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Original article

Detection of phytopathogenic fungi that cause leaf spots in Ecuadorian sugarcane EC-08 variety

Identificación de hongos fitopatógenos causantes de manchas foliares en caña de azúcar ecuatoriana variedad EC-08

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ABSTRACT: The importance of identifying phytopathogenic fungi in sugarcane is crucial for establishing effective control strategies that ensure the sustainability and production of this important grass. The study aimed to identify the fungal causal agents that cause necrotic leaf spots in the EC-08 variety of sugarcane. After disinfection, the samples were inoculated in potato dextrose agar (PDA) culture medium. Pure cultures were obtained for molecular analysis by extracting deoxyribonucleic acid (DNA) and internal transcribed spacer (ITS). In addition, a metagenomic analysis was performed using parallel mass sequencing metabarcoding (ITS), and the sequences were identified using BLAST. The results of the ITS molecular analysis yielded 100 % pure DNA in the amplified fragments. Agarose gel electrophoresis at 1 % visualized bands for the species: *Curvularia petersonii* (535 bp), *Aspergillus arachidicola* (536 bp), and *Rhinocladiella* sp. (529 bp) based on SANGER sequencing readings, while the taxonomic profile result detected 100 % *Fusarium pseudoanthophilum* and 97 % *Nigrospora osmanthi*. In conclusion, ITS barcoding and metabarcoding methods enabled the identification of the phytopathogenic fungi *C. petersonii*, *A. arachidicola*, *Rhinocladiella* sp., *F. pseudoanthophilum*, and *N. osmanthi* as causes of leaf necrosis in this cultivar.

Key words: Agriculture, ecosystem, disease, phytopathology, pathogen.

RESUMEN: La identificación de hongos fitopatógenos en caña de azúcar es fundamental para establecer estrategias de control eficaces, que garanticen la sostenibilidad y la producción de esta importante gramínea. El objetivo del estudio fue identificar los agentes fúngicos causantes de las manchas necróticas en las hojas de caña de azúcar variedad EC-08. Tras la desinfección, las muestras de hojas con síntomas fueron sembradas en un medio de cultivo agar papa dextrosa (PDA). Se obtuvieron cultivos puros para el análisis molecular mediante la extracción de ácido desoxirribonucleico (ADN) y espaciador transcrito interno (ITS). Además, se realizó un análisis metagenómico utilizando metabarcoding de secuenciación masiva paralela (ITS), y las secuencias se identificaron utilizando BLAST. Los resultados del análisis molecular ITS arrojaron un 100 % de ADN puro en los fragmentos amplificados. La electroforesis en gel de agarosa al 1 % visualizó bandas para las especies: *Curvularia petersonii* (535 pb), *Aspergillus arachidicola* (536 pb) y *Rhinocladiella* sp. (529 pb) según las lecturas de secuenciación SANGER, mientras que el resultado del perfil taxonómico detectó un 100 % de *Fusarium pseudoanthophilum* y un 97 % de *Nigrospora osmanthi*. En conclusión, los métodos de código de barras ITS y metacódigo de barras permitieron identificar los hongos fitopatógenos *C. petersonii*, *A. arachidicola*, *Rhinocladiella* sp., *F. pseudoanthophilum* y *N. osmanthi* como causas de la necrosis foliar en este cultivar.

Palabras clave: agricultura, ecosistema, enfermedad, fitopatología, patógeno.

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INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) cultivation is one of the most important in Ecuador, due to its contribution to the agricultural economy and high sugar production (1). Despite this, leaf necrosis, a common condition affecting yield and quality, has become a major threat in recent decades (2). Identifying the pathogens that cause this disease is essential for effective crop management and sustainable production (3).

These phytopathogenic fungi are responsible for various diseases, so their early detection for timely management would prevent significant yield losses (4). The Ecuadorian Sugarcane Research Center (CINCAE, according its acronyms in Spanish) has played a fundamental role in improving sugarcane production (5). However, its focus has been more oriented towards agronomy and genetic improvement, which has created a significant gap in research on phytopathogenic microorganisms affecting this crop.

Limited information exists on phytopathogens in new sugarcane varieties, which limits the establishment of effective control management (6). Leaf necrosis manifests as localized or generalized leaf death, affecting plant photosynthesis and can lead to secondary effects such as growth arrest, wilting, and even plant death (2). A multidisciplinary approach involving microbiology, phytopathology, and molecular biology can generate a deeper understanding of the interactions between sugarcane and its pathogens (7).

Ecuador's ecosystem diversity, combined with climatic variations and poor agricultural practices, creates a favorable environment for the emergence of new phytopathological threats (8). Understanding the dynamics of fungal diseases and their impact on crop health is essential for developing effective control strategies (9). Therefore, establishing a relationship between observed symptoms and the causal pathogens is essential (10).

Various fungal species are associated with a variety of diseases that can cause losses in grasses; *Aspergillus* sp., known for its mycotoxin production, affects grass quality and soil health (11). In contrast, *Curvularia* sp. is associated with diseases causing leaf spots, reducing yield in corn and wheat (12). *Rhinocladiella* sp. has also become a pathogen of high interest due to its incidence in grass crops, causing plant stress and affecting their physiology (13).

Likewise, *Fusarium* sp. is a phytopathogen that has attracted the interest of the scientific community, mainly due to its impact on agricultural crops, especially those of economic importance such as sugarcane (14). This pathogen is associated with root rot, wilting, and yellowing of plants, ultimately leading to yield losses (15). Current research has shown that *Fusarium* not only affects plant health but also compromises food safety through the production of its harmful mycotoxins (16). *Nigrospora osmanthi* is a phytopathogenic fungus causing significant losses in grass crops (17). It has the ability to adapt to diverse environmental conditions, which presents challenges for pathogen control (18).

In this context, the sustainability of sugarcane cultivation in Ecuador and the livelihoods of farmers are at risk, underscoring the need to prioritize microbiological and phytopathological studies aimed at identifying the pathogens affecting this crop and mitigating their impact. The study aimed to identify the causal fungal agents associated with necrotic spots on sugarcane leaves of variety EC-08.

MATERIALS AND METHODS

Sugarcane leaves with symptoms of leaf spots from cultivar EC-08 (Figure 1) were collected at the "Agrícola Cepeda S.A." farm, located in La Puntilla, La Troncal canton, Cañar province, Ecuador, georeferenced at coordinates (2°26'18.74" S, 79°23'45.54" W), at an altitude of 110 m a.s.l. The property covers an area of 450 hectares and is characterized by completely flat topography and silty-loam soils. Leaf samples with necrosis symptoms were taken from four different plots.

The samples were processed at the Microbiology Laboratory of the Faculty of Science and Engineering of the State University of Milagro (UNEMI, according its acronyms in Spanish). Plant sample preparation consisted of cutting the tissues into 3 cm² pieces, washing them with neutral liquid soap, and rinsing them with distilled water. Next, the samples were disinfected with 2 % sodium hypochlorite (NaClO) for 2 minutes, followed by consecutive rinses in sterile water, and then placed in 70 % ethanol for 1 minute.

Figure 1. Foliar lesions on sugarcane leaves (variety EC-08)

Subsequently, the samples were transferred to a sterile safety cabinet (BIOBASE FH1200), where they were cut into 5 mm² fragments at the leaf vein and inoculated onto Petri dishes containing potato dextrose agar (PDA) at a dose of 39 g·L⁻¹. The plates were incubated at 28 °C for 10 days for subsequent purification.

Fungal mycelia from the initial cultures were transferred to new PDA Petri dishes according to their colorimetric characteristics for purification. These were incubated at the same temperature for 14 days. This process resulted in four plates with pure strains for genus identification through morphological characterization using the lactophenol blue staining technique, followed by species identification using molecular techniques.

Three of the purified cultures, coded H700, H701, and H769, were molecularly identified by DNA extraction, amplification of the internal transcribed spacer (ITS) region, conventional sequencing, assembly of polymerase chain reaction (PCR) products, and database searching. The procedure was carried out as follows:

DNA extraction was performed using conventional methods (19) with approximately 100 mg of sample. DNA integrity and quality were assessed by microvolume spectrophotometry and visualization on 1 % agarose polyacrylamide gel. At a concentration of 20 ng·µL⁻¹, the DNA was diluted for PCR amplification using ITS1/ ITS4 primers (20). The final PCR product was purified and sequenced using the Sanger method. The obtained sequences were cleaned and assembled using Geneious bioinformatics software (version 11.1.2). The assembled sequences were compared with the NCBI GenBank database for taxonomic identification using the BLAST tool to find similarities.

A taxonomic profile analysis of the ITS region was performed using high-throughput massive sequencing (metabarcoding) on purified samples labeled H631 and H710. This analysis included DNA extraction and ITS metabarcoding, as detailed below:

Approximately 500 mg of each fungal mycelium isolated from the affected leaf tissue sample was used for DNA extraction using conventional methods. The quality of the extracted DNA was assessed by spectrophotometry using a NanoDrop. DNA integrity was verified by horizontal electrophoresis on 1 % agarose gel. After quality assessment, the target sequences were amplified using ITS primers (CTTGGTCATTTAGAGGAAGTAA and TCCTCCGCTTATTGATATGC) specific for fungi. The resulting PCR products were purified, quantified, and pooled for library preparation. Libraries were quality-controlled, and those meeting the criteria were processed for sequencing on the Illumina platform. The resulting reads were cleaned, assembled, and counted using bioinformatics tools. Chimeric sequences were removed, and the final sequences were identified using BLAST against the UNITE fungal database with a 97 % identity threshold. The results were visualized using Krona charts.

RESULTS AND DISCUSSION

Figure 2 shows the morphological characterization of the phytopathogenic fungi isolated from samples of necrotic leaf tissue of sugarcane.

The morphological characterization of the isolates allowed the identification of important structures for genus-level approximation of the isolated fungi (*Curvularia* sp. and *Aspergillus* sp.) when compared with taxonomic codes from published literature. *Curvularia* sp. exhibited cottony-textured, dark melanized growth, with dark dematiaceous hyphae and septate macroconidia-like structures; its conidia are curved and composed of unequal cells. *Aspergillus* sp. showed granular, white-textured growth, with lighter hyaline hyphae, conidiophores bearing vesicle-shaped conidial heads, and small, round conidia.

These characteristics are consistent with those reported in the scientific literature. *Curvularia* has fast-growing mycelium and multiseptate macroconidia with a prominent curved central cell (11), while the genus *Aspergillus* presents hyaline morphology characterized by radially arranged phialides (11). These phenotypic features allow an approximation of the causal agents of diseases, facilitating the establishment of management and control plans for agricultural crops of high economic value to farmers.

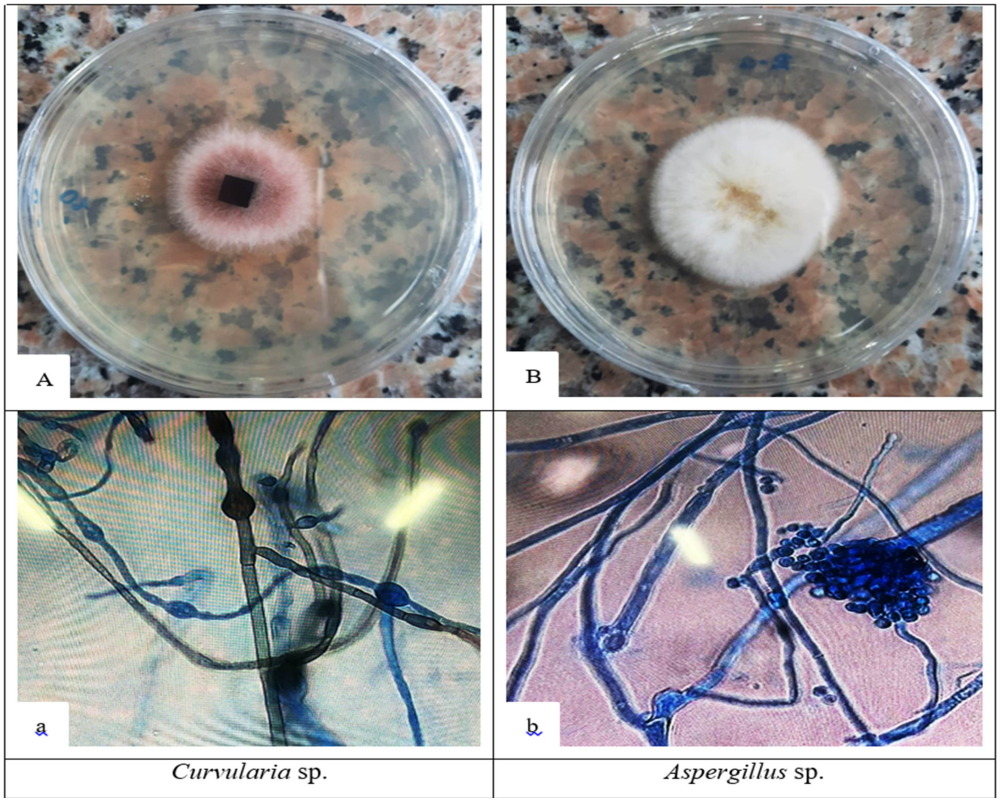
Molecular characterization of the isolated fungal strains using the ITS method confirmed the presence of high-quality DNA suitable for the amplification process (Figure 3).

Bands of 536 bp (H700), 535 bp (H701), and 529 bp (H769) were observed for the ITS marker. Assembled sequences from Sanger sequencing reads enabled fungal species identification, as shown in Table 1.

Molecular identification allows precise confirmation of the causal agent affecting this crop in Ecuador. In this study, three predominant fungal species were identified using the ITS barcoding method: *A. arachidicola*, *C. petersonii*, and *Rhinocladiella* sp. This method is recognized for its effectiveness in the taxonomic identification of fungal organisms, enabling accurate determination of the species involved in pathogenicity (21).

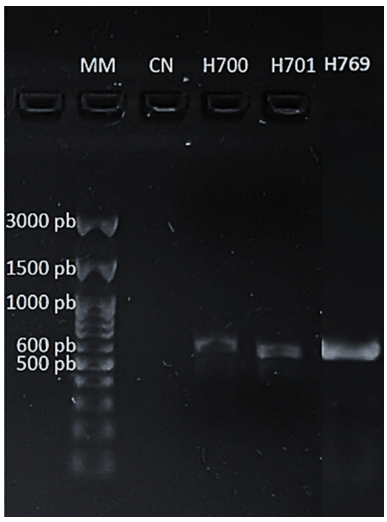
The presence of *A. arachidicola* is particularly significant, as this fungus is associated with the production of toxic compounds that can severely affect crop and food safety. Published research has shown that the presence of this fungus in crops can lead to substantial economic losses due to reduced sugarcane yield and quality (22). Therefore, accurate pathogen identification is essential for implementing effective management strategies.

C. petersonii is known as a phytopathogenic fungus that contributes to leaf necrosis in grass cultivars. It has been demonstrated that this fungus can negatively affect photosynthesis and, consequently, reduce plant biomass production (23). Curvularia spp. represents a challenge for agriculture and underscores the need to monitor and manage the environmental conditions that favor its proliferation (24).



A: top view of the fungus Curvularia sp. B: top view of the fungus *Aspergillus* sp. a: hyphae and conidia of *Curvularia* sp. b: hyphae, conidiophores, and conidia of *Aspergillus* sp

Figure 2. Morphological characterization of the phytopathogenic fungi isolated from samples of necrotic leaf tissue of sugarcane



MW: molecular weight marker in base pairs (bp). CN: negative control ITS

Figure 3. 1 % agarose gel showing polymerase chain reaction (PCR) products. Internal transcribed spacer (ITS) fragments

The presence of *Rhinocladiella* sp. among the identified fungal isolates indicates a diversity of organisms potentially involved in the disease. It has been reported that it can cause various plant pathologies (25, 26). These findings highlight the need for an integrated approach to fungal disease management, including continuous surveillance and the use of molecular identification methods.

Sample H710 was subjected to a taxonomic profile analysis of the ITS region using high-throughput massive sequencing (Table 2).

The adequate concentration and quality of DNA obtained from the fungal isolates for library preparation and subsequent metagenomic sequencing via ITS metabarcoding are evident. The BLAST search results for the identification of fungi at the genus and species level are presented in Krona charts (Figures 4 and 5) for the phylum Ascomycota, allowing an interactive analysis of the results.

The results indicate that sample H710, within the phylum Ascomycota, showed 100 % predominance of the species *Fusarium pseudoanthophilum*, while sample H631 showed 97 % predominance of *Nigrospora osmanthi*.

Table 1. Summary of the results of molecular characterization using the ITS fragment

Code	DNA Quality (%)	Organism	Gene	Identity (%)	Accession	Coverage (%)	E-value
H700	100	*Aspergillus arachidicola*	ITS	100.00	MF668184.1	100	0.0
H701	100	*Curvularia petersonii*	ITS	99.68	NR_158448.1	100	0.0
H769	98	*Rhinocladiella* sp.	ITS	98.00	HQ022439.1	100	0.0

Table 2. Results of DNA extraction and massive sequencing

Code	Concentration (ng/µl^{-1})	$A260 / A280$	$A260 / A230$	Total reads	OTUs*	Number of sequences
H710	2756,9	2,05	2,12	80,146	155	75,294
H631	698,2	2,01	1,98	37,763	120	37,564

OTUs (Operational Taxonomic Units)*

Figure 4. Phylum *Ascomycota* identified through ITS metabarcoding analysis of sample H710

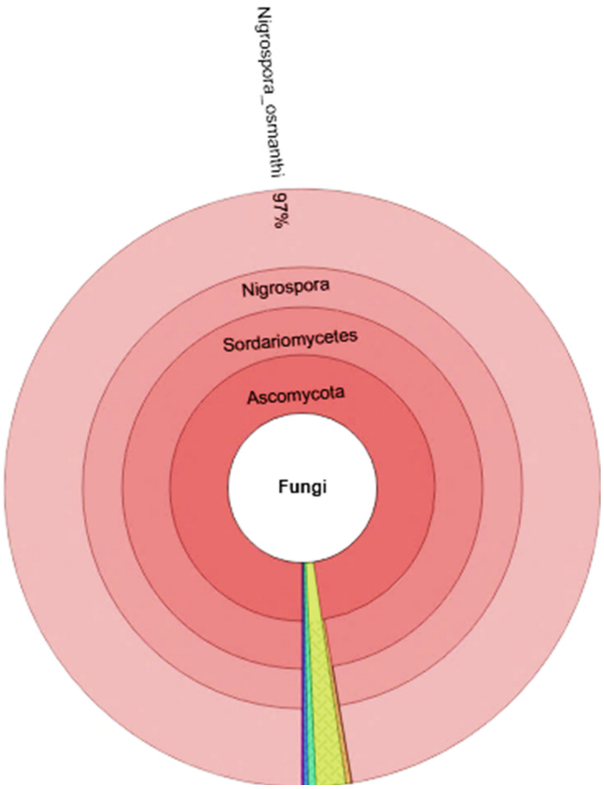


Figure 5. Phylum Ascomycota identified through ITS metabarcoding analysis of sample H710

The results obtained for sample H710 highlight the relevance of this fungus in the plant context. *F. pseudoanthophilum* has been recognized for its ability to cause diseases in crops (specific crops could be mentioned to illustrate the breadth of its impact or its proximity to sugarcane), underscoring the importance of its identification for effective phytopathogen management. Research has documented that this fungus can be associated with wilting and leaf reddening in several plant species, negatively affecting agricultural production (27).

N. osmanthi is a phytopathogenic fungus identified as a pathogen in various agricultural crops (28). This fungus belongs to the genus *Nigrospora*, which includes several species known for their ability to cause significant damage to plants (29). The identification and characterization of *N. osmanthi* are crucial for understanding its impact on agriculture and developing effective management strategies (17).

These findings contrast with the general predominance of the phylum *Ascomycota*, which includes many of the major plant pathogens. The predominance of *Fusarium* in this context reinforces the need for specific monitoring and control approaches focused on this genus, especially in agricultural systems where plant health is critical (30).

These results differ from the general predominance of the phylum *Ascomycota*, which encompasses many key plant pathogens. The dominance of Fusarium highlights the need for targeted monitoring and control strategies centered on this genus, particularly in agricultural systems where plant health is of critical importance (30).

CONCLUSIONS

The ITS metabarcoding technique allowed obtaining high-quality DNA, which facilitated the amplification and subsequent identification of the fungal species present in the analyzed samples, demonstrating the effectiveness of molecular techniques for accurately identifying pathogenic fungi.

The sequences obtained by Sanger sequencing confirmed the existence of the specific species *A. arachidicola*, *C. petersonii*, and *Rhinocladiella* sp., providing relevant information on the diversity of pathogenic fungi in the leaf tissues of the studied sugarcane variety EC-08.

Massive sequencing analysis of samples H710 and H631 revealed a 100 % predominance of *F. pseudoanthophilum* and 97 % predominance of *N. osmanthi*, respectively, within the phylum *Ascomycota*. Therefore, these species would be responsible for causing leaf necrosis in the sugarcane crop.

The information derived from this research has important implications for crop management. However, precise identification (which requires pathogenicity tests and completion of Koch's postulates), here it is merely an assumption of the pathogenic fungi could facilitate the development of effective control strategies to improve crop health. It is suggested to evaluate the degree of pathogenicity of these causal agents.

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