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 Smal 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, Bream, Alawaidy and Ashkar (Muhisen, 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
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 Puffo Research Center, 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 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% hexadecytrimethylammonium 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′-TCCGTAGGTAAACCTGCGG-3′, which hybridizes at the end of 18S rDNA and ITS4: 5′-TCTTCCGCTTAATGATATGC-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.

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

*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 10 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).

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 at very low frequencies.
(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

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

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Amplified products/ bp with ITS primers</th>
<th>Restriction fragments/ bp (profile patterns)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>EcoRI</td>
</tr>
<tr>
<td>Alternaria alternata</td>
<td>305</td>
<td>240+60 (B**)</td>
</tr>
<tr>
<td>A. citri</td>
<td>150</td>
<td>150 (A)</td>
</tr>
<tr>
<td>Aspergillus clavatus</td>
<td>575</td>
<td>150 (A)</td>
</tr>
<tr>
<td>A. niger</td>
<td>531</td>
<td>150 (A)</td>
</tr>
<tr>
<td>A. terreus</td>
<td>580</td>
<td>253+200+107 (C***)</td>
</tr>
<tr>
<td>Penicillium expansum</td>
<td>580</td>
<td>454+121 (B)</td>
</tr>
<tr>
<td>P. glabrum</td>
<td>600</td>
<td>490+109 (B)</td>
</tr>
</tbody>
</table>

*A Type with one undigested fragments pattern; **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 Smal (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. calvatus which belonged to the C Type pattern with three fragments after digestion with Smal. 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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