Comparative study on date palm (*Phoenix dactylifera L.*) leaf spot fungal pathogens *Nigrospora oryzae* and *N. sphaerica*

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ABSTRACT

Date palm diseases is 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. Recently, in Iraq, several fungal pathogens have been isolated from heavily infected date palm leaves exhibiting symptoms of leaf spot; most abundantly, different species of Nigrospora. The present study was aimed at the characterization of these 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

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).

Dates are well known as a good source of energy attributed to their rich content of nutrients, mostly carbohydrates and dietary fibre, certain essential vitamins and minerals

such as iron, potassium, calcium and low level of sodium and fats (Thabet et al. 2010; Dayani *et al.,* 2012).

Many bacterial, fungal and other pathogens have been well studied on date palm; fungal pathogens are considered as one of the most serious pathogens and cause a significant reduction in date palm growth, development and production (El-Hassani *et al.,* 2007; 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. (2006) were able to isolate and identify the species of N. oryzae from heavily infected date palm leaves with leaf spot disease in 2006, and in 2011-2012 they 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).

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

1-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.

2-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.

3-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, a 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].

4-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 waster 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).

5-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

6-Extracellular enzyme analysis

The most important enzymes of both Nigrospora spp. were assayed as below:

6-1- 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.

6-2- Protease activity

The protease activity of N. oryzae and N. sphaerica was assayed following the procedures described by Amirrita et al. (2012) on GYPA 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 GYPA 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 proteolyitc activity and was measured by taking the average of three directions on each Petri dish.

6-3-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

1-Morphological and molecular characterisation of Nigrospora species

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 (Fig. 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 (Fig. 2 A and B).

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 (Fig. 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 (Fig. 2 D).

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 (Fig. 3).

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.

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

The results of susceptibility test of five different date palm cultivars which were 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. 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. 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 (Fig. 4). N. oryzae and N. sphaerica were consistently recovered from lesion tissues and reidentified fulfilling Koch's postulates.

3-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, Fig. 5). It's noteworthy that N. oryzae was the most active in the cellulase and protease analysis compared to of N. sphaerica.

DISCUSSION

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). 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.,* 2006). 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.,* 2006; 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).

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.,* 2006, 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.,* 2006, 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 were larger in response to N. sphaerica (up to 20 µM) compared to N. oryzae (up to 16 µM). 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 belong to the genera of Alternaria, *Aspergillus* and *Penicillium* (Henry *et al.,* 2000; Konstantinova *et al.,* 2002; Pashley *et al.,* 2012; Abass, 2013b).

The susceptible test showed that the species of N. oryzae was the most aggressive on all 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 in the species of N. oryzae compared to N. spaherica. 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).

The degradative enzymes produced by plant fungal pathogens are crucial factors in the pathogenesis involving several biological functions such as host specificity, deterioration of 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 moniliforme, F. graminearum and F. semitectum (Abass, 2005; Ahmad *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).

CONCLUSIONS

Our results indicated that the morphological characteristics, based on the diameter of conidia of Nigrospora spp. are 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.

References

Abass, M.H. 2005. Extracellular enzymatic activity of pathogenic fungi of date palm (*Phoenix dactylifera* L.) and CYCAS (Cycas revolute L.). Basra J. of Date Palm Res. 4(1-2): 1-10.

Abass, M.H., Hameed, M.A., and AL-Sadoon, A. 2006. Survey of fungal leaf spot disease of date palm (*Phoenix dactylifera* L.) in Shaat- Alarab orchards/Basra and evaluation of some fungicides. Iraqi J. Biol. 5, 1–20.

Abass, M.H., AL-Abadi, U.A.M. and AL-Kaby, A.M.S. 2007. The efficiency of Henna leaves extracts and some fungicides to reduce the fungal contamination of date palm (*Phoenix dactylifera* L.) tissue culture. Iraqi J. Biotech. 6(2):1-40.

Abass, M.H., Hameed, M.A. and Ahmed, A.N. 2013. First report of Nigrospora sphaerica (Sacc.) Mason as a potential pathogen of date palm (*Phoenix dactylifera* L.). Can. J Plant Pathol. 35(1):75-80.

Abass, M.H. 2013a. Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi

tissue culture laboratories. Emirate J. Food and Agric. doi: 10.9755/ejfa.v25i11.15351.

Abass, M.H. 2013b. A PCR ITS-RFLP method for identifying fungal contamination of date palm (*Phoenix dactylifera* L.) tissue cultures. African J of Biotech. 12(32): 5054-5059.

Ahmed Y., Hameed, A. and Ghaffar, A. 2006. Enzymatic activity of fungal pathogens in corn. Pak. J. Bot. 38(4): 1305-1316

AL-Juboory, H.H. 2005. First identification of Omphalia root rot disease of date palm in Iraq. Iraqi J. Agric. Sci. 36(4): 101-106.

Amirrita, A., Sindhu, P., Swetha, J., Vasanthi and Kanna, K.P. 2012. Enumeration of endophytic fungi from medicinal plants and screening of extracellular enzymes. World J. Sci. Tech. 2(2): 13-19.

AL-Shahib, W. and Marshal, R.M. 2003. The fruit of the date palm: it's possible uses as the best food for the future. Int. J. Food Sci. Nutr. 54:247-259.

AL-Sheikh, H. 2009. Date-Palm fruit spoilage and seed-borne fungi of Saudi Arabia. Res. J. Microbiol. 4(5):208-213.

Bachillor, N., and Ilage, L. 1998. Etiology of stem bleeding disease of coconut in Philippines. J. Crop. Sci. 23, 42–50.

Dayani, O., A. Khezri and Moradi, A.G. 2012. Determination of nutritive value of date palm by-products using in vitro and in situ measurements. Small Rum. Res. 105:122-125.

EL-Hassani, M., EL-Hadrami, A., Fauad, D., Mohamad, C., Barka, E.A., and EL-Hadrami, I. 2007. Biological control of Bayoud disease in date palm. Selection of microorganism inhibiting the causal agent, inducing defense reaction. Environ. Exp. Bot. 59, 224–234.

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.

Food and Agriculture Organization 2011. FAOSTAT-agriculture.http://faostat.fao.org/site/567/default.aspx#ancor.

Fukushima, T., Tanaka, M., Gohbara, M., and Fujimois, T. 1998. Phytotoxicity of three lactones from Nigrospora sacchari. Phytochem., 48, 625–630.

Henry, T., Iwen, P.C. and Hinrichs, S.H. 2000. Identification of *Aspergillus* species using Internal transcribed Spacer regions 1 and 2. J. Clin. Microbiol. 38(4):1510-1515.

Konstantinova, P., Bonants, P.J.M., Gent-Pelzer, P.E., Zouwen, P. and Bulk, R. 2002. Development of specific primers for detection and identification of Alternaria spp. In carrot material by PCR and comparison with blotter and plating assays. Mycol. Res. 106(1): 23-33.

Matsushima, T. 1975. Icones Microfungorum a Matsushima lectorum. Kobe, Japan. 1–209, Plates 1–405.

Mew, T.W., and Gonzales, P. 2002. A Handbook of Rice Seed borne Fungi. Los Banos, Philippines: International Rice Research Institute (IRRI) and Enfield, NH: Science Publishers, Inc. 83 pages.

Pashley, C.H., Fairs, A., R.C. Free and Wardlaw, A.J. 2012. DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology. Fungal Biol. 116:214-224.

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

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

Sierra, G. 1957. A simple method for detection of lipolytic activity of microorganism and some observation on the influence of contact between cells and fatty substrates. Antooni Van Leauwenhoek 23(1): 15-22.

Sulieman, A., Abd Elhafies, I.A. and Abdelrahim, A.M. 2012. Comparative study of five Sudanese date palm (*Phoenix dactylifera* L.) fruit cultivars. Food Nutr. Sci.3, 1245-1251.

Tan T., Zhang, NM., Xu, J. and Zhang, J. 2004. Optimization of culture conditions and properties of lipase from *Penicillium* camembertii. Thom PG-3.Proc. Biochem. 39:1495-1502.

Thabet, I., Francis, F., De Paw, E., Besbes, S., Attia, H., Devanne, C. and Blecker, C. 2010. Charcterisation of proteins from date palm sap (*Phoenix dactylifera* L.) by a protein approach. Food Chem. 123:765-770.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 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.

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.

Zaid, A. (Ed.). 2002. Date Palm Cultivation. Rev. Ed. FAO, Rome.

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

Figures

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

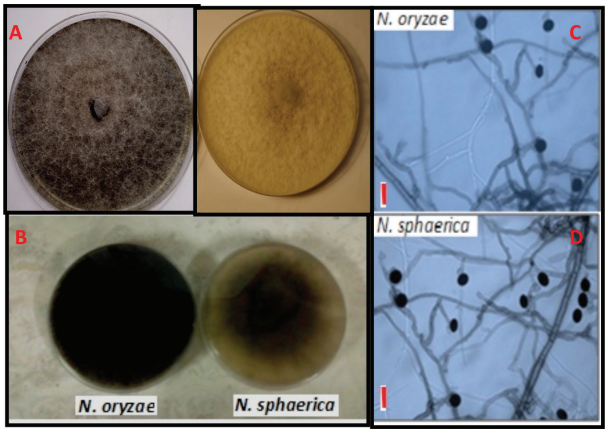

Fig.2 A. 7 days growing culture of N. oryzae and N. sphaerica on PDA plate. B. Reverse growth of N. oryzae and N. sphaerica on PDA plate. C. Microscopic features of N. oryzae . D. Microscopic features N. sphaerica. Bar 20 µm.

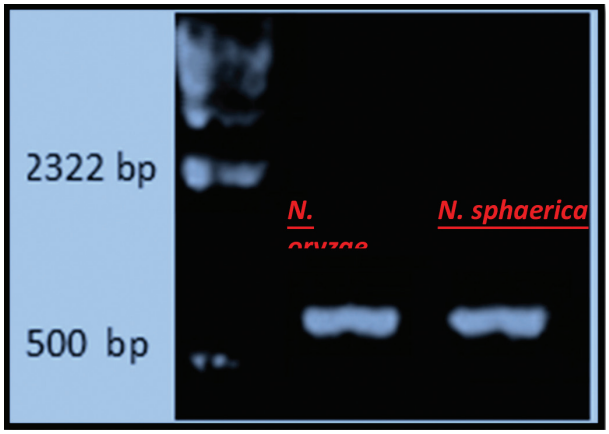

Fig. 3. PCR products of DNA from N. oryzae and N. sphaerica with ITS primers. Lane 1, Lambda HindIII DNA marker; lane 2, N. oryzae (515 bp); lane 3, N. sphaerica (500 bp). The sizes of both fragments were estimated by comparison with lambda HindIII DNA marker (Gene Ruler) and the computer program of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

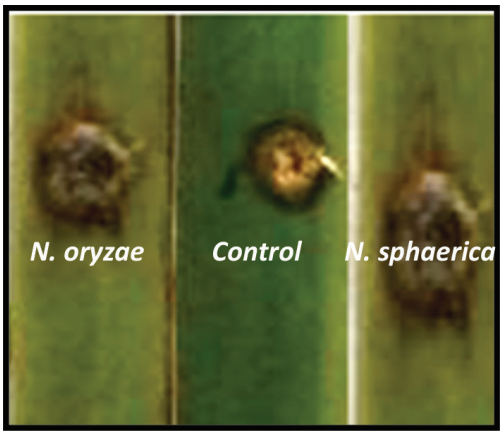

Fig. 4. Infection procedure of N. oryzae and N. sphaerica on date palm detached leaves.

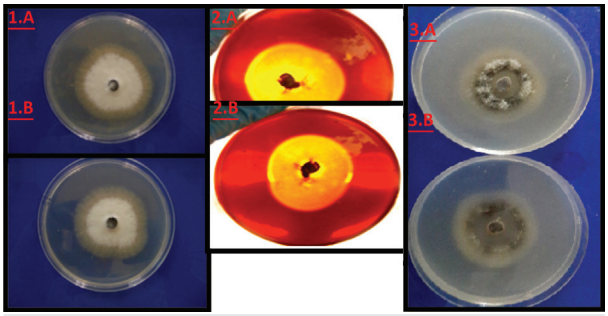

Fig. 5. Results of different enzymatic activity of N. oryzae and N. sphaerica.

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.

Tables:

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

Date Palm cultivar	Fungal species		Average of cultivar
	N. oryzae	N. sphaerica	
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	1.80a*	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 test.

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

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

R.G.: Radial Growth, Z.D.: Zone Diameter, E.A.: Enzyme Activity. + Active; - Inactive.