Studies on the Detection of *Xanthomonas citri* by Phage Technique and the Surface Sterilization of Unshu Orange for Export to the United States

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In July, 1967, the United States revised Foreign Quarantine No. 28 and lifted her ban on the importation of Japanese Unshu organe (*Citrus reticulata* var. *Unshu*) with the provision of manifold safeguards. One of the safeguards is the surface sterilization of fruits before packing and another is the bacteriophage test of each shipment just prior to export in order to ensure freedom from citrus canker bacteria (*Xanthomonas citri* (HASSE) DOWSON). Inclusion of these two safeguards necessitated Japanese Plant Protection Service to work out authorized standard procedures upon the basis of experimental data. The authors, under such circumstances, undertook experiments on certain prerequisite aspects of the phage technique to be applied to the detection of *Xanthomonas citri* from Unshu orange fruits and on the effect and use of a chemical selected for the surface sterilization. Results presented in this report provided one basis upon which the current working procedures have been implemented.

Bacteriphage can reproduce itself only in the presence of the living host bacteria. The host range of a phage is usually species specific but, more often than not, it is strain specific. If, therefore, a phage with a known host specificity is available, the presence of its host bacteria in a given sample can be detected by adding the phage and then assaying whether or not its multiplication occurs. Two *Xanthomonas citri* phages with a wide host range are known to exist in Japan. These two have been designated by the junior author as phages CP_1 and CP_2 (Wakimoto, 1967). A clear-cut differenciation exists in the host specifity of these two phages and practically all of the *Xanthomonas citri* isolates are found to be sensitive to either of them. It is reasonably surmised, therefore, that the combined use of these two phages should cover the detection of nearly all strains of citrus canker bacteria that may happen to contaminate the fruit of export Unshu orange. Consequently, the present studies dealt with the trend in multiplication, level of detection and stability of these two phages.

For the surface sterilization of orange fruits, sodium hypochlorite and sodium orthophenylphenate were selected. These were selected, not for their prospective

effect on *Xanthomonas citri*, but primarily because they are an only choice out of the list of FDA that can be approved for use to the treatment of fruits in the United States at the moment. Experiments were undertaken on the effect of the chemicals in relation to concentration and some properties. The possible injury to the treated fruits was also tested.

MATERIALS

Phages and bacterial isolates — Phages CP_1 and CP_2 and the corresponding senstive strains of *Xanthomonas citri* isolate N6101 and N6119 (Wakimoto, 1967) were used throughout the experiments. Phages were stored by freeze drying under vacuum and recovered as was needed in potato semi-synthetic liquid medium (PS medium). For the phage assay, bacterial cultures of 2 to 4 days old on potato semi-synthetic agar slant were prepared into suspension of ca. 10^8 cells/ml and 2 ml each of this suspension was used for one plate for the plaque formation. PSA medium (agar added PS medium) was used for plating while PS medium was employed for the multiplication of phages under shaking.

Surface disinfectants — Sodium hypochlorite solutions were prepared from Antiformin (commercial sodium hypochlorite formulation of 6–8% or 10% available chlorine). Sodium orthophenylphenate solutions were prescribed from Dowicide-A furnished from the Dow Chemicals. For the dilution of chemicals, distilled water was used throughout.

EXPERIMENTAL METHODS AND RESULTS

A. Detection of *Xanthomonas citri* by phage technique

1. Multiplication of phages CP_1 and CP_2.

Two loopfuls of *Xanthomonas citri* were suspended into 6 ml of PS medium. This suspension was equally divided into two centrifuge tubes. One of the tubes was heated to 95°C for 10 minutes to kill the bacteria. Equal amount of CP_1 or CP_2 phage suspension was added at each tube. After gentle stirring for 5 minutes, the samples were centrifuged at 10,000 rpm for 5 minutes and the phage content of the supernatant was assayed by the plaque count method. Remaining samples were immediately resuspended and incubated under shaking at 25°C. Multiplication of phages was assayed every hour upto 5 hours after the addition of phages, each time after the same centrifugation.

As is shown in Table 1, the increase of phage CP_1 was recognized after two hours shaking and then showed a sharp logarithmic rise whereas that of phage CP_2 became apparent after three hours and the speed of its increase lagged far behind. Phages tend to decrease during the first one hour with phage CP_1 and the first two hours with phage CP_2. This is evidently due to the advancement in the adsorption of phages to cells of host bacteria. Heat sterilization of the sample reduced the amount of adsorption to a marked extent. This may be regarded as an indication of the presence of a selective or irreversible adsorption between the phages and the living host bacteria.

Table 1 Multiplication of phages CP_1 and CP_2 on respective host bacteria.

Phage	Bacterial isolate	Time after addition of phages					
		5 min.	1 hr.	2	3	4	5
CP_1	*X. citri* N6101	131	86	466	3,130	26,100	500,000
	Heated control	210	200	195	—	—	179
CP_2	*X. citri* N6119	87	16	8	123	364	792
	Heated control	669	673	—	—	—	670

Figures in the table show average plaque number per 0.1ml of supernatant.

From the results obtained, at least 5 hours shaking should be required for any reliable detection of canker bacteria from a sample given for the phage test.

2. Detection level of phage technique.

a) Phage CP_1: One slant of *X. citri* N6101 was suspended into 15 ml of sterilzied distilled water. One loopful of this suspension was transferred to 10 ml of PS medium and a serial dilution was made from this suspension. Five ml of each dilution was assayed by phage technique and, of the remaining suspension, 1 ml each of three replicate platings were made for the colony count. Colonies of X. *citri* were counted after 5 days incubation at 25°C.

b) Phage CP_2: One loopful of *X. citri* N6119 was suspended into 5 ml of PS medium and a serial dilution was made from this suspension. Three ml of each dilution was assayed by the phage technique and 0.1 ml of the remaining suspension was plated in three replication for the colony count.

As is shown in Table 2, the presence of the host bacteria at the concentration of 5 cells/ml could be detected with phage CP_1 while a positive increase of phage CP_2 was obtained at the population of ca. 10^5 cells/ml of the host bacteria. A lower detection level with phage CP_2 may be primarily due to the slow rate of multiplication as was demonstrated in the preceding experiment.

Table 2 Detection of *X. citri* by phage technique compared with dilution colony count.

Phage-Host bacteria	Method	Dilution of bacterial suspension							
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	PS
CP_1-*X. citri* N6101	Phage technique*	302	77	28	24	−32	−58	−8	−26
	Colony count/ml	N	610	61	5	0	0	0	0
CP_2-*X. citri* N6119	Phage technique*	4×10^3	—	86	—	−29	—	−2	−7
	Colony count/ml	ca. 10^6	—	ca.10^5	—	3.5×10^4	—	2.4×10^3	—

* Figures in the table show plaque number in increase or decrease after 5 hours shaking at 25°C.

3. Stability of phages CPI and CP_2.

a) Equal amount of CP_1 phage suspension was diluted into 10 ml each of PS medium, Ca^{++} added Vitamin-free casein hydrolysate medium (CaVfCh medium)

and sterilized distilled water. After 72 hours standing at 5°C, phage titre was assayed by the plaque count method. Results are given in Table 3. Phage CP_1 is quite stable in PS medium, less so in CaVfCh medium and quickly inactivates in distilled water.

b) PS suspension of phages CP_1 and CP_2 was stored at 5°C and the phage count was made from time to time with 0.1ml each of three replicate plates. Results are shown in Table 4. Both phages can be stored in PS medium without a fatal decrease in titre for one month at least.

c) Sodium hypochlorite was incorporated into 10 ml of PS medium, CaVfCh medium and distilled water at the concentration from 10 to 10,000 ppm. Equal number of phage CP_1 was pipetted into these preparations and, after 5 hours, phage titre was assayed by the plaque count method. As is seen from Table 5, phage CP_1 undergoes rapid inactivation in distilled water in the presence of sodium hypochlorite. Effect of the chemical seems to be slower and less hazardous in PS or CaVfCh medium.

5. Propagation of phages CP_1 and CP_2 added in mixture to senstive bacteria.

One loopful each of *X. citri* N6101 and N6119 was mixed in 10ml of sterilized water. This suspension was divided into two centrifuge tubes and one of the tubes was heated to 75°C for 10 minutes to kill the bacteria. Phages CP_1

Table 3 Stability of phage CP_1 in relation to suspension medium.

Phage count	Suspension medium		
	PS	CaVfCh	DW
Plaques/0.1 ml	737	467	174

Figures show average plaque number from three replicate plates.

Table 4 Stability of phages CP_1 and CP_2 in PS liquid medium.

Phage	Days of storage at 5°C						
	0	5	10	15	20	25	30
CP_1	541	—	—	441	513	528	463
CP_2	486	410	451	—	351	—	372

Figures show average plaque number per 0.1ml from three replicate plates.

Table 5 Effect of sodium hypochlorite on the inactivation of phage CP_1.

Suspension medium	Sodium hypochlorite concentration (ppm)				
	0	10	100	1,000	10,000
PS	692	691	638	600	21
CaVfCh	690	689	648	573	0
Distilled water	693	545	367	0	0

Figures show average plaque number/0.1ml from three replicate plates.

Table 6 Result of a model procedure for the detection of *X. citri* by phage technique.

Phage	Sample	Phage count		Test-Check
		Check	Test	
CP_1	Live bacteria	100	N*	N
	Heated control	186	181	−5
CP_2	Live bacteria	364	553	+189
	Heated control	512	501	−11

*N:too numerous to count. Test-Check> 0 indicates the detection of living bacteria.

and CP_2 were added in mixture to each of the tubes and their increase was assayed by the procedure illustrated in Figure 1.

As is presented in Table 6, the presence of both *X. citri* isolates was demonstrated by the significant increase of the corresponding phages. Since no factor for the increase of phage population is involved in the heat treated sample, inclusion of such a control should provide one basis upon which the validity of the phage increase can be assessed.

B. Studies on surface sterilization.

1. Bactericidal effect of sodium hypochlorite and sodium orthophenylphenate.

Bacterial suspension of ca. 10^{7-8} cells/ml was prepared with sterilized water. 0.1 ml of this suspension was pipetted into each of the concentration series of sodium hypochlorite and sodium orthophenylphenate. After 5 to 10 minutes standing at 20°C, surviving bacteria were counted by the conventional dilution cultures of the treated suspensions.

Results are given in Table 7–8. Both chemicals are effective on *X. citri* but sodium hypochlorite is found to be far superior to sodium orthophenylphenate.

Table 7 Effect of sodium hypochlorite

Concentration (ppm chlorine)	No. of *X. citri* colonies after 5 min. treatment
1,600	0
800	0
160	0
1.6	0
Untreated control	1,576

Table 8 Effect of sodium orthophenylphenate

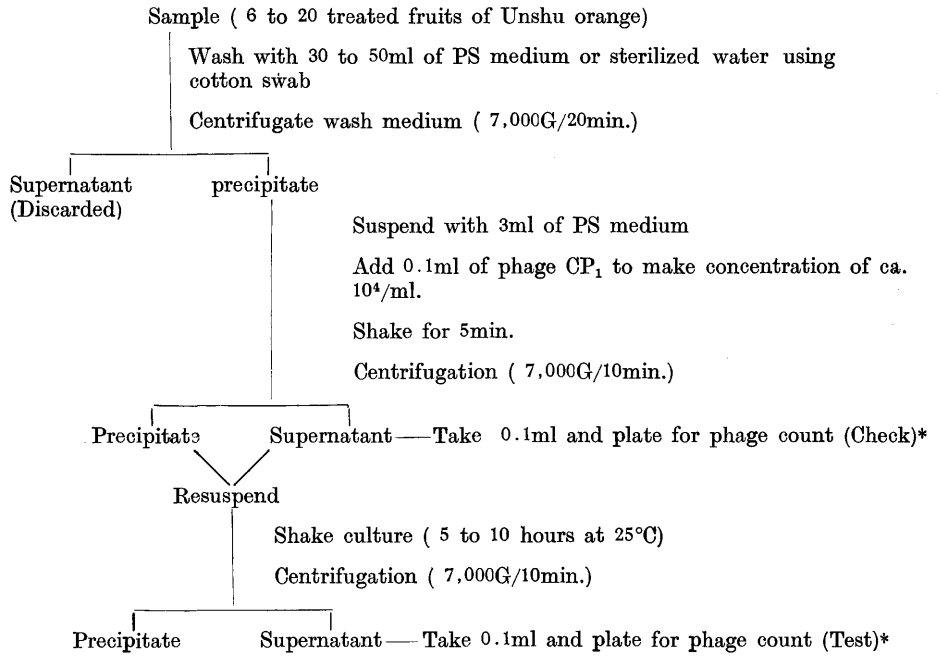
Concentration (ppm)	No. of *X. citri* colonies after treatment	
	5 min.	20 min.
1,000	0	0
800	0	0
600	0	0
400	0	0
200	101	294
100	906	1,030
Untreated control	1,476	1,848

2. Sterilization of contaminated fruits by sodium hypochlorite.

Fifty non-waxed Unshu orange fruits were uniformly sprayed with 50 ml of the bacterial suspension of *X. citri* N6101 which ranged from 10^5 to 10^8 cells/ml. Fruits thus inoculated were dried indoors for 1 or 2 days and then dipped for 2 minutes in 1 liter of sodium hypochlorite solutions. After dipping, fruits were gently rinsed with tap water, allowed to dry and assayed for the presence of viable bacteria by the phage technique described in Figure 1. This technique was developed by Katznelson and his coworker in 1951 and have been applied to the ecological studies of phytopathogenic bacteria (Katznelson and Sutton, 1951; Wakimoto, 1954).

Results are given in Table 9. Presence of some surviving bacteria was detected from the fruits inoculated with 10^8 cells/ml concentration. Little or no viable bacteria were detected from the fruits inoculated with the concentration range of 10^5 to 10^7 cells /ml. Preliminary experiments have shown that inoculated bacteria die quickly upon drying on the surface of fruits. Such tendency is also noticed in Table 9 between Experiment No. 2 and No. 3. Better yield of propagated phages

Figure 1 Detection by phage technique of *X. citri* from Unshu orange fruits after treatment with sodium hypochlorite.



* When the number of the plaques results in Test>Check, surviving bacteria are considered to have been present in the sample tested.

Table 9 Effect of sodium hypochlorite dipping on Unshu orange fruits inoculated with *X. citri* at various concentrations.

Experiment	Concentration of inoculum (cells/ml)	Concentration of sodium hxpochlorite (ppm chlorine)	Phage count			Result
			Check	$Test_1$	$Test_2$	
No. 1	10^8	600	—*	386	—	+
		400	—*	453	—	+
		200	—*	425	—	+
		100	—*	555	—	+
		Control	341	5,000	—	+++
No. 2	10^7	200	—*	156	153	—
		100	—*	155	163	±
		Control	153	170	1,498	+++
No. 3	10^7	200	—*	117	109	—
		100	—*	147	132	—
		Control	143	141	380	+
No. 4	10^6	100	289	287	—	—
		Control	263	5,000	—	##
No. 5	10^5	100	233	216	—	—
		Control	221	271	—	+

* As an equal amount of phages were added to each sample, phage counts for these checks are considered as nearly equal to the check of the corresponding control.
Fruits were treated one day after inoculation with the exceptions of Experiment No. 3 and No. 4 in which they were treated after two days and two hours, respectively.
$Test_1$: phage count after 5 hours, $Test_2$: phage count after 10 hours.

was seen in the former that was assayed one day earlier than the latter. Prominent phage yield from untreated control of Experiment No. 4 that was assayed two hours after inoculation can be cited as an additional indication of the poor viability of inoculated bacteria when compared with the controls of Experiment No. 2 and 3 in which a more concentrated inoculum was employed. In the light of these results, sodium hypochlorite dipping of two minutes at the concentration above 100 ppm chlorine should provide a satisfactorily effective method for the surface sterilization of Unshu oranges for export to the United States. The dipping time of 2 minutes seems to be essential because a trace of surviving bacteria was detected for an instant dipping even at the concentration of 500 ppm chlorine.

3. Injury of sodium hypochlorite dipping to Unshu orange fruits.

Ten fruits each of Unshu orange were dipped in sodium hypochlorite solutions of 250 to 2,000 ppm for 5 and 20 minutes, respectively. Five of the fruits were dried quickly by the dryer while the remaining five were immediately washed with tap water. Fruits thus treated were put into paper bags, kept at 20°C and examined every other day for the appearance of possible injury from the dipping treatment.

No sign of injury was discovered until after 20 days. Taste and flavor were not detectably affected. There seems to be little direct danger of sodium hypochlorite treatment in the range of satisfactorily effective concentration.

C. Some properties of sodium hypochlorite.

1. Relation between labelled chlorine content of Antiformin and sodium hypochlorite concentration.

A serial dilution was made of an Antiformin formulation on the basis of labelled chlorine content and the concentration of sodium hypochlorite in each dilution was measured by the sodium thiosulfate titration. As is shown in Figure 2, a perfect positive correlation was obtained between the available chlorine and the sodium hypochlorite concentration. Thus, the calculated concentration of chlorine can be used as a reliable index of the proportional concentration of sodium hypochlorite.

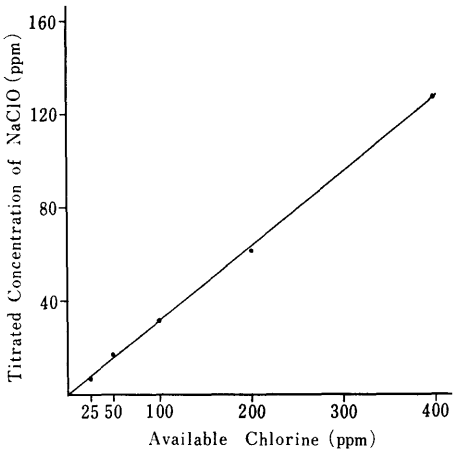


Figure 2. Relation between available chlorine and titrated sodium hypochlorite concentration

2. Effect of light and sitrring on the stability of sodium hypochlorite.

a) One liter each of 100 ppm chlorine solution was put in a beaker and subjected to the following conditions:

(1) Stand still indoors
(2) Continuous sitrring indoors
(3) Stand still outdoors (Cloudy weather)
(4) Exposure to ultraviolet light (15 cm away from 13 W light source)

(5) Stand still indoors in enamelled container

Concentration of sodium hypochlorite was analysed after 6 and 24 hours by the sodium thiosulfate titration.

b) Ten liters each of 100 ppm chlorine solution was placed in enamelled containers and exposed to the following conditions:

(1) Dark room

(2) Indoors

(3) Outdoors (Fine weather)

Sodium hypochlorite concentration was analysed at a regular interval until after 4 days.

Results of Experiment a and b are shown in Figures 3–4. Whereas the decomposition of sodium hypochlorite is accelerated by stirring the solution, it is by far the most susceptible to the effect of ultraviolet light. The prepared solution can stand storage for a few days without a fatal decrease in concentration. It is preferable, however, to use newly prepared solution and, in no case, the dipping treatment should be done outdoors under exposure to sunlight.

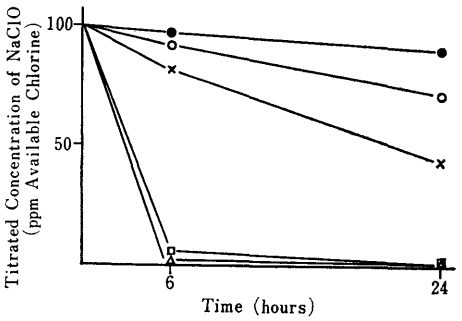


Figure 3. Effect of light and stirring on the decomposition of sodium hypochlorite (1 liter)
○ Stand still indoors
× Continuous stirring indoors
□ Stand still outdoors (Cloudy weather)
△ Exposure to ultraviolet light
● Stand still indoors in enamelled container

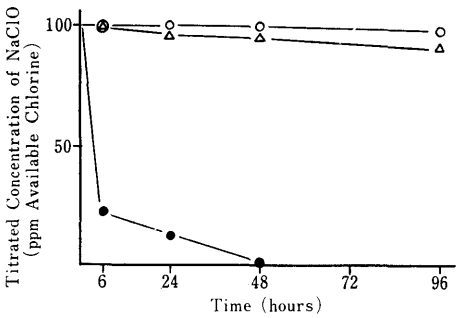


Figure 4. Effect of light on the decomposition of sodium hypochlorite (10 liters)
○ Dark room
△ Indoors in open enamelled container
● Outdoors in open enamelled container

3. Effect of fruit dipping on the concentration of sodium hypochlorite.

Five kilograms of Unshu orange fruits were repeatedly dipped for five minutes in Antiformin solution at 20°C and the possible decomposition of sodium hypochlorite was studied in relation to the accumulative amount of fruits treated. Five liters of 100 ppm chlorine solution was used in Experiment a, 10 liters of 100 ppm chlorine solution in Experiment b and 10 liters of 200 ppm solution in Experiment c. Results of these test are presented in Tables 10–11. Repeated dipping of fruits markedly reduced the sodium hypochlorite concentration. The following relations were obtained between the amount of dipped fruits and the concentration of sodium hypochlorite:

Y*=100 − 0.85X** (5 liters of 100 ppm solution)
Y =100 − 0.66X (10 liters of 100 ppm solution)
Y =200 − 0.60X (100 liters of 200 ppm solution)
*Y — Chlorine concentration (ppm), **X — Total amount of dipped fruits (kg)

It is evident from these results that a disadvantage of this chemical lies in the difficulty in maintaining a constant effective concentration. However, since this chemical does not induce fruit injuries upto more than 500 ppm, such difficulty should be overcome by using 200ppm solution and by supplementing calculated dosages at intervals to ensure the required minimum concentration level of 100 ppm.

Table 11 Effect of fruit dipping on the decomposition of sodium hypochlorite (10 liters)

Amount of f.uits treated (kg)	0	15	30	45	60	75
Titrated concentration of sodium hypochlorite (ppm chlorine)	100	92.7	78.0	70.7	65.9	—
	200	185	178	166	162	158

Table 10 Effect of fruit dipping on the decomposition of sodium hypochlorite (5 liters)

Amount of fruits treated (kg)	0	10	20	30	40	50
Titrated concentration of sodium hypochlorite (ppm chlorine)	100	94.2	83.7	76.7	67.4	58.1
Control	100	—	—	—	—	96.5

4. A handy estimation of Antiformin concentration.

Approximate concentration of any given sodium hypochlorite solution can be readily checked by a modified application of starch-iodine reaction. The procedure is given as follows:

(1) Take 5 ml of sample solution.
(2) Add 2 ml of 36% acetic acid.
(3) Dissolve 0.2 g of potassium iodide.
(4) Dip one end of a strip of starch paper and compare the intensity of the resulting starch-iodine coloration with that of a standard solution.

In performing this test, care should be taken to try the starch-iodine coloration immediately after dissolving potassium iodide. For the reliable comparison, it is also important to read the color within a few minutes after the onset of coloration. A relatively fine color gradation was obtained between the range of 10 to 100 ppm chlorine concentration. The concentration higher than 100 ppm can also be checked by adequately diluting the sample solution. This method can be favorably combined with the dipping of export Unshu organe to determine the time of supplying additional dosage or of the renewal of the dipping solution.

DISCUSSION

For the reliable dtection of *X. citri* from export Unshu orange fruits, an ideal phage preparation to be used for the test should lyse all the strains of *X. citri* that are present in Japan. Wakimoto (1967) has reported the presence of two representative phages CP_1 and CP_2. Out of 41 *X. citri* isolates collected from citrus areas throughout Japan, 20 belonged to CP_1 sensitive group, 19 were of CP_2 sensitive group while only

one was resistant to both phages. This provides a reasonable basis for the supposition that the combined use of phages CP_1 and CP_2 should cover the detection of nearly all the strains of *X. citri* in Japan. Occurrence in any higher rate of immune strains, however, will no dobut become a serious flaw to the application of phage technique. Therefore, a more thorough investigation on the distribution of phage strains of *X. citri* is now under way in which additional evidence is being accumulated in favor of the use of these two phages.

Both CP_1 and CP_2 phages showed a significant propagation in the presence of respective host bacteria. Propagation of CP_1 phage became apparent from 1 to 2 hours after addition of phages while that of phage CP_2 was recognized after 2 to 3 hours. In performing the phage test, therefore, zero time phage count (Check) must be done within one hour at the latest and, in view of the slow increase of phage CP_2, at least 5 hours shaking should be given before the second count (Test) is undertaken. Although a wide disparity exists between the detection levels with CP_1 and CP_2, this will not invalidate the phage technique because the host bacteria can be collected from any large quantity of sampled fruits by centrifugation.

Both phages were stable in PS medium and can stand cold storage without a fatal decrease in titre for one month. Inactivation by sodium hypochlorite is not hazardous in the range of 10 to 100 ppm within 5 hours after the addition of phages. Residual effect of this chemical on the phage inactivation can be precluded by one or two centrifugal washings of precipitated bacteria before the addition of phages.

For the surface disinfection of Unshu orange, sodium hypochlorite dip for 2 minutes was found to be satisfactorily effective at the concentration of 100 ppm chlorine. One disadvantage in the use of this chemical is a fairly rapid decomposition that accompanies fruit dipping. Unshu orange fruit, on the other hand, is not affected by the treatment upto more than 500 ppm concentration. Upon the basis of the present studies, it is recommended to use 200 ppm chlorine solution and to supplement calculated dosages at regular intervals in order to maintain the minimum effective concentration of 100 ppm. In view of the possible accumulation of dirt or organic impurities that are known to hasten the decomposition, fairly frequent renewal of the dipping solution would also be required. A modified starch-iodine test here described can be favorably combined with the dipping treatment for the determination of the time of supplementing additional dosages or of the time of the renewal of the whole solution.

SUMMARY

For the establihsment of a standard phage test procedure for the detection of *X. citri* from Unshu orange fruits to be exported to the United States, studies were made on some basic aspects that are prerequisite to the application of phage technique. Surface sterilization of Unshu orange fruits with sodium hypochlorite was also evaluated with special reference to its effect on *X. citri*, possible chemical injuries and some properties.

(1) Phages CP_1 and CP_2 showed a significant increase in the presence of respective host bacteria. Increase of phage CP_1 was recognized from 1 to 2 hours after addition of phages and followed a sharp logarithmic rise whereas that of phage CP_2 became apparent after three hours and the speed of increase lagged far behind that of phage CP_1. From this result, at least 5 hours shaking at 25°C should be required for the detection of *X. citri* from a sample given for the test.

(2) Presence of the host bacteria at the concentration of 5 cells/ml was detected with phage CP_1 while a positive increase of phage CP_2 was observed at the population of ca. 10^5 cells/ml.

(3) Both phages inactivated rapidly in distilled water. In PS medium, however, they could be stored at 5°C without a marked decrease in titre for one month.

(4) For the surface sterilization of Unshu orange, sodium hypochlorite dip of 2 minutes was satisfactorily effective at the concentration 100 ppm chlorine. By this treatment, some viable bacteria were detected from fruits sprayed with *X. citri* of 10^8 cells/ml but none from the fruits inoculated with less than 10^7 cells/ml concentration.

(5) The use of sodium hypochlorite in open outdoor condition must be avoided because of the hazardous decomposition that occurs under exposure to sunlight.

(6) Unshu orange fruits were not detectably injured by sodium hypochlorite dipping of upto more than 500 ppm chlorine.

(7) A notable decomposition of sodium hypochlorite also resulted from fruit dipping. Upon the relationship between sodium hypochlorite concentration and amount of fruits treated, the use of 200 ppm chlorine with additional dosing at intervals were recommended in order to ensure the effective concentration.

(8) A modified starch-iodine test can be favorably combined with the fruit treatment process for the determination of the time of supplementing additional dosages or of the time of the renewal of the whole solution.

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摘　　要

対米輸出ウンシュウミカンのファージ検定法ならびに表面殺菌法の検討

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カンキツ潰瘍病菌を対象とした対米輸出ウンシュウミカンのファージ検定について2～3の基礎的検討をおこなうとともに，主として次亜塩素酸ソーダによる果実の表面殺菌効果ならびにその使用方法を検討した。

(1) カンキツ潰瘍病菌のファージ CP_1 および CP_2 はそれぞれ寄主細菌の存在下に有意な増殖を示す。CP_1 ファージの増殖は添加後1～2時間内に認められ，対数的増加を示すが，CP_2 ファージの増殖は2～3時間にかけて明らかになり，その後の増加傾向も緩慢である。潰瘍病菌のファージ検定に当っては少なくとも5時間 (25°C) の振盪が必要と考えられる。

(2) 潰瘍病菌の純粋懸濁液を用い，振盪5時間の範囲で，ファージ法と希釈培養法による菌の検出を比較した結果，CP_1 ファージは細菌数 5個/ml でも有意な増加を示したのに対し，CP_2 ファージは 3.5×10^4 個/ml 以下では増加を認めなかった。

(3) 両ファージは PS 培地で冷蔵すれば1カ月間はほぼ安定に保存することができる。

(4) 潰瘍病菌を噴霧接種したウンシュウミカンに対し，次亜塩素酸ソーダ 100ppm (有効塩素)，2分間の浸漬は非常に有効であった。接種1日後の浸漬処理において，接種に用いた細菌濃度が 10^8 個/ml の場合には若干の生存菌が検出されたが，10^7 個/ml 以下の接種濃度では殺菌効果は完全であった。

(5) 次亜塩素酸ソーダは光線とくに紫外線によって著しく分解されるので，屋外での作業はさける必要がある。

(6) ウンシュウミカンは次亜塩素酸ソーダの 2,000 ppm 以下，5分間の浸漬では薬害を生じなかった。

(7) 次亜塩素酸ソーダは果実浸漬により分解が促進される。有効濃度 100ppm 以上を保持するには 200ppm 液を使用し，果実の浸漬量に応じ薬剤を補強するとともに，かなりひんぱんに薬液を更新する必要があると考えられる。

(8) チオ硫酸ソーダ法の原理を定性的に応用したヨードでんぷん比色法により，次亜塩素酸ソーダのおよその濃度を推定することができる。この方法は薬液の補強または更新の時期を決定するさいに補助的に利用すると有効であろう。