Journal of Microbiology (2014) Vol. 52, No. 5, pp. 422–426
DOI 10.1007/s12275-014-3366-z

The Role as Inoculum Sources of *Xanthomonas citri* pv. *citri* Surviving on the Infected Satsuma mandarin Fruits

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(Received Jul 9, 2013 / Revised Oct 28, 2013 / Accepted Dec 18, 2013)

Importing citrus fruits infected by Asiatic citrus canker caused by *Xanthomonas citri* pv. *citri* (*Xcc*) can act as an inoculum source for the disease epidemic in citrus canker-free countries. In this study, the pathogenicity of the causal agent of Asiatic citrus canker surviving on infected Satsuma mandarin fruits was evaluated. The washing solution of infected Satsuma mandarin fruits did not cause lesion formation on the citrus leaves. However, a typical citrus canker lesion was formed on the leaves after inoculation with higher concentrations of the inoculum from the washing solution (washing solution II). It indicated that the pathogenicity of the citrus canker surviving on the symptomatic Satsuma mandarin fruits was not changed. Scanning electron microscopic observation showed that the numbers of bacterial cells on the leaves of Satsuma mandarin which inoculated with the washing solution directly (washing solution I) was less compared to those of leaves inoculated with the washing solution II. This result supports that the pathogenicity of *Xcc* surviving on Satsuma mandarin fruits may not be changed but that the sucessful infection of citrus caker may depend on the concentration of the inoculum.

Keywords: bio-test, citrus canker, export, quarantine, scanning electron microscopy

Introduction

Citrus canker is one of the most devastating bacterial diseases affecting citrus production in the world (Civerolo, 1984; Gottwald *et al.*, 1993). This disease occurrence has also been reported in Japan and South Korea, in which moderately resistant Satsuma mandarin cultivars are mostly planted (Hyun *et al.*, 2003). Using windbreaks and applying antibiotics or copper sprays these countries have successfully controlled the disease.

Due to a strict quarantine in force in many countries, in general, only citrus canker free fruits may be exported to other countries (Koizumi, 1985; Rybak *et al.*, 2009; Centner and Ferreira, 2011; Jianwei *et al.*, 2012). Bacteria causing citrus canker surviving on infected citrus fruit can act as an inoculum for the spread of the disease. Recently, there were two studies concerning the transmittion of the citrus canker pathogen from infected to healthy fruit. Gottwald *et al.* (2009) has shown by a simulation experiment that canker infected fruit in cull piles could not act as a source of inoculum for dispersal of the disease. In another one, it was shown that citrus canker pathogen was not detected in rainwater collected beneath contaminated fruit in a field experiment in 2005 and 2006 in Japan (Shiotani *et al.*, 2009). These two studies revealed that citrus canker pathogen on the infected fruit may not act as an inoculum responsible for the disease spread.

Although citrus canker is not transmitted by infected fruits, the disease can be epidemic in the citrus orchard where protection activity is not performed. In the field, the canker bacterium is able to infect leaves, stems or/and fruit of the citrus tree (Schoulties *et al.*, 1987). However, very few studies have been carried out with Satsuma mandarin concerning the pathogenicity of citrus canker pathogen (Centner and Ferreira, 2011; Kositcharoenkul *et al.*, 2011; Golmohammadi *et al.*, 2012).

In this study, in order to illustrate the transmission possibility of the citrus canker by the infected fruits, bio-test was carried out on the citrus leaves by inoculation with the washing solution from the infected Satusuma mandarin fruits. Furthermore, the surfaces of the inoculated citrus leaves were observed using a scanning electron microscope.

Materials and Methods

Plant

The seedings of Satsuma mandarin (Sunmyeong®, Nongwoobio, Korea) were planted in plastic pots (Ø 25 cm, 40 cm high) filled with commercial soil (Tuksimi®, Nongwoogreentec, Korea) which was sterilized at 80°C for 4 h prior to use. The seedlings were grown in a greenhouse maintained at 60% relative humidity (RH), 25±1°C, with 4,000 lux illumination during day time and 20±1°C at night. The seedlings were watered every 3 days and fertilized once a week with 30 ml of a complex fertilizer Choroc Nala® (N-P-K, 30-10-10, Bokyung Nongsan, Korea) as recommended for commercial usage.

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Sections of the tops of the branches, containing 5–6 leaves were detached from the seedling with a sterile blade and were put in a flask containing distilled water. These branches were used for the bio-test.

Inoculation with different concentrations of *Xcc* and assessment of disease severity

To determine the minimum concentration of *Xcc* to induce citrus canker lesion, the bacterial suspension of different concentrations 1.0×10^4, 1.0×10^5, 1.0×10^6 and 1.0×10^7 CFU/ml were inoculated on the citrus leaves.

Adding 0.01% Tween 20 all bacterial suspension were sprayed onto upper and lower surfaces of the leaves of Satsuma mandarin. The inoculated plants were kept in a dew chamber maintained at 100% RH in the dark at 28 ± 1°C for 48 h and then transferred to a growth chamber maintained at 60% RH and at 23/28°C (night and day, respectively) until symptoms of canker appeared. The level of disease severity was determined by counting the lesion number per leaf. At 20 days after inoculation the lesions were counted by visual observation.

Inoculation with the washing solution

In order to determine the pathogenicity of *Xcc* isolated from the infected Satsuma mandarin fruits, three inoculums were prepared;

1) washing solution of the infected Satsuma mandarin (washing solution I). The infected Satsuma mandarin fruits were collected from an orchard on Jeju Island in Korea. Three symptomatic fruits containing 3–6 typical canker lesions were put in a plastic container and added with sterilized water until the fruits were submerged. They were shaken hardly in a shaker over 12 h. The washing solution was filtered through three folded Miracloth (Calbiochem, USA) and used as inoculums. Also, the concentration of the washing solution was measured by incubating the diluted washing solution on a semi selective medium (Dezordi *et al*., 2009).

2) higher concentration of the inoculum from the washing solution (washing solution II). The second inoculum was prepared from the washing solution. One hundred microliters from the washing solution was spread on the semi selective medium. The plates were incubated at 28°C for 48 h. One colony from the medium was separated and further smeared on tryptic soy agar (TSA) medium. Adding sterilized water on the TSA medium, the inoculum was prepared by adjusting the concentration to 1.0×10^7 CFU/ml.

3) as a positive control, same concentration of *Xcc* obtained from Korea Agricultural Culture Collection (KACC; No. 10443), Republic of Korea. The susceptibility of Satsuma mandarin against the bacterial isolation has been reported as middle resistant (Hyun *et al*., 2003).

The bacterial suspensions were inoculated on the leaves of Satsuma mandarin and counted the lesion number describe as above.

PCR-based identification of *Xcc* isolated from washing solution

To ensure *Xcc* from the selective medium, the separated bacterial isolates were identified by sequence analysis of ribosomal DNA. The bacterial isolaties were incubated in trytic soy broth (TSB) medium at 28°C for 48 h. Bacterial genomic DNA was extracted using the genomic DNA extraction kit (DNeasy Blood & Tissue Kit 56, QIAGEN^{TM}, Germany).

The intergenic spacer region (ITS) of rDNA was amplified using primers 2 (5′-CACGGGTGCAAAAAATCT-3′) and 3 (5′-TGGTGTCGTCGCTTGTAT-3′) (Hartung *et al*., 1993). PCR was carried out in a total volume of 40 μl containing 2 μl of total genomic DNA (5–10 ng/ml), 10 pmol of each primer, 1.5 mM KCl, 10 mM Tris-HCl (pH 9.0), 30 mM MgCl, 1% Triton X-100, 2.5 mM of each of the four dNTPs, and 5 units of Taq DNA polymerase.

All regents were mixed and heated to 95°C for 2 min. Thirty cycles of PCR were run at 95°C for 70 sec, 58°C for 60 sec, and 72°C for 60 sec followed by 72°C for 2 min. PCR products were visualized in 1% agarose gels stained with 0.001% ethidium bromide.

The PCR fragments were cut out from the gel and purified using a PCR Purification Kit (NuCleoSpin Extract II, MACHEREY-NAGEL, Germany) according to the manufacturer's protocol. The DNA was suspended in 20 μl of TE buffer and stored at -20°C.

Nucleotide sequences of PCR products were determined using a Genetic Analyzer (3130xl, Applied Biosystems, USA) with the primer 2 and 3. The resulting sequences were compared with sequences in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Scanning electron microscopy of the Satsuma mandarin leaves after bacterial inoculation

Leaf surfaces of Satsuma mandarin inoculated with washing solution I, washing solution II and *Xcc* obtained from KACC were observed at 1, 7, and 14 days after inoculation using a scanning electron microscope (SEM 435 VP, Leo 40 Electron Microscopy Ltd., UK).

The inoculated leaves were cut to 0.4×0.6 mm^2 using a sterile blade. Fixation, dehydration and embedding of the roots were performed according to Hayat (1989). The leaf samples were fixed in 2% (v/v) glutaraldehyde in 0.05 M phosphate buffer (pH 7.4) for 2 h. After washing with the same phosphate buffer for 10 min three times each, post fixation was performed in 2% (v/v) osmium tetroxide in phosphate buffer for 2 h at room temperature. After washing three times, the samples were dehydrated through an alcohol series (25, 50, 70, 90, and 100% two times for 30 min each). The samples were gently dried using a critical point drier (CPD 030, BAL-Tec). Samples were mounted on metallic stubs, gold-coated (~100 Å) with a sputter coater (Polaron Sputter Coat System, Model 5001, England) and viewed under SEM 435 VP at 20 kV. The numbers of bacteria on the randomly selected 10 images of the inoculated leaves were counted.

Statistical analyses

The evaluation of disease severity after inoculation of branches with various inoculums was carried out as 3 separate experiments at different (n=9).

The number of bacterial cells on the photographs by SEM and the lesion number of infected citrus leaves were statis-

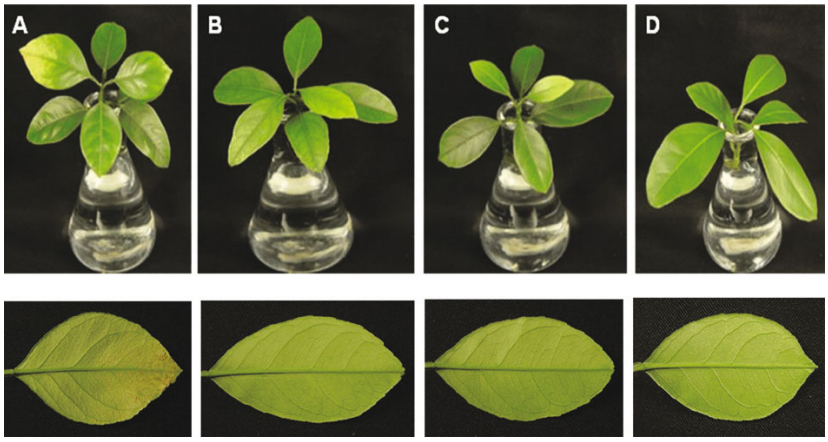

Fig. 1. Satsuma mandarin branches inoculated with the suspension of *Xcc* from KACC at different concentrations 1.0×10^7 (A), 1.0×10^6 (B), 1.0×10^5 (C), and 1.0×10^4 (D) CFU/ml. The photographs were taken at 20 days after inoculation.

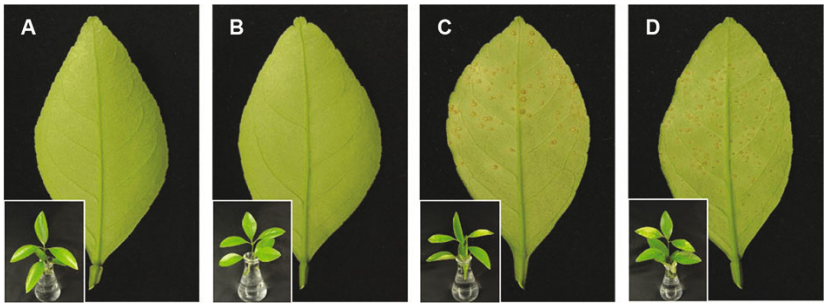

Fig. 2. Satsuma mandarin branches inoculated with H_2O (A), the washing solution from Satsuma mandarin fruits directly (washing solution I; B), the higher concentration of the inoculum from the washing solution (washing solution II; C) and the suspension of *Xcc* from KACC (D). The photographs were taken at 20 days after inoculation. The concentration of *Xcc* (C and D) was 1.0×10^7 CFU/ml.

tically analyzed using Duncan's multiple range tests (DMRT). Statistical analysis of the experimental data was conducted using the Statistical Analysis System (SAS institute, version 9.0).

Results and Discussion

This study aimed to illustrate whether a citrus canker infected Satsuma mandarin citrus fruit can act as a potential inoculum source. Prewashing the fruits could reduce surface bacterial populations and the packing line process also reduced canker lesion activity by as much as 50% compared to unprocessed fruits in Florida and Argentina (Gottwald *et al.*, 2009). No strains of *X. citri* pv. *citri* were recovered from the asymptomatic fruits harvested from severely diseased Satsuma mandarin trees growing in Japan (Shiotani *et al.*, 2009). These findings suggest that the risk of transmission by an infected fruits to healthy fruits is low. However, pathogenicity of the *Xcc* surviving on the surface of the infected fruits has not been tested yet.

Minimum concentration of *Xcc* to induce disease symptom

On the leaves of Satsuma mandarin inoculated with the bacterial suspension at the concentration of 1.0×10^7 CFU/ml typical citrus caker symptoms was observed 20 days after inoculation (Fig. 1). The bacterial suspension at the concentration of 1.0×10^6 CFU/ml could form the citrus caker symptom although the canker lesions were not apparently distinguishable (Fig. 1). However, lower concentration of the bacterial suspension could not form canker lesions on the leaves. These results indicate that the concentration of inoculum should be higher than 1.0×10^6 CFU/ml to induce lesions on the citrus leaves.

Bio-test by inoculation with the bacerial inoculums

No lesion was formed on the citrus leaves after inoculation with water and the washing solution I (Fig. 2A and 2B). Also, the concentration of *Xcc* in the washing solution was about 6.4×10^4 CFU/ml which was too low to induce symptom on the leaves (see Fig. 1). Therefore, these results indicate a rare possibility of transmitting citrus canker by contact

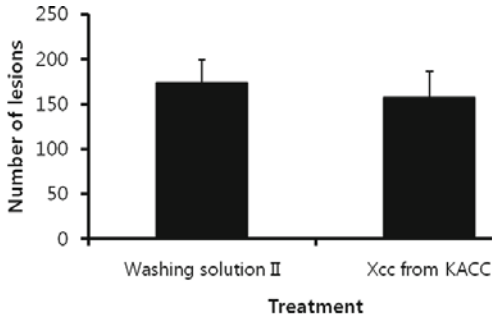

Fig. 3. Number of lesions on the leaves of Satsuma mandarin inoculated with the higher concentration of the inoculum from the washing solution (washing solution II) and the suspension of *Xcc* from KACC at 20 days after inoculation. No lesion was found on the leaves of Satsuma mandarin inoculated with the washing solution directly (washing solution I). The concentration of both inoculems were adjusted to 1.0×10^7 CFU/ml. The vertical bar means standard deviation from 3 replications of 9 branches of Satsuma mandarin.

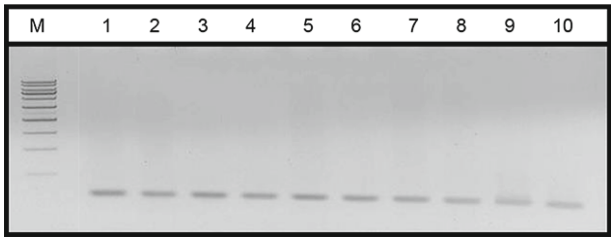

Fig. 4. Gel electrophoresis of PCR-amplified 16S/23S internal transcript spacer (ITS) regions from the bacterial isolates cultivating the semi selcetive medium (lane 1-10) using primer-2 (5′-ACGGGTGCAAAAAAT CT-3′) and primer-3 (5′-TGGTGTCGCTTGTAT-3′). M, 1 kb ladder (iNtRON Bio.).

with symptomatic fruits of Satsuma mandarin. However, a higher concentration of the inoculum from the washing solution (washing solution II) caused canker lesions on the citrus leaves (Fig. 2C). Similarly, canker lesions were also observed on the citrus leaves inoculated with *Xcc* from KACC (Fig. 2D). From these results it is suggested that the pathogenicity of the citrus canker pathogen on the infected citrus fruits may not be changed. However, it does not mean that citrus canker is able to transfer to healthy fruits from infected fruits, because the concentration of bacteria on most infected fruits is too low to induce lesions on the citrus leaves (Figs. 2B and 3).

There was no difference in the number of lesions caused by the washing solution II, and those from the KACC isolate (Fig. 3), indicating no difference in their pathogenicity.

These results was not coincident with a previous study concerning the pathogenicity of citrus canker pathogen on infected fruits in which no symptom was observed on the citrus leaves inoculated with the blending solution from infected fruits (Jin and Kang, 2006).

Identification of *Xcc* isolated from washing solution by PCR

Colonies from the washing solution which were grown on the semi selective medium were identified by sequence analysis of ribosomal DNA. The primers 2 and 3 for identification of *Xcc* were used successfully to amplify the ITS region of ribosomal DNA from the bacteria growing on the semi selective medium. Agarose gel electrophoresis of the amplified product from the bacteria showed a band each (Fig. 4). The sequencing results of PCR fragments were compared with the GenBank database using the BLAST algorithm. All bacteria amplified by PCR had 98% nucleotide similarity

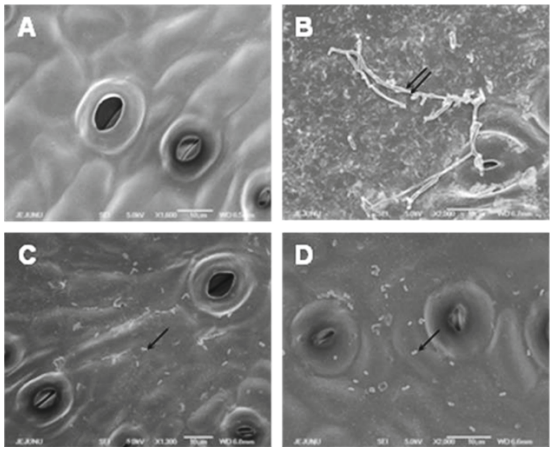

Fig. 5. SEM images of Satsuma mandarin leaves at 1 day after spraying with H_2O (A), inoculated with the washing solution from Satsuma mandarin fruits directly (washing solution I; B), inoculated with a higher concentration of the inoculum from the washing solution (washing solution II; C), and suspension of *Xcc* from KACC (D). There were some unidentified bacteria and hyphae of fungi on the leaves inoculated with washing solution from Satsuma mandarin fruits (double arrows). Some cells of *Xcc* were observed on the leaves inoculated with the inoculum from the washing solution or the suspension of *Xcc* from KACC (arrows). All bars = 10 μm.

with *Xcc* (data not shown). From these results it makes sure that the bacterial cells from the washing solution were *Xcc*.

Scanning electron microscopy on the Satsuma mandarin leaves after inoculation with the bacterial inoculums

On the leaf surface treated with sterilized water, no microorganism was found at 1 day after treatment (Fig. 5A). Some unidentified microorganisms, but not bacterial cells, were observed on the leaves inoculated with the washing solution I at 1 day after inoculation (Fig. 5B).

In contrast to that, some bacterial cells were observed on the leaves inoculated with the washing solution II (Fig. 5C). Similarly, the same amount of bacterial cells were observed on the leaves inoculated with the suspensions of *Xcc* from KACC (Fig. 5D) which caused canker symptoms on the leaves.

The population of the bacterial cells on the citrus leaves inoculated with the various inoculums was clearly shown by scanning electron microscopic observation. The SEM images revealed that some bacterial cells found either on the citrus leaves inoculated with the washing solution II or on

Table 1. Number of cells of *Xcc* on the leaves of Satsuma mandarin inoculated with the washing solution from fruits directly (washing solution I), the higher concentration of the inoculums from the washing solution (washing solution II), and the suspension of *Xcc* from KACC as a positive control, at different days after inoculation

Treatment[a]	Total numbers of *Xcc* after inoculation					
	1 day	Duncan's test[c]	7 days	Duncan's test	14 days	Duncan's test
Washing solution I	2.6 ± 3.5[b]	a	4.5 ± 3.8	a	40.8 ± 14.9	a
Washing solution II	49.6 ± 23.6	b	118.3 ± 22.7	b	135.9 ± 34.0	b
Suspension of *Xcc* from KACC	51.1 ± 19.6	b	114.1 ± 17.6	b	119.4 ± 31.0	b

[a] The higher concentration of the inoculums from the washing solution and the suspension of *Xcc* from KACC were adjusted to 1.0×10^7 CFU/ml.
[b] Means±SD from 10 randomly selected SEM images.
[c] Different letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

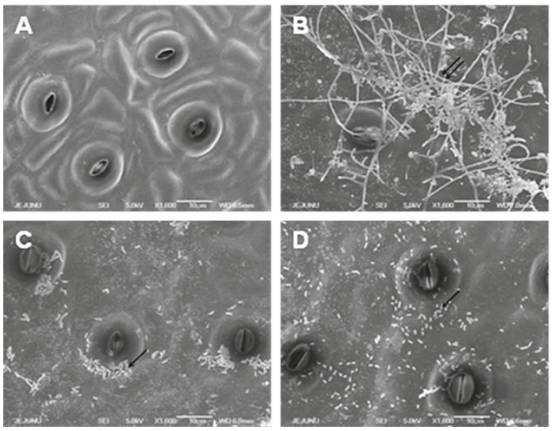

Fig. 6. SEM images of Satsuma mandarin leaves at 7 days after spraying with H_2O (A), inoculated with the washing solution from Satsuma mandarin fruits directly (washing solution I; B), inoculated with a higher concentration of the inoculum from the washing solution (wasing solution II; C), and suspension of *Xcc* from KACC (D). The unidentified bacteria and hyphae of fungi grew on the leaves inoculated with washing solution from Satsuma mandarin fruits (double arrows). A lot of *Xcc* were observed on the leaves inoculated with the inoculum from the washing solution and the suspension of *Xcc* from KACC (arrows). All bars = 10.

the leaves inoculated with suspension of *Xcc* from KACC at 1 day after inoculation (Fig. 5C, 5D, and Table 1). However, on the leaves inoculated with the washing solution I, only a few bacterial cells were found at the same time (Fig. 5B and Table 1).

At 7 days after spraying with sterilized water, neither fungi nor bacterial cells were observed to grow on the leaves (Fig. 6A). The unidentified fungi were broadly spread on the leaves inoculated with the washing solution from Satsuma mandarin fruits (Fig. 6B). However, the microorganism seemed not cause any disease symptom on the leaves (Fig. 2B).

Lots of bacterial cells expected as *Xcc* were found on the leaves inoculated with either the washing solution II, or the suspension of *Xcc* from KACC, at 7 days after inoculation (Fig. 6C, 6D, and Table 1). In terms of the infection structure no apparent difference was observed for leaves inoculated with either suspension.

Total numbers of *Xcc* were increased on the leaves inoculated with washing solution 14 days later (Table 1). However, no remarkable increase was found on the leaves inoculated with either washing solution II, or the suspension of *Xcc* from KACC (Table 1).

The difference in number of the bacterial cell was more apparent at 7 days after inoculation (Fig. 6 and Table 1) which was coincident with the induction of lesion on the citrus leaves after inoculation (Figs. 2 and 3). Therefore, it seems that the population of the bacterial cells in the washing solution from Satsuma mandarin fruits may play an important role for the successful infection to the host tissue.

Acknowledgements

This research was supported by Export Promotion Technology Development Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea and by a 'Grant for Export of Fresh Agricultural Products' of Animal, Plant and Fisheries Quarantine and Inspection Agency/Ministry for Food, Agriculture, Forestry and Fisheries, Rep. of Korea. We thank KACC for supplying the isolate *Xcc* No. 10443.

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