Efficacy of Bioindexing for Graft-Transmissible Citrus Pathogens in Mixed Infections

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ABSTRACT

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Biological indexing for graft-transmissible pathogens of citrus in the presence of additional pathogens was investigated. The probability for symptom expression, the efficacy of the bioindexing tests, and the number of citrus indicators required for pathogen detection were statistically evaluated. Multiple infections did not preclude symptom expression or reduce the diagnostic efficacy of the primary indexing hosts for *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), and Citrus tatter leaf virus (*Apple stem grooving virus*). Symptoms of Citrus vein enation virus (CVEV) and the diagnostic efficacy of Mexican lime were suppressed by the T30 group CTV isolates, but not by other CTV isolates tested. CPsV suppressed symptom expression and diagnostic efficiency of Dweet tangor and sweet orange for concave gum. The application of alternate bioassay hosts for indexing was also investigated. Dweet tangor, sweet orange, and *Citrus excelsa* are not typically used for bioindexing of CVEV, however, Dweet tangor and *C. excelsa* detected CVEV in single infections, whereas in sweet orange, CVEV was detected only when CPsV, concave gum, or citrus viroids were present. CTV was readily detected using the alternative indicator *C. excelsa*, whereas only shock reacting CPsV isolates were effectively indexed by Mexican lime.

Additional keywords: Clopper-Pearson confidence intervals

Accurate and reliable disease diagnosis is the basis for every successful program for the maintenance, importation, introduction, and commercial propagation of pathogen-free citrus budwood. Serological and/or molecular diagnostic techniques are available for some important virus and viroid pathogens causing citrus diseases such as tristeza (18), psorosis (2), and exocortis (19,24). However, detection of poorly characterized pathogens of citrus diseases such as cristicortis, impietratura, non-psorosis-associated ring spot, gummy bark, chlorotic dwarf, vein enation-woody gall, and concave gum is still based solely on biological indexing (11,20,25). Bioindexing coupled with laboratory-based diagnostic techniques offers the best available detection for viral citrus pathogens of uneven tree distribution and/or low titer (8,12,15,22).

Citrus budwood chosen for improvement by selection or importation of new varieties is often collected from field trees that harbor more than one graft-trans-

missible pathogen. In a budwood certification program, the detection of any pathogen automatically indicates the need to initiate disease containment and/or elimination procedures. The lack of detection of a component in a mixed infection is not critical as long as one pathogen is detected and proper follow-up testing is done. However, biological indexing used in surveys should reliably identify all pathogens present. If one pathogen masks the presence of another, the false negative result may lead to erroneous choice of susceptible citrus varieties and/or rootstocks as well as inappropriate procedures to establish disease-free material. It is also possible that the characterization of a pathogenic isolate as mild based on altered symptom expression because of mixed infections could lead to the field application of a potentially severe isolate as, for example, a cross protection agent.

Even though it has been recognized that symptom expression may be affected by interactions among different disease agents (3,14,16,23,29,30,32,33), the established protocols for biological indexing of graft-transmissible pathogens of citrus have been established without any experimental evaluation of the potential effects of mixed infections. The diagnostic accuracy of the citrus indicators traditionally has been evaluated using pure single inoculum sources or sources of poorly characterized pathogen content. Similarly, experimental

or statistical information dictating the recommended number of citrus indicators necessary for bioindexing has not been produced. Alternative indexing hosts have been recommended for the bioindexing of the known graft-transmissible citrus pathogens (20). However, there are no experimental studies evaluating the efficacy of the alternative indicators, especially in the presence of mixed infections.

The need for systematic study of the effect of mixed infections on the biological indexing of graft-transmissible pathogens of citrus has long been recognized, as in the early statement of Wallace (27) "...mixtures of tristeza and other viruses occur rather commonly, however, a thorough or complete experimental study has not been made of the effect of other viruses on tristeza symptoms." In the present study, effects on the diagnostic reactions of the bioindexing hosts, efficacy of the established biological indexing protocols, and theoretical limitations on these tests were evaluated in the presence of mixed infections. Ancillary data were also developed for the effective use of alternative indicators for the biological indexing of some graft-transmissible pathogens of citrus.

MATERIALS AND METHODS

Citrus pathogen properties and sources. Citrus is affected by a large number of virus, viruslike, and viroid pathogens, many of which are deleterious to tree health and fruit production (4,10,20,25). The graft-transmissible disease agents tested in this study (Table 1) are responsible for significant losses in yield and fruit quality as well as reduced tree vigor, while others have not been related to specific named citrus diseases of economic importance (10,21,26).

Inoculum sources for the citrus diseases were obtained from the disease collection of the Citrus Clonal Protection Program (CCPP) at the University of California, Riverside, and graft-inoculated to Pineapple sweet orange (*Citrus sinensis*) seedlings. Following 1 year of maintenance under standard greenhouse conditions, the sweet orange seedlings were held permanently in a screen house as reservoir hosts. The disease collection source trees were positive for single diseases as determined by repeated testing over the past 15 to 40 years. Since some of the CCPP disease isolates were collected prior to 1988, be-

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fore citrus viroids were characterized (9), the viroid status was uncertain. Tests for viroids were made by graft-inoculation of the viroid bioamplification host Etrog citron (*C. medica*) followed by total nucleic acid extraction and sequential polyacrylamide gel electrophoresis (PAGE) or slot blot hybridization (19,24). These tests confirmed that the nonviroid source trees of the disease collection were free of viroids.

Designations of the different pathogenic isolates correspond to the CCPP archives except for the VT group *Citrus tristeza virus* (CTV) isolate 65, which was provided by the California Central Eradication Agency (CCTEA). Information on the molecular characterization and classification of the CTV isolates into the T30 and VT groups was kindly provided by M. E. Hilf (personal communication).

Indexing hosts, inoculation procedures, and monitored symptoms. The citrus indicator plants used in this study were those routinely used for bioindexing in the CCPP (Table 1). Seedlings were grown in the University of California potting mix for citrus (17) with three seedlings per 4-liter plastic pot. In the case of Rusk citrange (*Poncirus trifoliata* × *C. sinensis*), buds were grafted on rough lemon (*C. jambhiri*) seedlings planted individually in the 4-liter pots. Test plants were maintained in the greenhouse at the temperature range of 20 to 24°C.

At the time of inoculation, indicator plants were cut back approximately 25 to 30 cm above the soil surface, leaving four to six buds above the inoculation site, allowing for development of young shoots. Plants were graft-inoculated by T-cut using bark patches (5,20). Two bark patches from each pathogen source were placed

into each indicator plant. The inoculations involved in a multiple infection were performed at the same time except for the experiments where the effect of a challenge inoculation was to be tested. When testing for the interactions of CTV and Citrus enation virus (CVEV) in Mexican lime (ML) (*C. aurantifolia*), the time between the budding of the first and the second pathogen in reciprocal tests was 15 days. Similarly, in tests of interactions of *Citrus psorosis virus* (CPsV) and concave gum in sweet orange (SwO) and Dweet tangor (DwT), the challenge inoculations were applied after symptom expression of the first pathogen. Immediately after inoculation with the challenge agent, the plant tissue displaying symptoms of the established pathogen was removed and symptoms of the double infection were monitored in the new growth. Clippers, budding knives, and razor blades used in the inoculation process were disinfested between treatments by dipping into 1% sodium hypochlorite solution.

Survival of the inoculum tissue graft after inoculation was considered evidence for transmission. Indicators were inspected weekly or at shorter intervals, depending on the progress of symptom development, for a period of 4 to 8 months. Symptoms did not diverge from what has been previously reported for the tested pathogens (5,11,20).

Statistical analysis. Data from 4 years of experimentation were combined for the statistical analysis. Preliminary experiments that involved a small number of indicators evaluated the effect of a broad range of different pathogen combinations. The significant biological interactions were further investigated using additional isolates and larger numbers of indicators. This experi-

mental approach produced differences in sample size among treatments.

The statistical analysis employed provided three distinct statistical values. The first, the *P* value, is based on experimental data and describes the probability for symptom expression in single or mixed infections in a specific indexing host. The second statistical value, $\Theta = 1 - (1 - P)^n$, includes the *P* value and *n*, the minimum number of inoculated indicators recommended for disease diagnosis (5,20); therefore, Θ evaluates the efficacy of the most common bioindexing practices. The third value, *n*, combines both *P* and Θ values for the estimation of the number of alternative indicators required for efficient disease diagnosis.

The collected experimental data are presented as the number of symptomatic indicators per total number of indicators inoculated. Inference for the *P* and Θ values was established by using confidence intervals at significance level 0.05 (7). Statistical calculations employed a spreadsheet algorithm developed in the UCR "Statistical Consulting Collaboratory" under the supervision of Daniel Jeske.

The confidence interval for *P* was defined by the Clopper-Pearson lower (*P_L*) and upper (*P_U*) boundaries (7). *P_L* is more significant on a practical level since it represents the lowest for the symptom expression probability. The sample size affects the span of the confidence interval for the *P* value, with smaller samples producing wider intervals and reduced *P_L*. Thus, in some instances, although all the inoculated indicators expressed symptoms, the estimated *P_L* values were low.

The confidence interval for the Θ value was defined by the lower boundary $\Theta_L = 1 - (1 - P_L)^n$ and the upper boundary $\Theta_U = 1$

Table 1. Graft-transmissible citrus pathogens used singly and in mixtures and citrus indexing hosts used for biological indexing

Disease agent	Genus	Isolate-variant ^a	Citrus indexing hosts	
			Common name	Scientific name
<i>Citrus tristeza virus</i> (CTV)	<i>Closterovirus</i>	T30 group: 514, 516 VT group: 519, 65	Mexican lime (ML)	<i>Citrus aurantifolia</i>
CTV-Seedling Yellows (CTV-SY)	<i>Closterovirus</i>	568 (severe) 554 (mild)	ML	
Citrus vein enation virus (CVEV)	— ^b	704	ML	
<i>Citrus psorosis virus</i> (CPsV)	<i>Ophiovirus</i>	205 (shock) 250 (lesion bark)	Dweet tangor (DwT) Pineapple sweet orange (SwO)	<i>C. reticulata</i> × <i>C. sinensis</i> <i>C. sinensis</i>
Concave gum (CG)	—	301	DwT SwO	
Citrus tatter leaf virus ^c (CTLV)	<i>Capillovirus</i>	100	Rusk citrange <i>Citrus excelsa</i> Citrange C-35	<i>Poncirus trifoliata</i> × <i>C. sinensis</i> <i>C. excelsa</i> <i>P. trifoliata</i> × <i>C. sinensis</i>
<i>Citrus exocortis viroid</i> (CEVd)	<i>Pospiviroid</i>	E-811	—	
<i>Citrus viroid I</i> (CVd-I)	<i>Apscaviroid</i>	Ia	—	
Citrus viroid II ^d (CVd-II)	<i>Hostuviroid</i>	IIb	—	
<i>Citrus viroid III</i> (CVd-III)	<i>Apscaviroid</i>	IIIb	—	

^a Isolate-variant numbers correspond to the Citrus Clonal Protection Program archives except CTV 65, which was provided by California Central Eradication Agency.

^b CVEV, probable *Luteovirus* (6).

^c Apple stem grooving virus.

^d Hop stunt viroid.

– $(1 - P_U)^n$. A bioindexing protocol that uses n indicators for the detection of a pathogen can be considered efficient if one of the n inoculated plants expresses symptoms. As in the case of the P value, the lower boundary Θ_L is more important, since it represents the lowest probability for a successful bioindexing protocol. Therefore, the Θ_L value was used as a percent measure for the efficacy of the bioindexing practices.

The value n for efficient disease diagnosis using alternative citrus hosts was estimated based on the hypothesis of at least one positively reacting indicator. The number of alternative indicators required for 99.0% disease diagnosis efficacy was determined by using the 95.0% confidence boundary for Θ_L and finding the smallest n such that $\Theta_L \geq 99.0\%$.

RESULTS

Efficacy of standard indexing hosts for biological indexing of citrus graft-transmissible pathogens. *CTV bioindexing by ML.* ML is the primary indexing host for both CTV and CVEV (Table 1) (5,20). The coinfection with CVEV and CPsV, concave gum (CG), Citrus tatter leaf virus (CTLV), or the mixture of the viroids *Citrus viroid-Ia* (CVd-Ia), CVd-IIb, CVd-IIIb, and *Citrus exocortis viroid* (CEVd) did not preclude symptom expression of either the T30 group CTV isolate 514 or the VT group CTV isolate 519 in the primary indicator ML (Table 2). In addition, the presence of CVEV in triple infections with CTLV and the citrus viroid mix did not affect CTV isolate 514 reactions in ML or symptom expression of the severe CTV-Seedling Yellows (CTV-SY) isolate 568 or

the mild CTV-SY isolate 554 (data not presented).

The lower probability (P_L) for symptom expression with CTV isolate 514 alone in ML of 76.8% was not reduced by coinfection with CVEV and CPsV. The reduction noted for CG, CTLV, and the viroid mix was due to the small sample size. A similar relationship was observed with the VT group CTV isolate 519, although there was a small reduction of the P_L value by the coinfection with CPsV (Table 2). The efficacy of CTV detection based on the recommended four ML is 95.6% or higher regardless of the coinfection treatment (Table 2).

CVEV bioindexing by ML. Mixed infections of CVEV with CTLV, the viroids CVd-Ia, CVd-IIb, CVd-IIIb, CEVd, or CG did not affect formation of enations, whereas coinfection with CPsV only slightly reduced the number of vein enation symptomatic indicators (Table 2). The efficacy of four ML for detection of CVEV is 95.6% or higher even in the presence of mixed infections.

The indicator ML is also the primary indexing host for CTV (Table 1) (5,20). The coinfection of CVEV with the severe (568) and the mild (554) CTV-Seedling Yellows isolates, as well as the VT group isolates CTV 519 and 65, did not preclude vein enation symptom expression, and the efficacy for detection of CVEV using four ML is at least 99.0% (Table 3).

However, in striking contrast, the symptom expression of CVEV was dramatically suppressed in the presence of the T30 group CTV isolates 514 and 516 (Table 3). The P_L value for vein enation symptom expression in the presence of these CTV isolates is significantly lower than the control (Table 3). The P_L value for CVEV expression was lower even when CVEV infection was established prior to CTV, and infection with CTV prior to CVEV inoculation had an effect similar to coinfection (Table 3). The efficacy for CVEV biological indexing using four ML was reduced by coinfection and superinfection treatments with the T30 group CTV isolates (Table 3). Isolate CTV 514 was also used in preliminary experiments that included triple infections of CVEV with combinations of CTV, CTLV, CPsV, and the citrus viroids. In every combination where CTV 514 was present, a significant reduction in CVEV symptom expression was recorded (data not presented).

CPsV bioindexing by SwO and DwT. Since the young leaf pattern symptoms for both the shock-reacting CPsV isolate 205 and the lesion bark isolate CPsV 250 were identical, data generated with both isolates were combined. The primary indexing hosts for CPsV, SwO and DwT, are also used for the detection of concave gum (Table 1) (5,20). The coinfection with concave gum did not preclude symptom expression of CPsV (Table 4), including

Table 2. Effects of additional pathogens in biological indexing of *Citrus tristeza virus* (CTV) and Citrus vein enation virus (CVEV) using the primary indicator Mexican lime (ML)

Treatment	Symptomatic/ inoculated	Lower probability (P_L) ^a for symptom expression	Efficacy of bioindexing using 4 ML (Θ_L %) ^b
CTV 514, T30 group	14/14	0.768	99.7
+ CVEV 704	70/70	0.949	100
+ CPsV 205/250 ^c	31/32	0.838	99.9
+ CG 301	9/9	0.664	98.7
+ CTLV 100	6/6	0.541	95.6
+ Citrus viroid mix	6/6	0.541	95.6
CTV 519, VT group	15/15	0.782	99.8
+ CVEV 704	43/43	0.918	100
+ CPsV 205/250	17/18	0.727	99.4
+ CG 301	9/9	0.664	98.7
CVEV 704	17/17	0.805	99.9
+ CTLV 100	6/6	0.541	95.6
+ Citrus viroid mix	6/6	0.541	95.6
+ CG 301	6/6	0.541	95.6
+ CPsV 205/250	23/26	0.698	99.2

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.976 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b Θ_L , lower probability boundary. Upper boundary was 100%.

^c CPsV, *Citrus psorosis virus*; CG, concave gum; CTLV, Citrus tatter leaf virus; citrus viroid mix, coinoculation with *Citrus exocortis viroid*, *Citrus viroid Ia* (CVd-Ia), CVd-IIb, and CVd-IIIb.

Table 3. Effects of different *Citrus tristeza virus* (CTV) isolates in biological indexing of Citrus vein enation virus (CVEV) using the primary indicator Mexican lime (ML)

Treatment	CVEV symp- tomatic/ inoculated	Lower probability (P_L) ^a for CVEV expression	Efficacy of CVEV bioindexing using 4 ML (Θ_L %) ^b
CVEV 704	17/17	0.805	99.9
CTV-Seedling yellows (CTV-SY)			
CVEV 704 + CTV-SY 568 (severe)	23/23	0.852	100
CVEV 704 + CTV-SY 554 (mild)	10/10	0.692	99.1
VT group			
CVEV 704 + CTV 519	42/43	0.877	100
CVEV 704 + CTV 65	52/52	0.932	100
T30 group			
CVEV 704 + CTV 514	11/70	0.081	28.7
CVEV 704, 15 days later + CTV 514	20/38	0.358	83.0
CTV 514, 15 days later + CVEV 704	8/44	0.082	29.0
CVEV 704 + CTV 516	7/46	0.063	23.1
CVEV 704, 15 days later + CTV 516	19/22	0.651	98.5
CTV 516, 15 days later + CVEV 704	0/14	0.000	0.00

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.264 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b Θ_L , Lower probability boundary. Upper boundary fluctuated between 70.6 and 100%.

both the shock reaction and the characteristic ring spot-type young leaf symptom, distinct from the concave gum induced oak-leaf pattern. The presence of CVEV, CTV, CTLV, and the citrus viroids only slightly reduced the number of symptomatic indicators in some cases (Table 4). Furthermore, detection of CPsV was not impaired by triple combination infections of CTV 514, CVEV, CTLV, and the citrus viroid mix (data not presented).

The probability for symptom expression for psorosis alone has a P_L value of 85.8% for SwO and 89.7% for DwT (Table 4). The coinfection treatments of CG and CVEV did not reduce these values significantly (Table 4). The efficacy of four SwO for detection of the CPsV is at least 95.5% regardless of the coinfection treatment, while that of four DwT is higher than 99.0% (Table 4).

CG bioindexing by SwO and DwT. The primary indicators for CG bioindexing are the same as those used for the bioindexing of CPsV (Table 1) (5,20). The presence of the shock-reacting CPsV isolate 205 significantly reduced the number of SwO and DwT expressing the oak-leaf pattern symptom of CG as well as the P_L value for CG symptom expression (Table 5). In addition, the presence of CPsV reduced the efficacy of CG diagnosis using four SwO or DwT (Table 5). The suppressive effect of CPsV over CG symptom expression was also evident with the superinfection of CG on established CPsV infection for both SwO and DwT (Table 5). The superinfection treatment of CPsV on the established CG infection has no negative effect on the CG symptom expression or diagnostic efficacy of SwO and DwT (Table 5).

The coinfection treatments of CTLV (data available only for DwT), CVEV, or the citrus viroids did not affect the symptom expression of CG in SwO or DwT, and CG can be efficiently diagnosed in mixed infections using four seedlings (Table 5). The coinfection with the T30 group (514), VT group (519), and the severe SY (568) isolate of CTV also did not affect symptom expression of CG in either indicator (Table 5). Despite the slight effect of CTV 519 on CG symptom expression in SwO, the efficacy for CG diagnosis with four inoculated indicators is high for both SwO and DwT, with the lowest probability (Θ_L) for at least one CG symptomatic plant greater than 99.0% (Table 5).

CTLV bioindexing by Rusk citrange. Due to the limitations caused by multiple inoculation grafts required and the propagation of the Rusk citrange indicator buds on rough lemon rootstocks, only a small number of plants were employed in the preliminary test. No effect was observed on the symptom expression of CTLV by coinfections with the T30 group CTV isolate 514, the shock-reacting CPsV isolate 205, CVEV, and the mixture of citrus viroids. Thus, no further investigation was pursued.

In addition to Rusk citrange, the CTLV indicators *Citrus excelsa* and Citrange-35 were also tested (Table 1). In replications during the 4 years of experimentation, the single inoculation with CTLV failed to induce symptoms, suggesting that these two seedling indicators were inadequate for diagnosis of

the tested CTLV isolate 100 under the conditions employed.

Efficacy of alternative indexing hosts for biological indexing of citrus graft-transmissible pathogens. The potential use of alternative citrus indicators to supplement the primary indexing hosts for the biological indexing of graft-transmissible

Table 4. Effects of additional pathogens in biological indexing of *Citrus psorosis virus* (CPsV) using the primary indicators sweet orange and Dweet tangor

Treatment	CPsV symptomatic/ inoculated	Lower probability (P_L) ^a for CPsV expression	Efficacy of CPsV bioindexing using 4 indicators (Θ_L %) ^b
Sweet Orange			
CPsV 205/250	24/24	0.858	100
+ CG 301 ^c	37/37	0.905	100
+ CVEV 704	51/53	0.870	100
+ Citrus viroid mix	6/6	0.541	95.6
+ CTV 514	6/6	0.541	95.6
+ CTLV 100	6/6	0.541	95.6
Dweet tangor			
CPsV 205/250	34/34	0.897	100
+ CG 301	43/43	0.918	100
+ CVEV 704	25/25	0.863	100
+ Citrus viroid mix	29/29	0.881	100
+ CTV 514	29/32	0.750	99.6
+ CTLV 100	29/32	0.750	99.6

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.980 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b Θ_L , lower probability boundary. Upper boundary was 100%.

^c CG, concave gum; CVEV, Citrus vein enation virus; citrus viroid mix, coinoculation with *Citrus exocortis viroid*, *Citrus viroid 1a* (CVd-Ia), CVd-IIb and CVd-IIIb; CTV, *Citrus tristeza virus*; CTLV, Citrus tatter leaf virus.

Table 5. Effects of additional pathogens in biological indexing of concave gum (CG) using the primary indicators sweet orange and Dweet tangor

Treatment	CG symptomatic/ inoculated	Lower probability (P_L) ^a for CG expression	Efficacy of CG bioindexing using 4 indicators (Θ_L %) ^b
Sweet orange			
CG 301	17/18	0.727	99.4
CG 301 + CPsV 205 ^c	11/37	0.159	49.9
CPsV 205 symptom expression, cut back + CG 301	10/43	0.118	39.4
CG 301 symptom expression, cut back + CPsV 205	28/28	0.877	100
CG 301 + CVEV 704	24/24	0.858	100
+ Citrus viroid mix	36/36	0.903	100
+ CTV 514	36/36	0.903	100
+ CTV 519	29/31	0.786	99.8
+ CTV-SY 568	24/24	0.858	100
Dweet tangor			
CG 301	21/21	0.839	100
CG 301 + CPsV 205	22/43	0.355	82.7
CPsV 205 symptom expression, cut back + CG 301	6/45	0.051	18.7
CG 301 symptom expression, cut back + CPsV 205	45/45	0.921	100
CG 301 + CTLV 100	15/15	0.782	99.8
+ CVEV 704	15/15	0.782	99.8
+ Citrus viroid mix	36/36	0.903	100
+ CTV 514	15/15	0.782	99.8
+ CTV 519	15/15	0.782	99.8
+ CTV-SY 568	15/15	0.782	99.8

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.470 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b Θ_L , lower probability boundary. Upper boundary fluctuated between 71.3 and 100%.

^c CPsV, *Citrus psorosis virus*; CVEV, Citrus vein enation virus; citrus viroid mix, coinoculation with *Citrus exocortis viroid*, *Citrus viroid 1a* (CVd-Ia), CVd-IIb, and CVd-IIIb; CTLV, Citrus tatter leaf virus; CTV, *Citrus tristeza virus*; SY, Seedling Yellows.

citrus pathogens has also been tested and statistically evaluated. Diagnostic performance under the biological pressure of mixed infections and the minimum number of indicators necessary for effective diagnosis were estimated.

CVEV symptoms expressed in DwT and SwO. Indicators DwT and SwO are the primary indexing hosts for CPsV and CG (Table 1) but also may be used for the biological indexing of CVEV (30). The minimum probability for at least one positive (Θ_L) vein enation reaction is 99.0% or higher with just three singly inoculated

CVEV seedlings of DwT (Table 6). Coinfection with CPsV, CG, CTV 514, and the viroids CVd-Ia, CVd-IIb, CVd-IIIb, and CEVd significantly reduced the number of vein enation symptomatic DwT and the efficacy of DwT as a CVEV indicator (Table 6).

The experimental probability for vein enation detection in SwO was low in single infections, however, in contrast to DwT, it improved with coinfection by CPsV, CG, or the viroids CVd-Ia, CVd-IIb, CVd-IIIb, and CEVd (Table 6). The T30 group CTV isolate 514 precluded vein enation symp-

tom expression in SwO (Table 6). The calculated n for SwO indicates it is useful only when CG or the viroid mixture is present (Table 6).

To determine if a specific viroid of the four used initially was active in the enhancement of CVEV detection by SwO, single viroids from the three different viroid genera were tested (Table 1). All three coinfection treatments increased the number of vein enation-symptomatic SwO plants (Table 6). The P_L value for vein enation expression was higher for all the coinfection treatments than the CVEV single infection, with CEVd providing the most significant increase (Table 6).

CTV and CVEV symptoms expressed in C. excelsa. *C. excelsa* is primarily used for the bioindexing of CTV (Table 1). However, inoculation of *C. excelsa* with the T30 group CTV isolate 514 resulted in the expression of both leaf cupping and stem pitting symptoms typical of CTV infection. Only three inoculated *C. excelsa* are sufficient for detection of CTV 514 with 99.0% probability (Table 7). The coinfection treatment with CPsV only slightly reduced the number of CTV-symptomatic plants, and CVEV had no effect on it (Table 7).

C. excelsa inoculated with CVEV expressed vein enation symptoms, and only three *C. excelsa* are needed to detect CVEV at the 99.0% level (Table 7). However, this efficiency was severely suppressed by coinfection with CPsV and CTV 514. The P_L value for vein enation expression in the presence of CPsV was reduced, whereas coinfection with the T30 group CTV isolate 514 totally precluded symptoms (Table 7).

CPsV symptoms expressed in ML. While ML is the primary indexing host for CTV and VE (Table 1) and typically is not used for CPsV, single infection with the shock-inducing CPsV isolate 205 produced positive disease reactions in ML. The estimated number n of indicators for 99% probability for detection was six (Table 7). Coinfection with CTV and CVEV reduced the probability for psorosis expression in ML and increased the minimum number of indicators necessary (Table 7). Low levels of detection of the non-shock-inducing CPsV isolate 250 (Table 1) indicated that ML is not consistent for all CPsV isolates (Table 7).

DISCUSSION

The question of efficacy of biological indexing of graft-transmissible pathogens of citrus in the presence of a complex pathogenic background has been addressed experimentally and analyzed from statistical and theoretical approaches.

The statistically estimated probability interval for symptom expression ($P_L - P_U$) characterized the degree of interference of additional pathogens on symptom expression. Symptom expression of CTV in the primary indexing host ML and CPsV in the

Table 6. Biological indexing for Citrus vein enation virus (CVEV) in the presence and absence of additional pathogens using the alternative indexing hosts Dweet tangor and sweet orange and estimated number of indicators required

Treatment	CVEV symptomatic/inoculated	Lower probability (P_L) ^a for CVEV expression	Indicators required (n) ^b
Dweet tangor			
CVEV 704	25/25	0.863	3
+ CPsV 205/250 ^c	4/7	0.184	23
+ CG 301	3/6	0.118	37
+ CTV 514	2/22	0.011	405
+ Citrus viroid mix	2/6	0.043	104
Sweet orange			
CVEV 704	15/39	0.234	18
+ CPsV 205/250	22/38	0.408	9
+ CG 301	15/15	0.782	3
+ Citrus viroid mix	19/22	0.651	5
+ CTV 514	0/6	0.000	—
+ CEVd	33/35	0.808	3
+ CVd-IIb	31/39	0.635	5
+ CVd-Ia + CVd-IIIb	31/51	0.461	8

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.292 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b n , required number of Dweet tangor or sweet orange for at least one symptomatic CVEV estimated from $1 - (1 - P_L)^n \geq 99.0\%$ at the confidence level of 0.05.

^c CPsV, *Citrus psorosis virus*; CG, concave gum; CTV, *Citrus tristeza virus*; citrus viroid mix, coinoculation with *Citrus exocortis viroid* (CEVd), *Citrus viroid Ia* (CVd-Ia), CVd-IIb, and CVd-IIIb.

Table 7. Biological indexing for *Citrus tristeza virus* (CTV), Citrus vein enation virus (CVEV), and *Citrus psorosis virus* (CPsV) in the presence and absence of additional pathogens using the alternative indexing hosts *Citrus excelsa* and Mexican lime and estimated number of indicators required

Treatment	Symptomatic/inoculated	Lower probability (P_L) ^a for symptom expression	Indicators required (n) ^b
<i>Citrus excelsa</i>			
CTV 514, T30 group	17/17	0.805	3
+ CPsV 205/250	13/14	0.661	5
+ CVEV 704	6/6	0.541	6
CVEV 704	26/26	0.868	3
+ CPsV 205/250	9/17	0.278	14
+ CTV 514	0/6	0.000	—
Mexican lime			
CPsV 205 (shock)	12/14	0.572	6
+ CTV 514	11/15	0.449	8
+ CTV 519, VT group	5/9	0.212	20
+ CVEV 704	9/17	0.278	14
CPsV 250 (lesion bark)	5/15	0.118	37
+ CTV 514	4/16	0.073	61
+ CTV 519	0/9	0.000	—
+ CVEV 704	0/14	0.000	—

^a P_L , Clopper-Pearson lower probability boundary. Upper boundary (P_U) fluctuated between 0.232 and 1. Span of the P_L to P_U interval varies with sample size. Small sample size results in a wide interval and therefore reduced P_L values.

^b n , required number of *C. excelsa* or Mexican lime for at least one symptomatic indicator estimated from $1 - (1 - P_L)^n \geq 99.0\%$ at the confidence level of 0.05.

primary indicators SwO and DwT was essentially unaffected by the presence of other tested pathogens. Similarly, most of the coinfection treatments did not reduce the probability of CVEV expression in Mexican lime. However, the presence of the T30 group CTV isolates in the primary indexing host ML inhibited CVEV diagnosis. Thus, the biological indexing of CVEV occurring in a mixed infection can be seriously impaired since the indicator ML is the primary indexing host for both pathogens. Furthermore, natural mixed infections of CTV and CVEV are commonly encountered, as both viruses are aphid transmitted (13,25,31). Even though only two CTV isolates were tested, it can be speculated that the effect of CTV on CVEV is group specific. Further investigation with additional CTV isolates is appropriate; however, the report that T30 CTV isolates have high levels of homology (1) supports the T30 group-specific interaction with CVEV. Formation of woody galls associated with wounds on rough lemon has also been used for the detection of CVEV (28). It would have to be determined if T30 group CTV isolates also suppress gall symptoms in the symptomless CTV host rough lemon. Bioindexing of CG becomes challenging since the presence of a shock-producing CPsV isolate reduced the probability for CG symptom expression as has been previously observed (23). In addition, both CPsV and CG induce the development of leaf patterns in young growth, which persist only for a few days.

The accuracy of bioindexing practices was evaluated from estimation of the probability interval ($\Theta_L - \Theta_U$) on the basis of at least one positive disease reaction. The recommended practice of using four ML for CTV detection and the use of four SwO or DwT for CPsV indexing is effective even with mixed infections. Similarly, the various coinfections with pathogens did not reduce the efficacy of the recommended practice of using four ML for detecting CVEV except when coinfection with T30 group CTV isolates is present.

Bioindexing of CG using the recommended four SwO or DwT was successful in coinfections with other pathogens except for CPsV. The shock-producing CPsV isolate reduced the probability for at least one CG reaction in the primary indexing hosts, thus jeopardizing detection of CG disease. This observation gains importance as the agents reducing the efficiency of citrus vein enation and CG detection share the same recommended biological indicators, and currently there are no well-accepted alternative diagnostic protocols available (6).

Alternative hosts to those commonly recommended for bioindexing were also tested and may have applications in some cases. This is documented by estimating the minimum number of indicators (n)

necessary for efficient biological indexing. DwT and SwO used together can be considered as valuable indicators for CVEV, as the former is the most sensitive in single infections, and where it is not as sensitive, in multiple infections, SwO is effective. Mixed infections did markedly increase the number of DwT required for accurate diagnosis. In contrast, mixed infections with CPsV, CG, and viroids potentiate vein enation symptom expression in SwO. This observation might explain the previously reported infrequent symptom expression of CVEV in SwO (30). These reports were made prior to the characterization of multiple citrus viroids. Therefore, if some of the CVEV sources carried citrus viroids, CVEV expression on SwO may have been enhanced as demonstrated in this study.

It has been previously reported that *C. excelsa* expresses CTV symptoms (20), and the presented results confirmed that *C. excelsa* is an efficient and practical alternative indexing host for CTV. *C. excelsa* can be used efficiently for CVEV detection only in single infections. Coinfections with CPsV and the T30 group CTV isolate 514 suppressed vein enation symptoms.

The suppression effect of CTV 514 on vein enation expression observed in *C. excelsa*, ML, SwO, and DwT suggested that the interaction between CVEV and T30 group CTV is not host dependent. CTV and CVEV are different viruses and cross protection is unlikely; however, since both viruses are phloem limited (25), interference in replication and/or gene silencing could be hypothesized.

The shock-producing CPsV isolate 205 induced disease symptoms in the alternative indicator ML. However, ML cannot be considered as an efficient indicator for CPsV since it did not react strongly to the other CPsV isolates tested and the estimated number of indicators necessary for efficient disease diagnosis is impractically high.

The experimental mixed infections employed in this study should be viewed as a model system that may not simulate naturally occurring long established complex infections where the equilibrium between pathogens differs from recent infections. However, the simultaneous inoculations assured that none of the pathogenic agents involved in any of the tested mixed infections had any advantage. In mixed infections where symptoms of one pathogen were suppressed, verification of the presence of the suppressed agent by a laboratory technique would be preferred. In the absence of quantitative detection methods for CVEV and CG, the systemic presence in a multiply infected indicator was ensured by inoculation with the suppressed pathogen prior to the challenge pathogen. When infection of CVEV or CG was ensured by the pre-inoculation treatment, suppression by CTV of CVEV expression was reduced and the CPsV over CG was

completely eliminated. This indicates that the variability of symptom expression might also be affected by timing in the initiation of infection.

These studies endorse nontraditional yet potentially valuable approaches and methods that can be applied in a program involving biological indexing of graft-transmissible pathogens of citrus. Two indexing hosts, ML and SwO, provide efficient diagnosis of at least four viral diseases. Using these indicators for preliminary screening can determine if further investment of facilities, labor, plant material, and time is necessary for the determination of specific diseases.

The statistical evaluation of the bioindexing protocol's efficacy provides a tool to customize diagnostic processes to specific needs. The number of indicators used for disease diagnosis can be adjusted for each disease based on the probability for symptom expression to optimize indicator usage and minimize the overall cost of diagnosis. The dynamics in the bioindexing process demonstrated here remain open to further improvement as different pathogens in various natural combinations are tested and the database is enlarged. Other factors such as low titer or uneven distribution of pathogens in field trees can also negatively affect the bioindexing tests. Such obstacles might be overcome with authenticated sampling methods or increased inoculations per indexing host. This might increase the probability for pathogen detection without necessarily increasing the optimized number of indicators.

An efficient program is essential for every country with commercial citrus production, particularly for those with limited capabilities or resources to conduct laboratory diagnostics that rely primarily on bioindexing for the diagnosis of graft-transmissible diseases.

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