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ARTICLE

Identification of Putative Mutations Potentially Associated with Fungicide Resistance in *Thecaphora frezii*, a Pathogen of Peanut Crops

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ABSTRACT

Thecaphora frezii (*T. frezii*) is the causal agent of peanut smut, a destructive disease responsible for substantial yield losses in Argentina and other peanut-producing regions. Management practices such as crop rotation, resistant cultivars, and fungicide applications have not prevented recurring outbreaks. Continuous fungicide exposure can exert selection pressure favoring resistant fungal populations.This study analyzed genetic variants in genes associated with four major fungicide classes: triazoles (*CYP51*), carboxamides (*SDHB, SDHC, SDHD*), strobilurins (*CYTB*), and benzimidazoles (*TUBB*). Protein-coding sequences from the *T. frezii* transcriptome and reference genome were compared with orthologous genes from related *Ustilaginales* species to detect amino-acid substitutions potentially related to resistance. Three conserved substitutions were identified in *CYP51* (R164K, K256R, I339L) and one in *SDHB* (H257R), while additional non-conserved variants occurred in *CYP51* (R336L), *SDHB* (R255A) and *SDHC* (A93I). No variants were observed in *SDHD*, *CYTB*, or *TUBB*.These results suggest that some *T. frezii* variants could reduce fungicide sensitivity, whereas others indicate that strobilurins and benzimidazoles may still be effective. Because the sample size was limited, our conclusions are exploratory. Nevertheless, this study provides the first genetic evidence of potential fungicide-resistance-related variants in *T. frezii*, emphasizing the importance of monitoring resistance evolution to ensure sustainable peanut-smut management.

INTRODUCTION

Peanut (*Arachis hypogaea* L.) is one of the most important oilseeds crops worldwide, valued for its nutritional quality and economic relevance. Globally, peanuts are cultivated on approximately 29 million hectares, with an annual production exceeding 49 million metric tons (FAO, 2021). In Argentina, peanut cultivation is concentrated in Córdoba province, which accounts for nearly 90% of the country's production (Ministerio de Agricultura, 2022). This crop is a key source of

edible oil, protein, and export revenue, yet its productivity is increasingly threatened by fungal diseases.

Thecaphora frezii (*T. frezii*), the causal agent of peanut smut, has become one of the most damaging pathogens affecting Argentine peanut crops. Since its first detection in the 1990s, the disease has expanded throughout major peanut-producing regions, causing significant yield and quality losses (Rago *et al.*, 2017; Cazón *et al.*, 2018). *Thecaphora frezii* replaces the peanut seed with a mass of dark teliospores within the pod, rendering it unmarketable and severely affecting seed viability.

Chemical control has been one of the main management strategies for peanut smut, alongside crop rotation and host resistance breeding (Paredes *et al.*, 2021). However, field reports have documented failures of fungicide treatments to control peanut smut, even when products with proven efficacy against other pathogens were applied (Paredes *et al.*, 2021). This limitation underscores the urgent need to understand possible mechanisms of fungicide resistance in *T. frezii* populations.

Fungicide resistance in plant pathogens often arises from point mutations in target-site genes that reduce fungicide binding efficiency (Lucas *et al.*, 2015; Yin *et al.*, 2023; Islam *et al.*, 2024). Examples include *CYP51* mutations conferring resistance to triazoles and *CYTB* mutations associated with strobilurin resistance (Wyand and Brown, 2005). Similarly, amino-acid substitutions in the *SDHB*, *SDHC*, and *SDHD* subunits of succinate dehydrogenase have been reported in *Botrytis cinerea*, *Alternaria alternata*, and *Cercospora beticola*, leading to resistance to SDHI fungicides (Sierotzki and Scalliet, 2013). The relatively low efficacy of SDHI fungicides against peanut smut (Paredes *et al.*, 2021) may reflect similar underlying mechanisms in *T. frezii*.

Because no close relatives of *T. frezii* have been extensively studied at the genomic level, comparative analyses were performed using data from phylogenetically related smut fungi in the order Ustilaginales, including *Ustilago maydis*, *Sporisorium reilianum*, and *Moesziomyces antarcticus*, as well as other well-characterized fungal pathogens such as *Cercospora beticola* and *Botrytis cinerea*. Although these latter species are taxonomically distant, they were included because they possess experimentally confirmed data on fungicide resistance mutations, providing valuable reference points for comparative analysis.

This study represents the first attempt to investigate potential genetic determinants associated with reduced fungicide sensitivity in *T. frezii*. By identifying mutations in genes encoding target proteins for triazoles, SDHIs, QoIs, and benzimidazoles, we aim to provide a molecular basis to explain field observations of reduced fungicide efficacy. The findings reported here contribute to the understanding of fungal adaptation and support the design of resistance-monitoring programs for peanut smut management.

MATERIALS AND METHODS

Sample Collection and Spore Isolation

Teliospores of *Thecaphora frezii* were collected in 2019 from hypertrophic peanut pods naturally infected in a single commercial field located at Criadero El Carmen, General Cabrera, Córdoba province, Argentina (32°49'44.2"S, 63°52'31.8"W). This region has a long history of peanut smut occurrence and fungicide use. According to local records, fungicides belonging to the triazole, SDHI, and strobilurin classes have been applied in this area since at least 2016 (Paredes, 2017). The collected material consisted exclusively of teliospores, confirmed by microscopic observation.

RNA Extraction

Teliospores from 20 infected pods were pooled, and total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA pellets were resuspended in 20 µL of RNase-free water and incubated at 55–60°C for 10 min. RNA integrity and purity were verified using agarose gel electrophoresis, a NanoPhotometer spectrophotometer (Implen), and quantification with the Qubit RNA assay kit and Qubit 2.0 Fluorometer (Life Technologies) (Díaz *et al.*, 2024).

Library Preparation and RNA-Seq

Complementary DNA (cDNA) libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB) at INDEAR (Agrobiotechnology Institute of Rosario, Argentina). mRNA was purified using poly-T oligo-attached magnetic beads, fragmented using divalent cations, and reverse-transcribed into first-strand cDNA using random hexamer primers. Second-strand synthesis was performed with DNA polymerase I and RNase H. The resulting cDNA was size-selected, adaptor-ligated, and enriched. Index-coded samples were clustered using the cBot Cluster Generation System with the TruSeq PE Cluster Kit v3-cBot-HS (Illumina). Sequencing was performed on an Illumina HiSeq 1500 platform, generating 2 × 150 bp paired-end reads.

Data Analysis

Raw reads were quality-checked using FastQC and assembled *de novo* using standard transcriptome pipelines. Read counts were normalized as counts per million (CPM), and gene expression levels were estimated. Differential expression analysis was conducted using a General Linear Model implemented in EdgeR (Robinson *et al.*, 2010) within the R environment (R Core Team, 2018).

Gene and Protein Selection

Genes associated with fungicide resistance in plant-pathogenic fungi were selected based on their well-established roles:

- Triazoles: Lanosterol 14α-demethylase (*CYP51*), a nuclear-encoded enzyme involved in ergosterol biosynthesis.
- Carboxamides (SDHIs): Succinate dehydrogenase subunits B, C, and D (*SDHB*, *SDHC*, *SDHD*), nuclear-encoded components of the mitochondrial respiratory complex II.
- Strobilurins (QoIs): Cytochrome b (*CYTB*), a mitochondrial gene encoding a central subunit of the electron transport chain.

- Benzimidazoles (MBCs): β-tubulin (*TUBB*), a nuclear gene essential for microtubule formation during cell division.

Protein sequences of *Ustilago maydis*, *Pseudozyma flocculosa*, *Kalmanozyma brasiliensis*, *Sporisorium reilianum*, and *Moesziomyces antarcticus* were retrieved from the NCBI protein database and used as queries in BLASTp searches to identify orthologous genes. Genomic data of *Thecaphora thlaspeos* (GenBank accession: GCA_900291925.1) (Courville *et al*., 2019) and *T. frezii* (ASM2628400v1) (Arias *et al*., 2023) were retrieved from NCBI. It is worth noting that *T. thlaspeos* is a urocystidial fungus within the Urocystidales, is phylogenetically distinct from *T. frezii*. Gene predictions were performed using AUGUSTUS, a web-based gene-prediction platform. Protein-coding genes analyzed in this study and their corresponding GenBank accession numbers are listed in Table 1.

Table 1. Accession numbers of genes associated with fungicide resistance in various species of the Ustilaginomycetes class, *Cercospora beticola* and *Botrytis cinerea*.

	GENES					
	CYP51	*CYTB*	*SDHB*	*SDHC*	*SDHD*	*TUBB*
Fungi	GenBank Accession Number [a]					
Cercospora beticola	XP_023450255.1	AMD11306.1	XP_023450315.1	ND	ND	ND
Botrytis cinerea	AAV35295.1	ACL50595.1	UJU85472.1	XJQ58198.1	UJU85486.1	AAB60296.1
Ustilago maydis	XP_011390148.1	YP_762699.1	XP_011386878.1	XP_011390581.1	XP_011390278.1	XP_011392194.1
Ustilago hordei	XP_041413325.1	AMA33514.1	XP_041415690.1	XP_041410447.1	XP_041413508.1	XP_041412583.1
Moesziomyces antarcticus	GAC74292.1	AMA33507.1	XP_014658568.1	XP_014653555.1	XP_014655830.1	XP_014657872.1
Kalmanozyma brasiliensis	XP_016291895.1	ND	XP_016295207.1	XP_016289580.1	XP_016293060.1	XP_016290680.1
Sporisorium reilianum	CBQ68278.1	CBQ72547.1	CBQ70235.1	CBQ68620.1	CBQ68458.1	CBQ70872.1
Thecaphora frezii (Genome)	ASM2628400v1 [b]		MH392473.1		ASM2628400v1 [b]	
Thecaphora frezii (Transcriptome)	UKG18589.1	ND	PV330449	PV330450	PV330451	UOP56877.1
Thecaphora thlaspeos (Genome)	GCA_900291925.1 [b]					
Pseudozyma flocculosa	XP_007876928.1	ND	XP_007878503.1	XP_007876648.1	XP_007882182.1	XP_007878351.1

[a]Abreviation: ND, no data

[b] Genome assembly accessions are provided where protein accessions are not available.

Protein Sequence Alignment and Variant Identification

Predicted protein sequences from *T. frezii* were aligned with orthologs from closely related fungi within the *Ustilaginales* and with sequences from *Cercospora beticola* and *Botrytis cinerea*, which have well-documented fungicide-resistance mutations (Secor *et al*., 2010; Fernández-Ortuño *et al*., 2012). Alignments were generated using Clustal Omega. Proteins were considered homologous if they shared ≥25% identity over >80 amino acids (Sander & Schneider, 1991). Amino-acid substitutions were evaluated based on conservation and physicochemical properties.

Effect of Amino Acid Substitutions

The structural and functional impact of identified variants was assessed using the HOPE web platform (Venselaar *et al*., 2010), which predicts changes in protein stability and function resulting from amino-acid substitutions.

RESULTS AND DISCUSSION

This study identified mutations in key *Thecaphora frezii* genes potentially associated with fungicide resistance. Analyses were based on transcriptome sequences obtained from teliospores and orthologous contigs of related fungi including *Ustilago*

maydis, *Sporisorium reilianum*, *Moesziomyces antarcticus*, *Cercospora beticola*, and *Botrytis cinerea*. The sample size was limited, which restricts definitive conclusions but provides valuable preliminary evidence for future research.

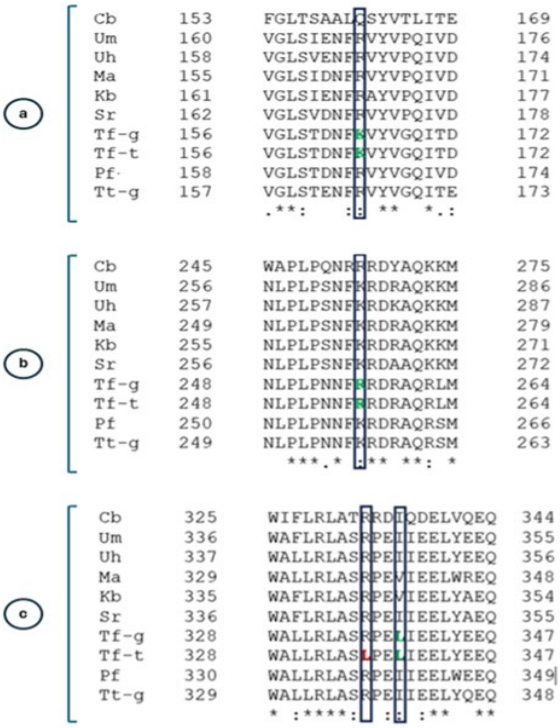

Figure 1. Alignment of *CYP51* amino acid sequences from *Thecaphora frezii* and different fungi. Panels a, b and c represent portions of the *CYP51* protein. Amino acids identified as conserved variations are shown in green, and non-conserved variations are shown in red. Cb: *Cercospora beticola*; Um: *Ustilago maydis*; Uh: *Ustilago hordei*; Ma: *Moesziomyces antarcticus*; Kb: *Kalmanozyma brasiliensis*; Sr: *Sporisorium reilianum*; Tf-g: amino acid sequence deduced from *Thecaphora frezii* genome; Tf-t: amino acid sequence deduced from *Thecaphora frezii* transcriptome; Pf: *Pseudozyma flocculosa*; Tt-g: amino acid sequence deduced from *Thecaphora thlaspeos genome*. Note: * indicates identical residues (fully conserved), : indicates highly conserved residues (similar properties), . indicates moderately conserved residues, (space) indicates no conservation (no similarity).

Variants in Triazole Resistance Genes (*CYP51*)

Three conserved variants (R164K, K256R, I339L) were identified in the *CYP51* gene (Figure 1). These substitutions involve residues with similar physicochemical properties and are thus unlikely to alter enzyme activity or fungicide binding significantly. A non-conserved substitution (R336L) was also detected exclusively in the transcriptome of *T. frezii*. This variant replaces a positively charged arginine with a hydrophobic leucine in the ligand-binding region.

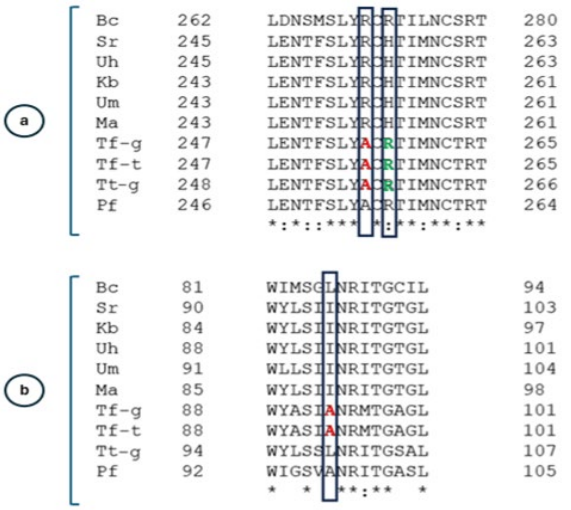

Figure 2. Alignment of SDH amino acid sequences from *Thecaphora frezii* and different fungi. Panels a and b correspond to the *SDHB* and *SDHC* protein regions, respectively. Amino acids identified as conserved variations are shown in green, and non-conserved variations are shown in red. Bc: *Botrytis cinerea*; Sr: *Sporisorium reilianum*; Kb: *Kalmanozyma brasiliensis*; Cb: *Cercospora beticola*; Uh: *Ustilago hordei*; Um: *Ustilago maydis*; Ma: *Moesziomyces antarcticus*; Tf-g: amino acid sequence deduced from *Thecaphora frezii* genome; Tf-t: amino acid sequence deduced from *Thecaphora frezii* transcriptome; Pf: *Pseudozyma flocculosa*. Tt-g: amino acid sequence deduced from *Thecaphora thlaspeos* genome Note: * indicates identical residues (fully conserved), . indicates moderately conserved residues, (space) indicates no conservation (no similarity).

Comparable substitutions, such as Y136F in *Blumeria graminis*, have been associated with azole resistance (Wyand & Brown, 2005). Moreover, silencing of the *CYP51* gene has been shown to alter fungal sensitivity to triazoles, confirming its central role in fungicide resistance (Koch *et al.*, 2013). Although triazole fungicides like cyproconazole have shown good control of peanut smut (>40% reduction in incidence; Paredes *et al.*, 2021), the R336L mutation could represent an early adaptation event that merits further monitoring.

Because *T. frezii* populations are exposed to fungicides in the field, such mutations may arise from natural genetic variability rather than direct selection. Further population-level studies will be necessary to determine whether these variants confer any fitness advantage under fungicide pressure.

Variants in Carboxamide Resistance Genes (*SDHB*, *SDHC*, and *SDHD*)

Two amino-acid substitutions were detected in *SDHB*: R255A and H257R (Figure 2). The latter aligns with the well-characterized H272R mutation in *Botrytis cinerea* associated with SDHI resistance (Fernández-Ortuño *et al.*, 2012). The R255A substitution, found near this position, replaces a positively charged residue with a small nonpolar alanine, potentially affecting local folding and hydrogen-bond networks. Similar structural effects have been correlated with partial loss of SDHI sensitivity in other fungi (Sierotzki & Scalliet, 2013).

One variant was identified in *SDHC* (A93I), substituting a small alanine for a bulkier isoleucine near a residue homologous to R87 in *B. cinerea* (XJQ58198.1), another site linked to SDHI resistance. No variants were found in *SDHD*.

The relatively poor performance of SDHI fungicides against peanut smut in Argentina (Paredes *et al*., 2021) could therefore be linked to these subtle alterations. However, reported mutation rates for SDHI resistance in other smut or related fungi remain low (10^{-6}–10^{-8} per generation) (Ishii & Hollomon, 2015), suggesting that *T. frezii* resistance, if present, is likely in an early stage of development.

Because our isolates were collected from a single field, these findings cannot be extrapolated to the entire pathogen population. Still, they underline the importance of regional monitoring for emerging resistant alleles.

Variants in Strobilurin and Benzimidazole Resistance Genes (*CYTB* and *TUBB*)

No amino-acid variants were detected in *CYTB* or *TUBB*. The absence of *CYTB* mutations suggests that *T. frezii* populations have not yet developed resistance to strobilurins. Indeed, strobilurin-based fungicides achieved the highest control efficacy in field trials (Paredes *et al*., 2021; Table 2). However, studies in other species, such as *Monilinia* spp. (Hily *et al*., 2011) and *Colletotrichum truncatum* (Rogério *et al*., 2024), have shown that single-nucleotide mutations in *CYTB* can confer QoI resistance after repeated exposure. Continuous monitoring of *T. frezii* populations will be crucial to detect early resistance signals.

Table 2. Relative efficacy of fungicide treatments against peanut smut under field conditions (adapted from Paredes *et al*., 2021)

Fungicide class	Active ingredient	Mode of action	Relative efficacy (%)	Reference
Triazole	Cyproconazole	Demethylation inhibitor (*CYP51*)	42.3	Paredes et al., 2021
SDHI	Boscalid	Succinate dehydrogenase inhibitor	18.7	Paredes et al., 2021
Strobilurin	Azoxystrobin	QoI inhibitor (*CYTB*)	63.5	Paredes et al., 2021
Benzimidazole	Thiophanate-methyl	β-tubulin inhibitor	22.9	Paredes et al., 2021

No variants were found in *TUBB*, which encodes β-tubulin, the target of benzimidazoles. Nevertheless, the low efficacy of benzimidazoles reported in vitro and in field trials (Paredes *et al*., 2021) may not necessarily indicate true resistance. It could instead reflect low pathogen sensitivity or limited fungicide uptake, as observed in *Fusarium graminearum* (Zhou *et al*., 2016). Furthermore, given our limited sample size, the absence of resistance mutations should be interpreted cautiously.

Comparative Insights and Broader Implications

Fungicide resistance is often multigenic and may involve the accumulation of multiple mutations affecting different components of the target pathway (Lucas *et al*., 2015). For example, multiple *CYTB* and *SDHB* variants have been reported to jointly confer resistance to QoI fungicides in *Puccinia horiana* (Matsuzaki *et al*., 2021). The presence of several amino-acid substitutions in *T. frezii* may therefore reflect natural diversity that, under sustained selection, could evolve toward higher resistance levels.

Notably, *Thecaphora thlaspeos*, a related urocystidial fungus, is not known to be exposed to agricultural fungicides. To our knowledge, no published evidence of fungicide selection pressure has been reported for *T. thlaspeos*, which supports the hypothesis that observed polymorphisms in *T. frezii* are the result of intrinsic genetic variability rather than selection.

Overall, these findings emphasize the necessity of adopting integrated disease management approaches combining resistant cultivars, crop rotation, and rational fungicide use. The detection of conserved and unique variants in *T. frezii* provides a starting point for future genomic surveys across different regions to evaluate the distribution and frequency of potential resistance alleles.

CONCLUSIONS

This study provides the first molecular evidence of genetic variation in fungicide target genes in *Thecaphora frezii*, the causal agent of peanut smut. Several amino-acid substitutions were identified in *CYP51*, *SDHB*, and *SDHC* genes that could potentially influence fungicide sensitivity, while *SDHD*, *CYTB*, and *TUBB* appeared conserved.

Although these findings suggest possible associations between specific variants and decreased fungicide efficacy, the small sample size limits the ability to establish definitive links between mutations and resistance phenotypes. Additionally, observed substitutions may arise from natural genetic variability rather than exposure to fungicide pressure.

Further studies integrating population sampling from multiple peanut-producing regions, combined with in vitro fungicide sensitivity assays and functional validation of these variants, will be essential to confirm their biological significance. Expanding genomic data for *T. frezii* and related smut fungi will also contribute to understanding the evolution of fungicide resistance in this group.

By identifying preliminary molecular markers and comparing them with orthologous sequences from related fungi, this work lays the foundation for future resistance-monitoring programs aimed at improving peanut smut management and reducing economic losses.

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