Evaluation of Citrus Cultivars Resistance to *Alternaria alternata*, the Causal Agent of Brown Spot Disease, Using RAPD-PCR

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ABSTRACT

Alternaria brown spot is one of the most important worldwide diseases of citrus. This disease caused by *Alternaria alternata* (Fr.:Fr.) Kessl, and causing serious economical losses in citrus yield. Citrus cultivars vary genetically in their degree of brown spot resistance. In this research, susceptibility of 13 citrus cultivars including "Orlando tangelo, Minnelo a tangelo, Clementine mandarin, Fortune, Page, Unesi, Kino, Local tangerine, Sweet orange (Thomson and Local orange), Sweet lemon, Lime and Sour orange to *Alternaria* brown spot was analyzed. The results revealed that Page, Orlando tangelo and Mineola tangelo were highly susceptible to *A. alternata* and showed severe brown spot symptoms on leaves. Based on Disease rating Index (DRI) these cultivars were placed in sensitive class (S). However, Sour orange and Clementine with the least DRI were designated as resistance varieties (R). In molecular method, the total DNA was extracted from leaves with CTAB-phenol chloroform method and DNA fragments were amplified by RAPD-PCR using 2 single, random 10mer oligonucleotide primers; AL3 and P12. In sour orange and Clementine mandarin an 850 bp fragment by P12 and a 1250 bp fragment by AL3 primer were amplified and there was not observed any fragment in these regions in other citrus cultivars. Based on results of molecular marker and pathogenicity test, sour orange and Clementine are resistance to *Alternaria* brown spot and have resistant gene (amplified by P12 and AL3 primers). This is the first report of resistant gene in citrus cultivars to *Alternaria alternata* in Iran.

Keywords: *Alternaria alternata*, Resistance gene, Citrus cultivars, Sour orange, RAPD-PCR

INTRODUCTION

Alternaria brown spot of mandarins is a disease of fruit and foliage, causing necrosis and defoliation in young shoots as well as fruit drops and rind blemishes. Brown spot causes severe losses in both semi-arid and summer-rainfall citrus-growing areas worldwide (Timmer et al., 2003). The disease is caused by a pathotype of the fungus *Alternaria alternata* (Fr.:Fr.) Keissl. that produces a
host specific toxin affecting mandarin hybrids, as well as other citrus. This disease was first reported in 1966 on Emperor Mandarin from Queensland, Australia (Pegg, 1966) and has expanded worldwide distribution considerably in recent years. It has been known for many years in the Turkey (Canihos et al., 1997), South Africa (Swart et al., 1998), Spain (Vicent et al., 2000), Italy (Bella et al., 2001), United states (Peever et al., 2002), China (Wang et al., 2010), Brazil and Argentina (Peres et al., 2003). This disease was first reported in Iran in 2006 on the tangerine hybrid cultivars (Minneola, Page and Fortune) and then on other citrus cultivars (Golmohammadi et al., 2006). Alternaria fungi cause several diseases in Citrus species, resulting in a substantial loss of production and a lower value product, which in many cases leading to serious financial loss. There are seven pathotypes of A. alternata including two pathotypes which produce host-selective ACT-toxin causing Alternaria brown spot and host-selective ACR-toxin causing Alternaria leaf spot (Akimitsu et al., 2003; Yago et al., 2011). Alternaria brown spot and leaf spot pathogens cause necrotic lesions on leaves and twigs, and lesions may expand rapidly due to the production of a host-specific toxin by the pathogens, often resulting in leaf drop and twig dieback (Akimitsu et al., 2003).

To prevent the development of this pathogen and to limit losses, treatment with chemical fungicides is a widely used procedure. However, such chemicals may produce serious problems, with residues on the fruit, appearance of fungicide resistant strains of A. alternata and their possible accumulation in human adipose tissue constituting an additional health threat (Vicent et al., 2009). An alternative way to fight against these infections might be to modulate the natural defense mechanisms of the plant. Some studies have described the mechanisms involved in the defense response of citrus against fungal infection (Lin et al., 2011). The susceptibility to A. alternata depends on the citrus species. Many studies have demonstrated that cultivars of C. reticulata and its hybrids, including Minneola tangelo and Orlando tangelo, and the hybrids Fortune, Nova and Lee are susceptible to the tangerine pathotype of A. alternata (Vicent et al., 2004). In contrast, the Satsumas (C. unshiu Mark. Marc.) and the Clementines (C. clementina Hort. ex Tan.) show a certain degree of resistance and other species, such as C. sinensis and C. limon (L.) Burm. are resistant to the pathogen (Gardner et al., 1986).

Molecular markers provide efficient and powerful tools for constructing genomic maps and tagging genes of interest for map-based cloning or marker-assisted selection. In citrus, several genes control important horticultural traits, including salinity and cold tolerance in Poncirus trifoliata (Deng et al., 1997), Citrus tristeza Virus and citrus nematode resistance in P. trifoliata (Ling et al., 2000; Weber et al., 2003) and Alternaria brown spot resistance in Clementine (Dalkilik et al., 2005). Localized linkage maps were constructed for the citrus tristeza virus resistance gene, Ctv, from P. trifoliata with RFLP (Fang et al., 1998) and the brown spot with RAPD (Dalkilic et al., 2005) marker systems. Genetic analysis showed that the Alternaria brown spot resistance is presumed to be controlled by a single recessive allele (Dalkilik et al., 2005) in citrus that could amplify using RAPD primes. Different citrus species are cultivated in Iran for fresh fruit or for processed products. The most severe fungal diseases of citrus in Iran are gummosis caused by species of the genus Phytophthora, and brown spot diseases by Alternaria pathotypes. Some of these cultivars have certain degrees of resistance to citrus pathogens.
(Golmohammadi et al., 2006). The objectives of this study were (i) to evaluate the reaction of commercial citrus cultivars to brown spot pathogen under in vitro and (ii) to localize resistance gene in citrus cultivars using RAPD fragments.

**MATERIALS AND METHODS**

**Plant materials**

Young leaves (about 50% of leaf development) of citrus cultivars including Orlando tangelo, Clementine mandarin (C. clementina), Mineola tangelo (C. paradisi × C. reticulata), Fortune (C. tangerina × C. clementina), Page (Minneola × C. clementina), Sour orange (C. aurantium), Sweet orange (Thomson, C. sinensis), Unesi mandarin, Kino, Local mandarin (C. reticulata), Local orange, Sweet lemon and Lime were obtained from field of citrus germplasm bank of Iranian Citrus Research Institute, Ramsar, Iran.

**Fungal isolates and pathogenicity test**

Pathogenic single-spore isolates of A. alternata isolated from infected leaves and fruits of citrus cultivars from north regions of Iran were used for inoculation. These isolates were identified morphologically and cultivated on PDA medium at 25°C to serve as inoculums. The pathogenicity of selected isolates was determined using the spray inoculation assay of Kohmoto et al., (1991). Briefly, isolates were grown in potato dextrose broth (PDB), and mycelial mats were washed three times with sterile water to prepare conidia. The pathogenicity of each isolate was determined by spray inoculation of a conidial suspension (1×10^5 conidia/ml) on detached leaves from greenhouse grown citrus cv. Page. Detached leaves were placed in 2 ml microcentrifuge tube filled with water by inserting petioles into water and sealing with parafilm. Inoculated leaves were incubated in a moist chamber for 48 h at 25°C in the dark, and the development of necrotic spots on the leaves was monitored.

**Leaves inoculation and measurement of growth**

To study the *in vivo* growth of the fungus, leaves were washed and their surface were disinfected using spraying with 90% ethanol and placed in microcentrifuge tubes filled with water by inserting petioles into water. Four similar leaves were used for each cultivar. Detached leaves were inoculated with 10^5 conidia ml^{-1} of 10-day old A. alternata. For the inoculation assays, two drops of this suspension (40 μl) were placed on the surface of each leaf and controls were inoculated with sterile-distilled water. Inoculated leaves were incubated for 3 days at 25°C with 85% relative humidity. The disease severity was assessed by visual inspection as described by Peever et al. (2002): 0 = no necrotic lesions; 1 = 1-20% leaf spot; 2 = 21-40% leaf spot; 3 = 41-60% leaf spot; 4 = 61-80% leaf spot; 5 = necrotic lesions spread over the leave. The experiment was repeated 2 times for each treatment.

**DNA extraction**

Total DNA was extracted from leaves using 1.5% hexadecyltrimethylammonium bromide (CTAB) according to Dalkilik et al., (2005) with minor modification. Leaves were ground in 18 × 150-mm glass test tubes with 2 mL lysis buffer (1% CTAB, 1% polyvinyl pyrrolidone (PVP), 1.4 M NaCl, 20 mM EDTA, 10 mM Tris-HCl (pH 8.0), and 0.2% mercaptoethanol).

Samples were transferred into sterile 2.0-mL Eppendorf tubes that were
centrifuged at 1200 g for 5 min at 4 °C. The supernatant (700 μL) was transferred to sterile 2-mL Eppendorf tubes. Equal volumes (700 μL) of phenol-chloroform iso-amyl-alcohol (25:24:1) were added to the tubes and the supernatant collected after centrifugation at 1200 g for 5 min at 4 °C. A second phenol - chloroformiso - amyl-alcohol (25:24:1) extraction was performed, the supernatant (500 μL) transferred to sterile 1.5-μL Eppendorf tubes, 2.5 volumes (1000 μL) of 95% ethanol was added to the samples and the DNA precipitated at 4 °C overnight. The DNA was recovered by centrifugation at 4000 g for 15 min. The DNA samples were rinsed with 70% ethanol, dried at room temperature, and re-suspended in 100 μL 1 M TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNase (Cinagen Co., Iran) was added (1 μL of 500 ng mL–1), and the samples were incubated at 37 °C at least 1 h in incubator. DNA concentrations were determined by samples compared with standard uncut lambda DNA on a 1% agarose gel and total DNA stored at -20 °C.

Isolation of resistance gene in citrus cultivars

DNA fragments were amplified by using 2 single, random 10 - mer oligonucleotide primers, AL3 and P12. These primers were previously selected from 294 RAPD primers for isolation of resistance gene in citrus cultivars by Dalkilic et al., (2005). Each 15 μL of PCR reaction mixture contained 2 mM MgCl2, 200 μM of 10X dNTPs mixture, 0.8 μM 10- mer primer, 1 unit Taq DNA polymerase (Cinagen, Tehran, IRL), 50 ng genomic DNA, and distilled water. The PCR amplification program used was: 94 °C for 2 min, then 94 °C for 1 min, 35 °C for 1 min, 72 °C for 2 min, for 43 cycles. The amplification products were separated on 1% agarose gel, prepared with 1X TAE buffer (40 mM Tris-acetate, 1mM EDTA), and electrophoreosed.

RESULTS

Pathogenicity tests were performed by artificially inoculating leaves of Minneola hybrid by single spore isolate A. alternata. In pathogenicity assay, test fungus was significantly pathogenic on Page leaves and high degree of brown spot symptoms was observed. The results obtained following artificial inoculation of the citrus leaves with A. alternata showed significant variation among citrus cultivars in detached leaves inoculation method (Figure1).

In this study the Disease Ratio Index was designated for comparing of cultivars reaction to brown spot and definition as 0 for resistant, 1, 2 and 3 for moderate resistant, 4 for moderate susceptible and 5 for susceptible reactions (Table 1). The results revealed that the Mineola tangelo, Fortune mandarin and Page were highly susceptible to artificial inoculations with A. alternata and based on Disease Rating Index (DRI) were indexed as sensitive class (S). However, Clementine mandarin and Sour orange were resistance to A. alternata and no disease symptoms were observed in inoculated leaves. In this assay orange cultivars, Local and Thomson, were moderately susceptible and showed slight symptoms of disease. Based on DRI orange cultivars were placed in MR class. Other cultivars with different reactions were mentioned in Table 1.

For isolation of homologous resistance gene in citrus cultivars, two RAPD primers AL3 and P12, were used. Separation of PCR product in 1% agarose gel showed that these primers could amplify different fragment from genomic DNA of cultivars. Based on the available information, AL3 and P12 amplify
specific fragment from genomic DNA in resistance citrus cultivars with 1250 and 850 bp respectively. Results of present research showed that referred gene was amplified only in Sour orange and Clementine mandarin and no amplified fragments were observed in these regions in other cultivars (Figure 2 and Figure 3).

In vitro assessment of Sour orange and Clementine showed high resistance reaction to test fungus which is in agreement with PCR analysis. Molecular DNA markers have been applied in citrus to find resistance genes. These genes were previously reported in Poncirus trifoliata against Citrus Tristeza Virus, and in Clementine mandarin against A. alternata. According to researches, this is the first report of homologous resistance gene for Alternaria brown spot in Sour orange.

### Table 1. Comparison of citrus cultivars based on reaction to the Alternaria alternata causal agent of Brown spot disease

<table>
<thead>
<tr>
<th>Citrus cultivars</th>
<th>Leaf necrosis (%)</th>
<th>Assessment index (%)</th>
<th>Disease ratio index</th>
<th>Resistance class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sour Orange</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>R</td>
</tr>
<tr>
<td>Orlando tangelo</td>
<td>75</td>
<td>61-80</td>
<td>4</td>
<td>MS</td>
</tr>
<tr>
<td>Mineola tangelo</td>
<td>85</td>
<td>81-100</td>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>Page</td>
<td>93</td>
<td>81-100</td>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>Fortune mandarin</td>
<td>85</td>
<td>81-100</td>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>Unesi mandarin</td>
<td>65</td>
<td>61-80</td>
<td>4</td>
<td>MS</td>
</tr>
<tr>
<td>Clementine mandarin</td>
<td>0.0</td>
<td>0</td>
<td>0</td>
<td>R</td>
</tr>
<tr>
<td>Local mandarin</td>
<td>53</td>
<td>41-60</td>
<td>3</td>
<td>MR</td>
</tr>
<tr>
<td>Kino</td>
<td>38</td>
<td>21-40</td>
<td>2</td>
<td>MR</td>
</tr>
<tr>
<td>Sweet Orange</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thomson</td>
<td>17</td>
<td>1-20</td>
<td>1</td>
<td>MR</td>
</tr>
<tr>
<td>Local</td>
<td>25</td>
<td>21-40</td>
<td>2</td>
<td>MR</td>
</tr>
<tr>
<td>Lime</td>
<td>21</td>
<td>21-40</td>
<td>2</td>
<td>MR</td>
</tr>
<tr>
<td>Sweet lime</td>
<td>19</td>
<td>1-20</td>
<td>1</td>
<td>MR</td>
</tr>
</tbody>
</table>

R: Resistance; MR: Moderately Resistance, MS: Moderate Susceptible, S: Susceptible

![Figure 1](image-url) **Figure 1.** In vitro efficacy of citrus cultivars susceptibility to brown spot pathogen, *Alternaria alternata*. Detached leaves were inoculated with 40 μl of 10^7 conidia ml^-1 suspension of *A. alternata* and incubated for 3 days at 25 °C in moist chamber. A, Control including Page mandarin
leaf sprayed with distilled water; B, Page mandarin; C, Orlando tangelo; D, Mineola tangelo; E, Fortune mandarin; F, Sour orange; G, Unesi mandarin; H, Local mandarin, I, Sweet Orange (Thomson); J, Sweet Lime

![Image](https://www.SID.ir)

Figure 2. Amplification of resistance gene in citrus cultivars, Sour orange and Clementine mandarin, using AL3 oligonucleotide primer. M: 1Kb DNA Ladder; SO, Sour Orange; MT, Mineola tangelo; OT, Orlando tangelo; CM, Clementine mandarin; F, Fortune mandarin; P, Page mandarin; UM, Unesi mandarin; LM, Local mandarin; K, Kino mandarin; T, Sweet orange (Thomson); LO, Local Sweet orange; L, Lime; SL, Sweet Lime.

![Image](https://www.SID.ir)

Figure 3. Amplification of resistance gene in citrus cultivars, Sour orange and Clementine mandarin, using P12 oligonucleotide primer. M: 1Kb DNA Ladder; MT, Mineola tangelo; SO, Sour Orange; OT, Orlando tangelo; F, Fortune mandarin; P, Page mandarin; UM, Unesi mandarin; LM, Local mandarin; K, Kino mandarin; T, Sweet orange (Thomson); CM, Clementine mandarin; LO, Local Sweet orange; L, Lime; SL, Sweet Lime.

**DISCUSSION**

Inoculation tests with virulence isolate of *A. alternata* showed a wide variation in susceptibility among the citrus cultivars. Gardner *et al.* (1986) indicated that the apparent resistance or susceptibility to the ACT host-selective toxin can be influenced by the bioassay method. The inoculation method used in this work is similar to the method used by Kohmoto *et al.* (1991) which is considered useful as a means of discarding potential hosts. Kohmoto *et al.* (1991) proposed that many citrus cultivars are very sensitive to toxins but
their susceptibility to the pathogen is different. *Alternaria* brown spot pathogen of *A. alternata* produce Host-selective toxin ACT-toxin. The selective toxicity of HSTs is correlated with the pathogenicity reactions of the fungi that produce them and HSTs are demonstrated pathogenicity factors (Masunaka et al., 2005). In this research, *A. alternata* could growth on detached leaves of Page cultivar and produced severe symptoms of brown spot in pathogenicity test.

Resistance to *Alternaria* brown spot in commercial citrus cultivars significantly lowered the incidence of disease and reduced the adverse effects of fungi on plants, but it did not prevent all infections. In the present work, Clementine and Sour orange were not affected by the N21 isolate used in this study. In molecular experiment, AL3 and P12 primers could amplify homologous gene related to this resistance reaction. On comparison to, other mandarin cultivars, Page and Mineola tangelo, were highly sensitive to *A. alternata*. Kohmoto et al., (1991) demonstrated that Clementine was not susceptible to *A. alternata* pv. *citri* when inoculated with isolates from Australia. These authors also found that *Citrus temple* and *Citrus deliciosa* were both resistant to the disease. *Citrus deliciosa* was also resistant when inoculated with an isolate from Florida. Others mandarin cultivars (Mineola tangelo, Orlando tangelo, Page and Fortune) revealed divers susceptibility reaction to the pathogen. In previous research indicated that these cultivars were highly sensitive to *Alternaria* toxin (Kohmoto et al., 1991).

Under natural conditions in Iran brown spot disease was so far recorded on many citrus cultivars specially Minneola mandarins. According to Golmohammadi et al. (2006), in artificial inoculations the host range of *A. alternata* is broader than that under natural infection conditions. Our results, with tested citrus, agree with those from Spain and with some differences. The oranges Local and Thomson were moderately resistant in inoculation with our strain while they were slightly susceptible in the Spanish experiments. Peever et al. (2002) determined the quantitative differences in virulence among isolates from different citrus growing areas of the world, and found statistically significant differences. Although the host range varied in each country there were similarities such as the disease symptoms and basic host range (Vicent et al., 2004).

In conclusion Clementine mandarin and Sour orange are very resistant to *Alternaria* brown spot and molecular marker can identify and isolate resistance gene in these cultivars. Therefore Clementine and Sour orange could use in breeding *Alternaria* resistant cultivars. In addition to, the resistance response of mandarin cultivars should be tested further with different *A. alternata* isolates, to be used in sexual hybridizations for transferring *Alternaria* resistance gene from the resistant cultivars to newly improved commercial cultivars.

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REFERENCES


