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Identification Of *Nigrospora Sphaerica* As A Causal Agent Of Fruits Rots Disease In Basrah Governorate-Iraq

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Abstract

The date palm is infected with many fungal pathogens that cause a problem in the quality and quantity of date production. The fungus *Nigrospora sphaerica* was isolated from date fruit that show symptoms of fruit rot in the orchards of Abu Al-Khaseeb and Al-Hartha in Basrah Governorate, southern Iraq. The results of this study showed that the fungus can infect the fruits of ten date palm cultivar such as (Zahdi, Barhi, Bream, Khadrawi, Shukar, Khasab, Sayer, Hillawi, Leilwi and Deri) and cause fruit rot disease during the Kimri and Khalal stages of date palm fruits, with the highest infection severity in the cultivar Shukar 37.33 % during the Kimri stage, and it reached 33.67 % in the Khasab cultivar during the Khalal stage. While the lowest rate of infection severity was 13% in the Barhi cultivar during the Kimri stage, while it reached 11% in the Barhi and Khadrawi cultivars during the Khallal stage. During the study, it was found that the moisture content of the date fruit has an important role in increasing the severity of infection during the Kimri and Khalal stages, where the percentage of moisture at its highest levels reached 83.67 and 72.67 %, respectively, in the Shukar cultivar, followed by the Leilwi and Khasab cultivars, where the severity of infection increased with the high moisture content of the fruits. The total tannin content ranged from 0.46 to 0.96 mg/100g for date palm fruits in Kimri stage and ranged from 1.06 to 2.56 mg/100g in Khalal stage.The results of fungicides screening that the fungicide Score was the best fungicide in decreasing the disease index as it reached 1.67 compared with control which reached 77.33 %.

Keywords: *Nigrospora sphaerica*, date palm fruit, fruit rot.

2. Introduction

Date palm (*Phoenix dactylifera* L.) is one of the most cultivated fruit trees around the world, was cultivated as early as 4000 B.C. since it was used for the construction of the temple of the moon god near Ur in Southern Iraq - Mesopotamia (Popenoe, 1973). Iraq's position declined from the first to the fifth among the countries producing dates (FAO, 2018).

The date palm is infected with many different diseases, Date palm diseases cause a reduction in the quality and quantity of dates production in Iraq, and a number of pathogens have been first recorded in Iraq on date palm trees as *Thielaviopsis paradoxa, Bipolaris australiensis, Phoma*

glomerata, Phoma leveilli and *phoma costaricensis* were considered as first record as causal agent of leaf spot disease on date palm in Iraq (fayad and mania, 2008., Manea et al , 2021). *A. alternata* has ability to cause the shoot holes disease on date palms (manea, 2013). *Fusarium solani* as a causal fungus of wilt and death disease on date palm tissue culture off shoots (Al-Saad et al, 2018. In 2007, date palm trees surveyed in Upper Egypt, the samples were collected from symptomatic fruits in four localities (Assiut, Qena, Sohag and El-Minia, Governorates), the major diseases noticed were fruit spots caused by *F. proliferatum* and *A. niger* fungi (Farrag and Abo-Elyousr, 2011).

The secondary metabolites of plants play an important role in protecting plants from infection with pathogens, and tannins are among the most important oligomeric or polymeric flavonoids compounds that help protect plants and thus resist the occurrence of diseases in them, which may be stored primarily in inactive forms (phytoantibenzenes) also known as proanthocyanidins (Sharma, 2019., War et al, 2012). Tannins can inhibit the invasion and growth of many Mycopathogens such as *Penicillium digitatum* (Zhu et al, 2019), *Alternaria alternata*, *Botrytis cinerea* and *Candida albicans* (Brighenti et al, 2021), *Fusarium oxysporum* and *Phytophtora infestans* on tomato plants (Dakole et al, 2016).

Several chemical fungicides have been tested against some pathogens of leaf spot disease. Evaluation of five fungicide revealed that score (difenoconazole) inhibited completely the radial growth of tested fungi, field experiment showed that difenoconazole, Mancozab and Penconazole reduced the infection rate (r) for 0.032, 0.044 and 0.049 cm/day respectively compared to 0.098 cm/day for control treatment. (fayyadh and Mania, 2008). Mizeb S, Score and Carbendazim chemical fungicides were showed in vitro high level of inhibition in fungal radial to three isolates of *Alternaria alternata* that causing Fruit rot in date palm. (Alasadi et al,2006).

This study aimed to isolation the causal agent of date fruit rot *Nigrospora sphaerica* and its phenotypic and molecular diagnosis, and detect the effect of moisture content of date palm fruits and tannins on the severity of fruit rot disease caused by the pathogen and the possibility of its chemical control.

3. Materials and Methods

3.1. Sample collection and fungal isolation

Field sampling were conducted from May to in date palm orchards located in Abu Al-Khaseeb and Al-Hartha in Basrah provinces south of Iraq. These fruits were washed with tap water to get rid of the dust, then dried by filter papers and were cut into small pieces about 0.5 cm and then its surface sterilized with a 10% sodium hypochlorite solution for two minutes, then transferred to sterile distilled water, then dried by sterile filter paper before transferring to dishes containing sterilized Potato Dextrose Agar (P.D.A) sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes and supplemented with 250 µg/ml chloramphenicol. Four pieces of the infected tissue were placed in each Petri dish and the dishes were incubated at 25°C ±2 for a period of 4-6 days. The pathogenic fungi were purified on the P.D.A medium for the purpose of microscope and morphological

identification for the level of genus and species. The isolates were kept in slant containing sterilized P.D.A and kept in the refrigerator until use.

3.2. Morphological characterization

Conidia and conidiophores were collected from thought colonies growing on the PDA medium in the dishes for identification. Pathogen fungal colonies were taken per infected fruits sample of ten cultivars (Barhi, Bream, Khadrawi, Sayer, Maktum, Hillawi, Khasab, Leilwi, Zahdi and Shukar). Conidial features such as presence or absence of chasmothecia, shape, color, size and for conidiophores such as size, shape, foot-cell dimensions and number of conidia per chain were recorded for each colony isolate were measured by visualization with the light microscope (40 x and 100 x objectives; Olympus, Japan). Morphological identification were carried out according to following key (Ellis, 1971; Ellis, 1976).

3.3. Molecular identification

DNA extraction were done according to gSYNCTM DNA Extraction Kit. The partial ITS region was amplified using the primers ITS-1/ITS-4 V: TCCGTAGGTGAACCTGCGG: R: TCCTCCGCTTATTGATGATGC Polymerase chain reaction (PCR) was conducted and the PCR products were electrophoresed on a 1.2% agarose gel according to the method described by Abd-Elsalam et al. (2003). DNA sequencing was performed at the Korean company Macrogen. The obtained sequences were compared with *N. sphaerica* isolates from other related sequences deposited in GenBank using BLAST search.

3.4. Sensitivity of some cultivars of date palm fruits to infection with the fungus *Nigrospora sphaerica*

This study was conducted on ten cultivars of date palm fruits which are Barhi, Bream, Khadrawi, Sayer, Maktum, Hillawi, Khassab, Leilwi, Zahdi and Shukar . Ten fruits of each cultivar were taken in the Kimri and Khalal stages and sterilized well with 10% NaOCl solution for two minutes and then washed with sterile distilled water to get rid of traces of the substance Sterilized, then dried with sterile filter paper, and after drying it was divided into two groups, one group had wounds of equal size near the Fruit cap using a sterile needle, and another section was left without wounds. Part of the fruits (wounded and unwounded) were inoculated by spraying them with *N. sphaerica* spores at a concentration of 10^5 spores/ml using a hemocytometer, and another part of the fruits sprayed with sterile distilled water serve as a control treatment for the purpose of comparison. Each of ten fruits were placed in a sterile glass beaker hole was tightly closed with a piece of aluminum foil. Then all the beakers containing the fruits were placed in an incubator at a temperature of 25 C˚. For ten days, where the fruits were extracted and all examined separately, disease incidence and severity were calculated. The experiment was carried out at a rate of three replications for each treatment.

3.5. Efficacy of some fungicides in controlling date fruits rot disease

Date fruit of Sayer cultivar in kimri stage was prepared, fruits were washed with distilled water and sterilized with 10% of Naocl for two minute, a small wound was made in each of them near

the fruit cap, using a sterile inoculation needle. Then all fruits (10 fruits for each treatment), each ten fruits were placed in a sterile glass flask individually and then with spore suspension, the pathogen spores were sprayed at a concentration of 10^5 spores/ml, then the flasks were placed in an incubator at a temperature of 25 °C after their erlenmeyer flasks hole were covered with cotton and aluminum foil. On the second day, the fruits were treated with a recommended concentration of fungicide, all the flasks were extracted and the fruits (except the comparison) were treated with the pesticides to be tested for their effectiveness and according to the recommended concentrations for each pesticide shown in Table (1). The flasks, after being covered with cotton and aluminum foil, were returned to the incubator at the same temperature used previously. All flasks were incubated at 25 ˚C for 10 days and then the flasks were extracted from the incubator and the fruits were examined and the severity of the infestation was recorded. The experiment was conducted with an average of three replicates for each treatment.

Table 1: Fungicides used with recommended concentrations

Fungicide Product Name	Active ingredient	Recommended concentration
Topsin M	Thiophanate methyl	1 g/L
Score 250 EC	Difenoconazole	0.5 g/L
Vacomil-MZ 72%	Mancozeb 64% W/W Metalaxyl 8% W/W	2 g/L
Mizeb S	Mancozeb 80%	2 g/L
Kumulus® DF	Sulfer 800g/Kg	3 g/L

3.6. Moisture content

The moisture content of dare palm fruit samples was measured by placing 10 g of the fruits in an electric oven at a temperature of 70 °C for 72 hours until the weight was stable. The percentage of moisture content was calculated using the following equation:

$$\text{Moisture content} = \frac{Sample\ wet\ weight - Sample\ dry\ weight}{Sample\ wet\ weight} \times 100$$

3.7. Determination of tannins concentration in fruits

The tannins were estimated according to the method described by A.O.A.C (1975), when 7.5 gm of the fruits of all date palm cultivars tested in the study was taken, and after washing and drying well, they were placed in an electric mixer for the purpose of mashing, then placed in a 250-ml glass beaker and 75 ml of distilled water was added to it. Subsequently, the sample was heated at 30-45°C, the solution was cooled and placed in a standard 125ml beaker and volume up to mark, then volume up to mark, take 5 ml of the extract after filtering it using filter paper and then put it in a 1 liter beaker and add to it 12.5 ml of Indigo carmine solution with 375 ml of distilled water and then potassium permanganate was added by burette until the color of the solution turned golden yellow. The number of milliliters of potassium permanganate was

calculated. The extract was titrated and called A, then 50 ml of the extract was mixed with 0.5 g of animal charcoal for ten minutes in a closed container, then filtered through filter paper and 5 ml of the filtrate was taken with 12.5 ml of Indigo carmine dye and 375 ml of distilled water with potassium permanganate according to the number of milliliters Permanganate and the letter B was given only on the basis of that, the amount of tannins in the fruits according to the following equation:

$$\text{Amount of tannins \%} = \frac{(A-B)\times solution\ titrate\times 0.0035\times Dilution}{Weight\ of\ sample\times 0.1\ M}\times 100$$

Where: A = the number of milliliters of potassium permanganate titrated to the solution before adding the animal charcoal, B = the number of milliliters of potassium permanganate titrated to the solution after adding the animal charcoal.

3.8. Statistical analysis :

The randomized complete design (RCD) was used in in carrying out all experiments, and the GenStat software was used to do an analysis of variance in the results, and the least significant difference (LSD) tests ($P < 0.05$) were used to compare the means between treatments (Gomez and Gomez, 1984).

4. Results and discussion

4.1. Morphological characterization and Molecular identification

Nigrospora sphaerica Fungal was isolated from date palm fruit with fruit rot symptoms in Basrah, the fungal isolates were identified on the basis of its morphological and microscopic characteristics such as colony growth pattern, pigmentation and conidial morphology. The colonies of fungi pathogen were initially white to light gray (Figure 1-A), and gradually turned dark gray with age due to the formation of spores reaching 9 cm in 6 day at 25°C ±2. Hyphae hyaline, smooth, branched, septate (Figure 1-B). The conidiophores were micronematous or semi-macronematous, hyaline to pale brown, smooth, extensively branched, flexuous or straight, reduced to conidiogenous cells pale brown, subspherical, determinate, monoblastic, 6–12 µm diam. produces one lateral or terminal conidia are smooth, black opaque, aseptate, 15–20 µm in diameter under microscope (Figure 1B). The characteristics of *N. sphaerica* in this study are consistent with those described in the taxonomic keys (Ellis, 1971; Ellis, 1976) and with published descriptions of *N. sphaerica* (Wang et al. 2017., Zheng et al. 2021).

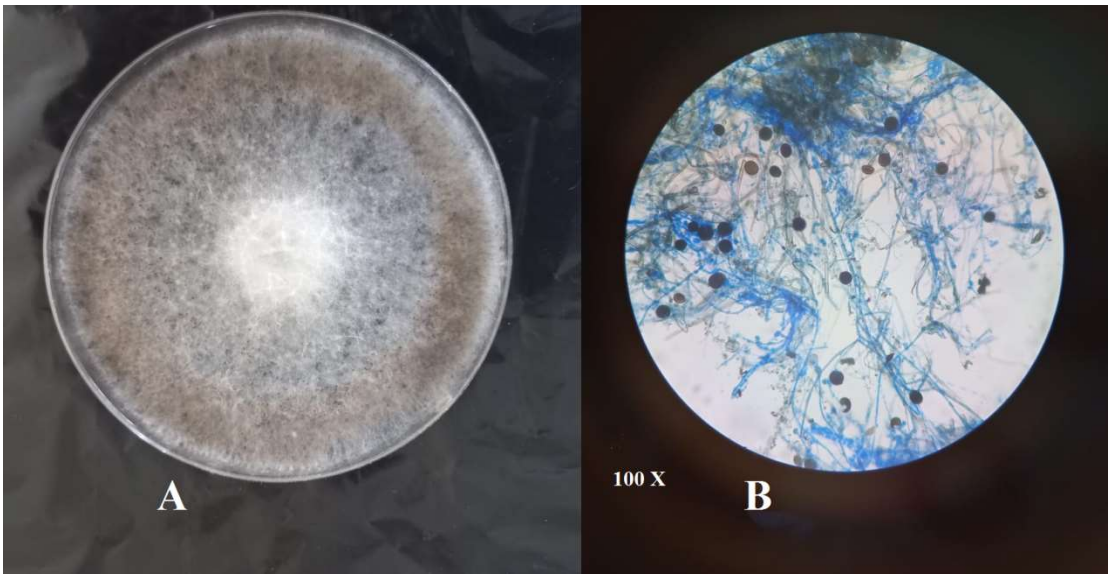

Figure 1: A= Mycelium of *Nigrospora sphaerica* on PDA
B = Hyphae, Conidia and Conidiogenous of *N. sphaerica* under microscope 100X

4.2. Molecular identification

For the molecular identification, the internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 and ITS4 (Abd-Elsalam et al.2003), and PCR products from *N. sphaerica* was sequenced and deposited in GenBank (Accession No. KP730001). The lengths of the ITS PCR products from pathogen isolates ranged 567 bp.

The BLAST analyses of fungal isolates showed 99.79% similarity with (MK808074.1, ON754217.1) and showed 98.59% with (MW319713.1, MT683265.1, HQ631070.1, OK047752.1, MN215818.1) similarity to the *Nigrospora* spp. isolates reported in other studies. This confirms the correctness of the microscopic diagnosis that the pathogenic fungus is *N. sphaerica*.

4.3. Susceptibility of some date palm cultivars to infection with the fungus *Nigrospora sphaerica*

Symptoms of the water-soaked spot on date palm fruits caused by *N. sphaerica* were observed in Abu Al-Khaseeb and Al-Hartha palm orchards in Basrah governorates in southern Iraq although the pathogen *Nigrospora* sp. was observed attacking palm fruit in KSA.(Elarosi et al,1983)
Fayyadh et al. (2016) were able to isolate *N. sphaerica* from symptoms associated with leaf blight on date palms.

Symptoms on fruits were started after seven days from inoculation with the appearance of yellowish-orange water soaked spot on the fruits in the Kimri stage, followed by drying starting from the end part to cover the entire fruits after fifteen days.

Pathogenicity was verified on fruits of ten different date palm cultivars (Zahdi, Barhi, Bream, Khadrawi, Shukar, Khasab, Sayer, Hillawi, Leilwi and Deri), inoculated fruits had symptoms similar to those originally observed on affected fruits, and the control treatment remained asymptomatic. The previous study indicates that *N. sphaerica* cause date palm leaf blight. (Fayyadh et al, 2016).

Table 2 data showed that date palm varieties differ in their susceptibility to be infected with *N. sphaerica* as disease severity was 42.00, 37.33, 34.33, 33.67, 24.33, 23.33, 17.67, 14.00, 13.00 and 12.33% for cultivars Khasab, Shukar, Deri, Leilwi, Hillawi, Sayer, Zahdi, Khadrawi, Barhi and Bream respectively, and reached at Khala stage 33.67, 29.33, 28.00, 24.33, 20.33, 18.33, 12.00, 11.00, 11.00 and 10.67 % for cultivars Khasab, Shukar, Deri, Leilwi, Hillawi, Sayer, Zahdi, Barhi, Khadrawi and Bream, respectively. In all cases, the control treatments of all cultivars remained asymptomatic.

The results of this study showed that the fruits of Khasab were more sensitive compared to other varieties, while the fruits of the cultivar Bream were the most resistant to the pathogen in both Kimri and Khalal stages. Previous studies indicate that many pathogens that affect the vegetative parts of the date palm such as *Alternaria alternata, A. tomato, A. burnsii*, *Fusarium incarnatum-equiseti, F. brachygibbosum*, *Nigrospora lacticolonia*, *Didymella microchlamydospora* and *Curvularia subpapendorfii* (Al-Nadabi etal, 2020). Al-Hassan and Abbas (1983) indicated that the rate of infection with the fungus *Thielaviopsis paradoxa* differed according to the date cultivar and its stage of maturity.

The results showed (Table 2) that the high moisture content in the date fruits have important role in the high severity of infection during the Kimri and Khalal stages. The highest moisture content in the Kimri stage was in the cultivars Shukar, Leilwi, Khasab, Hillawi and Deri, which amounted to 83.67, 83.67, 82.67, 82.33 and 82.33%, respectively, as well as in the Khalal stage of 72.67, 72.33, 70.67, 69.67 and 69.33 %, respectively, and these date palm cultivars with high moisture content showed high sensitivity to infection by *N. sphaerica* fungus. The role of high moisture content in facilitating the infection of date palm fruits pre and post-harvest has been reported (Lobo et al., 2014). The high moisture content of the fruit makes it a good target for invasion with many fungi such as *Mycosphaerella, Cladosporium, Mucor, Fusarium, Eurotium, Aspergillus, Penicillium, Rhizopus* and *Alternaria* (Djerbi, 1983; Gherbawy, 2001).

Previous studies indicated the possibility of *N. sphaerica* to produce ability to producing hydrolytic extracellular enzymes such as amylase, xylanase and carboxymethyl cellulase (CMC-ase) (Farouk et al, 2020), and producing cellulases that degrade cellulose material (Ayob and Simarani, 2016),

Thus, it is possible that the increase in moisture content in the affected date fruits is due to hydrolytic extracellular enzymes

In general, date trees can grow in saline soils and tolerate high temperatures and low humidity as compared to many other plants.

Zaied and dewet (2002) proposed to the high moisture affects the date fruits and makes them conducive to infection with rot fungi. and this is what is observed in Basrah Governorate, as the majority of fungal diseases were concentrated in the date palm orchards closest to the banks of the Shatt al-Arab River south of Iraq.

During the study of the relationship between the severity of infestation and tannins concentration of date palm fruits (Table 2), it was noted that the severity of infestation and tannin concentration differed according to the cultivar, this difference was significant in the ten cultivars. The total tannin content ranged from 0.46 to 0.96 mg/100g for date palm fruits in Kimri stage and ranged from 1.06 to 2.56 mg/100g in Khalal stage. All tested cultivars showed higher tannin content in fruits with lower severity of infection with pathogenic fungi, except for the Leilwi and Shukar cultivars, the severity of infection in the Kimri stage was 33.67% in the Leilwi cultivar, while the content of tannins reached 0.80 mg/100g and reached 2.06 mg/100g in the Khalal stage. In the Shukar cultivar, the severity of infection in the Khalal stage was 29.33%, and the tannin content reached 2.56 mg/100g at its highest levels compared to other cultivars. In another study, tannins were tested as antioxidant compounds in five cultivars of date palms during development and maturation stages, the activities of the antioxidant enzymes increased from the hababouk through to the Kimri and/or the Khalal stage and the antioxidant capacity was highly positively correlated with the concentration of antioxidant compounds in most cultivars (Awad et al, 2011). Date syrup is rich in tannins (357 mg/100 g), which are known potent antioxidants to *Escherichia coli* and *Staphylococcus aureus*.

The results (Table 2) showed that the concentration of tannins at their highest levels in the Kimri stage in the cultivars (Zahdi, Barhi, Bream, and Khadrawi) reduced the severity of the infection as well as in the Khalal stage. As for the cultivars (Khasab, Sayer, Deri and Hillawi) it is noted that the low concentration of tannins in the Kimri stage encouraged the high severity of the infection. The high concentration of tannins did not appear to have an important role in reducing the severity of infection for Hillawi and Leilwi cultivars in the Khalal stage, as the severity of infection were 24.33 and 33.67%, respectively, while the concentration of tannins was 1.67 and 2.06 mg/100 g, respectively.

This discrepancy in tannin concentrations and its relationship to the severity of infection caused by *N. sphaerica* in Kimri and Khalal stages may indicate that the effect of tannins depends on the cultivar and one the development stage to another, and that the relationship of water moisture content has a direct effect in increasing the severity of infection, the higher moisture content, the more severe the infection. Previous studies indicated that the activities of antioxidant enzymes for five cultivars of date palm, catalase, polyphenol oxidase and peroxidase were activated from hababouk to Kimri stages and then, declined at the ripening stages (Awad et al, 2011).

Table 2. Pathogenicity of *N. sphaerica* on different date palm cultivars

Cultivars	Severity of infection %		Moisture content %		Tannins conc. mg/100g	
	Kimri stage	Khalal stage	Kimri stage	Khalal stage	Kimri stage	Khalal stage
Zahdi	*17.67	12.00	67.00	61.67	0.73	1.62
Barhi	13.00	11.00	70.33	62.00	0.96	1.23
Bream	12.33	10.67	71.00	63.33	0.76	1.30
Khadrawi	14.00	11.00	73.33	66.00	0.83	1.83
Shukar	37.33	29.33	83.67	72.67	0.73	2.56
Khasab	42.00	33.67	82.67	72.33	0.46	1.13
Sayer	23.33	18.33	80.33	68.33	0.53	1.20
Hillawi	24.33	20.33	82.33	69.33	0.56	1.67
Leilwi	33.67	24.33	83.67	70.67	0.80	2.06
Deri	34.33	28.00	82.33	69.67	0.63	1.06
L.S.D.	0.667	0.333	0.333	0.333	0.100	0.033

* Each number represents three replicates

4.4. Efficacy of some fungicides in protecting date fruits from infection with of *N. sphaerica*

Five fungicides (Topsin M, Score, Vacomil-mz 72%, Mizeb S and Kumulus ®DF) were tested against date fruit rot disease on the fruits of the date palm cultivar Sayer during the Kimri stage. Figure (3) showed the high inhibitory ability of the fungicide Score against date fruit rot disease caused by *N. sphaerica* fungus by reduced disease index on the wounded date fruits, which reached 1.67 %, followed by Mizeb S fungicide which reached 4.67 % compared to the non-treated fruits (control), which reached 77.33 %.

Many fungicides have been used to control various date palm diseases as Score, Rizolex-T, Topsin M70, Ridomil Gold Plus and Kemazed caused the highest reduction in the incidence of inflorescences rot and black scorch disease caused by *Thielaviopsis paradoxa* and leaf base rot diseases caused by *Botryodiplodia theobromae* (El-Morsi et al, 2012). Fungicides Ortiva 250 SC, Naturame , Phyton-27 and Score 250 EC reduced by varying rates the severity of Black Scorch disease in date palms, Score fungicide showed the highest inhibition of pathogen mycelial growth (Saeed et al, 2016). Application of Cidely® Top fungicide significantly inhibited the *Fusarium solani* mycelial growth in vitro and reduced sudden decline syndrome (SDS) disease development on date palm seedlings (Alwahshi,2019). Fungicide Score significantly impeded in vivo studies of the mycelial of *T. punctulata* with 73% efficiency (Alhudaib et al, 2022). Manea (2013) indicated the efficacy of the pesticides Score, Mizeb S, and Vacomil MZ72 in inhibiting the infection of the

pathogenic fungus *A.alternata* that causes Leaves shoot hole disease on date palm, While when using the pesticide Vacomil MZ 72, the average number of holes was 0.25 hole/leaf, which is a very low percentage compared to the control. This suggests that Score and Mizeb S fungicides may avail as candidate fungicides against *N. sphaerica*.

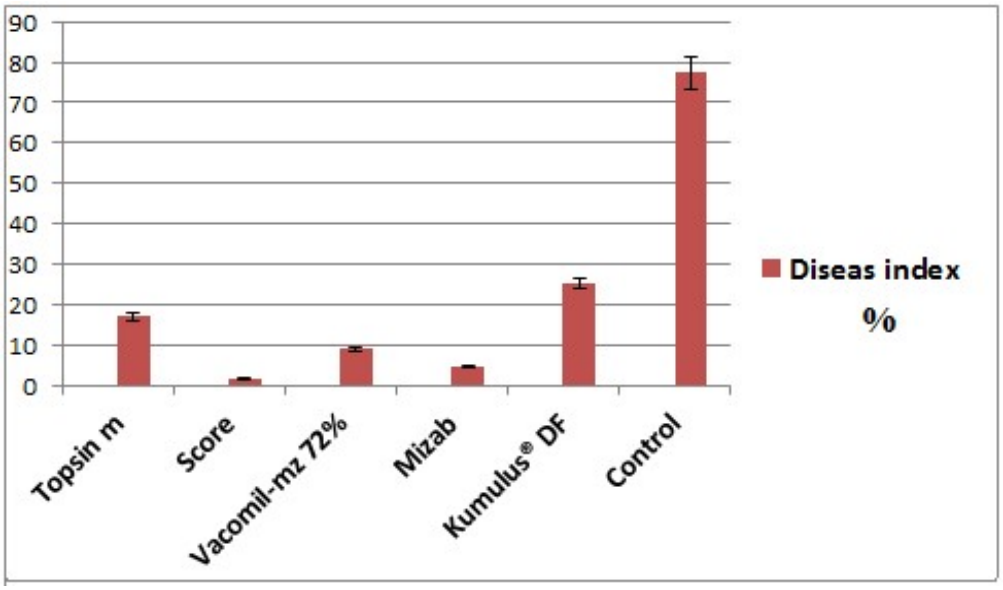

Figure (3): Effect of fungicides on date palm fruit disease caused by *N. sphaerica*

5. Conclusions

The result of this study indicates that the fungus *N. sphaerica* was the causal agent of date palm fruit rot disease. Morphological and Molecular identification was identical of *N. sphaerica* fungal associated with date palm fruit rot disease, ITS DNA sequencing revealed their identities. Pathogenicity experiments on ten cultivars of fruits (Zahdi, Barhi, Bream, Khadrawi, Shukar, Khasab, Sayer, Hillawi, Leilwi and Deri)

and The role of moisture content and tannins have been studied, which have a role in infecting susceptible varieties of the disease, and the ability of Score and Mizeb S fungicides reduced date palm fruit rot disease index on the wounded date fruits.

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