



RESEARCH ARTICLE

Isolation and molecular identification of a new *Sordaria fimicola* and *Nigrospora sphaerica* from stem of *Prunus armeniaca*. L. tree

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Abstract

The sooty canker disease was studied in some orchards of the Karbala and Babylon provinces to understand the reasons for the decline of apricot trees (*Prunus armeniaca* L.) in some areas of Karbala Governorate, specifically Al-Husayniyah District (Al-Qantara Al-Bayda area and Bani Hassan stream) and Awn In the province of Babylon (the first and second ponds, the Umm al-Hamam area, Bustan and Hor Hussein). The isolation results showed the presence of *Sordaria fimicola* with the highest frequency of 37 % followed by *Nigrospora sphaerica* with frequency rates of 23 %, sequencing new *S. fimicola* and *N. sphaerica* isolates that have been registered at the Gene Bank. Pathogenicity test results showed canker on the seedlings one month after the inoculation process the length of the canker was 1.25 and 0.9 cm for both fungi sequentially and significantly different from comparison treatment. The pathogen was isolated and greenhouse inoculation and diagnosed based on their morphological, molecular and pathogenicity.

Keywords: *Nigrospora sphaerica*; polymerase chain reaction; *Prunus armeniaca*; *Sordaria fimicola*

Introduction

The genus *Prunus armeniaca* L. (Sub-family Prunoideae) is an economically important fruit crop that is widely planted throughout Iraq. Apricot fruits are used fresh or dried, or manufactured and desirable because they contain high nutritional value (1). The highest average yield per tree of apricot was estimated in Baghdad (40.10 g) and the least of it was in Karbala (20.21 g) (2). Apricot trees suffer from many pathogens and are among the most prominent factors specific to the cultivation, especially fungal, leading to the canker of their trunks and thus deterioration of the wood quality produced and deformed as well as the death of branches, stains its leaves (3). Apricot trees are attacked by a variety of fungi, causing the canker stems and dieback and wilting, which leads to the weakening of the cytosolic wood and coloured brown than cracking the bark longitudinally and death of branches because of mycotoxin secretion (4). Molecular biology was used as an effective method for diagnosing many fungal isolates and quickly determine disease. It is one of the ways to explain the relationships between fungal species, distinguish between closely related species with some morphological differences and distinguish strains within a single species (5).

Although the fungi were recorded on many plant families, However, there are many fungi that were not recorded on the apricot tree in Karbala Province, Iraq. The aim of this research was to isolate the fungi associated with apricot plant stem and leaves and identify the most frequent fungus than molecularly diagnosed.

Materials and Methods

Study area and sample collection

This study was conducted in selected apricot (*P. armeniaca*) orchards located in Karbala Province, Iraq, during the spring and early summer of 2020. Field visits were carried out to identify symptomatic trees exhibiting signs of stem canker, including bark necrosis, gummosis and cracking. Samples were collected from 15 mature apricot trees with clear symptoms, Samples were collected from deteriorating apricot trees in various orchards in Karbala Governorate (Al-Qantara Al-Bayda area in Al-Husayniya district and Bani Hassan stream) Babylon (the first and second Al-Baraka districts, the Umm Al-Hamam area, Bustan and Hor Hussein in the Al-Musayyib district). Bark tissues were cut from the advancing margins of the canker lesions using sterile blades and immediately transferred into sterile plastic bags, then stored in a cooler box during transport to the laboratory.

Fungal isolation and culture conditions

The collected bark fragments were first rinsed under running tap water to remove surface debris, then surface-sterilised by immersion in 1 % sodium hypochlorite solution for 1 min, followed by 70 % ethanol for 30 sec and rinsed thrice with sterile distilled water. The sterilised tissues were dried on sterile filter paper and placed on Petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin sulfate at a concentration of 50 mg/L to suppress bacterial growth (6). The plates were incubated at 25–27 °C for 5–7 days under dark conditions.

Emerging fungal colonies were subcultured onto fresh PDA plates to obtain pure isolates. Morphological characterisation was conducted based on macroscopic features (colony colour, texture, growth rate) and microscopic traits (spore shape, size, presence of reproductive structures) using a compound light microscope.

Molecular identification using polymerase chain reaction

DNA extraction

Take approximately 50–100 mg of the fungal growth to be diagnosed, growing on PDA medium. Genomic DNA was extracted from freshly grown fungal mycelia (5–7 day-old cultures) using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Mycelial mats were scraped from PDA cultures, frozen in liquid nitrogen and ground to a fine powder using a sterile mortar and pestle prior to extraction.

Polymerase chain reaction amplification of internal transcribed spacer region

The internal transcribed spacer (ITS) region of the rDNA was amplified using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (5). Polymerase chain reactions were prepared in a total volume of 25 μ L containing 12.5 μ L of Ready-To-Go™ PCR Beads (GE Healthcare, USA), 1 μ L (5 pmol) of each primer, 2 μ L of genomic DNA template (50–100 ng) and 8.5 μ L of nuclease-free water. Amplification was performed in a thermal cycler using the following program: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

Gel electrophoresis and sequencing

Polymerase chain reaction products were visualised using 1.5 % agarose gel electrophoresis stained with ethidium bromide. Bands were documented using a UV transilluminator. Successful amplifications were purified and sent for sequencing to Macrogen Inc. (Seoul, South Korea). Resulting sequences were aligned and compared with known fungal ITS sequences in the NCBI GenBank database using the BLAST tool. Phylogenetic analysis was performed using MEGA6 software with the neighbor-joining method and bootstrap analysis (1000 replicates) (7). Sequences of confirmed isolates were submitted to GenBank and assigned accession numbers MW338650 (*S. fimicola*) and MW338661 (*N. sphaerica*).

The stability of candidate reference genes (TEF1- α , GAPDH and β -Tubulin) was evaluated using the geNorm software package. The program calculates the gene stability measure (M) based on the average pairwise variation of a particular gene with all other reference genes tested. A lower M-value indicates higher expression stability. Genes were ranked and the 2 most stable genes were selected for subsequent normalisation of target gene expression.

Pathogenicity test

Plant material and inoculation method

Healthy *P. armeniaca* seedlings (18 months old), uniform in size and free from visible disease symptoms, were obtained from a certified nursery and maintained in polyethylene greenhouse conditions at the Faculty of Agriculture, University of Kerbala. During the day (growth the temperature, up to 25 °C and Relative Humidity 60–70 %).

Inoculation was conducted by artificially wounding the bark using a sterile 4-mm cork borer to remove a disc of bark (without penetrating the xylem). Mycelial plugs (5 mm diameter) taken from

the active margin of seven-day-old PDA cultures were placed directly onto the wound. Each inoculated site was covered with sterile moist cotton and sealed with Parafilm to maintain humidity and prevent contamination (6). Three branches were inoculated per seedling and 3 seedlings were used per fungal isolate (Each repeater has ten experimental units). Control plants received sterile PDA plugs without fungal inoculum. Koch's hypotheses were applied.

Symptom assessment and re-isolation

After 30 days, the length of the resulting cankers was measured using a digital caliper. The mean lesion length was calculated for each treatment. To fulfill Koch's postulates, fungi were re-isolated from the inoculated sites and identified morphologically to confirm the causal relationship.

Statistical analysis

Canker lengths were analysed using one-way analysis of variance (ANOVA) and means were compared using the least significant difference (LSD) test at a 5 % significance level using SPSS software (version 23.0). Pathogenicity was considered confirmed if statistically significant canker were produced and the inoculated fungi were successfully re-isolated.

Results and Discussion

Symptom observation and field diagnosis

Visual assessment of apricot (*P. armeniaca*) trees in surveyed orchards revealed striking pathological features, as presented in (Fig. 1). The left panel depicts a close-up of the tree trunk, exhibiting a distinct canker characterised by sunken bark, dark discoloration and cracking. The margins of the canker appear raised and blackened, while the central zone is visibly exposed with clear signs of tissue decay. Additionally, the affected area shows evidence of dried exudate, consistent with gummosis. In the right panel, a whole-tree view illustrates widespread canopy dieback, leaf wilting and a general decline in tree vigortypical symptoms of systemic infection or prolonged vascular impairment. Such manifestations strongly suggest the presence of stem canker disease, a condition known to be caused by a range of fungal pathogens that invade woody tissues through wounds or natural openings (5). The observed cracking and gummosis are classical signs of defense responses in stone fruit trees, where gum production is triggered to isolate infected tissues and impede pathogen spread (8). However, when the infection overwhelms plant defenses as appears evident in the case shown the result is progressive bark death and compromised structural integrity of the stem. These field observations are in line with previous reports from Karbala province, where *Botryosphaeria obtusa*, *Cytospora* spp. and more recently *S. fimicola* and *N. sphaerica* were implicated in similar symptomatology (3, 4). Gummosis, while non-specific, is often an early warning sign of deeper vascular or cambial infections. In particular, *N. sphaerica* is known to generate bioactive compounds that can suppress host defenses, contributing to lesion expansion and systemic decline (9). The apparent progression from localised canker (as seen in the trunk) to whole-canopy decline (as shown in the right image) underscores the importance of early detection and control. Canker expansion not only girdles the affected branches but also disrupts water and nutrient flow, leading to drought-like symptoms even under normal irrigation. If left untreated, such infections can culminate in total tree death, translating into substantial economic losses for orchard owners. These



Fig. 1. Local type of symptoms on *Prunus armeniaca* trees.

observations laid the groundwork for further diagnostic steps including fungal isolation, PCR-based molecular identification and pathogenicity assays. The distinct morphology of the lesions and their distribution on the tree serve as crucial indicators for selecting appropriate tissues for laboratory analysis.

Isolation and morphological identification of *Sordaria fimicola* and *Nigrospora Sphaerica*

Fungal isolation from symptomatic apricot stems yielded multiple colonies, among which *S. fimicola* was consistently recovered at a frequency of 37 %, making it one of the dominant fungal species associated with the observed canker symptoms (Fig. 2a). Colonies on PDA appeared dark brown to black with a dense, woolly texture and concentric zonation. Growth was moderate in rate and the reverse side of the colony showed intense pigmentation, typical of the *Sordaria* genus.

Microscopic examination confirmed the presence of morphological features characteristic of *S. fimicola*, a member of the class Sordariomycetes. The fungus produced obpyriform, darkly pigmented perithecia aggregated in clusters (Fig. 2d & e). Asci were cylindrical, containing eight mature ascospores arranged in a uniseriate or biseriate manner. Notably, the ascospores were olive to dark brown in colour, ellipsoidal with rounded ends and displayed a distinct germ pore at the basal end (Fig. 2b & c). Rosettes of asci were clearly observed (Fig. 2f), which is another diagnostic hallmark of *S. fimicola*. These morphological features are in line with the classical descriptions provided by previous researchers who reported the presence of *S. fimicola* on *Acer palmatum*, highlighting its thick-walled perithecia and deeply pigmented ascospores as distinguishing characteristics (9, 10). While *S. fimicola* is traditionally classified as a coprophilous or saprophytic fungus commonly isolated from decomposing plant matter or animal dung, its recovery from active canker on apricot stems suggests a possible ecological shift or opportunistic pathogenic behaviour.

The *N. Sphaerica* fungus was isolated at a high rate of 23 %. The white woolly colonies grow rapidly. Colonies at first white with small, shining black conidia easily visible under a lower-power dissecting microscope, later brown when sporulation is abundant. Conidiophores semi-macronematous, branched, flexuous, colourless to brown, smooth. Conidia solitary, with a violent discharge mechanism, acrogenous, simple, spherical or ellipsoidal, compressed dorsiventrally (Fig. 3a–c) (11).

The consistent isolation of *S. fimicola* and *N. Sphaerica* from necrotic stem tissues, coupled with its distinct colony morphology

and reproductive structures, supports its role as a potentially pathogenic organism in apricot canker disease. However, mere presence does not confirm pathogenicity; thus, further molecular identification and pathogenicity tests are essential to ascertain its causal relationship with the disease symptoms.

Molecular identification using polymerase chain reaction and phylogenetic analysis

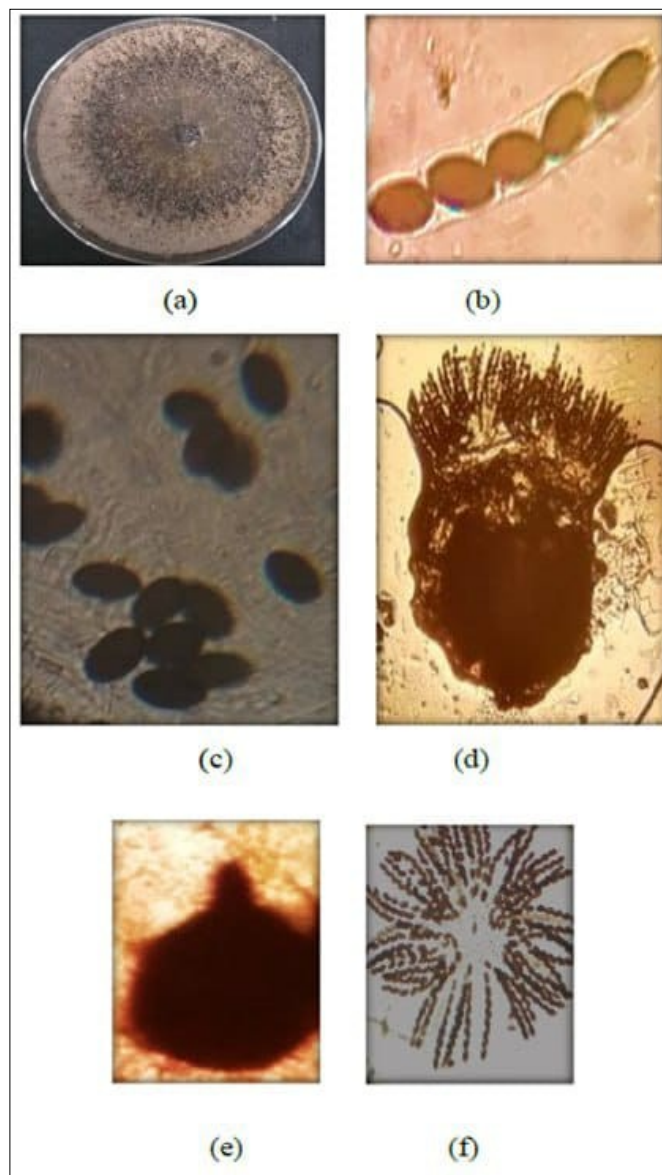


Fig. 2. *Sordaria fimicola*. a: Culture on medium; b: Ascus with 5 ascospores; c: Masses of mature ascospores; d: Perithecium and asci; e: Perithecium with distinct neck; f: Rosettes of asci.

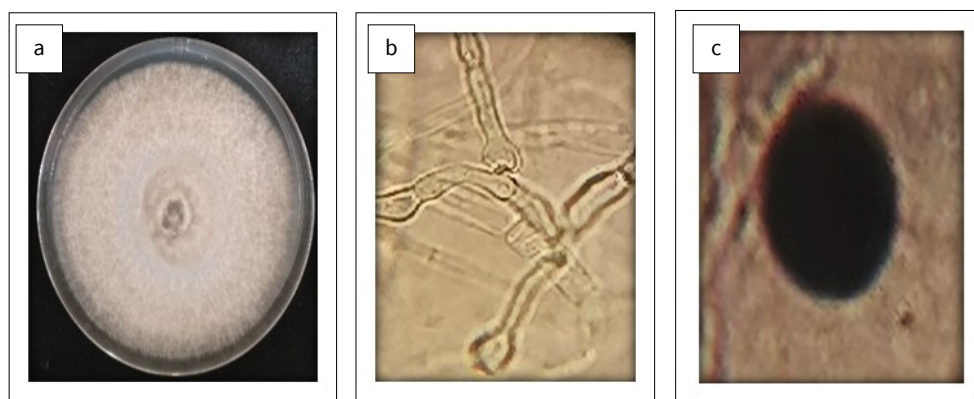


Fig. 3. *Nigrospora sphaerica*. a: The colony culture on PDA; b: Mycelia; c: Spores.

A genetic method was used to verify the initial identification grounded on morphological features. Specifically, the ITS region inside the ribosomal DNA of the fungal isolates derived from apricot canker tissues was amplified by PCR. Widely known in fungal taxonomy, the ITS region is particularly useful for exact species-level identification as it shows significant variation across species yet remains somewhat constant between individuals of the same species (12). The ITS sequences of both isolates *S. fimicola* and *N. sphaerica* were successfully amplified using the ITS1 and ITS4 primer pair and the PCR products were sequenced and submitted to the NCBI GenBank database under the accession numbers MW338650 for *S. fimicola* and MW338661 for *N. sphaerica*. Fig. 4 shows the results of the nitrogenous base sequence analysis, indicating that isolate number three belongs to the fungus *S. fimicola*, isolate number 7 belongs to the fungus *N. sphaerica*.

The obtained sequences were subjected to BLAST analysis to compare them with previously reported sequences in the database. The *S. fimicola* isolate from Karbala (MW338650) shared a high degree of similarity with isolates from South Korea (e.g., MN341414, MN341412), as shown in the phylogenetic tree (Fig. 5). The topology of the tree reveals that the Iraqi isolate clusters closely with the South Korean strain MN341414, suggesting a conserved lineage and confirming the identity of the isolate as *S. fimicola*. This close genetic relationship also suggests possible wide ecological

plasticity of the species across different geographic regions and hosts. Similarly, the phylogenetic tree of *N. sphaerica* (Fig. 6) places the Iraqi isolate (MW338661) within a well-supported clade alongside strains from China (MW077080 and MW077072). Interestingly, the Philippine isolates (MT645657 and MT597827) formed a separate branch, indicating potential regional divergence or subspecies variation within *N. sphaerica*. The genetic proximity between the Iraqi isolate and Chinese strains might be explained either by environmental similarities that have moulded similar evolutionary features in both areas and by a shared ancestral lineage.

By use of molecular technologies, verifying the identity of these fungi not only validates the first morphological identification but also emphasises the significance of ITS-region analysis in fungal classification. As shown in many previous investigations, the ITS region has long been seen as a potent tool for differentiating fungal species, particularly those invading woody plants (13). The ITS sequencing was crucial in this study to precisely identify 2 fungus species not often found in connection with apricot disease. *Sordaria fimicola* is hardly known on live woody hosts (14), but its genetic grouping with previously identified isolates from other nations gives great support for its identification. Furthermore, the evolutionary insights obtained from this study offer a wider viewpoint on the possible dissemination and development of fungal infections outside of local borders. With continuous

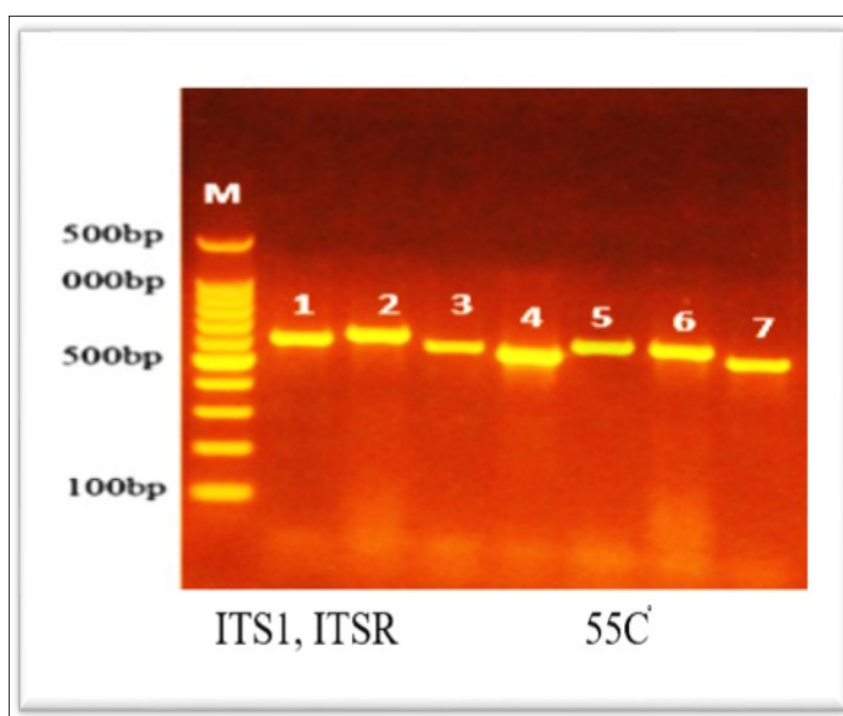


Fig. 4. The resulting bands from electrophoresis and their sizes (base pairs, bp) are indicated on the left side of the DNA ladder.

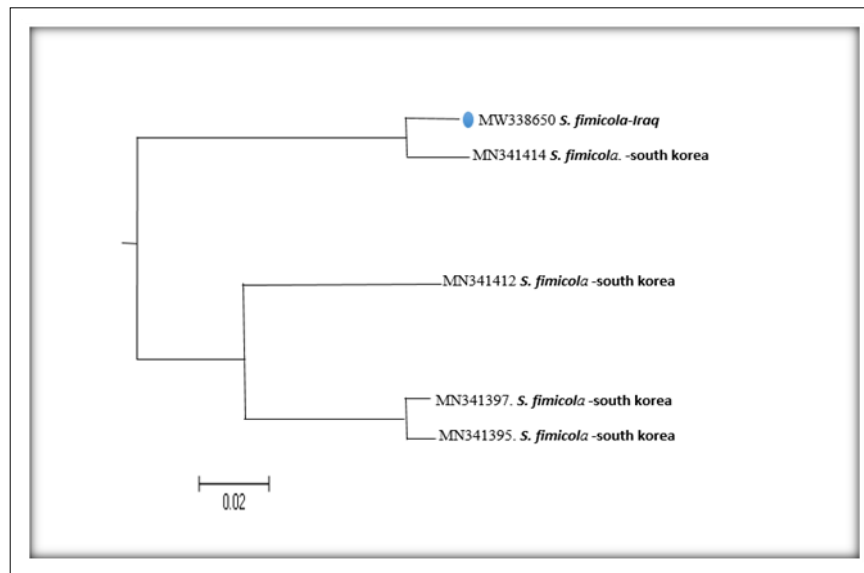


Fig. 5. The genetic tree of fungus *Sordaria fimicola*.

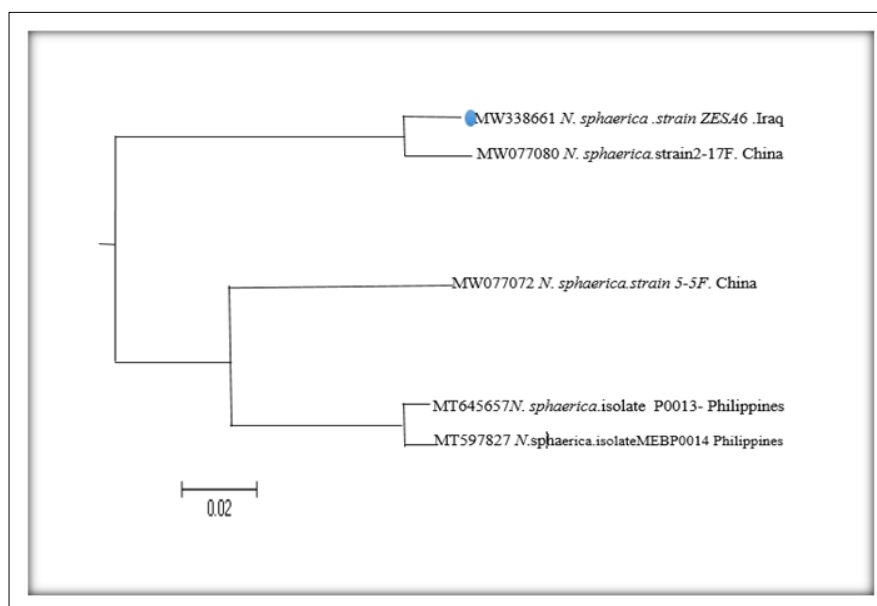


Fig. 6. The genetic tree of fungus *Nigrospora sphaerica*.

worldwide changes including climate change, growing international commerce and changing agricultural methods, molecular monitoring becomes ever more important for early detection and management of such infections in fruit crops.

Pathogenicity test and confirmation of Koch's postulates

To assess the pathogenic potential of the fungal isolates recovered from apricot trees, an artificial inoculation test was conducted under controlled greenhouse conditions. After 30 days of infection, measurable canker lesions were observed in the inoculated seedlings, confirming the ability of both *S. fimicola* and *N. sphaerica* to induce disease symptoms. As shown in Fig. 7, *S. fimicola* caused the most severe lesions, with a mean canker length of 1.25 cm, followed by *N. sphaerica* with a mean canker length of 0.9 cm. Control treatments, which involved wounding the plant without introducing fungal inoculum, produced no visible cankers, confirming that the disease symptoms were attributable to the tested fungi.

The observed difference in canker length between *S. fimicola* and *N. sphaerica* was statistically significant ($p \leq 0.05$), indicating variable degrees of virulence among the isolated

pathogens. The higher pathogenicity of *S. fimicola* suggests that, although historically considered a saprophyte, it may act as a primary pathogen under conducive conditions. This aligns with earlier reports who identified *S. fimicola* as an endophytic pathogen of peach trees in Iran, previous researchers documented its association with canker symptoms on *Acer palmatum* (15, 11).

Nigrospora sphaerica induced discrete and quantifiable cankers, therefore confirming its pathogenic behaviour even if its degree of aggressiveness was lower than that of *S. fimicola*. Literature often describes this species as an endophyte or latent pathogen that may become virulent under situations of stress-inducing stimuli recorded its existence in hosts such as *Moringa oleifera* and many palm plants, indicating to its capacity to synthesis phytotoxic chemicals that could help in breaking plant defences and starting infection (9). The findings of this investigation confirm that interpretation suggests that *N. sphaerica* may function as a secondary pathogen under favourable settings or contribute to the complexity of diseases syndromes.

Strong proof that satisfies Koch's postulates comes from the formation of cankers only in inoculation plants and effective re-

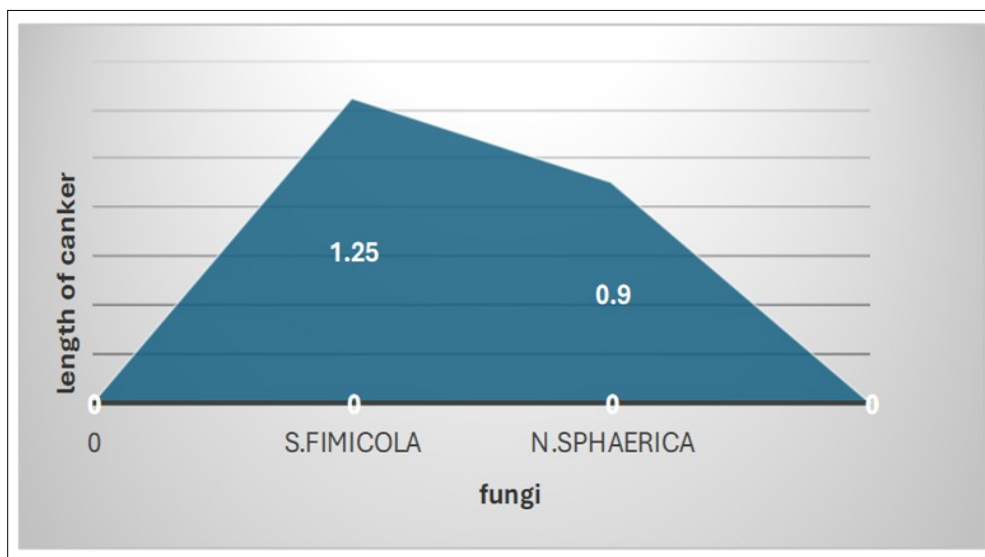


Fig. 7. Test of pathogenicity ability after one month of field inoculation.

isolation of both fungus species from the affected tissues. This proves that *S. fimicola* and *N. sphaerica* are real causes of canker disease in apricot, not only pollutants. Especially, this is the first officially recorded instance of this fungus linked to apricot stem cankers in Iraq.

Routine pathogen surveillance and early diagnostic initiatives are desperately needed given the growing reports of gummosis and canker in apricot orchards all around Karbala. Moreover, the capacity of *S. fimicola* usually considered as a saprophyte to change into a pathogenic form emphasises the need of investigating environmental and physiological stress elements that can support such a change in ecological behaviour.

The increase in fungal diseases on apricot trees may be due to environmental stress resulting from increased reliance on chemical pesticides or failing to reduce water stress, more efficient and precise irrigation systems (such as drip irrigation) should be used instead of flood irrigation. In addition to other reasons too numerous to mention, environmental stress creates an ideal environment for the faster and more intensive growth and reproduction of fungal spores, thus leading to the selection of more virulent fungal strains capable of adapting to harsh conditions (such as drought resistance or high temperatures). Therefore, the increased occurrence of fungal diseases due to environmental stress has greatly complicated the management of Iraqi apricot orchards and imposed negative effects on the strategies employed.

Conclusion

Two fungal species *Sordaria fimicola* and *Nigrospora sphaerica* were effectively identified from apricot trees showing canker symptoms in orchards in Karbala, Iraq in this work both morphological description and molecular analysis with ITS-PCR verified their identification; the obtained sequences were entered into the GenBank database. Strong proof of their contribution in disease progression comes from pathogenicity tests verifying their capacity to induce stem lesions. These results underline the need of ongoing surveillance and diagnostic campaigns in impacted areas as well as the possible appearance of certain fungus as pathogenic hazards in apricot farming.

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Authors' contributions

SAA was responsible for field sampling, fungal isolation and conducting laboratory experiments, including morphological identification and pathogenicity tests. ZAM contributed to molecular analysis, sequence data interpretation and preparation of the phylogenetic trees. Both authors collaborated equally in designing the study, analysing the results, writing the manuscript and approving the final version for submission.

Compliance with ethical standards

Conflict of interest: The authors declare they do not have any conflict of interest.

Ethical issues: None

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