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Characterization of *Alternaria* Isolate from Organically-Grown Tomato (*Lycopersecon esculentum* L.) in Relation to the Fruit Antioxidant Compounds

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Environmental pollutionas a consequence of irrational usage of fungicides is an immense problem bothering the well-being of human kind globally. The real risk is the possibility of such pollutants to ultimately find their way to human food via food chain. Recently, a growing concern regarding food safety has increased in response to the high demand for organic products. Organic tomato cultivation gainedgreat preference and acceptability for consumers. However, contamination of fresh fruits and vegetables with a variety of postharvest fungi is an essentialproblem which requires immediate measures to be taken seriously for the sake of maintaining human health.The main objective of this work is to study the susceptibility of organically-cultivated tomato fruit to post harvest pathogenic fungi and to find out whether there is relationship between total phenolic compounds, ascorbic acid content and antioxidant capacity of tomato fruit and the appearance of fungal growth. Furthermore, DNA-based method, Random amplified polymorphic DNA technique (RAPD) was used to fingerprint and assess the genetic informationabout thefungal isolate. The current results revealed that different tomato varieties showed different susceptibility levels to infection by postharvest pathogenic fungi besides different amounts of accumulated total phenolic compounds, ascorbic acid contentand antioxidant capacity. Furthermore, higher concentrations of total phenolic compounds,ascorbic acid content andantioxidant capacity were detected in the small size tomato fruit, which might point to absence of fungal growth. Furthermore, morphological characteristics showed that the isolate detected from infected tomato fruit was *alternaria alternata,* finger print (RAPD) technique showed clear DNA bands for the isolate which indicated that the opa04 was an efficient primer that supported the amplification by producing unique patterns of banding for the *A. alternata* isolate. Further molecular work is needed to provide more authenticated information regarding the isolate.

Key words: *Lycopersecon esculentum*, antioxidants, *alternaria alternata*, RAPD

Tomato is one of the most widely grown vegetables in the world. Tomato fruit production has been mainly dependent on fungicides usage, but growing concerns about food safety and environmental pollution have increased the demand of organic products all over the world. Organic cultivation of tomato is one of the factors that are becoming major decision-making in tomato preferences for many consumers[1]. The total area of organically certified land in Saudi Arabia is expanding fast as more farmers are converting to organic production due to increase in consumer awareness. The most important organic crops in Saudi Arabia are vegetables with acultivation area of about 8.373 hectare[2]. Tomato is an excellent source of many secondary metabolites important for human health such as folate, ascorbic acid, flavonoids, chlorophyll, ²-carotene and lycopene[3]. Phenolic compounds are synthesized by plants during normal development and also in response

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to stress conditions. Generally, phenolics are characterized by a benzene ring and hydroxyl group which can be converted into lignin. Light enhances the biosynthesis of phenolic substances in chloroplast, but they tend to accumulate in vacuoles in relatively high amounts or deposit in secondary cell wall as lignin[4]. The interest in polyphenols as antioxidants is focused on flavonoids[5]. Flavonoids can interfere with several free radical-generating systems and their reactive hydroxyl groups via oxidation to more stable and less reactive radical compounds[3]. Recent studies reported that the regular consumption of tomato has been associated with decreased risk of chronic degenerative diseases due to the presence of antioxidant compounds such as carotenoids, particularly ascorbic acid and phenolics, particularly flavonoids as well as higher levels of potassium since it is an important factor in lowering blood pressure [3-6]. A comparative study of vegetables produced in organic farming showed predominately lower susceptible to pests and diseases and were characterized by higher contents of defense-related secondary metabolites [7]. Furthermore, Losses due to postharvest disease that leads to reductions in fruit quantity and quality may occur any time during postharvesthandling, from harvest to consumption. *Alternaria alternata* is a common saprobe opportunistic pathogen affecting many cultivated plants in the field and during post-harvest storage of fruit and vegetables [8-9]. The pathogenicity factor of *A. alternata* with respect to tomato plant infection lies in the production of host-specific AAL-toxin that is capable of inducing cell death[10]. To date, little is known about the direct interaction of infection of tomato fruit by *A. alternata*and certain fruit compounds accumulation; nevertheless, plant might reactwith pathogenic attack by accumulation of compounds that increase plant resistance to infection. Soluble sugars, especially sucrose, glucose and fructose can be increased in tomato fruit due to infection pressure in order to restrict the accumulation of reactive oxygen species [11]. On the other hand, the response of phenolic compounds to pathogenic attack is based mainly on defense response that is characterized by the early and rapid accumulation of phenolics at the infection site[12]. Moreover, ascorbic acid contributes on the response to pathogenic attack through the control of biosynthesis of hydroxyproline-containing protein, which is involved in the cross-linking of the cell wall in response to injury and extension genes[13]. Furthermore, several methods have been used to illustrate the variation among species of parasitic fungi such as morphology of spores and colonies, extracellular protein profiles, pathogencity and growth requirements[14] . Nevertheless, variation among fungal strains and species using Random Amplified Polymorphic DNA (RAPD) which is described by Williams *et al.*[15] is an easy tool to detect polymorphism in a largenumber of samples at relatively low cost. It is a modification of PCR (polymerase chain reaction) that allows revealing polymorphism within completelyunknown samples without the need of probe hybridization or DNA sequencing[16].The present study aimed to gain knowledge about antioxidant compounds accumulation in different tomato cultivars cultivated under organic farming conditions in relation to infection by post-harvest pathogen. Besides,a PCR-based DNA marker was used for pathogenic microorganism characterization.

MATERIALS AND METHODS

Plant Materials

Two different varieties of tomato fruit were obtained from an organic farm in Diyrab, Riyadh, KSA on the same harvest day in January 2013. One variety with small size (weight 5.5g),cherry and other one with a big size (weight 41.1 g). Some of the collected samples were stored at "50°C for further analysis. The rest of the samples were kept at room temperature (25° C) in the department of biology laboratory and observations were recorded every two days regarding appearance of any fungal growth. For the fruit quality measurements, frozen samples were freeze-dried (Epsilon2-40, Christ, Germany) for four days, then the samples were milled in a speedy mixer (KRUPS, Speedy Pro Plus F7247010F, France). Afterwards, samples were milled to a fine powder up to a particle size of 0.2 µm for further analysis.

Determination of ascorbic acid, total phenolic compounds and antioxidant capacity

Ascorbic acid was measured by titration from fresh fruits according to Albrecht [17]. The total phenolic compounds content was determined with

Folin-Ciocalteu´s phenol reagent from the freeze-dried material by spectrophotometer according to Singleton and Rossi [18] and the antioxidant capacity was determined using the Ferric Reducing Antioxidant Potential (FRAP) assay which works with iron for oxidation according to Binoy et al [19]. Three replicates for each sample were performed regarding the laboratory analysis.

Detection of fungal growth

Tomato samples with symptoms of fungal growth were collected and taken to the microbiology lab for further investigations. The isolation of the pathogen was carried out by cutting the pieces, at the edge of contaminated and healthy tissue [20]. Thereafter, fragments were transferred on potato dextrose agar (PDA) and incubated at 25°C for three days. Growth characteristics of cultures were studied on PDA.

Fungal isolation and DNA extraction:

The selected isolate was grown on potato dextrose (PD) medium and allowed to grow for 7 days at 28ºC. After the incubation, the mycelium was scraped off for DNA extraction. Total DNA was extracted by DNeasy Plant Mini Kit (QIAGEN)[21].

Characterization of isolate by RAPD:

RAPD analysis was carried according to Gherbawy [22] and Parak *et al*.[23], 25¼l PCR reaction, containing 1¼l of template DNA, 12,5 ¼l KAPA Taq ready mix, 10.5 ¼l water and 1 ¼l of primer OPA04 (5'- AAT CGG GCT G-3').

The amplification was carried out as follows:

The thermal cycling program was as follows: 4 minutes initial denaturation at 95 C, followed by 45 cycles of 9 seconds denaturation at 95 C, 25 seconds 40 C, 90 seconds with final extension at 72 C for 4 minutes. A negative control using water instead of template DNA was included in the amplification process. DNA fragments were separated by electro-phoresis in 1.5% (w/v) agarose gel containing ethidium bromide (0.5 g/ml) using 1×TBE buffer. Visualization was performed in UV transilluminator and the images were captured with DOC PRINT system (VilberLour-mat, USA).)The lengths of the amplification products were estimated by comparison with a 100 bp DNA ladder.

RESULTS AND DISCUSSION

Tomato is an excellent source of many secondary metabolites[3].The present study revealed signiûcant variations in the content of fruit antioxidant compounds between the investigated varieties. The individual compounds and their relationship to fruit weight were presented in table 1. Total phenolic compounds, ascorbic acid content and antioxidant capacity were detected in both varieties which had the same trend compatible with[19,24] who studied different tomato varietiesin conventional and organic farming system. Variations between studied varieties were observed in the content of the total phenolic compounds, ascorbic acid and antioxidant capacity; many different studies showed that the secondary metabolites of tomato fruit differ according to different varieties[24]. Our results indicated that, cherry tomato had the higher content of total phenolic compound (894.1 ± 8.3), ascorbic acid (325.5 ±5.2)mg g^{-1} fresh weight and antioxidant capacity (70.85±1.5)mmol Fe^{2+} kg^{-1} fresh weight compared with the big size tomato(Table 1).Similar results were also detected by[19,24,25] who stated that cherry tomatoes with a low fruit weight represent a better source of antioxidants compounds than fruits of a larger size. The antioxidant capacity was

Table 1. Fruit total phenolic compounds, ascorbic acidand antioxidant capacity for two different tomato varietiesgrown organically at Diyrabfield. Riyadh, Saudi arabia.

Antioxidant capacity (mmol Fe^{2+} kg^{-1} fresh weight)	Ascorbic acid(mg g^{-1} fresh weight)	Total phenolic compounds(mg g^{-1} fresh weight)	Fruit size(g)	Genotype
70.85 ± 1.5 a	325.5 ±5.2 a	894.1 ± 8.3 a	05.5 b	Cherry tomato
53.35 ± 3.3 b	172.4 ±7.1 b	489.7 ± 9.2 b	41.1 a	Big size tomato

Data are expressed as mean ± SD. Different letters within a column indicate signiûcant differences in theTukey test;±=5%.

higher in the cherry tomato where the total phenolic compound was higher. This might support the information that the total phenolics are important contributors to the total antioxidant activity[26]. Measurement of antioxidant capacity in an organic solvent extract, e.g. in acetone or dichloromethane, as in this study, is usually well correlated to carotenoid and lycopene concentrations, as carotenoids are part of the lipophylic extract [27]. On the other hand, tomato with a big size 31.1 g showed fungal growth 10 days after harvest, but cherry tomato (5.5 g) showed no growth although both tomato varieties were kept in the same area at the same room temperature. Variations among different varieties were also observed by Mohammed *et al.*[27] who studied 28 organic tomato cultivars that differ in their susceptibility to fungal infection in the field by *Phytophthora infestans*. Interestingly, the total phenolics compounds and antioxidant capacity had higher concentrations on the cherry tomato which showed no fungal growth compared with a big size one, same result was also described by Jinsin[27] who stated that small tomato fruits had higher resistance to disease. Differences in plant susceptibility to disease could be attributed to possible variations in the growth type, physiological and genetic attributes besides differences in the accumulation of plant secondary metabolites[28]. Early study[29] reported that cultivar resistant to fungi infection expressed much higher levels of phenolics and oxidative enzymes. Further observation [30] found that ascorbic acid content was higher in tomato cultivars resistant to tomato yellow leaf curl virus than the non-resistant ones. These observations might explain the absence of the fungal growth in cherry tomato since resistant to fungi infection was related to much higher levels of phenolics and oxidative enzymes which could create an inhibiting environment in and around the infected tissues, comprising very reactive phenylpropanoid free radicals and lignifications process[29]. Another study revealed that the elevation of the antioxidant capacity of plant is expected to increase their tolerance to pathogens or a biotic stress[31].

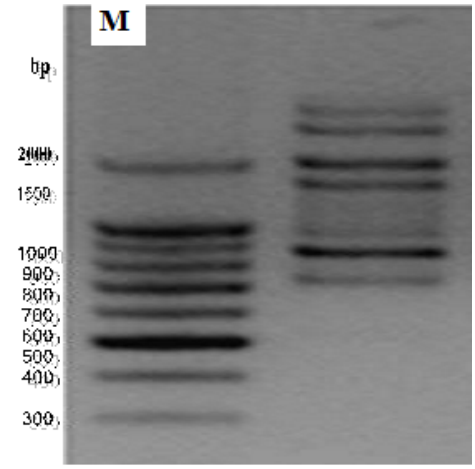

Fig. 1. Random ampliûed polymorphic DNA patterns obtained by using the primer OPA04 (5'- AAT CGG GCT G-3') from *Alternaria alternata* isolate. The results showed that the tested primer recorded amplification with tested isolate;it amplified 8 DNA bands.100 bp DNA ladder

Furthermore, one of the major causes of poor fruit quality during storage and transport are diseases caused by phytopathogenic fungi[32]. According to the morphological characteristics isolated fungus from tomato fruit in this study was identified, it considered as the hardest step in the detection process[33]. *A. alternata* was determined following the growth characteristics on PDA Initially; the mycelium was hyaline that turned to grey- brownish, multicelled, with cottony texture and white margin. Findings about mycelium colour was in agreement with results of different studies [34,35,36] who noticed mycelium of *Alternaria spp*., as grayish to green, brown and deep olive grey in colour in infected watermelon, chilli, gerbera and tomato respectively. Furthermore, *A. alternata* isolated from contaminated tomato fruit was confirmed by comparing the amplified DNA fragments. In the present study, Amplification of genomic DNA of the isolates of endophytic *Alternaria alternata* which was performed Using opa04 primer that produced 8 DNA bands. All the bands were clear and reproducible, and the size ranged from 750-3000 base pairs. No visible fragment was produced below 750 bp or above 3000 bp. Our results indicated that the opa04 was an efficient primer that supported the amplification by producing unique patterns of banding for *A. alternata* isolate.

CONCLUSION

In light of the present findings it was obvious that the size of tomato fruit is an important

parameter to be considered in fruit selection with regard to fruit quality attributes. More interestingly the size of the fruit was found to be strongly correlated with the degree of susceptibility to fungal growth. Seemingly, it would be quite helpful to investigate the mechanisms underlying the secret between fruit size and susceptibility level. Answers to such enquiries would no doubt enable the most appropriate of tomato varieties for the purpose of breeding selection.

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