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Pathogenicity investigation of contaminated fungal isolates of tissue culture on date palm seedlings *(Phoenix dactylifera* L.*)* in the greenhouse

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Abstract

This study was carried out in the laboratories and greenhouse of the date palm research center at the University of Basrah. The aim of this study is to evaluate the pathogenicity of fungi and their filtrates that cause tissue culture contamination on seedling of date palm under greenhouse condition. The results of the isolation fungi from date palm tissue cultures showed that there were many fungal contaminations, which included *Alternaria alternata*, *Fusarium* sp., *Aspergillus niger*, *As. fumigatus*, *Cladosporium herbarum*, and *Rhizopus stolinfer*. Also the results showed that the most frequent fungus was *Alternaria alternata* with 33%, followed by *Fusarium* sp.,with 26%, and the least frequent fungus was *Rhizopus stolinfer* with 1%. The pathogenicity assay of these isolated fungi on date palm seedlings showed that all of these tested fungi were nonpathogenic against these plantlets, except the isolates of *A. alternata,* which recorded a 28%, followed by *Fusarium* sp., with a 16%, while the other isolates were not recorded. The efficiency of fungal surpeninat on date palm seedlings showed the superiority of the fungal surpeninat of *A. alternata* and *Fusarium* sp. Other fungi recorded discoloration symptoms on the roots of date palm seedlings by about 12 and 8%, respectively. The study's findings indicate that these polluting fungi have pathological effects on tissue culture growth, but the greatest effect of fungal contamination is concentrated on their secretion of toxins and other metabolic products, which also compete for nutrients in the tissue culture medium, resulting in weak growth and deterioration of tissue growth.

Keywords: Date palm, Tissue culture technique, fungal contaminations, pathogenicity.

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Introduction

The technique of plant tissue culture contributes greatly to obtaining large numbers of plants to be propagated, especially rare ones, within a short period of time, providing an economic return for workers in this field. This technique is characterized by the fact that tissue-propagated plants are free from the presence of various pathogens, especially viruses, as well as having identical genetic characteristics to the source from which they were taken. Also, high-quality varieties can be produced by growing them on special culture media (Al-Gamide, 1995; Al-Wasel, 2002). Contaminating of tissue cultures is one challenge facing workers in this field, as it leads to the failure of the growth of large numbers of tissue cultures because of the damage caused by these contaminated microorganisms through the secretion of toxic substances or enzymes secreted, as well as their competition for vegetative growth in the elements. In addition, some of these contaminated microorganisms are pathogenic or can intrude on tissue growths, which leads to damaging the growths of plant tissues. The sources of contamination for tissue cultures are multiple, the most important of which are the cultivated part, the culture vessels, the tools used in preparing the cultured part, the hands of the workers, or the materials and tools used in the preparation laboratory, such as the culture media and the transplanting room (Leifert and Cassells, 2001; Sharaf-eld and Weathers, 2006; Tanprasert and Reed, 1997).Fungal contamination represents 10–35% of the sources of microbial contamination. Several fungal species were isolated from contaminated nutrient media after the process of cultivating plant parts. A study conducted on six different date palm cultivars, Umm Al-Dahn, Al-Shuwaithi, Al-Brem, Al-Barhi, Al-Halawi, and Al-Sayer, in the stage of embryonic callus production found that the majority of the fungal contamination isolates were *Alternaria alternaria*, *Fusarium* sp., and *Aspergillus* sp. (Hameed and Abass, 2006). In the study conducted by Abass et al. (2007), they were able to isolate many fungal genera such as *Alternaria alternate*, *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp., and the percentage frequency of these fungi was about 21, 32, 54, and 45%, respectively. A recent study by Jassim et al. (2021) showed there are several fungi contaminating the cultures formed from the callus of date palm that were isolated, and these isolates include *Alternaria alternata*, *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp., and their frequency rates were 45, 32, 17, and 6%, respectively. The objective of the study was to

evaluate the pathogenicity of contaminated fungi and their filtrates in the field against the growth of offshoots of date palm.

Material and Methods

Isolation and identification of fungi contaminating tissue cultures

Containers of contaminated tissue cultures were brought from the tissue culture laboratory at the date palm research center. A single spore method was adopted to isolate and diagnose contaminated fungi from tissue cultures. Parts of the contaminated growth were separately suspended in 10 ml of distilled water and mixed well for 15 minutes and vortexes. Suspension was serially diluted from 10^{-1} to10^{-6}. 0.1 ml was pipette out onto sterile Petri dish (diameter 9 cm) containing 20 mL of sterile Potato dextrose ager (PDA) medium(the antibiotic tetracycline was added at a rate of 250 mL. L) before the solidification of the medium stirred the mixture to homogenize inside the dish. All petri dishes were incubated at 27 °C for 72 hours. Then each fungal colony was purified by being transferred to a Petri dish containing the sterile PDA medium, and incubated at the same temperature. After 5-7 days of incubation, the percentage of the frequency of each fungus was calculated according to the following equation: % of frequency = [number of fugue appearances / total number of fungal isolates] x 100. The isolated fungi diagnosed according to the keys of Barnet and Hunter (2005) and Ellis (1971). Fungi were maintained on of PDA medium and kept in the refrigerator until use.

Inoculum preparation and pathogenicity.

The broth medium consisting of potato extract and dextrose(PD Broth) was prepared. Antibiotic chloramphenicol was added at a rate of 250 mg/L. The broth medium was autoclaved at 105 °C/cm^2 for 20 minutes. After the temperature of the culture media decreased, each flask was inoculated with a 0.5 cm disc taken from the edge of the colony of the purified fungi at the age of 5-7 days. The flasks were incubated at 27°C with shaking every 72 hours to allow the fungal inoculum to spread. After twenty days, the flasks were taken out, and fungal suspensions were filtered for each fungus using whatmman-No4 filter paper. The filtrate of isolated fungi was repeated after 48 hours. The filtrates are kept in the refrigerator until used in the subsequent experiments. Soil was prepared, consisting of a mixture of peat moss and mixed soil in a ratio of 1:2, sterilized with an autoclave and re-sterilized after 72 hours. The sterilized soil was placed in sterilized plastic pots (2 kg.). Seeds of Halawi cultivar were taken and washed with running

water. Then sterilized with a 10% commercial sodium hypochlorite solution for three minutes, and washed several times with sterile distilled water to get rid of the traces of the sterilized substance. The sterilized seeds were placed in a small sterile plastic container. A little sterile distilled water was added to it, and covered with a layer of cotton. and All containers were placed in the incubator at 28–30 ^{o}C. Add sterile distilled water when need it to prevent drying until seeds germination. The germinated seeds were transplanting into pots containing sterilized soil, with five seedlings for each pot. All pots were watered with sterile distilled water, And placed under the conditions of the greenhouse until there were two leaves on the plantlets (the seedling age is approximately six months). Pots were arranged into two groups. First, irrigated with the fungal suspension inoculum at a concentration of 1.6×10^{6} spores/ mL. Second, pots were irrigated with the fungal filtrate, control treatment(without add any fungus) was irrigated with sterilized water (60 mL per pot). For 60 days, the fungal inoculum and their filtrate were added to each fungus treatment (three pots for each treatment) and recorded the changes that occurred on seedlings (Al-Ani et al., 2012).The infection percentage was calculated according to the following equation: [Number of healthy seedlings–Number of infected seedlings/Number of healthy seedlings] x 100. The disease severity was recorded according to the Abduo et al. (2003) scale, which consists of five degrees: 0 = healthy plants, 1 = discoloration of 1-25% of the total roots, 2 = discoloration of 26–50% of the total root, 3 = discoloration of 51–75% of the root system with wilt of half of the leaves, 4 = 100% of the root system and wilting of all leaves. The disease severity was determined using the following formula: Disease severity (%) = [Σ(disease rate × number of plantlets with same rate) / total number of plantlets × maximum value of disease scale] × 100

Results

Isolation and identification of fungal contaminated tissue cultures

The results of isolation and diagnosis revealed that there are many fungal associated with contaminated tissue culture growth. The fungi were diagnosed after purification on PDA media depending on the morphological and physiological characteristics according to Ellis (1971) and Barnett and Hunter (2005). The result showed that the most frequent fungus was *Alternaria alternata* with 33%, followed by *Fusarium* sp. about 26%, *Aspergillus flavus* about 17%, *As. niger* about 14%, and the least frequent fungus was *Rhizopus stolonifer* about 1.7% (Fig. 1and Table 1). According to the infection severity test, all isolated fungi are weak or nonpathogenic. *A. alternata* and *Fusarium* sp., had the highest percentages of infection, at 12 and 8%, respectively,

and the severity of infection reached one degree, according to the degree of infection scale, while the remaining fungi had rates of infection ranging from 0–2.1%. The fungus *Aspergillus* sp. (verscolor section strain) also showed an improvement in the growth of palm seedlings by 1.8% compared to the control treatment (without adding fungi). The results of fungal filtrates test on those seedlings showed a weak effect of these filtrates on its growth., t The presence of colored areas on parts of the roots of those seedlings was appear. The most effect fungus al was *A . alternata*, *Fusarium* spp., and *A. flavus* with percentages of colored areas on the roots about 28, 20 and 18%, respectively(Table 2). *Aspergillus* sp (verscolor section strain) caused an improvement in the growth of seedlings treated with fungal filtrate compared to the control treatment.

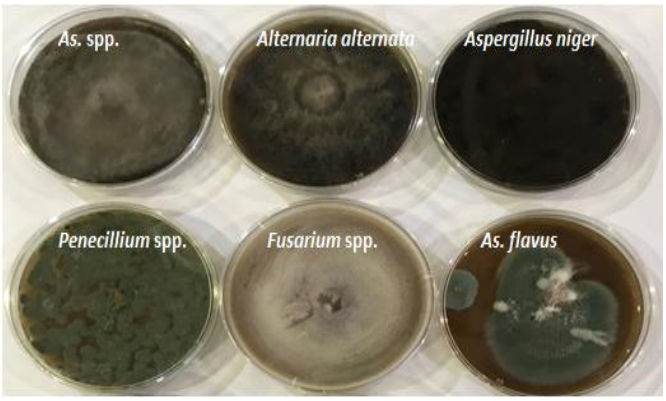

Fig. 1. The most contamination fungi that isolated from date palm tissue culture

Table 1: The percentage frequency of contamination fungal isolates from tissue culture of date palm

Contamination Fungal	% fungal Frequency
Alternaria alternate	33
Fusarium sp	26
Aspergillus flavus	17
Aspergillus niger	14
Aspergillus sp	2.8
Penicillium sp	3.2
Cladosporium herbarum	2.3
Rhizopus stolonifer	1.7

Table 2: Pathogenicity test of isolated fungi on date palm seedlings under greenhouse conditions.

Contamination Fungal Isolates	% infection fungal	Infection degree	% disease incidence	% root discoloration
Alternaria alternate	12	1	16	28
Fusarium sp.	8	1	14	20
Aspergillus flavus	2.1	1	0	18
Aspergillus niger	1	1	0	4.6
Aspergillus sp.	0	0	0	0
Penicillium sp.	0	0	0	0
Cladosporium sp.	0	0	0	0
Rhizopus stolonifer	0	0	0	0

Discussion

The results of the study indicated that the most isolation and diagnosis of many fungi was accompanied with tissue culture growths caused damage, death, as well as rotting.. The results study agree with many other researchers. Hameed and Abass (2006) found that many fungi that were isolated when they are studying six cultivars of date palms, namely Umm Al-Han, Al-Shuwaithi, Al-Brim, Al-Barhi, Al-Halawi, and Al-Sayer, these fungi are *A. alternate*, *A. niger*, and *As. clavatus.* Also, Abass (2007) found that the most contaminating fungal isolates for tissue cultures of date palm are *A. niger*, *Penicillum* sp., and *A.alternata*, and the percentages of these fungi are about 27, 25, and 18%, respectively. In a recent study, found that the most fungal contaminants of date palm tissue cultures were *A. alternate*, *Fusarium* sp., *Aspergillus* sp., and *Penicillum* sp. with frequencies of 45, 32, 17 and 6%, respectively (Jassim et al.2021). The results study carried out by Odutayo et al. (2007) on a number of tissue cultured plants, namely cassava (*Manihot esculenta*),banana(*Musa paradisiaca*) and kenaf (*Hibiscus cannabinu*), showed that there are number of fungi isolated from various sources inside the laboratory, including tools used in dissection, samples, development, and others, and most of the isolated fungi were *Al. alternate*, *As. niger*, *As. fumigatus*, *F. oxysporum*, *Cladosporium* sp, *Rhizopus nigricans*, and *F. culmorum.* Fungal pathogens differ in their ability to cause infections to plants through secrete of different chemical compounds, such as enzymes and the production of toxins, growth regulators or complex sugars. These compounds determine the ability of the pathogen to cause disease to the plants ((Agrios, 2005).There are several species related to the *Alternaria* genus may be found

growth in different places all over the world. However, opportunistic infections make up a major part of it. Plants that are economically important, such as legumes, vegetables, and fruits, are susceptible to a variety of diseases caused by *A. alternaria*(Agrios,2005). The ability of *A. alternata* to cause diseases is due to the producing of many metabolic compounds such as enzymes and toxins. There are several specialized enzymes that make the fungus highly pathogenic to many plant hosts. These include extracellular degradation enzymes, which include the enzymes protease and cellulose (Barka-Golon, 2008; Noser et al., 2011).The important pathogenic toxins produced by the fungus *Al. alternaria* include alteranol (AOH), alteranol monomethylether (EME), AM-toxin, AF-toxin, and ACT-toxin (Guo et al., 2019; Ito et al., 2004). The results of this study are consistent with the findings of many researchers regarding the ability of the fungus *Al. alternata* to cause many diseases on several plants. According to Goetz and Dugan (2006), *Alternaria* spp. are harmful to coniferous plants and can be found in their rhizospheres. Pomerleau and Nadeau(1960) identified Alternaria spp. fungi as the culprit for the withering of 20–65% of fir seedlings in nurseries. Similar results were documented by James and Woo (1987), who discovered that seedlings of coniferous plants treated with *Alternaria alternate* totally wilted away. Many studies indicated that the causes of leaf spot diseases on date palms are numerous; however, the fungal genus *Alternaria alternata* was identified as one of the most important causative pathogens(Al-Zubaidi, 2005; Ahmed, 2011). A recent study revealed that after artificial inoculation with the pathogen, the seedling of date palms presented symptoms typical of Alternaria leaf spots, which were identical to those in the natural field (Jassim, 2017). *Fusarium* spp., are characterized as being pathogenic to many plant families, causing various diseases such as root rot, Damping off seedling, and wilt diseases. (Hadi, 2019; Dia et al., 2004). *Fusarium* spp., grows well in culture media rich with sugar substances. Blesa et al. (2008), they showed that the presence of sugar in the culture medium makes the fungus stimulate the production of T2-toxin, and the presence of starch stimulates the fungus to produce the enzymes amylase and cellulase (Bluhm and Woloshuk, 2005; Di-Pietro et al., 2001). Therefore, the presence of various of *Fusarium* spp., as contaminated fungus in the culture medium used in tissue culture technique to compete with tissue growth for nutrients, in addition to the secretion of various enzymes and toxins (Abass et al.2007). Finally, many vitro pathogens limit growth and/or kill plants growing *in vitro* just by altering the growth medium composition. Even harmful organisms *in vivo* are assumed to affect tissue cultures mostly through their metabolism in the media rather than direct parasitism of plant tissue (Leifert and Cassells, 2001).

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أختبار أمراضية الفطريات الملوثة المعزولة من الزراعات النسيجية في بادرات نخيل التمر(*Phoenix dictylifer*a L.) في ظروف البيت البلاستيكي

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الخلاصة

أجريت الدراسة في مختبرات والبيت البلاستيكي لمركز ابحاث النخيل– جامعة البصرة. هدفت الى تقييم امراضية الفطريات الملوثة المعزولة و رواشحها على بادرات نخيل التمر في الاصص البلاستيكية. أوضحت نتائج العزل والتشخيص الى وجود العديد من الفطريات الملوثة للزراعات النسيجية لنخيل التمر وكان اكثر الفطريات ترددا الفطر *Alternaria alternata* بنسبة بلغت 38% تلاه الفطر spp *Fusarium* بتردد بلغ 2% اما اقلها ترددا فهو الفطر *Rhizopu stolonifer* بنسبة بلغت 1% . اظهرت نتائج اختبار المقدرة الامراضية للفطريات المعزولة ورواشحها على بادرات نخيل التمر ان جميعها ضعيفة أو غير ممرضه تجاه بادرات النخيل المزروعة في الاصص باستثناء عزلة الفطر *Alternaria alternata* فقد سجلت مقدرة امراضية بلغت 28% تلتها عزلة الفطر spp *Fusarium* بنسبة بلغت 16% في حين لم تسجل بقية العزلات الفطريه اية تاثيرات امراضية على بادرات النخيل . بينت نتائج اختبار تاثير الرواشح الفطرية على بادرات نخيل التمر الى تفوق الراشح للفطرين *A. alternata* و spp *Fusarium* على بقية الرواشح الفطرية الاخرى حيث اظهرت اعراض تلون على المجموع الجذري لبادرات النخيل بنسب مئوية بلغت 12 و8% على التوالي من المجموع الجذري. يتضح من نتائج الدراسة الى وجود تاثيرات امراضية لهذة الفطريات الملوثة على النموات النسيجية الا ان التاثير الاكبر افرازها للسموم والمنتجات الايضية الاخرى وتنافسها على العناصر الغذائية الموجودة في الوسط الزرعي المستخدم في الاستزراع النسيجي مما يؤدي الى ضعف وتدهور النموات النسيجية.

الكلمات المفتاحية: نخيل التمر، تقانة زراعة الانسجة، التلوث الفطري، الامراضية.