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**Isolation and identification of Aspergillus sp. from decayed Medjool date fruit**

**Report submitted by**

**By**

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**Bet Dagan April 2019**

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The purpose of this study was to identify the Aspergillus species in Medjool date fruit showing internal symptoms (black rot) and test the capability of representatives of the identified isolates to produce mycotoxins.

Fruit suspected to be infected (40 fruit, and infected fruit (40 fruit) showing external typical symptoms of Aspergillus black rot was obtained from a pack house located in the Arava region (total of 80 fruits). The actual fruit showing internal black rot symptoms among the fruit tested was **88.7%** (87% in the first group, 90% in the second group).

Isolation of Aspergillus from infected fruit:

Each fruit was cut aseptically into two halfs and flesh tissue (0.5 x 0.5 cm ) with or without symptoms was taken from 4 locations in each fruit and placed on petri dishes contained PDA + chloramphenicol at 250 mg/l to prevent growth of bacteria. Plates were then incubated at 26oC for 4 days to allow growth of the fungus.

In 100% of the isolations, a growth of typical Aspergillus cultures was observed. Morphological and microscopic test was also performed to each isolate to confirm that the culture showing characteristic structures such as conidiophores of Aspergillus sp. A total of 100 isolates were selected and transferred to fresh PDA-chloramphenicol medium to obtain pure cultures.

Molecular identification of Aspergillus isolates:

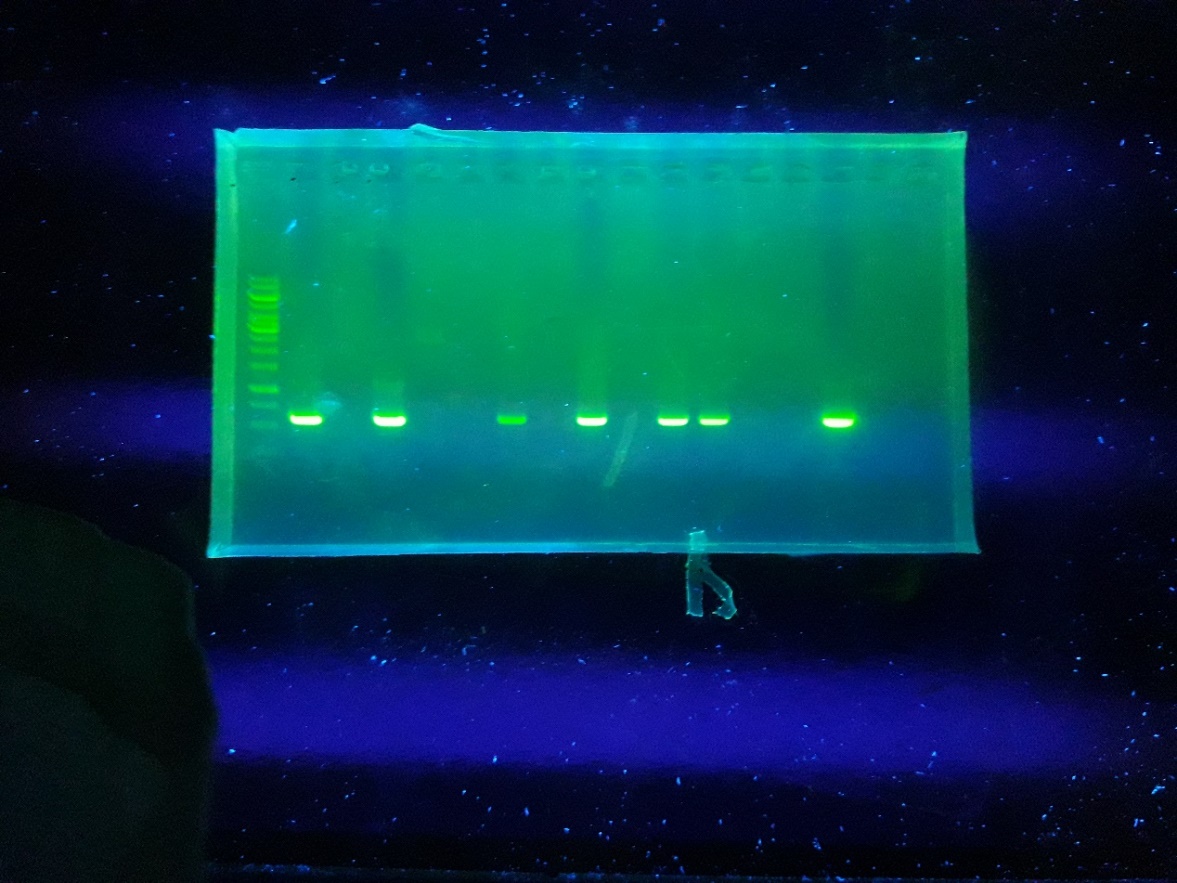
To Identify the Aspergillus species, each one of the 100 isolates was grown in liquid medium to obtain mycelial mats. Spores were aseptically removed using sterile bacteriological loop and suspended in 1 ml of PDB-chloramphenicol and incubated for 2 days on orbital shaker for 2 days at 26oC. Mycelial mats were pelleted by centrifugation (10,000 rpm for 10 min) and washed twice with distilled sterile water. DNA was then extracted from each isolate using commercial kit.

DNA templates were used to amplify the ITS region of the rDNA using the following set of primers:

ITS 1: 5’-TCC GTA GGT GAA CCT GCG G-3’

ITS 4: 5’-TCC TCC GCT TAT TGA TAT GC-3’

Following PCR, a product a size of about 550 bp was obtained each DNA template (Fig. 1).



**Fig. 1**: PCR products of the ITS region of the rDNA of Aspergillus spp.

To identify each isolate, PCR products were sent out for sequencing by Macrogen Inc (Netherlands). Each of the sequences were then blasted to known ITS sequences deposited public gene bank (NCBI).

The following are the results of the blast:

**Table 1:** Blast results of the ITS region sequences obtained from all

Aspergillus isolates.

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| **Isolate** | **Identification** | **Similarity** |
| **Yasp 1** | *Aspergillus niger* | 99% |
| **Yasp 2** | *Aspergillus niger* | 98% |
| **Yasp 3** | *Aspergillus niger* | 99% |
| **Yasp 4** | *Aspergillus niger* | 99% |
| **Yasp 5** | *Aspergillus niger* | 99% |
| **Yasp 6** | *Aspergillus niger* | 98% |
| **Yasp 7** | *Aspergillus niger* | 99% |
| **Yasp 8** | *Aspergillus niger* | 98% |
| **Yasp 9** | *Aspergillus niger* | 99% |
| **Yasp 11** | *Aspergillus niger* | 99% |
| **Yasp 14** | *Aspergillus niger* | 99% |
| **Yasp 15** | *Aspergillus niger* | 99% |
| **Yasp 16** | *Aspergillus niger* | 99% |
| **Yasp 17** | *Aspergillus niger* | 80% |
| **Yasp 18** | *Aspergillus niger* | 88% |
| **Yasp 19** | *Aspergillus niger* | 99% |
| **Iasp 2** | *Aspergillus niger* | 94% |
| **Iasp 3** | *Aspergillus niger* | 98% |
| **Iasp 4** | *Aspergillus niger* | 98% |
| **Iasp5** | *Aspergillus niger* | 83% |
| **Iasp 6** | *Aspergillus niger* | 99% |
| **Iasp12** | *Aspergillus niger* | 99% |
| **Iasp13** | *Aspergillus niger* | 99% |
| **Iasp14** | *Aspergillus niger* | 98% |
| **Iasp15** | *Aspergillus niger* | 98% |
| **Iasp16** | *Aspergillus niger* | 99% |
| **Iasp17** | *Aspergillus niger* | 98% |
| **Iasp21** | *Aspergillus niger* | 99% |
| **Iasp22** | *Aspergillus niger* | 82% |
| **Iasp23** | *Aspergillus niger* | 99% |
| **Iasp25** | *Aspergillus niger* | 85% |
| **Iasp26** | *Aspergillus niger* | 99% |
| **Iasp28** | *Aspergillus niger* | 99% |
| **Iasp29** | *Aspergillus niger* | 98% |
| **Iasp30** | *Aspergillus niger* | 99% |
| **Iasp31** | *Aspergillus niger* | 99% |
| **Iasp32** | *Aspergillus niger* | 98% |
| **Iasp33** | *Aspergillus niger* | 97% |
| **Iasp34** | *Aspergillus niger* | 99% |
| **Iasp37** | *Aspergillus niger* | 98% |
| **Iasp39** | *Aspergillus niger* | 99% |

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| **Sasp 1** | *Aspergillus niger* | 99% |
| **Sasp 2** | *Aspergillus niger* | 99% |
| **Sasp 3** | *Aspergillus niger* | 99% |
| **Sasp 4** | *Aspergillus niger* | 99% |
| **Sasp 5** | *Aspergillus niger* | 99% |
| **Sasp 6** | *Aspergillus niger* | 99% |
| **Sasp 7** | *Aspergillus niger* | 99% |
| **Sasp 9** | *Aspergillus niger* | 92% |
| **Sasp 10** | *Aspergillus niger* | 99% |
| **Sasp 12** | *Aspergillus niger* | 99% |
| **Sasp 13** | *Aspergillus niger* | 99% |
| **Sasp 14** | *Aspergillus niger* | 99% |
| **Sasp 15** | *Aspergillus niger* | 94% |
| **Sasp 16** | *Aspergillus niger* | 98% |
| **Sasp 17** | *Aspergillus niger* | 99% |
| **Sasp 18** | *Aspergillus niger* | 99% |
| **Sasp 19** | *Aspergillus niger* | 99% |
| **Sasp 20** | *Aspergillus niger* |  |
| **Sasp 22** | *Aspergillus niger* | 99% |
| **Sasp 23** | *Aspergillus niger* | 98% |
| **Sasp 27** | *Aspergillus niger* | 99% |
| **Sasp 29** | *Aspergillus niger* | 99% |
| **Sasp 31** | *Aspergillus niger* | 99% |
| **Sasp 34** | *Aspergillus niger* | 99% |
| **Sasp 36** | *Aspergillus niger* | 85% |
| **Sasp 40** | *Aspergillus niger* | 87% |

As showed in Table 1, all isolates were identified as ***Aspergillus niger*** with high similarity percentage to available sequences in the gene bank. No other Aspergillus species was identified in the fruit sample tested in this study.

Analysis and quantification of ochratoxin A production

To evaluate the potential of mycotoxin production, 10 isolates were selected for analysis for their potential to produce ochratoxin A (OTA) – the most important mycotoxin in *Aspergillus nigre* group (black Aspergilli).

Ten *Aspergillus niger* isolates (isolated from dates: Sasp 7, 9, 10, 12, 13, 14, 17, 18, 19, 20) were sub-cultured on three different solid media – PDA, YES (yeast extract sucrose (15%)) and MEA (malt extract agar) – and incubated for 10 days at 28℃. Ochratoxin A (OTA) accumulation was evaluated in five 1-cm-diameter homogenized agar medium discs. Three replicates were performed for each experiment. Samples were weighed and crushed in 50 ml tubes. OTA was extracted with 2 ml methanol by shaking for 1 h in an orbital shaker (200 rpm) and centrifuging for 5 min at 8,800x*g*. The supernatant was filtered through a 0.22-μm PTFE filter before injecting into the column. Quantitative analysis of OTA was performed by UHPLC-FLD (Waters, USA) using a Zorbax Eclipse XDB-C18 3.5 μm (4.6x150 mm) column (Agilent Technologies, Santa Clara, CA, USA). A mobile phase consisting of acetonitrile:water:acetic acid (99:99:2, v/v/v) completely separated OTA under the optimum HPLC conditions. The flow rate was 0.8 ml/min. The fluorescence detector was set up at excitation and emission wavelengths of 330 and 450 nm, respectively. OTA was identified according to its retention time (7.6 min) by comparison with a commercial standard, and quantified by measuring the peak area according to a standard curve. The detection limit was 0.2 μg/l. OTA was not detected at any tested sample, suggesting that all examined *A. niger* isolates are not producing OTA.

No OTA was detected in all isolates grown on the different media. This result clearly show that *Aspergillus niger* strains isolated from Medjool date fruit are not capable of producing OTA.

**Fig. 2**: Pictures of the morphological diversity of Aspergillus isolates obtained from infected dates fruits

