

## Evaluation of Potential of Mandarin Hybrid 'Shiranuhi' against inoculation of Bacterial Canker Disease Pathogen (*Xanthomonas axonopodis* pv. *citri*) in Citrus Field in Jeju Island

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This study was carried out to evaluate potential of Shiranuhi cultivar against inoculation of causal pathogen of citrus canker, *Xanthomonas axonopodis* pv. *citri* in Jeju Island by comparing degree of susceptibility of fruits and leaves/twigs, and analysis of incidence of canker disease. Progression of symptom, disease incidence, and percent area of lesion were surveyed for evaluation of resistance. In greenhouse condition, symptoms of bacterial citrus canker progressed more rapidly in sweet orange, a moderately susceptible cultivar, than in other four cultivars (satsuma mandarin, 'Kiyomi', 'Shiranuhi' and 'Yuzu'). At 20 days after inoculation, disease severity was the highest in sweet orange ( $5.0 \pm 0.0$ ), and all tested leaves were distorted or had dropped. On the other hand, 'Yuzu' showed the lowest disease severity ( $2.6 \pm 0.47$ ), followed by 'Kiyomi' ( $4.0 \pm 0.0$ ), 'Shiranuhi' ( $4.0 \pm 0.82$ ), and satsuma mandarin ( $4.3 \pm 0.47$ ). Percent area of lesion per leaf 30 days after inoculation was the highest in sweet orange ( $8.31 \pm 1.78$ ), followed by satsuma mandarin ( $1.51 \pm 1.25$ ), 'Shiranuhi' ( $1.39 \pm 0.94$ ), and 'Kiyomi' ( $1.1 \pm 0.9$ ), while the lowest was in 'Yuzu' ( $0.26 \pm 0.17$ ). In field condition, percentage of diseased leaf in 'Shiranuhi' was very low,  $5.2 \pm 2.9$ , compared with sweet orange,  $71.0 \pm 11.5$ , while that of satsuma mandarin and 'Kiyomi' were  $6.9 \pm 7.0$  and  $4.3 \pm 2.0$ , respectively. Percentages of diseased leaf was higher ( $17.4 \pm 7.1$ ) than that of diseased fruit ( $3.2 \pm 2.5$ ) in severely diseased trees of Shiranuhi cultivar, and the disease was not observed on twig in open field condition. Lesion sizes on leaves and fruits in open field condition were  $4.1 \pm 2.2$  mm<sup>2</sup> and  $5.1 \pm 5.6$  mm<sup>2</sup>, respectively, while those in greenhouse condition were  $8.7 \pm 5.7$  mm<sup>2</sup>,  $10.4 \pm 9.2$  mm<sup>2</sup> and  $5.6 \pm 2.6$  mm<sup>2</sup> on leaves, fruits and twigs, respectively. The disease was observed in 5.6% out of total 107 farmers Shiranuhi fields under polyethylene film house, and average percentages of diseased tree in 31 fields of Shiranuhi cultivar and adjacent satsuma mandarin

fields were 0.02% and 14.8%, respectively. Average percentage of diseased fruit was 1.6% in satsuma mandarin which was not observed in any one of all the 31 Shiranuhi farmers fields. Therefore, it was concluded that 'Shiranuhi' cultivar is not potential against causal pathogen of citrus canker disease in Jeju Island because the cultivar has similar resistance as satsuma mandarin which occupies over 95% of total 25,000 ha in Jeju Island in polyethylene film houses protected from outside.

**Keywords :** Citrus bacterial canker disease, disease incidence, inoculum potential, resistance, Shiranuhi, *Xanthomonas axonopodis* pv. *citri*

The Asiatic citrus canker (canker A), caused by *Xanthomonas axonopodis* pv. *citri* (syns. *X. compestris* pv. *citri*), is the most widespread and severe disease. The canker decreases fruit marketability, can decrease crop yields by causing premature fruit drop, and can induce severe defoliation on susceptible cultivars (Gottwald and Graham, 2000). Resistance of *Citrus* species and the *Citrus* hybrids with their relatives to this disease has been investigated in the United States and Japan (Gottwald et al., 1993; Koizumi and Kuhara, 1982) since the first description of the bacterial citrus canker. Koizumi and Kuhara (1982) and Matsumoto and Okudai (1988) used pinprick inoculation and measured lesion size and bacterial populations in lesions for evaluation of citrus cultivars for their resistance to Asiatic citrus canker. This technique was further applied to determine the susceptibility of *Citrus* species and hybrids to *X. axonopodis* pv. *citrumelo* (Graham et al., 1990). However, there are some restrictions because the pinprick inoculation of leaves bypass bacterial penetration through stomatal openings by creating a wound on the leaf epidermis and introducing a high concentration of bacteria into the leaf mesophyll tissue (Egel et al., 1991). Graham et al. (1992) assessed the susceptibility of citrus cultivars to the bacterial canker disease by stomatal characteristics,

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lesion number and the degree of growth of bacteria in tissues. Stall et al. (1982) attributed the decrease in susceptibility to citrus canker with leaf age to the development of mesophyll resistance to bacterial multiplication in the mature leaf. Although there has been an accumulation of substantial information regarding the genetics, biology, assay of disease resistance of citrus, resistance to citrus canker remains unclear in some varieties because new varieties have been continuously bred and assay of resistance against bacterial canker has not been completely established.

'Shiranuhi' cultivar bred by crossing 'Kiyomi' tangor and 'Nakano No. 3' ponkan in 1972 by Kuchinotsu Branch, Fruit Tree Research Station (Matsumoto, 2001) has been cultivated in polyethylene film house in about 1,330 farmers fields with a total area of 621 ha in Jeju island. The number of cultivating farmers is rapidly increasing in recent years. However resistance of the cultivar against bacterial canker is not known yet, though it was reported (Koizumi and Kuhara, 1982) that parents of 'Shiranuhi' cultivar, 'Kiyomi' tangor, and 'Nakano No. 3' ponkan are resistant to bacterial canker.

This study was carried out to evaluate the potential of Shiranuhi cultivar against inoculation of pathogen of citrus canker disease in Jeju Island by comparing its resistance, degree of susceptibility of fruit, and leaves/twigs, and analysis of incidence of canker disease on Shiranuhi cultivar. Progression of symptom, disease incidence, and percentage of area of lesion were surveyed for evaluation of resistance. To determine the possibility of influx of the pathogen from outside, the incidence of bacterial canker disease on 'Shiranuhi' cultivar was surveyed in the farmers fields under polyethylene film house and adjacent satsuma mandarin open field.

## Materials and Methods

**Preparation of bacterial culture and plant materials.** Bacterial strain *X. axonopodis* pv. *citri* Xa-2 isolated from satsuma mandarin leaves, which showed typical canker symptom in Korea, was used in this study. Inoculum was prepared by 24-hour cultured bacteria in PSG broth (bacto peptone 10 g, sucrose 10 g, sodium glutamate 1 g/L) at 28°C with 180 rpm and harvested by centrifugation (8,000 rpm). Then, pellet was suspended in 0.01 M K-phosphate buffer (pH 7.2), adjusted optical density of cells suspension by spectrophotometer to approximately  $2 \times 10^8$  cfu/mL, and inoculum density was confirmed by plating on PSG agar medium. Three- or four-year-old seedlings of tested citrus plants were routinely grown in a greenhouse at 18-32°C. The plants were pruned to stimulate production of uniform flushes of new leaves.

**Assay of disease severity on leaves in greenhouse condition.** Bacterial suspensions were sprayed using lab-spray bottle on leaves of one-half mature size of five cultivars; sweet orange (*C.*

*sinensis* 'Lane late'), satsuma mandarin (*C. unshiu* 'Miyakawa wase'), 'Yuzu' (*C. junos*), 'Shiranuhi' ['Kiyomi' tangor  $\times$  'Nakano No. 3' ponkan (*C. reticulata*)] and 'Kiyomi' ['Miyakawa wase' (*C. unshiu* Marc.)  $\times$  'Trovia' (*C. sinensis* Osbeck)], and then the plants were kept in glass greenhouse at 18-32°C. The plants were covered with plastic bags for 3 days after inoculation for infection. Disease occurrence was examined daily 3 days after inoculation, and disease severity was scaled based on the following indices: 0 = no symptom; 1 = first symptom (very small pustule); 2 = yellowish halo around the pustule; 3 = prominent pustule; 4 = reddish or brownish pustule and appearance of the necrotic tissue; and 5 = leaf distortion or leaf drop. Percentage of area of lesion per leaf was assayed by using 'ASSESS' image analysis software for plant disease quantification, APS press, 30 days after inoculation except for some leaves of sweet orange which had dropped. The dropped leaves were assayed immediately after dropping. Three plants were used per cultivar, and five leaves per plant which showed the severest symptoms per plant were assessed.

**Assay of disease severity on leaves in open field condition.** Three- or four-year-old seedlings of the five cultivars grafted onto potted trifoliate orange were used. They were positioned around the severely diseased 'Natsudaidai' (*C. natsudaidai*) plants before shooting. Disease incidence was surveyed after 3 months when the leaf growth was fully grown. Five plants were assayed per cultivar.

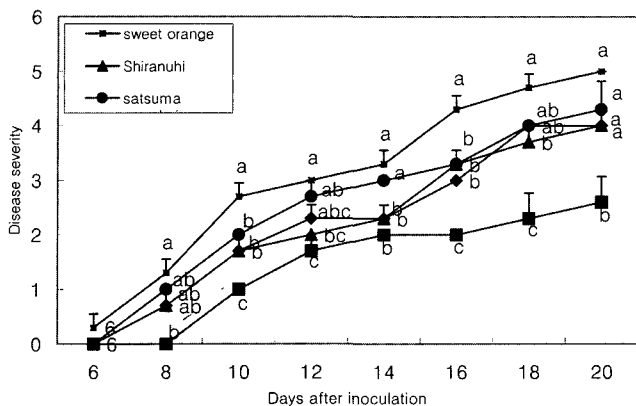
**Susceptibility of fruits and leaves/twigs.** To assess the susceptibility of bacterial canker disease in 'Shiranuhi' cultivar, the rate of disease incidence and lesion size in leaf, fruit, and twig were surveyed in severely diseased ten trees in the open field at three locations middle of September 2003. To evaluate those in the greenhouse condition, bacterial suspensions were sprayed on the leaves at half expansion leaf stage and on fruits 1 month after petal fall. The lesion size was assessed on leaves, fruits, and twigs 3 months after inoculation. The lesion size was assessed from typical symptom on five leaves and fruits (three fruits in the greenhouse) per one tree by using 'ASSESS' image analysis software for plant disease quantification, APS press.

**Incidence of the bacterial canker disease on 'Shiranuhi' cultivar in the farmers field.** The incidence of bacterial canker disease was surveyed in 107 farmer's polyethylene film houses in three districts of Jeju Island in late September 2003. All the trees in house were surveyed.

**Comparison of incidence of citrus bacterial canker disease on 'Shiranuhi' cultivar and adjacent satsuma mandarin field.** To assess the role of polyethylene film house as protector against influx of bacterial pathogens from outside, disease incidence was investigated on 'Shiranuhi' cultivar in 31 farmers' fields under polyethylene film houses and adjacent satsuma mandarin in open field late September 2003. Forty trees of satsuma mandarin per farmer's field were randomly selected within 100-m radius of 'Shiranuhi' polyethylene film house and the fruits were surveyed.

## Results

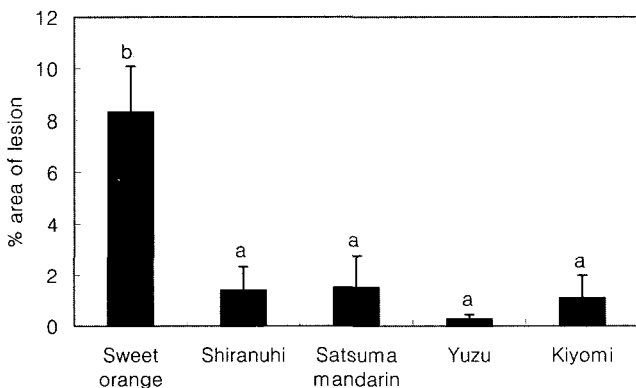
**Disease severity assay in greenhouse condition.** In



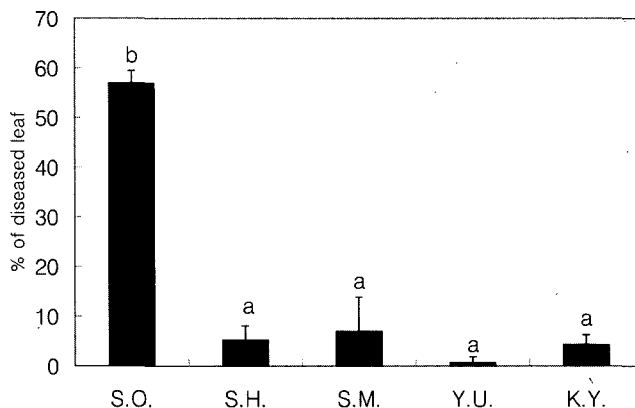
**Fig. 1.** Disease progressive curve of *Xanthomonas axonopodis* pv. *citri* on five citrus cultivars in greenhouse condition. Disease severity: 0 = no symptom; 1 = first symptom (very small pustule); 2 = yellowish halo around the pustule; 3 = prominent pustule; 4 = reddish or brownish pustule and appearance of the necrotic tissue; 5 = leaf distortion or leaf drop. Different letters indicate significant differences ( $p < 0.05$ ) according to Duncan's multiple range test.

greenhouse condition, the first symptom was observed 6 days after inoculation in sweet orange, and 10 days after inoculation in 'Yuzu'. Disease severity was the highest in sweet orange,  $5.0 \pm 0.0$ , and all tested leaves were distorted or had dropped 20 days after inoculation. On the other hand, 'Yuzu' showed the lowest disease severity ( $2.6 \pm 0.47$ ), followed by 'Kiyomi' ( $4.0 \pm 0.0$ ), 'Shiranuhi' ( $4.0 \pm 0.82$ ), and satsuma mandarin ( $4.3 \pm 0.47$ ) (Fig. 1). Percent area of lesion per leaf was assessed 30 days after inoculation. Percent area of lesion was the highest in sweet orange ( $8.31 \pm 1.78$ ), followed by satsuma mandarin ( $1.51 \pm 1.25$ ), 'Shiranuhi' ( $1.39 \pm 0.94$ ), and 'Kiyomi' ( $1.1 \pm 0.9$ ), while the lowest was in 'Yuzu', ( $0.26 \pm 0.17$ ) (Fig. 2).

**Disease severity assay on leaves in open field condition.**



**Fig. 2.** Percent (%) area of lesion per leaf of five citrus cultivars 30 days after inoculation in greenhouse condition. The percent (%) area of lesion was assayed by using 'ASSESS' image analysis software for plant disease quantification, APS press. Different letters indicate significant differences ( $p < 0.05$ ) according to Duncan's multiple range test.



**Fig. 3.** Incidence of citrus canker on five citrus cultivars in field condition. S.M. = satsuma mandarin; S.H. = 'Shiranuhi'; K.Y. = 'Kiyomi'; Y.U. = 'Yuzu'; S.O. = sweet orange. Means followed by the same letter are not significantly different according to Duncan's multiple range test ( $p = 0.05$ ).

Percentage of diseased leaf was very high in sweet orange ( $71.0 \pm 11.5$ ), but other four cultivars showed relatively low disease incidence of  $6.9 \pm 7.0$ ,  $5.2 \pm 2.9$ ,  $4.3 \pm 2.0$  and  $0.6 \pm 1.2$  in satsuma mandarin, 'Shiranuhi', 'Kiyomi' and 'Yuzu', respectively (Fig. 3).

**Susceptibility of fruits vs. leaves/twigs.** To assess the susceptibility to the citrus canker disease of fruit, leaf, and twig of 'Shiranuhi' cultivar, disease incidence and lesion size in leaf, fruit, and twig was surveyed. Percentage (%) of diseased leaf was,  $17.4 \pm 7.1$ , higher than that of fruit ( $3.2 \pm$

**Table 1.** Occurrence of bacterial canker disease on leaf, fruit, and twig of 'Shiranuhi' cultivar in open field condition<sup>a</sup>

Percent (%) of diseased leaf, fruit and twig		
Leaf	Fruit	Twig
$17.4 \pm 7.1$ b <sup>b</sup>	$3.2 \pm 2.5$ a	$0.0 \pm 0.0$ a

<sup>a</sup> Surveyed on severely diseased 10 plants at 3 locations in Jeju Island middle of September 2003 when the growth of fruit was almost completed.

<sup>b</sup> Numbers within a column followed by different letters are significantly different ( $p = 0.05$ ) according to Duncan's multiple range test.

**Table 2.** Lesion size of bacterial canker disease on leaf, fruit and twig of 'Shiranuhi' cultivar

Cultural condition	Lesion size (mm <sup>2</sup> ) <sup>a</sup>		
	Leaf	Fruit	Twig
Open field	$4.1 \pm 2.2$ a <sup>b</sup>	$5.1 \pm 5.6$ a	- <sup>c</sup>
Greenhouse	$8.7 \pm 5.7$ a	$10.4 \pm 9.2$ a	$5.6 \pm 2.6$ a

<sup>a</sup> Lesion size was assayed by using 'ASSESS' image analysis software for plant disease quantification, APS press.

<sup>b</sup> Numbers within a column followed by different letters are significantly different ( $p = 0.05$ ) according to T-test or Duncan's multiple range test.

<sup>c</sup> No occurrence.

**Table 3.** Incidence of bacterial canker disease on 'Shiranuhi' cultivar in the farmer's fields under polyethylene film houses

Percent (%) of diseased tree	Number of farmer's field			
	Seogwipo City	Nam-Jeju	Buk-Jeju	Total
0	44	29	28	101
<0.5	2	1	2	5
0.5-1.0	1	0	0	1

**Table 4.** Comparison of incidence of citrus bacterial canker disease on 'Shiranuhi' cultivar in polyethylene film house and adjacent satsuma mandarin field<sup>a</sup>

Plant	Average percent(%) of	
	Diseased tree	Diseased fruit
Satsuma mandarin	14.8	1.6
Shiranuhi	0.02	0.0

<sup>a</sup> All trees of 'Shiranuhi' cultivar were surveyed in 31 farmer's fields under polyethylene film house and 40 trees of satsuma mandarin each field were randomly selected and surveyed in satsuma mandarin open field within 100-m radius of 'Shiranuhi' cultivar cultivating house.

2.5), but disease symptom was not observed on twig in open field condition (Table 1). Also in open field condition, lesion sizes were  $4.1 \pm 2.2 \text{ mm}^2$  and  $5.1 \pm 5.6 \text{ mm}^2$  on leaf and fruit, respectively. Meanwhile lesion sizes were  $8.7 \pm 5.7 \text{ mm}^2$ ,  $10.4 \pm 9.2 \text{ mm}^2$ , and  $5.6 \pm 2.6 \text{ mm}^2$  on leaf, fruit, and twig in greenhouse condition, respectively (Table 2).

**Incidence of bacterial canker disease on 'Shiranuhi' cultivar in the farmer's fields.** A total of 107 out of 1,330 farmer's fields under polyethylene film houses were surveyed. The disease was observed in six farmer's fields, of which five had percentage of diseased tree lower than 0.5% while one had 0.6% (Table 3).

**Comparison of incidence of citrus bacterial canker disease on 'Shiranuhi' cultivar and adjacent satsuma mandarin field.** The incidence of citrus bacterial canker disease on 'Shiranuhi' cultivar in polyethylene film houses and satsuma mandarin in adjacent open field was surveyed. Average percent of diseased tree were 14.8 and 0.02% in satsuma mandarin and 'Shiranuhi' fields, respectively, and the disease was observed in only one of 31 'Shiranuhi' fields. Average percentage of diseased fruit was 1.6% in satsuma mandarin and the disease was not observed in fruit in all of 31 'Shiranuhi' farmer's fields (Table 4).

## Discussion

'Shiranuhi' cultivar has been cultivated in about 1,330 farmer's fields in 621 ha in Jeju Island, and the number of cultivating farmers is rapidly increasing. To evaluate the

potential of 'Shiranuhi' cultivar against inoculation of bacterial canker disease pathogen in citrus field in Jeju Island, resistance to the disease was evaluated by comparing progression of symptom, disease incidence, and percent area of lesion with that of other cultivars. The degree of susceptibility of fruits vs. leaves/twigs was determined, and disease incidence on 'Shiranuhi' cultivar in polyethylene film houses and adjacent satsuma mandarin open fields was surveyed. Lesion size, stomatal characteristics, and growth of pathogen in tissue were analyzed by wound inoculation such as pinprick inoculation (Koizumi and Kuhara, 1982) or nondisruptive stomatal inoculation by using adequate pressure (Graham et al., 1992) to evaluate resistance to bacterial canker of *Citrus* species and their hybrids.

In this study, progression of symptom, disease incidence, and percent area of lesion were assessed by spray inoculation for evaluation of resistance. In the assay for the progression of symptom, sweet orange, satsuma mandarin and 'Yuzu' which were susceptible, moderately resistant and highly resistant, respectively (Graham et al., 1992; Koizumi and Kuhara, 1982) were statistically differentiated 8-18 days after inoculation. But, the differentiation was not clear 20 days after inoculation which may be due to completion of disease progression. Graham et al. (1992) concluded that leaf characteristics such as leaf growth stage that influence infiltration of the leaf surface by water and congestion of tissue are more important determinants of bacterial penetration than genotypic differences in density and structure of stomata, and Gottwald and Graham (1992) reported that leaves are highly susceptible to stomatal infections only from the two-thirds to full expansion stage. These reports suggest that leaf characteristic such as leaf growth stage is important determinants of bacterial penetration. In this study, resistances of cultivars used could be differentiated at half expansion leaf stage (not presented). 'Shiranuhi' cultivar showed resistance to bacterial canker disease, similar to satsuma mandarin and Kiyomi which are known to have resistance (Koizumi and Kuhara, 1982). This result was expected by considering that the parents of 'Shiranuhi' cultivar, 'Kiyomi' tangor and 'Nakano No. 3' ponkan, are both resistant to bacterial canker.

The bacteria in leaf and twig lesions are probably the most epidemiologically significant inoculum for secondary infections. It was reported that internal *X. axonopodis* pv. *citri* population sizes reach  $10^6$  to  $10^7$  cfu/lesion whatever the lesion size throughout spring, summer, and fall, and decrease drastically to  $10^2$  to  $10^4$  cfu/lesion during the winter season according to lesion age (Pruvost et al., 2002; Stall et al., 1980). Rainwater-mediated release of the bacteria was slower from old than from young lesions (Timmer et al., 1991). Based on these data, we think that susceptibility of leaves and fruits based on lesion size do

not affect the potential against inoculation of bacterial canker disease pathogen.

It was known that the majority of new canker infections occurred within approximately 579 m (1,900 ft) of known source trees. As a result, a new regulation, the "1,900-ft rule" was put into practice in Florida in late 1999, removed and destructed diseased citrus trees along with all citrus trees within a 1,900-ft radius of a diseased tree (Gottwald et al., 2000). However, we think that this rule can not be applied to Jeju citrus because satsuma mandarin occupies over 95% of total 25,000 ha in Jeju island, it is resistant to bacterial canker and wind block trees are well planted. Myung et al. (2003) recently reported that citrus canker disperse within 7.5 m from inoculum sources in nursery plots surrounded Japanese cedar plants. In addition, 'Shiranuhi' cultivar, of which cultivation in the farmer's fields is rapidly increasing in recent years, is grown only in polyethylene film houses protected from outside.

Up to now the bacterial canker disease did not commonly occur on 'Shiranuhi' cultivar, which has been maintained in polyethylene film houses except trees for non-commercial purposes such as garden tree. However, this year, the canker disease occurred in 6 of 107 surveyed farmer's fields though the symptom was observed only on leaves and the percent diseased tree in the field was lower than 1.0%. The all six farmer's fields were damaged by the previous year's typhoon, destroying the film in polyethylene houses. Therefore, the occurrence of bacterial canker in the six farmer's fields could be attributed to the damaged polyethylene film houses which allowed the influx of pathogen from outside. Average percent of diseased tree were 0.02% and 14.8 % in 31 farmer's fields under polyethylene film houses of 'Shiranuhi' cultivar and adjacent satsuma mandarin open field, respectively, and average percent of diseased fruit were 0.0% and 1.6%, respectively. Though percent of diseased tree was over 20% in some satsuma mandarin open fields, there were no diseased trees in the adjacent polyethylene film houses of 'Shiranuhi' cultivar. These results suggest that polyethylene film house for the cultivation of 'Shiranuhi' should be protected from influx of bacterial canker pathogen from outside.

Based on the above results, it can be concluded that 'Shiranuhi' cultivar is not important as rate of resistance to citrus canker disease is similar to that of satsuma mandarin cultivar which occupies over 95% total of 25,000 ha in Jeju Island. Additionally, satsuma mandarin has been cultivated only in polyethylene film houses which can play role to protect the pathogen to enter green house from outside of the greenhouse.

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