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Isolation of Pathogenic Fungi (*Alternaria alternata, Fusarium oxysporum, and Penicillium sp.*) Associated with Palm Tree Diseases in Bani Waleed

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Abstract: This study aimed to identify and isolate pathogenic fungi from palm trees (*Phoenix dactylifera* L.) in Bani Walid. Samples were collected and examined in a laboratory at Bani Waleed University. Samples were sectioned into 0.5–1 cm pieces, washed and sterilised with 5% sodium hypochlorite for 3 minutes, rinsed three times in sterile distilled water, and dried on sterile filter papers. Pure cultures of the resulting fungi were obtained from subcultures on sterilised Potato Dextrose Agar and incubated at 25 ± 2 °C for 4-5 days. Fungal colonies were identified morphologically and microscopically according to taxonomic standard procedures. Three fungal species were identified (*Alternaria alternata, Fusarium oxysporum,* and *Penicillium sp.*), and their presence was found to be associated with leaf spot symptoms, al'bayoud, root rot, and other diseases in palm trees. To assess the susceptibility of palm seedlings to fungal infection, two varieties of date palm seeds (Dagla and Saidi) were inoculated with fungal suspensions ($1x10^6$ conidia ml) obtained from diseased palm samples. Results showed that fungi caused disease infections on inoculated seedlings, indicating the potential for further research.

Keywords: (Date palm frond, seedlings, fungi, palm diseases, *Phoenix dactylifera* L.)

Introduction

The cultivation of date palms (*Phoenix dactylifera* L.) is widespread in the Libyan Sahel regions, which are considered one of the most suitable areas for producing excellent varieties of dates due to high rates of rain, humidity, and low temperatures. The southern regions of Libya are considered the most productive areas for dry dates [3], due to the palms' adaptability to high temperatures, drought, salinity, and other challenging environmental conditions, they grow in sandy, calcareous, and saline soils [34]. The number of palm species worldwide is 2,500, including more than 2,000 in the Arab world (about 600 in Iraq, more than 400 in the Kingdom of Saudi Arabia, more than 450 in North Africa, and 392 in Libya). The rate of date production and cultivation in Libya has increased in recent years, estimated at 188.6 thousand tons in 2016, compared to 85 thousand tons in 1990 [31]. Date palm trees in Libya have grown extensively and increased from 24.6 thousand hectares in 1990 to 36.5 thousand hectares in 2016 [46]. Libya produces 2.93% of the production of the Arab countries, as its production reached 133.5 thousand tons during the year 2002 [14]. The date palms are infested with disease-causing microbes and affected by various pests [20]. Date palms are infested by agricultural insects with up to 280 pests, including various insect pests and bacterial and fungal pathogens [48]. Fungal infections of fruits and palm trees caused significant crop losses in the agricultural industries [8]. One of the most important obstacles that can limit the expansion of palm cultivation are various plant diseases. Therefore, this research was conducted for the purpose of identifying the most important plant diseases that affect palm trees in Bani Walid, Libya.

2. Materials & Methods

2.1. Plant sample collection and diagnosis:

Samples showing symptoms of diseases were collected from palm trees on different farms in Bani Walid. The parts that showed symptoms of disease were cut using sterilised pruning shears. The samples were placed individually in sterile polyethylene bags and brought to the laboratory of the Plant Protection Department

at the Faculty of Agriculture, Bani Waleed University. An initial examination was conducted in order to determine the cause of the disease, whether the pathogen was fungal, insect, or other.

2.2. Culture and media of fungi

For culturing fungi, the culture media of Potato Dextrose Agar (PDA) was used aseptically according to the manufacturer's instructions to culture fungal isolates. The antibiotic chloramphenicol was mixed with PDA for sterilisation, a few drops per litter.

2.3. Isolation and identification of fungi

Infected palm frond specimens were sectioned with sterile scissors into 0.5–1 cm pieces. The specimens were washed with tap water, placed in a sterile petri dish, and sterilised with 5% sodium hypochlorite for 3 minutes using sterile forceps. The pieces of samples were rinsed three times in sterile distilled water and dried on sterile filter paper. Five replicates were used for each palm frond sample. The samples were transferred to 9-cm petri-sterile dishes containing sterilised potato dextrose agar (PDA), 4-5 pieces per plate. The plates were incubated at 25 ± 2 °C under light for 4-5 days. Colonies of different colors and shapes were observed on the plates 5 days post incubation. Pure cultures of fungi colonies were obtained by transferring and subculturing the fungal hyphae onto new petri dishes containing PDA. Isolates were then incubated at a temperature of 25 ± 2 °C for 5-7 days. Isolated fungi were identified according to the approved taxonomic criteria described in relevant literature [11], [35], [36], [39].

2.4. Testing the susceptibility of palm seedlings (Al-Daqla and Al-Saidi varieties) to infection with isolated fungi

To test the susceptibility of palm seedlings to the fungal infection of *Alternaria alternata* and *Fusarium oxysporum*, two varieties of date palm seeds, namely Dagla and Saidi, were planted in pots containing sterilised peatmoss soil. The seeds were sterilised with 30% alcohol for 10 minutes and soaked in water for two days before planting. The seeds of each variety were planted separately, with 5 seeds per pot and 10 replicates for each variety. The pots were placed under the shade of a tree until the seeds germinated with regular irrigation. After 30 days of planting, all the planted seeds of both varieties grew, and the seedlings were preserved for use in fungal tests.

2.4.1. Fungal isolates and culture

Fungal cultures were grown on Saborauds Dextrose Ager with yeast extract (SDAY) and incubated at 27 °C under light for 7 days. The suspensions of *Alternaria alternata* and *Fusarium oxysporum* were prepared by adding 10 ml of distilled water to a petri dish containing pure fungal culture and then filtering with a piece of sterile gauze.

Fungal spores were collected by tapping them over a clean glass funnel into 30 ml sterilised glass-capped vials. The spores were then air-dried for 12 hours overnight in a safety cabinet with air flow.

The suspensions of fungi were prepared by adding 5 mg of fresh dry spores to 5 ml of distilled water to the final concentrations. Fungal suspensions were diluted to final concentrations of $1x10^6$ conidia/mL. Final conidia concentrations were determined by direct count using a hemocytometer.

2.4.2. Plant inoculation with fungal suspensions to evaluate pathogenicity

The pathogenicity experiment was conducted for *Alternaria alternata* and *Fusarium oxysporum* on two varieties of three-month-old date palm seedlings. Palm seedlings were injected at the base of the stem with a suspension of fungal spores at a concentration of $1x10^6$ conidia ml using a medical syringe. Five replicates were used per treatment and fungal isolate. Control treatments were injected with sterile distilled water. The seedlings were covered with plastic bags for three days post-inoculation to provide appropriate humidity. Inoculated seedlings were kept under light at room temperature (24–27 °C). The pathological symptoms of each fungus were observed 15, 30, 45, and 60 days post-inoculation with both fungi. The dead seedlings were re-isolated to confirm the infection by the same isolates that were used for the inoculation experiments.

3. Results and discussions

3.1. Phenotypic morphological examination:

An initial morphological examination of the infected plant samples was performed, and the results showed that the infected fronds turn pale yellow from the base of the leaf to the top with drooping leaflets, giving them the appearance of a wet feather and dwarfing, malformed fronds. The leaves were dissected transversely, and the anatomical results showed a reddish-brown coloration of the internal tissues with black spots (Fig. 3.1.c). Culturing the diseased samples on sterilised potato dextrose resulted in colonies of different

colors and shapes being observed on the plates 5 days post-incubation.

Fig 3.1. samples of palm fronds showing leaf spot symptoms.

3.2. Microscopic examination of isolated fungi from infected palm trees

Light microscope results showed three fungi associated with the symptoms of leaf blight in palm trees (*Alternaria alternata, Fusarium oxysporum, and Penicillium sp*) isolated from samples of infected palm fronds obtained from different farms in Bani Walid (Figs. 3.2, 3.3, and 3.4). All isolated fungi showed germination and growth on PDA culture media after 3-5 days of isolation. Colonies of different colors and shapes were observed on the plates. Light microscope results showed that the isolated fungi produced conidia and a single short germ tube after 3 days of isolation, and all fungi showed extensive mycelial growth of germinated spores after 5 days post-isolation. The description of fungi under a light microscope was as follows:

3.2.1. *Fusarium oxysporum* f.sp. albedinis

This fungus has many strains of economic importance across various crops, some of which are more ferocious than others. This fungus is characterised by the formation of three different types of spores: The first type is small conidia consisting of one or two cells at most, and large conidial spores are characterised by being crescent-shaped and consisting of three or more cells. The third type is chlamydial, which is characterised by having thick walls and may be peripheral or interstitial [6], [7], [16], [17], [22], [39].

This study has observed that pure colonies of *Fusarium* sp. grew on infected palm fronds 5 days post-incubation (Fig. 3.2.c, d). A pure colony of *Fusarium* sp. was obtained by transferring and subculturing fungal hyphae onto new petri dishes containing PDA, and the fungus grew, forming a cottony shape with a white color (Fig. 3.2.b).

Microscopic examination showed the conidia spores were single and crescent-shaped with one or two cells, while the large macroconidia spores were divided into three to five septa (Fig. 3.2.a). Chlamydial spores were observed singly or in short, terminal, or brown chains. These characteristics of the fungus match those that cause Fusarium wilt [6], [18], [26], [30], which is one of the most important diseases of palm trees. Its symptoms appear on the affected fronds, which turn gray and white. This disease can cause deterioration in the affected palm trees and cause them to dry out within a limited period of time. These results are consistent with what was mentioned by [15], [18]. This disease affects the leaves of trees, turning them white; for this reason, local farmers called it (al' bayoud).

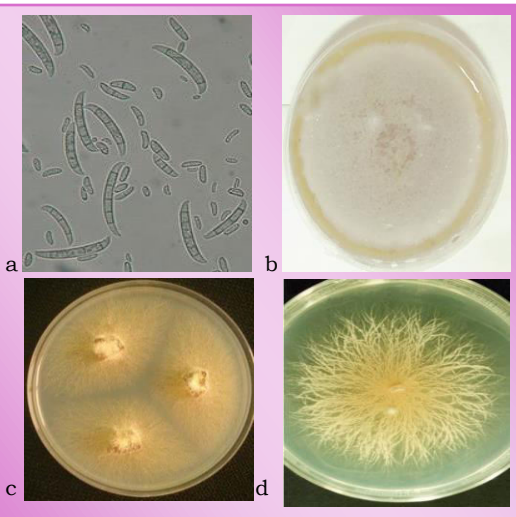

Fig 3.2. (a) Fungal spore under a light microscope. (b) Pure colonies of *Fusarium oxysporum* re-isolated from inoculated seedlings with fungal suspensions ($1x10^6$ conidia ml) obtained from diseased palm samples. (c, d) *Fusarium* sp. grew on infected palm fronds grown on PDA 5 days post-incubation.

An examination of the roots of infected palms was performed, and the results revealed that their color had changed to brown. Also, when infected leaves were dissected, the results showed that the color had changed from inside to brown and became black.

3.2.2. *Alternaria alternata*

Microscopically, it was observed that the mycelium was divided into transverse and longitudinal septa. The conidial spores are pear-shaped, brown to dark olive in color, and branched with numerous buds that carry the conidial spores in long chains (Fig. 3.3.a). The fungus grows very quickly on infected palm fronds, forming black colonies with round edges (Fig. 3.3.b). These descriptions are consistent with many research studies [23], [37], [38], [41]. *Alternaria* species can be broadly split into two categories: large-spore species and small-spore species. One of the small-spore species is A. alterata, and there are a wide variety of presumed host-specific plant pathogens that have been described as pathotypes [9]. This fungus causes wilt, root rots, fruit rots, and brown leaf spot disease in date palm trees [4], [8], [41], [44]. The fungus produces a short, dark-colored carrier that carries conidia in branched chains or singly on infected tissue. In general, the conidial spores have a long tip [8].

Fungus spores are spread by wind or rainfall, and the germination of conidial spores occurs when moisture is available. Dry environmental conditions with wind allow the conidia spores to be released and spread from infected plants to healthy ones. The availability of moisture is necessary for the fungus to germinate, and the optimum temperature that allows conidial spores to germinate is more than 20 °C [4]. Fungal spores attack old frond leaves or damaged fronds through wounds, and fungal mycelium is formed in the affected tissues. The conidial spores formed by the fungus are dark in color and divided into transverse and longitudinal walls [23]. Symptoms appear on the leaflets in the form of brown bands with a dark edge, covering a large area of the leaflets. The size of the spot ranges from 5–15 cm.

Fig. 3.3: (a) Conidia of *Alternaria alternata* isolated from infected palm fronds grown on PDA. (b) colony of *Alternaria alternata* grown on infected palm fronds. (c) *A. alternata re*-isolated from inoculated seedlings with fungal suspensions ($1x10^6$ conidia ml) obtained from diseased palm samples.

3.2.3. *Penicillium* sp.

The fungus was isolated from infected palm fronds grown on PDA (Fig. 3.4.a). The results showed that *Penicillium* sp. exhibits rapid growth and invades the petri dishes in about 4 days post incubation.
A pure colony of *Penicillium* sp. was obtained by transferring and subculturing fungal hyphae onto new petri dishes containing PDA (Fig. 3.4.c).

Microscopic results showed that *penicillium* sp. produces multiple spores on long stalks called conidiophores (Fig. 3.4.b). Most species of *Penicillium* appear yellow, greenish, and blue on various agars [31] (Fig. 3.4.a).

Penicillium sp. causes soft roots on injured date fruits. It is widespread in North Africa and other countries. Fungal spores enter the fruit tissue through injuries on the surface. The spores can spread from infected dates to healthy ones.
It was reported that *Penicillium* sp., isolated from the soil surrounding the roots of the date palm, was found to be antagonistic against *Fusarium oxysporum* f. sp. albedinis, thus offering protection to date palms from the disease [7]. In addition, [25] mentioned that Penicillium sp., isolated from diseased

date palm samples, was classified as a non-pathogenic fungus. However, [27] stated that *Penicillium expansum* causes postharvest blue mold in fresh date palm fruit. [5] reported that *Penicillium* sp. has been reported to cause fruit rot on date palms.

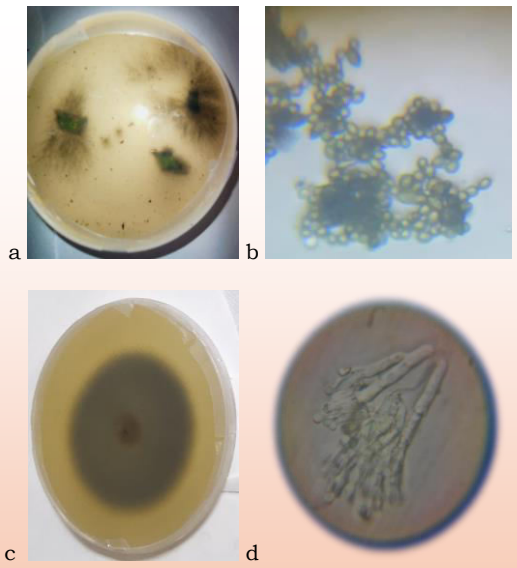

Fig. 3.4: (a) colony of *Penicillium* sp. grew on infected palm fronds. (b, d) *Penicillium* sp. slide showing conidia spores. under microscope. (c) Pure colonies *Penicillium* re-isolated and grown on PDA.

3.3. Testing the susceptibility of palm seedlings (Al-Daqla and Al-Saidi varieties) to infection with isolated fungi.

Planting two varieties of date palm seeds (Dagla and Saidi) resulted in 100% germination of both varieties after 30 days. Inoculation of three-month-old palm seedlings with the pathogenic fungi *Alternaria alternata* and *Fusarium oxysporum* showed the production of leaf spot symptoms 30 days post-inoculation with the fungi. The symptoms of inoculated palm seedlings were brown discoloration at the bases of the stems, wilting of the seedlings, and longitudinal spots with black edges 5–10 mm and 3-5 mm in diameter (Fig. 3.1.e). Therefore, an additional experiment was conducted by re-isolating the dead seedlings and examining the fungi from the inoculated seedlings, and the microscopic results showed the same fungi used in the infection process (Figs. 3.2.b, 3.3.c).

The variation in fungal species in their infection and the occurrence of spot diseases is due to the variation in their ability to produce cellulose and quinine hydrolytic enzymes, as well as to the variation in their ability to produce mycotoxins [2], [29].

Conclusion

In this study, three fungi, *Alternaria alternata, Fusarium oxysporum*, and *Penicillium sp.*, were isolated from samples of infected palm fronds obtained from different farms in Bani Walid. Many studies have shown that these fungi are pathogenic for date palms and are associated with leaf spot symptoms in palm trees [4], [8], [13], [41]. It was mentioned that *Alternaria alternata* is one of the most widespread fungi in the field on palm trees; it causes fruit rot disease [41], [44], [45].

[5], [45], [12] reported that species of *Alternaria*, *Fusarium*, and *Penicillium* have been reported to cause fruit rots of date palm and date palm leaf spot disease. *Alternaria* sp. attacks fruits before maturity, whereas *Penicillium* sp. and *Fusarium* sp. attack dates after harvest [12]. [28] analysed the fungal microbial load of date fruit in the pulp and peel. They have reported that *Alternaria* and *Penicillium* species were the most abundant genera in both parts of the date fruit. [1] stated that *Fusarium sp.* causes palm pollen disease. [40], [47] studied palm diseases in different cities in Libya, including Sabha, Wadi Al-Shati, Murzuq, and Benghazi. They reported that these fungi cause diseases in palm trees. [4] studied palm varieties in Oman and reported many of their fungal diseases, including black blight, diplodia, inflorescence rot, and leaf blight diseases. Isolated fungi from infected palm trees showed symptoms of leaf spot disease, starting with light brown, and over time, with the development of the disease, they turned to dark brown and black spots [10], [32]. In conclusion, this study has reported that both isolated fungi caused disease infections on inoculated seedlings, indicating the potential for further research.

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