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Detection of *Xanthomonas axonopodis* pv. *citri* on Satsuma Mandarin Orange Fruits Using Phage Technique in Korea

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A phage technique for detection of *Xanthomonas axonopodis* pv. *citri*, a causal bacterium of canker on Sastuma mandarin fruits was developed. Phage and ELISA techniques were compared for their sensitivity for detection of *Xanthomonas axonopodis* pv. *citri* on orange fruits. Both of techniques revealed a similar efficiency for the bacterial detection; the pathogenic bacteria were observed in pellet from the fruits with over one canker spot with below 2 mm in diameter. In field assays, the increase of phage population (120%) on surface of the fruits related to the disease development one month later indicated that the bacterial pathogens inhabit on the surface. The procedure will be effectively used for detection of only living bacterial pathogen on fruit surfaces of Satsuma mandarin and for the disease forecasting.

Keywords **:** phage detection, Satsuma mandarin, *Xanthomonas axonopodis* pv. *citri*

Citurs canker, caused by *Xanthomonas axonopodis* pv. *citri* (syn. *X. campestris* pv. *citri*, group A) was serious in numerous citrus-producing countries worldwide (Civerolo, 1984). Symptoms of citrus canker include erumpent and corky lesions on all aerial parts of mature citrus trees including leaves, stems, and fruits (Schoulties et al., 1987). A wide variety of *Citrus* spp. and relatives in Rutaceae have reported as hosts of the bacterial pathogen. Inter- and intra-country movement of citrus fruits and seedlings infected has strictly been regulated to preclude entry of the disease into the disease-free areas (Anonymous, 1990; Koizumi, 1985). Therefore, the development of reliable procedures for the diagnosis of living bacterial pathogen has been a priority to export the orange fruits.

Techniques using antiserum and DNA for detection of *X. axonopodis* pv. *citri* have been developed (Alverez et al., 1992; Hartung, 1993). However, these techniques detect both of living and dead bacterial pathogens. For detection of living bacterial pathogens on plant materials, phage technique is effective because phages only reproduce in the presence of the living host bacteria. Phage technique is more rapid, simple and effective than conventional time-consuming procedure, which phage lysis zones usually become visible within 18-24 hrs of incubation. Moreover, host specificity for a species or subspecific group of bacteria have been used for rapidly identifying animal and plant pathogens (Anderson and Willians, 1956; Billing, 1963 &1970), and extensively for studying epidemiology of human pathogens (Anderson and Williams, 1956) and plant pathogenic bacteria (Gross et al., 1991; Myung, 2003).

One group of *X. axonopodis* pv. *citri* phage designated as CPK distributed in Korea (Myung et al., 2001). Based on specificity of CPK, Myung et al. (2001 & 2002) presented that two lysotypes was distributed and lysotype I predominated about 97% in Korea. Practically, because of predominant existence of lysotype I, CPK should detect almost of the causal agents that attach to plant materials. In this study, a technique using phage for detection of the living bacterial pathogens on the surface of Satsuma mandarin fruits was developed based on the information on distributions of phage and its hosts in Korea. The phage technique and ELISA method were compared for detection of the pathogens on the fruits. In field, using the phage technique, we showed that citrus canker development was related with preexistence of the bacterial pathogens on the fruit surface.

Materials and Methods

Preparation of bacterial cell and phage. Bacteria and phages were cultured as previously described (Myung et al., 2001). Briefly, strain (BC1) of *X. axonopodis* pv. *citri* were routinely cultured at 27°C on peptone sucrose agar (PSA). For phage propagation, the bacterial cells cultured within 24 hrs at 27°C were suspended in peptone sucrose broth (PSB) or Wakimoto, and phages were multiplicated at 25°C above 5 hrs. The concentration of phage in the supernatant was measured in a previous day. Phage concentration in the supernatant stored at 4°C was adjusted

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to 6×10^3 plaque forming unit (pfu)/ml in 0.1 ml by a serial dilution with PSB.

Preparation of bacterial cells from fruits. Diseased or non-infected fruits were collected from citrus nurseries and greenhouse at Seogyupo station, National Institute of Subtropical Agriculture, in Jeju islands, respectively. Satsuma mandarin fruits (2 Kg) with one canker spot (below 2 mm in diameter) were washed in 2,000 ml sterile distilled water and equally divided into two parts. One part was applied for indirect ELISA, and another for phage test. Each part was centrifuged at 12,000 rpm for 30 minutes. The pallets were used as samples for detection of *X. axonopodis* pv. *citri* on the fruits. Each test was done twice.

Detection of pathogens using phage and ELISA methods. Samples for ELISA were prepared as follows; the pellets from the centrifugation described above were resuspended in 3 ml of Wakimoto Potato Semisynthetic Broth (WPSB). Indirect ELISA method for detection of the bacterial

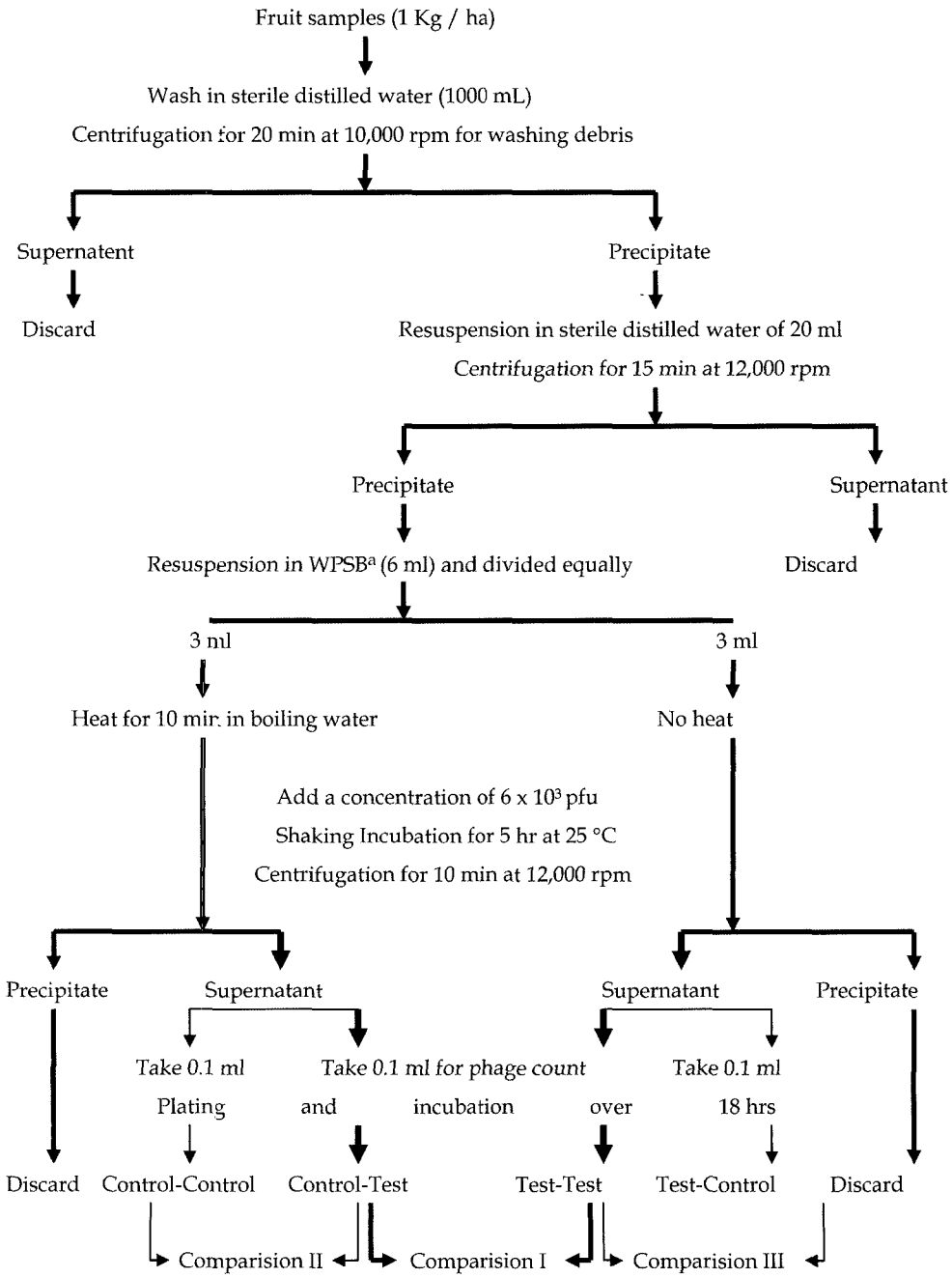

Fig. 1. Scheme for detection of *Xanthomonas axonopodis* pv. *citri* by using phage. [a]Wakimoto Potato Semisynthetic Broth.

pathogen using commercial kit from Agdia™ was performed by the procedure according to manufacturer's instruction. One hundred microliters of the samples resuspended were dispensed into each well of a 96-well microplate, and added 100 μl of coating buffer into wells. Each well was blocked with 5% bovine serum albumin in phosphate buffered saline (BSA/PBS). Serum samples were diluted 1:200 with 2.5% BSA/PBS containing 0.05% Tween 20. After one hour incubation at room temperature, enzyme conjugate was applied and the plates were incubated for another one hour. After washing, PNP solution was added and the reaction stopped by adding 50 μl of 3 M sodium hydroxide. Results were evaluated after reading optical density at 405 nm using automatic ELISA reader (Molecular Devices, USA).

Phage test were performed as followed the bolded lines indicated in Fig. 1. The procedure consisted of three steps. The first step was a procedure that bacterial cells were collected from the surface of Satuma mandarin fruits; the fruits are washed with sterile distilled water, and then the washed materials were centrifuged for 20 min at 10,000 rpm for bacterial collection. The second step was a procedure for multiplication of phage; an aliquot of three ml equally divided from the pellet was resuspended in 6 ml of WPSB. One part was heated in boiling water to kill bacterial pathogens in the suspension, which was used as a control. After the two parts were adjusted to a concentration of 6×10^3 pfu of phage, those were shaking-incubated to make multiplication of phage in bacterial cells. The third step is a procedure that the multiplied phages are measured. To measure the phage multiplied in its host cells, 0.1 ml of the cell suspension was taken after centrifugation of cultures of the second step above. The supernatant from each part was mixed with Wakimoto's potato soft agar medium and poured into a plate, following incubation for 18 hrs at 25°C. Bacterial pathogens on fruit samples were determined by comparing the number of plaque on 'Test-Test' plate originated from the supernatant of the second step (no heated culture) with that of 'Control-Test' from the supernatant (heated in boiling water). Existence of pathogens on fruit samples was confirmed when the plaque number of 'Test-Test' was more than that of 'Control-Test'.

Field assays. Field assays were performed at nine cirrus nurseries, which citrus canker had not developed in August and September. Phage tests were performed one or two treatment(s) per the test. The tests were performed as described above. Disease development was observed at late of October. The fruits of Satsuma mandarin spread a bacterial concentration of 1×10^5 cfu/ml were used for positive control. Detached fruits from nurseries were air-dried at room temperature and used for the phage test described above.

Results and Discussion

We primarily developed phage technique, a procedure indicated as bold lines in Fig. 1 for detection of *X. axonopodis* pv. *citri* on fruit of of Satuma mandarin orange based on previous reports (Myung et al., 2001 and Myung et al., 2002). Phage and ELISA techniques were compared based on detection sensitivity of the bacterial pathogens on fruits of the Satuma orange (Table 1). Using phage technique, the bacterial pathogens within surface-washing materials from the fruits (1 kg) including one diseased fruit with one canker spot below than 2 mm in diameter could be detected. The result was similar to that tested using ELISA, indicating that phage technique for detecting citrus canker

Table 1. Detection *of Xanthmonas axonopodis* pv. *citri* on Satsuma mandarin orange fruits using phage and ELISA techniques[a]

Methods	Number of diseased fruits[b]			
	10	5	1	0
ELISA	2/2[c]	2/2	2/2	0/6
Phage	2/2	2/2	1/2	0/6

[a]Fruit sample (2 Kg) in sterile distilled water of 2 L was washed and divided into two parts, which were used for ELISA and phage tests.
[b]A diseased fruit had one canker spot below than 2 mm in diameter.
[c]Number of positive reaction/test number.

Table 2. Detection of *Xanthomonas axonopodis* pv. *citri* on Satsuma mandarin orange using phage technique and citrus canker development one month later in fields

Date	Plot	Treatment	pfu/plate		%[a]	Disease development[b]
			Control	Test		
Late Aug	I	1	138	183	132	+
		2	153	305	199	
	II	1	137	164	120	+
		2	153	238	156	
	III	1	185	202	109	−
		2	179	200	111	
	IV	1	178	198	111	−
		2	170	193	114	
	V[c]	1	175	UC[d]	NT	+
Late Sept	VI	1	169	174	102	−
	VII	1	167	171	102	−
	VIII	1	193	192	99	−
	IX[c]	1	180	UC[d]	NT	+

[a](plaque number on test plate/plaque number on control plate)×100.
[b]Observed one month later after phage tests.
[c]Artificial inoculation (1×10^5 colony forming unit/mL).
[d]Uncountable.

pathogens was as effective as ELISA method. Furthermore, detection of bacterial pathogens using this technique is more effective than ELISA in that the phage technique detects only living bacterial cells and is inexpensive.

In field assays, it was possible to detect the pathogenic bacterial pathogens and to predict disease development in the citrus plots using phage technique. Phage population in the washing materials from the fruits of the plots that citrus cankers were developed one month later increased to 120% (Table 2). This result indicates that disease occurrence can be forecasted based on population change of bacterial pathogens on fruits, supporting the previous study (Myung et al., 2003). The disease did not develop when a ratio of control to test was below 99 to 114%, indicating that there might be experimental error in the course of the test. Thus, for make up for this error, we adced procedures for the comparison II and the comparison III in Fig. 1. Experimental error was determined by comparing the plaque number of 'Control-Control' with that of 'Control-Test' (comparison II) or 'Test-Control' with 'Test-Test' (comparison III). In comparison II and comparison III, when the numbers of 'Control-Control' and 'Test-Control' were more/less than that of 'Control-Test' and 'Test-Control', respectively, we regarded the test as the error, and the test was tried again.

Since 1993, this procedure have been used for detection of *X. axonopodis* pv. *citri* on Satsuma mandarin fruits for exporting to the United States of America at the Jeju branch of National Plant Quarantine Service.

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