First Report of Citrus Scab on Trifoliate Orange (*Poncirus trifoliata*)

**Cheol Woo Choi**1*, Jae Wook Hyun1*, Rok Yeon Hwang1, Jae Sin Park2, and Kyung Eun Jung1

1Citrus Research Institute, National Institute of Horticultural and Herbal Science, RDA, Jeju 63607, Korea
2Jeju Special Self-Governing Province Agricultural Research & Extension Services, Jeju 63556, Korea

Citrus scab symptoms were observed on leaves of trifoliate orange (*Poncirus trifoliata*) in open field of Citrus Research Institute, National Institute of Horticultural and Herbal Science. Typical scab pustules were formed and they were elevated protuberances form and light brown to grey. The pathogens were isolated from the symptomatic leaves and then were identified to *Elsinoë fawcettii* by morphological characteristics, pathogenicity and PCR assay. The morphological characteristics of colonies formed very slow-growing, pulvinate, or raised and deeply fissured, gummy to mucoid or tomentose colonies on potato dextrose agar medium. The pathogens were pathogenic to leaves of Satsuma mandarin, lemon, sour orange and grapefruit with typical scab symptoms. In PCR assay, specific amplified of products of 717 bp with Efaw-1 and 384 bp with Efaw-2 were observed from trifoliate isolates and *E. fawcettii* as reference but not from *E. australis*. This is first report of trifoliate orange being positive for citrus scab disease.

**Keywords:** Citrus, Scab disease, Trifoliate orange

Trifoliate orange (*Poncirus trifoliata*) is a member of the family Rutaceae and used as rootstock to citrus tree in the citrus production worldwide. Citrus scab disease is one of the predominant fungal diseases distributed in many citrus-producing countries worldwide in humid zones except Mediterranean regions. It has been reported that citrus scab may affect lemons, grapefruit, and some tangerines and their hybrids as well as rootstock species such as rough lemon, sour orange, rangpur lime, and carrizo citrange (Timmer, 2000).

Two species of scab pathogens have been recognized as *Elsinoë fawcettii* and *E. australis* causing citrus scab and sweet orange scab respectively. *Elsinoë fawcettii* are differentiated into six pathotypes as follows: Florida Broad Host Range (FBHR), Florida Narrow Host Range (FNHR), Lemon, Jingeul, tryon’s and Satsuma mandarin, Rough lemen, Grapefruit, Clementine (SRGC). *E. australis* are into two pathotypes: Sweet orange and Natsudaidai (Hyun et al., 2009).

Typical scab symptoms were observed on leaves of trifoliate orange in open field of Citrus Research Institute (Fig. 1A, B). The symptoms showed typical scab pustules formation and elevated protuberances with light brown to grey. The symptomatic leaves of trifoliate oranges were collected in research open field of Citrus Research Institute in Jeju Island and then the casual pathogens were isolated from the leaves the using method reported previously (Hyun et al., 2001). The lesions of diseased leave were washed for 1 min in 75% ethanol and then 1 min in 1% sodium hypochlorite followed by rinsing in sterile water. After drying, scab pustules were scraped with a scalpel blades to deposit flakes of diseased leaves onto potato dextrose agar (PDA; 12.0 g of potato dextrose broth and 15 g of agar in 1 liter of distilled water). The colonies on PDA medium typically formed very slow-growing, pulvinate, or raised and deeply fissured, gummy to mucoid or tomentose colonies (Hyun et al., 2001). The colonies were developed after incubated for 10 days at 25°C. The morphological characteristics of colonies were similar to...
E. fawcetttii. These isolates were named as trifoliate 1-1, trifoliate 1-2, trifoliate 2, trifoliate 4, and trifoliate 5, respectively. To confirm the pathogenicity of the causal pathogen, satsuma mandarin, lemon, sour orange, and grapefruit seedlings were pruned to stimulate production of uniform flushes of new leaves and maintained at 25°C in growth room. When leaves were one-fourth of mature size, the new leaves of them were inoculated with these isolates. Each colony of these isolates was mashed with a steel spatula on petri dish and then cultured in 50 ml of sterilized water at 180 rpm in a rotary shaking incubator at 25°C for 24 hr for sporulation (Hyun et al., 2015). Before spraying, conidial suspensions in sterilized water were filtered through the mira-cloth and counted using a hemacytometer. These conidia were hyaline (Fig. 2A). Three-week-old leaves of hosts were sprayed with conidial suspension (1×10^6 conidia/ml) and covered with plastic bags to keep moist and maintained at 25°C in a 65% humid chamber for 16 hr:8 hr light:dark condition. The pathogenicity test was conducted twice. All the inoculated leaves had scab typical symptoms, pustules and elevated protuberances with light brown to grey. Pathogenicity of these pathogens were consistent with E. fawcetttii (Table 1). Additionally, at 2 days post inoculation on water agar (WA; 15 g of agar in 1 liter of distilled water), hyphae of isolates, germinated from each conidium, were light-colored and grown in all direction (Fig. 2B, C) (Wang et al., 2009).

To confirm identification of these isolates, PCR assay was conducted with primer sets Efaw-1, targeting OPX-8-A and Efaw-2, targeting internal transcribed spacer, for E. fawcetttii (Hyun et al., 2007). Total genomic DNA of each fungal isolate was extracted using Qiagen extraction kit (Germantown, MD, USA) following the manufacturer’s instructions and DNA samples were eluted in 100 μl of elution buffer and stored at −30°C. Genomic DNA of E. fawcetttii and E. australis were used as reference. The PCR was performed in a final volume of 20 μl of reaction mixture containing 1 μl template DNA, 1 U DNA Taq DNA polymerase, 1.0 μM of forward and reverse primers, 250 μM of each dNTP, 1× PCR buffer (20
mM Tris-HCl, pH 8.4 and 50 mM KCl) and 15 mM MgCl$_2$. PCR reactions were carried out as follows: pre-denaturation for 5 min at 94°C, 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 57°C, extension for 30 sec at 72°C, and final extension for 5 min at 72°C. The PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide and then photographed under UV light using a Davinchi-Gel Gel Imaging System (Davinchi-K Inc., Seoul, Korea). In result of PCR assay, specific amplified products of 717 bp with Efaw-1 and 384 bp with Efaw-2 were observed from trifoliate isolates and \textit{E. fawcettii} as reference but not from \textit{E. australis} (Fig. 3). In conclusion, pathogens isolated from scab disease symptomatic leaves of trifoliate orange were identified as \textit{E. fawcettii} FBHR pathotype by morphological characteristics, pathogenicity test and PCR assay. This is the first report of scab disease on trifoliate orange.

**Table 1.** Pathogenicity of trifoliate orange isolates on differential host varieties in greenhouse

<table>
<thead>
<tr>
<th>Location</th>
<th>Isolate</th>
<th>Host</th>
<th>SM</th>
<th>RL</th>
<th>SO</th>
<th>GF</th>
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<tbody>
<tr>
<td>Jeju</td>
<td>Trifoliate 1-1</td>
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<tr>
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<td>Trifoliate 1-2</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<tr>
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<td>Trifoliate 5</td>
<td>TO</td>
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<tr>
<td>Hyun et al. (2009)</td>
<td>\textit{Elsinoë fawcettii}</td>
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<td>+</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>\textit{Elsinoë australis}</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

SM, satsuma mandarin; RL, rough lemon; SO, sour orange; GF, grapefruit; TO, trifoliate orange.

*Symbols: +, pathogenic; –, nonpathogenic.

**Fig. 3.** Specific detection of \textit{Elsinoë fawcettii} from trifoliate orange scab pathogens using Efaw-1 and 2 primer sets for \textit{E. fawcettii}. Lane M, 1-kb DNA ladder (Takara); lane 1, SM 24-4 (\textit{Elsinoë fawcettii} FBHR pathotype); lane 2, Ea-2 (\textit{E. australis} sweet orange pathotype); lane 3, trifoliate 1-1; lane 4, trifoliate 1-2; lane 5, trifoliate 2; lane 6, trifoliate 4; lane 7, trifoliate 5.

**Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

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