

Morphological, molecular and pathological study on *Nigrospora oryzae* and *Nigrospora sphaerica*, the leaf spot fungi of date palm

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Abstract:

Date palm diseases are rising as an important concern during the last decades. The fungal pathogens of date palm are considered as the most serious problem causing significant reductions of growth, development, and production of date palm. The present study was aimed at the characterization of two *Nigrospora* species, isolated from different cultivars of date palm, based on morphological, molecular and pathological characteristics. In the current study, the identity of both *Nigrospora* species have been revealed to be as *N. oryzae* and *N. sphaerica* on the basis of their morphological characteristics and molecular analysis of the Internal Transcribed Spacer (ITS) region. Results showed that both pathogens were found to be true pathogens on different date palm cultivars. Compared to *N. Sphaerica*, *N. oryzae* was more aggressive on the following cultivars: Al-Sayer, Hillawi, Zahdi, Leloy and Kantar. After 30 days post-inoculation, the overall average lesion diameter was 1.85 cm in response to the artificial infection with *N. oryzae*, whereas infection with *N. sphaerica* produced 1.42 cm lesions. Al-Sayer cv. was the most susceptible, among the tested cultivars to both *Nigrospora* species; the lesion diameter was 2.50 cm, in contrast with cv. Leloy, 1.10 cm, which showed the lowest level of susceptibility. The extracellular enzymatic activity of both pathogens revealed that *N. oryzae* surpassed *N. sphaerica* in the production of cellulase and protease enzymes; whereas, lipase enzyme activity was absent in both fungi. The high enzymatic activity and virulence of *N. oryzae* on different date palm cultivars were approved in contrast with the species of *N. sphaerica*.

Keywords: Date palm, Enzyme activity, Leaf spot disease, *Nigrospora oryzae*, *Nigrospora sphaerica*, morphological, molecular and pathological characteristics.

Introduction

Date palms (*Phoenix dactylifera* L.) are monocotyledon, dioecious plants, and one of the most cultivated palms around the world (Abass, 2013a). Date palm trees are cultivated in different regions worldwide, especially in Middle East, North Africa, Central and North America, Southern Europe, Pakistan and India (Zaid, 2002, Alshahib and Marshall, 2003). World production of date is estimated to exceed 7.5 million tons in 2009; the Arabian Peninsula contributes over one third of world total dates production (FAO, 2011). Date palm is considered as one of the most ancient cultivated palm trees in the world providing fruit (dates) as a food source for thousands of years (Sulieman *et al.*, 2012).

Fungal pathogens are considered as one of the most serious pathogens causing a significant reduction in date palm growth, development and production (El-Hassani *et al.*, 2007, Abass *et al.*, 2013).

In Iraq, date palm cultivation encounters several constraints among which the wide spread of fungal diseases presenting a serious threat for growth and development of date palm (Abass *et al.*, 2007). Several important fungal pathogens have been isolated and identified as a causal agent of damaging diseases, including leaf spot disease (*Alternaria*, *Graphiola*, *Pestalotia*, *Microsphaerella* and *Phoma*), inflorescence rot (*Mauginiella scattae*), neck bending (*Ceratocystis paradoxa*), root rot and fruit rot (*Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium*) (Al-Juboory, 2005, Abass *et al.*, 2007, Al-Sheikh, 2009). Most of these diseases have been concentrated in the date palm orchards nearest to the river banks, such as Shaat-Al-Arab River in Basra province where the high level of humidity could contribute to the spread of these fungal infections (Abass *et al.*, 2013).

Different species of the genus *Nigrospora* have been isolated and identified as a true endophytic pathogen on numerous plants. For examples, the species of *N. oryzae* (Berk and Broome) Petch is hosted by rice (Rice grain spot disease) and maize (Maize root rot) (Mew and Gonzales, 2002, Saunders and Kohn, 2008). Whereas, *N. sphaerica* (Sacc.) Mason has been isolated from decayed banana fruits (Esposito *et al.*, 1962) and spotted leaves of blueberry plants (Wright *et al.*, 2008).

Both of these two *Nigrospora* species were reported to infect and cause disease in date palm. Abass *et al.* (2007) isolated and identified *N. oryzae* from heavily infected date palm leaves with leaf spot disease in 2007. They also reported the species of *N. sphaerica* as a true pathogen of date palm trees which exhibited severe symptoms of leaf and stem spot diseases (Abass *et al.*, 2013), most severe symptoms were observed on young date palm trees, the leaves and stem spot disease was most serious on these young trees.

The present study aimed at the separation of these two species of *Nigrospora* genus according to their morphological, molecular and pathological levels on different cultivars of date palm.

Material and Methods

Fungal isolates

N. oryzae and *N. sphaerica* were isolated from heavily infected date palm leaves with spot symptoms. Most leaves were collected from cvs. Al-Sayer and Hillawi. The isolation was conducted on a Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol at 25 ° C according to Abass *et al.* (2013). Briefly, heavily infected leaves of date palm were brought to the laboratory and sectioned into small pieces of 1-2 cm², and sterilized with sodium hypochlorite (10% of commercial chlorox), subsequently rinsed in distilled water and placed on PDA plates.

Morphological identification of N. oryzae and N. sphaerica

The hyphae and conidia were examined in 7-d old colonies grown on PDA plates. The morphological identification was performed according to Matsushima (1975). Specimens were examined using a Zeiss AxioLab compound optic light microscope (AxioLab.A1, Fisher Scientific, Germany). Micrometric data was based on measurement of 100 individual spores, hyphae and conidiogenous cells.

Extraction and purification of fungal DNA

The procedures used for fungal genomic DNA extraction, purification and ethanol precipitation were according to Zolan and Pukkila (1986). Briefly, single-spore cultures were placed on Potato Carrot Agar (PCA) medium at 25 °C for 7 days. The mycelium and conidia were collected (approximately 10 g) and ground with liquid nitrogen at room temperature, then extracted with 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, tubes were then centrifuged 5 min at 13000 rpm. The aqueous phases were recovered into fresh tubes containing isopropanol and followed by a second centrifugation for 1 min. The DNA pellets were resuspended in 300 µL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

Primers description and PCR amplification

Universal primers (ITS1 and ITS4) were selected for molecular identification of *Nigrospora* species. The sequences of primers were: ITS1: 5': TCCGTAGGTGAACCTGCGG-3', which hybridizes at the end of 18S rDNA and ITS4: 5': TCCTCCGCTTATTGATATGC-3', which hybridizes at the beginning of 28S rDNA (White *et al.*, 1990). The Polymerase Chain Reaction (PCR) was carried out in 0.2-mL polypropylene tubes with a total mixture of 50 µL consisting of a 4 ng of gDNA template, 5 µL of 10× polymerase buffer, 8 µL of dNTPs (1.25 mM), 1 µL of *Taq* DNA polymerase (Roche) and 1 µL of each primer, and distilled water up to 50 µL.

The thermal cycler used was equipped with a heated lid (M. J. Research Inc., Waltham, Massachusetts, USA). The PCR cycle was set up as follow: 5 min initial denaturation and enzyme activation at 95°C, followed by amplification for 35 cycles at 95°C for 1 min, 55° C for 1 min and 72° C for 1 min with a final extension at 72° C for 10 min (Rodrigues *et al.*, 2011).

The PCR products were resolved by horizontal electrophoresis in a 2% agarose gel after staining with ethidium bromide (approximately 0.2-0.5 µg/mL). The PCR products were sequenced and analyzed by comparison with all available sequences in the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) using the Basic Alignment Sequence Tool (BLAST): (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Susceptibility of different date palm cultivars to the infection with Nigrospora species

Five date palm cultivars (Al-Sayer, Hillawi, Zahdi, Leloy and Kantar) were chosen (because of their heavily infection symptoms of leaf spot) to determine the level of their susceptibility to the artificial infection with *N. oryzae* and *N. sphaerica* under the laboratory conditions. Following the procedures of Abass *et al.* (2013) using mycelium plug inoculation on detached healthy date palm leaves. Briefly, five pieces of leaves (approximately 1.5 cm in length) per cultivar were surface-sterilized and rinsed in sterile distilled water five times. A wound of 0.5 cm diameter and 0.5 cm depth was made by a sterilized cork borer, and a 0.5 cm mycelial plug from *N. oryzae* and *N. sphaerica* colony grown on PDA was placed inside the wound and sealed with parafilm. A sterile PDA plug (0.5 cm) served as a negative control was used. The inoculated wounded leaves were placed in 200 mL flasks containing 20 mL sterilised distilled 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 according to Bachillor and Ilage (1998). The reisolation of the pathogen from the inoculated leaves, to fulfil Koch's postulates, was conducted on PDA plates as described above. The current test was repeated twice to confirm the results, the average of these experiments were considered for analysis.

Extracellular enzyme analysis

The most important enzymes of both concerned *Nigrospora* spp. were assayed as shown here below:

1- Cellulase activity

N. oryzae and *N. sphaerica* were grown on YEPA (0.1 g yeast extract, 0.5 g peptone, 16 g agar in 1 litre of distilled water) supplemented with 0.5% (w/v) N-carboxymethyl cellulose. Each plate was incubated at 25 °C. The plates (9 cm diameter) were flooded with 5 mL of Congo red (0.1%) and then destained with sodium chloride (1%) for 15 min. The clear zones

around the colonies were measured by taking the average of three directions on each Petri dish.

2- Protease activity

The protease activity of *N. oryzae* and *N. sphaerica* was assayed following the procedures described by Amirrita *et al.* (2012) on GYP medium (1 g glucose, 0.1 g yeast extract, 0.5 g peptone, 16 g agar in 1 litre of distilled water) amended with gelatine (0.4% w/v). Both GYP and gelatine were sterilized separately by autoclaving for 20 min. Saturated aqueous of ammonium sulphate was used (5 mL/ plate) to flood the cultures. The saturation of ammonium sulphate was done by dissolving a 75 g of ammonium sulphate in 100 mL of distilled water. The clear halo around the colonies indicating the proteolytic activity and was measured by taking the average of three directions on each Petri dish.

3-Lipase activity

The procedure of Sierra (1957) was followed to determine the lipase activity of *N. oryzae* and *N. sphaerica*. Briefly, the medium of Peptone Agar Medium (PAM) (10 g peptone, 5 g NaCl, 16 g agar in 1 litre of distilled water) supplemented with sterilized Tween 20 at 1% (v/v) was inoculated with fungal colony plugs of 0.5 cm of tested species and incubated at 25 °C. The clear halo indicating the lipase activity

Results and Discussion

Morphological and molecular characterisation of both Nigrospora species under investigations

Both *N. oryzae* and *N. sphaerica* were isolated from heavily infected date palm leaves with spot symptoms, most leaves were collected from cvs. Al-Sayer and Hillawi (Figure 1). After 7 days of culture on PDA plates, both species of *Nigrospora* grew rapidly and produced white colonies, initially, and then became brown to dark brown due to the abundance of sporulation (Figure 2 A and B).



Figure 1. Leaf spot disease symptoms on A and B Al-Sayer cv. C and D Hillawi cv.

The species of *N. oryzae* produced a single-cell conidium of 14 -16 μM in diameter; each conidium was born on hyaline vesicle at the tip of the conidiophore of 4.5-6.0 μM . The conidium shape was ranging from spherical to black subspherical with the hyphae diameter at 7 -9 μM (Figure 2 C).

The species of *N. sphaerica*, a single-cell conidium was produced at the attenuate apex of conidiophores which were 7-9 μM in diameter, spherical to oblate, solitary, black with smooth-walled and about 19 -20 μM as a diameter. The diameters of hyphae were 8 -11 μM (Figure 2 D).

Regarding the disease of leaf spot, several fungal genera have been isolated and identified as a true pathogen on date palm in Iraq, including: *Alternaria*, *Pestalotio* *Mycosphaerella*, *Phoma* and *Nigrospora* (Abass *et al.*, 2007, 2013). Two different species have been found to be a leaf spot pathogen which belongs to the genus of *Nigrospora*. *N. oryzae* and *N. sphaerica* (Abass *et al.*, 2007, 2013). In the present study, both species of *Nigrospora* were successfully grown in vitro and exhibiting rapid proliferation on PDA plates at 25° C. However, the morphological examination showed that the sizes of conidia and conidiophores as well as the hyphae diameter could be a reliable parameter for discriminating between these two species. Most importantly, the conidia diameter which was larger in response to *N. sphaerica* (up to 20 μM) compared to *N. oryzae* (up to 16 μM).

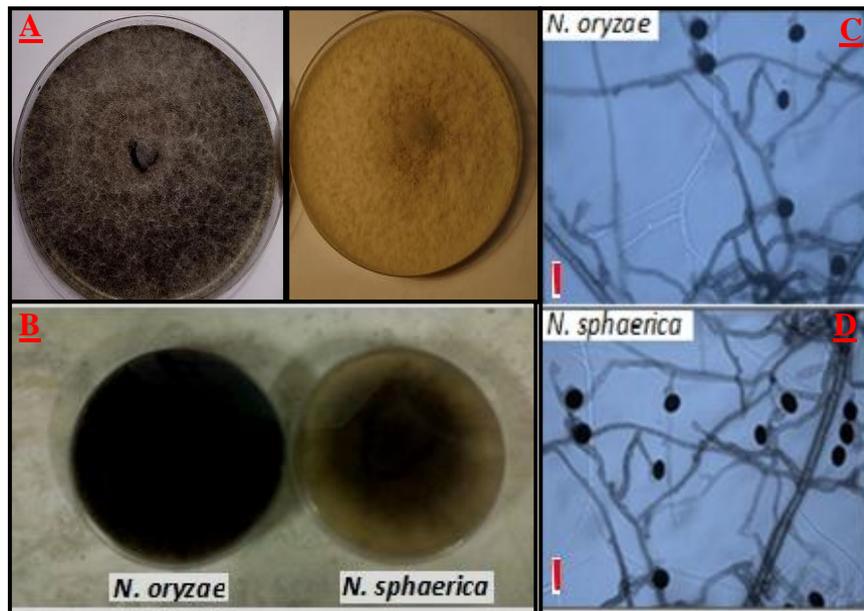


Figure 2 A. 7 days growing culture of *N. oryzae* and *N. sphaerica* on PDA plate. B. Reverse growth of *N. oryzae* and *N. sphaerica* on PDA plate. C. Microscopic features of *N. oryzae* . D. Microscopic features *N. sphaerica*. Bar 20 μ m.

The results of molecular characterization of *Nigrospora* species emphasizing on the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA) with ITS1 and ITS4 primers showed that the ITS sequence analysis had a 99% of identity with a total of ~515 bp for *N. oryzae*, and ~500 bp for *N. sphaerica* (Figure 3).

The molecular identification with ITS primers (ITS1/4) revealed the identity of both pathogenic species of *Nigrospora*. The sequence data alongside with BLAST search proved the identity (99%) to be *N. oryzae* and *N. sphaerica* thus confirmed the morphological identification. The Internal transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) has a great importance in confirmation of fungal identification; both ITS primers ITS1 and ITS4 were used to amplify these regions which compass the 5.8S coding sequence situated between large and small units (White *et al.*, 1990). The ITS sequencing method has been implied widely for discrimination between many closely related species belonging to the genera of *Alternaria*, *Aspergillus* and *Penicillium* (Henry *et al.*, 2000; Konstantinova *et al.*, 2002; Pashley *et al.*, 2012, Abass, 2013b).

On the basis of morphological characterization and molecular analysis of ITS region, the identity of *Nigrospora* species was revealed to be as *N. oryzae* and *N. sphaerica*.

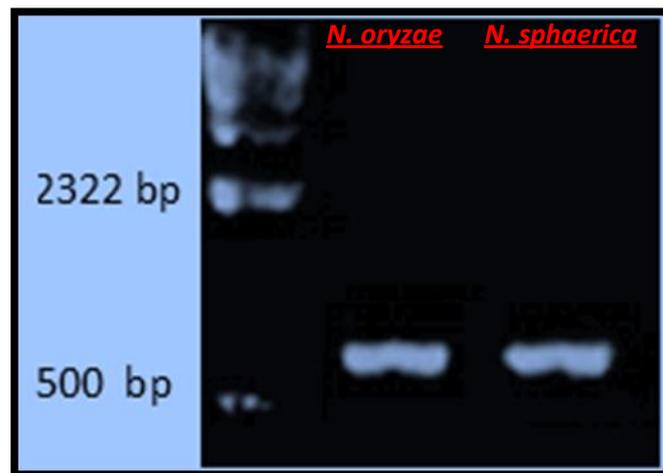


Figure 3 PCR products of DNA from *N. oryzae* and *N. sphaerica* with ITS primers. Lane 1, Lambda HindIII DNA marker; lane 2, *N. oryzae* (515 bp); lane 3, *N. sphaerica* (500 bp). The sizes of both fragments were estimated by comparison with lambda HindIII DNA marker (Gene Ruler) and the computer program of Photocapt MW software 10.0, Vilber Lourmat.

Susceptibility test of five date palm cultivars to the infection with Nigrospora species

The results of susceptibility test of five different date palm cultivars i.e., Al-Sayer, Hillawi, Zahdi, Leloy and Kantar, proved the ability of both tested species of *Nigrospora* to induce spot symptoms on all tested cultivars after artificial inoculation at laboratory. Generally, *N. oryzae* was more aggressive species on all detached healthy leaves of date palm cultivars compared to *N. sphaerica* (Table 1). The overall average of lesion diameter was 1.83 cm in leaf treated with *N. oryzae* with a significant difference than the *N. sphaerica* species. The symptoms of leaf spot developed as an oval to spherical shape with a green blackish centre. Al-Sayer cultivar was the most susceptible among tested cultivars to the artificial infection with both *Nigrospora* species where the lesion diameter was 2.50 cm with a significant difference than other cultivars results. In contrast cv. Leloy showed the lowest level of susceptibility showing 1.1 cm-lesions after 30 days of inoculation; whereas, all tested cultivars in negative control remained symptomless during the incubation period up to 30 days post-inoculation (Figure 4). *N. oryzae* and *N. sphaerica* were consistently recovered from lesion tissues and reidentified fulfilling Koch's postulates.

The susceptible test showed that the species of *N. oryzae* was the most aggressive on all five tested date palm cultivars, compared to *N. sphaerica*. The most susceptible reactions were observed with Al-Sayer and Zahdi cultivars, in contrast with Leloy cultivar which showed the lowest level of susceptibility for both species of *Nigrospora*. The high level of pathogenicity in the artificial inoculation with *N. oryzae* on date palm detached leaves could be attributed to the enzymatic and toxic activity of the pathogen, which might be higher in the species of *N. oryzae* compared to *N. sphaerica*. Several toxins have been isolated and identified from the culture filtrate of *Nigrospora*, such as lactones, most importantly; phomalactone which induced water-soaked lesion of tested leaves (Fukushima *et al.*, 1998).

Table 1. Lesion diameter of different date palm cultivars (cm) caused by two species of *Nigrospora*

Date Palm cultivar	Fungal species		Average of cultivar
	<i>N. oryzae</i>	<i>N. sphaerica</i>	
Al-Sayer	2.90	2.10	2.50a
Hillawi	1.60	1.25	1.40c
Kantar	1.30	1.50	1.40c
Leloy	1.20	1.00	1.10d
Zahdi	2.15	1.25	1.70b
Average of fungal species	1.83a*	1.42b	

* Means within each column followed by the same letter are not significantly different at the $P < 0.01$ level as determined by Duncan's multiple range tests.

Figure 4. Infection procedure of *N. oryzae* and *N. sphaerica* on date palm detached leaves.

Extracellular enzymatic activity of *N. oryzae* and *N. sphaerica*

The two species of *Nigrospora* spp. were screened for the activity of their extracellular enzyme, including cellulase, protease and lipase. Both *N. oryzae* and *N. sphaerica* showed positive results for cellulase and protease enzyme assay, while no indication for any activity with lipase assay in *Nigrospora* species (Table 2, Figure 5). It's noteworthy that *N. oryzae* was the most active in the cellulase and protease analysis compared to of *N. sphaerica*.

The degradative enzymes produced by plant fungal pathogens are crucial factors in the pathogenesis involving several biological functions such as host specificity, deterioration of food, and decomposition of organic matter (Ahmed *et al.*, 2006).

The present study shows positive results of cellulase and protease activity in the culture media. Both *N. oryzae* and *N. sphaerica* produced cellulase and protease enzymes but the highest activity was observed in the cultures of *N. oryzae*. This variation could be attributed to the level of virulence of *N. oryzae* which was more aggressive on all tested date palm

cultivars in contrast with *N. sphaerica*. It was reported that the host specificity as well as fungal virulence could be one of the explanations of the variations in the enzymatic activity of different plant fungal pathogens such as *Mauginiella scattae*, *Fusarium moniliform*, *F. graminearum* and *F. semitectum* (Abass, 2005; Ahmed *et al.*, 2006).

No detection of any lipase activity in both species of *Nigrospora* when Tween 20 was used as a substrate for lipase enzyme assay. Numerous published paper showed the suitability of Tween 20 as an appropriate substrate for lipase assay in solid medium (Tan *et al.*, 2004; Amirita *et al.*, 2012). The negative result of lipase was reported in different plant fungal pathogens such as *Thialoviopsis paradoxa* (Abass, 2005).

Table 2. Extracellular enzyme assay of *N. oryzae* and *N. sphaerica*.

<i>Nigrospora</i> species	Lipase activity (mm)			Protease activity (mm)			Cellulase activity (mm)		
	E.A.	Z.D.	R.G.	E.A.	Z.D.	R.G.	E.A.	Z.D.	R.G.
<i>N. oryzae</i>	-	-	45.0	+	15.0	53.0	+	20.0	50.0
<i>N. sphaerica</i>	-	-	35.0	+	10.0	45.0	+	10.0	45.0

R.G.: Radial Growth, Z.D.: Zone Diameter, E.A.: Enzyme Activity.

+: Active, -: Inactive.

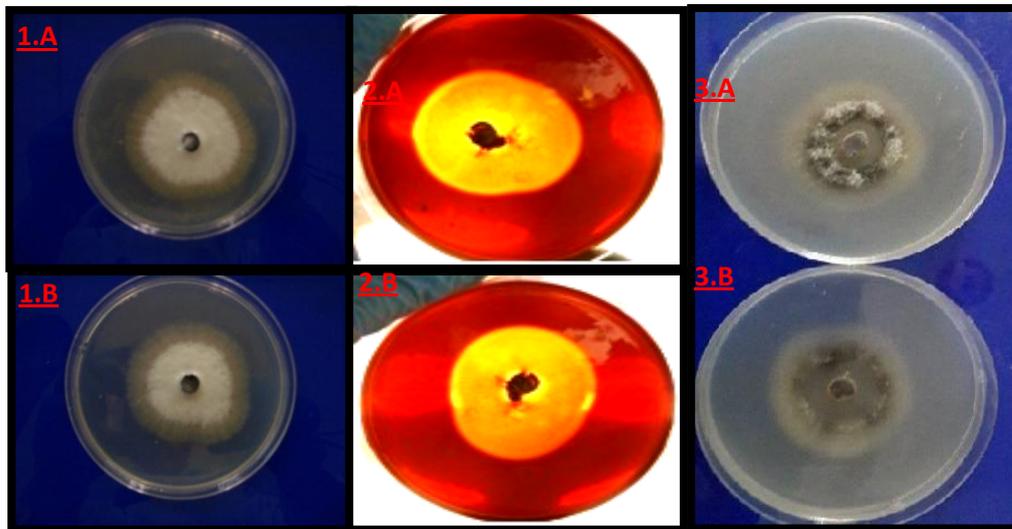


Figure 5. Results of different enzymatic activity of *N. oryzae* and *N. sphaerica*.

1. Protease activity: A. *N. oryzae*, B. *N. sphaerica*. 2- Cellulase activity: A. *N. oryzae*, B. *N. sphaerica*. 3- Lipase activity: A. *N. oryzae*, B. *N. sphaerica*.

Conclusions

Our results indicated that the morphological characteristics, based on the diameter of conidia of *Nigrospora* spp. under study were reliable features for fungal identification on the species level. The morphological characterisation was confirmed by ITS sequences and proved the identity of *N. oryzae* and *N. sphaerica*. The susceptibility test of different date palm cultivars revealed higher levels of virulence of *N. oryzae* compared to *N. sphaerica*.

The variation of enzymatic activity of cellulase and protease between the two species of *Nigrospora* may suggest an explanation for the significant differences in their pathogenicity on date palm detached leaves. The high level of virulence of *N. oryzae* could be correlated with the high enzymatic activity. Further investigations focusing on toxicological and histological aspects will help to better understand the nature of pathogenicity of *Nigrospora* species.

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