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Mohammed H. Abass [a] , Muhammed A. Hameed [a] & Alaa Naser Ahmed [a]
[a] Date Palm Research Centre, Basra University, Basra, Iraq
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Disease report/Rapport des maladies

First report of *Nigrospora sphaerica* (Sacc.) Mason as a potential pathogen on date palm (*Phoenix dactylifera* L.)

MOHAMMED H. ABASS, MUHAMMED A. HAMEED AND ALAA NASER AHMED

Date Palm Research Centre, Basra University, Basra, Iraq

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Abstract: *Nigrospora sphaerica* was isolated from date palm leaves which showed severe symptoms of leaf and stem spot. Pathogenicity tests revealed that several isolates of this fungus reproduced symptoms on the cultivar 'Al-Sayer'. The susceptibility of five different cultivars of date palm showed that 'Halawii' and 'Al-Sayer' were the most susceptible to infection compared with other cultivars such as 'Barhee', 'Zahdi' and 'Khadrawii'. Morphological and molecular characteristics confirmed the identification of *N. sphaerica*. The phytotoxicity of the culture filtrates were investigated, and shown to induce severe symptoms on date palm leaves.

Keywords: date palm, *Nigrospora sphaerica*, pathogenicity

Résumé: *Nigrospora sphaerica* a été isolé à partir de feuilles de dattier qui affichaient de graves symptômes de la tache des feuilles et des tiges. Des tests de pathogénicité ont montré que plusieurs isolats de ce champignon reproduisaient les symptômes sur le cultivar 'Al-Sayer'. La susceptibilité de cinq cultivars différents de dattier a montré que 'Halawii' et 'Al-Sayer' étaient les plus réceptifs à l'égard de l'infection, comparativement à d'autres cultivars comme 'Barhee', 'Zahdi' et 'Khadrawii'. Les caractères morphologiques et moléculaires ont confirmé l'identification de *N. sphaerica*. La phytotoxicité du filtrat des cultures a été étudiée et elle nous a permis de démontrer qu'elle reproduisait de graves symptômes sur les feuilles de dattier.

Mots clés : dattier, *Nigrospora sphaerica*, pathogénicité

Introduction

The date palm (*Phoenix dactylifera* L.) is one of the most important cultivated palm trees worldwide. The economical importance is attributed to several reasons, especially the nutritional value of their fruit, including the presence of amino acids, vitamins, carbohydrates and minerals (Bokhary, 2010). Different bacterial, fungal, nematode and other pathogens of date palm have been well studied. The fungal diseases of date palm in particular cause serious problems, including reduced growth and development of the trees. These pathogens include *Fusarium oxysporum* Schlecht, the causal agent of Bayoud (El-Hassani *et al.*, 2007), *Phytophthora* sp., the causal agent of Belaat (Howard & Carpenter, 1993); *Ceratocystis paradoxa* (Dale) Moreau, the causal agent of bending head (Elliot *et al.*, 2004), and species of *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium*, the causal agents of fruit rot (Food and Agriculture Organization, 2004). As well, species of *Alternaria*, *Graphiola*, *Pestalotia*, *Microsphaerella* and *Phoma* are the causal agents of leaf spots (Sheir *et al.*, 1982; Abass *et al.*, 2006).

The genus *Nigrospora* has been well studied as a plant endophytic pathogen (Fukushima *et al.*, 1998). The species *N. oryzae* (Berk & Broome) Petch has been reported as a pathogen of rice leaves and causes grain spot disease (Mew & Gonzales, 2002) and this fungus was isolated from the roots of maize plants

Correspondence to: M. H. Abass. E-mail: mohammedha1974@yahoo.com

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(Saunders & Kohn, 2008), while the species *N. sphaerica* (Sacc.) Mason has been reported as a fruit decay pathogen of banana (Esposito *et al.*, 1962). In addition, the species *N. sphaerica* is widely known as a leaf spot pathogen on several hosts, including liquorice medical plant (*Glycyrrhiza glabra*), on which severe leaf spot symptoms were induced by artificial inoculation with this pathogen (Verma & Gupta, 2008). In blueberry plants (*Vaccinium corymbosum* L.), it was found that *N. sphaerica* was able to produce leaf spot, twig and shoot blight symptoms (Wright *et al.*, 2008).

There is no previous work regarding *N. sphaerica* as a causal agent of the black spot disease of date palm. Therefore, the present study was aimed at isolation, pathogenicity and molecular identification of several isolates of *N. sphaerica*.

Materials and methods

Isolation of N. sphaerica

Several infected leaves of three cultivars of date palm, namely 'al-Sayer', 'Hillawi' and 'Breem', which showed evident symptoms of leaf spot, were collected from different areas in Basra province and brought to the laboratory. The isolation procedure from infected leaves was done according to Keith *et al.* (2006). The symptomatic leaf tissues were sectioned into small pieces, approximately 1–2 cm^2, and surface-sterilized by dipping in a 10% solution of sodium hypochlorite (as 1% commercial Chlorox) for 5 min and rinsed several times in distilled water, and placed on the surface of potato dextrose agar supplemented with 150 mg L^{-1} chloramphenicol and kept at 25 °C for several days. The isolates of the fungus were kept as single spore transfers according to Vitale & Polizzi (2005).

Morphological characterization and identification of N. sphaerica

The morphological examination of hyphae and conidia was performed on colonies of the isolates grown previously on PDA at 25 °C for 7 days. The morphological characterization was done according to Matsushima (1975). Observations of each isolate were done using a Zeiss Axiolab compound light microscope by using a bright-light optic. Micrometric data in the light microscope is based on measurement of 50 individual spores, hyphae and conidiogenous cells.

Molecular analysis

DNA extraction. A single-spore culture was grown on PCA (Potato Carrot Agar) medium at 25 °C for 4 days. The mycelium and conidia were transferred into a mortar and pestle and ground with liquid nitrogen at room temperature, and transferred into a 1.5 mL Eppendorf tube containing 600 μL extraction buffer [1% hexadecyltrimethylammonium bromide, 0.7 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol], vortexed and incubated at 60 °C for 30 min. An equal volume of chloroform-isomyl alcohol (24:1, v/v) was added and the mixture was spun in a microcentrifuge for 5 min. The aqueous phase was transferred into a fresh tube containing isopropanol and another centrifugation was done for 1 min. The pellets were dissolved in 300 μL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] and RNase treatment and ethanol precipitation were done as described (Zolan & Pukkila, 1986).

Amplification of N. sphaerica *DNA by polymerase chain reaction (PCR)*

A standard 50 μL volume was used for all PCR reactions in a 0.2 mL polypropylene tube and contained 4 ng of DNA template, 5 μL of 10× polymerase buffer, 8 μL of dNTPs (1.25 mM), 1 μL of *Taq* polymerase (Promega) and 1 μl of each primer. The thermal cycler used in this experiment was from MJ Research, PTC-100. The PCR conditions were: 95 °C for 1 min, 55 °C for 1 min, 72 °C for 10 min. The primer sequences were: ITS1: 5': TCCGTAGGTGAACCTGCGG-3', which hybridizes at the end of 18S rDNA and ITS4: 5': TCCTCCGCTTA TTGATATGC-3', which hybridizes at the beginning of 28s rDNA (Rodrigues *et al.*, 2011). The resulting products were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide. Purification of PCR products were done according to the kit instructions (QIAGEN, UK). The PCR product was sequenced and analysed using the Blast tool of NCBI (http://www.ncbi.nlm.nih.gov).

Pathogenicity test

'Al-Sayer' cultivar of date palm was used in this experiment. Four pieces (approximately 1.5 cm in length) of 'Al-Sayer' leaves were surface-sterilized and rinsed in distilled water as mentioned previously. A 0.5 cm diameter wound was made by a sterilized cork borer, and a 0.5 cm mycelial plug from a *N. sphaerica* colony (on PDA) was placed in the wound and sealed with parafilm to maintain a good level of humidity. A 0.5 cm of PDA plug was used as a control treatment. The inoculated leaves were placed in flasks containing 20 mL water and kept at 25 °C for 30 days. The development of symptoms was monitored and the diameters of resulting necrotic lesions

around the wound were measured acceding to Bachillor & Ilage (1998). The reisolation of the pathogen from the inoculated leaves was done on PDA plates as mentioned above to fulfil Koch's postulates. The pathogenicity test was repeated twice to confirm the results, as well as on living date palm trees of 'Al-Sayer' located at Basra University, Basra-Iraq. The procedure of Wright *et al.* (2008) was followed. In brief, a conidial suspension of 10^8 mL^{-1} of sterile distilled water was sprayed onto the foliage of seven non-wounded trees; an additional seven non-wounded trees were sprayed with sterile water to serve as control treatments. All of the treated leaves were sealed with plastic bags for 24 h post-inoculation to maintain a good level of humidity. Disease symptoms were recorded and reisolation from infected tissues was done to satisfy Koch's postulates.

Susceptibility of several date palm varieties to the fungus N. sphaerica

An isolate of *N. sphaerica* was chosen for further studies because of its severe effect on 'Al-Sayer'. The methodology above was followed to determine the level of susceptibility of 'Al-Sayer' compared with 'Hillawi', 'Zahdi', 'Barhee' and 'Khadrawi' to artificial inoculation by *N. sphaerica*. A total of five leaf pieces were used and the experiment was conducted four times. Lesion diameters were measured after 30 days post-inoculation and averaged.

The phytotoxicity of N. sphaerica *culture filtrate*

The culture filtrate was prepared by growing an isolate of *N. sphaerica* in a 2 L Erlenmeyer flask containing 30 g glucose, 3 g $NaNO_3$, 1g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 1 g yeast extract and water up to 1 L. The cultures were kept at 25 °C on a rotary shaker (180 rpm for 10 days) and the culture filtrate was then collected and freeze-dried. The procedure of Fukushima *et al.* (1998) was followed with some modification. Briefly, the crude filtrate was dissolved in 70% acetone and 0.1% Tween 80. Puncture wounds were performed on 'Al-Sayer' leaves, and a 5 μL droplet of the crude extract were placed on each wound, the leaves were kept at 25 °C for 7 days and the diameters of necrotic lesion were measured. A control treatment was done by application of a solution of 70% of acetone and 0.1% of Tween 80 only.

Statistical analysis

The results of the susceptibility and phytotoxicity tests were statistically analysed by one-way analysis of variance (ANOVA) with triplicates for each treatment. All statistical analyses were performed with Minitab version 15 statistical software. Differences were considered significant when $P < 0.05$. Tukey's test was applied when one-way ANOVA revealed significant differences.

Results

Fungal isolation and morphological characteristics

A total of 20 morphologically similar isolates of *Nigrospora* sp. were isolated from diseased date palm leaves with severe symptoms of black spot disease from different varieties of date palm ('Sayer', 'Hillawi' and 'Breem'). All of these isolates were selected and grown on PDA plates at 25 °C. Some other fungi were seen on the PDA plates used for isolation from the infected leaves and stems, including unidentified species of *Aspergillus*, *Epicoccum*, *Penicillium* and *Ulocladium.*

The colony characteristics of *Nigrospora* were as follows: colonies expanding on PDA plates rapidly at 25 °C, at first white then changing to brown to black due to the abundance of sporulation (Fig. 1*c*). The microscopic features included: conidiogenous cells on superficial hyphae, lateral or terminal, swollen, ampulliform, 8–11 μm in diameter, hyaline, with a single conidium at the attenuated apex. Conidia were spherical or oblate, solitary, black,

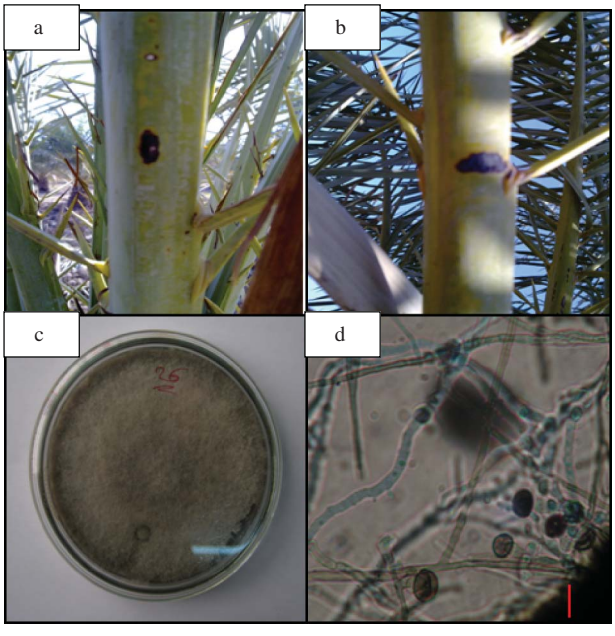

Fig. 1 **a**, Stem spot disease symptoms on date palm 'Sayer'. **b**, Stem spot disease symptoms on 'Breem' variety. **c**, Pure culture of *N. sphaerica* on PDA plate. **d**, Microscopic features of *N. sphaerica* grown in pure culture on PDA. Bar: 20 μm.

opaque, smooth-walled with an equatorial furrow with 17–20 μm in diameter (Fig. 1*d*). A total of 50 spores were used for examination.

PCR amplification and sequence analysis

The results of PCR amplification of the ITS region showed the expected size of PCR fragment which was ∼500 bp. Fifteen isolates of *Nigrospora* showed the expected size, while the other five isolates did not show any PCR products with ITS primers (Fig. 2). The results of sequence datasets for both ITS 1 and 4 (∼ 500 bp) showed a 99% identity to the fungus *Nigrospora sphaerica* (GenBank accession number: HQ608063.1). The sequence data analysis and the morphological observations strongly confirmed the identity of this fungus as *N. sphaerica*.

Pathogenicity tests

The results of pathogenicity tests (by using mycelium plug inoculation on detached date palm leaves) showed the ability of several isolates of *N. sphaerica* to induce mild to severe disease symptoms (necrosis) on inoculated leaves of 'Al-Sayer'. The most severe symptoms were apparent as a spot with a diameter of 2.1 cm after 30 days of incubation. The pathogenicity test on living date palm trees using conidial suspensions of 10^8 conidia mL^{-1} proved the ability of this fungus to induce the symptoms of leaf spot after 30 days, with an average of 1.8 cm diameter of induced spots. The symptoms of disease were obvious as oval to spherical shaped spots and definite grey to black coloured centre (Fig. 1*a*, *b*). No evident symptom was seen in non-inoculated trees which remained symptomless. The pathogen (*N. sphaerica*) was re-isolated from diseased tissues fulfilling Koch's postulates. The susceptibility test of several date palm varieties showed that 'Al-Sayer' was the most sensitive to *N. sphaerica* inoculation, with lesion diameter of 3.4 cm, followed by 'Hillawi' and 'Zahdi', with lesion diameters of 1.7 and 1.8 cm, respectively, while the lowest level of susceptibility was seen in 'Barhee', which was 1.2 cm.

The phytotoxicity of N. sphaerica *culture filtrate*

A crude extract from the most virulent isolate of *N. sphaerica* was used for the detection of phytotoxicity on the sensitive variety of date palm 'Al-Sayer'. This experiment was carried out by a special leaf puncture assay according to Fukushima *et al*. (1998). The results showed that the first necrosis symptom appeared after 72 h of treatment (5 μl of the crude extract); the symptoms were slight necrosis at the edges of the wounds.

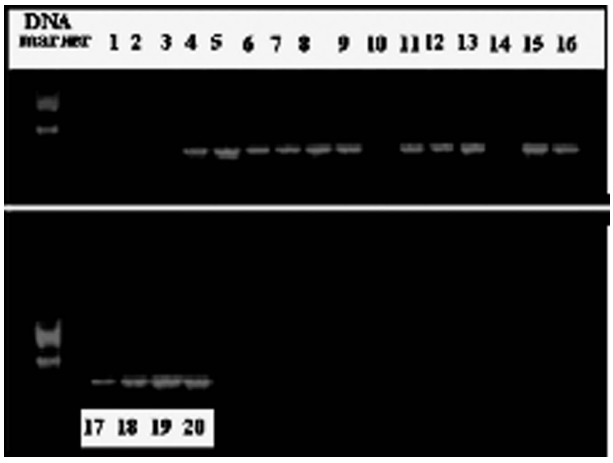

Fig. 2 PCR products of 20 isolates of *Nigrospora* sp. DNA with ITS primers. Fifteen isolates showed a 500 bp positive fragment; lanes 1, 2, 3, 10 and 14 = negative isolates.

Fig. 3 Phytotoxicity of *N. sphaerica* culture filtrate on 'Sayer' leaves. **a**, control treatment; **b**, treated leaf.

The diameters of necrotic lesions extended rapidly at day 5 after inoculation and reached ∼1.25–1.5 cm, while at day 7 after inoculation, more significant phytotoxic effect was evident on the leaf of 'Al-Sayer', with necrosis diameter of 2.2–2.5 cm (Fig. 3).

Discussion

In the present study, a new potential pathogen of date palm, *N. sphaerica*, has been described originating from date palm with leaf spots in Iraq. Several heavily infected date palm trees were observed with severe symptoms of

leaf and stem spot, especially in the orchards nearest to the bank of the Shaat-Alarab river in Basra province, and this can be explained by the high level of humidity which is required for infection. The identification of this new pathogen was based on morphological characteristics and DNA sequence data by using internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). The sequenced data and the Blast search showed the identity (99%) of 15 isolates of the fungus to be *N. sphaerica* (Gen Bank accession No. HQ608063.1) – the highest nucleotide similarities were considered as the closest relative of the query sequence. While the other five isolates did not show any PCR products (see Fig. 2) with ITS primers, their morphological characteristics seem to be similar to the species *N. oryzae*. The most important difference between these two species is the size of the spore which is larger in *N. sphaerica* (about 17–20 μm) compared with *N. oryzae* (14–15 μm).

To fulfil Koch's postulates, 'Al-Sayer' date palm was used, and the results of pathogenicity experiments confirmed that the fungus *N. sphaerica* is the causal agent of date palm leaf spot disease. Inoculation experiment resulted in induction of mild to severe disease symptoms; most severe symptoms were evident after 30 days of incubation. The reisolation of the pathogen from the infected tissues was done on PDA plates, and the microscopic examination of the fungus proved that the fungus *N. sphaerica* is the true pathogen of date palm leaf spot. Inoculated leaves showed symptoms similar to those observed in date palm orchards naturally. To the best of our knowledge, this is the first record of *N. sphaerica* as a date palm pathogen in Iraq.

The results from this work are in good agreement with the results of many studies showing severe symptoms of leaf spots on different hosts following artificial inoculation with *N. sphaerica* (Soylu *et al.*, 2011; Zhang *et al.*, 2011). *Nigrospora sphaerica* is the second species to be involved in leaf spot disease of date palm; the first species is *N. oryzae* (Abass *et al.*, 2006). *Nigrospora sphaerica* is also known as a leaf spot pathogen on several hosts worldwide, such as leaf blight pathogen on Cucuma (*Cucuma wenyujin*) in China (Zheng *et al.*, 2011) and leaf spot on Chinese wisteria in Turkey (*Wisteria sinensis*).

The direct phytotoxic effect of *N. sphaerica* metabolites was examined on 'Al-Sayer' leaves by using a leaf-puncture assay; the dose of 5 μL of the crude extract was used and the results revealed the significant effect on date palm leaves at 7 days post-inoculation. A 2.5-cm diameter necrotic lesion was seen on inoculated leaves. This phytotoxic effect of *N. sphaerica* culture filtrate can be attributed to the presence of lactones; the purified phomalactone was able to induce water-soaked lesion on leaves 24 h after application at 1000 ppm (Fukushima *et al.*, 1998). The relationship of this pathogen with other date palm leaf spot pathogens and their control requires further investigation.

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