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Evaluation of Shiranuhi, a Hybrid of Kiyomi Tangor and Nakano No. 3 Ponkan, for Resistance to Citrus Canker in Growth Chamber

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Citrus canker disease is caused by bacteria *Xanthomonas axonopodis* pv. *Citri*. Shiranuhi cultivar, a hybrid of Kiyomi tangor and Nakano No. 3 ponkan was evaluated for resistance to citrus canker based on initiation of disease, percent area of lesion infected and growth rate of bacteria in the leaf under growth chamber condition. Significant differences between susceptible plant and resistant plants were observed in these assays. Resistant plants showed delayed disease symptoms compared to the susceptible plants after spray inoculation of the pathogen. The resistant verities, satsuma, yuzu, and Shiranuhi showed symptoms after six days where as susceptible, mexican lime showed the symptoms just after three days of inoculation. 18 days after inoculation, percent area of lesions developed on leaf and disease severity differed significantly in susceptible and resistant plants, and were ranked as follows: mexican lime > early satsuma = Shiranuhi = yuzu ($P < 0.01$). However, 30 days after inoculation, percent area of lesion was further differentiated into resistant and highly resistant plants. That was ranked as follows: sweet orange > early satsuma = Shiranuhi = Kiyomi > yuzu ($P < 0.01$). These results indicate that host reaction to the bacterial was more distinct when the disease developed for a longer period. Growth rates of a citrus canker bacterium during 16 40 h also were distinct after infiltration into leaves of susceptible and resistant plants, and were ranked as follows: sweet orange > early satsuma = Shiranuhi = Kiyomi = yuzu ($P < 0.01$). Based on these results, we concluded that Shiranuhi is resistant to citrus canker as compared to Kiyomi, early satsuma, and yuzu.

Keywords : Evaluation, resistance, Shiranuhi, *Xanthomonas axonopodis* pv. *citri*.

Citrus bacterial canker disease (CBCD, "citrus canker), caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. campestris* pv. *citri*, group A) has been a serious problems in several citrus-producing regions worldwide (Civerolo, 1984). Citrus canker occurs endemically in Jeju island of Korea, where satsuma mandarin is commercially cultivated (Myung et al., 2001, 2003).

Host of the bacterium includes a wide variety of *Citrus* spp. and relatives in Rutaceae (Peltier and Frederich, 1924). Many of *Citrus* species, hybrids, and cultivars have been recorded tobe resistant to citrus canker (Gottwald et al., 1993; Koizumi, 1981; Peltier and Frederick, 1924). Recently, areas for commercial cultivation of Shiranuhi, a hybrid of 'Kiyomi' tangor and 'Nakano No. 3' ponkan (Matsumoto, 2001) have been increased in Jeju islands, but resistance of the hybrid to citrus canker has not yet been evaluated.

Plant resistance to citrus canker has been evaluated based on changes of lesion extension and/or bacterial population after pin-prick or carborundum-rub inoculation on leaves (Egel et al., 1991; Graham and Gottwald, 1989; Graham et al., 1990; Koizumi and Kuhara, 1982). Percent area of lesion formed within a given period may be differentiated into resistant and susceptible plants (Lamari, 2002).

In this study, we have evaluated resistance of Shiranuhi plant to citrus canker, which compared the plants with susceptible and resistant ones on initial incidence of citrus canker, percent area of lesion and disease severity, and growth rate of *X. axnonopodis* pv. *citri* infiltrated in leaf in a growth chamber.

Materials and Methods

Bacterial culture and selection of Rif^r strain. Bacterial strain BC1 (Myung et al., 2001 & 2002) of *Xanthomonas axonopodis* pv. *citri*, was used as inoculum source, which was isolated from satsuma mandarin leaves. For assay of bacterial multiplication in leaf, rifampicin-resistant strains (Rif^r) were selected from the culture that was incubated at 28°C and 250 rpm for two days in the PS broth supplemented with rifampicin (Bacto peptone 10 g, sucrose 10 g, sodium glutamate 1 g, distilled water 1 L, and rifampicin 50 μg/mL). The bacterial cells were harvested by centrifugation from 16-hr-old shaking culture (28°C, 250 rpm) in PS broth medium amended with rifampicin (50 μg/mL). Pellet of the bacterial cells was washed twice with 0.01 M potassium phosphate buffer (pH 7.2) and adjusted spectrophotometrically to approximately 2×10^8 colony forming units (cfu)/mL using 0.01

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M potassium phosphate buffer (pH 7.2). Inoculum concentration was confirmed by plating onto the supplemented PS agar medium.

Plant materials. *Citrus* species and hybrids used in this study were sweet orange 'Lane late' (*C. sinensis*) or mexican lime (*C. aurantifoli*) as susceptible, Kiyomi (*C. unshiu* × *C. sinensis*) and early satsuma (*Citrus unshiu*) as resistant, yuzu (*C. junos*) as highly resistant based on Koizumi (1981) and Peltier et al. (1924). Two–three-years-old seedlings grafted onto potted trifoliate orange were cultivated at 18-30°C in a greenhouse, and pruned to stimulate synchronous leaf flushes. The seedlings were transferred to a growth chamber, and inoculated after acclimatization in a growth chamber. The growth chamber was controlled at 28°C and RH of 75%.

Spray inoculation. Bacterial suspension (BC1) was sprayed on over half expanded-leaf of host plants with lab-spray bottle, and the inoculated plants were cultivated in a growth chamber. Seedlings with the 10-15 immature leaves were covered with plastic bags to facilitate infection for three days after spray inoculation. The plants had been daily examined until first disease symptom occurred after the inoculation, and then the bags were removed.

Assay for disease severity. In a growth chamber, disease severity on two plants per each host (mexican lime, Shiranuhi, early satsuma and yuzu) was examined at 3, 5, 6, 7, 12, 15, and 18 days after inoculation, and recorded as percentage of number of infected leaves per number of leaves examined (Myung et al., 2003).

Bacterial growth-rate in leaf. Suspensions of the Rif' strain in 0.01 M potassium phosphate buffer (pH 7.2) were infiltrated into over half expanded-leaves of sweet orange 'Lane late', Shiranuhi, early satsuma, Kiyomi, and yuzu with a syringe that gause was removed at several points. Bacterial populations were measured at times of 0, 16hr, and then every 24-hr for seven days. Three 0.85-cm-in-diameter leaf disks were randomly removed from within the infiltrated area. The leaf disks were macerated with one ml of the sterile potassium phosphate buffer (pH 7.2) in a sterile mortal and pestle, and dilution plated onto PS agar amended with rifampicin (25 μg/mL). Bacterial populations were expressed as colony-forming unit (cfu)/a disk in one ml of 0.01 M potassium phosphate buffer (pH 7.2). The populations were transformed into growth rate of a bacterial cell in leaf for 24 hr as follows:

$GR = (A_t - A_{t-1})/A_{t-1}$ where GR is the growth rate, A_t is a bacterial population at t time, and A_{t-1} is a bacterial population at t-1 time.

Percent area of lesion infected. Percent area of lesion infected per leaf on susceptible and resistant hosts was compared at 18 day and 30 day after spray inoculation. At 18 day, infected leaves were detached from mexican lime, Shiranuhi, early satsuma and yuzu, and at 30 day from sweet orange, Shiranuhi, early satsuma, Kiyomi and yuzu. Percent area of lesion infected per leaf was assayed using ASSESS: image analysis software for plant disease quantification (Lamari, 2002) according to instruction manual.

Statistical analysis. Growth rate in leaf and percent area of lesion data for each data were compared by ANOVA; if the *F* test was significant at $\alpha = 0.01$ level, means were compared by LSD procedure ($\alpha = 0.01$) for each data. The General Linear Model (GLM) and the Least Significant Difference (LSD) procedures were run using SAS (Statistical Analysis Systems, Cary, NC, USA).

Results

Disease development. Disease incidence occurred three days earlier for susceptible than resistant plants, and continued to occur up to 7-13 days after inoculation (Fig. 1). On Shiranuhi, initial occurrence of citrus canker was six days later inoculation, which was concurrent on resistant plants, early satsuma, Kiyomi and yuzu. Disease incidence ceased earlier on resistant plants as follows: after 7 days on yuzu, and after 13 days on Shiranuhi and early satsuma. All of leave on mexican lime were infected 11 days after inoculation.

On 18 days after inoculation (Fig. 1), disease severity differed significantly in susceptible and resistant plants ($P < 0.01$), but was not further differentiated among resistant and highly resistant plants. Disease severity was ranked as follows: mexican lime > early satsuma = Shiranuhi = yuzu ($P < 0.01$).

Percent area of lesion infected per leaf. Different percent-area of lesion infected was evident among susceptible and resistant hosts (Fig. 2). On Shiranuhi, percent area of lesion was similar to that on resistant plants, early satsuma and Kiyomi 18 days after inoculation. Percent areas of lesion differed significantly on highly resistant and resistant plants, when citrus canker developed for a longer period (Fig. 2A); however, the percent areas did not significantly differed among the resistant plants, developed for a shorter period ($P < 0.01$). At 18 day, the lesions were ranked as follows: mexican lime > Shiranuhi = early satsuma = yuzu, and at 30 day, sweet orange > Shiranuhi = early satsuma = Kiyomi > yuzu ($P > 0.01$).

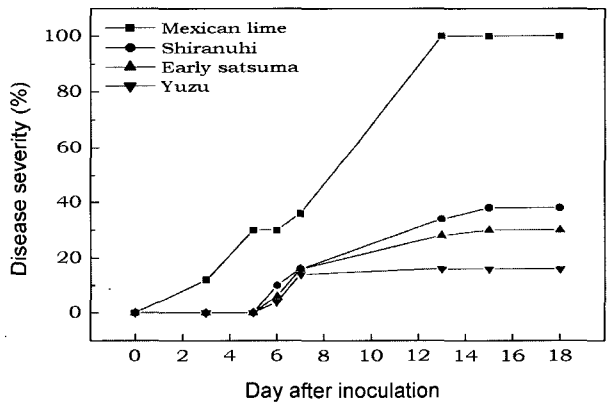

Fig. 1. Disease severity on different plants after spray inoculation with BC1 of *Xanthomonas axonopodis* pv. *citri* in growth chamber. Plants followed by unlike letters have significantly different ($P < 0.01$) disease severity according to Students' t-test of LSD procedure.

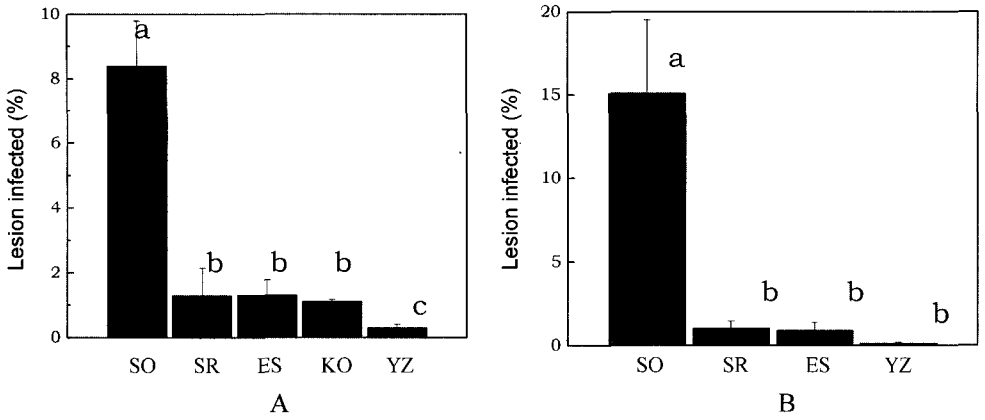

Fig. 2. Percent area of lesion infected per leaf on different hosts after spray inoculation with strain BC1 of *Xanthomonas axonopodis* pv. *citri* in growth chamber. A; percent lesion infected 30 days. B; percent lesion infected 18 days. SO = sweet orange 'Lane Late', ML = mexican lime, SR = Shiranuhi, ES = early satsuma, KO = Kiyomi, and YZ = yuzu. Host plants followed by unlike letters have significantly different ($P < 0.01$) growth rates in leaf according to Students' t-test of LSD procedure.

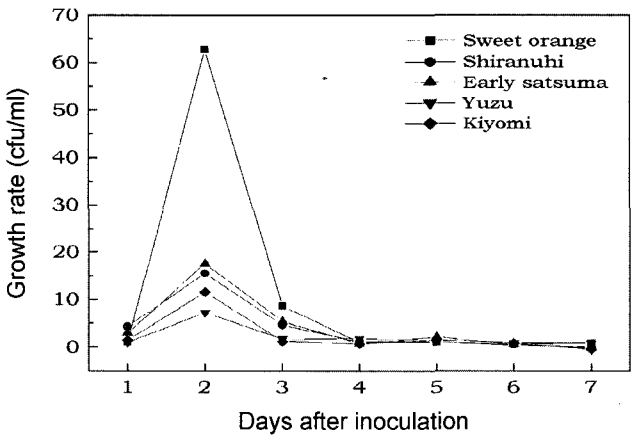

Fig. 3. Growth rate of *Xanthomonas axonopodis* pv. citri (BC15) in leaves of *Citrus* plants and hybirds after infiltration with in growth chamber. Plants follwed by unlike letters have significantly different ($P < 0.01$) grwoth rates in leaf according to Students' t-test of LSD procedure.

Gowth rate of a bacterial cell in leaf. Growth rate in leaf on all hosts tested increased sharply during a period of 16 40 hrs after infiltration with strain BC15 of *X. axonopodis* pv. *citri*, and then the rate decreased constantly to remain at near zero until seven days (Fig. 3). Growth rate differed distinctly between susceptible and resistant plants. The rate was higher for sweet orange than Shiranuhi, early satsuma, Kiyomi, and yuzu, and ranked as follows: sweet orange > early satsuma = Shiranuhi = Kiyomi = yuzu ($P < 0.01$).

Discussion

We revealed that Shiranuhi is as resistant to citrus canker as Kiyomi, early satsuma, and yuzu in growth chamber. Citrus canker bacteria enter through the plant's stomatal openings or through wounds of the plant. Stomatal infections in uninjured plants occur less on resistant plants than on susceptible plant, because bacterial ingress is restricted by difference in stomatal structure among them (McLean and Lee, 1921, 1922). Koizumi, (1981) and Koizumi and Kuhara (1982) found that lesion expansion is less in resistant plants than in susceptible plants. Slower lesion development in resistant plant is due to a series of plant reaction that meristematic tissue surrounding the degenerated tissue elicited by infection with citrus canker bacteria (Koizumi, 1977, 1979), eventually results in less lesion size in resistant plants. Lesions naturally formed by *X. axonopodis* pv. *citri* can be noticed with a hand lens 7 days later and with the naked eyes 14 days later (Stall and Seymour, 1983). These results could explain that disease severity was less in resistant plants, and initial disease incidence was delayed three days in resistant plants.

Graham et al. (1990) observed that sizes of lesion infected with *X. axonopodis* pv. *citri* were not distinctly different at 40 days among the resistant and susceptible plants tested. In this study, we showed that percent area of lesion could discriminate on resistant and susceptible. Moreover, when lesion develops for a longer period, resistant plants were further differentiated into resistant plant and highly resistant plant by the percent area. This result suggested that lesion stop expanding earlier on resistant plant on citrus canker. The percent area is more effective approach to evaluate host resistance to citrus canker because the area of lesion includes lesion development and number of lesion infected per leaf.

We found that growth rates of a bacterial cell in leaf during 16-40 hr were distinct on resistant and susceptible plants. Citrus canker bacteria start to multiply in intercellular spaces after introduction into host tissue (Koizumi

1977, 1979). Increase in bacterial cell number is less on resistant plant because fibrillar materials coagulate in intercellular space and ensheath the bacterial cells (Koizumi 1977, 1979). Whereas bacterial population is larger on susceptible plants, and lesion develops faster (Egel et al.; 1991; Graham and Gottwald, 1989; Graham et al., 1990; Koizumi, 1977; Koizumi, 1981; Koizumi and Kuhara, 1982; Peltier and Frederick, 1924). Population of *X. axonopodis* pv. *citri* in lesion sustains a concentration of 10^6 to 10^7 cfu during growing season of citrus plant (Pruvost et al., 2002; Stall et al., 1982). These results indicated that canker bacteria infected into susceptible plant multiple more rapidly in a limited area because of less structural restriction described above. Thus, we inferred that resistant and susceptible plants could be determined by differences of bacterial growth rate in leaf during 16-40 hrs. Maintenance of near zero in the growth rate during three- seven days might be due to lack of available nutritional materials in the area of susceptible and resistant plants. In addition, we suggested that growth rate of the bacteria in leaf can be used to distinguish resistant plants from susceptible ones.

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