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Association of *Geotrichumcitri-Aurantii* with Citrus Fruitsdecay in Iran

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Abstract

An outbreak of citrus sour rot disease occurred on citrus fruits in northern of Iran during theautumn season of 2012. Isolations were made from rotted fruits on PDA and CMA media. Afungus consistently isolated from the diseased fruits formed white-to-cream colonies on themedia. Pathogenicity was confirmed by needle-stab inoculation at the fruits. Based onmorphological, pathogenicity and intergenic transcribed spacer (ITS-PCR) region and ITS-RFLPanalysis, all isolates were identified as *Geotrichumcitri-aurantii,* the causal agent of sour rot ofcitrus. Several cultivars of citrus fruits were evaluated for their susceptibility to the causal agent.Tested fruits were ranked for their susceptibility to the *G. citri-aurantii*by determining diseaseseverity based on mean lesion size. Citrus were classified into three groups using Duncan'smultiple range test, include: most susceptible: (cvs: Onshiu and Ponkan), moderately susceptible: (cvs. Clementine, Page and King), oranges (cvs. Thomson Navel and Washington Navel), sweetlime and Grapefruit (cv. Marsh), and the least susceptible: Valencia and Sour orange. Based onthe results obtained here, the disease severity would be in correlation with the fruits firmness. Toour knowledge this is the first report of *G. citri-aurantii*causing sour rot on citrus fruit from Iran.

Keywords: Citrus sour rot, Detection,Geotrichum, Pathogenicity, ITS-PCR, ITS-RFLP

Introduction

Citrus grows well in northern and southern parts of Iran because of freezing conditions are notenough to kill the tree. Iran is 9th largest citrus producer with the production of 4000.02 thousandtons annually (GoleinandAdoli, 2011). Citrus provides employment for many people through thecountry. Citrus fruits can be attacked by several pathogens in orchard, storage, and transportation.Sour rot of citrus fruits was first described by Smith (1917) and designated as *Oosporacitriauranti*(Ferr.) Sacc.etSyd. Then *Geotrichumcandidum*Link ex Pers (synonym *Geotrichumcitri-aurantii*(Ferraris) R. Cif. and F. Cif) was proposed by Butler et al. (1988) (asexual form) and*Galactomycescitri-auranti*Butler (Sexual form). However sour rot is a postharvest disease butdisease outbreak occurred in the field because of heavy rainfall during the autumn season of2012. The *G. citri-auranti*is a common inhabitant of citrus soils causes losses of lemon (*Citruslimon*Burm, f.), mandarin (*C. reticulate* Blanco), and orange (*C. sinensis*(L.)) worldwide (Plaza et al., 2004; Smilanickand Sorenson, 2001; El-mougay et al., 2008). The organism is soil wind and splash-borne in soil particles to surfaces of fruit in the tree canopy. Healthy citrus fruits areinfected by *G. citri-auranti*through injuries, particularly deep injuries that extend into the albedo(Powell 1908). The development of rot is dependent on turgid tissues, wet and rainfall seasonsand temperature >10^{o}36 C (Brown and Eckert, 1988; Cohn et al., 1991; Liu et al., 2009). Moistenvironmental condition and unsuitable harvest and packing methods led to spread propaguls(artroconidia) of *G. citri-aurantii*. However loss caused by sour rot is comparatively small, it maybe large in some years and areas (Eckert 1959). Since oranges are stored for long periods innorthern Iran, sour rot is more common on oranges than other citrus fruits. Susceptibility of citrusfruits to sour rot has been reported in association with ripeness and over ripeness of the fruits andlong storage (Baudoinand Eckert, 1982). Disease incidence is also a function of inoculums densityand number of wounds per fruit (Bancroft et al., 1984).The disease seems to be spread quicklyand there is cause for

concern if diseased fruits are found among healthy. Rotted and maceratedtissue containing mycelium and spores of the fungus spreads the decay from infected 45 to healthy46 fruit by fruit fly in orchards or storage.

To date several studies have discussed regardingpostharvest decay of fruits and vegetables, but few data about sour rot have been published. Theobjective of this study was identification of the causal agent of the disease and determining the susceptibility of some citrus cultivar to the pathogen.

Materials and Methods

Samples of citrus fruits (*Citrus sinensis* L)) with a light brown to yellow color and exhibitingextensive water soaked lesions suspected to be infected by *G. citri-aurantii*were collected from arange of citrus orchards and brought into the laboratory for analysis during autumn season of2012. The fruits surface were disinfected with 10% of commercial NaClo for 3 min. Small pieces (0.5-1 cm) were placed onto corn meal agar (CMA) and potato dextrose agar (PDA) media. Purified cultures werestored in test tube on PDA medium at 25^{o} C. Pathogenicity tests were performed after disinfectingof the inoculation surface with 70% ethyl alcohol, by dipping a steel rod with a 1-mm-wide and2-mm-long tip into the inoculum suspension (1×1010arthroconidia L-157) and making a single puncture in each fruit with the rod (Smilanick et al., 2008).

The intergenictranscribed spacer region PCR (ITS-PCR) was done as described by Masayuki et al. (2008) with ITS1 and ITS4 primers for five isolates. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under UV light.The ITS-PCR products then were digested with Hinfɪ restriction enzyme, and electrophoresed on 4% MetaPhor® agarose gel To compare citrus cultivars reaction to sour rot caused by *G. citri-auranti*, an experiment was conducted in a completely randomized design with 11 cultivars as treatments in four replications. For each cultivar, a total of 16 fruits were used, each treatment consisting of 12 inoculated and four un-inoculated as controls on detached fruits (Mirzaee et al., 2009). Fruits were ranged for disease severity by measuring length and width of each lesion three days after inoculation (Biggs and Miller, 2004). The results were analyzed using the analysis of variance (ANOVA). Mean lesion diameter data were compared by Duncan's Multiple Range Test (MSTAT-C) at $p < 0.01$.

Results and Discussion

In pathogenicity test fungus caused severe disease reaction on citrus fruits tested and was practically identical. The fungus re-isolatedfrom fruits which inoculated in pathogenicity test, confirming Koch's postulates. The test wasrepeated three times to confirm our diagnosis (Hernández-Montiel et al., 2010**).** *G. citri-aurantii*was detected in most samples. All isolates showed similar morphological characteristics such asgrowth on autoclaved lemon juice. In pathogenicity test, light brown to yellow color and extensive water soaked lesions (Figure 1; A) exhibiting large amount of artroconidia with mycelia on fruit surface after 10 days in humid condition were the main characteristics of the fungus(Figure 1; B). Artroconidia were mostly cylindrical and some oval (Figure 1; C). Mycelia white incolors with dichotomous branching on PDA medium (Figure 1: D), characteristics of *G. citriaurantii*(Gente et al., 2006).

An amplified fragment of ca. 370 bp was obtained from all strains by amplification of ITS1 and ITS4 primers (Figure 2). When the ITS-PCR productswere digested with Hinfɪ restriction enzyme, two bands of ca. 190/180bp migrated in 4%MetaPhor® agarose gel (Fig. 3). On the basis of the phonotypical characteristics and results ofPCR-based ITS analysis, and the ITS-PCR with HinfIdigestion, all isolates were identified as *G.citri-aurantii*as described by Masayuki et al. (2008). One day after fruit inoculation, decay symptoms were observed on inoculated fruits. Controlfruits showed no symptoms. The causal agent was re-isolated from the inoculated fruits. Fruit rot ranged from 5.1 cm in diameter for *Citrus unshiu*Marcow (cv. Onshiu) to 0.8 cm for*Citrus aurantium*L. (Sour orange). The former is considered the most susceptible cultivar and thelatter the least. Based on Duncan's multiple range test, citrus cultivars were classified into threegroups: most susceptible: mandarins (cvs: Onshiu and Ponkan), moderately susceptible: mandarin (cvs. Clementine, Page and King), oranges (cvs. Thomson Navel and WashingtonNavel), sweet lime and Grapefruit (cv. Marsh), and the least susceptible: Valencia and Sourorange (Table 1). In spite of the low number of citrus cultivar examined here, it is concluded thatsignificant variations observed in aggressiveness of the fungus. This result is important because itshows that particular pathogen that displays the disease symptoms on wide host range can serve asa powerful repository of inoculums. It is clear that asexual reproduction is a dominant character in*G. citri-aurantii*. The citrus fruits susceptibility to sour rot has been demonstrated incorrelation with fruit ripeness and over-ripeness (Daubeny et al., 1980; Kvikliene et al., 2006;Blazec et al., 2007). Fruit firmness is also related to postharvest time and fruit maturity in citrusvarieties (Olmo et al., 2000). Firmness of citrus fruits which predispose the citrus fruits to attack by rotting fungi has been correlated with the degradation of the cell wall (Olmo et al., 2000). Poor sanitary conditions, fruit damage during harvest and uncutbranches touching the soil surface in citrus groves may contribute to fungal

infection and spread(Mirzaee et al., 2009). The sour rot disease is considered similar to other postharvest diseases inmanagement, so disease management protocols which are used for other postharvest diseasescould be used for sour rot management. Routine controlling measures include pruning citrus treebranches touching the soil surface, separating diseased fruit from healthy, harvest the fruit carefully to minimize injuries, preventing fruit contact with the soil, delay in harvest until later in the day reduce peel moisture and fruit turgidity that enhance injury. Fruit should be harvestedprior to excessive mature stage. Application of preventive fungicides is recommended for diseasemanagement (Skaria et al., 2003). *G. citri-aurantii*should be considered a serious threat to thecitrus industry especially in warm and humid autumn season in the region.This result suggests different responses of citrus species examined here to citrus sour rot. This isprobably because of differences in the chemical constitution or physical structure of the peels ofthe different species (Suprapa et al., 1995). It is demonstrated that some mineral nutrients such as calcium has a great effect on improving fruit firmness, cell wall composition, boost fruit resistance to mechanical injuries andreducing the occurrence of decay (Kader andRolle, 2004). In contrast high nitrogen and excesswater supply to plants reduce fruit firmness and increase susceptibility to mechanical damage anddecay by postharvest fungi.

Table 1: Disease severity of mean lesion diameter in different citrus cultivars inoculated with *Geotrichumcitri-aurantii.*

Cultivar	Species	Lesion diameter (cm)
Unshiu	*Citrus unshiu*Marcow	5.11[a]
Ponkan	*Citrus reticulate* Blanco	4.8[a]
Clementine	*Citrus Clementina*hortex Tanaka	4.1[b]
Thomson Navel	*Citrus sinensis*(L.) Osbeck	3.92[b]
Page	*Citrus reticulate* Blanco	3.38[bc]
Sweet lime	*Citrus Limettioides* Tanaka	2.73[c]
King	*Citrus nobilis*Lour.	2.28[cd]
Marsh	*Citrus paradise*Macfad	1.82[cd]
Washington Navel	*Citrus sinensis*(L.) Osbeck	1.59[cde]
Valencia	*Citrus sinensis*(L.) Osbeck	1.2[f]
Sour orange	*Citrus aurantium*L.	0.8[fg]

Data shows the mean of observations of four replicates per cultivar. Different letters represent significant differences among means according to the DMRT. Standard errors (SE) and coefficient of variation (CV) = 0.124 and 9.8% respectively.

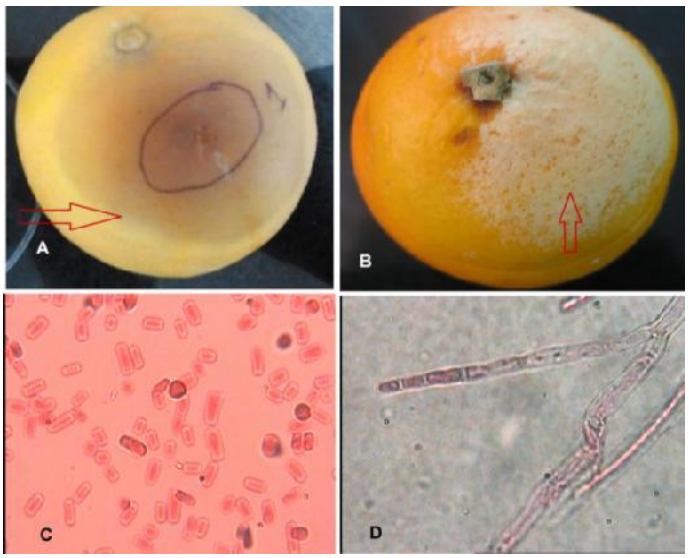

Figure 1 Morphological characteristics of sour rot caused by *G. citri-aurantii*.
A: Symptoms of sour rot on cv. Thomson Navel (arrow) and B: sign of fungus on cv. Washington Navel (arrow) 10 days after inoculation. C: Arhtroconidia of *G. citri-aurantii*mostly cylindrical and some oval. D: Dichotomous branching of *G. citri-aurantii.*

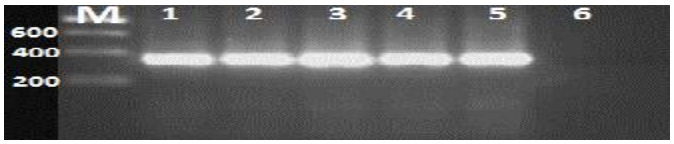

Figure 2. Polymerase chain reaction banding pattern of the ITS of *G. citri-aurantii*isolates.M; 200bp DNA ladder, lane 1-5; citrus isolates, lane 6: negative control without DNA.

Figure 3. ITS-RFLP banding pattern of *G. citri-aurantii*digested with HinfI restriction enzyme. M; Molecular marker 100 bp; Lane 1-5; *G. citri-aurantii*.

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