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NATURAL OCCURRENCE OF *ALTERNARIA* TOXINS IN POMEGRANATE FRUIT AND THE INFLUENCE OF SOME TECHNOLOGICAL PROCESSING ON THEIR LEVELS IN JUICE

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Alternaria species produce several mycotoxins that are of particular health concern. The natural occurrence of three *Alternaria* toxins; alternariol (AOH), alternariol methyl ether (AME), and tenuazonic acid (TA) in pomegranate fruit was considered. *A. alternata* and *A. tenuissima* were identified by analysis of partial sequence of ITS-region. All studied strains produced high quantities of AOH in vitro on rice. *A. tenuissima* produces high quantities of AME and TA compared with *A. alternata*. In rotten tissues AME was the highest determined toxin with frequency percentage of 95.6%, followed by AOH and TA. All toxins were detected in the healthy tissues surrounding the infected tissues but at low levels. No visible changes were noted in *Alternaria* toxins after pasteurization of pomegranate juice, but they appeared after clarification. In conclusion, pasteurization and/or clarification are not sufficient to reduce *Alternaria* toxins in juice. The removal of the rotten parts does not ensure excluding *Alternaria* toxins.

Keywords: pomegranate, *Alternaria*, alternariol, alternariol methyl ether, tenuazonic acid, juice

Pomegranate (*Punica granatum*, Punicaceae) has high nutritional and medical value mainly due to its exceptional and unique sensory and nutritional properties (Viuda-Martos et al., 2010). The most destructive disease observed on pomegranate trees causes a leaf blotch and fruit spot (Ezra et al., 2010). Fruit symptoms are small, conspicuous, dark brown spots, initially circular and becoming irregular. *Alternaria alternata* has been reported as a postharvest disease causal agent of pomegranate in Greece (Pantidou, 1973). Black rot of the fruit core is starting from the calyx region, while the hard leathery rind appears healthy and fruit remains firm. Although this disease has been detected previously in Greece (Pantidou, 1973), it was noticed again only recently, probably due to the recent expansion of pomegranate cultivation and changes in weather conditions. Therefore, another report of *A. alternata* causing fruit decay in pomegranate orchards in Greece has been published in 2008 (Tziros et al., 2008). Moreover, fruit rot caused by *Alternaria* sp. has previously been reported in the USA and in Mexico (Farr et al., 2007). In 2009, study on integrated management of *Alternaria alternata* causing rot of pomegranate fruit has been conducted (Dahiwale et al., 2009). In 2010, the first report on black spot disease of pomegranate caused by *A. alternata* on pomegranates in Israel has been published as a new disease. Symptoms have been seen on

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the leaves and fruit, but no damage to the inner edible tissue has been found (Ezra et al., 2010).

Alternaria toxins have been found in fruit, such as apples, blueberries, cherries, and several citrus fruit. *Alternaria* species produce more than 70 phytotoxins. Some toxins, such as AOH and AME, are non-host-specific toxins and are concern for public health. These toxins are described to induce harmful effects in animals, including teratogenic, mutagenic, and clastogenic effects in various in vitro systems (Barkai-Golan, 2008). TA is toxic to chicken embryos and can cause haemorrhage and death in mice (Da Motta & Soares, 2000b). AOH, AME, and TA have been found in different fruit, such as olives, mandarins, apple, and peaches (Robiglio & López, 1995; Pose et al., 2010).

To the best of our knowledge, there are little information on the pomegranate fruit infection with fungi, and no available information about the presence of mycotoxins in pomegranate fruit or their fresh and processed juices. The pasteurization is the most commonly used preservation technique to extend the shelf-life of juices. However, this process may have adverse effects on sensory and nutritional properties of juices, particularly colour quality of anthocyanin containing juices, which is undesirably lost during thermal process (Patras et al., 2010). Therefore, the consumer demands for freshly squeezed fruit juices, but such products are susceptible to spoilage and thus have a limited shelf-life (Buzrul et al., 2008). In the case of the preparation of juice from fruit contaminated with toxin-producing fungi, there is a high probability for the transfer of these toxins to the consumers.

The aim of the current study was to: i) determine the species of *Alternaria* causing black spots resulting in mouldy heart of pomegranate fruit collected from local market of Taif City, KSA, ii) identify their ability to produce AOH, AME, and TA in rotten fruit, and iii) evaluate the effect of pasteurization and/or clarification during juice preparation on the presence of these mycotoxins.

1. Materials and methods

1.1. Sample collection and cultural technique

Healthy-appearing and black-spotted pomegranate fruit were collected from the local market of Taif City, KSA. Pieces of the healthy and rotten tissues from the inner surface were placed onto malt agar (MA) with penicillin G and chloramphenicol (MApc, 75 mg l^{-1} MA) and incubated at 30 °C for 5 days. Pure cultures were obtained by transferring hyphal tips to MApc. Isolates were maintained on MApc at 4 °C, and identified by macroscopic and microscopic observations. Identification of isolated fungi was carried out based on the book of Pitt and Hocking (1997).

1.2. Molecular identification

Fungal strains were cultured in 100 ml Erlenmeyer flasks containing 20 ml potato dextrose broth (PDB) for 5 days using a rotary shaker (30 °C, 150 r.p.m.). The mycelium was collected by filtration and ground to fine powder in liquid nitrogen. The genomic DNA of fungal isolates was extracted using the Fungi/Yeast Genomic DNA Isolation Kit (Norgen, Biotek Corp., Canada). From the nuclear rDNA, the ITS regions (ITS1 and ITS2) and the 5,8S rRNA gene were amplified by PCR using the primer set ITS1 (5′ tccgtaggtgaacctgcgg 3′) and ITS4 (5′ tcctccgcttattgatatgc 3′) (White et al., 1990). PCR protocol described by Gardes and Brund, (1993) was applied. The PCR product was excised from the ethidium bromide-stained

gel and purified using a purification kit (QIAquick Gel Extraction Kit, Qiagen) according to the manufacturer's protocol. All of sequenced strains were identified by similarity searches with ITS sequences at the website of NCBI database. The nucleotide sequence data obtained in the current work were submitted to the EMBL Nucleotide Sequence Database (Table 1).

Table 1. Identification of *Alternaria* strains by alignment with the sequences of fungi in the NCBI database

Proposed identity	Accession No.	Identity according to CBS (%)	Database accession No.
Alternaria tenuissima TUAa1	HG974556	100	KT598351.1
Alternaria tenuissima TUAa2	HG974557	100	JX406579.1
Alternaria tenuissima TUAa3	HG974558	100	KC415611.1
Alternaria alternata TUAa4	HG974559	100	KP638335.1
Alternaria alternata TUAa5	HG974560	100	KF380822.1
Alternaria alternata TUAa6	HG974561	100	JF835808.1
Alternaria tenuissima TUAa7	HG974562	100	KF907249.1

1.3. Preparation of pomegranate juice

Two sets of pomegranate juices were prepared in the present study (Fig. 1). The first was prepared from healthy-appearing fruit and the second set was prepared from the rotten fruit after removing the infected tissues. Raw pomegranate juice (RPJ) was extracted as described by Rinaldi and co-workers (2013). Pomegranate fruit were washed with tap water and manually separated into arils. The RPJ was extracted from arils by electric fruit crusher. The extracted juice, having a deep-red colour, was prefiltered with a stainless steel filter. Then it was stored in a refrigerator at –20 ºC and defrosted before use. Clarified pomegranate juice (CPJ) was obtained by treating the RPJ with 15 µl l^{-1} pectolytic enzymes solution (Erbslöh Geisenheim, Germany) for 120 min at 25 ºC (Rinaldi et al., 2013). Pasteurization of pomegranate juice was achieved in glass bottles (200 ml) at 95 ºC for 7 min by immersion in a hot water bath. The juices were then cooled under tap water (Elhariry et al., 2011). Pasteurized pomegranate juices were named as PRPJ for pasteurized raw pomegranate juice and PCPJ for pasteurized clarified pomegranate juice. H and I letters were added to the abbreviations of juices prepared from healthy-appearing and rotted fruit, respectively.

1.4. In vitro production of Alternaria *toxins*

The ability of fungal isolates to produce mycotoxins was in vitro determined (Pose et al., 2010).

1.5. Alternaria *toxins in the pomegranate tissues and juices*

Alternaria toxins; AHO, AME, and TA were determined in rotted and surrounding healthy tissues. Also, these toxins were assayed in pomegranate juices prepared from healthy appearing fruit and from rotted fruit after removing infected tissues. Extraction and determination of mycotoxins were achieved as described by Da Motta and Soares (2000a).

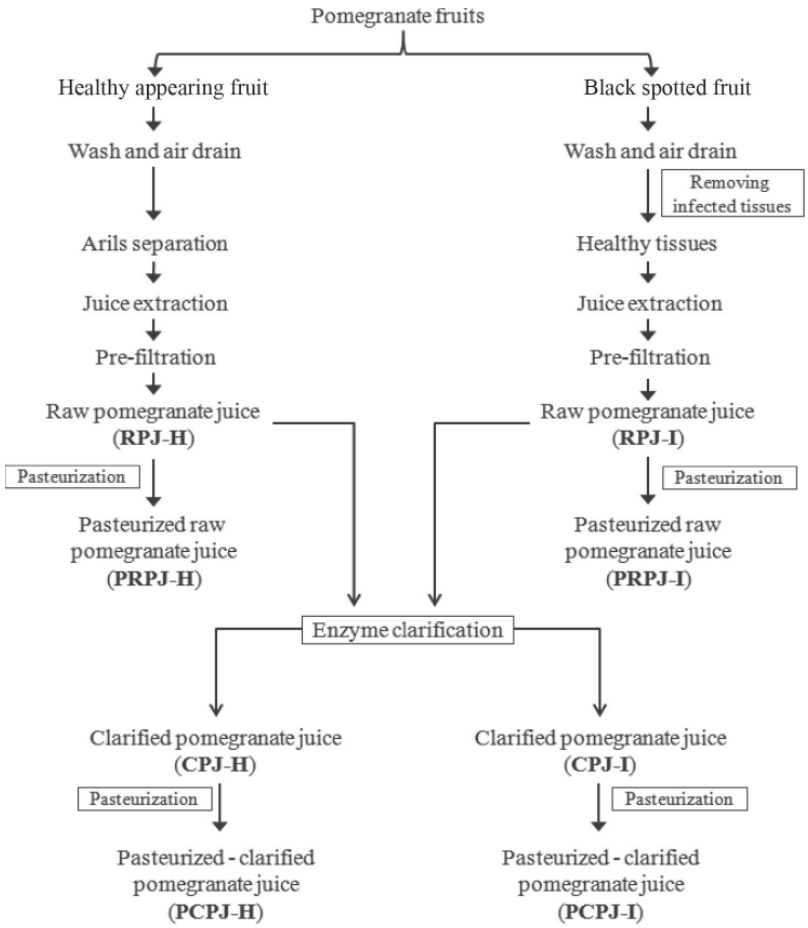

Fig. 1. Production scheme of pomegranate juices

1.6. Statistical analysis

All data were statistically evaluated by ANOVA test (P=0.05), performed with the Statistical Analysis Tool of Microsoft Excel 2003, Microsoft Office Professional Edition 2003 (Microsoft Co., Santa Rosa, CA, USA).

2. Results and discussion

2.1. Isolating and identifying Alternaria *sp.*

One hundred pomegranate fruit were collected from the local market of Taif City, KSA. All fruit were investigated for presence of fungal infection symptoms. Eighty-seven percentages of fruit appeared as healthy fruit with normal leathery exocarp. Twenty-three percentages of fruit appeared with black spot symptoms ranging from a single spot to more than 25% of the fruit surface (Fig. 2). Few spots consisted of a green-yellow halo surrounding a necrotic

lesion. Damage to fruit was observed to the peel surface. Some fruit appeared with small reddish brown circular spots. Black spot symptoms have been noted on fruit, ranging from a single spot to more than 50% of the fruit surface (Ezra et al., 2010).

Fig. 2. Black spots infected pomegranate fruit (A, B, and C) and inside rotten tissues (D, E, and F). Black spots are singly distributed on the leathery exocarp or aggregated surrounding fruit-stalk. In some fruit, small black spots were distributed from calyx to fruit-stalk.

Both healthy-appearing and infected fruit were cut and investigated from the inside (Fig. 2). No fungal infection symptoms were noticed inside the healthy-appearing fruit. However, in the black-spotted fruit, the infection symptoms appeared on the edible parts (arils and seeds) in some fruit, whereas, in others both edible and non-edible parts were affected. Arils of infected fruit became pale, light brown, or dark brown. The fleshy mesocarp of some infected fruit appeared in light brown to dark brown. Moreover, the pulp membrane was also infected in some fruit and became dark brown to black (Fig. 2). Fungal colonies emerging from symptomatic tissue had morphology and conidia typical of *Alternaria* spp. Seven *Alternaria* isolates obtained from the edible parts were subjected to molecular identification by determining the partial sequence of the rDNA ITS region. All of sequenced strains were identified by similarity searches with ITS sequences in the NCBI database. The obtained results of the molecular identification ensured the presence of seven *Alternaria* strains belonging to two species; *A. alternata* (3 strains) and *A. tenuissima* (4 strains) with 100% identity percentage (Table 1). The obtained sequences of the seven strains were deposited to

the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries. The accession numbers and similarity percentage of the submitted sequences are illustrated in Table 1.

2.2. In vitro mycotoxin production by Alternaria *strains*

The seven identified *Alternaria* strains were tested for their ability to produce AOH, AME, and TA in rice at 25 ºC (Table 2). The strains were classified to low (+), moderate (++), and strong (+++) producer of mycotoxins according to the classification categories described by Pose and co-workers (2010). All studied strains produce high quantities of AOH (strong-AOH producers). *A. tenuissima* strains were strong-AME producers and moderate-TA producers, whereas *A. alternata* strains displayed moderate and low production of AME and TA, respectively. *A. tenuissima* strains showed high ability to form AME and TA compared with *A. alternata* strains. On the other hand, the level of TA toxin was produced in lower amounts compared with the quantities of AOH and AME produced by all tested strains. Generally, high percentage (100%) of the isolated strains showed the ability to form all studied *Alternaria* toxins at different levels. These results were in accordance with those demonstrated in previous works by *Alternaria* species isolated from other fruit. Robiglio and López (1995) isolated eleven *A. alternata* strains from mouldy core disease in "Red Delicious" apples. They stated that most of the isolates had the ability to produce AOH and AME toxins in the whole fruit. Recently, Pose and co-workers (2010) mentioned that 85.7% of *Alternaria* strains, isolated from "Moldy Heart" affected peaches, produced TA toxin, whereas 100% of the strains were able to form AOH and AME toxins in rice at 25 ºC. In another work, only 64% of *Alternaria* strains isolated from tomato fruit produced TA (Pose et al., 2004).

Table 2. Mycotoxin production in rice at 25 °C by *Alternaria* strains isolated from inner infected tissues of pomegranate fruit

Strains	*Alternaria* toxins		
	AOH	AME	TA
A. tenuissima TUAa1	+++	+++	++
A. tenuissima TUAa2	+++	+++	++
A. tenuissima TUAa3	+++	+++	++
A. alternata TUAa4	+++	++	+
A. alternata TUAa5	+++	++	+
A. alternata TUAa6	+++	++	+
A. tenuissima TUAa7	+++	+++	++

+: Low; ++: Moderate; +++: Stronger; This classification was suggested by Pose and co-workers (2010) as follows:
AOH (alternariol): +: 0.04–0.15 mg g^{-1}, ++: 0.16–0.27 mg g^{-1}; +++: over 0.28 mg g^{-1}.
AME (alternariol methyl ether): +: 0.07–0.24 mg g^{-1}; ++: 0.25–0.41 mg g^{-1}; +++: over 0.42 mg g^{-1}.
TA (tenuazonic acid): +: 0.01–0.03 mg g^{-1}, ++: 0.04–0.07 mg g^{-1}; +++: over 0.08 mg g^{-1}.

2.3. Alternaria *toxins in the pomegranate tissues*

The natural co-occurrence of *Alternaria* toxins was determined in the inner tissues of healthy-appearing and black-spotted pomegranate fruit (Table 3). All healthy-appearing pomegranate fruit (87 samples) were negative to *Alternaria* toxins. On the other hand, all tested rotted tissues (23 samples) and most of healthy tissues of the black-spotted fruit (18 out of 23

samples) proved positive to at least one *Alternaria* toxin investigated. This result indicates that *Alternaria* toxins can be distributed from the rotted tissues to the surrounding healthy tissues in black-spotted pomegranate fruit. The natural co-occurrence of all three tested toxins was recorded in 12 out of 23 rotted tissues and in only 2 out of 23 healthy tissues, whereas co-occurrence of two toxins was recovered from 7 and 5 rotted and healthy tissues, respectively.

Table 3. Natural co-occurrence of *Alternaria* toxins in healthy-appearing and black-spotted pomegranate fruit

Pomegranate sample	Total number of tested samples	No. of samples containing *Alternaria* toxins				No. of positive samples
		3 toxins	2 toxins	1 toxin	No toxin	
Healthy-appearing fruit	77	0	0	0	0	0
Black-spotted fruit	23					
Rotten tissues		12	7	4	0	23
Healthy tissues[a]		2	5	11	5	18

[a]: surrounding the rotten tissues

Table 4 shows the range and frequency percentage of the individual toxins in the pomegranate fruit. None of the investigated toxins was detected in the healthy-appearing pomegranate fruit. In the rotted tissues of black-spotted fruit, AME toxin was the highest determined toxin (2.14–32.02 ng g^{-1} d.w.) with frequency percentage of 95.6%, followed by AOH and TA toxins. In the healthy tissues surrounding the rotted tissues, all toxins were also found but at lower levels compared with that measured in the rotted tissues (Table 4). This means that *Alternaria* toxins are not only found in the infected tissues, but also diffuse into the surrounding tissues at low levels. This finding is in agreement with those mentioned by Robiglio and López (1995). They have demonstrated that *Alternaria* toxins in apple fruit were not restricted to the rotted area, which was characterized by abundant fungal hyphae, but could be also recovered from the surrounding tissues. Another previous work had demonstrated that mycotoxins are released by the fungi into the surrounding substrate and contamination of agricultural products is therefore possible (Brzonkalik et al., 2011).

Table 4. Natural occurrence of *Alternaria* toxins in healthy-appearing and black-spotted pomegranate fruit

Sample (No.)	*Alternaria* toxins					
	AOH		AME		TA	
	Range[a]	Frequency%[b]	Range[a]	Frequency%[b]	Range[a]	Frequency%[b]
Healthy-appearing fruit (87)	ND	0	ND	0	ND	0
Black-spotted fruit (23)						
Rotten tissues	1.86–19.20 ±0.02	78	2.14–32.02 ±0.02	95.6	1.11–13.71 ±0.01	69.5
Healthy tissues[c]	0.71–4.44 ±0.01	43.5	0.91–6.38 ± 0.01	61	0.47–4.22 ±0.01	17.4

AOH: alternariol; AME: alternariol methyl ether; TA: tenuazonic acid; ND: not detectable under studied conditions
[a]: calculated per gram dry weight of tissue (ng g^{-1} d.w.) ± standard deviation
[b]: (No. of samples containing toxin/total no. of tested samples)×100
[c]: surrounding the rotted tissues

2.4. Alternaria *toxins in pomegranate juices*

AOH, AME, and TA toxins were determined in two juice sets prepared from healthy-appearing (H) and rotten (I) fruit. The effect of pasteurization and/or clarification on the level of *Alternaria* toxins was also studied (Table 5). None of the examined toxins was observed in the pomegranate juice set prepared from healthy-appearing fruit (RPJ-H, PROJ-H, CPJ-H, and PCPJ-H). In the pomegranate juice set prepared from the healthy tissues of the black-spotted fruit, all *Alternaria* toxins were detected at different concentrations. In raw pomegranate juice (RPJ-I) AOH, AME, and TA toxins were measured at level of 3.4, 4.6, and 1.65 ng g^{-1} d.w., respectively. No significant differences were recorded in the tested *Alternaria* toxins after pasteurization (PRPJ-I) (Table 5). However, clarification process led to significant increase in the level of all tested toxins in the clarified pomegranate juice (CPJ-I). There is no significant effect of pasteurization on the level of *Alternaria* toxins in the clarified juice (PCPJ-I, Table 5).

Table 5. Alternaria toxins in pomegranate juices

Juice*	*Alternaria* toxins[a] (ng g^{-1}d.w.)		
	AOH	AME	TA
RPJ-H	ND	ND	ND
PRPJ-H	ND	ND	ND
CPJ-H	ND	ND	ND
PCPJ-H	ND	ND	ND
RPJ-I	3.40^{b}±0.01	4.65^{b}±0.01	1.65^{b}±0.02
PRPJ-I	3.14^{b}±0.02	4.49^{b}±0.02	2.65^{b}±0.02
CPJ-I	4.24^{a}±0.03	5.84^{a}±0.02	3.24^{a}±0.01
PCPJ-I	4.85^{a}±0.02	6.07^{a}±0.03	3.92^{a}±0.01

AOH: alternariol; AME: alternariol methyl ether; TA: tenuazonic acid; ND: not detectable under studied conditions. Values in the same column with different letters differ significantly ($P<0.05$, n=3)
*: Juice types were described in Figure 1; [a]: calculated per gram dry weight of juice

To our best knowledge, there are no previous studies available on the natural occurrence of *Alternaria* toxins or the effect of pasteurization and clarification on these toxins in pomegranate juice. Many previous studies demonstrated that the toxic fungal secondary metabolites are thermally stable, therefore they might be transferred from contaminated raw-foods into the final products (Malachova et al., 2011). This statement could explain the insignificant effect of pasteurization on the level of *Alternaria* toxins in pomegranate juices.

The increasing amounts of *Alternaria* toxins after the clarification step could be explained by the presence of masked mycotoxins such as *Alternaria* toxin-glucoside, which is considered a mycotoxin derivative (Mikula et al., 2013). These masked mycotoxins may be degraded during the clarification step using the pectolytic enzymes solution, resulting in the release of the free AOH, AME, and TA toxins.

3. Conclusions

AHO, AME, and TA toxins were present in the rotten and healthy parts of the black-spotted pomegranate fruit. In rotten tissues, AME toxin was the highest determined toxin with frequency percentage of 95.6%, followed by AOH and TA. The removal of the rotten parts does not ensure the complete elimination of *Alternaria* toxins. Pasteurization and/or clarification were not sufficient to reduce *Alternaria* toxin concentrations in pomegranate juice. Therefore, close attention should be paid to the preparation of fruit juice to ensure its safety.

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