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Detection of *Xanthomonas axonopodis* pv. *citri* on Citrus Fruits Using Enzyme-Linked Immunosorbent Assay

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Detection of *Xanthomonas axonopodis* pv. *citri* (Xac) on citrus fruits for exporting is usually made by bacteriophage test (BPT) to demonstrate the pathogen-free status. BPT has rather time-consuming and complicate procedures for dealing with massive samples to be inspected. In this study, enzyme-linked immunosorbent assay (ELISA) was applied to detect Xac on fruits, and compared with BPT. In ELISA, positive reactions occurred in the bacterial densities of 3×10^5 cells/ml or more. To detect the bacterial infection on citrus fruits with a density of lower than 3×10^5 cells/ml, the bacterial suspensions were mixed with fruit rinse water and incubated in broth medium. Ordinary peptone sucrose broth (PSB) was not a proper medium for increasing Xac density specifically enough to be detect by ELISA. On the other hand, modified PSB (MPSP) amended with Fe-EDTA (0.25 g/l) and 2.5% potato-dextrose broth sufficed to differentiate uninfected and infected citrus fruits by ELISA after 24 h incubation of the fruit rinse water. Using various citrus samples from infected and uninfected fields, efficiencies in detecting Xac on fruits were compared between ELISA and BPT. For infected fruits samples, ELISA detected Xac by 100%, while BPT by about 44%, indicating that the detection efficiency was improved by 23.5% by ELISA, compared to BPT. In addition, ELISA has simpler procedures for testing and is less time-consuming than BPT, suggesting that ELISA may be accurate and simple method to detect Xac on citrus fruits.

Keywords : bacterial citrus canker, bacteriophage detection, ELISA, *Xanthomonas axonopodis* pv. *citri*.

Bacterial citrus canker caused by *Xanthomonas axonopodis* pv. *citri* (Xac) originating from Southeast Asia is known to distribute in Asia, Middle and South America, Papua New Guinea in Australia, and some countries of Africa (Hayward and Waterson, 1964). The disease occurs on leaves, branches and fruits. Early disease symptoms are small and round yellow lesions, which expand up to 1 cm in diameter (Whiteside et al., 1989). Unshu citrus (*Citrus reticulata* var. *unshu*) which is a moderate resistant variety growing in Jeju province, Korea, may not have such symptoms when the pathogen infects later than 6 weeks after leaf expansion (Koizumi, 1971: Koizumi and Kuhara, 1982: Kuhara, 1978). On the other hand, susceptible varieties and species, orange, lemon and grapefruit that are cultivated in the USA, Australia, and Europe can be damaged severely when the disease epidemic occurs (Stall and Civerolo, 1991).

Bacterial canker of citrus is the most important disease for quarantine in citrus-growing areas of western countries. In the USA, the disease was found firstly in Florida from imported trifoliate orange seedlings from Japan in 1912 (Berger et al., 1914). The disease had spread to five states adjacent to Florida. Expenses for eradicating the disease were about 6 million dollars, and about 270 thousand mature trees and 3 million seedlings were burnt up. In 1984, the disease outbroke again, and 25 million dollars was used for its eradication by 1986. In 1992 and 1995, citrus canker occurred again in Florida, and the eradication program is still undergoing (Anonymous, 1999). Citrus canker was also eradicated from Australia (Curry, 1989), New Zealand (Dye, 1969) and South Africa (Doidge, 1929). Eradication programs are in progress in Brazil and Uruguay (Stall and Civerolo, 1991).

In Korea, citrus fruits have been exported especially to the USA since 1995. When exporting, bacteriophage test is used to detect the pathogen infection just before harvest of citrus fruits, and as a result of the test, the fruits produced in pathogen-free areas have been allowed for exporting (Kang, 1999). However, the bacteriophage test is rather complicated in dealing with a large amount of samples at the same time, and needs a long time and many laboratory equipments, which may be technically difficult to meet the expected demand of export from 2001. Therefore, there are needs somehow to develop an accurate and easy-to-use

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method for the detection of the disease. This study was conducted to develop a method of Xac detection from citrus fruits that can be an alternative to the bacteriophage test.

Materials and Methods

Citrus canker pathogen. Three pathotypes of Xac were collected; pathotype A from Jeju Branch of National Plant Quarantine Service (NPQS), and pathotype B and C from Division of Plant Pathology, National Institute of Agricultural Science and Technology. Antibody to Xac and enzyme conjugate sets which were proceeded by indirect enzyme linked immunosorbent assay (ELISA) were bought from Agdia company (Elkhart, Indiana, USA).

Sensitivity of ELISA to the citrus canker pathogen. The bacterial pathogens were cultured on peptone-sucrose agar (PSA) (bactopeptone: 5 g, $Na_2HPO_412H_2O$: 2 g, $Ca(NO_3)_24H_2O$: 0.5 g, sucrose: 15 g, agar: 20 g, distilled water: 1 l) at 28°C for 24 h. The bacterial cultures were suspended in sterilized distilled water (SDW), and adjusted to the optical density (O.D.) of 0.250-0.265 under a turbidimeter, which was corresponding to about 3×10^8 cells/ml, and then serially diluted with SDW to 3×10^4 cells/ml. Each diluted bacterial suspension was subjected to ELISA by following the usual procedures of indirect ELISA. Also using a half volume of each diluted bacterial suspension, bacterial cells were killed by treating the suspension in boiling water for 10 min, and subjected to the ELISA test as described above.

Detection of Xac in low population densities by ELISA. To detect Xac on fruits with low population densities indetectable *per se* by ELISA, the population increase was made by culturing the bacterial suspension (mixture of the pathogen and fruit rinse water) with initial densities of 3×10^5 cells/ml to 3×10 cells/ml in broth medium. Firstly, the bacterial suspension of 0.1 ml was inoculated in 10 ml peptone-sucrose broth (PSB) and cultured for 24 h or 48 h at 28°C in a shaking incubator (120 rpm). After culturing, each bacterial culture was tested by ELISA, following the procedures described above. Secondarily, PSB was modified to increase Xac density in a short period time by adding 2.5% potato-dextrose broth (PDB) and various amounts of Fe-EDTA (0.1-1 g/l) in PSB. The population growth was examined visually 24 h after culturing.

Detection of Xac in rinse water from fruit samples by ELISA. As population densities of Xac on fruits are usually low regardless of its infection, fruit rinse water from citrus fruits was incubated in PSB or modified PSB (MPSB) amended with 2.5% PDB and 0.25 g/l Fe-EDTA to increase its density. Fruit samples were obtained infected or uninfected fields in Jeju province, Korea. The samples were rinsed with 500 ml of SDW, and centrifuged for 15 min at 10,000 rpm for concentration. The pellets were resuspended with 5 ml of SDW, and the resulting suspensions (0.1 ml) were inoculated in 10 ml PSB or MPSB and cultured for 24 or 48 h at 120 rpm in the shaking incubator. After culturing, the culture suspensions were tested by ELISA as described above.

Comparison of detection efficiencies between ELISA and bacteriophage test (BPT). Eighteen citrus fruit samples were collected from infected and uninfected fields at three locations in Jeju province, Korea. Fruit rinse water was used for ELISA and BPT. For each fruit sample, 1 kg of citrus fruits were rinsed with 500 ml of SDW, and the rinse water was centrifuged for 15 min at 10,000 rpm. The resulting pellet was resuspended in 20 ml of SDW (fruit rinse water suspension).

For ELISA, 1 ml of the suspension was inoculated in MPSB and incubated for 24 h at 28°C in the shaking incubator as described above. The MPSB cultures were tested by ELISA following the usual procedures.

For BPT, the remaining (19 ml) suspension was centrifuged again at 10,000 rpm, the pellet obtained was resuspended with 6 ml of SDW, and divided equally into two parts; one as control and the other as test samples. Control samples were heated for 10 min in an 100°C water bath, but test samples were not treated. The suspension was added with 1 ml of bacteriophage suspension (1×10^5 plaques), and incubated 4 h at 120 rpm in the shaking incubator. After incubation, the suspension was centrifuged at 10,000 rpm and the supernatant was subjected to BPT as follows. One ml of the supernatant, 15 ml of PSA and 2 ml of Xac suspension were mixed and poured in Petri plates, incubating for 24 h at 28°C in the incubator. After incubation, bacteriophage plaques formed on the plates were counted. A test sample was counted to have a positive reaction when the number of bacteriophage plaques were 20% higher than the control check. Otherwise, the reaction was considered negative.

Results

Sensitivity of ELISA in detecting Xac. The suspensions of *Xanthomonas axonopodis* pv. *citri* (Xac) containing 3×10^5 cells/ml or more showed positive reactions in ELISA whether the bacterial cells were killed by heat treatment or not (Table 1). There were no significant differences in the ELISA reactions among the three pathotypes. The optical densities representing the intensities of serological reaction

Table 1. ELISA of live and dead cells of *Xanthomonas axonopodis* pv. *citri* pathotypes A, B and C

Cell status	Density (cells/ml)	Pathotype A		Pathotype B		Pathotype C	
		O.D.[a]	Reaction	O.D	Reaction	O.D	Reaction
Live	3×10^8	4.686	+[b]	4.690	+	4.635	+
	3×10^7	4.098	+	3.494	+	3.927	+
	3×10^6	1.561	+	1.245	+	1.726	+
	3×10^5	0.362	+	0.323	+	0.220	+
	3×10^4	0.126	–	0.115	–	0.127	–
Dead	3×10^8	4.685	+	4.542	+	4.680	+
	3×10^7	4.718	+	4.717	+	4.609	+
	3×10^6	3.099	+	2.244	+	4.641	+
	3×10^5	0.601	+	0.585	+	1.410	+
	3×10^4	0.121	–	0.150	–	0.214	–
Control		0.109					

[a] O.D.; Optical density at 405 nm by the ELISA reader.
[b] +; Positive, –; Negative.

Table 2. ELISA of the cultures of *Xanthomonas axonopodis* pv. *citri* (Xac) with different initial densities in rinse water[a]

Initial bacterial density	24 h culture		48 h culture	
	O.D.[b]	Reaction	O.D.	Reaction
3×10^5	0.148	–[c]	0.641	+
3×10^4	0.239	–	0.583	+
3×10^3	0.111	–	0.450	+
3×10^2	0.195	–	0.373	+
3×10^1	0.200	–	0.814	+
Control	0.152			

[a] Peptone sucrose broth was used for the culture of Xac in fruit rinse water.
[b] O.D; Optical density at 405 nm by the ELISA reader.
[c] +; Positive, –; Negative.

were increased with the increased bacterial densities in all Xac pathotypes tested.

Detection of Xac in low population densities by ELISA. PSB was used to increase Xac density as a way to increase detection ability by ELISA for Xac on citrus fruits of which the population density is usually low. However, culturing Xac of low densities in PSB gave no specific population increase to discriminate the initial population densities by ELISA (Table 2). Regardless of the initial bacterial density, bacterial cultures incubated for 24 h showed negative reactions, while those incubated for 48 h positively reacted. The reaction intensity as expressed by optical density (O.D.) was not correlated with the initial Xac density.

PSB medium was modified for specific population increase of Xac in a short period of time (24 h) by adding potato dextrose broth and Fe-EDTA. Contrary to PSB which had little population increase for 24 h of culture, the pathogen grew well in the medium supplemented with 0.1 to 1 g/l of Fe-EDTA. The pathogen grew best in the medium with 0.25 g/l of Fe-EDTA, and the bacterial population increased more with the addition of PDB (data not shown).

Detection of Xac in rinse water from fruit samples by ELISA. The rinse water from citrus fruits from different sources was also cultured with in PSB for 24 h or 48 h, showing the same results as above. The cultures of 24-hour incubation reacted negatively, but those of 48-hour incubation reacted positively even though fruits were collected from an uninfected field (Table 3).

However, when the fruit rinse water was incubated in MPSB (amended with 0.25 g/l Fe-EDTA and 2.5% PDB) for 24 h, ELISA of the culture showed positive reactions to fruits from infected fields, but negative reactions to those from uninfected field (Table 3).

Comparison of detection efficiencies between ELISA and BPT. Two detection methods, ELISA and BPT were compared in their sensitivity to the citrus canker pathogen using the same fruit samples. All 9 samples from the infected fields showed positive reactions in ELISA, and one sample from the uninfected fields showed positive reactions (Table 4). In BPT, only 4 out of 9 samples from the infected fields had positive reactions, while the other samples negatively reacted to BPT whether they were obtained from infected or uninfected fields.

Discussion

The bacterial canker of citrus is an important disease for quarantine especially in citrus-growing areas. In Korea, citrus fruits have been exported to the USA since 1995, but not have been allowed for exporting to five orange-growing states (California, Florida, Texas, Arizona and Louisiana). The pathogen should not be detected on exporting fruits in beforehand inspection by an authorized detection method. Detection of the bacterial canker pathogen on the citrus leaves using bacteriophage test was firstly described by Obata (1974), and the method has been used to demonstrate canker-free citrus fields in Japan. In Korea, citrus fruits are tested also by the bacteriophage method developed by

Table 3. ELISA of the cultures of fruit rinse water in different media for the detection of *Xanthomonas axonopodis* pv. *citri*

Sample		PSB[a]				MPSB[b]	
		24-h culture		48-h culture		24-culture	
Field status	Fruit status	O.D.[c]	Reaction	O.D.	Reaction	O.D.[b]	Reaction
Infected	Infected	0.124	–[d]	0.861	+	0.415	+
	Uninfected	0.135	–	0.941	+	0.283	+
Uninfected	Uninfected	0.189	–	0.556	+	0.142	–
Control		0.107				0.109	

[a] PSB: peptone sucrose broth
[a] MPSB: modified peptone sucrose broth (amended with 0.25 g/l Fe-EDTA and 2.5% potato-dextrose broth)
[c] O.D; Optical density at 405 nm by the ELISA reader.
[d] +; Positive, –; Negative.

Table 4. Comparison of two methods, ELISA and bacteriophage test, for detecting *Xanthomonas axonopodis* pv. *citri* on citrus fruits from different fields

Location	Fruit status	Field	ELISA		Bacteriophage		
			O.D.[a]	Reaction	No. of plaques		Reaction
					Control	Test	
Haean (Jeju)	Infected	A	2.536	+[b]	119.3	115.0	−
		B	1.881	+	124.3	118.7	−
		C	1.428	+	116.0	150.0	+
	Uninfected	D	0.408	−	120.0	126.0	−
		E	0.277	−	135.3	144.3	−
		F	0.318	−	129.7	126.7	−
Aewool (Bukjeju)	Infected	G	3.205	+	127.7	160.5	+
		H	3.305	+	120.0	167.0	+
		I	1.555	+	131.3	234.3	+
	Uninfected	J	0.353	−	133.6	146.7	−
		K	0.810	+	124.0	133.3	−
		L	0.325	−	164.3	164.0	−
Namwoon (Namjeju)	Infected	M	3.248	+	130.0	153.7	−
		N	3.187	+	140.7	130.3	−
		P	3.255	+	156.7	151.0	−
	Uninfected	Q	0.389	−	148.7	149.7	−
		R	0.286	−	139.7	155.7	−
		S	0.348	−	130.7	145.0	−
Negative control			0.210				

[a] O.D.; Optical density at 405 nm by the ELISA reader.
[b] +; Positive, −; Negative.

Myung et al. (1995), which is similar to that used in Japan. However, the bacteriophage test is rather complicate and time-consuming to deal with a large number of samples because of its complex and time-requiring procedures.

ELISA is an immunoassay method progressed recently for detection of bacterial pathogens from seeds and plant materials. Detection of Xac by ELISA was reported by Civerolo and Fan (1984), which was used for quantitative diagnosis of Xac in a range of 10^5-10^6 cfu/ml. However, detection efficiency of ELISA is limited by the level of the pathogen population, and dependent upon the immunological properties of antiserum used. In our study, when Xac densities were not less than 3×10^5 cell/ml, a positive ELISA reaction occurred (Table 1). The Xac population densities of rinse water from infected leaves in the field varied from 10^4 to 10^6 cfu/ml (Stall et al., 1980). However, leaves with old lesions had much lower (0 to 10^3 cfu/ml) bacterial cells (Timmer et al., 1991). Timmer et al. (1991) suggested that the bacteria might not be released from the old lesions because of the suberization of the lesions. Therefore, ELISA of fruit rinse water without additional Xac population increase may not be sufficient to detect the low populations of Xac, which may be dependent upon disease severity, fruit age, etc.

We tried to increase population density of the pathogen by incubating rinse water of the citrus fruits in broth medium. The cultures incubated in PSB for 24 h did not give a population density enough to be detected by the ELISA test, but all the cultures incubated for 48 h positively reacted. When the 24-h cultures were plated on King's B agar, most colonies are not Xac but fluorescent pseudomonads (may be antagonistic) that probably affected the growth of Xac at the early stage. Xac is an aerobic bacterium (Krieg and Holt, 1984), and ferrichrom, an Fe-binding compound, is found in all aerobic bacteria, which is a component of the hydrogen- and electron-carrier system (Neidhardt et al., 1990; Guirad and Smell, 1981). Bakker et al. (1990) mentioned that fluorescent pseudomonads released siderophore which is functioning as carrier and chelating material of iron, inhibiting iron uptake of Xac. In our study, the increase of fluorescent pseudomonads may affect the respiration of the bacterial canker pathogen in the liquid medium at the early stage of the bacterial growth.

On the other hand, Xac grew well in MPSB that added with 0.25 g/l of Fe-EDTA and 25 g/l of PDB in PSB, so that ELISA could give positive reaction to the 24-h culture of

rinse water from infected fruits. This is probably because the addition of Fe-EDTA reduced the harmful effect of fluorescent pseudomonads on inhibiting iron uptake of Xac.

When the MPSB cultures of fruit rinse water were used for ELISA, it is assumed that 17 out of 18 samples had correct responses, showing the correct infection status. However, in bacteriophage test (BPT), 13 samples might represent correctly Xac infestation on citrus fruits. In this respect, the ELISA test was 23.5% higher in detection efficiency than the BPT. Bacteriophage is a parasitic agent specific to certain bacteria, and may differently react depending on the source and longevity or growth conditions of the host (Goto and Starr, 1972). However, in ELISA, the serological reaction to the bacterial population/bacterial protein is consistent and is not changed due to the bacterial source (Seattler et al., 1989: Simbert and Krieg, 1981). Even dead bacterial cells could be detected by ELISA as efficiently as live cells. This suggests that the ELISA test should be a more appropriate way for quarantine of importing countries than the BPT.

In terms of time and labour for the detection procedures, BPT is relatively more time-consuming and laborious than ELISA. Major components for time saving in ELISA were the absence of additional two times of centrifugation, and bacteriophage culture. If ELISA is applied to test export citrus fields and if we should test more than 100 samples, then, to some extend, we expected 71.4% of time can be saved compared to BPT.

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