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REVIEW ARTICLE

Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi tissue culture laboratories

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Abstract

The date palm is one of the most important economic species of the palm family, grown mainly for its fruits (dates). Nowadays there is increased demand for date palm fruits around the world. To meet this demand, several propagation methods have been utilized, among them micropropagation which has been used in Iraq and many other countries for large-scale multiplication of date palm. Micropropagation faces several constraints; one is microbial contamination which represents a major challenge to the initiation and maintenance of date palm micropropagation laboratories. In recent years, two major groups of contaminants have been identified and isolated from different date palm tissue culture laboratories in Iraq. The first group is fungi. Several fungal species have been isolated and identified as contaminants; most predominant are: *Aspergillus niger*, *Alternaria alternata* and *Penicillium* spp. The second group is bacteria; predominantly of the genera Bacillus, *Staphylococcus* and *Proteus*.

Key words: Date palm, In vitro, Microbial contamination, Tissue culture

Introduction

Date palm is one of the most popular fruit trees in Middle Eastern countries. World production of dates was estimated to be more than 7.5 million metric tons in 2009, from a total harvested area of 1.3 million ha. Most of the world's production of dates is provided by countries in the Middle East and North Africa (FAO, 2011).

Two different explanations have been proposed to explain the meaning of the scientific name of the date palm (*Phoenix dactylifera* L.). The first is from the Phoenician term "Phoenix" which means date palm; while the species name "dactylifera" is derived from the Greek word (daktous) which means fingers (Linne, 1734). The second explanation is based on the mythological Egyptian bird, the Phoenix, said to live for more than 500 years, and then is reborn after a fire. Regarding the species name meaning, this is derived from Hebrew word "dachel" and describes the fruit shape (Popenoe, 1938).

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Nutritional value of dates

Dates are a good source of energy and are rich in nutrients. Date fruits consist of 70% carbohydrates; mostly as sugars, and 15-30% water. Dates are also a source of different minerals including iron, potassium and calcium, with low levels of sodium and fat (Al-Rawi et al., 1967; Thabet et al., 2010; Dayani et al., 2012).

Date palm production and consumption in Iraq

Iraq is one of the top ten date producers in the world; between 1991 and 2001 Iraq contributed a total of 7.5% of world date production (FAO, 2011).

Date palm has been of socioeconomic importance to the population of Iraq for a very long time. Many traditional and new date industries are widely distributed in Iraq, especially in the Middle and Southern regions of the country. Most of these industries are entirely dependent upon dry and soft date types. A majority of the dates produced are consumed locally because of an increased domestic demand for date palm fruit. Not only is the date palm fruit of economic importance, but date palm leaves are used in traditional industries to make products such as mats, fans, baskets and crates (El-Hadrami and Al-Khayri, 2012).

Propagation of date palm

Seed propagation

The oldest method of date palm propagation is by seed, also known as a sexual propagation. This method is not ideal for propagation because of the nature of date palm itself. Date palm is a dioecious species (male and female flowers are borne on different plants), meaning that the half of the progeny is male and the other half female (Jain, 2012). In addition, the quality of fruits produced by female seedling trees is of inferior quality, as compared to clonal plants. Finally, another disadvantage is the heterozygous nature of seedling date palms, which exhibit a high level of variation within progeny (Mater, 1986).

Off shoot propagation

A second method of propagation is by means of offshoots, asexual or vegetative propagation, which is true-to-type to the parent palm and with the capacity of flowering 2-3 years earlier than seedling date palms. The problem with this method of propagation is that each palm produces a limited number of offshoots, borne only at the early stage of life (10-15 years) after planting (Popenoe, 1938; Jain, 2012).

Tissue culture

The third procedure is the tissue culture propagation, also referred to as micropropagation or *in vitro* multiplication (Aaouine, 2003; Al-Khayri, 2005, 2007). The importance of this technique can be attributed to several advantages, including: (1) large scale of production (mass production), (2) no seasonal effect on the propagation procedures, because the entire propagation procedures are done under controlled conditions in the laboratory, (3) production of healthy females (disease and pest-free) and (4) production of genetically uniform plants, i.e. true-to-type (Mater, 1986; Omer et al., 1992; Al-Ghamdi, 1993; Letouze et al., 1998; Al-Wasel, 2001; Kriaa et al., 2011). Date plant tissue culture techniques are employed successfully to clone other economically important palms, such as coconut (*Cocos nucifera*) and African oil palm (*Elaeis guineensis*) (George et al., 2007).

In Iraq, several institutes, both commercial and governmental, are working on micropropagation of elite date palm cultivars, such as Sherafy, Al-Sayer, Hilawi, Khasab, Um Al-Dihin, Barhi, Kantar, Shwaythee, Breem, Alawaidy and Ashkar (Muhsen, 2006; Al-Khalifa et al., 2009; Al-Najm, 2009; Jasim et al., 2009; Al-Meer and Jameel, 2011).

Challenges to date palm micropropagation

Micropropagation of date palm is a difficult procedure and faces numerous challenges. Microbial contamination is rapidly becoming one of the most important constraints, which can occur in any stage of the tissue culture (Leifert and Waites, 1992). Date palm explants are exposed to microbial infection at all stages of tissue culture, contamination coming with the explants themselves, or occurring during the propagation procedures (Al-Mussawi, 2010).

Different explanations have been proposed about the causes of microbial contamination of date palm tissue culture. One explanation is the method used to sterilize the explants, tools and equipment. Improper methods and insufficient amounts of disinfectant are the most common problems in tissue culture. Another problem is external contamination of the explants, which comes from contaminated tools, equipment and workers in the preparing and culturing of media (George, 1993). Composition of the tissue culture medium is a good source of nutrients for microbial contaminants, with all the essential requirements to support their growth and development (Odutayo et al., 2007).

The major adverse effects of microbial contamination on date palm tissue culture are degradation and browning of infected tissues caused by the release of substances into the medium, such as degrading enzymes (cellulase, phenol oxidase and others) as well as toxins (Hameed and Abass, 2006). Microbial contamination leads to wasted time, effort and material, and contributes to severe economic losses. Total losses due to the microbial contamination are about 3-15% in most tissue culture laboratories around the world; chief contaminants being fungal, bacterial and yeast agents (Leifert et al., 1994).

The present review attempts to shed light on the major groups of date palm tissue culture contaminants in Iraq, their identification, negative effects and the best methods to minimize their impact on the growth and development of date palm tissue culture.

Microbial contamination of date palm tissue culture

Two major groups of date palm tissue culture contaminants have been identified in Iraqi date palm tissue culture laboratories: fungi and bacteria.

Fungal contamination

Fungi represent a major group of date palm tissue culture contaminants in Iraq, and this can be explained by their ability to grow anywhere, outdoors or indoors, when sufficient moisture is

available (at least 60% relative humidity) and there is a source of suitable food, which stimulates their growth and development. Therefore, one can expect to isolate fungi from contaminated building materials (wherever there are damp dust particles, paint, wallpaper, carpet and other substrates) (Flannigan and Morey, 1996). Fungal contamination (including the most common fungi and yeasts) cause various types of damage to tissue cultures such as increasing turbidity, changing the pH of the medium, as well as cell destruction (Hameed and Abass, 2006). A survey of fungal contamination of different date palm cultivars during embryogenic callus production, showed that the fungi *Aspergillus niger*, *A. clavatus* and *Alternaria alternata* were the most predominant species as contaminants of six different date palm cultivars, including Um Al-Dihin, Shwaythee, Breem, Barhi, Hilawi and Al-Sayer (Hameed and Abass, 2006). Another two-year study by Abass et al. (2007), showed that the fungi *Aspergillius niger*, *Penicillium* sp. and *Alternaria alternata* were the most commonly isolated contaminants with frequencies of 27, 25 and 18 %; respectively, while the lowest frequency was seen with *Aspergillius terreus*. A recent study conducted by Al-Mayahi et al. (2010) identified different fungal genera from contaminated date palm tissue cultures such as *Aspergillius niger*, *Chaetomium atrobrunneum*, *Penicillium* sp. and *Fusarium* spp.

A summary of all papers published in Iraq (Table 1) indicates that both *Aspergillius niger* and *Alternaria alternata* were the most predominant fungi in the contaminated tissue cultures of date palm. *Aspergillius niger* is a filamentous ascomycete fungus that is ubiquitous in different environments and the most common member of the microbial communities found in soil, air and many other environments (Samson et al., 2002). Hence, its saprophytic activity with a wide range of hydrolytic and oxidative enzymes enables this fungus to grow wherever there is a suitable source of food and moisture (Schuster et al., 2002). The *Aspergillius niger* fungus appears to be a strong contaminant of tissue culture in Iraq.

Alternaria alternata is the second most important contaminant of date palm tissue culture in Iraq. It is a cosmopolitan saprophyte fungus found in soil, plants, as well as in the air (Shelton et al., 2002). Results of a study by Hameed and Abass (2006) revealed a high level of hydrolytic activity of phenol oxidase and cellulase in vitro for several date palm tissue culture fungal contaminants including *Aspergillius niger* and *Alternaria alternata*. For phenol oxidase activity, *Aspergillius niger* showed the highest in vitro activity with a total of 18.60 mm as an activity zone, while the filamentous fungus *Alternaria alternata* showed the highest level of activity of cellulase enzyme with a total of 11.50 mm as a zone of extracellular activity. All of these features along with the saprophytic behavior of both contaminant fungi could be a good explanation for their devastating role on date palm tissue culture in Iraq (Figure 1).

Table 1. Predominant fungal species of date palm cultivars propagated *in vitro*.

Reference	Fungal species	Date palm cultivar
Hameed and Abass (2006)	*Alternaria alternata*	Al-Sayer,
	Aspergillus niger	Barhi
	Aspergillus clavatus	Breem
	Scytalidium lignicola	Hilawi, Um Al-Dihin, Shwaythee
Abass et al. (2007)	*Alternaria alternata*	Al-Sayer
	Alternaria citri	Ashkar
	Aspergillus niger	Barhi
	Aspergillus terreus	Breem
	Cladosporium sp.	Hilawi
	Epicoccum sp.	Um Al-Dihin
	Penicillium spp.	Sherafy, Shwaythee
Al-Mayahi et al. (2010)	*Alternaria alternata*	Alawaidy
	Aspergillus niger	Kantar
	Chaetomium atrobrunneum	Khatraway
	Eurotium amstelodami	Shwaythee
	Penicillium spp.	
	Fusarium sp.	

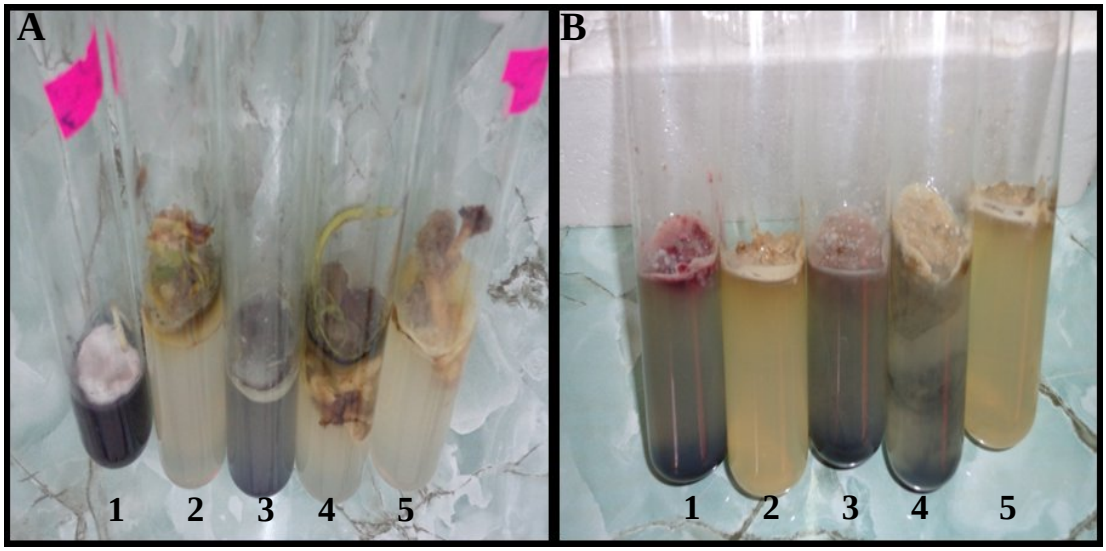

Figure 1. Fungal and bacterial contaminants of date palm tissue cultures.
A) Fungal species:1. *Penicillium* sp.; 2. *Alternaria* sp.; 3. *Chaetomium* sp.; 4. *Aspergillus niger*; 5. *Aspergillus terreus*.
B) Bacterial species: 1. *Staphylococcus aureus*; 2. and 3. *Proteus* sp.; 4. and 5. *Bacillus subtillis*.

Bacterial contamination

Bacterial contamination is an important problem in date palm tissue culture laboratories in Iraq, and is not easy to eliminate by surface sterilization, with some difficulties in determining the source of contamination. Several species of bacterial contaminants have been purified using general standard bacteriological methods and identified by biochemical tests such as Gram stain, motility, gelatinase, oxidation/ fermentation and oxidase (Al-Mussawi, 2010; Al-Dosary et al., 2011). The highest rates of occurrence of different genera of bacteria were reported with contaminated date palm tissue culture. The most unusual bacterial species were found to be *Bacillus subtillus*; *Staphylococcus aureus* and *Proteus* sp. (Al-Hadethy et al., 2007; Al-Mussawi 2010; Al-Dosary et al., 2011). The high percentage of occurrences for the abovementioned species were found to be associated with most tissue cultured date palm cultivars at different stages of micropropagation (Figure 1).

Regarding *Bacillus subtillus*, a gram-positive bacterium with the ability to produce a tough and protective endospore, this bacterium is able to tolerate extreme environment conditions (Nakano and Zuber, 1998). *Bacillus subtillus* is commonly found in different environments, such as soil, the normal human gut, in the air and so on. *Staphylococcus aureus* is widely known to be the second most serious bacterial contaminant of date palm tissue culture (Al-Hadethy et al., 2007; Al-Dosary et al., 2011); it is a gram-positive coccal bacterium, facultative anaerobic and considered as one of the most common skin and nasal passage flora. More than 20% of the human population have been recorded as long-tem carriers of this bacterium (Hong et al., 2009).

The genus *Proteus* belongs to the Proteobacteriaceae family; it is a gram-negative bacterium, and widely known as a contaminant of tissue culture laboratories around the world. Several species has been isolated and identified from contaminated tissue culture samples, such as *Proteus vulgarius* from the laboratories of tissue cultures in Southern Nigeria (Odutayo et al., 2007).

All of the abovementioned cosmopolitan bacteria can be isolated from plant debris, human skin, as well as the tables and walls of tissue culture laboratories (Odutayo et al., 2007). Therefore, different sources of contamination are present in the preparation and incubation rooms, and the indoor and outdoor air of date palm tissue culture laboratories. All of these factors together account for the high isolation frequencies of these bacteria from different laboratories in Iraq. Intensive care should be taken at each individual step of the date palm tissue culture process in order to prevent or avoid contamination.

Table 2. Antibiotic used in date palm micropropagation.

Reference	Type of antibiotic	Concentration mg/L	Date palm cultivar
Al-Kaby (2004)	Nystatin Griseofulvin Streptomycin	25-50	Ashkar
Al-Mussawi (2010)	Gentamycin Streptomycin	50	Al-Sayer Breem Kantar
Al-Dosary et al. (2011)	Amoxicillin Gentamicin Chloramphenicol	50	Al-Sayer Breem Kantar

Preventing and Reducing Contamination

Several approaches from around the world have been used to prevent or reduce fungal and bacterial contamination in date palm micropropagation. Approaches vary according to the incorporation of chemical agents or their tested concentrations that significantly prevent or reduce the growth and spread of microbial contaminants, and allows a substantially normal growth and development of date palm tissue (Hameed and Abass, 2006; Abass et al., 2007).

Different antibiotics and fungicides have been incorporated into the medium to control microbial contaminants (Table 2), sometime in combination, but otherwise alone. Most of chemical agents are effective at preventing bacterial and fungal growth when applied as prophylactics in date palm tissue culture medium, and other types of agents should be used to disinfect explants (Al-Mayahi et al., 2010).

For the selection of antibiotic and fungicide, care must be taken to best understand the effectiveness, regarding the type of antibiotic and fungicide, as well as the concentration. The most effective antibiotic and fungicide should be soluble, stable, suitable for combination treatment, inexpensive, a nonresistance inducer, produce high antimicrobial activity and not be harmful to human health (Al-Kaby, 2004; Hameed and Abass, 2006; Odutayo et al., 2007; Al-Dosary et al., 2011).

Selection of an antibiotic or fungicide is dependent on the type of microbial contamination present in the culture medium. Therefore, proper identification of the contaminant bacteria and fungi is essential and should be followed by preliminary testing for antimicrobial activity of both antibiotics and fungicides at different concentrations to select the most appropriate. Another test which may be necessary and needs to be explored, is the phytotoxicity (side effects) of chemical agents on date palm tissues, before adding them to the culture medium, especially for combination treatments which are more likely to be phytotoxic. Knowledge of the effects and side effects of both antibiotics and fungicides on bacteria and fungi, as well as on the date palm tissue cultures, is a crucial factor for controlling microbial contamination and recovering healthy plants.

Sterilization methods for in vitro cultures

Most of the attention and effort regarding sterilization has been carried out to obtain axenic explants. Several procedures and methods have been utilized in Iraq to avoid microbial contamination and produce contaminant-free cultures in Iraq. Unfortunately, the majority of these procedures are focused on the laboratory, whereas more attention should be paid to the donor plants in the farm field before selection, which is no less important. Treatment of donor plants with contact and systemic chemical agents may be necessary to obtain clean explants. The systemic fungicide Benlate is increasingly seen as a good option to decrease possible internal fungal contamination, either on the farm or in the laboratory. For surface sterilization of date palm explants, sodium hypochlorite (NaOCl) has been widely used as a disinfectant solution in Iraq; concentrations of 20% of NaOCl and 1 ml/l of Tween 20 were used successfully for this purpose (Muhsen, 2006; Al-Khalifa et al., 2009; Al-Meer and Yassen, 2010; Al-Mayahi et al., 2011).

Control of bacterial contamination

Several antibiotics have been used successfully to control bacterial contamination in date palm tissue culture (Table 2). Al-Kaby (2004) showed that the screening of three antibiotics (Nystatin, Griseofulvin and Streptomycin) at concentrations of 25 and 50 mg/l had no obvious effect on the embryogenic callus or somatic embryos of cv. Ashkar. Hence, a significant inhibition role was

seen in their effect to reduce and prevent contamination of date palm tissue culture from 20 % as a total contamination percentage to 0-10 % with antibiotic treatments. Also, treatments of Amoxicillin, Gentamicin and Chloramphenicol gave high protection efficiency for date palm callus. The most important results were seen with the treatment with Chloramphenicol at a concentration of 50 mg/l, which inhibits growth of *Bacillus*, *Staphylococcus* and *Proteus* bacteria, followed by the Gentamicin antibiotic (Al-Dosary et al., 2011).

Control of fungal contamination

Several fungicides have been used in Iraqi tissue culture laboratories to prevent or control fungal contamination. Al-Kaby (2004) showed that a concentration of 0.5-1 g/l of both fungicide Carbendazim and Score succeeded in decreasing the total percentage of contamination without any side effects on the growth and development of date palm tissue culture.

A study by Abass et al. (2007) revealed the positive effect of Benlate fungicide at a concentration of 1 g/l to inhibit the growth of contaminant fungi in vitro. Also, no obvious effects were seen on the callus of different date palm cultivars, such as Ashkar, Al-Sayer, Sherafy and Shwythee. Similar results were seen concerning the neutral effect of Benlate on both the growth and development of date palm tissue culture. Benlate treatment decreased the contamination percentage from 25% in control treatment to 1.65%. Similar findings were observed regarding the activity of Benlate in controlling the most common fungal contaminants including *Aspergillus niger*, *Alternaria alternata* and *Penicillium* spp. (Al-Mayahi et al., 2010).

Conclusion

Microbial contamination remains a serious threat to date palm tissue culture at different laboratories in Iraq. Several fungal and bacterial species have been isolated and identified during all stages of date palm micropropagation. Many practical steps are taken to maintain sterile techniques to greatly reduce the contamination of tissue culture, including use of antibiotics and fungicides. The cost of using fungicides and antibiotics in date palm tissue culture media, especially for large-scale propagation, should be considered along with their potential benefits in controlling microbial contamination and achieving better results.

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