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First report of *Colletotrichum nymphaeae* causing anthracnose on argan (*Argania spinosa.* L) in Morocco

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Abstract: This study presents the identification of *Colletotrichum nymphaeae* for the first time as the causal agent of anthracnose on argan fruits (*Argania spinosa* L.) in Morocco. The affected fruits were collected from Dar Chef Province (Ouazzane region). The pathogen was isolated and identified using morphological and molecular characterization. The pathogenicity tests were also carried out to confirm its ability to induce disease symptoms on argan fruits. The results showed fungal colonies that produce abundant orange conidial masses. The microscopic examination showed cylindrical, hyaline, unicellular conidia similar with known morphology of *C. nymphaeae.* Pathogenicity tests confirmed Koch's postulates. The typical anthracnose symptoms were developed on inoculated fruits, with larger lesions developing on wounded tissues (average diameter: 1.64 cm) compared to unwounded tissues (0.66 cm). However, control fruits were asymptomatic. Molecular identification using the internal transcribed spacer (ITS) region of rDNA showed 99.83% similarity with *C. nymphaeae* isolate from China (GenBank: ON793164.1). Phylogenetic analysis grouped the Moroccan isolate (OP363901) within a well-supported clade alongside Asian and Central American *C. nymphaeae* species complex, confirming its taxonomic status within the *C. acutatum* species and highlights its genetic divergence from related species such as *C. acutatum, C. lupini, and C. godetiae.* This finding raises an important phytopathological concern, given the economic importance of argan oil production in Morocco. The wide host diversity and environmental adaptability of C. nymphaeae suggest its potential impact as an exotic threat to argan agroecosystems. The study highlights the urgent need for integrated disease management strategies and subsequent future research including multilocus sequencing in order to better understand the population structure and epidemiology of *C. nymphaeae* in Morocco.

Keywords: Anthracnose, *Colletotrichum nymphaeae*, Koch postulate, Morocco, province of ouazzane.

Introduction

The argan tree (*Argania spinosa* L.) is an endemic tree specific to the arid zone in southwestern Morocco, has remarkable socio-economic and ecological importance in comparison to other trees found in dry and semi-dry regions of the country. The cultivation of this tree in these regions is due to a particular adaptation to the climate of the region but also for an economic interest for argan oil production and other derivatives from seeds used for cosmetics and beauty, those products are a vital source of income for local populations in addition, its cultivation in these areas contributes to soil preservation and desertification control. However, phytopathological features of this species, particularly the fungal pathogen impacting its health and productivity, remain underexplored.

Rieuf (1962) documented initial work on fungi associated with the argan trees. Their work reported a number of fungi, saprotrophic as well as parasitic, which infest woods, twigs and fruits of argan tree in southwestern Morocco. This work triggers many other studies into the fungal biodiversity interacting with this species.

Fungal susceptibility of argan trees has been confirmed in several studies, Bakry et al. (2009) reported the presence of *Pestalotiopsis clavispora* in cultivated nurseries of argan plants which suggests a possible threat to young trees even at early stages of development.

Later, Sellal et al. (2019) conducted a comprehensive study on fungal species affecting both leaves and fruits of argan trees and reported several genera including *Alternaria alternata*, *Mucor* sp., *Rhizopus stolonifer*, *Epicoccum nigrum*, *Curvularia lunata*, *Drechslera australiensis*, *Aspergillus niger*, *A. fumigatus*, *Cladosporium pistacinaerensis*, and *Pestalotia* sp. These findings highlighted the diversity of fungi present in the phyllosphere and carposphere of the argan tree.

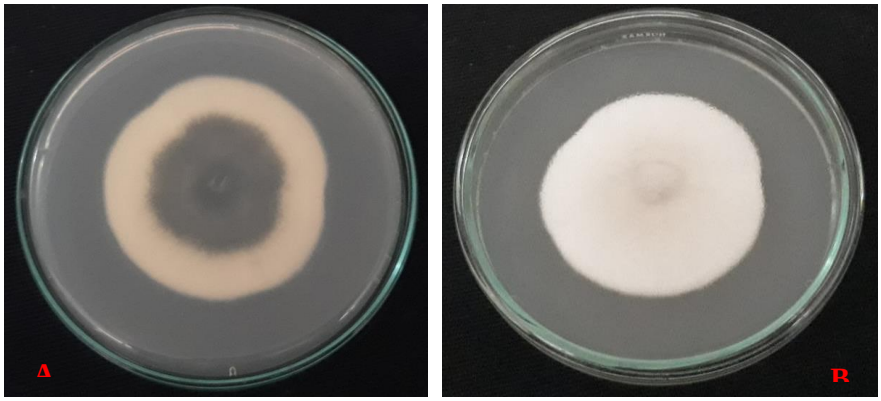

Figure 1. Colony morphology of *Colletotrichum nymphaeae* isolate (GenBank accession OP363901) grown on Potato Dextrose Agar (PDA) at 28°C for 7 days (**A**) Upper surface of the colony showing a concentric growth pattern with a dark gray center surrounded by a lighter outer ring. (**B**) Reverse side of the colony displaying a uniform pale coloration with a faint central pigmentation.

More recently, Bouchar et al., (2022) documented the presence of two Basidiomycetes *Auricularia auricula-judae Wettstei* and *Fomes fomentarius* as well as two Ascomycetes, *Hysterium pulicare*, *Pers fries* and *Trematosphaeria pertusa* Pers growing on argan tree trunks. These results emphasized the need for further investigation into the diversity of fungi infestating the argan tree as well as their ecological implications.

In this context, the present study focused on the identification of fungi affecting the argan tree, to our knowledge this is the first recorded case of a disease in argan fruits caused by the pathogenic fungus *Colletotrichum nymphaeae* isolated from symptomatic fruits in the Dar Chef province (Ouazzane region), the fungus was identified using both morphological and molecular methods, and its pathogenicity was confirmed through Koch's postulates.

This research will contribute to the best understanding of fungal pathogens affecting *Argania spinosa*, but also highlight the important considerations regarding the management of emerging diseases that could threaten the sustainability and economic importance of *Argania spinose* oil production in Morocco.

Results

Isolation and morphological identification of what

Colonies on PDA were initially white, turning gray with age, and produced pink-orange conidial masses. Conidia were fusiform to cylindrical, hyaline, unicellular, measuring approximately 0.73 µm in length and 0.5 µm in width (n = 40) **(Fig. 1) (Fig. 2).**

Molecular identification and phylogenetic analysis

The ITS sequence of the Moroccan isolate (OP363901) showed 99.83% similarity with a Chinese *C. nymphaeae* isolate (ON793164.1). Phylogenetic analysis placed the Moroccan isolate within a well-supported clade (bootstrap value: 70 %) of *C. nymphaeae* strains, distinct from *Colletotrichum*, *C. acutatum*, *C. godetiae*, and *C. lupini* (**Fig. 3**).

Pathogenicity tests

Inoculated Argan fruits exhibited anthracnose symptoms (s orange spore masses and sunken lesions) after 7 days. Lesion diameters were 1.64 cm and 0.66 cm on non-wounded fruits. Control fruits showed no symptoms. The pathogen was successfully re-isolated from infected tissues, completing Koch's postulates (**Fig. 4).**

Discussion

This study provides the first confirmed report of *Colletotrichum nymphaeae* as the causal agent of anthracnose in argan fruits (*Argania spinosa* L.) in Morocco. The pathogen was isolated from infected fruits collected in the Dar Chef Province (Ouazzane region) and identified using several approaches that integrated morphological, pathogenicity, and molecular analyses.

Members of the Ascomycete fungus genus *Colletotrichum* are of critical phytopathological significance. They are widely distributed and are well known to be responsible for anthracnose diseases in numerous economically significant crops worldwide (Wharton and Diéguez-Uribeondo, 2004).

In Morocco, their presence has been documented on several hosts including olive trees (Achbani et al., 2013; Chliyeh et al., 2014; Msairi et al., 2017, 2020) and strawberries (El Alaoui et al., 2021; El Kaissoumi et al., 2018; Mouden et al., 2016), leading in many cases to serious fruit rot and economic loss (De Silva et al., 2017).

C. nymphaeae fungi was identified on the basis of distinctive morphological characteristics: white colonies that gradually turned gray with abundant orange conidial masses. Under microscopic examination the fungi revealed fusiform to cylindrical, smooth, hyaline, unicellular conidia with rounded or slightly tapering ends, as described previously by Damm et al. (2012) and later validated and confirmed by Ma et al., (2022) and Wang et al. (2022) in China.

Pathogenicity tests clearly satisfied Koch's postulates, as inoculated argan fruits formed the same characteristic as sunken necrotic lesions, especially prominent in wounded fruits (1.64 cm in diameter) compared to unwounded ones (0.66 cm).

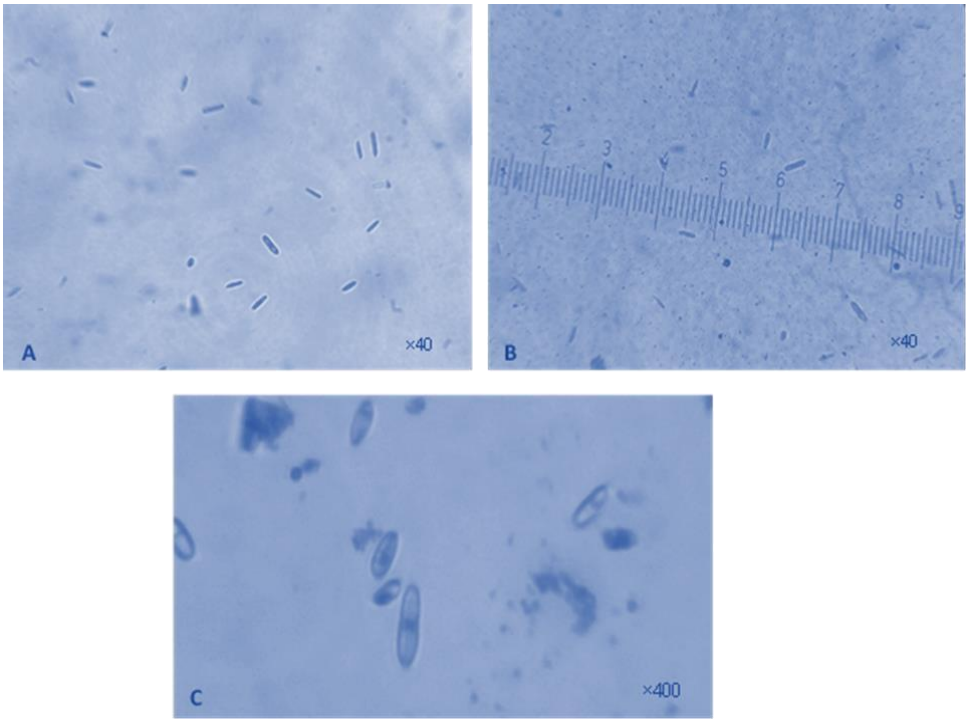

Figure 2. Microscopic observation of conidia produced by the Moroccan isolate of *Colletotrichum nymphaeae*. (**A, B**) Conidia viewed under light microscope at 40× magnification (A micrometric scale is included for size reference (**B**). The spores appear hyaline, unicellular, smooth-walled, and smooth-walled, mostly cylindrical in shape. (**C**) Detailed view at 400× magnification , conidia show fusiform to cylindrical morphology with rounded ends. Spore dimensions averaged 12.73 × 4.5 µm (n = 40).

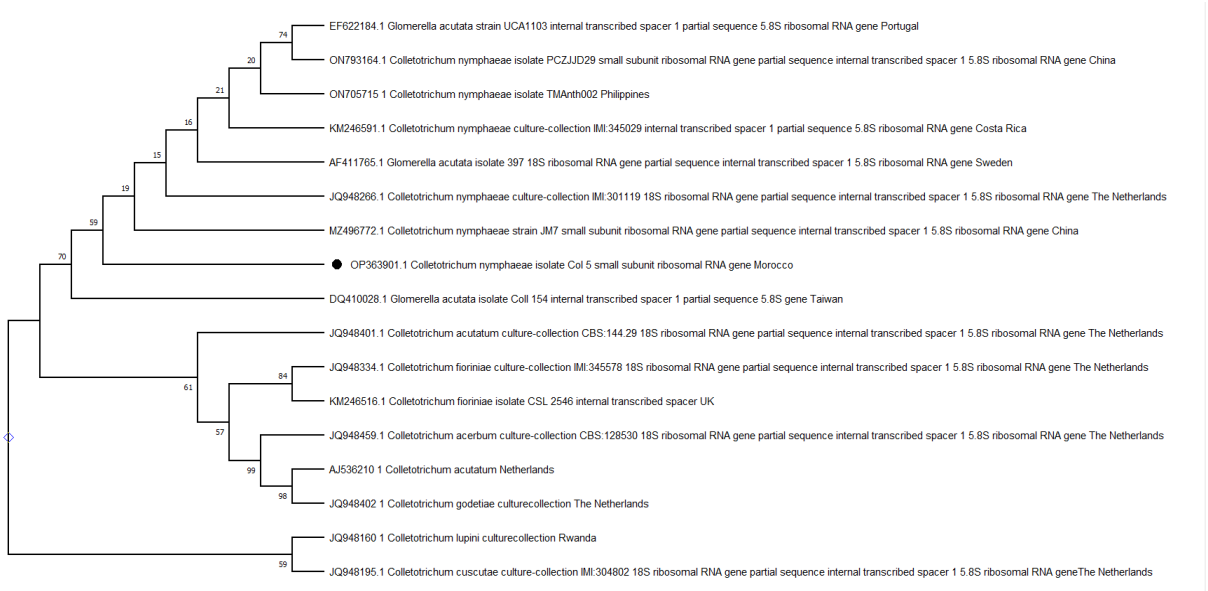

Figure 3. A phylogenetic tree constructed using the Neighbor-Joining method *reconstructed from* ITS rDNA sequence of the isolate identified in this study and related taxa *in* GenBank. *bootstrap values are shown at branch points to indicate clade support. The Moroccan isolate from this study (GenBank accession* ***OP363901****) is marked with a black circle (●) and forms part cluster within a well-supported clade of C. nymphaeae isolates from China, the Philippines, and Costa Rica.*

Orange gelatinous spore masses were often observed visible at the lesion sites, and the pathogen was invariably recovered, verifying its causal nature.

ITS-based molecular identification showed 99.83 % DNA sequence identity with the reference isolate of the Chinese strain, *C. nymphaeae* (GenBank: ON793164.1). Phylogenetic analysis placed the Moroccan isolate within a robustly supported monophyletic clade (bootstrap 70 %) along with other *C. nymphaeae* isolates from Asia, including those from China and the Philippines. This low degree of intraspecific variation suggests a shared ecological niche or recent common ancestry. The Moroccan isolate was differentiated from related taxa such as (*C. acutatum, C. lupini, C. godetiae*), validating its place in the *Colletotrichum acutatum s.l.* complex. While ITS sequencing was adequate for proper species-level classification in the given instance, more research applying multilocus sequencing (e.g., GAPDH, ACT, CHS-1, TUB2) are needed for deeper phylogenetic relationships resolution.

The detection of *C. nymphaeae* on argan fruits is of significant value due to its socio-economic importance in this region and also for the production of argan oil in Morocco. *C. nymphaeae* is known to infect various hosts such as olives, apples, walnuts,

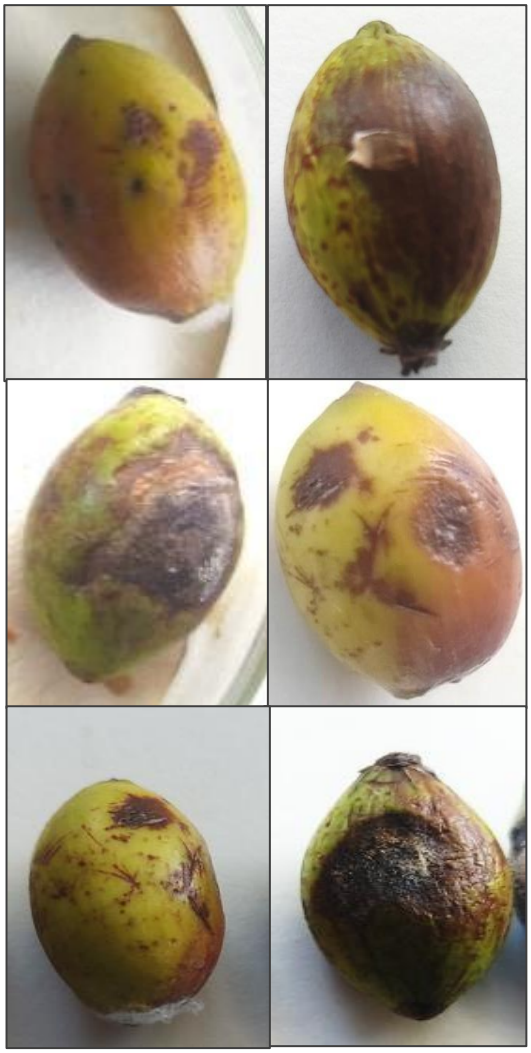

Figure 4. Symptoms of anthracnose on argan fruits (Argania spinosa L.) caused by *Colletotrichum nymphaeae* (isolate OP363901) ten days after inoculation., typical signs of anthracnose began to appear on the argan fruits. The infected fruits show dark, sunken necrotic lesions, which are circular to irregular in shape and frequently exhibit coalescence. Some lesions are associated with soft rot and gelatinous orange spore masses, indicating active sporulation. Lesions vary in size and severity across fruits, and several exhibit discoloration or partial mummification. These symptoms are consistent with anthracnose disease progression. Control fruits (not shown) remained healthy and symptom-free under identical conditions.

strawberries, tomatoes, and peaches (Abo-Elwafa et al., 2023; Antelmi et al., 2019; Chechi et al., 2019; C. H. Kim et al., 2020; Y. Kim et al., 2019; Liu et al., 2023; Talhinhas et al., 2018; Tan et al., 2022). It has been reported in Portugal, Italy, South Korea, China, Brazil, and Iran (Karimi et al., 2017; Moreira et al., 2019; Rockenbach et al., 2016; Velho et al., 2014). Its broad host spectrum and ecological flexibility elevate its threat level, particularly in the diversified Moroccan agroecosystems.
The Moroccan isolate's similarity to Asian strains raises questions about its geographic origin and possible potential modes of introduction, including international trade, contaminated plant material, or human-mediated dispersal. This highlights the need of biosecurity and early detection systems in argan cultivation. In the future, there is an urgent need to implement integrated disease management (IDM) practices, such as periodic orchard surveillance, improved hygiene practices, elimination of infected material, judicious fungicide use, and development of biocontrol methods. The findings from this study provide a critical foundation for sustainable disease management in argan-based agroecosystems.

Materials and Methods

Sample collection and fungal isolation

Argan fruits showing visible anthracnose symptoms were collected from the Dar Chef Province. Fruits were first washed under running tap water, then surface-sterilized with 75% ethanol for one minute. After rinsing with sterile distilled water, they were left to air-dry on sterile filter paper. Small sections of the sterilized fruit tissues were placed on Potato Dextrose Agar (PDA) media and incubated in the dark at 28°C. Emerging fungal colonies were sub-cultured to obtain pure isolates for subsequent analyses.

Morphological characterization

Fungal isolates were examined for their colony morphology, as well as the shape, size, and microscopic features of their conidia, following standard mycological protocols described by Smith and Black (1990).

DNA Extraction, PCR amplification, and sequencing

After a 10-day incubation period on Potato Dextrose Agar (PDA), the fungal isolate was preliminarily identified as *Colletotrichum nymphaeae* based on the morphology of its colonies and conidia. 10–40 mg of freeze-dried mycelium was ground using a sterile mortar and pestle. Genomic DNA was then extracted using the OmniPrep Kit for Fungi (**G-Biosciences, USA; Cat. #786-399**) following the manufacturer's instructions. DNA yield and purity were assessed using a NanoPhotometer N50 (**Implen GmbH, Germany**). The internal transcribed spacer (ITS) region, including the 5.8S rDNA gene, was amplified by PCR reaction using the universal primers ITS1 (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990).

Each PCR reaction (20 µL total volume) contained 11 µL of Milli-Q water, 2 µL of 10× PCR buffer, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.2 µL of x-VITA Taq DNA polymerase (**Labbox, Spain; Cat. #TAQP-S05-001**), 1 µL of each primer, and 1 µL of genomic DNA. Amplification was performed in a MultiGene OptiMax Thermal Cycler (**Labnet International, USA; Cat. #TC9610-230**) using the following program: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute and a final elongation step at 72°C for 7 minutes and 25 seconds.

PCR products were visualized on 1% agarose gels stained with innoQ DNA stain (**Labbox, Spain; Cat. #GDYE-001-500**). DNA bands were visualized under UV light using the Enduro GDS Gel Documentation System (**Labnet International, USA; Cat. #GDST-1302**). Positive amplicons were purified using the ExoSAP-IT PCR Product Cleanup Reagent (**Affymetrix, USA**) and sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (**Applied Biosystems, USA**) on an ABI PRISM 3130XL Genetic Analyzer using a POP-7 polymer.

DNA sequence alignment and phylogenetic analysis

The ITS rDNA sequence obtained from the isolate was deposited in the GenBank database under the accession number OP363901**,** a BLASTn analysis was performed to search for sequence similarity with others accessions in the databse. For phylogenetic construction, multiple sequence alignment was performed using the ClustalW algorithm implemented in MEGA11 software (Tamura et al., 2021). The phylogenetic tree was constructed using the Neighbor-Joining (NJ**)** method (Saitou & Nei, 1987), with 1,000 bootstrap replicates to evaluate the robustness of the inferred clades.

Koch's postulates and pathogenicity assessment

To evaluate the pathogenic potential of the *Colletotrichum nymphaeae* isolated, two distinct inoculation experiments were conducted with healthy nursery-grown Argania spinosa seedlings and freshly harvested fruits**.** The methods were modified from existing protocols developed for *C. lupini* and *C. acutatum* in olive pathosystems (Chliyeh et al., 2014; Msairi et al., 2017, 2020), and to fulfill Koch's postulates.

Spray inoculation protocol for argan seedlings

For inoculum preparation, a conidial suspension was obtained from 7-day-old PDA cultures of C. nymphaeae. Mycelial plugs (5 mm diameter) were rinsed thrice with sterile distilled water to release conidia; the resulting suspension was then filtered through sterile muslin cloth to remove hyphal fragments.

Using a hemocytometer, Spore concentration was adjusted to 1×10^6 conidia/mL. To enhance the uniformity and adherence of the spray, the suspension was supplemented with 0.05% Tween 20 and 0.05% gelatin. Twelve-month-old, disease-free argan seedlings were uniformly sprayed with this conidial suspension using a sterile handheld atomizer. As a control, another group of seedlings was sprayed with sterile distilled water containing the same concentrations of Tween 20 and gelatin. All plants were maintained under high-humidity conditions (moist chamber) at 24°C, with a 12 h light/dark cycle. The seedlings were monitored daily over a 10-day period for the appearance of any disease symptoms.

Fruit inoculation under wounded and non-wounded conditions technique

Healthy, mature argan fruits were carefully surface-sterilized in 75% ethanol for 1 minute, rinsed with sterile distilled water, left to dry on sterile absorbent paper. To simulate natural infection and evaluate the role of tissue damage on disease development, two types of inoculation were carried out with and without wounding.

- Wounded fruits (n = 20) were gently punctured to a depth of approximately 2 mm using a sterile needle and inoculated with 5 mm diameter mycelial plugs from actively growing mycelium.
- Non-wounded fruits (n = 20) received the same mycelial plugs, but directly on the surface.
- Control fruits (n = 20) were treated in the same way but with sterile PDA plugs instead of fungal cultures.

All fruits were placed in sterile Petri dishes containing three layers of moistened sterile filter paper to ensure a humid environment. The dishes were sealed and incubated at 24°C under continuous light for eight days. After incubation disease symptoms were evaluated by measuring the diameter of lesions (cm), recording the severity of symptoms, and re-isolating the pathogen from symptomatic tissues. No symptoms were observed in the control group. However, *C. nymphaeae* was successfully re-isolated from symptomatic fruits, but not from control tissues confirming Koch's postulates.

Conclusion

To the best of our knowledge this is the first confirmed report of *Colletotrichum nymphaeae* as the causal agent of anthracnose in argan fruits in Morocco. Using a combination of pathogenicity tests, morphological characterization, and

molecular analysis such as sequencing the ITS region and the phylogenetic analysis, the fungus isolate was identified, the Moroccan isolate (GenBank accession OP363901) shared 99.83% sequence identity with the reference isolate from China and clustered within a strongly supported monophyletic clade of *C. nymphaeae* strains, thereby confirming its taxonomic identity. Pathogenicity tests indicated the ability of the fungus to induce substantial necrotic lesions in both wounded and unwounded argan fruits under controlled laboratory conditions, which confirms its pathogenic effect. The re-isolation of the pathogen was successful from infected tissues confirmed fulfillment of Koch's postulates and validated its pathogenic role. These findings indicate the vulnerability of argan fruits to a previously undocumented pathogen, suggesting that *C. nymphaeae* might infect other hosts and be environmentally very versatile. The emergence of this pathogen is especially alarming considering the economic and ecological value that argan cultivation brings to the local population. Anthracnose could severely damage fruit quality and quantity, putting at risk not only the argan oil industry but also the livelihoods that depend on it, particularly for the local population. In order to minimize these risks, we recommend the incorporation of integrated disease management (IDM) practices such as regular orchard surveillance, early detection using molecular methods, timely use of fungicide, symptomatic tissues pruning, and rigorous sanitation procedures.
In conclusion, this study provides a critical baseline data on a newly emerging fungal threat to argan cultivation and highlights the urgent need for coordinated research and management strategies for sustainability and productivity of this economically important species.

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Consent to publish declaration:
Not applicable.

Availability of Data and Materials
The data generated and analyzed during this study are not publicly available to protect the anonymity of the interviewees. However, the article presents sufficient anonymous verbal excerpts to illustrate the results. Sequence data have been submitted to GenBank under the accession number OP363901.**Ethics approval and consent to participate**
Not applicable

Authors' contributions
Soukaina Maazouzi drafted the initial, Soukaina Msairi participated in development work carried out in the laboratory, Moulay Abdelaziz El Alaoui conducted data analysis and contributed to the interpretation of results, Zineb Sellal contributed to sampling in the field, Allal Douira provided overall supervision and approved the final version of the manuscript, Karima Selmaoui and Amina Ouazzani Touhami read and approved the final version of the manuscript.

Competing interests
We have no competing interests to declare.

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