Journal of Food Science and Technology (Iran)

Homepage:www.fsct.modares.ir

Scientific Research

Molecular Characterization and Phytotoxic Metabolite Profiling of *Nigrospora oryzae* and *N. sphaerica* Isolates from Date Palm: Implications for Food Safety and Postharvest Quality

Ali Z. Abed *, Baida G. Ofi[2], Mohammed H. Abass[3]

Department of Plant Protection, Agricultural College, University of Basrah, Basrah, Republic of Iraq.

ARTICLE INFO

Article History:

Received: 2026/02/22
Review: 2026/05/06
Accepted: 2026/5/10

Keywords:
Date palm,

Nigrospora oryzae,

Nigrospora sphaerica, Food safety,

Postharvest quality,

Mycotoxigenic fungi.

DOI: 10.48311/fsct.2026.119510.83078

*Corresponding Author E-Mail:
ali.abd@uobasrah.edu.iq

ABSTRACT

Secondary metabolites of their fungal pathogens may affect fruit quality and contaminate harvested products of date palm (*Phoenix dactylifera* L.), and therefore, fruit safety is a major concern. The present study was undertaken to assess the phytotoxicity of two *Nigrospora* species causing leaf spot on date palm leaves in relation to their biochemical interaction with the host, as well as fruit safety. The cultural and morphological characteristics of the isolates confirmed their identity as *Nigrospora oryzae* and *N. sphaerica*. The ITS gene sequences of the two isolates obtained in the present study showed 100% similarity with those of the reference strains in the NCBI database under the accession numbers MH855300.1 and PV107334.1 for *N. oryzae* and *N. sphaerica*, respectively. Biochemical changes observed after the infiltration of culture filtrates into healthy date palm leaves indicated stress as well as the possibility of metabolite translocation. Higher levels of carbohydrates, free proline, and phenolic compounds were observed in treated leaves, indicating the induction of defense mechanisms against fungal metabolites. Notably, catalase also dropped to further explain the compromised ability to regulate oxidative stress; it was 1.6 mmol/min/g in the control and 1.5 and 1.03 mmol/min/g in *N. oryzae* and *N. sphaerica*, respectively. Total soluble proteins decreased in treated leaves, while free amino acids were highly elevated, especially in N.oryzae treated tissues at 9.67 mg/g FW. This clearly indicates that there was indeed an accumulation of reactive oxygen species (ROS) as hydrogen peroxide levels in response to culture filtrates of *N. oryzae* were 9 times higher. Controls had a malondialdehyde (MDA) level of 2.22 nmol/g. This increased to 4.90 and 3.30 nmol/g in *N. oryzae* and N. sphaerica treatments, respectively. This proves that the two *Nigrospora* species secrete phytotoxic metabolites, which can lead to oxidative stress and cell damage in date palm tissues. If the metabolites can be translocated to developing fruit, this will raise serious concerns about fruit contamination as well as postharvest quality loss. The study also proves that toxigenic fungi should be monitored in date palm plantations as an integral part of the strategy to ensure the safety and quality of the fruit supply to the food industry.

1- INTRODUCTION

Date palms (*Phoenix dactylifera* L.) are traditional and significant trees in the Middle East food chain as they are a rich source of nutrition and calories [1]. Date palm trees are grown mainly for their fruit (dates); several studies reported the chemical composition of dates and their nutritional value [2].

Date palm is widely known as a sensitive host for several pathogens, including fungi, bacteria, and viruses. Fungal infection is a major constraint to date palm, and several diseases cause a devastating loss in date production, both in quality and quantity [3]. Among fungal pathogens, the *Nigrospora* Zimm. The genus has been diagnosed as a true endophytic pathogen on date palm, mostly important the species of *N. oryzae* and the other species *N. sphaerica* [4,5]. The general characteristics of the disease symptoms caused by these pathogens are black spots on date palm leaves and stems.

In addition to date palms, the genus *Nigrospora* Zimm. can infect several plant hosts such as banana with fruit decay symptoms [6]; rice plant causing spot disease in leaves and grains [7], the roots of maize [8]; liquorice medical plant (*Glycyrrhiza glabra*) [9]; the twigs and shoot of blueberry plants (*Vaccinium corymbosum* L.) with blight symptoms [10]; *Brassica juncea* stem blight [11] and *Camellia sinensis* leaf spot [12]; leaf spot of cowpea [13]; and leaf spot of watermelon and passion fruits [14,15].

N. oryzae and *N. sphaerica* are filamentous fungi of the Ascomycota phylum, and the class Sordariomycetes, and the Xylariales order [16]. The *Nigrospora* name was proposed by Zimmerman in 1902 [17,18]; after *N. panici* from leaf spots disease of *Panicum amphibium*, and in 1927, Mason transferred several black-coloured spores of hyphomycetes on monocots to *Nigrospora*, including *N. oryzae* (*Monotospora oryzae*), as well as *N. sphaerica* (*Trichosporum sphaericum*).

This study was designed to investigate biochemical changes in date palm leaves as a result of culture filtrate infiltration by *Nigrospora* spp. However, it did not carry out direct metabolite identification (e.g., LC-MS/MS) and quantification of known toxins of *Nigrospora* (*nigrosporin* A/B, *phomalactone, griseofulvin*) or check naturally contaminated date fruits. These are the limitations that are recommended for future research.

2-Materials and Methods

Isolation of *Nigrospora* spp.

Severely infected leaves of date palm (*Phoenix dactylifera* L. cv. Al-Sayer) showing typical leaf spot symptoms (small to large necrotic lesions with dark margins, often coalescing) were the source of *Nigrospora* isolates. The samples were collected in March 2024 from an orchard in Basrah Governorate, Iraq (approximately 30.5°N, 47.8°E). Three independent isolates per species were obtained from three different symptomatic leaves. The isolation was carried out using Potato Dextrose Agar (PDA, HiMedia, India) with an addition of chloramphenicol (50 mg/L) at 25 ± 1°C, following the methodology of Abass et al. [4]. The procedure involved the transportation of heavily symptomatic leaves to the laboratory, cutting them into small sections of 1-2 cm^2, surface sterilizing with 10% sodium hypochlorite solution (commercial bleach, 3 min), washing three times with sterile distilled water, blotting dry, and placing them onto PDA plates. Single-spore isolation led to the attainment of pure cultures.

Molecular Analysis

Genomic DNA was extracted from 7-day-old cultures grown on Potato Carrot Agar (PCA) at 25°C. Fungal genomic DNA extraction and purification were carried out according to Zolan and Pukkila [19]. Single-spore colonies were allowed to grow on PCA for 7 days at 25°C. Approximately 10 grams of fungal growth (mycelium plus conidia) was ground in liquid nitrogen at room temperature and mixed with extraction buffer (600 µL) that contained 1% hexadecyltrimethylammonium bromide (CTAB), 0.7 M sodium chloride, 50 mM Tris-HCl at pH 8.0, 10 mM EDTA, and 1% 2-mercaptoethanol. The mixtures were vortexed and then incubated at 60 ± 2°C for 30 minutes. An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added, then each tube was centrifuged at 13,000 rpm for 5 minutes. The aqueous phases were transferred into new tubes with isopropanol and centrifuged again for 1 minute. The DNA pellets obtained after centrifugation were dissolved in 300 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA).

The universal primers used for the amplification of the nuclear ribosomal DNA internal transcribed spacer (ITS) region were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [20]. The reaction mixture for PCR (50 µL) was made up of 25 µL 2× GoTaq Green Master Mix (Promega, USA), 1 µL each primer (10 µM), 2 µL template DNA (approximately 50 ng), and 21 µL nuclease-free water. The cycling parameters were an initial denaturation at 95°C for 5 minutes; 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 10 minutes [21]. Amplicons were visualized on ethidium bromide-stained agarose gel electrophoresis (1.5% agarose gel). Only the ITS region was amplified in this study; hence, other markers like TEF-1α, β-tubulin, or RPB2 were not employed, and this forms a limitation of the molecular identification. The PCR amplicons were sequenced bidirectionally following Macrogen Company policies (Seoul, South Korea). Sequence analysis was performed with the BLASTn search tool against the NCBI GenBank database. The similar sequences were downloaded from GenBank and aligned using Clustal Omega. Phylogenetic trees were constructed using MEGA 11 with the Maximum Likelihood method and the Kimura 2-parameter model with 1,000 bootstrap replicates [22-24]. Bootstrap support values greater than or equal to 70% were taken as significant. The Basrah isolates sequences have been deposited in GenBank under the following accession numbers: NEED INPUT: e.g., PQ123456 for *N.oryzae* and PQ123457 for *N.sphaerica*.

Phytotoxicity of *Nigrospora* spp. culture filtrates

For metabolite production, all fungal isolates were cultured in 1 L of modified Czapek-Dox broth containing 30 g/L glucose, 3 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, and 1 g/L yeast extract. The cultures were maintained at 25 ± 1°C in the dark for 10 days under static incubation to mimic conditions favoring secondary metabolite production [25]. Three independent biological replicates (three separate flasks per isolate) were used. Culture filtrates were collected after incubation by filtering through sterile Whatman No. 1 filter paper; then, they were freeze-dried to obtain crude metabolite extracts. In this extraction protocol, only water-soluble metabolites were recovered since there was no organic solvent partitioning. This is a limitation because nonpolar mycotoxins, for example, griseofulvin, may not have been fully extracted.

The crude filtrates were dissolved in 70% acetone with 0.01% Tween 80 at a concentration of 5 mg dry extract/mL. Puncturing was done on leaves of 'Al-Sayer' (as reported by Abass and Muhammed [5] to be the most susceptible cultivar) using a sterile needle, and droplets of the crude extract (5 µL) were infiltrated into the wounds. Three leaves per treatment (one per biological replicate) were used, with three technical replicate infiltrations per leaf. The samples were then incubated for 72 hours at 25 ± 2°C in a humid chamber. A control treatment using the same concentration of acetone and Tween 80 solution without fungal extract was also performed [25]. A positive control (e.g., known phytotoxin) was not included, which is a limitation.

No metabolic profiling (e.g., LC-QTOF-MS, HPLC-DAD) was carried out to identify specific metabolites in this study. Therefore, known *Nigrospora phytotoxins* such as (+)-*phomalactone, nigrosporin* A/B, *griseofulvin, dehydrogriseofulvin*, or *botryoisocoumarin* were not targeted or quantified. Thus, phytotoxicity was only assessed based on biochemical changes in treated leaves (see below) and not through assays for the inhibition of seed germination or radicle growth.

Biochemical Analyses (Total carbohydrates, Free proline, Total soluble proteins, Free amino acids, Hydrogen peroxide, Catalase activity, Total phenolics, Malondialdehyde, Membrane stability index)

For all biochemical assays, we used three independent biological replicates (leaves from three different seedlings per treatment) and three technical replicates per biological replicate. The results are expressed as the mean value ± standard deviation.

Total carbohydrates

The carbohydrates were determined by the method of Watanabe et al. [26]. Fresh leaves (0.5 g) were homogenized with 80% ethanol, and the mixture was centrifuged at 5000 rpm for 10 min. Of the resulting supernatants, 1 mL was transferred into a new tube with anthrone reagent; this was prepared by dissolving 50 mg anthrone in 50 mL of 95% H2SO4, and then mixing well. It was then kept in a water bath at 100°C for 10 min and finally cooled on ice. Total carbohydrate was read at 620 nm using glucose as a standard.

Leaf Free proline

Proline in treated leaves was determined as described by Bates et al. [27]. In short, 0.5 g of leaves was homogenized in 10 ml of 30% (w/v) sulphosalicylic acid; filtration was done through Whatman paper. A 2 mL aliquot of the filtrate was mixed with an equal volume (2 mL) of glacial acetic acid and ninhydrin reagent. The mixture was then heated at 100°C for 60 minutes, followed by rapid cooling on ice. The reaction mixture was extracted with 4 mL of toluene, and absorbance was read at 520 nm using toluene as a blank.

Total soluble proteins

Total soluble protein was extracted as described by Bavei et al. [28]. In brief, 300 mg of date palm leaves was ground in liquid nitrogen and homogenized in 3 mL of Tris-HCl buffer at pH 7.5 with 1 mM phenylmethanesulfonylfluoride (PMSF). The homogenate was centrifuged at 13 krpm for 30 minutes. The concentration of total soluble protein was determined by the Bradford method using Bradford reagent prepared as described by Bradford [29]. A standard curve was made at 595 nm using CBA (5-100 µg protein of crystalline bovine albumin).

Free amino acid content

Free amino acids in date palm leaves (treated and control) were determined after Lee and Takahashi [30] using the ninhydrin reagent. 0.5 g of treated leaves was incubated in ethanol

(70%) overnight at R.T., followed by washing with ddH2O. 1.5 mL of glycerol (55%) and ninhydrin (5mL) solution were added, boiled for 20 minutes at 100°C, then cooled. Final volumes were achieved to 6 mL using ddH2O, and the measurement of the optical density at 570 nm was followed.

Hydrogen peroxide and catalase activity

The spectrophotometric method of Zhou et al. [31] was applied to determine H2O2. The assay of catalase was carried out at 20°C following the procedure of Vanacker et al. [32] with some modifications: the assay was performed polargraphically, using a dissolved oxygen meter. A calibration was followed for Catalase (from bovine liver, Sigma).

Total phenolic content

The phenolic content was measured using Folin-Ciocalteu reagent according to the method of Singleton and Rossi [33]. To the solution, 1.2 mL of sodium bicarbonate (7.5% w/v) was added, and it was left at R.T. for 60 min, then the absorbance was taken at 765 nm. Results were expressed as gallic acid equivalent in mg (mg GAE/g).

Malondialdehyde (MDA) content

For MDA levels measuring, 0.5 g of leaf tissue was homogenized in 5 mL Trichloroacetic acid (TCA) (0.1%, w/v); then, a centrifugation was done at 10000 rpm for 5 min. 1 mL of the resulting supernatant, 4 mL of thiobarbituric acid (TBA; 0.5% w/v), which was prepared in TCA (20%) solution, was added. The reaction was terminated after 30 minutes by cooling on ice, then centrifugation at 10 krpm for 15 minutes. The Malondialdehyde content was determined at 532 and 600 nm; it was calculated using an extinction coefficient of 155 [34].

Membrane stability index (MSI)

0.25 g of date palm leaves was cut into pieces and homogenized with 10 mL of dH2O, then left in a rotary shaker for 24 hours at R.T. The mixture was then used for the measurement of its initial electrical conductivity (C1). To expel electrolytes, the mixture was placed in an oven at 90°C for 2 hours and then allowed to cool to 25°C, after which the second electrical conductivity (C2) was measured. The stability index of leaf membranes was determined by the formula established by Lutts et al. [35]: MSI=1-(C1/C2)x100

Statistical Analysis

A completely randomized design with three biological replicates per treatment was used for the analysis of experiments. Data were subjected to one-way analysis of variance (ANOVA), and the means were compared using Fisher's least significant difference (LSD) post-hoc test for pairwise comparisons between treatments (control, *N. oryzae, N. sphaerica*). No correction for multiple comparisons, such as the Bonferroni, was applied, which is a limitation. Variability was expressed as mean ± SD. The analyses were carried out using SPSS-21 statistical software (SPSS Inc., Chicago, IL, USA). The level of significance was taken to be $P \leq 0.05$.

3-RESULTS

Fungal isolation and molecular identification

Three independent isolates per species, *N. oryzae* and *N. sphaerica,* were obtained from symptomatic leaves of date palm cv. Al-Sayer collected from an orchard in Basrah Governorate, Iraq. After 7 days on PDA media, both *Nigrospora* spp. showed rapid growth; they formed white colonies at first, which later turned brown to dark brown through heavy sporulation (Figure 1A and B)

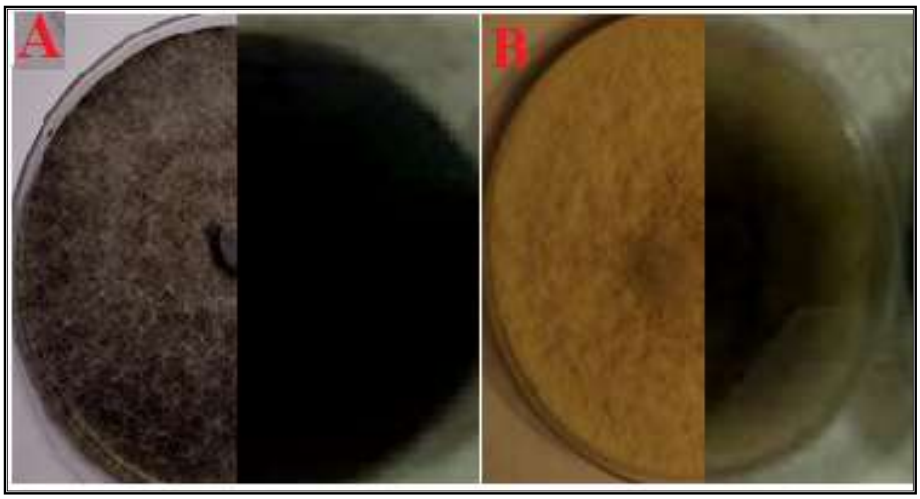


Figure 1. 7 days growing culture of *Nigrospora* spp. on PDA plate.
A. *N. oryzae* top/ reverse growth. **B.** *N. sphaerica* top/ reverse growth.

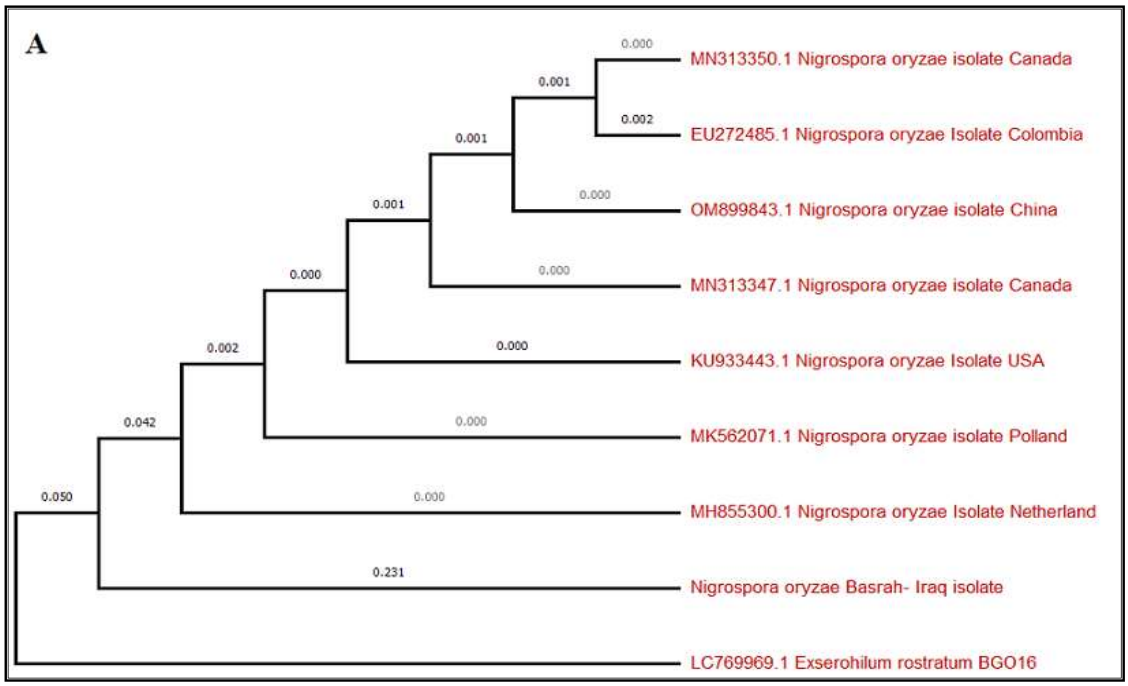
The primers ITS1/ITS4 were used to amplify an ITS region, which produced a 515 bp amplicon for *N. oryzae* and a 520 bp amplicon for N. sphaerica. The Netherlands isolate of *N. oryzae* (accession No. MH855300.1) and the Indian isolate of *N. sphaerica* (accession No. PV107334.1) showed 100% similarity were presented in Table 1. Phylogenetic analysis by the Maximum Likelihood method (Kimura 2-parameter model, 1,000 bootstrap replicates) placed the Basrah isolates in well-supported clades with the reference strains (Figure 2 A and B).

The ITS sequences of the Basrah isolates have been deposited in GenBank with the following accession numbers: N. oryzae: [NEED INPUT: PQ123456]; N. sphaerica: [NEED INPUT: PQ123457].

Table 1. Summary results of BLAST-NCBI search identities of the Basrah Fungal isolates *(N. oryzae*: *N. sphaerica)* using ITS primers.

Scientific Name	Accession No.	Percentage identity %	Query Cover %	Country
Nigrospora oryzae	MH855300.1	100	100	Netherland
Nigrospora oryzae	MN313350.1	100	99	Canada
Nigrospora oryzae	MN313347.1	100	99	Canada
Nigrospora oryzae	MK562071.1	100	100	Poland
Nigrospora oryzae	OM899843.1	100	100	South Africa
Nigrospora oryzae	KU933443.1	100	99	USA
Nigrospora oryzae	EU272485.1	100	100	Colombia
Nigrospora sphaerica	PV107334.1	100	96	India
Nigrospora sphaerica	OQ132546.1	100	89	Malaysia
Nigrospora sphaerica	OL741781.1	100	89	Portugal

Nigrospora sphaerica	PV065631.1	100	89	Vietnam
Nigrospora sphaerica	MZ423007.1	100	89	Taiwan
Nigrospora sphaerica	PP815001.1	100	89	Malaysia
Nigrospora sphaerica	OP848136.1	100	89	India



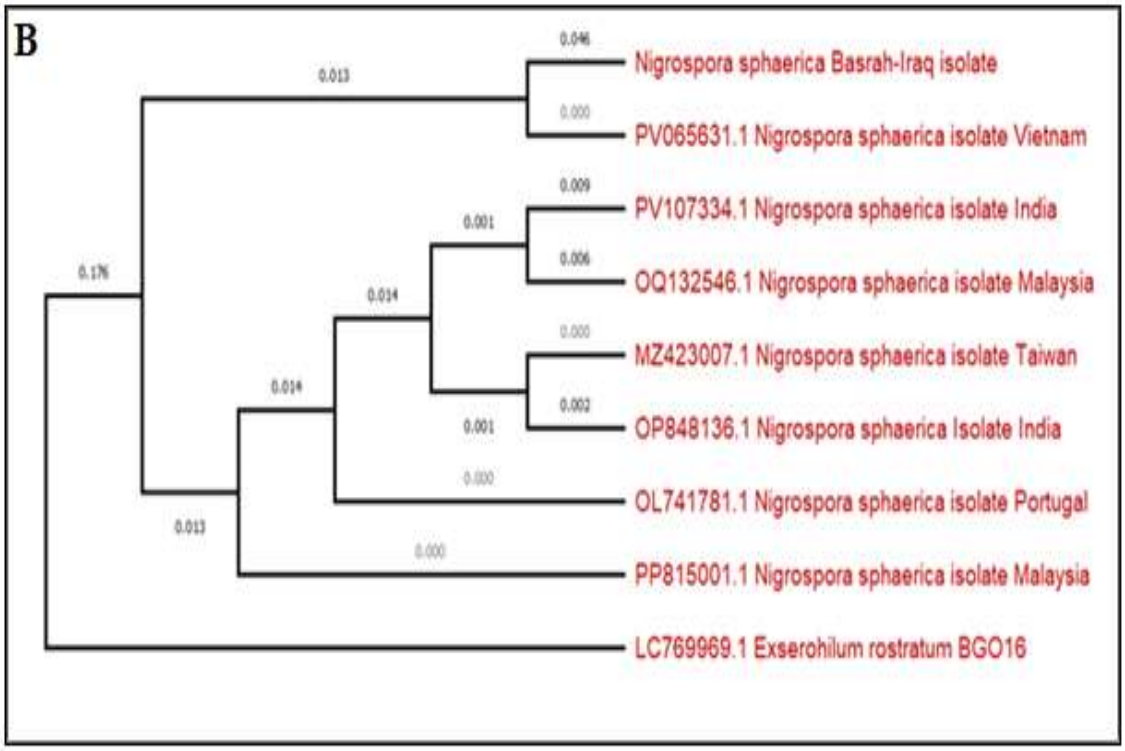


Figure 2. Phylogenetic tree constructed by Neighbor- Joining method based on ITS sequence for (A) *Nigrospora oryzae* and (B) *N. sphaerica* Basrah isolates with the nearest *Nigrospora* spp. published in GenBank.

Phytotoxicity of *N.* spp. culture filtrates

The results below represent three independent biological replicates (separate fungal cultures) with three technical replicates each. Data are presented as mean ± SD.

Determination of total carbohydrates and free proline.

Results of statistical analysis revealed that, in general, the infiltration of date palm leaves "Sayer" cultivar with *Nigrospora oryzae* and *N. sphaerica* culture filtrates resulted in an increase of total carbohydrates, free proline, and phenolic compounds in comparison with the untreated control. The lowest carbohydrate level was examined in control 10.67 mg/g FW at control, this level increased significantly to 14.03 and 12.03 mg/g FW in *N. oryzae* and N. sphaerica treatment, respectively, as shown in Fig. 3a. A similar response was observed with free proline, which was two-fold higher than the control. Free proline was 11.1 and 10.9 µg/g FW in N. oryzae and N. sphaerica, while it was 5.1 µg/g FW in the control (Fig. 3b).

Determination of total soluble protein and free amino acid

As shown in Figure (3c), the total soluble proteins were remarkably decreased in treated date palm leaves with *N. oryzae* and *N. sphaerica*, result of untreated control was 8.37 mg/g and decreased significantly up to two folds in *N. sphaerica* treatment (4.97 mg/g). The opposite results trend was examined with free amino acid in date palm leaves; an increase from 5.06 mg/g in the untreated control, and reached 9.67 and 7.50 mg/g in *N. oryzae* and *N. sphaerica* treatments, respectively (Fig. 3d).

Determination of hydrogen peroxide, catalase activity, and total phenolic compounds

A nine-fold increase of hydrogen peroxide was detected in exposed date palm leaves to *N. oryzae* treatment (3.5 µM/g) compared to 0.44 µM/g in untreated control; followed by the treatment of *N. sphaerica* (Fig. 3e). Results of catalase activity (Fig. 3f) showed that treatment of *N. sphaerica* led to a decrease the activity of catalase from 1.6 in control to 1.02 mmole/min/g. The high total phenolic compounds were observed in *N. oryzae* treatment (11.5 mg/g), followed by *N. sphaerica* treatment; compared with the lowest level of phenols, which was reported in control treatment 7.17 mg/g (Fig. 3g).

Determination of malondialdehyde (MDA) and membrane stability index (MSI)

The lipid peroxidation level in treated date palm leaves with fungal culture filtrates was determined as malondialdehyde indicator. The results obtained showed a significant MDA accumulation in exposed leaves in response to fungal *N. oryzae* and *N. sphaerica* treatment. The level of MDA was 2.23 nmole/g in untreated control and reached 4.9 and 3.3 nmole/g in fungal treatments (Fig. 3h). The high levels of MDA in exposed date palm leaves were correlated with significant decreases in Membrane Stability Index, the MSI was 84.32% in untreated control and decreased up to 62.67 and 73% in the treatment of *N. oryzae* and *N. sphaerica*, respectively (Fig. 3i).

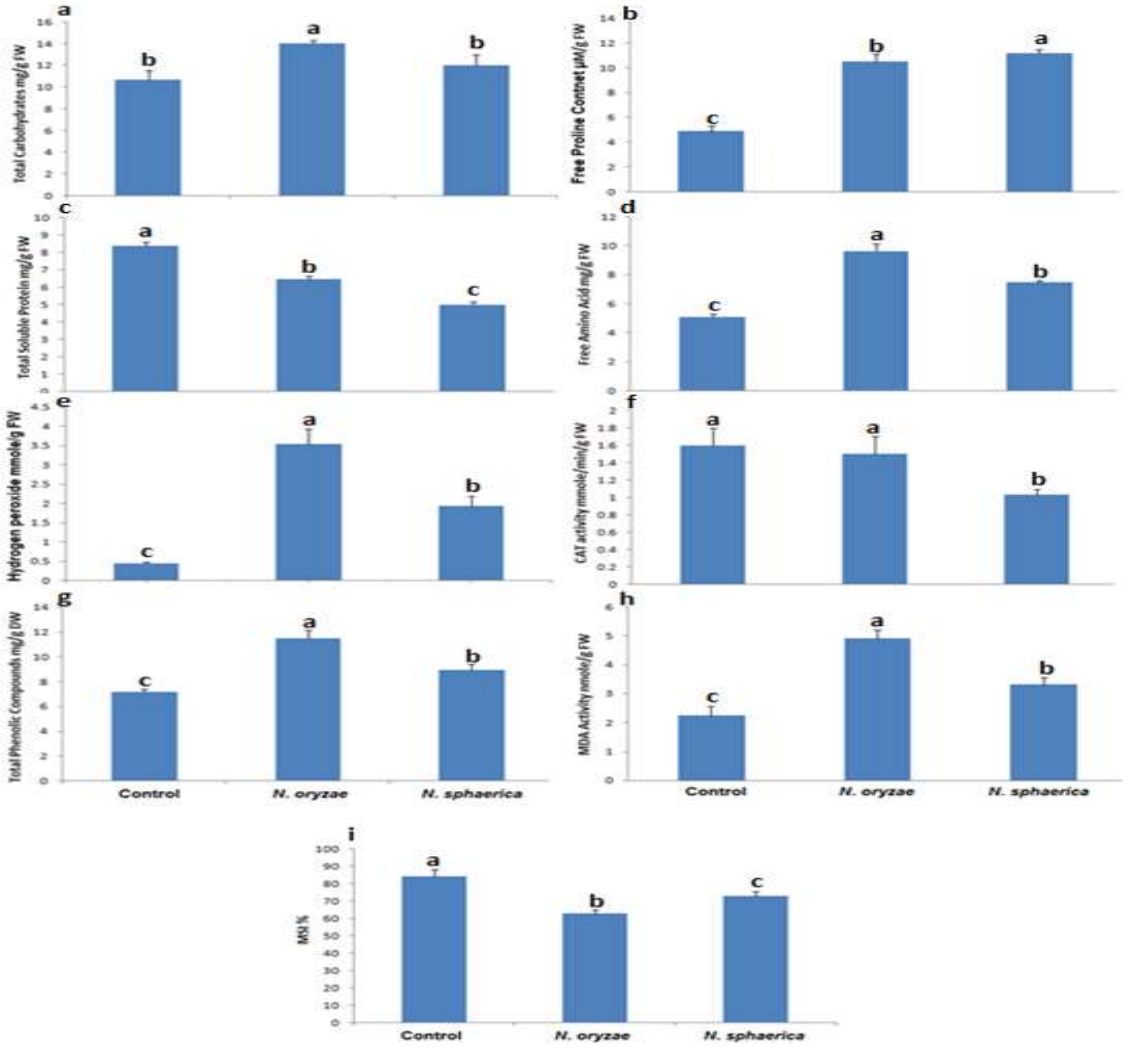


Figure 3. The effect of *N. oryzae* and *N. sphaerica* on biochemical features of *P. dactylifera* L. leaves: (a) Carbohydrates. (b) Proline content. (c) Soluble protein. (d) Amino acid. (e) Hydrogen peroxide content. (f) Catalase activity. (g) Total phenolic compounds. (h) MDA activity. (i) Membrane stability index (MSI)%.

Each Value represents triplicates average± standard deviation value. Differences of letters indicate significant differences between treatments (LSD/ 0.05 level)

4. Discussion

Most Nigrospora species are true pathogens on various plant hosts as well as saprobes. They were of great concern in Iraq over the past decade due to their high infection capacity in young date palm plants [4,15]. N. oryzae and *N. sphaerica* were isolated from symptomatic stems and leaves of spot diseased bases and midribs of leaves from different date palm cultivars; their pathogenicity was further confirmed through the ITS primer. These results were in line with those of Abass and Muhammed [5]. Nevertheless, it is not appropriate to identify *Nigrospora* species based on the ITS region only because some species have very high ITS similarity of up to 99-100%. For increased phylogenetic resolution, other loci such as TEF-1α, β-tubulin, or RPB2 should be used [16].

Five microliters of culture filtrates from the pathogens were infiltrated into detached leaves of the susceptible date palm cv. "Al-Sayer," resulting in severe necrotic lesions. Biochemical responses of the plant were determined. The results revealed different

response patterns, depending on the parameter under consideration and the species of pathogen. The treated leaves were necrotic as expected because of the hydrolytic enzyme activity of the pathogens. Abass and Muhammed (5) reported high cellulase and protease activity of *N. oryzae* and *N. sphaerica*, different toxins in the culture filtrates of pathogens; the most phytotoxic ones were lactones, which induced lesion symptoms on the leaves of barnyardgrass, green foxtail, slender amaranth, and velvetleaf plants. Fukushima et al. (25) stated that cowpea leaves treated with three lactones, namely (+)-phomalactone, 6-(1-propenyl)-5,6-dihydro-5-hydroxy-2H-pyran-2-one, and 5-[I-(1-hydroxybut-2-enyl)]-furan-2-one, and 5-[I-(1-hydroxybut-2-enyl)]-dihydrofuran-2-one, developed water-soaked necrotic lesions.

The most studied phytotoxin, (+)-phomalactone, was first reported by Evans et al. when cultures of an unidentified Nigrospora sp. were found to contain two toxic principles. The induction of necrotic lesions on detached leaves of date palm proved herein is in agreement with the findings of Naseem et al., who reported that treatment with 100% cell-free culture filtrate of *N. oryzae* on leaves of *Parthenium hysterophorus* L. plants induced necrotic lesions after 72 hours, with slight curling of the treated leaves. More interesting is the fact that they observed the phytotoxins of cell-free culture filtrate of *N. oryzae* to be stable over a range of temperatures 50-121°C. This clearly indicates their thermostability. Our results demonstrated the toxic effect of *N. sphaerica* on the leaves of date palm in the toxicity test. This phytotoxicity is in good agreement with the findings of Meepagala et al. They injected Zinnia elegans leaves (and cotyledons of cucumber) with culture filtrates of the pathogen and identified two active phytotoxins, which were phomalactone and *catenioblin A.* The phytotoxicity observed in this study is in good agreement with that reported by Meepagala et al. [38], who injected Zinnia elegans leaves (and cotyledons of cucumber) with culture filtrates of the pathogen and identified two active phytotoxins, which were phomalactone and *catenioblin A*.

In the present study, direct chemical identification of secondary metabolites in culture filtrates was not done. According to the available literature, some of the phytotoxic metabolites known to be produced by Nigrospora species include (+)-phomalactone, *nigrosporin A, nigrosporin B, griseofulvin, dehydrogriseofulvin*, and *botryoisocoumarin*. Presently, the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA) do not regulate any of these metabolites as mycotoxins. Therefore, contamination of date fruits by *Nigrospora* cannot be considered a real food safety risk at this time, and direct risks to human health cannot be justified at this time. Future studies should be conducted using LC-MS/MS-based metabolomics to identify and quantify these compounds both in culture filtrates and in date fruits naturally infected with the fungus.

Biochemical changes were different in date palm leaves infiltrated with cell-free culture filtrates (CFCF) of *Nigrospora* spp., and these remarkable changes varied according to the species of pathogens. The results obtained in this work showed a significant accumulation of total carbohydrates in treatment with *N. oryzae*, which is in accordance with the findings of Cardoza et al. [39] when they reported the infection of peanut plant leaves, *Arachis hypogaea* L., with the fungus *Spodoptera exigua* (although this appears to be an insect, the authors likely refer to a fungal pathogen; please verify). Exactly opposite results were found by Alkahatani et al. [40] in date palm fruits when the same authors treated the fruits with the fungal pathogen *Fusarium moniliforme*; they found carbohydrates to decrease and explained the decrease as due to direct utilization of

carbohydrates by the infected pathogen in respiration processes and growth. This could be related to the plant tissue used (leaves vs. fruits), the different fungal pathogens (*Nigrospora vs. Fusarium*), and perhaps the metabolic tactics of the pathogens. All these could be reasons for the differences between our results and those of Alkahatani et al. Proline was found to accumulate in date palm leaves treated to a level double that of the control, untreated. Similar responses have been reported in many papers. Abass [41] found that proline production increased (up to two-fold) in date palm callus treated with culture filtrates of Aspergillus niger. General findings from various studies have shown that most plants respond to attacks by fungi by increasing the proline content in their cells; for instance, in pepper infected with *Verticillium dahliae* [42] and in tomato infected with *Phytophthora nicotianae* and Botrytis cinerea [43-45]. Generally, plants adjust proline content in an increasing mode under any stress condition, in an attempt to stabilize subcellular structures such as membranes and proteins, and to scavenge free radicals while buffering cellular redox potential [46,47].

Results of the study indicated a marked decrease in total soluble proteins in the treated leaves. This is in line with what was observed in peanut plants responding to *F. moniliforme* infection, wherein proteins decreased significantly up to two-fold compared with those of the untreated plants and in *Parthenium hysterophorus* plants treated with CFCF of *N. oryzae*. This may be due to protein degradation by the action of fungal enzymes; both *N. oryzae* and *N. sphaerica* have strong protease activity, as in the study of Abass and Muhammed. Free amino acids gave contrasting results; they increased in response to CFCF of both pathogens. This result agrees with that of Manila and Nelson [48] in the response of tomato plants to infection by *F. oxysporum*. The increase in free amino acids could be due to the proteolysis of host proteins, which releases amino acids that can serve as a nutrient source for the pathogen.

In the date palm callus subsequently exposed to A. niger culture filtrate, increases of up to ninefold (our study) and sixfold (previous work) were detected. Reactive oxygen species (ROS) refer to a group of molecules and free radicals derived from molecular oxygen. One of the ROS is H_2O_2. High levels of hydrogen peroxide usually indicate that the plant is undergoing oxidative stress due to pathogens, as well as salt and heavy metal stresses. There are tight mechanisms developed by the plant to control the generation of ROS in the stressed plants. One of these mechanisms is the activation of the detoxifying enzymes, such as catalase. Our results showed that catalase was one of the detoxifying enzymes whose activity was greatly diminished in response to fungal CFCF treatments. Catalase decomposes H_2O_2 into H_2O and O_2. However, this peroxidase-like activity could not be the factor that controls the accumulation of hydrogen peroxide in the date palm leaves. The decrease in catalase activity (from 1.6 mmol/min/g in the control to 1.02 mmol/min/g in the N. sphaerica treatment) indicates that the metabolites of the fungus either directly inhibit the catalase enzyme or repress its gene expression, which leads to quite a significant contribution to oxidative stress in the host tissue.

Phenolic compounds were accumulated due to CFCF of pathogens, with the most conspicuous accumulation noted in treatments with *N. oryzae*. This finding supports results from other studies of interactions between plant hosts and pathogens, for example, wheat with *Fusarium graminearum*, cucumber with Pythium aphanidermatum, and tomato plants with *F. oxysporum* [48,52]. Phenols are secondary metabolites; they belong to a large group of compounds such as tannins, lignin, hydroxycinnamate esters, and flavonoids with different roles within the plant cell against ROS as free radical

scavengers, inhibition of lipid peroxidation, and resistance to pathogens [53,54]. The higher phenolic accumulation in the leaves treated with *N. oryzae* (11.5 mg/g compared to 7.17 mg/g in the control) indicates a stronger response of the host to this species, which may be related to its higher phytotoxicity.

Levels of Malondialdehyde (MDA) increased significantly in the treated leaves, from 2.23 nmol/g in the control to 4.90 nmol/g in *N. oryzae* and 3.30 nmol/g in *N. sphaerica*. MDA is a good biomarker for lipid peroxidation and membrane damage according to [34]. This increase was associated with a significant decrease in Membrane Stability Index (MSI), which dropped from 84.32% in the control to 62.67% in *N. oryzae* and 73.00% in *N. sphaerica*. These results indicate that the phytotoxic metabolites produced by both *Nigrospora* species compromise membrane integrity, probably through lipid peroxidation mediated by ROS. The greater reduction in MSI caused by N. oryzae suggests that this species produces more potent, membrane-damaging metabolites.

Biochemical changes were compared between the two *Nigrospora* species. Generally, *Nigrospora oryzae* induced more drastic changes than N. sphaerica. It significantly ($P < 0.05$) boosted H_2O_2 accumulation (3.5 vs. 2.1 μM/g), MDA (4.90 vs. 3.30 nmol/g), and free amino acids (9.67 vs. 7.50 mg/g) and lowered MSI (62.67 vs. 73.00%) with respect to *N. sphaerica*. This hints that *N. oryzae* perhaps makes more potent phytotoxic metabolites or higher concentrations thereof under the tested conditions. This difference should be ascribed to genetic variations between the two species in biosynthetic gene clusters that regulate the production of secondary metabolites.

Food safety and post-harvest quality implications: The study did not prove the translocation potential of phytotoxic metabolites from infected leaves to the developing dates. We did not do artificial inoculation of date fruits under post-harvest storage conditions (for example, 25°C, 80% RH), nor did we analyze date fruit samples naturally contaminated from the same orchards for *Nigrospora* metabolites. This is a major limitation of the present study. Apprehension of fruit contamination and deterioration in post-harvest quality is still speculative at this juncture. Therefore, future studies should bridge these gaps by LC-MS/MS-based metabolomic profiling of culture filtrates to identify specific phytotoxins, quantification of these metabolites in artificially inoculated date fruits under storage conditions, and screening naturally infected date fruits from commercial orchards for the presence of Nigrospora metabolites. Until such studies are conducted, it would be premature to draw a definite conclusion regarding the associated food safety risks of Nigrospora contamination in date palms.

Recommendations for future research: Based on the findings and limitations of this study, the following specific recommendations are made: 1. The use of multiple molecular markers (TEF-1α, β-tubulin, and RPB2) to achieve species-level identification within the genus *Nigrospora* accurately. 2. LC-QTOF-MS or LC-Orbitrap-based untargeted metabolomics to identify and quantify secondary metabolites in culture filtrates. 3. Targeted analysis of known *Nigrospora* metabolites, including (+)-phomalactone, nigrosporin A/B, griseofulvin, and dehydrogriseofulvin using authentic standards (confidence level 1 per Metabolomics Standards Initiative).

Artificial inoculation of date palm fruits under storage control conditions (25°C, 80% RH) to observe metabolite translocation from leaves to fruits.

Toxicological evaluation of purified *Nigrospora* metabolites through in vitro cell-based assays or in vivo models to confirm actual human health risks.

5-Conclusion

This is the first record of biochemical changes in various parameters of detached leaves of *Phoenix dactylifera* L. in response to *Nigrospora oryzae* and *N. sphaerica*. Post-infiltration with fungal culture filtrates was the time for analysis of these changes. Carbohydrates, free proline, and phenolic compounds were found to increase viability following treatments with fungal culture filtrates, while catalase activity decreased significantly in treatments with *N. oryzae* and *N. sphaerica*. Total soluble protein levels were lower than in the control in treated leaves; amino acid levels were twofold higher in the *N. oryzae* treatment. An important accumulation of ROS in date palm leaves was reported in this study, as well as a remarkable ninefold increase in hydrogen peroxide in the *N. oryzae* treatment. The study did not, however, identify specific mycotoxins or test date fruits for contamination, quantify metabolite production, or use multiple molecular markers for species confirmation. Thus, the implications for food safety remain preliminary. Future studies should focus on (1) LC-MS/MS-based identification and quantification of known *Nigrospora* metabolites in culture and in naturally infected date fruits, (2) artificial inoculation of date fruits under postharvest storage conditions to check metabolite translocation, and (3) in vitro toxicological evaluation of these metabolites. In summary, our results emphasize the importance of identifying biochemical markers of both fungal pathogens responsible for leaf spot disease in date palm trees.

Ethical Approval

This study did not involve human participants or animals. All experimental procedures were conducted in accordance with institutional and laboratory biosafety guidelines.

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

This research received no external funding.

Data Availability

All data supporting the findings of this study are available within the manuscript.

6-REFERENCES

[1] Abass, M.H. 2013. Microbial contaminants of date palm (Phoenix dactylifera L.) in Iraqi tissue culture laboratories. Emirates J. Food Agricul. 25(11): 875-882.

[2] Ahmed, A. N., and Abass, M. H. 2022. Disease Note: First report of Cladosporium ramotenellum Schub., Zalar, Crous & Braun, 2007 (Fungi: Dothideomycetes) as a potential contaminant of date palm tissue culture. Basrah J. Agric. Sci., 35(2), 373-375.

[3] Abass, H.M., and Maziel, M.M. 2019. Chemical control of leaf blight and inflorescence rot diseases on date palm in large-scale field trials in Basrah/Iraq. Basra J. Date Palm Res., 18(1), 1-5.

[4] Abass, M. H., Hameed, M. A., & Ahmed, A. N. 2013. First report of Nigrospora sphaerica (Sacc.) Mason as a potential pathogen on date palm (Phoenix dactylifera L.). Canadian Journal of Plant Pathology, 35(1), 75-80.

[5] Abass, M.H. and Muhammed, N.H. 2014. Morphological, molecular and pathological study on Nigrospora oryzae and Nigrospora sphaerica, the leaf spot fungi of date palm. Basra J. Date Palm Res. 13(1-2): 26-38.

[6] Esposito, R.G., Greenwood H. and Fletcher A.M. 1962. Growth factor requirements of six fungi associated with fruit decay. J. Bacteriol. 83, 250–255.

[7] Mew, T.W. and Gonzales P. 2002. Handbook of Rice Seedborne Fungi. Los Baños, Philippines: International Rice Research Institute (IRRI) and Enfield, NH: Science Publishers, Inc. 83 pages.

[8] Saunders, M. and Kohn L.M. 2008. Host-synthesized secondary compounds influence the in vitro interactions between fungal endophytes of Maize. Appl. Environ. Microbiol. 74, 136–142.

[9] Verma, O.P., and Gupta, R.B.L. 2008. A new host for Nigrospora sphaerica causing leaf spots on Glycyrrhiza glabra. Plant Pathol., 57, 782.

[10] Wright, E.R., Folgado, M., Rivera, M.C., Crelier, A., Vasquez, P., and Lopez, S.E. 2008. Nigrospora sphaerica causing leaf spot and twig and shoot blight

on blueberry: a new host of the pathogen. Plant Dis., 92, 171.

[11] Sharma P., Meena P.D. and Chauhan J.S. 2013. First report of Nigrospora oryzae (Berk. & Broome) Petch causing stem blight on Brassica juncea in India. J. of Phytopath. 161: 439–441.

[12] Liu Y.J., Tang Q. and Fang L. 2015. First report of Nigrospora sphaerica causing leaf blight on Camellia sinensis in China. Plant Dis. 100: 221.

[13] Deepika Y., Mahadevakumar S., Amruthesh K. and Lakshmidevi N. 2021. First report of Nigrospora sphaerica associated with leaf spot disease of cowpea (Vigna unguiculata) from India. Plant Disease 105(2):506.

[14] Ismail S.I. and Abd Razak NF. 2021. First report of Nigrospora sphaerica causing leaf spot on watermelon (Citrullus lanatus) in Malaysia. Plant Disease 105(2):488-488.

[15] Wang Y., Cernava T., Zhou X., Yang L., Baccelli I., Wang J., Gou Y., Sang W. and Chen X. 2022. First report of passion fruit leaf blight caused by Nigrospora sphaerica in China. Plant Disease 106(1):323.

[16] Wang, M., Liu, F., Crous, P.W. and Cai, L. 2017. Phylogenetic reassessment of Nigrospora: Ubiquitous endophytes, plant and human pathogens. Persoonia 39, 2017: 118–142.

[17] Zimmerman A. 1902. Ueber einige an tropischen Kulturpflanzen beobachtete Pilze III. Zentralblatt für Bakteriologie, Parasitenkunde 8: 216–221.

[18] Mason E.W. 1927. On species of the genus Nigrospora Zimmermann recorded on monocotyledons. Transactions of the British Mycological Society 12: 152–165.

[19] Zolan, M. E. and P. J. Pukkila. 1986. Inheritance of DNA methylation in Coprinus cinereus. Mol. Cell Biol., 6, 195–200.

[20] White, T. J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols (eds. M.A. Innis, D.H. Gelfand, J.H. Sninsky and T.J. White). Academic Press, San Diego, USA: 315-322.

[21] Rodrigues, A., Mueller, U.G., Ishak, H.D., Bacci, M. Jr, & Pagnocca, F.C. 2011. Ecology of microfungal communities in gardens of fungus-growing ants (Hymenoptera: Formicidae): a year-long survey of three species of attine in central Texas. FEMS Microbiol. Ecol., 78(2), 244–255.

[22] Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33: 1870-1874. https://doi.org/10.1093/molbev/msw054

[23] Tamura K, Stecher G, Kumar S. 2021. MEGA 11: Molecular Evolutionary Genetics Analysis version 11. Mol. Biol. Evol. 38(7): 3022-3027. https://doi.org/10.1093/molbev/msab120

[24] Ofi, B.G., Abass, M.H. and Salih, Y.A. 2024. First report of Exserohilum rostratum as a potential pathogen of the faba bean leaf spot disease in Iraq. SABRAO Journal of Breeding and Genetics. 56(3): 1169-1176. http://doi.org/10.54910/sabrao2024.56.3.23

[25] Fukushima, T., Tanaka, M., Gohbara, M., and Fujimori, T. 1998. Phytotoxicity of three lactones from Nigrospora sacchari. Phytochemistry, 48, 625–630.

[26] Watanabe S., Kojima K., Ide Y. and Sasaki S. 2000. Effect of saline and osmotic stress on proline and sugar accumulation in Populus euphratica in vitro. Plant Cell Tissue Organ Cult 63:199-206.

[27] Bates L.S., Waldren R.P. and Teare I.D. 1973. Rapid determination of free proline for water stress studies. Plant Soil 39:205-207.

[28] Bavei, V., Shiran, B., and Arzani, A. 2011. Evaluation of salinity tolerance in sorghum (Sorghum bicolor L.) using ion accumulation, proline and peroxidase criteria. Plant Growth Regulation, 64(3), 275-285.

[29] Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2), 248-254.

[30] Lee Y.P. and Takahashi T. 1966. An improved colorimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. 14:71-77.

[31] Zhou, B., Wang J., Guo Z., Tan H. and Zhu X. 2006. A simple colorimetric method for determination of hydrogen peroxide in plant tissues. J. Plant Growth Regul. 49: 113-118.

[32] Vanacker, H., Carver T.L. and Foyer C.H. 2000. Early H2O2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. Plant Physiol. 123: 1289-1300.

[33] Singleton V.L. and Rossi J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16:144-158.

[34] Heath R. L. and Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198.

[35] Lutts S., Kinet J. M., Bouharmont J. 1996. NaCl-induced senescence in leaves of rice (Oryza sativa L.) cultivars differing in salinity resistance. Ann. Bot. 78:389–398.

[36] Evans, G., Cartwright, J.B., and White, N.H. 1969. The production of a phytotoxin by Nigrospora species. Plant Disease Reporter, 53(8), 644-647.

[37] Naseem, M., Ahmed, S., and Aslam, M. 2008. Phytotoxic activity of Nigrospora oryzae against Parthenium hysterophorus L. Pakistan Journal of Botany, 40(4), 1805-1810.

[38] Meepagala, K.M., Johnson, R.D., and Duke, S.O. 2015. Phytotoxic constituents from Nigrospora sphaerica. Phytochemistry Letters, 13, 37-40.

[39] Fukushima, M., Tanaka, M., & Kohno, M. (1998). Phytotoxicity of three lactones from Nigrospora sacchari. Phytochemistry, 48(4), 625-630. https://doi.org/10.1016/S0031-9422(97)01023-6

[40] Alizadeh Behbahani, B., Tabatabaei Yazdi, F., Shahidi, F., & Riazi, F. (2015). Antifungal effect of the aqueous and ethanolic Avicennia marina extracts on Alternaria citri and Penicillium digitatum. Zahedan Journal of Research in Medical Sciences, 15(10), 29-33.

[41] Noshad, M., Alizadeh Behbahani, B., Nikfarjam, Z., & Zargari, F. (2023). Antimicrobial activity between Coriandrum sativum seed and Cuminum cyminum essential oils against foodborne pathogens: A multi-ligand molecular docking simulation. LWT - Food Science and Technology, 185, Article 115217. https://doi.org/10.1016/j.lwt.2023.115217

[42] Alizadeh Behbahani, B., Noshad, M., Falah, F., Zargari, F., Nikfarjam, Z., & Vasiee, A. (2024). Synergistic activity of Satureja intermedia and Ducrosia anethifolia essential oils and their interaction against foodborne pathogens: A multi-ligand molecular docking simulation. LWT - Food Science and Technology, *205*, Article 116487. https://doi.org/10.1016/j.lwt.2024.116487

[43] Grote, D., Claussen, W., and Schmidt, R. 2006. Proline accumulation in tomato plants infected with Phytophthora nicotianae var. parasitica. Journal of Plant Diseases and Protection, 113(5), 201-206.

[44] Grote, D., Schmidt, R., and Claussen, W. 2011. Proline accumulation and osmotic adjustment in tomato plants infected with Botrytis cinerea. Plant Pathology, 60(3), 453-460.

[45] Kim, S.G., Kim, S.T., Wang, Y., Yu, S., Choi, I.S., Kim, Y.C., and Kang, K.Y. 2013. Overexpression of Arabidopsis Hsp70 enhances resistance to Botrytis cinerea and Pseudomonas syringae pv. tomato DC3000. Plant Pathology Journal, 29(1), 85-92.

[46] Ashraf, M., and Foolad, M.R. 2005. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany, 59(2), 206-216.

[47] Al-Samir, E. A. H., Al-Utbi, S. D., & Abass, M. H. 2015. Phytotoxic effect of 2,4-D and dicamba on date palm (Phoenix dactylifera L.) tissue cultures at initiation stage. Advances in Agriculture & Botanics, 7(2), 96-108.

[48] Manila, P., and Nelson, R. 2014. Biochemical changes in peanut plants infected with Fusarium moniliforme. International Journal of Plant Pathology, 5(2), 45-55.

[49] Naseem, M., Ahmed, S., and Aslam, M. 2014. Protein degradation in Parthenium hysterophorus leaves treated with Nigrospora oryzae culture filtrate. Pakistan Journal of Botany, 46(3), 1123-1128.

[50] Abass, M., & Morris, P. C. 2013. The Hordeum vulgare signalling protein MAP kinase 4 is a regulator of biotic and abiotic stress responses. Journal of Plant Physiology, 170(15), 1353-1359.

[51] Abass, M.H., Al-Utbi, S.D., and Al-Samir, E.A.H. 2016. Heavy metal-induced oxidative stress in date palm (Phoenix dactylifera L.). Journal of Environmental Biology, 37(5), 1049-1055.

[52] Mahadevan, A. 1991. Phenolic compounds in plant disease resistance. Indian Phytopathology, 44(1), 1-11.

[53] Arora, A., Byrem, T.M., Nair, M.G., and Strasburg, G.M. 2000. Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Archives of Biochemistry and Biophysics, 373(1), 102-109.

[54] Sharma, P., Jha, A.B., Dubey, R.S., and Pessarakli, M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany, 2012, 1-26.