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Molds Associated with Olive Fruits Infested with Olive Fruit Fly (*Bactrocera oleae)* and their Effects on Oil Quality

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Abstract

Olive is the most widely grown fruit tree in Jordan; it is annually attacked by the olive fruit fly, *Bactrocera oleae* (Rossi), whose larvae usually cause great economic losses in fruit yield. *Alternaria solani, Aspergillus niger, Cladosporium herbarum, Fusarium solani, Penicillium digitatum, P. italicum* and *Rhizopus stolonifer* were found associated with the fly infestation with a sample frequency ranging from 6.7-33.3%. *Penicillium digitatum* was the most dominant species. All molds were vulnerable for a hot water treatment at 50 and 70ºC as indicated by their low spore germination and colony-forming unit except for *P. digitatum* and *P. italicum* which were the most heat-tolerant. When the olive oil was inoculated with pure cultures of these molds, the mold fungi were able to colonize olive oil. *Rhizopus stolonifer* was the greatest colonizer and besides *Alternaria solani*, both had obviously reduced the oil peroxide value over the control without greatly affecting the oil free fatty acid content.

Keywords: *Bactrocera oleae*, Jordan, *Olea europea* L., Olive fruits, Spore germination.

1. Introduction

Olive, *Olea europea* L., is the most widely grown fruit tree in Jordan occupying about 77% of the total area planted with fruit trees and 34% of the whole planting area covering now more than 130 thousand hectares (Anonymous, 2013). Olive plantation is a traditional part of the Jordanian agriculture (Al-Shdiefat *et al*., 2006; 2009) having a social, economic and environmental importance; it occupies the same position in the surrounding Mediterranean Basin countries (Loumou and Giourga, 2003).

Olive fruits are a valuable commodity worldwide; they are consumed as whole, as a table olive that is stuffed and sliced and as olive oil produced by milling fruits that must be prepared using safe conditions based on international olive oil standardization (Tokuşoğlu *et al.,* 2012).

Contamination of the fruits by hazardous microorganisms may occur through insect pest infestation, when they fall on the ground or by workers during handling (Asehraou *et al.,* 1992; Chliyeh *et al.,* 2014). One of the most important insect pests that usually attack olive in Jordan is olive fruit fly (*Bactrocera oleae* (Rossi) formerly *Dacus oleae* (Diptera: Tephritidae) whose larvae usually cause great annual economic losses in fruit yield through making fruit tunnels and enhancing the infection of fungal molds (Al-Raddad and Mustafa, 2008).

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in humans and other animals (Bennett and Klich, 2003). Many types of mycotoxins are produced by mold fungi including aflatoxin, citrinin, alkaloids, fumonisins, ochratoxin A, patulin, trichothecenes, and zearalenone (Abarca *et al*., 2003; Abrunhosa *et al*., 2002; Bennett and Klich, 2003). Mycotoxins could act as nephrotoxic teratogenic, immunotoxic, genotoxic, mutagenic and carcinogenic agents and cause other human health hazards, all of which lead to life-threatening diseases (Creppy *et al.,* 1985; El Adlouni *et al.,* 2000; Bhat *et al.,* 1997).

Some olive mills usually use hot water to wash olive fruits before processing, which may drastically raise milling temperature. The milling temperature is kept under 27°C which is crucial to olive oil quality. Sometimes, hot water is added to olive paste during oil extraction for increasing the oil release from the tissues especially from olive fruits yielded under rainfed and non-supplementary irrigation conditions. If the temperature exceeds 27°C, the more volatile aromas are lost and oil oxidation rate is increased, thus reducing oil quality. The chemical content of polyphenols, antioxidants, and vitamins of the oil is also reduced by higher temperatures

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(Boskou, 2006) but these temperatures may have the advantage of controlling molds associated with olive fruit.

Therefore, the present study was conducted to isolate and identify the various mold fungi that are usually associated with olive fruit decay, to evaluate their ability of resisting hot temperature and colonizing olive oil and their influence on some oil quality parameters.

2. Material and Method

2.1. Mold Isolation, Culture and Identification

About 200 fully-mature and ripped olive fruits, previously infested with olive fruit fly, showing internally mold growth, were collected during Nov. 2014 from some trees belonging to some olive cultivars; Nabali, Nassohi, Rassei and Nabali Mohassan grown at the Agricultural Research Station (31° 16' N, 35° 45' E and ca 920 meters above sea level) of Faculty of Agriculture, Mutah University, Karak, Jordan. The part of olive fruit sample, showing the mold growth (0.5cm-in-diamter piece), was cut off and transferred into potato dextrose agar medium-containing Petri dish, 3 pieces of the same fruit/ plate (Asehraou *et al.*, 1992). All culture dishes were then incubated at 25ºC in darkness in a growth chamber for one week. A week later, all fungal colonies growing out from the pieces were identified under 100 and 400X slide stereoscopic microscope.

2.2. Mold Type Frequency

The percentage of each identified mold species was determined by counting the number of fruits showing positive isolation per fungal species divided by the total number of collected samples and multiplied by 100%.

2.3. Influence of Hot Water on Mold Spore Germination

To estimate the influence of hot water treatments at different temperatures on mold growth and development indicated by Colony Forming Unit (CFU) and spore germination %, a sterile tap water was heated to 30, 50 and 70±5ºC using a hot plate. A half ml-volume of hot water was transferred into one ml-in-volume test tubes (3 replicates per a treatment) and inoculated with a drop of fungal suspension made by washing the mold culture plate for each of the isolated mold fungi (Table 1) with a sterile tap water and set at a concentration of about 30000 spores per ml. Then, the test tubes were set at the same temperature for 2 minutes. One drop of water containing mold spores was taken from each test tube and added on a slide and spore germination % was then determined by counting the number of germinated spores. A 0.25 ml of each test tube was transferred into potato dextrose agar medium-containing Petri dish (3 plates/ a treatment). All culture dishes were then incubated at 25±2°C in darkness in a growth chamber for one week. After that, the number of emerging fungal colonies was counted per plate and the CFU/ml was determined per a plate and the average of CFU/ml for each treatment was calculated.

2.4. Mold-Olive Oil Interactions

A fresh virgin olive oil (10ml-volume per a 15ml glass test tube) was inoculated with a loopfull of each mold (Table 1) plate culture (3 replicates per treatment). All mold-inoculated oil test tubes were incubated at 25±2°C in darkness in a growth chamber for about three months. Percentage of mold colonization (in terms of mycelial growth and sporulation) of the oil was estimated; oil Peroxide Value (PV) and the percentages of Free Fatty Acids (FFA%) were measured using standard methods (IUPAC, 1979; Wong, 1989)

3. Results and Discussion

Table 1 shows that seven species of mold fungi were isolated from olive fruits infested with olive fruit fly; *Alternaria solani* (causes brown rot), *Aspergillus niger* (black mold), *Cladosporium herbarum* (sooty mold), *Fusarium solani* (fruit rot), *P. digitatum* (green mold), *P. italicum* (blue mold) and *Rhizopus stolonifer* (causes soft rot) with a sample frequency ranging between 6.7-33.3%. Among them, *Penicillium digitatum* was the most frequent mold species isolated from the fruit samples (Table 1).

Table 1. Fungal species associated with olive fruit rot that were isolated and identified *in vitro*:

Fungal species	Sample Frequency %
Alternaria solani Sor.	10.0*
Aspergillus niger v. Tieghem	13.3
Cladosporium herbarum Fr.	16.7
Fusarium solani (Mart.) App. Et Wr.	13.3
Penicillium digitatum Sacc.	33.3
Penicillium italicum Wehmer	06.7
Rhizopus stolonifer (Her.) Vuill	20.0

* Over 200 rotted fruits with apparent mycelial growths were collected during November 2014.

At 50ºC hot water treatment, there was a significant reduction in CFU/ml and in the spore germination % of the mold fungi except for *P. digitatum* and *P. italicum* where no significant reduction in the spore germination % at 50ºC was recorded with 30ºC (Table 2). At the highest hot temperature of water (70ºC), all fungi showed remarkably lower CFU/ml and spore germination % than those at 30ºC except *P.digitatum* and *P. italicum* that gave more than 50% spore germination at 70 ºC (Table 2).

All mold fungi were able to colonize olive oil (Table 3) and the range was from 5.7-36.2%. Mold colonization % of olive oil was significantly the highest for *Rhizopus stolonifer* while the lowest value was for *Aspergillus niger*.

Peroxide Value (PV) was significantly lower in the olive oil treated with *Alternaria solani* and *Rhizopus stolonifer* than that in the oil treated with the other fungi or in non-treated oil (Table 3).

The olive oil treated with *P. digitatum* and *Rhizopus stolonifer* had significantly higher FFA% than the oil treated with *P. italicum.* The increase or decrease caused by fungi in FFA% over the untreated control was not significant (Table 3).

Table 2. Influence of hot water treatments on colony forming unit (CFU) and spore germination % of some mold fungi associated with olive fruits:

Fungal species	CFU/ml			Spore germination %		
	30 ºC	50 ºC	70 ºC	30 ºC	50 ºC	70ºC
Alternaria solani	36[1] a[2]	0 b	0 b	96 A	33 B	0 C
Aspergillus niger	61 a	37 b	33 b	66 A	10 B	5 B
Cladosporium herbarum	26 a	10 b	0 c	78 A	28 B	0 C
Fusarium solani	14 a	0 b	0 b	84 A	34 B	5 C
Penicillium digitatum	71 a	50 b	32 c	97 A	70 A	51 B
Penicillium italicum	111 a	39 b	51 b	98 A	83 A	60 B
Rhizopus stolonifer	47 a	6 b	0 b	76 A	0 B	0 B

[1] Average of three replicates per a treatment.

[2] Means within rows per a parameter followed by the same letter are not significantly different at 0.05 probability level.

Table 3. Effects of mold fungi on peroxide value (PV) and free fatty acid percentage (FFA%) of olive oil:

Fungal species	Mold colonization%	PV	FFA %
Alternaria solani	18.6[1] bc[2]	22.0 bc	1.00 ab
Aspergillus niger	05.7 d	31.0 ab	0.86 ab
Cladosporium herbarum	10.0 cd	30.0 abc	1.03 ab
Fusarium solani	15.3 c	28.0 abc	1.03 ab
Penicillium digitatum	23.7 b	28.5 abc	1.13 a
Penicillium italicum	26.4 b	26.2 abc	0.63 b
Rhizopus stolonifer	36.2 a	18.8 c	1.10 a
Negative Control	00.0 d	36.2 a	0.88 ab

[1] Average of three replicates per a treatment.

[2] Means within columns per a parameter followed by the same letter are not significantly different at 0.05 probability level.

4. Discussion

Seven species of mold fungi were identified from olive fruits followed the infestation of olive fruit fly including *Alternaria solani, Aspergillus niger, Cladosporium herbarum, Fusarium solani, P. digitatum, P. italicum* and *Rhizopus stolonifer* with a sample frequency ranging between 6.7-33.3%. The low rate of colonization could be due to the occurrence of some natural inhibitors in the olive oil, such as some polyphenols (Ruiz-Barba *et al.*, 1990; Asehraou *et al.*, 1992).

Penicillium digitatum was the most dominant mold and was frequently isolated. *Penicillium crustosum, P. roqueforti* and *P. viridicatum* were the dominant flora of black table olives and the most frequently encountered species in Aegean and Marmara areas of Turkey (Tokuşoğlu *et al.*, 2012). Three of the mold genera identified in the present study, *Aspergillus, Penicillium* and *Fusarium*, are considered the most frequent toxigenic fungi in Europe (Creppy, 2002). *Penicillium* and *Aspergillus* represented the majority of mesophilic fungi isolated Moroccan olive and olive cake (Roussos *et al*., 2006).

Most of the isolated molds were vulnerable to hot water treatment at 50 and 70ºC as indicated by the great reduction in their spore germination and colony forming unit. This is a valuable result since some olive mills usually use hot water to wash olive fruits, which has an additional value through its detrimental effect on spore germination and fungal growth. Physical, chemical and microbiological characteristics of olive oil can be affected by pre-milling storage time and the storage manner of the oil (El Haouhay *et al.*, 2015). Generally, olives are very sensitive to physical damage and alterations caused by the presence and activity of microorganisms like molds, yeast and bacteria (Gutiérrez *et al.*, 2009; Asehraou *et al*., 1992; El Haouhay *et al.*, 2015). The seven isolated mold fungi were able to colonize olive oil through growth and spore production. Among them, *Rhizopus stolonifer* was the greatest colonizer and had obviously reduced PV of the oil and the same effect was also caused by *Alternaria solani.* When olive fruits were inoculated with some mold fungi, fungal infection had indirectly resulted in a significant increase in the extracted oil acidity and PV. However, there was no significant difference in the acidity and PV among different fungal isolates (Torbati *et al.*, 2014).

Acidic pH even slight is most probably caused by the double action of the lipolytic microorganisms and/or lipases, which may release FFA. This can be supported by the high PV, which may indicate the level of the lipolysis (Asehraou *et al*., 1992). There was no considerable change in FFA three months after inoculating olive oil with different molds. Perhaps, a longer period of incubation may lead to significant changes in the oil FFA. Free fatty acids are related to the lipolytic action of some microorganisms, which are generally active inhibitors against most microorganisms (Asehraou *et al*., 1992).

Storage of olive fruits and oil under uncontrolled environmental conditions had a drastic effect on the quality and shelf life of olive oil as indicated by the increase in its acidity and oxidation (Chliyeh *et al*., 2014; El Haouhay *et al*., 2015). Besides, a significant and important reduction of polyphenol contents and antioxidant capacity were detected in olive oil. The development of molds on olives is responsible for the poor nutritional quality of olives because molds can disturb the synthesis of fatty acids (Biasone *et al.*, 2012; El Adlouni *et al*., 2000).

Insect pest infestation and bad storage conditions of olive fruits may enhance the activity of other microorganisms besides molds, e.g. *Clostridium* spp., *Pseudomonas* spp. and *Enterobacter* spp. (Angerosa *et al.*, 1999). Olive oil produced under these adverse conditions not only leads to poor quality but could also put human health at risk. Mold growth on black table olives may be prevented by treating the fruits with sorbic acid, methyleugenol and spice essential oil where sorbic acid was the most efficient (Kivanç and Akgül, 1990).

Therefore, it is essential to have an effective management of the conservation of olives before and after

harvest to avoid fungal mold infection and its adverse effects. Effective management of olive fruit fly would reduce its fruit infestation and secondary mold infections, which would improve olive oil yield and quality.

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