, ochratoxin by *P. verrucosum* and *A. ochraceus*, diplodiotoxin by *Stenocarpella* spp., deoxynivalenol (DON), and zearalenone by *F. graminearum* (Table 1). Substantial levels of ear rot infection and mycotoxin accumulation have been reported in maize in Europe, North and South America, North Africa, Asia and East Africa. The presence of mycotoxins in pre-harvest maize is of big concern, because they cause health diseases in both humans and livestock that ingest contaminated grains (Mukanga et al., 2010 & Thapa et al. 2021).

Table 1: Major fungal pathogens associated with maize ear rot, their secondary metabolites, including mycotoxins and potential effects on human and animal health.

Fungal pathogens	Toxins/ Metabolites	Effects on Human & Animals	References
<i>Aspergillus</i> spp	Aflatoxins (B1, B2, Ochratoxin A)	Potent hepatocarcinogenic, immunosuppressive	(Chen et al., 2023)
<i>Fusarium graminearum</i>	Deoxynivalenol, Zearalenone	Gastrointestinal, immunological, reproductive	(Thapa et al. 2021)
<i>Fusarium verticillioides</i>	Fumonisin (B1, B2 and B3)	Hepatotoxic, nephrotoxic	(Mesterhazy 2024)
<i>Penicillium</i> spp	Roquefortine C, Penitrem A, Cyclopiazonic acid, Patulin	Neurotoxin, immunosuppressive, Hepatotoxic	(Frisvad & Samson, 2004.; Perrone & Susca, 2017; Visagie et al., 2014)
<i>Trichoderma</i> spp	Harzianic acid, Peptaibols	-	(Pfordt et al., 2020; Steindorff et al., 2026)
<i>Talaromyces</i> spp	Red polyketides metabolites, Purpactins, Talaroconvolutins	-	(Lei et al., 2022)
<i>Epicoccum</i> Spp	Epicorazine A&B, Flavipin & polyketide	-	(Deshmukh et al., 2014)
<i>Thielaviopsis</i> spp	Produce phytotoxic compounds	-	(Ayika et al., 2024)

<i>Stenocarpella</i> spp	Diplodiatoxin, Chaetoglobosins (cytotoxic, actin- binding)	Immuno suppressive, Livestock poisoning	(Alberts et al., 2016)
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2.2 *Fusarium* species associated with maize

Several *Fusarium* species, including *F. graminearum*, *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, *F. temperatum*, *F. boothii*, *F. meridionale*, and *F. temperatum* have been reported as maize ear rot pathogens (Deressa et al., 2025). These species can infect maize ears alone and can also co-occur, which can aggravate disease severity and mycotoxin accumulation (Munkvold, 2003; Santiago et al., 2020). Co-infection with *F. proliferatum* or *F. graminearum* with *F. verticillioides* leads to higher fumonisin levels and significant kernel colonization, suggesting that these fungi may enhance their pathogenicity and metabolic activity synergistically (Logrieco et al., 2002). Similar synergy has been recorded between *F. verticillioides* and *A. flavus*, where co-infection leads to raised levels of both fumonisins and aflatoxins, causing high food safety risks due to combined mycotoxin contamination. *F. verticillioides* is the most commonly isolated species across global maize production systems, particularly dominating in tropical and subtropical regions (Munkvold, 2003). This species has been found in symptomatic and asymptomatic maize kernels (Lanubile et al., 2017). In contrast, *F. graminearum* is more common in temperate regions, such as Europe and North America, and is mainly associated with *Gibberella* ear rot under cool and wet conditions. (Oldenburg et al., 2017). Other species, including *F. subglutinans* and *F. temperatum*, are found less frequently but are increasingly identified through multilocus sequencing approaches targeting genes such as *tefla* and *rpb2* (Santiago et al., 2020; Scaufaire et al., 2011).

2.3 Other fungal pathogens associated with maize

2.3.1 *Aspergillus*

Aspergillus species, especially *A. parasiticus* and *A. flavus*, rank among the major fungal pathogens causing maize ear rot. These fungi infect maize in the field and during storage, particularly when kernels are damaged or exposed to heat stress. They produce aflatoxins (B1, B2, G1, G2), which are some of the most potent natural carcinogens (Chen et al., 2023). Aflatoxin B1 is classified by the International Agency for Research as a Group 1 carcinogen on Cancer and is

linked to liver cancer, immune system suppression, impaired growth in children, and deadly aflatoxicosis outbreaks in humans and animals (Wu et al., 2014). Recent research indicates that climate-related increases in temperature and drought have escalated the incidence of *Aspergillus* ear rot across Africa, Asia, and the Americas, positioning *A. flavus* as a leading ear-rot pathogen in maize production globally (Cotty & Jaime-Garcia, 2007; Mutiga et al., 2015).

2.3.2 *Penicillium*

Penicillium species are more frequently implicated in maize spoilage during storage rather than in field infections. Species such as *P. crustosum* and *P. verrucosum* are commonly isolated from stored maize, particularly where moisture levels are high and drying is insufficient. These fungi thrive in cooler, humid environments and are major contributors to grain deterioration during storage. Some *Penicillium* species produce mycotoxins like ochratoxin A, which is concerning due to its nephrotoxic and carcinogenic effects. The presence of *Penicillium* in maize is thus largely linked to post-harvest storage conditions and environmental factors, especially in systems lacking effective post-harvest management (Frisvad & Samson, 2004).

2.3.3 *Trichoderma*

Trichoderma is notable because it is typically regarded as a beneficial biocontrol agent; however, recent research has identified certain strains, particularly *T. afroharzianum*, as maize pathogens (Pfordt et al., 2020). A 2020 report from Europe described *T. afroharzianum* ear rot as an emerging disease in maize. Subsequent studies revealed that pathogenic strains could reduce cob weight, impair grain quality, and severely diminish germination rates. Furthermore, pathogenicity was not confined to *T. afroharzianum*; species such as *T. asperellum*, *T. atroviride*, and *T. guizhouense* were also found to cause disease in some instances (Steindorff et al., 2026). Phylogenetic analyses using *tefl* and *rpb2* genes did not distinctly separate pathogenic from non-pathogenic isolates (Pfordt et al., 2020).

2.3.4 *Stenocarpella*

Stenocarpella species, especially *S. maydis* and *S. macrospora*, are key pathogens causing Diplodia ear rot of maize. Infected ears will have white to gray colonies of fungus at the base of the cob and move up the cob to yield light-weight, discolored kernels. These fungi have secondary metabolites such as diplodiatoxin, chaetoglobosins, associated with immunosuppression, loss of weight gain and respiratory disorders in ruminants that feed on contaminated feed. (Alberts et al., 2016). *Stenocarpella* infections typically occur as single, dominant infections,

although co-infections with *Fusarium* species have been observed in humid field environments. Because they can persist in crop residues, *Stenocarpella* spp. continue to pose significant risks in no-till and residue-retaining maize production systems.

3. Material and methodology

3.1 DNA sequence processing & phylogenetic analysis

Fungal isolates were subjected to molecular characterization using Sanger sequencing of the translation elongation factor 1-alpha (*tefl*- α) and RNA polymerase II second largest subunit (*rpb2*) genes for *Fusarium* and *Trichoderma*. β tubulin (*tub2*) gene was used for *Penicillium* species.

3.1.1 Sequence quality assessment

Raw sequence chromatograms from *rpb2* & *tefl* based on *Fusarium* isolates, from *rpb2* & *tefl* based on *Trichoderma* and from beta tubulin for *Penicillium* isolates obtained from Sanger sequencing were inspected visually to assess sequence quality. Low-quality regions, ambiguous base calls, and sequencing artefacts were identified and manually trimmed to ensure accuracy of downstream analyses. Sequence editing and quality assessment were performed using DNASTAR SeqMan Pro (version 17), following standard procedures for chromatogram evaluation and base-calling correction. For sequence quality control, similar approaches have been widely applied in fungal molecular identification studies (O'Donnell et al., 2015; Toohey-Kurth et al., 2020).

3.1.2 Consensus sequence generation

Forward and reverse reads were trimmed and assembled into contigs in DNASTAR, SeqMan Pro v17 (Madison, WI, USA) to generate high-confidence consensus sequences for each isolate. Consensus sequences were exported in FASTA format and saved individually. This method aligns with standard Sanger sequence processing protocols used in molecular taxonomy and phylogenetic studies (Singh & Bhatia, 2016).

3.1.3 Species identification using BLAST

Consensus sequences underwent preliminary species identification through Nucleotide Basic Local Alignment Search Tool (BLAST) searches against the National Center for Biotechnology Information (NCBI) nucleotide database. The closest matches, along with their percentage identity and query coverage, were documented to support downstream phylogenetic analysis.

3.1.4 Sequence alignment

Consensus FASTA sequences were aligned using AliView v1.26 (Larsson, 2014), using the MUSCLE algorithm for multiple sequence alignment. The alignments were visually inspected to ensure correct codon positioning and to remove poorly aligned or ambiguous regions. MUSCLE is widely used for accurate and computationally efficient alignment of protein-coding genes in phylogenetic studies. The final alignments were exported in FASTA format for downstream phylogenetic analysis.

3.1.5 Phylogenetic analysis

The curated FASTA alignment files were used as input for IQ-TREE v2 (Minh et al., 2020) executed through Windows PowerShell. The best-fit nucleotide substitution model was selected automatically using Model Finder, and phylogenetic reconstruction was performed under the maximum likelihood framework. The best-fit model TIM2e+R3 for *tefl*-based *Fusarium* phylogeny, TIM2+R6 for *rpb2*-based *Fusarium* phylogeny, K3Pu+F+I+R3 for *tefl*-based phylogeny of *Trichoderma*, TIM3e+R5 for *rpb2*-based phylogeny of *Trichoderma* and TNe+I+R2 for phylogeny of *Penicillium* was used. Branch support was assessed using 1000 ultrafast bootstrap replicates (UFBoot). IQ-TREE is widely used for robust phylogenetic inference in fungal systematics (Minh et al., 2020). Moreover, the same steps were repeated for *Trichoderma* and *Fusarium*, with each gene (*tefl* and *rpb2*) analysed separately to generate individual phylogenetic trees.

Furthermore, in addition to single-gene analysis, concatenated phylogenetic trees were also made using an online tool (ACES, MES concatenation) by merging the corresponding *tefl* and *rpb2* sequences for each isolate, excluding reference sequences. The fused alignment was then inspected in Aliview, exported as a FASTA file and subjected to the same phylogenetic workflow in IQ-TREE (Minh et al., 2020).

3.2 Co-culture assay

3.2.1 Selection of fungal isolates

Four fungal isolates were selected based on phylogenetic identification and were assigned laboratory codes for traceability throughout the experiments. These included *F. graminearum* (19-8:2_B1), *F. verticillioides* (7:3_A1), *A. flavus* (33-22:2_A2) and *P. crustosum* (26-15:2_B1). These isolates were selected due to their relevance to maize contamination and their known mycotoxigenic potential.

3.2.2 Preparation of culture media

Four different media were prepared for fungal cultivation for optimized spore production.

3.2.3 Potato dextrose broth

Commercial Potato Dextrose Broth (PDB; Difco™, Becton, Dickinson and Company, Sparks, MD, USA) powder was used according to the manufacturer's instructions by dissolving 39 g in 1 L of distilled water. The medium was mixed thoroughly and autoclaved. After cooling, the medium was inoculated with selected fungal isolates and incubated in the dark at 25 °C on a shaker at 100 rpm for a week.

3.2.4 Carboxymethyl cellulose broth

Carboxymethyl Cellulose (CMC) broth was prepared by dissolving 15 g of carboxymethyl cellulose in approximately 300 mL of water with continuous magnetic stirring due to its low solubility. After partial dissolution, 1 g of NH_4NO_3 , 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1 g of yeast extract were added. The final volume was adjusted to 1 L with Milli-Q water, mixed thoroughly and autoclaved. Once cooled, it was inoculated with the fungal isolates and incubated under shaking conditions.

3.2.5 Mung bean extract broth

Yellow mung beans were soaked in water for 2 hours, then boiled until the water turned yellow-brown. The extract was filtered, adjusted to 1 L, autoclaved, and transferred to 250 mL flasks. After cooling, the broth was inoculated with fungal strains and incubated under shaking conditions.

3.2.6 Musli oat meal agar media

A 14 g of Musli oat meal and 10g of agar were dissolved in 1 L of water, and then autoclaved. After autoclaving, this medium was poured into Petri plates, followed by the inoculation of selected fungal strains, which were incubated at 25 °C for a week.

3.2.7 Preparation of spore suspension

After 7 days of incubation in liquid media, fungal cultures were processed to obtain clean spore suspensions. The entire broth culture from each flask was passed through sterile Mira cloth to remove mycelial fragments and retain only conidial fractions. The filtrate containing spores was collected in sterile tubes.

Spore suspension was determined using haemocytometer. A 10 µL of the filtrate was loaded onto the haemocytometer chamber, and spores were counted under a light microscope following the standard counting procedures. Based on the initial counts, the suspensions were diluted with distilled water to get the final concentration of 1×10^5 spores/mL for all isolates. This normalized inoculum was utilized in all the subsequent pathogenicity assays.

3.2.8 Inoculation of maize seeds

For the pathogenicity assay, all the treatments were set up using five biological replicates, each replicate consisting of a Petri plate containing 50 seeds. The seeds were autoclaved with small amount of water to soften enough, which facilitated fungal penetration and nutrient access for the pathogens during colonization.

Standardized spore suspensions at a concentration of (1×10^5 spores/mL) were applied at a total volume of 4 mL per plate, distributed according to the treatment combinations. The combinations were designed based on our hypothesis to evaluate whether the selected fungi exhibit antagonistic or synergistic interactions during co-infection, particularly in relation to infection spread and mycotoxin production (Table 2). The treatment groups included:

- Single isolates: 4 mL from one isolate
- Dual combination: 2 mL + 2 mL from each isolate
- Triple combination: 1.33 mL $\times$ 3 from each isolate
- Quadruple combination: 1 mL $\times$ 4 from all four isolates

Table 2: Individual and co-inoculation of maize kernels for in-vitro co-culture assay

<i>Fusarium verticillioides</i> (FV)
<i>Fusarium graminearum</i> /Meridionale (FG)
<i>Aspergillus flavus</i> (AF)
<i>Penicillium crustosum</i> (PC)
FV+FG
FV+AF
FG+AF
FV+PC
FG+PC
AF+PC
FV+FG+AF
FV+FG+PC
FV+PC+AF
FG+PC+AF
FV+FG+AF+PC

FV = *Fusarium verticillioides*; FG = *Fusarium graminearum*/F. meridionale; AF = *Aspergillus flavus*; PC = *Penicillium crustosum*.

After inoculation, plates were sealed with Parafilm and incubated at 25 °C in complete darkness. After a week of incubation, the samples were harvested and transferred into corresponding pre-weighed 50 mL Falcon tubes, then placed at -70 °C for two days to ensure complete freezing, and subsequently freeze-dried.

3.2.9 DNA extraction

Genomic DNA was isolated from the freeze dried samples using CTAB extraction method. A precisely weighed small subsample from each treatment was utilized, yielding high- quality genomic DNA through CTAB method. A sample was collected and placed into a 1.5 mL Eppendorf tube. To each tube, 700 µL of 3% CTAB solution was added, followed by vortexing to homogenize the sample. The tubes were then incubated at 65 °C for 30 minutes, with intermittent shaking. After incubation, the tubes were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was transferred to a new Eppendorf tube. Subsequently, 600 µl of chloroform was added, the mixture was vortexed, and centrifuged at 13,000 rpm for 15 minutes. The supernatant was transferred to a fresh 1.5 mL tube. An equal volume of chloroform was added, vortexed, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was moved to a new tube, then an equal volume of isopropanol was added, mixed gently, and the sample was precipitated at -20 °C for 20 minutes. The sample was centrifuged at 13,000 rpm for 20 minutes, the supernatant was discarded, and the pellet briefly air-dried. The pellet was washed with 200 µL of 70% ethanol, centrifuged at 13,000 rpm for 5 minutes, and the

supernatant discarded. Finally, the pellet was dried and dissolved in 50 µL of TE buffer. DNA concentration was measured using a Nanodrop.

3.2.10 Polymerase chain reaction

Gradient PCR was conducted using various primer sets over an annealing-temperature range of 50–65 °C to assess primer specificity. Additional PCR assays were conducted using species- and toxin-associated primer pairs supplied by the supervisors (Table 3). The primer pairs included Tri13DON-F/Tri13DON-R for detecting DON chemotypes within the *Fusarium graminearum* species complex; aflP-F/aflP-R for detecting the aflatoxin-biosynthesis-associated *aflP* gene in *Aspergillus flavus*; Fg16F/Fg16R for detecting members of the *F. graminearum* species complex; Bt2a/Bt2b for amplifying the β -tubulin (*tub2*) region of *Penicillium* species; VER1/VER2 for identifying *F. verticillioides*; and Fum5F/Fum6R for detecting the fumonisin-biosynthesis-associated *FUM1* region in *Fusarium* species.

PCR amplification was performed using DreamTaq™ PCR Master Mix (Thermo Fisher Scientific). The thermal-cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. The PCR products were separated by agarose gel electrophoresis and visualised under ultraviolet light to determine whether products of the expected sizes were obtained.

Table 3: List of Primers that were used in this study

Target species/group	Selected primers	Sequence (5'–3')	Expected amplicon size	Annealing temperature
<i>Fusarium graminearum</i> species complex	Fg16F	CTCCGGATATGTTGCG TCAA	Approximately 400–500 bp, commonly 450 bp	55 °C
	Fg16R	GGTAGGTATCCGACA TGGCAA		
<i>Penicillium</i> spp.	Bt2a	GGTAACCAAATCGGT GCTGCTTTC	Approximately 450–550 bp; size can vary among species	55 °C
	Bt2b	ACCCTCAGTGTAGTGA CCCTTGGC		
<i>Fusarium verticillioides</i>	VER1	CTTCCTGCGATGTTTC TCC	578 bp	55 °C

	VER2	AATTGGCCATTGGTAT TATATATCTA		
<i>Fusarium meridionale/ F. graminearum</i>	Tri13 DON F Tri13 DON R aflP F	CATCATGAGACTTGTK CRAGTTTGGG GCTAGATCGATTGTTG CATTGAG	282bp	50 °C
<i>Aspergillus flavus</i>	aflP R	GACCAATACGCCCAC ACAG CTTTGGTAGCTGTTTC TCGC	301bp	55 °C

3.3 Statistical analysis

Data analysis was carried out using R software (version 4.4.1; R Core Team, 2024). Frequency and distribution of the fungal species were summarized using descriptive statistics, at provincial, district and maize variety levels. Fungal species occurrence data were summarized in contingency tables, and chi-square test of association was done using the stats package to test the significance of the difference in the distributions of the different species among provinces, districts and maize varieties. The strength of statistically significant associations was measured with Cramer's V, computed from the package companion. All statistical analysis was conducted at the 5% significance level (i.e. at a significance level of $P < 0.05$). Frequency tables and graphical summaries were used to present the results to aid comparison and interpretation of the distribution of fungal species between the study areas.

4. Results

4.1 *rpb2* and *tef1*-based phylogeny of *Fusarium* isolates

The *rpb2*-based phylogeny representing both isolates from this study and reference sequences identified four *Fusarium* species complexes. This includes the *F. graminearum* species complex (FGSC), the *F. incarnatum-equiseti* species complex (FIESC), the *F. fujikuroi* species complex (FFSC), and the *F. oxysporum* species complex (FOSC). FGSC consisted of *F. graminearum*, *F. meridionale*, *F. asiaticum*, and *F. boothii* together with 170 isolates from this study. Within this complex, *F. graminearum* and *F. meridionale* were the dominant species, comprising 133 of the isolates. The FFSC encompassed 188 isolates potentially identified as *F. verticillioides*, *F. subglutinans* and *F. andiyazi*. Additionally, 11 isolates from FIESC and 8 from FOSC consisted of *F. incarnatum*, *F. equiseti* and *F. oxysporum*, respectively. Several subgroups were present within the large clade; some contained only study isolates, and others included both study and reference sequences, with varying bootstrap values, such as 85%, 90% and 67%. Additionally, within this broad cluster of species complexes, multiple subgroups were observed with lower bootstrap values (Figure 1).

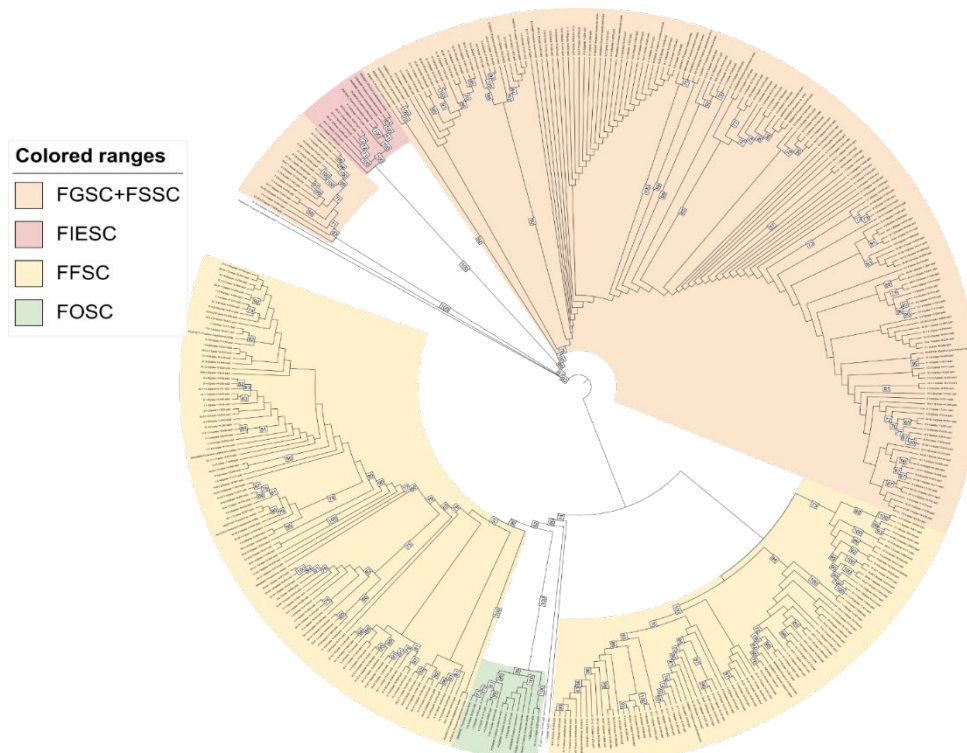


Figure 1: Phylogenetic tree based on *rpb2* sequences of *Fusarium* isolates. Major clades are shown in different colors with Bootstrap (BS) values (Created by Author)

TEF1-based phylogenetic analysis revealed similar results to those based on *rpb2*-based phylogeny (Figure 2). The analysis recovered the same major complex (FGSC), though this complex was supported by low bootstrap values of 39%.

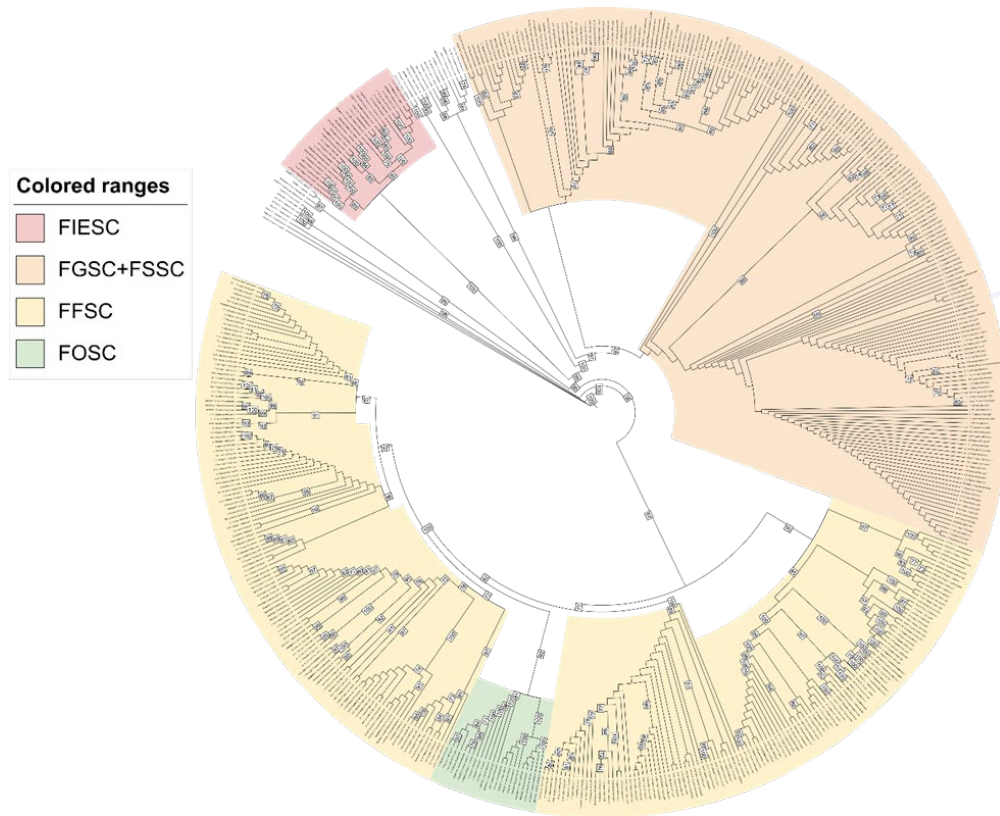


Figure 2. Phylogenetic tree of *Fusarium* isolates based on *tef1*. Major clades are shown in different colors (Created by Author)

4.2 Concatenated *rpb2* and *tef1* phylogeny

A phylogenetic tree based on concatenated *rpb2* and *tef1* gene sequences further resolved the relationships among the observed *Fusarium* isolates into better-supported clades corresponding to major species complexes. The isolates clustered according to their representative species complexes, including FFSC, FIESC and FOSC. Within this tree, *F. verticillioides* grouped among FFSC, while *F. subglutinans* also formed part of this complex. *F. graminearum* clustered within the FGSC clade, whereas *F. oxysporum* formed a separate discrete lineage consistent with FOSC.

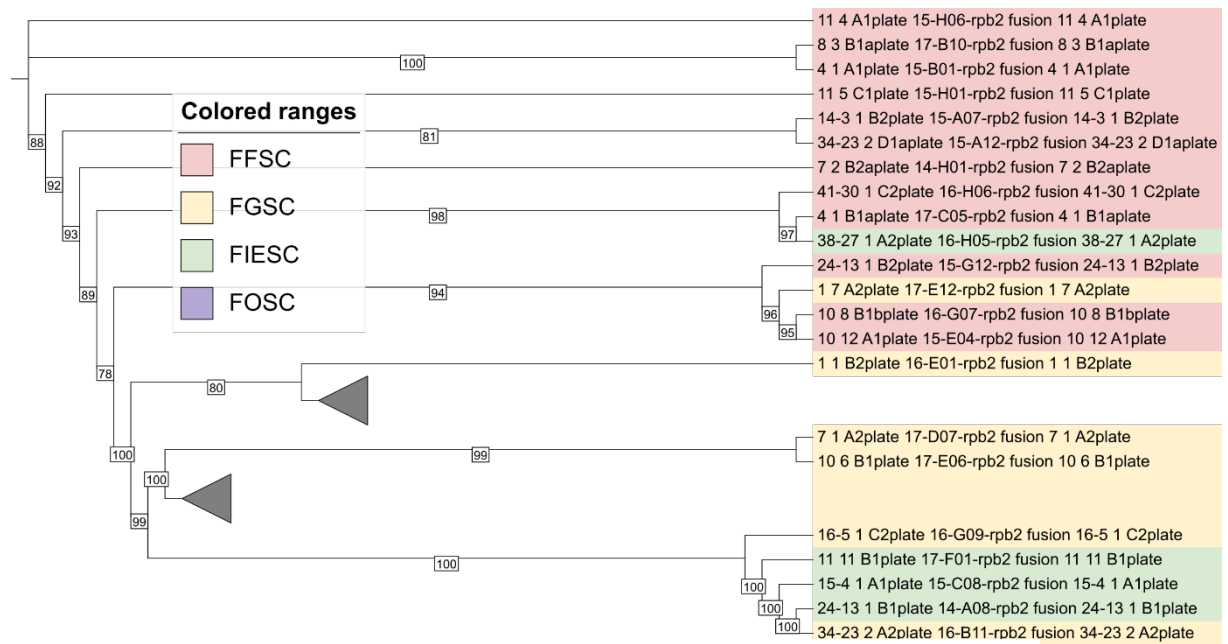


Figure 3: Collapsed phylogenetic tree based on concatenated *rpb2* and *tef1* sequences of *Fusarium* isolates. Major clades are shown in different colors (Created by Author).

4.3 *tef1* and *rpb2*-based phylogeny of *Trichoderma*

The *tef1* and *rpb2*-based phylogenetic analysis of *Trichoderma* resulted in the identification of eight *Trichoderma* species. Fourteen isolates from this study were placed outside the main cluster as an outgroup. The largest clade corresponded to *T. paratroviride* representing 64 isolates from this study with a 76% BS value. A distinct group corresponding to *T. nordicum* containing four isolates from this study supported by a bootstrap value of 100%, was recovered. Another clade represented as *T. gamsii* group included three isolates from this study and was supported by a 98% bootstrap value. Furthermore, four and six isolates formed a cluster with *T. asperelloides* and *T. afroharzianum*, respectively, with a strongly supported bootstrap value of 100% (Figure 4). Five of the isolates formed a separate cluster without any reference sequence included.

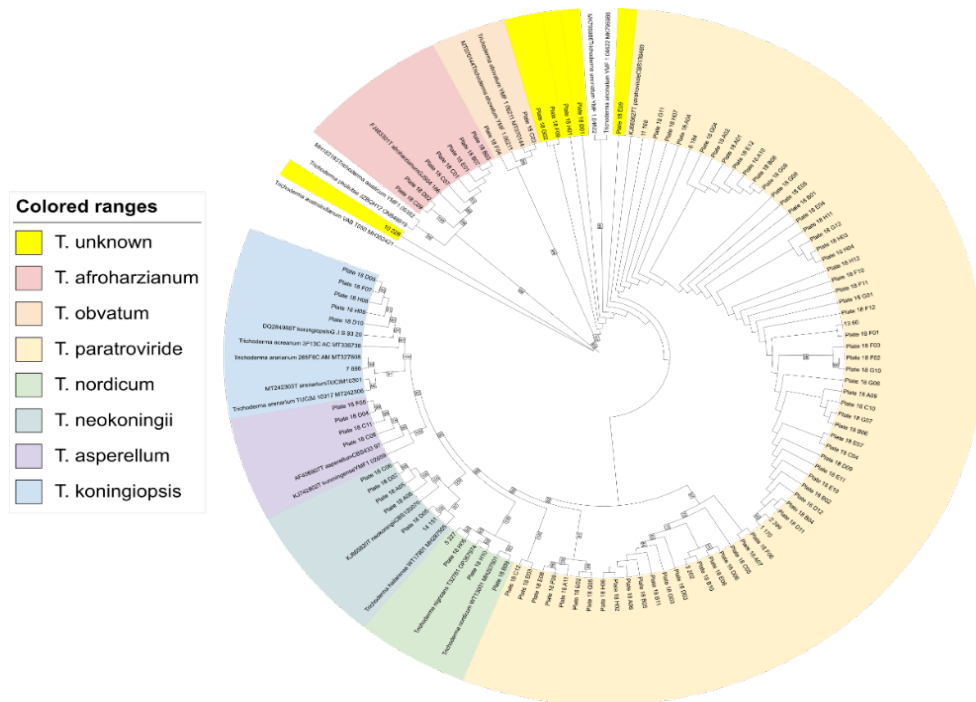


Figure 5: Maximum likelihood tree based on *tef1* sequences of *Trichoderma* isolates (Created by Author)

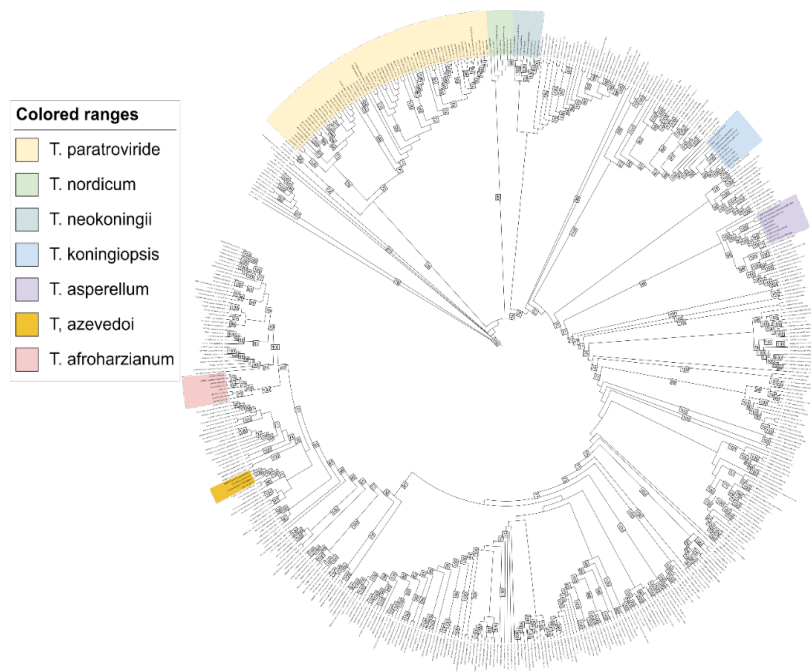


Figure 5: Maximum likelihood tree based on *rpb2* sequences of *Trichoderma* isolates. Major clades are shown in different colors with bootstrap (BS) value (Created by Author).

4.4 Concatenated *rpb2*+ *tef1* tree for *Trichoderma*

The collapsed concatenated tree based on *rpb2* and *tef1* sequences from the isolates of this study resolved seven major clades within *Trichoderma*. One cluster contained isolates belonging to the *T. afroharzianum* group, while another distinct cluster represented the *T. asperelloides* group. A mixed cluster was also observed comprising of *T. acreanm*, *T. dorothisopsis* and *T. arenarum*. Additionally, a well-supported clade including *T. gamsii* and *T. paratroviride* complex was seen. All the major clusters were supported with 100% bootstrap support (Figure 6).

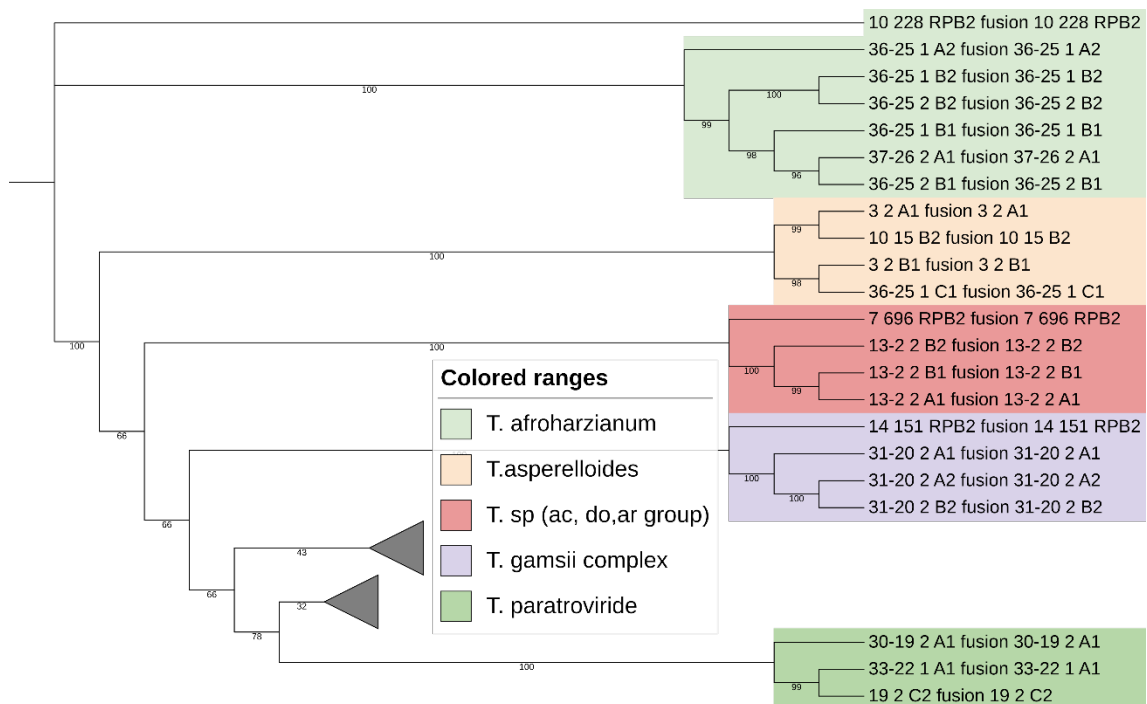


Figure 6: Collapsed maximum likelihood tree based on concatenated *tef1* and *rpb2* sequences of *Trichoderma* isolates. Major clades are shown in different colors with bootstrap support (Created by Author).

4.5 β -tubulin phylogeny of *Penicillium*

The phylogeny of β -tubulin identified four *Penicillium* species. The most abundant was *P. crustosum*, comprising 22 isolates. This was followed by three isolates of *P. brevicompactum* with a bootstrap value of 94%; four isolates of *P. lanosocoeruleum* supported by a 96% bootstrap value; and three isolates of *P. polanicum* supported by a 78% bootstrap value (Figure 7).

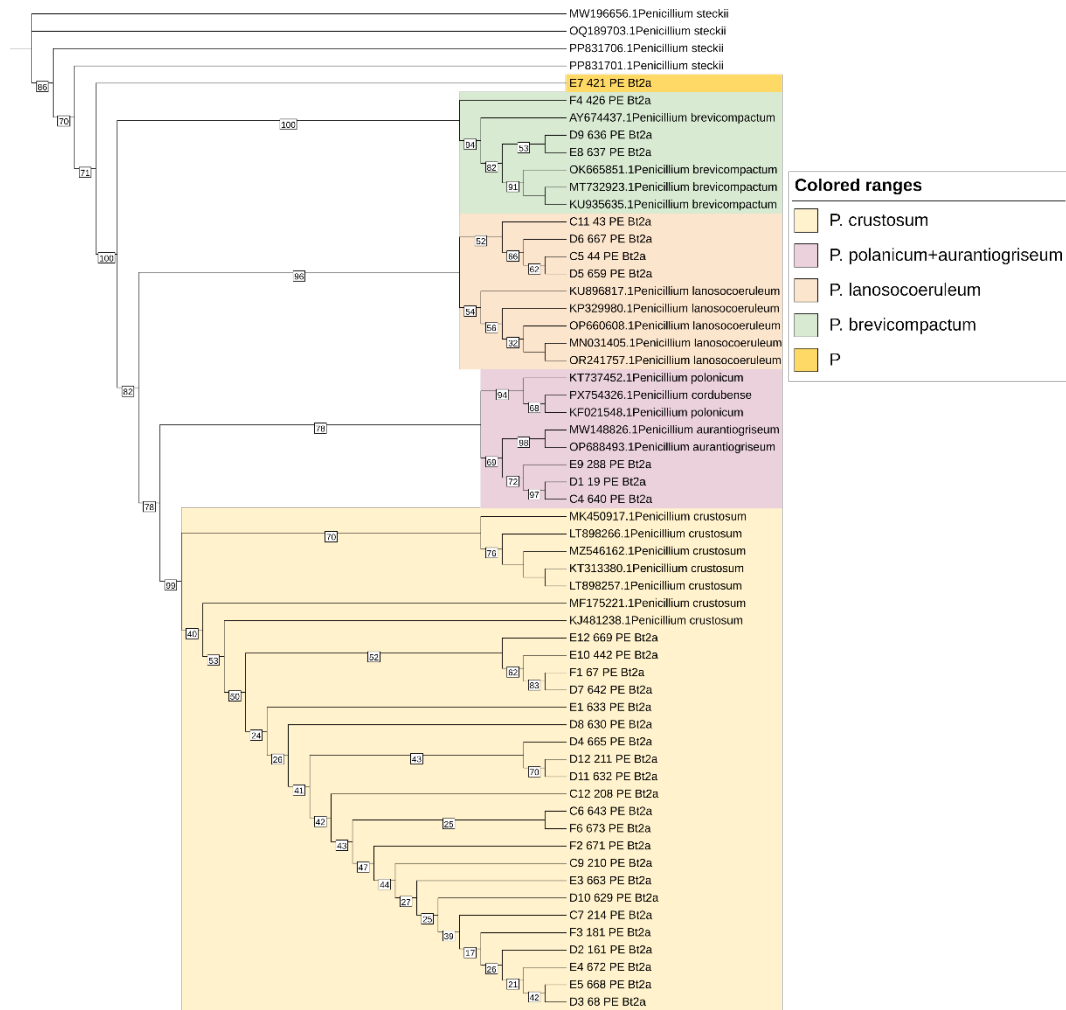


Figure 7: Maximum likelihood phylogenetic tree based on β -tubulin sequences of *Penicillium* from this study (Created by Author)

4.6 Distribution of maize ear rot pathogens is determined by geography and maize variety

A correlation analysis was performed to test whether the distribution of fungal isolates purified from maize ear rot was associated with geography (provinces and districts) and maize variety in Rwanda. The chi-square analysis revealed highly significant associations between fungal species composition and both province ($\chi^2 = 675.29$, $p < 0.001$, Cramer's $V = 0.589$) and district ($\chi^2 = 2647.00$, $p < 0.001$, Cramer's $V = 0.441$). The effect size was strong for provinces and moderate-to-strong for districts, indicating that geographic location had a substantial influence on fungal community composition (Table 4). For example, *F. meridionale* was most abundant in the Southern Province, whereas *Mucor spp.* occurred predominantly in the Eastern Province. This indicates that indicate that

geographic location significantly influenced the distribution of fungal species in maize samples.

Table 4: Association between fungal species distribution and explanatory variables

Variable		χ^2 Value	p-value	Cramer's V	Effect Size
Province	×	675.29	<0.001	0.589	Strong
Species					
District	×	2647.00	<0.001	0.441	Moderate– Strong
Species					
Variety	×	2000.70	<0.001	0.393	Moderate
Species					

Note: Monte Carlo simulation (10,000 replicates) was used for all chi-square tests because of sparse contingency tables and low expected frequencies

The variety × fungal species contingency analysis revealed considerable variation in fungal occurrence among maize varieties. Variety RHM 1409 was predominantly associated with *F. meridionale*, while P0017 showed high frequencies of *F. meridionale*, *F. subglutinans*, and *F. graminearum*. The chi-square analysis demonstrated a highly significant association between maize variety and fungal species occurrence ($\chi^2 = 2000.70$, $p < 0.001$, Cramer's V = 0.393), suggesting that maize genotype contributed significantly in determining the occurrence and composition of maize ear rot pathogens (Table 5).

Table 5: Distribution of fungal isolates across maize variety

Variety	Dominant species
RHM 1409	<i>Fusarium meridionale</i> (59), <i>Fusarium subglutinans</i> (13), <i>Trichoderma paratroviride</i> (12)
P0017	<i>Fusarium meridionale</i> (28), <i>Fusarium subglutinans</i> (17), <i>Fusarium graminearum</i> (14)
TUBURA	<i>Fusarium andiyazi</i> (11)
WH403	<i>Mucor spp.</i> (19), <i>Fusarium spp.</i> (11)
WH507	<i>Fusarium spp.</i> (10)
WH629	<i>Trichoderma spp.</i> (7)

4.7 *In-vitro* assays

4.7.1 PCR (Polymerase chain reaction)

DNA extraction through the CTAB method yielded good concentrations from all samples. Gradient PCR was conducted using various primer sets over a range (50–65 °C) of annealing temperatures to assess primer specificity, resulting in clear amplification patterns for the tested primer pairs. Additionally, a separate set of PCR tests with toxin-specific primers produced distinct amplicons in samples containing the corresponding toxin-related genes. Overall, the amplification results confirmed consistent DNA quality across samples and enabled reliable detection of both general and toxin-associated markers.

PCR amplification using Tri13DON and AFL Primer sets failed to produce the expected specific amplicons in some samples. Gel electrophoresis showed no amplification or non-specific bands, indicating inconsistent detection under the tested conditions. FUM1 was amplified in *F. verticillioides* consistently across different temperatures (Figure 8, wells 1, 2, 3, 4), confirming detection of fumonisin-associated genes. Moreover, no amplification within Fg16N primer in mixed samples showed its specificity for *F. graminearum* only. Amplification of β -tubulin across all isolates confirmed its role as housekeeping gene. In contrast, the VER1 primer set generated clear, distinct bands corresponding to the expected size. The lack of prominent non-specific products confirmed the specificity of this primer for the target isolates.

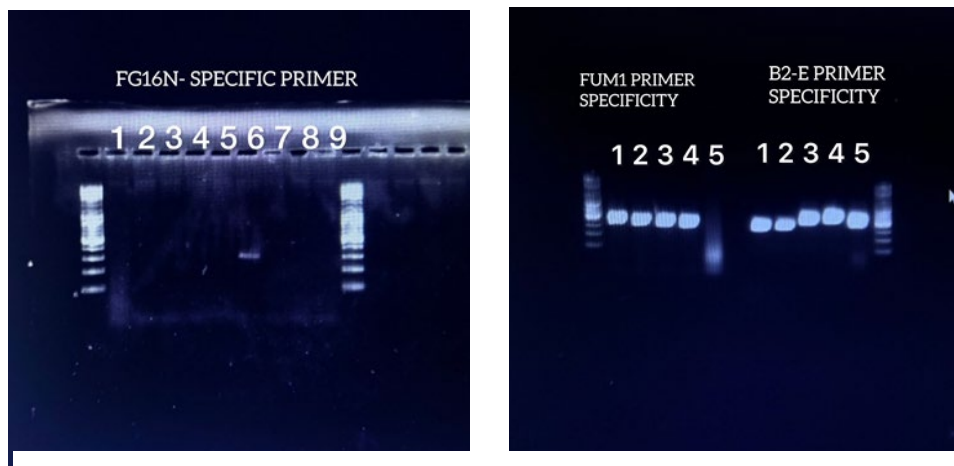


Figure 8: Testing PCR primers specificity for fungal isolates

5. Discussion

The multilocus phylogenetic analyses provided species-level resolution among the fungal isolates associated with maize in Rwanda. Across all genetic markers, *Fusarium* isolates consistently clustered into the major species complexes that are globally recognized as dominant maize ear rot pathogens, including the FGSC, FFSC, FOSC, and FIESC. The dominance of FGSC and FFSC in both single-gene and combined phylogenetic trees corresponds with global findings that identify these complexes as the main agents of maize ear rot and mycotoxin production (Logrieco et al., 2021; O'Donnell et al., 2013). High bootstrap values across *rpb2*, *tef1*, and concatenated datasets demonstrate the robustness of these loci for resolving *Fusarium* diversity (Jabłońska et al., 2020) at the species complex level. The detection of multiple subclusters within each complex indicates genetic variation and potential geographic structuring among the isolates from Rwanda, a pattern similarly reported in other maize-producing regions of Africa (Deressa et al., 2025). Although *tef1* and *rpb2* sequences demonstrated strong resolution of the major *Fusarium* species complexes, such as FGSC and FFSC, their capacity to distinguish species-level differences was limited. Although many isolates could be assigned to species complexes, the precise species boundaries within these groups remained unresolved. These observations align with previous studies (O'Donnell et al., 2004). Therefore the present results showed both the strengths and limitations of *tef1* and *rpb2*. Yilmaz et al. (2021) showed that combining *tef1* and *rpb2* with additional loci such as *rpb1*, β -tubulin (*tub2*), and calmodulin (CMDA) significantly enhanced phylogenetic resolution and allowed for the identification of cryptic taxa that were indistinguishable with fewer markers. These results indicate that the unresolved relationships observed in this study likely stem from the limited phylogenetic information provided by just two loci, rather than a lack of biological diversity among the isolates.

Multilocus approaches are increasingly considered best practice across fungal systematics, not only in *Fusarium*. The phylogenetic analyses of *Trichoderma* revealed clearly defined species-level groupings, with *T. paratroviride* identified as the predominant lineage in both *rpb2* and *tef1* datasets. Additional clades corresponding to *T. afroharzianum*, *T. asperelloides*, *T. nordicum*, and *T. gamsii* were consistently detected, illustrating the ecological diversity characteristic of *Trichoderma* populations in maize and soil ecosystems (Jaklitsch & Voglmayr, 2015). The combined *rpb2*+*tef1* phylogeny further reinforced species delineations, with all principal complexes receiving strong bootstrap support, in line with prior multilocus studies highlighting the robust discriminatory capacity of these markers in *Trichoderma* taxonomy (Chaverri et al., 2015). Notably, several isolates grouped into clusters lacking corresponding reference sequences, indicating the possible existence of underrepresented or novel lineages, an

occurrence commonly observed in tropical agricultural systems where *Trichoderma* diversity is often underestimated (Steindorff et al., 2026).

The β -tubulin phylogeny of *Penicillium* resolved multiple species-level clades, with *Penicillium crustosum* representing the largest cluster. This species is commonly linked to stored cereals and frequently reported in warm, humid grain storage environments (Visagie et al., 2014). Additional clades corresponding to *P. brevicompactum*, *P. lanosocoeruleum*, and *P. polonicum* indicate that *Penicillium* colonization in Rwandan maize involves several coexisting species rather than a single dominant strain (Frisvad & Samson, 2004). The existence of these distinct groups underscores the likelihood of multiple mycotoxin-producing species cohabiting within the same production system, corroborating global reports of *Penicillium* diversity in cereal-based environments (Visagie et al., 2014).

Overall, the multilocus phylogenetic analyses reveal that the fungal community associated with maize in Rwanda comprises multiple phylogenetically distinct lineages within the genera *Fusarium*, *Trichoderma*, and *Penicillium*. The identification of several species complexes and subclusters supports the notion that fungal diversity is geographically structured and encompasses both field-associated and storage-associated fungal populations. These results offer a molecular basis for understanding the distribution, ecological functions, and potential pathogenic or antagonistic interactions of fungi in maize production systems in Rwanda.

The statistical analysis showed that fungal distribution was significantly affected by both geographic location and maize variety. The observed association between fungal species and maize variety in this study indicates that the host genotypes play an important role in shaping maize ear rot pathogenesis. This aligns with findings of Magarini et al. (2023), who showed that maize genotypes exhibit varying susceptibility to *F. graminearum*, attributable to genetic differences in resistance mechanisms. *Fusarium* ear rot resistance is considered a quantitative trait controlled by multiple genes and the interaction between genotypes and environment, resulting in fungal colonization across different maize varieties (Magarini et al., 2023). Furthermore, Mesterhazy (2024) highlighted that morphological features such as kernel architecture, husk coverage and biochemical defence responses contribute to the capacity of maize varieties to inhibit fungal infection and reduce mycotoxin contamination. Therefore, the dominance of *F. meridionale*, *F. subglutinans* and *F. graminearum* in P0017 likely reflects differences in host susceptibility among the maize varieties examined. Additionally, the geographic variation observed across provinces and districts suggests that environmental factors also influence maize ear fungal community composition, as climatic variables like precipitation, temperature and humidity are known to affect *Fusarium* species distribution (Munkvold, 2003; 2017). Collectively, these results highlight that both host genotypes and

geographic factors are crucial in determining fungal populations in maize and should be integrated into breeding programs and disease management strategies aimed at mitigating fungal infections and mycotoxin contamination.

The *in vitro* pathogenicity assay effectively created controlled conditions for both single and co-infections, allowing early colonization of maize seeds by maize ear rot pathogens. While biomass and disease symptoms were not measured, the consistent detection of fungal DNA across all treatments indicates that the inoculation method and incubation environment successfully supported pathogen establishment on the seed substrate. Similar seed-based pathogenicity models have been used to investigate infection in *Fusarium*-maize interactions, with autoclaved seeds providing an appropriate nutrient source for fungal growth (Munkvold, 2017). The successful amplification of fungal DNA in all treatments confirms the growth of both single and co-inoculated pathogens. The PCR detection of toxin-related genes further supports the assay's suitability for assessing whether co-infecting pathogens activate mycotoxin biosynthesis, despite the absence of toxin quantification. PCR-based identification of genes linked to fumonisin, trichothecene and other toxins is commonly used as an early indicator of toxigenic potential in *Fusarium*-infected maize (Bluhm et al., 2004). In summary, this assay provides a reproducible molecular platform for future studies testing the hypothesis that co-infection affects mycotoxin production. Previous studies has demonstrated that interactions among co-infecting *Fusarium* species can either enhance or suppress toxin gene expression depending on the species involved (Ferrigo et al., 2016). By producing high-quality DNA and verifying the detection of toxin-associated markers, this assay plays a foundation for combining pathogenicity testing with quantitative toxin measurements in forthcoming research.

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Popular Science Summary

Fungal diseases pose a significant threat to maize production, as some fungi can reduce crop yield and quality as well as producing toxins known as mycotoxins. Effective control of these diseases requires an understanding of the species of fungi present, their relationships and distribution in different growing areas.

The aim of this study was to use DNA-based identification tools to analyse the fungal species associated with ear rot in maize in Rwanda. Samples of the fungi were taken from various maize-growing locations to ascertain their identification and relationships between different fungal groups. The study also examined the interaction between selected fungal pathogens when grown together in a laboratory setting and investigated the variation in fungal presence between sites and maize varieties. The findings indicated that maize ear rot in Rwanda is associated with various fungal communities. Various strains of *Fusarium* fungi, which are among the most significant maize ear rot pathogens worldwide, as well as various *Penicillium* and *Trichoderma* species, were detected. The study demonstrated that multiple regions of DNA could be used to reliably identify closely related fungal species, thereby improving our understanding of fungal diversity.

Laboratory experiments showed that different fungal pathogens could grow successfully both alone and together. Further research is required to investigate the potential interactive effects of fungi on disease development and mycotoxin production, building on these experiments. The study also revealed differences in the fungal communities across provinces, districts, and maize varieties. This suggests that environmental factors and maize variety influence the presence of fungi.

This study provides valuable data on the diversity and distribution of maize ear rot fungi in Rwanda. These results could facilitate the monitoring of future diseases, the study of fungal interactions, and the development of measures to mitigate the risk of fungal diseases and mycotoxins in maize production.

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