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A cost-effective and simplified protocol for fungal DNA extraction using silica-based grinding, without liquid nitrogen or lyophilization

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Abstract. Efficient DNA extraction from filamentous fungi is often hindered in developing countries because of the limited availability of liquid nitrogen and lyophilization, which are widely used for breaking down the fungal cell walls. While the production and storage of liquid nitrogen pose significant environmental concerns owing to their high carbon footprint and associated costs, the adoption of lyophilization is restricted by its substantial operational expenses. To address these challenges, a cost-effective and accessible CTAB-based DNA extraction protocol was developed, utilizing silicon dioxide as an abrasive, along with mortar and pestle grinding. This approach eliminates the dependency on liquid nitrogen and lyophilization. DNA was successfully extracted from the mycelia of *Colletotrichum* sp.1, *Colletotrichum* sp. 2, and *Penicillium* sp. using the developed protocol. Spectrophotometric quantification revealed high average DNA concentrations. Purity was assessed using A260/280 and A260/230 absorbance ratios, which fell within the recommended range, indicating minimal contamination and high-quality DNA. DNA integrity was further confirmed by PCR amplification using ITS1 and ITS4 primers, producing expected amplicons of 594 bp for both *Colletotrichum* species and 585 bp for *Penicillium*. This protocol provides a reliable and affordable alternative for DNA extraction from fungal mycelia, enabling broader accessibility to laboratories with limited resources.

Key words: *Colletotrichum* spp., CTAB-based protocol, fungal DNA extraction, PCR amplification, *Penicillium* sp., silicon dioxide, spectrophotometric quantification.

INTRODUCTION

Phytopathogenic fungi pose a major threat to global food security, causing up to 30% losses in crop yields through disease and spoilage. In addition, mycotoxin-producing and

food-spoiling fungi further compromise the availability of safe and fresh foods (Avery et al., 2019). Rapid and accurate identification of fungal pathogens is therefore essential for effective disease management and mitigation strategies. Beyond pathogen detection, fungal identification plays a pivotal role in understanding species diversity, ecological interactions, and the potential of fungi in biotechnological applications. However, distinguishing closely related or cryptic fungal species remains challenging due to the limitations of traditional morphological methods, which are often subjective, poorly reproducible, and ineffective in the absence of diagnostic structures.

To overcome these constraints, molecular approaches based on DNA analysis (notably PCR and sequencing) have become reference tools. However, the effectiveness of these methods heavily depends on the quality of the extracted DNA. Fungal DNA extraction remains a challenging technical step due to the rigid and complex cell wall of fungi, composed of polysaccharides such as chitin and glucans, which resist lysis (Zhang et al., 2010). Obtaining pure, intact, and usable DNA for molecular analysis remains a critical challenge.

Traditional extraction protocols often rely on expensive techniques that require specialized equipment, such as lyophilization, mechanical grinding with glass beads, liquid nitrogen, sonication, or microwave treatments (DeScenzo & Harrington, 1994; Bir et al., 1995; Lo et al., 1997; Williams et al., 2001; Haugland et al., 2002; Jin et al., 2004; Aamir et al., 2015). Although effective, these methods are often inaccessible in resource-limited laboratories due to their high cost and associated logistical constraints.

The use of liquid nitrogen highlights these difficulties: in addition to its high cost, its use requires specific infrastructure (cryogenic tanks, safety equipment), which is often lacking in under-equipped or remote laboratories. Furthermore, regular supply of liquid nitrogen is difficult to ensure in underserved areas (Agbagwa et al., 2012; Quiñones et al., 2024). Additionally, its environmental impact, due to its high carbon footprint, further complicates its use. These constraints significantly hinder access to molecular biology technologies, especially in developing countries (Cenis, 1992; Tapia-Tussell et al., 2006; Yuan et al., 2023; Gölen & Akar., 2024).

Mechanical grinding with glass beads also illustrates the challenges of conventional extraction methods. While it can be performed manually at low cost, automated bead-beating devices are typically used in laboratories to achieve rapid and uniform homogenization. These devices require financial investment, regular maintenance, and strict safety precautions to prevent glass fragment projection and cross-contamination (Bürgmann et al., 2001; Kermode, 2003; Wang et al., 2023). Manual grinding, although inexpensive, is labor-intensive and produces variable fragment sizes, potentially reducing the efficiency of cell lysis and the quality of extracted DNA. Combining bead-beating with other physical treatments has been shown to improve DNA yield and purity, providing a reliable alternative while remaining more economical than commercial kits (Schurig et al., 2023). Therefore, cost, safety, and reproducibility justify the classification of glass bead grinding as a specialized extraction technique in fungal DNA protocols.

Furthermore, commercial kits based on silica columns, such as the Qiagen DNeasy Plant Pro Mini, have been developed to provide fast and standardized protocols (Conlon et al., 2022a). While these kits are generally effective, they often still require

liquid nitrogen during sample preparation. Moreover, their cost remains prohibitive for many laboratories, and their performance is not always superior to simpler methods using classical reagents, which are often more economical and adaptable to specific needs (Tsuruta et al., 2001; Habibi et al., 2022; Buljević et al., 2025).

In light of these challenges, it is necessary to develop DNA extraction protocols that are simple, effective, and accessible. These methods should eliminate the need for complex equipment while ensuring sufficient DNA quantity and purity for molecular analysis.

The protocol presented in this study offers a promising alternative based on the use of a cetyltrimethylammonium bromide (CTAB) buffer, silicon dioxide as an abrasive, and manual grinding with a mortar and pestle - eliminating the need for liquid nitrogen, lyophilization, and costly commercial kits. When tested on the mycelia of *Colletotrichum* sp.1, *Colletotrichum* sp. 2, and *Penicillium* sp., this method consistently yielded DNA of high purity and concentration, suitable for reliable PCR amplification. This protocol offers a reliable, cost-effective, reproducible, and robust alternative for fungal DNA extraction, making it particularly well-suited for laboratories with limited resources.

MATERIALS AND METHODS

Fungal materials

A total of nine isolates representing three fungal species (*Colletotrichum* sp.1, *Colletotrichum* sp.2, and *Penicillium* sp.) known to infect citrus trees and fruits in Morocco, were used in this study. These isolates are maintained in the fungal collection of the Laboratory of Virology at the National Institute of Agronomic Research (INRA) in Kenitra, Morocco. The corresponding isolate codes are provided in Table 1 and Fig. 1.

Preparation of fungal mycelia

A liquid culture medium composed of 2% malt extract (2 g per 100 mL), and 0.2% yeast extract (0.2 g per 100 mL) was used according to Descenzo & Harrington (1994) to promote optimal production of fresh mycelium. Fragments taken from the edges of monosporic colonies of *Colletotrichum* sp.1, *Colletotrichum* sp. 2, and *Penicillium* sp., grown on Potato Dextrose Agar (PDA) medium, were used to inoculate the liquid medium (Fig. 2, b and e). The isolates were transferred into 25 mL of liquid medium in 125 mL Erlenmeyer flasks and incubated under orbital shaking at 134 rotations per minute (rpm), at a controlled temperature between 20 and 25 °C, under continuous light (Fig. 2, a–c).

The incubation period, ranging from 14 to 20 days depending on the amount of mycelium obtained (Fig. 2, d–f), was followed by harvesting for DNA extraction. The mycelial mats were collected by filtration using sterile gauze compresses and a funnel (Fig. 2, g and h), then carefully rinsed with sterile distilled water. They were wrapped in sterile paper towels and aluminum foil (Fig. 2, i), then stored at 4 °C for 10 days to ensure complete drying (Fig. 2, j and k), (Fig. 2, l).

Table 1. Quality and Purity of DNA Extracted from *Colletotrichum* and *Penicillium* Isolates: DNA Concentration and Absorbance Ratios (A260/280, A260/230) for *Colletotrichum* sp. 1 *Colletotrichum* sp. 2 and *Penicillium* sp. as measured by a NanoDrop 2000 spectrophotome

Genotype name	Isolate	Sample weight (mg)	DNA concentration (ng μl^{-1})		A260/280		A260/230	
			Our method	Without silica	Our method	Without silica	Our method	Without silica
Colletotrichum sp. 1	HaBe1	40 mg	457.65	2.2	1.703	1.58	1.832	0.13
	HaBe2	40 mg	374.64	1.8	1.980	1.46	1.845	0.12
	HaBe3	40 mg	421.14	1.5	1.850	1.52	1.876	0.08
	Mean ± SE	40 mg	**417.81 a ± 24.01**	**1.83 a± 0.28**	**1.844 a ± 0.080**	**1.52 a ± 0.05**	**1.851 b ± 0.013**	**0.08 a ± 0.04**
Colletotrichum sp. 2	AfMo1	40 mg	975.55	3.4	1.822	1.57	2.032	0.10
	AfMo2	40 mg	1,541.60	4.2	2.001	1.58	2.116	0.22
	HaGr1	40 mg	1,276.57	3.7	1.902	1.54	2.124	0.17
	Mean ± SE	40 mg	**1,264.57 b ± 163.52**	**3.77 b ± 0.36**	**1.908 a ± 0.052**	**1.53 a ± 0.03**	**2.091 a ± 0.029**	**0.16 a ± 0.05**
Penicillium sp.	HaGr2	40 mg	1,275.00	4.8	1.916	1.58	2.043	0.25
	ImMr1	40 mg	1,072.80	5.6	1.875	1.47	2.107	0.48
	ImMr2	40 mg	1,176.50	7.2	1.892	1.56	2.046	0.33
	Mean ± SE	40 mg	**1,174.77 b ± 58.38**	**5.87 c ± 1.23**	**1.894 a ± 0.012**	**1.54 a ± 0.03**	**2065 a± 0.021**	**0.35 b ± 0.10**
ANOVA (*p*-value)			0.0019**	0.0021**	0.7066 ns	0.5905 ns	0.0005***	0.0192ns
Two-way ANOVA (*p*-value)	Method		1.57E-09***		6.29E-07***		1.94E-16***	
	Species		1.13E-04***		0.492 ns		1.05E-04***	
	Interaction (Method × Species)		1.21E-04***		0.930 ns		0.0202	

ns: not significant ($p \geq 0.05$) ; $p < 0.05$: significant; ** $p < 0.01$: very significant; *** $p < 0.001$: highly significant.

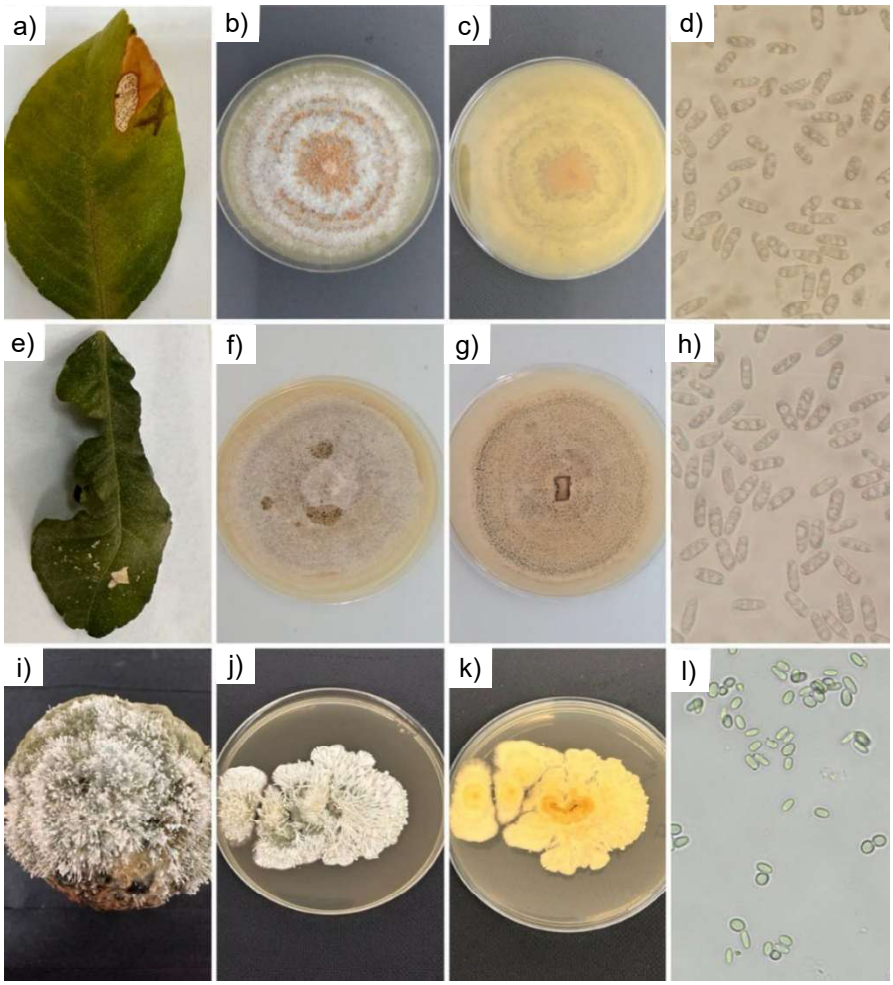

Figure 1. Foliar or fruits Symptoms and Colony Morphology of *Colletotrichum* and *Penicillium*. Species isolated: (a–d) *Colletotrichum* sp.1 *isolate HaBe1* from *Citrus limon* in Bouknadel: a) Anthracnose symptoms on *Citrus limon*, manifested as circular necrotic lesions, brown in color, with lighter areas in the center; b) Colony morphology from the upper surface: circular colonies, smooth in the center, with a color transition from white-cream to light orange at the center; c) Colony underside, almost white, with slight orange coloration at the periphery, showing conidial structures; d) Elongated conidia, cream to yellow in color, observed after 7 days of incubation at 25 °C on PDA medium, under an optical stereoscope. (e–h) *Colletotrichum* sp. *2 isolate AfMo1* from *Citrus limon* in Bouknadel: e) Anthracnose symptoms on *Citrus limon*, showing circular brown lesions with central lighter areas; f) Colony morphology from the upper surface: circular, dense colonies, with colors ranging from light brown to beige; g) Colony underside, with dense, light, slightly brown mycelium, indicating spore production; h) Oval conidia, cream to yellow in color, observed after 7 days of incubation at 25 °C on PDA medium, under an optical stereoscope. (i–l) *Penicillium* sp. *isolate HaGr2* from *Citrus limon (L.) Burm. f.* in El Menzeh Kenitra: i) Symptoms on *Citrus limon*: lesions covered with a blue-gray mass of spores and coremies, with white stalks and mycelium at the lesion periphery; j) Colony morphology from the upper surface: cottony colonies, initially white, turning blue-gray, with a velvety surface; k) Colony underside, pale or orangish yellow, with visible conidiophores and high spore production; l) Blue-gray conidia, spherical to oblong in shape, arranged in chains, observed after 10 days of incubation at 25 °C on PDA medium, under an optical stereoscope (Photographs by H. Benzahra, H. Grijja & I. Mrabti).

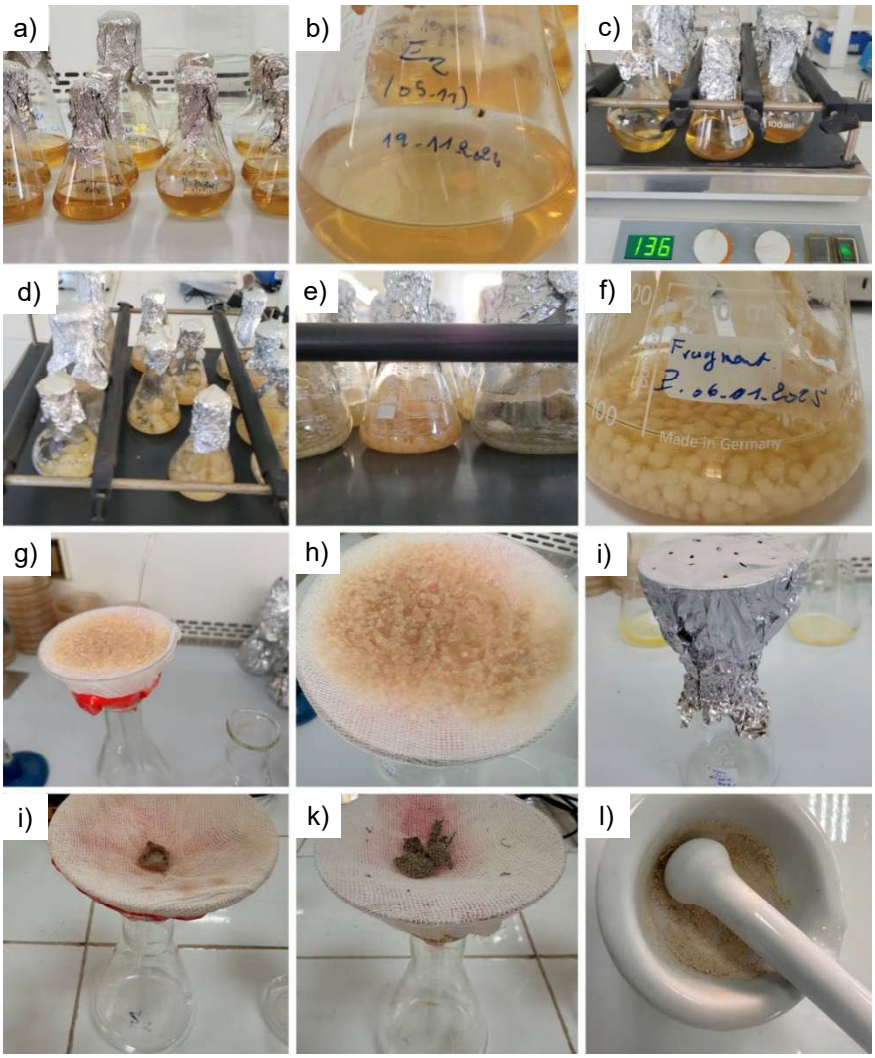

Figure 2. Steps of the liquid culture process and mycelium preparation of *Colletotrichum* spp. for genomic DNA extraction.

(a–b) Inoculation of a liquid medium with fragments taken from monosporic colonies grown on PDA. c) Incubation of cultures in Erlenmeyer flasks on an orbital shaker at 134 rpm, at a controlled temperature between 20 and 25 °C, under continuous light; (d–f) Mycelial growth over a period of 14 to 20 days depending on the isolate; (g–h) Mycelium harvested by filtration using sterile gauze pads and a funnel, followed by rinsing with sterile distilled water; i) Drying of the mycelium wrapped in sterile paper towels and aluminum foil; (j–k) Appearance of the dried mycelium after 10 days at 4 °C; l) Grinding of the dried mycelium with a mortar and pestle in the presence of silicon dioxide to obtain a fine, homogeneous powder suitable for DNA extraction.

DNA Extraction Protocol

DNA extraction from fungal mycelium was performed using a modified CTAB protocol adapted from Doyle & Doyle (1990). After complete drying of the mycelial mats, 40 mg of mycelium was ground into a fine powder using a mortar and pestle in the

presence of 10 mg of commercially obtained silicon dioxide powder (Sigma-Aldrich®, Germany), sterilized prior to use and employed as an abrasive to enhance cell disruption, for approximately 2–3 minutes. Then, 1 mL of CTAB buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP) containing 1% 2-mercaptoethanol was added to the ground mycelium using a mortar and pestle to ensure uniform dispersion in the lysis buffer. The resulting mixture was transferred to 2 mL Eppendorf tubes and centrifuged at 5,000 rpm for 1 minute to separate the silica particles from the supernatant containing the DNA. Subsequently, 500 µl of the supernatant were carefully collected and transferred into a new 1.5 mL Eppendorf tube, to which 15 µl of Proteinase K (20 mg mL^{-1}) were added; the mixture was homogenized by vortexing and incubated at 65 °C for 20 minutes to ensure the digestion of residual proteins. The samples were then quickly cooled on ice, followed by the addition of 500 µl of chloroform:isoamyl alcohol (24:1)and thorough vortexing. After centrifugation at 13,000 rpm for 10 minutes, 400 µl of the upper aqueous phase were carefully transferred to a new tube, and 240 µl of cold 2-propanol were added, with gentle inversion of the mixture twice to ensure complete homogenization. The samples were incubated at -20 °C for 60 minutes to promote DNA precipitation, then centrifuged at 13,000 rpm for 20 minutes to recover the DNA pellet. The pellet was washed with 1 mL of 70% ethanol, centrifuged again at 13,000 rpm for 10 minutes, and the remaining ethanol was carefully removed. Finally, the pellet was air-dried for 15 to 20 minutes, then resuspended in 30 µl of RNase- and DNase-free water, making the DNA ready for downstream applications.

To evaluate the efficiency of our optimized method, a modified comparative protocol was carried out. This protocol used only a classical CTAB extraction, without the key steps of our technique, namely without grinding in the presence of silicon dioxide. In this modified comparative protocol, the mycelial mats were collected from the liquid medium and then vacuum-filtered through Whatman No. 54 filter paper (Whatman Paper, Maidstone, England). The collected mats were dried for 14 to 20 days between paper towels, according to Descenzo & Harrington (1994). They were then transferred into microtubes containing 1 mL of CTAB buffer and incubated at 65 °C for 20 minutes to allow chemical lysis. Subsequent steps of chloroform:isoamyl alcohol extraction, centrifugation, isopropanol precipitation, ethanol washing, and final resuspension were performed as in the main protocol.

Quantitative and qualitative analysis of the extracted DNA

The quantification of DNA, as well as the evaluation of its purity and integrity, was carried out using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplifications targeting the internal transcribed spacer (ITS) region of fungal ribosomal DNA were performed using the forward primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and the reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). Each reaction, in a final volume of 25 µl, contained 1.5 µL 10x mi-*Taq* only buffer, 0.2 mM of each dNTP, 0.4 µM of each primer, 0.6 U of mi-*Taq* only DNA polymerase (Metabion, Germany), and 50 ng of extracted DNA.

The amplification program included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles consisting of denaturation at 95 °C for 30 seconds, primer annealing at 50 °C for 30 seconds for the isolates of the two *Colletotrichum* species and

at 49 °C for those of *Penicillium* sp., and extension at 72 °C for 1 minute. A final extension was performed at 72 °C for 7 minutes. Reactions were carried out using a Bioer thermal cycler (Hangzhou Bioer Technology Co., Ltd., China). Aliquots (10 µl) of PCR amplicons were resolved on a 1% agarose gel in 1× Tris-Acetate-EDTA (TAE) buffer, stained with 0.5 µg mL^{-1} ethidium bromide, and visualized on a Gel Doc imaging system (Bio-Rad, CA, USA). Fragment sizes were determined by reference to a 100 bp DNA Ladder (New England Biolabs, MA, USA).

Statistical analysis

Statistical analyses were performed using SPSS (IBM SPSS Statistics 22.0). DNA yield, purity (A260/280 and A260/230 ratios), and overall quality obtained from two extraction methods (here developed, with silica and standard, without silica.) applied to three fungal species (*Colletotrichum* sp.1, *Colletotrichum* sp.2, and *Penicillium* sp.) were analyzed by two-way analysis of variance (ANOVA) including species, extraction method, and their interaction. Species effects within each extraction method were assessed using simple main effects from the same model.

Results are presented as mean ± standard error (SE), with $p < 0.05$ considered statistically significant and ns indicating non-significant differences.

RESULTS AND DISCUSSION

The electrophoretic profiles of DNA extracted using the modified protocol showed highly satisfactory results, with clear, and well-defined bands; however, slight smearing was observed on agarose gels, suggesting a possible low level of DNA degradation (Fig. 3, a). Three technical replicates per species generated consistent profiles: *Colletotrichum* sp.1 isolates (HaBe1 to HaBe3), *Colletotrichum* sp. 2 (AfMo1, AfMo2, HaGr1), and *Penicillium* sp. (HaGr2, ImMr1, ImMr2), confirming the reproducibility and robustness of the protocol. *Colletotrichum* sp. 1, *Colletotrichum* sp. 2 and *Penicillium* sp. showed strong, intense bands comparable to the 1 kb marker, corresponding to DNA fragments larger than 10,000 bp.

To evaluate the efficiency of the method, a comparative protocol based on classical CTAB extraction, omitting silica-assisted grinding, was implemented. The results showed very low yields, high variability between replicates, and, in several cases, insufficient DNA quantities for reliable analyses (Table 1; Fig. 3, b). The obtained electrophoretic bands were weaker and slightly diffuse, accompanied by smearing, suggesting the presence of residual contaminants (Fig. 3, b). This comparison highlights the superiority of the silica-assisted protocol in terms of both DNA yield and quality.

DNA yields, quantified by NanoDrop 2000 spectrophotometry, and purity, assessed by A260/A280 and A260/A230 ratios, are summarized in Table 1. The addition of silica to dry mycelium significantly improved yield and purity from 40 mg of fungal material: Colletotrichum sp.1 showed 417.81 ± 24.01 ng μL^{-1} with silica versus 1.83 ± 0.28 ng μL^{-1} without, while Colletotrichum sp.2 and Penicillium sp. reached 1,264.57 ± 163.52 ng μL^{-1} and 1,174.77 ± 58.38 ng μL^{-1} with silica, respectively. The A260/A280 (1.703–2.001) and A260/A230 (1.851–2.091) ratios confirmed low protein and organic contamination, whereas values without silica were consistently lower.

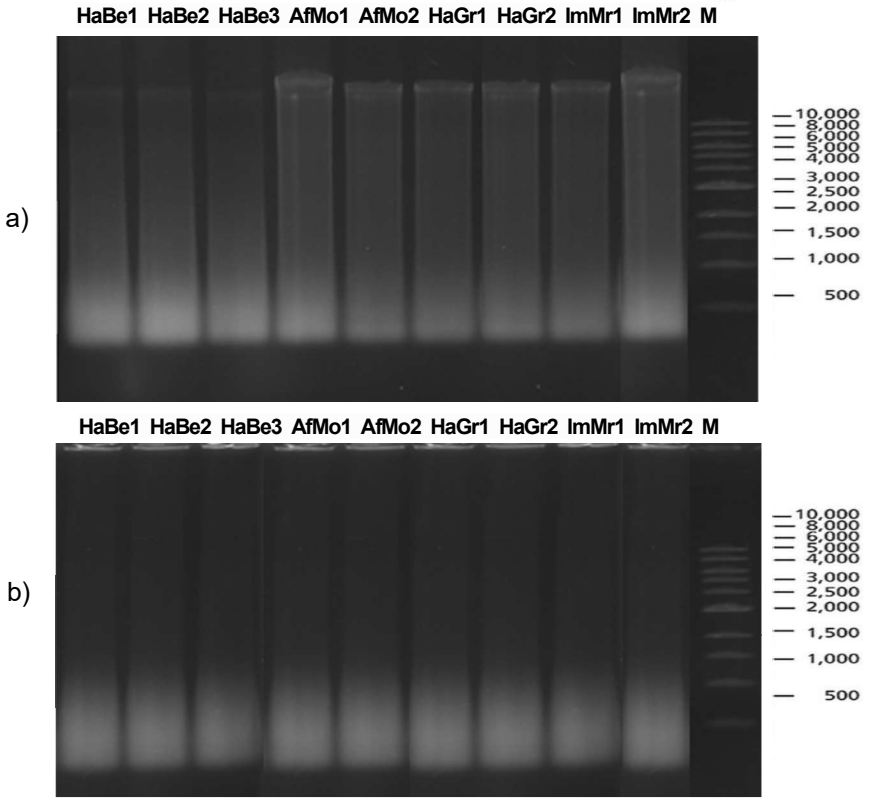

Figure 3. Agarose gel electrophoresis of total DNA extracted from *Colletotrichum* sp.1 isolates (lanes HaBe1, HaBe2, and HaBe3) and *Colletotrichum* sp.2 isolates (lanes AfMo1, AfMo2, and HaGr1), as well as three *Penicillium* sp. isolates (lanes HaGr2, ImMr1, and ImMr2). DNA extraction was performed using two methods: a) our extraction method with silica; b) control method without silica. M: 1 kb DNA marker.

Two-way ANOVA revealed that the extraction method (with vs. without silica) had a highly significant effect on DNA yield ($p < 0.001$) as well as on both purity parameters (A260/A280 and A260/A230, $p < 0.001$). Significant species effects were observed for DNA concentration and A260/A230 ($p < 0.001$), but not for A260/A280 ($p = 0.492$). Significant method × species interactions were also detected for DNA concentration ($p < 0.001$) and A260/A230 ($p = 0.020$), indicating that silica efficiency varies among fungal species. These results demonstrate that, although silica consistently improves DNA extraction, the extent of this improvement depends on the species studied.

Figs 4 and 5 illustrate the PCR amplification products obtained from the extracted DNA. The electrophoretic profiles of the amplifications were clear, reproducible, and sufficiently polymorphic to allow reliable isolate differentiation. These experimental results suggest that the optimized protocol enhances both DNA yield and purity and provides DNA of sufficient quality for advanced molecular analyses.

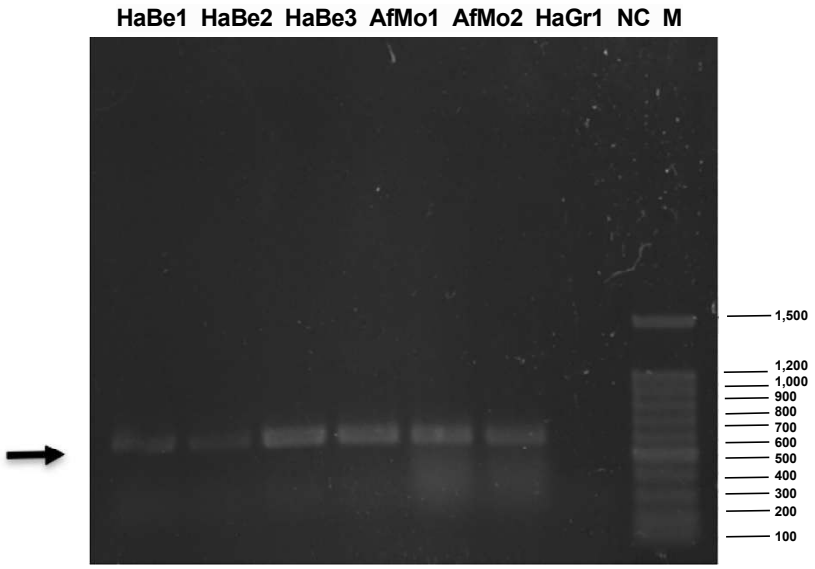

Figure 4. Agarose gel electrophoresis of the ITS region amplified using primers ITS1 and ITS4. Lanes HaBe1, HaBe2, and HaBe3 correspond to *Colletotrichum* sp.1 isolates; lanes AfMo1, AfMo2, and HaGr1 correspond *Colletotrichum* sp. 2. isolates. NC: negative control; M: 100 bp DNA ladder.

To place these findings in a broader methodological context, a comparative evaluation with previously published fungal DNA extraction studies was conducted, focusing on DNA yield and purity. DNA extraction from filamentous fungi remains technically challenging due to the rigidity of fungal cell walls and the abundance of polysaccharides and secondary metabolites that interfere with DNA recovery (Al-Samarrai & Schmid, 2000; Sambrook & Russell, 2001). Consequently, reported DNA yields vary widely depending on fungal species, biomass characteristics, and extraction strategy. CTAB-based methods are among the most widely used protocols. For example, Noor Adila et al. (2007) reported DNA yields of approximately 816 ng mg^{-1} of mycelium when extracting genomic DNA from the oleaginous fungus *Cunninghamella bainieri*, while maintaining acceptable purity ratios. Similarly, Voigt et al. (1999) demonstrated that CTAB effectively removes polysaccharides and produces DNA suitable for PCR and restriction enzyme digestion, although these methods generally require multiple steps and extended handling time. Mechanical disruption approaches have also been widely adopted. Aamir et al. (2015) reported DNA yields ranging from 300 to 1,150 ng mg^{-1} of fungal biomass using bead beating combined with phenol-chloroform extraction. While effective, such approaches often require specialized equipment and increase the risk of DNA shearing or laboratory contamination (Manen et al., 2005; Sharma et al., 2012).

In the present study, the silica-assisted protocol yielded approximately 313 ng mg^{-1} of dry mycelium for *Colletotrichum* sp.1, 948 ng mg^{-1} for *Colletotrichum* sp.2, and 881 ng mg^{-1} for *Penicillium* sp. These values fall within, and in some cases approach the upper range of, yields reported for conventional CTAB- and bead-based protocols.

Importantly, these yields were achieved using a simplified procedure requiring limited starting material and fewer processing steps, while maintaining DNA purity ratios comparable to those obtained using more complex and resource-intensive methods. Beyond yield, DNA purity is a critical determinant of downstream applicability. Several conventional protocols yield sufficient quantities of DNA but often suffer from residual contamination by proteins, polysaccharides, or organic solvents (Wilfinger et al.,1997; Biswas & Biswas, 2011; Japelaghi et al., 2011). In contrast, the silica-assisted method consistently produced DNA with A260/A280 and A260/A230 ratios within optimal ranges, confirming its suitability for enzymatic applications.

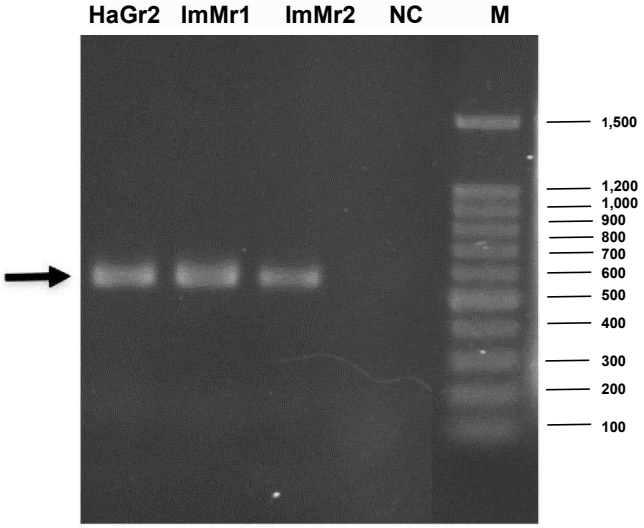

Figure 5. Agarose gel electrophoresis of the ITS region amplified using primers ITS1 and ITS4 from three *Penicillium* sp. isolates (lanes HaGr2, ImMr1, and ImMr2), showing a 585 bp amplicon. NC: negative control; M: 100 bp DNA ladder.

These experimental findings prompted a detailed analysis of the mechanistic role of silica in enhancing fungal cell disruption and DNA recovery. This treatment facilitates the disruption of resistant cell walls and the release of intracellular contents. After grinding and centrifugation, well-defined pellets composed of silica and cellular debris form, accompanied by a clear supernatant, ensuring sharp phase separation. In the absence of silica, mycelial aggregates remain suspended, limiting process efficiency and increasing the risk of incomplete lysis. Silica thus acts both as a mechanical abrasive and stabilizer, promoting homogeneous and effective grinding. This observation directly explains the higher DNA yields and purity obtained with silica-assisted extraction.

Optimization of of DNA extraction method from filamentous fungi that the addition of silicon dioxide to the dryed mycelium significantly improved the efficiency of the grinding process. This treatment contributed to a more effective disruption of the cell wall, which is known for its mechanical resistance, thereby facilitating the release of intracellular contents. Following grinding and centrifugation, this approach resulted in well-defined pellets composed of silica and cellular debris, along with a clear

supernatant, indicating good phase separation. In contrast, in the absence of silica, grinding was more challenging: mycelial aggregates remained suspended in the lysis buffer, floating and moving with the vortex created by the pestle. This compromised the efficiency of the process and increased the risk of incomplete cell disruption. This issue did not occur when the mycelium was mixed with silica, as the fragments rapidly settled at the bottom of the tube, trapped between the pestle and the tube wall. This mechanical arrangement promoted direct and sustained contact between the pestle and the sample, facilitating the breakdown of the mycelial structures. Consequently, the addition of silica acted both as a mechanical abrasive and as a stabilizer for the positioning of the mycelium, ensuring a more homogeneous, faster, and efficient grinding process. These mechanistic insights highlight why the optimized protocol consistently produces high-quality DNA suitable for downstream analyses.

In contrast to many fungal DNA extraction methods reported over the past decades (Nijs et al., 1996; Manen et al., 2005; Saitoh et al., 2006a; Biswas & Biswas, 2011; Japelaghi et al., 2011; Kelly et al., 2012; Sharma et al., 2012; Conlon et al., 2022b; Dar et al., 2025a), traditional protocols for filamentous fungi extraction are generally complex and require sophisticated laboratory equipment. These methods often rely on liquid culture followed by grinding in liquid nitrogen to break the rigid cell walls. Even alternatives that do not use liquid nitrogen often require multiple, lengthy steps, including the use of extraction columns, lyophilization, or thermal shocks alternating between extreme temperatures (from −80 °C to +60 °C). These requirements make it difficult to apply such methods in laboratories with limited resources, particularly in regions with unstable electricity supplies. By comparison, the simplicity of our method reduces dependency on specialized infrastructure.

Moreover, some DNA extraction methods yield DNA of sufficient quality only for specific applications such as PCR (Cenis, 1992; Liu et al., 2000), while others require expensive equipment, including commercial DNA extraction kits, thus limiting their accessibility in resource-constrained laboratories (Portela et al., 2025). For example, the Qiagen DNeasy Plant Pro Mini kit allows rapid and efficient fungal DNA extraction with minimal handling (Conlon et al., 2022b; Dar et al., 2025b). However, its high cost and reliance on specialized equipment, due to the mandatory pre-treatment of mycelium by grinding in liquid nitrogen, pose major obstacles for many laboratories. Using such methods requires not only the commercial kit but also a complete infrastructure to handle liquid nitrogen safely, including cryogenic containers, pre-cooled mortars or grinders, and personal protective equipment such as cryogenic gloves, safety goggles, insulating aprons, and proper ventilation systems. These technical and safety requirements significantly limit the feasibility of these protocols in resource-limited settings. These limitations further underscore the advantages of the present silica-assisted method.

The use of sand as a grinding agent, as described by Weiland (1997) and Yalong et al. (2016), can be effective for breaking down fungal mycelium. However, it presents several limitations. Natural sand may contain organic impurities such as microbial or plant residues, which can introduce exogenous DNA and compromise the reliability of molecular analyses (Al-Samarrai & Schmid, 2000; Feng et al., 2010). Its heterogeneous chemical composition, including potential traces of heavy metals or mineral salts, may inhibit enzymatic activity during PCR or digestion reactions (Ish-Horowicz & Burke,

1981; Sambrook & Russell, 2001). In addition, variability in particle size can affect the efficiency and reproducibility of mechanical disruption (Yang et al., 2016). Finally, standard sand sterilization procedures do not always eliminate thermostable chemical or organic contaminants, which may affect the reproducibility of DNA extractions across different batches or laboratories (Saitoh et al., 2006b). These limitations highlight the importance of using purified and standardized sand, or alternatively, materials with similar mechanical properties but higher chemical inertness. The use of purified silica in our protocol avoids these pitfalls and ensures reproducible results.

The DNA extraction method employed in this study stands out for its simplicity, speed, and low cost. It requires neither liquid nitrogen, nor lytic enzymes, nor specialized equipment, making it particularly well-suited for laboratories with limited resources. All extractions were performed in standard 1.5 to 2 mL microtubes, simplifying handling, minimizing logistical costs, and enabling rapid implementation without complex infrastructure. This approach offers an efficient alternative to conventional protocols, while ensuring an optimal balance between yield, purity, and reproducibility. The results demonstrate that the extracted DNA is of high quality, stable, and amplifiable, suitable for PCR, sequencing, and phylogenetic studies. These observations confirm that the method provides a reliable, versatile, and cost-effective solution for a wide range of applications in fungal molecular biology. Overall, the findings emphasize that silica-assisted extraction is both practically feasible and broadly applicable, especially in resource-limited settings.

CONCLUSIONS

This study presents a simple, efficient, and cost-effective CTAB-based protocol for the extraction of high-quality genomic DNA from fungal mycelia, without the use of liquid nitrogen, lyophilization, or commercial kits. The incorporation of silicon dioxide as an abrasive during the grinding of fungal mycelia significantly enhances cell wall disruption, leading to higher DNA yield and purity for the three studied fungal species, including *Colletotrichum* spp. and *Penicillium* sp. The extracted DNA exhibited strong integrity with limited fragmentation, and excellent amplifiability, as confirmed by ITS-region PCR. These findings demonstrate the method's reliability and reproducibility, particularly in resource-limited settings where access to advanced laboratory infrastructure is constrained. Compared to conventional protocols, this approach offers a sustainable and accessible alternative for fungal molecular diagnostics and phytopathological research.

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