A New Immunoassay for *Xanthomonas campestris* pv. *citri* and Its Application for Evaluation of Resistance in Citrus Plants

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ABSTRACT

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A selected antibody enzyme immunoassay (SAEIA) for detection of *Xanthomonas campestris* pv. *citri* (cause of citrus bacterial canker) was developed, in which a rabbit antiserum specific for *X. c. citri* strain QN8201, immobilized cell fragments of *X. c. citri* strain QN7501, and β-D-galactosidase–labeled goat anti-rabbit immunoglobulin G were used. The SAEIA was specific for strains of *X. c. citri* and showed very little cross-reaction with single strains of *X. c.* pv. *pruni* and *X. c.* pv. *oryzae*, or with several strains of other microorganisms. The detection minimum of the SAEIA was 50 cells per assay for five strains of *X. c. citri*. SAEIA was used for quantitative comparison of the resistance of 10 kinds of citrus species. Populations of QN7501 in citrus leaves were measured at 1, 4, and 7 days after pinprick inoculation. Leaf homogenate samples did not significantly interfere with this analysis. With resistant citrus species, populations in leaves at 7 days after inoculation were two to eight times lower than those in susceptible citrus.

Citrus bacterial canker (CBC), caused by *Xanthomonas campestris* pv. *citri* (Hasse) Dye, is a potentially serious disease of most major citrus cultivars worldwide (3,18,31,32). In Japan, the disease is widespread, and most native citrus species are resistant to the pathogen. Recently, at the Fruit Tree Research Station in Kuchinotsu, Japan, efforts have been made to produce new citrus cultivars of economic value that are resistant to CBC.

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These efforts have been hampered by the time period required for the detection of resistance (17,19,23). With field observations, more than 100 days were required between inoculation and determination of resistance level, and the test could be conducted only once a year.

Rapid and reliable detection of pathogenic microbes is of great concern in clinical medicine, immunology, and microbiology. The application of serological methods for the detection and identification has been studied extensively (7,8,20,30). However, most have concluded that serological methods are more suitable for classification than for identification (9,25,26). Because of the high specificity of serological reactions, it has been difficult so far to develop an immunoassay method that is specific for most or all strains of a single bacterium and yet will not cross-react with related species.

We have been studying new techniques, selected antibody enzyme immunoassay (SAEIA) methods, which have been specific for all tested strains of seven different microbial species (13–16; *unpublished*). We previously introduced the idea of reducing the binding activity of the tracer (enzyme) to antibody in a competitive immunoassay. The use of a small amount of the low, cross-reacting antibiotic tobramycin instead of kanamycin as the enzyme-labeled tracer allowed us to develop a new method for the general detection of five kinds of kanamycin group antibiotics (11,12). With the new method, assay sensitivities of kanamycin and its analogs were increased between 8.5 and 191,000 times that measured by the usual method of using the enzyme-labeled kanamycin as the tracer (12). The new quantitative working ranges of all kanamycin group antibiotics were similar. The success of the general detection method prompted us to study a general detection method for microbial species, which we have done since 1980. After extensive trials, the idea of reducing the binding activity of the tracer to antibody in an immunoassay has been further developed so as to be applicable to the identification and the quantitative measurement of several microbial species. Here we report on the application of the SAEIA for detection of *X. c. citri* strains and for rapid evaluation of the resistance of various citrus species.

MATERIALS AND METHODS

Chemicals and antibodies. β-D-Galactosidase (GAL)-labeled goat antibody

specific for rabbit immunoglobulin G (IgG) was prepared according to a previous method (34). Other chemicals were of reagent grade.

Bacteria. Five strains of *X. c. citri* were isolated from sweet orange (*Citrus sinensis* (L.) Osbeck) at the Kuchinotsu Fruit Tree Research Station (Table 1). *X. c. citri* was maintained on Wakimoto's semisynthetic medium (17). Strains of other organisms listed in Table 1 were stock cultures in our laboratory, and each of them was cultured in heart infusion broth (Difco) before use.

Preparation of bacterial cell fragments. Saline suspensions of *X. c. citri* were disrupted by a sonifier (Branson Sonic Power, model 185E, Danbury, CT) at 60 W for 3 min in an ice-water bath. Strain QN8201 cell fragments (1 mg/ml) were used for the immunogen, and strain QN7501 cell fragments (50 μg/ml) were used to prepare the solid-phase antigen.

Immunization. Two white rabbits were given subcutaneous and intramuscular injections of 1 mg of QN8201 cell fragments (161,000 cfu) suspended in 1 ml of saline plus an equal volume of Freund's incomplete adjuvant. Four booster injections were given at biweekly intervals at one half the dose used for the first injection. The rabbit was bled from the ear vein 2 wk after the final injection, and the antiserum specific for QN8201 (anti-XC) was prepared by the method cited elsewhere (10) and stored at −30 C.

Quantification of bacterial populations. The number of colony-forming units of *X. c. citri* QN7501 in the logarithmic growth phase in a heart infusion broth was determined by dilution plating on Wakimoto's semisynthetic medium. An aliquot containing a known number of cells was removed from the broth, lyophilized, and weighed with a dry weight equivalence of 161 cfu/ng.

Buffers used. Buffer A consisted of 10 mM Tris-HCl buffer, pH 8.5, 10 mM NaCl, and 10 mM NaN_3; buffer B, 60 mM sodium phosphate buffer, pH 7.4, 10 mM ethylenediaminetetraacetate (EDTA), 1% (w/v) bovine serum albumin,and 0.1% NaN_3; buffer C, 20 mM sodium phosphate buffer, pH 7.0, 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, and 0.1% NaN_3.

SAEIA method. To coat wells of a microtiter plate (Immunoplates II, Nunc, Roskilde, Denmark), we flooded the plates with 200 μl of a sonicated suspension of strain QN7501 in buffer A (50 μg/ml). After 1 hr at 25 C, the suspension was removed from the plates, and 300 μl of buffer B was added to them; the plates were then held at 25 C for 1 hr to prevent nonspecific absorption. The bacterial cell fragment–coated wells were filled with 100 μl of cell fragments of QN7501, a homogenate of inoculated citrus leaves, or phosphate-buffered saline as a control. Immediately 100 μl of anti-XC diluted 10,000-fold in buffer B was added to each well. The plates were incubated at 25 C overnight and once again rinsed with buffer B. Next, 200 μl of GAL-labeled goat anti-rabbit IgG antibody was added to each well, and the plates were incubated at 25 C for 3 hr. The wells were then washed three times with 200 μl of buffer C. The strength of the GAL-labeled goat anti-rabbit IgG activity, 1.2 units per liter of GAL activity, was calculated from 1 unit of GAL activity defined as the amount that hydrolyzed 1 μmol of substrate per minute.

The enzyme conjugate bound to each well was then reacted with 0.2 ml of 0.1% *o*-nitrophenyl-β-D-galactoside in buffer C at 25 C for 3–8 hr to produce a color change. The enzymatic activity was stopped by the addition of 25 μl of 1 M glycine-NaOH buffer, pH 10.6. The color intensity in each well was measured with an enzyme-linked immunosorbent assay analyzer set at 414 nm (SLT Lab Instruments, Salzburg, Austria).

Cross-reaction value. The relative amount of a microbial strain required to reduce the initial binding of antibodies in anti-XC to solid-phase QN7501 by one half was calculated from the dose response curves determined by the SAEIA procedure, in which the mass of the strain QN7501 was arbitrarily taken as 100% (1).

Host plants. Citrus cultivars included Yuzu (*C. junos* Sieb. ex Tan.), Hassaku (*C. hassaku* Hort. ex Tan.), Hyuganatsu (*C. tamurana* Hort. ex Tan.), Ponkan (*C. reticulata* Blanco), Satsuma mandarin (*C. unshiu* Markovich), Kawano natsudaidai (*C. natsudaidai* Hay.), Valencia orange (*C. sinensis*), Clementine mandarin (*C. reticulata*), Fukuhara orange (*C. sinsensi*), and Clement (*C. paradisi* Macfady. × *C. tangerina* Hort. ex Tan.) Each leaf was at least 10 mo old when tested.

Inoculation. For inoculations, 25 needles, 0.5 mm in diameter, were arranged in a 5 × 5 array 2 mm apart in a rubber stopper so that the points protruded by about 3 cm. The needles were dipped in a bacterial suspension (2×10^9 cfu/ml) and then pressed to the adaxial side of the leaf. Each leaf was inoculated in this manner on both sides of the main vein in three places. The needles were touched lightly to the leaf

Table 1. Cross-reaction values of seven strains of *Xanthomonas campestris* and other microorganisms determined by the selected antibody enzyme immunoassay (SAEIA) with the working range for each *X. c.* pv. *citri* strain

Strain of *X. campestris*	Cross-reactivity[a] (%)	Working range (cfu/assay)
X. c. pv. *citri*		
QN7501	100.0	50–16,000
QN8201	101.0	50–16,000
QN8202	320.0	50–16,000
QN8206	500.0	16–16,000
QN8208	700.0	16–16,000
X. c. pruni		
M19	0.001	
X. c. oryzae		
7705	0.001	

[a] The following organisms gave values of <0.001 in the SAEIA: *Escherichia coli* K-12, *E. coli* ATCC 25922, *Pyricularia oryzae* 033 TH 68-126, *P. oryzae* 047 F 67-57, *Fusarium oxysporum* f. sp. *cucumerinum* F2005, *F. roseum* F3301, *Streptomyces scabies* Obama, *S. scabies* Aino, *Staphylococcus aureus* ATCC 6538P, *S. epidermidis* ATCC 155, *Vibrio cholerae* El Tor 85P4, *V. cholerae* El Tor 85P6, *Streptococcus mutans* HS-6, *S. mutans* JC 2.

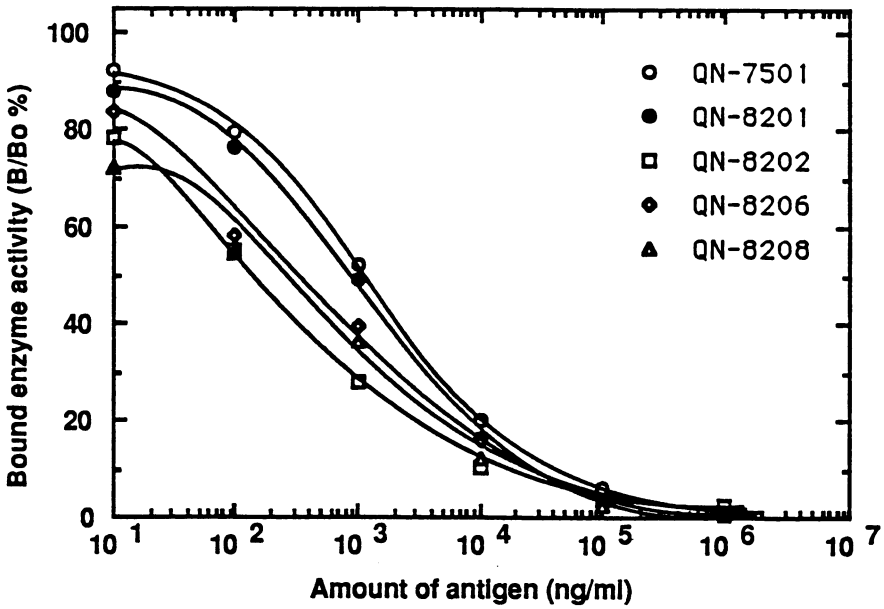

Fig. 1. Typical dose response curves of selected antibody enzyme immunoassay for five strains of *Xanthomonas campestris* pv. *citri*: QN7501, QN8201, QN8202, QN8206, and QN8208. The curves show the amount of bound enzyme activities for various log doses of the competing *X. c. citri* (B) as a percent of bound enzyme without competing antigen (B_0).

surface, so that only superficial wounds were created. Each leaf was then placed adaxial side up on a sterile, wet filter paper in a petri plate and stored at 28 C for the required period.

Sampling. A circle punch (10 mm in diameter) was used to remove sections of the inoculated wounded site at 1, 4, and 7 days after inoculation. Each leaf disk was added to 1 m of buffer B in a glass tube and then homogenized for 2 min. The homogenate in the tube was further treated by sonication at 60 W for 3 min in an ice bath. The resulting suspension was assessed by the SAEIA.

RESULTS

SAEIA. Similar quantitative working ranges were observed for the standard dose response curves in the SAEIA for five strains of *X. c. citri*, although they showed minor differences in their sensitivities (Fig. 1). The limits on detection of bacteria were between 3 ng (483 cfu) and 10 μg (1.61 million cfu) per milliliter. The detection minimum was 0.3 ng (48 cfu), since 0.1 ml was used for an assay sample, and 1 ng of the dry cells of QN7501 was found to contain 161 cfu. For practical purposes, the working range was arbitrarily set between 3 and 300 ng/ml, based on the precision data for the SAEIA (Table 2). The intraassay (five assays performed at the same time) and interassay (five independent assays) coefficients of variation between concentrations of 3 and 300 ng of the bacterial cells at five different levels each were 2.3–12.9% and 8.2–18.1%, respectively (Fig. 1; Table 2).

Specificity. With the limited number of bacterial strains tested so far, the scope of the assay was limited to *X. c. citri* species, and very low sensitivities given by cross-reaction values were observed for other bacterial and fungal species (Table 1).

Interference by leaf homogenate. The figures of the dose response curves of strain QN7501 measured for sample either with or without 10 mg/ml of citrus leaf homogenate are similar, showing that contamination of the homogenate interfered little with the SAEIA (Fig. 2). Populations of QN7501 in citrus leaves were determined with use of less than 10 mg/ml of the leaf homogenate as assay samples.

The growth of *X. c. citri* in citrus leaves. Any particular symptom was not observed with the leaves inoculated with strain QN7501 during the 7-day incubation period. At 1 day after the inocu-

Table 2. Detection of *Xanthomonas campestris* pv. *citri* QN7501 by selected antibody enzyme immunoassay

	Bacterial concentration		
	Added (ng[a]/ml)	Estimated[b] (ng/ml)	CV (%)
Intraassay[c]	3	2.97 ± 0.09	3.0
	10	9.95 ± 0.32	3.2
	30	29.50 ± 0.67	2.3
	100	98.10 ± 3.70	3.8
	300	310.0 ± 40.5	12.9
Interassay[d]	3	2.95 ± 0.24	8.4
	10	9.92 ± 0.82	8.2
	30	29.50 ± 2.86	9.7
	100	100.50 ± 10.7	10.5
	300	312.6 ± 56.6	18.1

[a] One nanogram of lyophilized cells of QN7501 contains 161 colony-forming units.
[b] Values represent the mean ± SD of a total of five assays.
[c] Assays performed at the same time.
[d] Assays performed independently.

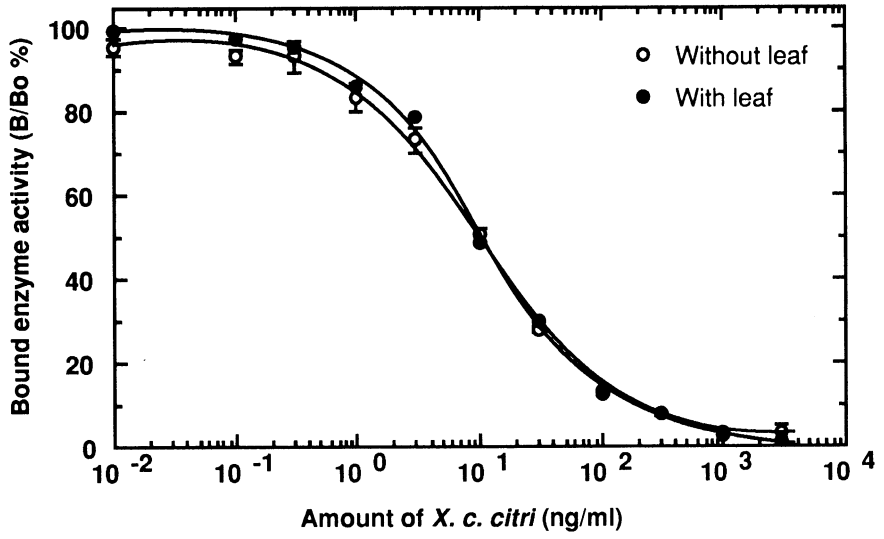

Fig. 2. Dose response curves of selected antibody enzyme immunoassay (SAEIA) for QN7501 either with or without the presence of 10 mg/ml aliquot of citrus leaf homogenate assessed by the SAEIA.

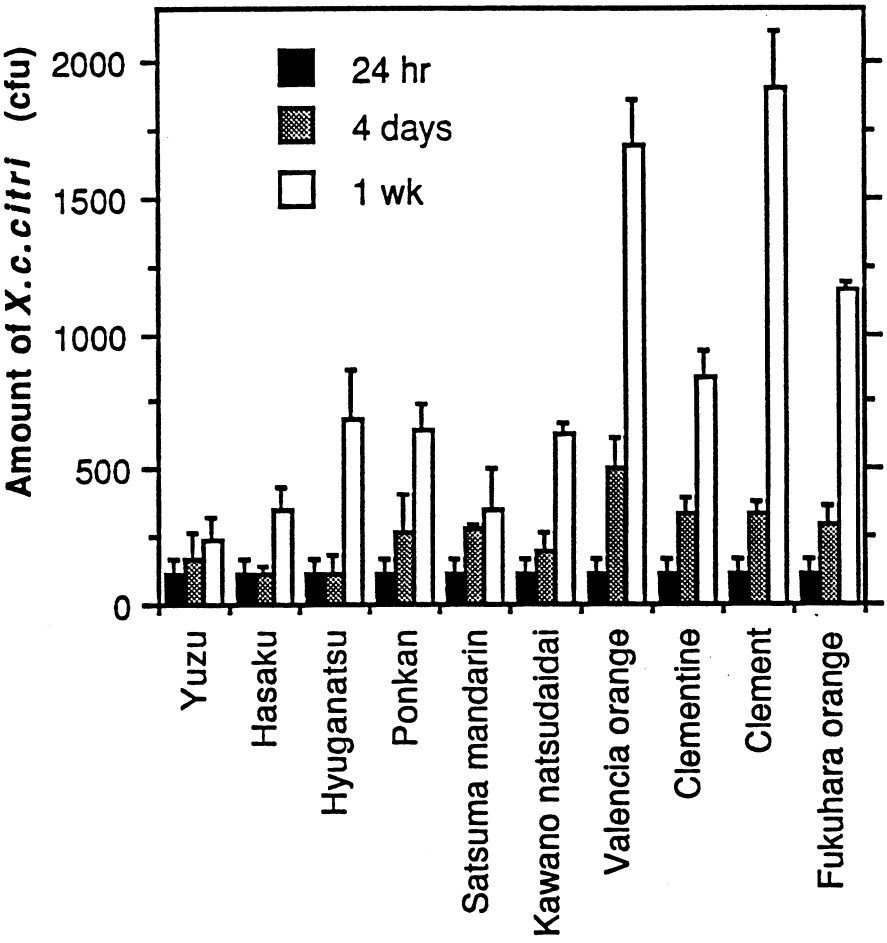

Fig. 3. Colony-forming units of *Xanthomonas campestris* pv. *citri* QN7501 in leaves of 10 kinds of citrus trees assessed by the selected antibody enzyme immunoassay method 24 hr, 4 days, and 1 wk after the pinprick inoculation of overnight culture broth of QN7501.

lation, all the assay values assessed by the SAEIA were similar, suggesting that the amount of bacteria applied was constant, that the technique was repeatable, and that very little growth of the pathogen had occurred. Four days postinoculation seemed sufficient time for the evaluation of the resistance of citrus plants to CBC. The data obtained for the samples 1 wk postinoculation made the evaluation easy, especially for citrus species highly resistant to CBC (Fig. 3).

DISCUSSION

An antiserum of a bacterium is highly specific to its immunogenic strain, and serological methods have been used for classification but not for identification of microbial species (9,25,26). *Escherichia coli* (Migula) Castellani and Chalmers, for example, can be divided into more than a million subclasses, because it has 164 O antigens, 103 K antigens, and 60 H antigens. Moreover, new ones are being added constantly (25,26). Immunoassay methods have shown rapid progress recently (2,35), and a number of bacterial immunoassays have been reported (4–6,21,22,24,27–29,36), including the one for *X. c. citri* with a quantitative working range of 10^5–10^6 cfu/ml (4). The scope of immunoassays has been limited by the immunological properties of antiserum used (2,35). Bacterial immunoassays developed by the classical assay principle, which detect the serological reaction of antiserum to antigen, have been specific to the immunogenic strain of antiserum used. Sensitivities and working ranges of the assay for different strains of a bacterium usually differ up to more than 100,000 times (4–6,15,21,22,24,27–29,36). This forbade the use of a microbial immunoassay for unknown specimens. Few immunoassays generally detect different strains of a bacterium with the same working range for each strain.

The SAEIA is based on the principle of competition between free and bound bacterial fragments for the antibodies contained in an aliquot of rabbit anti-XC serum. The competitive binding of rabbit antibodies to the solid-phase antigen was traced in the SAEIA. After aqueous reaction mixture was discarded, rabbit antibodies bound to the solid-phase antigen were reacted with GAL-labeled goat anti-rabbit IgG. The amount of the bound second antibody, which was parallel with that of rabbit antibodies, was assessed by enzyme activity of the bound GAL-label.

Antisera specific to QN8201 (anti-XC) was elicited in rabbits. A new enzyme immunoassay, which allowed the detection of five strains of *X. c. citri*, has been developed using anti-XC serum, QN7501 cell fragment–coated immunoplate as the solid-phase antigen, and GAL-labeled goat anti-rabbit IgG as an indicator.

There were three reasons for selecting the strain QN7501 as the solid-phase antigen. First, it possessed sufficient affinity to anti-XC. Second, an important component of SAEIA is the use of a different strain for solid-phase antigen than the one used for the immunogen. Only antibodies specific for common epitopes of *X. c. citri* in anti-XC serum were selected and used in the assays of strains QN7501 and QN8201 (13,33). If common epitopes may also be contained in every strain of *X. c. citri*, then the SAEIA would be the general detection method for the bacterium. Third, and most important, QN7501 adequately reduced the binding reactivity of the tracing reaction. The selected antibodies must be essentially specific to the epitopes contained in cells of the immunogenic strain QN8201, such that most of their reactions to the corresponding epitopes in cells of the solid-phase strain QN7501 should consist of cross-reactions with low bindings. In fact, all *X. c. citri* strains tested could compete with the reduced tracing reaction. The use of QN7501 kept the reduction in the reactivity of the tracing reaction slight enough so that distantly related bacterial species such as *X. c. pruni* and *X. c. oryzae* hardly competed with the tracing reaction. Selection of other strains such as QN8202, QN8206, or QN8208 as the solid-phase antigen was not studied, because the selection of QN7501 worked well, though extensive trials were needed to find the appropriate solid-phase strains with other microbes (*unpublished*).

The five strains of *X. c. citri* tested were sensitive within the same working range, 3 ng to 1 μg cells per milliliter (Table 1). In the SAEIA methods that we have studied to date, the working range for all strains of a microbial species have been similar (13–16; *unpublished*), as with the general immunoassay method of homologous antibiotics (12).

With the equation of 1 ng of dried culture of QN7501 equal to 161 cfu, we calculated that the detection limit was approximately 50 cfu per assay. The scope of the assay was limited to strains of *X. c. citri*. Single strains of *X. c. pruni* and *X. c. oryzae*, closely related but different pathovars, showed only cross-reaction values of 0.001%, which means their sensitivities by the SAEIA are 0.00001 of that of QN7501. Very low values of less than 0.001% were observed for other microbial species tested (Table 1). Thus, the SAEIA technology is applicable not only to parasite (14) and fungus (14,15) species but also to bacterial species, as with the SAEIA for *Vibrio cholerae* 01 species (16).

The SAEIA could also be used to compare pathogen populations in leaves of citrus trees as a test for resistance. Direct correlation between pathogen populations and histological changes in diseased tissues of citrus have been reported (18,19). The citrus leaf homogenate, up to 10 mg/ml, did not interfere with the SAEIA. Difference in pathogen populations associated with host resistance could be detected in homogenates of single 10-mm leaf disks within 4 days after pinprick inoculation. However, because population increases appeared correlated with level of resistance, tests conducted at 7 days after inoculation allowed for more precise comparisons among highly resistant citrus species, such as Yuzu, Hassaku, and Hyuganatsu (Fig. 3). In addition, handling of this test is simpler than that of dilution plate analysis of pathogen populations in wound sites. Only a single leaf of any type of citrus was needed for population determinations taken at three different times. The present results obtained for the comparative resistance of various citrus trees are well in accord with those obtained by Koizumi and Kuhara (19) and Matsumoto and Okuda (23), who measured the size of CBC lesions on leaves of natural citrus trees that had been inoculated with QN7501. In these tests, more than 100 days was required for them to obtain an accurate evaluation of the resistance of each citrus tree. Finally, the new SAEIA test can be performed at any time, whereas the original test was limited in practice to only once a year in Japan.

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