

Full Length Research Paper

A PCR ITS-RFLP method for identifying fungal contamination of date palm (*Phoenix dactylifera* L.) tissue cultures

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Morphological and molecular procedures have been used in the study to characterize the most abundant fungal contaminants of date palm tissue cultures. *Aspergillus* species were found to be the most frequently isolated species, followed by the species of *Alternaria* and *Penicillium*. Internal Transcribed Spacer (ITS) products of the predominant fungi were analyzed by PCR amplification with ITS1 and ITS4 primers. The results of BLAST search of the ITS sequences revealed the identity of the fungal species. Three species were found to belong to the genus *Aspergillus* as *Aspergillus clavatus*, *Aspergillus niger* and *Aspergillus terreus*, while, two species were found for the genus *Alternaria*-with *Alternaria alternata* and *Alternaria citri*, and two species for the genus of *Penicillium*-with *Penicillium expansum* and *Penicillium glabrum*. The restriction fragment length polymorphism (RFLP) of the ITS amplicons was used to discriminate between fungal species and provides an alternative method to sequencing ITS products; both restriction endonucleases *EcoRI* and *SmaI* were used to digest ITS products, and three types of fragment patterns were detected: A type (one undigested fragment), B type (two fragments type) and C type (three fragments type). This is the first study which used ITS sequence and ITS-RFLP techniques as a rapid and reliable procedure for identification of date palm fungal contamination in the laboratories of tissue cultures in Iraq.

Key words: Molecular identification, internal transcribed spacer (ITS), restriction fragment length polymorphism (RFLP) analyses, fungal contamination, date palm.

INTRODUCTION

Date palm is one of the most popular fruit trees in Middle Eastern countries. Total 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. One third of the world production of dates is provided by the Arabian Peninsula (FAO, 2011). Iraq is one of the top ten date producers in the world; between 1991 and 2001, Iraq contributed a 7.5% of the total world dates production (FAO, 2011). For date palm propagation, different methods have been used such as seed propagation, offshoot propagation and tissue culture method (Abass, 2013). The tissue culture propagation is also known as micropropagation or in vitro multiplication (Aaouine, 2003; Al-Khayri, 2005, 2007). In Iraq, many publications

reported on the methodology (or applicability) of tissue culture of elite date palm cultivars, including 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 Yassen, 2010). Date palm in vitro multiplication is a difficult technique 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). Fungal contamination

(including the most common fungi and yeasts) is considered as the most abundant contaminants of tissue culture and 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; Odutayo et al., 2007; Al-Mussawi, 2010). A survey of fungal contamination of different date palm cultivars during embryogenic callus production, revealed that the fungi *Aspergillus niger*, *Aspergillus clavatus* and *Alternaria alternata* were the most abundant contaminants of six different date palm cultivars, including Um Al-Dihin, Shwaythee, Breem, Barhi, Hilawi and Al-Sayer (Hameed and Abass, 2006).

Abass et al. (2007) showed that the fungi *A. niger*, *Penicillium* sp. and *A. alternata* were the most commonly isolated contaminants, while the lowest frequency was seen with *Aspergillus terreus*. Several approaches have been incorporated for the identification of fungal contaminants; generally, the majority of published articles have focused on the morphological methods at the macro- and microscopic levels (Hameed and Abass, 2006; Abass et al., 2007; Al-Mayahi et al., 2010). The morphological characterization of fungal species is mainly based on the differences in the morphological features including colony colour, size and shape of conidia and spores, size and shape of appressoria, presence of setae and the telomorph, and many other features (Abass et al., 2007). There are numerous difficulties facing the morphological characterization of fungal species, one of these difficulties is the stability of the morphological features which are influenced by environmental factors and cultivation media, as well as, the overlap of these features among fungal species, especially the most closely taxa (Martinez-Culebras and Ramon, 2007; Rubila et al., 2008; Diguta et al., 2011). All of these constraints can be overcome by the profits of molecular identification which offers several possibilities in distinguishing fungal species dependent on the genetic diversity of isolated fungi (MacLean et al., 1993; Martin and Rygielwicz, 2005; Abass et al., 2013).

Inter and intraspecific variations between fungal species could be analyzed by different approaches, such as Random Fragment Length Polymorphism (RFLP), random amplification of polymorphic DNA (RAPD), ribosomal RNA (rRNA) and DNA (rDNA) sequence analysis, and many other procedures (Gardes et al., 1991; Horton and Bruns, 2001; Martin and Rygielwicz, 2005; Abass et al., 2013). Using the DNA which is coding for the ribosomal RNA gene complex (rDNA) has a great importance in fungal identification; the 16S, 5.8S and 28S fungal rRNA subunits are separated by internal transcribed spacer (ITS) sequences (Freifelder, 1983). Both, ITS1 and ITS4 primers are amplifying the highly variable region flanked by these primers that encompass the 5.8S coding sequence and situated between small and large subunit coding sequence of the ribosomal operon (White et al., 1990).

The main objective of the current research was to establish a molecular assay for the species specific detection and identification of fungal contamination by identifying ITS regions of the most predominant fungi associated with the elite cultivars of date palm tissue cultures in Iraq.

MATERIALS AND METHODS

Fungal isolation

Contaminated date palm tissue cultivars with fungi were collected from the laboratories of Date Palm Research Centre- Basra University during the period of the 1st March 2012 to 1st September 2012. Most of the contaminated samples represented the following cultivars: Al-Sayer, Barhi, Breem, Hilawi and Shwaythee. The isolation of fungi was performed on the surface of fresh potato dextrose agar (PDA) supplemented with 150 mg l⁻¹ chloramphenicol at 30°C for several days. The observations of each isolates were done using a Zeiss Axio lab compound light microscope supplemented with bright-light optic according to Abass et al. (2013). The morphological characterization and identification were done according to Elliot (1917) and Ellis (1971).

DNA extraction and purification

The protocols of DNA extraction, purification and ethanol precipitation were done as described in Zolan and Pukkila (1986). Briefly, single-spore cultures were placed on potato carrot agar (PCA) medium at 30°C for 7 days. The mycelium and conidia were transferred into a mortar and pestle and ground with liquid nitrogen at room temperature, and transferred into a 1.5 ml Eppendorf tube containing 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-isoamylalcohol (24:1, v/v) was added, tubes were then centrifuged 5 min at maximum speed. The aqueous phases were recovered into a fresh tubes containing isopropanol and another centrifugation was done for 1 min. The final DNA pellets were obtained by dissolving in 300 µL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

Primers description

Primers were selected to amplify ITS1 and ITS2 regions of the most predominant fungi in contaminated date palm tissue cultures. Both universal ITS1 and ITS4 were chosen for the molecular identification of the contaminants. 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).

PCR amplification

PCR was carried out in 50 µL reaction volume in a 0.2 ml polypropylene tube and contained 40 ng of DNA template, 5 µL of 10 × polymerase buffers, 8 µL of dNTPs (1.25 mM), 1 µL of *Taq* DNA polymerase (Roche) and 1 µL of each primer. The thermal cycler used in this research was equipped with a heated lid, the model was MJ Research, PTC-100. An initial denaturation and enzyme activation step of 5 min at 95°C was done, followed by amplification for 35 cycles at the following PCR conditions: 95°C for

Table 1. Fungal contaminants associated with date palm tissue cultures.

Fungal contaminant	Percent of isolation*	Fungal species	Percent of isolation**
<i>Alternaria</i> spp.	21.70	<i>A. alternata</i>	83.40
		<i>A. citri</i>	16.60
<i>Aspergillus</i> spp.	49.80	<i>A. clavatus</i>	20.00
		<i>A. niger</i>	55.70
		<i>A. terreus</i>	24.30
<i>Penicillium</i> spp.	20.50	<i>P. expansum</i>	73.18
		<i>P. glabrum</i>	26.82
<i>Cladosporium</i> spp.	5.00		
<i>Epicoccum</i> spp.	3.00		

*Percent of isolation of the total isolates; **percent of isolation within the genera of *Alternaria*, *Aspergillus* and *Penicillium*.

1 min, 55°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min. All of the PCR products were stored at 4°C until used (Rodrigues et al., 2011).

Amplicons electrophoresis and sequencing

PCR products were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The PCR products were sequenced and analyzed by comparison with all available sequences in the NCBI (<http://www.ncbi.nlm.nih.gov>) using the BLAST: basic alignment sequence tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

ITS-RFLP analyses

PCR products with ITS primers were digested with the restriction enzymes *EcoRI* and *SmaI* (MBI; Fermentas) as described in Diguta et al. (2011). The restriction enzymes were selected after ITS sequence analysis in the Serial Cloner software, allowing differentiation among fungal species. The digestions were performed overnight following the manufacturer's instructions. RFLP analyses were done in 2% agarose gel and the sizes of each fragment were estimated by comparison with DNA ladder (100 bp DNA marker, Gene Ruler) and the computer program of Photocapt MW software 10.0, Vilber Lourmat.

RESULTS

Fungal contamination of date palm tissue cultures

The results of isolation and identification of contaminated date palm tissue cultures according to the morphological features (Elliot, 1917; Ellis, 1971) indicated that the genus *Aspergillus* was the most predominant fungal genera to be associated with date palm tissue cultures, with a total percent of isolation reaching 49.8% (Table 1), followed by the genera: *Alternaria* and *Penicillium* while the genera *Cladosporium* and *Epicoccum* were isolated

at very low frequencies.

Molecular characterization of fungi

The most predominant fungal genera (*Aspergillus*, *Alternaria* and *Penicillium*) were selected for molecular identification to the species level by using ITS1 and ITS4 primers as described by White et al. (1990), based on amplification of the ITS1 and ITS2 regions of ribosomal RNA. The results from gel electrophoresis showed evident differences among genera depending on the sizes of amplified fragments, and these differences provide a good tool to discriminate between genera (Figure 1). For the genus *Aspergillus*, three different fragments were seen as follow: 531, 575 and 580 bp, while two different fragments of sizes 305 and 150 bp were seen with genus *Alternaria*, finally, two fragments of sizes 580 and 600 bp were seen within the genus *Penicillium*. The ITS sequences for the contaminated fungi were analyzed in the GenBank of NCBI, the results of BLAST search revealed the identity of each predominant fungi. For the genus *Aspergillus*, three different species were identified as *A. clavatus* (EF670001.1), *A. niger* (KC119204.1) and *A. terreus* (EF592171.1), with identity 99 to 100%, while, two different species were identified belonging to the species *Alternaria* which were *A. alternata* (KC134318.1) and *A. citri* (AF397051.1) (99% of identity), and two species of the genus *Penicillium* were identified as *P. expansum* (JX869559.1) and *P. glabrum* (JX421729.1) (99% of identity).

In order to provide an alternative technique for fungal discrimination, which is not based on sequencing, we applied the RFLP technique following the amplification of the ITS region. The amplicon products of ITS were digested with two restriction endonuclease enzymes



Figure 1. PCR amplicons of DNA from the predominant contaminating fungal species of date palm tissue cultures with ITS primers. Lane 1, Lambda HindIII DNA marker; lane 2, *Aspergillus* sp. 1 (575 bp); lane 3, *Aspergillus* sp. 2 (531 bp); lane 4, *Aspergillus* sp. 3 (580 bp); lane 5, *Penicillium* sp. 1 (580 bp); lane 6, *Penicillium* sp. 2 (600 bp); lane 7, *Alternaria* sp. 1 (305 bp); lane 8, *Alternaria* sp. 2 (150 bp).

(*EcoRI* and *SmaI*). The profile obtained after digestion with restriction endonucleases were classified into three patterns: *A* Type, which was characterized by one undigested fragment; *B* type, which was characterized by two fragments pattern ranging in size from 60 to 532 bp, and *C* Type, which was characterized by a three fragments pattern. ITS-RFLP based on *EcoRI* digestion of the species of *A. alternata*, *A. niger*, *P. expansum* and *P. glabrum* followed the *B* type pattern, while no detectable digestion was seen in case of the other fungal species (Table 2). Similar results were obtained with the *SmaI* digestion of the ITS products. The results of ITS-RFLP showed that the fungal species of *A. clavatus* followed the *C* Type patterns, while no digestions were seen with the species of *A. alternata* and *A. citri* which followed *A* Type profile (Table 2).

DISCUSSION

The identification of microbial contaminants has a great importance in date palm tissue culture and many other plant tissue cultures, because the accurate identification of fungal contaminant species provides a guide to choose suitable methods for their control (Abass et al., 2013). Fungal contamination of different elite cultivars of date palm propagated by tissue culture technique represents the major group of microbial contamination—and can lead to severe consequences on the whole process of tissue culture, such as the degrading of the tissue cultures and the death of infected cultures. In the current study, several fungal species were identified and relative percentages of their isolation revealed their abundance in association with some elite cultivars of date palm

including Al-Sayer, Barhi, Breem, Hilawi and Shwaythee cultivars. *A. niger* was the most predominant fungal species among the community of fungal contaminants, followed by *A. alternata* and *Penicillium expansum*. These results are in a good agreement with many published reports about the abundance of these contaminants in association with date palm tissue cultures (Abass et al., 2007; Al-Mayahi et al., 2010). The highest isolation frequencies of these contaminants could be attributed to their saprophytic behavior, as well as a wide range of hydrolytic and degrading enzyme production such as cellulose, phenol oxidase and many other enzymes which enable these contaminants to grow rapidly on the growing media (Hameed and Abass, 2006). Hence, the culture medium which is used in micropropagation of date palm provides the fungi with all essential requirements to support their own growth (Odutayo et al., 2007).

The ITS sequencing was a rapid and reliable method to identify different species of date palm fungal contamination; many published reports have proved the effectiveness and reliability of ITS products as a tool of fungal discrimination including the species of *Aspergillus*, *Alternaria* and *Penicillium* (Henry et al., 2000; Konstantinova et al., 2002; Martinez-Culebras and Ramon, 2007; Diguta et al., 2011; Mmbaga et al., 2011; Pashley et al., 2012). The digestion with restriction endonucleases *EcoRI* and *SmaI* following the ITS amplification, indicated that both restriction endonucleases were sufficient to recognize the fungal contaminants (*Aspergillus*, *Alternaria* and *Penicillium*) of date palm tissue culture at species level. Moreover, the ITS-RFLP successfully separated the fungal species into three patterns according to the number of fragments. The

Table 2. Summary results of ITS-RFLP of the contaminants fungal species of date palm tissue cultures.

Fungal species	Amplified products/ bp with ITS primers	Restriction fragments/ bp (profile patterns)	
		<i>EcoRI</i>	<i>SmaI</i>
<i>Alternaria alternata</i>	305	240+60 (B**)	305 (A*)
<i>A. citri</i>	150	150 (A)	150 (A)
<i>Aspergillus clavatus</i>	575	575 (A)	253+200+107 (C***)
<i>A. niger</i>	531	286+244 (B)	409+121 (B)
<i>A. terreus</i>	580	580 (A)	446+133 (B)
<i>Penicillium expansum</i>	580	454+121 (B)	532+43 (B)
<i>P. glabrum</i>	600	341+250 (B)	490+109 (B)

*A Type with one undigested fragments patter; **B Type with two fragments pattern; ***C Type with three fragments pattern.

target of the *EcoRI* (GAATTC) existed in the sequence of *A. alternata*, *A. niger*, *P. expansum* and *P. glabrum*, and was not present in the *A. citri*, *A. clavatus* and *A. terreus*, compared with the target of *SmaI* (CCCGGG) which was found to be present in the sequence of *A. clavatus*, *A. niger*, *A. terreus*, *P. expansum* and *P. glabrum*, and not found in the species of *Alternaria* (Table 2). The fragment profile is a good tool for fungal discrimination apart from sequencing. Most of the isolated fungi belonged to the B Type pattern, with an exception of species *A. clavatus* which belonged to the C Type pattern with three fragments after digestion with *SmaI*. The ITS-RFLP procedure is widely used as a rapid and reliable technique for fungal identification, especially for the close taxa within the genera of fungi such as *Aspergillus*, *Alternaria* and *Penicillium* (Martinez-Culebras and Ramon, 2007; Diguta et al., 2011; Mmbaga et al., 2011).

In conclusion, the ITS-RFLP of the PCR products with ITS primers described here provides an easy, fast and reliable method for fungal identification in association with date palm tissue cultures, compared to the classic morphological characterization which requires more time and efforts. The molecular identification depending on both inter- and interspecies variations enables better identification of the fungal contaminants of date palm micropropagation, and may help to develop the proper treatments for decreasing the impact of these contaminants on the growth and development of date palm tissue cultures, thus, decreasing the loss caused by fungal contamination.

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