Problems with Interpretation of Serological Assays in a Virus Survey of Orchid Species from Puerto Rico, Ecuador, and Florida

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ABSTRACT

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Leaf samples collected in May 1990 from wild and cultivated orchids in Puerto Rico were tested for odontoglossum ringspot virus (ORSV), cymbidium mosaic virus (CymMV), tobacco mosaic virus common strain, tobacco mild green mosaic virus, two strains of cucumber mosaic virus, and cymbidium ringspot virus (CymRSV) with sodium dodecyl sulfate immunodiffusion, enzyme-linked immunosorbent assay (ELISA), and/or Western blot (immunoblot) procedures. Leaf tissue from orchids cultivated in Gainesville, FL, and from the wild in Ecuador were similarly tested. No virus was detected in the 277 wild orchids, and only ORSV, CymMV, or both ORSV and CymMV were detected in 20, 73, and 22 cultivated orchids, respectively, from Puerto Rico and Florida. Several orchid plants gave ELISA reactions greater than three times the negative control with all the virus antisera tested. Other methods did not confirm the presence of virus in these plants, however. Indeed, several preimmune sera also reacted with some of these plants. Caution must be used in interpretation of low ELISA values even when these reactions are clearly greater than those of uninfected controls. These results illustrate the need to utilize more than one diagnostic technique before discarding a valuable orchid plant.

Additional keyword: tomato ringspot virus

Orchid cultivation began in Europe more than 200 years ago. Since then, odontoglossum ringspot virus (ORSV) and cymbidium mosaic virus (CymMV) have become widespread in both cultivated species and hybrids (26). The natural origin of these two viruses, however, remains unknown. Neither virus has been detected in wild orchids surveyed in Florida, Guatemala, French Polynesia, and several other locations in both the New World and Old World tropics (20,22,25,26).

Strains of tobacco mosaic virus (TMV) are reported to infect orchids (5,11), yet little information is available regarding the incidence of these viruses in either cultivated or wild orchids. In contrast, high incidences of tobacco mosaic virus common strain TMV-U_1, and the closely related tomato mild green mosaic virus (TMGMV), occur in cultivated members of Gesneriaceae that grow intermixed with orchids in the wild and are often cultivated together in greenhouses (27).

Cucumber mosaic virus (CMV), an aphidborne virus, has been reported to infect cultivated orchids (7,12). This virus occurs naturally in Puerto Rico, infecting various weeds such as *Commelina* spp. (1); however, no information exists regarding its occurrence in wild orchids.

Although cymbidium ringspot virus (CymRSV) has been used in cytological and recombinant DNA studies involving *Nicotiana* spp., there apparently are no reports of CymRSV-infected orchids since its discovery in 1962 (2,9).

The objectives of this study were to determine if CymMV or other viruses can be found in wild orchids in Puerto Rico and to survey both wild and cultivated orchids for the presence of ORSV, TMV-U_1, TMGMV, CMV, and CymRSV. Samples from cultivated orchids grown in Florida and plants collected from the wild in Ecuador were included in this study for comparison.

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MATERIALS AND METHODS

Sources of plant materials. Leaf samples were collected from 257 wild orchids representing 25 genera and 35 species from eight forest reserves throughout Puerto Rico in May 1990 (Table 1). Both terrestrial and epiphytic orchids were collected from habitats that ranged from coastal arid forests at sea level to rain forests at altitudes exceeding 3,000 m. Additionally, 129 and 20 cultivated orchid samples were collected from Puerto Rico and Gainesville, FL, respectively (Tables 1 and 2).

Twenty leaf samples were also taken from orchids that had been collected from the wild in Ecuador and grown in isolation for less than 1 year in Gainesville, FL (Table 2). The provinces from which these plants were collected include Los Rios, Azuay, Imbabura, Morona-Santiago, Carchi, Bolivar, El Oro, Zamora-Chinchipe, and Esmeraldas. All these plants were epiphytic with the exception of *Phragmipedium*.

Leaf tissue was indexed with antisera for ORSV and CymMV (21) as well as for TMV-U_1 and TMGMV. TMGMV was formerly referred to as the U_2 strain of TMV (27). Antisera prepared by Jacono (10) to two Florida isolates of CMV were used in this study. One isolate (CMV-CD) was from *Commelina diffusa* Burm. f. (10) and the other (CMV-48) was from *Cucurbita pepo* L. (15). CymRSV antiserum was provided by Agdia Inc. (Elkhart, IN).

Electron microscopy. Leaf extracts were negatively stained with 2% uranyl acetate and examined with a Hitachi 600 electron microscope to detect virus particles (3).

Manual inoculation. Herbaceous dicotyledonous indicator plants were inoculated with 600 mesh Carborundum as the abrasive and 0.02 M sodium phosphate buffer pH 7.2. Thirty, 20, and 15 seedlings of *Cymbidium*, *Cattleya*, and *Phalaenopsis* orchid hybrids, respectively, were inoculated by dipping a wooden toothpick into an extract from CymRSV-infected *Nicotiana benthamiana* Domin. (prepared by triturating leaf tissue in 0.02 M sodium phosphate buffer, pH 7.2) and wounding the test plants by piercing the leaf surfaces. Ten, 5, and 5 *Cymbidium*, *Cattleya*, and *Phalaenopsis* hybrid seedlings, respectively, were similarly inoculated with CymMV as controls to confirm the effectiveness of the inoculation procedure.

SDS immunodiffusion. The sodium dodecyl sulfate (SDS) immunodiffusion serology procedures were those described by

Purcifull and Batchelor (14), using 0.8% Nobles agar, 0.5% SDS, and 1.0% NaN_3.

ELISA. Orchid leaf samples were triturated in 10 parts carbonate egg pyrrolidone (CEP) extraction buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.02% polyvinyl pyrrolidone-40, bovine serum albumin 0.2%, pH 9.6), and tested by indirect enzyme-linked immunosorbent assay (I-ELISA) (24) for all the viruses mentioned above except CymRSV. All sera and preimmune sera except CymRSV were collected from New Zealand White rabbits immunized at the University of Florida. Preimmune sera from rabbits immunized against viruses used in this study were used as controls whenever possible. Six additional preimmune sera were also used. Sigma whole molecule goat anti-rabbit alkaline phosphatase conjugate and p-nitrophenyl phosphate substrate were used in all assays except assays for CymRSV. The CymRSV antiserum was provided by Agdia Inc. (Elkhart, IN) in a horseradish peroxidase enzyme system. Antigen-trapped direct ELISA (antigen-trapped D-ELISA) (19) was used to test for CymRSV. Alkaline phosphatase was used in I-ELISA, and sample absorbance readings were made at a wavelength of 405 nm. Horseradish peroxidase was used for the antigen-trapped D-ELISA and sample absorbance readings were made at a wavelength of 490 nm. All absorbance readings were made after a 30-min incubation at room temperature.

Healthy controls included *Vanilla pompona* Schiede or *V. planifolia* Andr., *Gongora sp.*, and unspecified hybrids of *Cattleya*, *Cymbidium*, and *Phalaenopsis*. Samples were compared with specific genera when possible or with the average of the pooled absorbance values (A_{405}) of all the healthy orchid controls. I-ELISA absorbance values (A_{405}) representing the average of three wells per sample, and antigen-trapped D-ELISA A_{405} values representing the average of two wells per sample, were considered potentially positive if the absorbance values were equal to or greater than three times the healthy values (16).

Certain samples that produced high A_{405} values in I-ELISAs when tested against several different sera, including preimmune serum, were tested against six additional preimmune sera, to determine if the reactions were nonspecific in nature.

Orchid tissue samples that produced A_{405} values equal to or greater than two times healthy orchid A_{405} values when tested against CymRSV antiserum were triturated and used to inoculate the herbaceous hosts, *Gomphrena globosa* L., *Helianthus annuus* L., *Nicotiana tabacum* L. 'Xanthi NC,' *N. benthamiana*, *Ocimum basilicum* L., *Phaseolus vulgaris* L. 'Kentucky Wonder,' *Pisum sativum* L. 'Dwarf Gray Sugar,' *Vigna unguiculata* L. Walp. subsp. *unguiculata* 'Knuckle Purple Hull,' and *Zinnia elegans* Jacq. plants. These plants were

Table 1. Wild-collected and commercial orchid genera and species collected in Puerto Rico and indexed for viruses by sodium dodecyl sulfate immunodiffusion serology

Collection source	Total plants	No. species	ORSV[a]	CymMV[a]
Wild-collected				
Susua Forest Reserve	20	6	0	0
Guajataca Forest Reserve	45	13	0	0
Cambalache Forest Reserve	12	4	0	0
Rio Abajo Forest Reserve	1	1	0	0
Maricao Forest Reserve	70	16	0	0
El Yunque National Park	44	7	0	0
Carite Forest Reserve	16	7	0	0
Guanica Forest Reserve	8	1	0	0
Commercial orchids				
Cattleya alliance	72	NA[b]	20	63
Cyrtopodium punctatum (L.) Lindl.	2	NA	0	1
Dendrobium hybrid	6	NA	0	3
Epidendrum radicans Pav. ex Lindl.	2	NA	0	2
Epidendrum sp.	1	NA	0	0
Encyclia cochleata (L.) Dressler	1	NA	0	1
Neobenthamia sp.	1	NA	0	1
Oncidium altissimum (Jacq.) Sw.	2	NA	0	1
Oeceoclades maculata (Lindl.) Lindl.	2	NA	0	0
Phalaenopsis hybrid	4	NA	0	2
Vanda alliance	7	NA	0	7
Vanda teres (Roxb.) Lindl.	4	NA	0	4
Vanilla planifolia Andr.	14	NA	0	0
Vanilla pompona Schiede	1	NA	0	0
Vanilla sp.	10	NA	0	0

[a] ORSV = Odontoglossum ringspot virus; CymMV = Cymbidium mosaic virus.
[b] Not applicable.

Table 2. Cultivated and/or wild-collected orchids from Puerto Rico and Ecuador[a]

			ORSV			CymMV[c]		
Collection source[b]	No. species	No. plants	SDS-I	I-E	EM	SDS-I	I-E	EM
Wild-collected								
Ecuador	19	19	–	–	–	–	–	–
Puerto Rico	18	40	–	–	–	–	–	–
Cultivated orchids in Gainesville								
Cattleya Rembrandt 'Tenny'[d]	NA	1	–	+	–	+	+	+
Cattleya hybrid ?[d]	NA	1	–	+	–	+	+	+
Cattleya guttata Lindl.[e]	NA	1	–	–	–	–	–	–
Cymbidium findlaysonianum Lindl. × *Grammatophyllum scriptum* (L.)Bl.[d]	NA	1	–	+	–	+	+	+
Cymbidium hybrid?[e]	NA	1	–	–	–	–	–	–
Cymbidium findlaysonianum Lindl.[d]	NA	1	–	+	–	+	+	+
Dendrobium hybrid?[d]	NA	1	–	+	–	+	+	+
Epidendrum anceps Jacq.[e]	NA	1	+	+	–	+	+	+
Gongora quinquinervis Ruiz & Pav.[e]	NA	1	–	–	–	+	+	+
Laeliocattleya [e]	NA	1	–	–	–	–	–	–
Oncidium maculatum Urb.[d]	NA	1	–	+	–	+	+	+
Phalaenopsis Carousel[e]	NA	1	+	+	+	+	+	+
Phalaenopsis Zauberot 'Lemforder'[f]	NA	1	–	+	–	+	+	+
Pholidota sp.[d]	NA	1	–	+	–	+	+	+
Stanhopea sp.[d]	NA	1	–	+	–	+	+	+
Stanhopea sp.[d]	NA	1	–	+	–	+	+	+
Vanda hybrid ?[f]	NA	1	–	+	–	+	+	+
Vanda hybrid ?[d]	NA	1	–	–	–	+	+	+
Vanilla planifolia Andr.[e]	NA	1	–	–	–	–	–	–
Vanilla pompona Lindl.[f]	NA	1	–	+	–	–	–	–

[a] Indexed for viruses by sodium dodecyl sulfate immunodiffusion (SDS-I), indirect enzyme-linked immunosorbent assay (I-E), Western blot (immunoblot), host range, and electron microscopy (EM) techniques
[b] Wild-collected orchids were not tested for odontoglossum ringspot virus (ORSV) or cymbidium mosaic virus (CymMV) with Western blotting.
[c] Western blotting was not used to test for CymMV; – = no precipitin lines observed in SDS-I plates, A_{405} values less than three times the healthy values in I-E and no bands or particles observed in Western blotting and EM, respectively; + = precipitin lines observed in SDS-I plates, A_{405} values greater than three times the healthy values virus in I-E and bands or particles observed in Western blotting and EM, respectively; NA = not applicable.
[d] Tested for ORSV with Western blotting. No virus detected.
[e] Not tested for ORSV with Western blotting.
[f] Tested for ORSV with Western blotting. Virus was detected.

reported by Hollings et al. (9) to be susceptible to CymRSV. Tissue from these herbaceous hosts was then tested for CymRSV with antigen-trapped D-ELISA as described above.

Reciprocal tests between TMV-U_1, TMGMV, ORSV, and the two CMV strains were conducted to detect heterologous reactions.

Western blots. The Western blotting (immunoblotting) procedure was a modification of that described by Towbin et al. (17), with a Bio-Rad Mini-Protein II electrophoresis cell and Bio-Rad Trans-Blot electrophoretic transfer cell. Leaf tissue triturated and boiled for 90 s in dissociating solution (19% Tris-HCl, 1.25 M, pH 6.8, 19% SDS [10% solution], 4% 2-mercaptoethanol, 10% sucrose, 48% glass distilled H_2O), was electrophoresed on 10% SDS–polyacrylamide gel electrophoresis mini gels for 30 min at 200V constant voltage. Separated proteins in the gel were transferred to nitrocellulose membranes by electroblotting for 60 min at 100V. Washed membranes were incubated at room temperature in a 1:50 dilution of healthy sap in blocking solution consisting of 5% powdered skim milk in Tris-buffered saline and 0.1% Tween 20 (TSBT), and incubated for 60 min at room temperature in a 1:1,000 dilution of antiserum or preimmune serum in blocking solution. Membranes were then washed and incubated for 60 min at room temperature in a 1:1,000 dilution of alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO) in blocking solution. After three washes in TBST, the conjugate was detected with a solution of 0.33 mg of nitro blue tetrazolium (NBT: 75 ml of NBT in 0.025 ml of H_2O + 0.75 ml of DMF [N,N-dimethyl formamide]) per ml and 0.175 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP: 50 mg in 1 ml of DMF) per ml, in 15 ml of substrate buffer (O.1 M NaCl, 0.1 M Tris, and 5 mM $MgCl_2$, pH 9.5).

RESULTS

SDS immunodiffusion. Based on SDS immunodiffusion results, 20 of the 149 cultivated orchids were infected with ORSV, 73 with CymMV, and 22 with both viruses (Tables 1 and 2). ORSV, CymMV, TMV-U_1, and CMV-CD were not detected in any of the 277 wild orchids from Puerto Rico and Ecuador by SDS immunodiffusion serology (Tables 1 and 2). Likewise, neither TMV-U_1, TMGMV, CMV-CD, nor CMV-48 was detected by this technique in any of the 149 cultivated orchids from Puerto Rico and Florida.

CymMV and ORSV. CymMV or ORSV was detected in 15 of the 20 orchids cultivated in Florida and tested by I-ELISA (Table 2). A_{405} values of the 15 CymMV-infected samples ranged from 1.224 to 2.999, compared with values of 0.209 to 0.331 (mean = 0.284) for the healthy plants. For ORSV, A_{405} values (0.382 to 2.348) in excess of three times those of the healthy orchid values (0.098 to 0.148) were obtained in 14 of the 20 samples. However, ORSV was confirmed by SDS immunodiffusion and Western blot serology in only two of these 14 samples, which had mean A_{405} values of 1.721 and 2.348. Respective positive control ranges for CymMV and ORSV were 0.627 to 1.617 (mean = 0.965) and 0.439 to 0.671 (mean = 0.596). The mean A_{405} value for healthy orchids was 0.119.

TMV-U1 and TMGMV. Six cultivated orchids from Florida and nine wild-collected orchid samples from Puerto Rico and Ecuador had I-ELISA A_{405} values at least three times higher than those of the healthy orchid controls when tested against TMV-U_1 or TMGMV antisera. Absorbance values of these samples ranged from 0.205 to 1.440 for TMV-U_1 and 0.214 to 0.520 for TMGMV. Respective A_{405} positive control value ranges for TMV-U_1 and TMGMV were 1.552 to 1.936 (mean = 1.797) and 1.723 to 1.934 (mean = 1.848). Respective negative controls were 0.061 to 0.066 (mean = 0.0633) and 0.070 to 0.102 (mean = 0.088). All of the cultivated samples that produced high A_{405} values when tested against TMV-U_1 or TMGMV also gave high A_{405} values when tested against ORSV in ELISA. This is presumably due to cross-reactivity between antisera and antigens of these related tobamoviruses (19). In the SDS immunodiffusion tests, none of the cultivated or wild-collected samples formed precipitin lines when tested against either TMV-U_1 or TMGMV antisera. Precipitin lines were observed only when the cultivated samples were tested against ORSV antiserum.

CMV. None of the A_{405} values of the wild-collected orchids tested for CMV-CD by I-ELISA were equal to or greater than three times those of the healthy orchid controls. Absorbance values in excess of three times those of the healthy orchids were observed, however, in four of the 20 cultivated samples tested for this virus. Respective A_{405} values of the samples were 0.115, 0.147, 0.091, and 0.542, whereas healthy A_{405} values ranged from 0.011 to 0.047 (mean = 0.029). However, the four cultivated orchids that produced high A_{405} values in ELISA did not react when tested by SDS immunodiffusion or Western blot procedures. None of the wild or cultivated orchids reacted with CMV-48 antiserum in SDS immunodiffusion, I-ELISA, or Western blot serology.

CymRSV. CymRSV was not detected in orchid samples from Puerto Rico nor did this virus infect manually inoculated orchid seedlings. Absorbance values (A_{490}) that ranged from 0.049 to 0.116 and were two or more times greater than those of healthy orchids, were obtained in five of the 20 cultivated, six of the 19 wild-collected Ecuadorian, and one of the 40 wild-collected Puerto Rican samples. Absorbance values of the CymRSV positive controls in *N. benthamiana* ranged from 1.901 to 2.235 (mean = 2.068) whereas healthy orchid A_{490} values ranged from 0.006 to 0.043 with a mean of 0.030.

None of the nine herbaceous species developed symptoms after inoculation with triturated tissue from the aforementioned orchids that reacted with CymRSV antiserum. Moreover, low A_{490} values (0.014 to 0.096) were obtained when leaf tissue from these plants was tested for CymRSV by antigen-trapped D-ELISA. Corresponding healthy control A_{490} values of the nine herbaceous species inoculated in this experiment were also low (0.024 to 0.072). In contrast, A_{490} values of the CymRSV positive controls in *N. benthamiana* in this experiment ranged from 2.079 to 2.104 (mean = 2.091).

In a separate test, consistently low A_{490} values were observed when extracts from tissues of CymRSV-inoculated *Cattleya*, *Phalaenopsis*, and *Cymbidium* seedlings were tested by antigen-trapped D-ELISA; mean A_{405} values were 0.010, 0.005, and 0.006, respectively. Corresponding mean values of the noninoculated orchids were 0.010, 0.007, and 0.005, respectively. The mean absorption value of *N. benthamiana* plants inoculated with CymRSV was 1.662.

Of the seedlings inoculated with CymMV to test the efficacy of the CymRSV inoculation procedure, one of five *Cattleya* plants and five of five *Phalaenopsis* plants had high mean A_{405} values (0.785 and 0.672, respectively) when tested by I-ELISA. None of the 10 *Cymbidium* plants gave high ELISA values (mean = 0.032) for CymRSV. Respective healthy A_{405} values were 0.045, 0.022, and 0.023. Also, flexuous rod-shaped particles characteristic of CymMV were observed on electron microscope grids prepared from each of the samples that produced high A_{405} ELISA values.

A_{405} values in healthy orchid leaf extracts. The nine wild-collected orchid samples from Ecuador and Puerto Rico that had relatively high A_{405} values in ELISAs with TMV-U_1, TMGMV, CMV, CymMV, ORSV, and CymRSV antisera as well as preimmune serum were tested against six additional preimmune sera. The healthy controls were those described earlier. Very low A_{405} values, which did not exceed three times the healthy values, were observed for single specimens of *Oeceoclades maculata* and *Encyclia cochleata*. In contrast, an unidentified *Epidendrum* sp. and an *Epidendrum anceps* each reacted slightly (2.5 times healthy) with one of the preimmune sera tested. The *Pleurothallis domingensis* sample reacted with two of the six preimmune sera (4.3 and 7.3 times healthy). The *Epidendrum ciliare* (15.7, 8.7, and 4.6 times healthy), *Xylobium colleyi* (7.3, 5.8, and 3.6 times healthy), and one of the *Scaphyglottis* sp. (9.3, 6.2, and

4.3 times healthy), reacted with three of the six preimmune sera, and a second *Scaphyglottis* sp. reacted with five of the six preimmune sera (44.7, 33.3, 3.0, 7.3, and 5.0 times healthy). While none of these elevated reactions exceeded 0.200, all were greater than three or more times the healthy control values.

DISCUSSION

Neither ORSV nor CymMV was detected in any of 277 leaf samples collected from terrestrial and epiphytic orchids growing wild in Ecuador and Puerto Rico when tested by SDS immunodiffusion. In contrast, ORSV and CymMV were detected by the same method in 22 and 122, respectively, of the 149 samples collected from cultivated orchids in 10 commercial and private greenhouses in Florida and Puerto Rico. Similar results were reported by Zettler et al. (26), and Wisler et al. (22), who indexed cultivated and wild-collected orchids from Florida and Guatemala as well as several other locations in the New World and Old World tropics. While ORSV and CymMV presumably occur naturally in some populations of orchids, we were unable to identify either Puerto Rico or Ecuador as a location of such natural infections. A CymMV-infected *Encyclia cochleata* was collected from the Maricao Forest Reserve in Puerto Rico prior to this survey. However, this plant may have been in cultivation. It was much larger than the native specimens of this species we encountered at the Maricao Reserve and was found along the roadside in an unrooted condition. CymMV was not detected in any of the 27 leaf samples collected from wild orchids, including 16 other *Encyclia cochleata* plants, growing within 100 yards of the location where the infected plant was reportedly collected nor was it detected in any of the 43 samples collected in other locations within this Forest Reserve (Table 1).

The only report of a virus detected in a wild population of orchids is that by Yao et al. (23), who detected tomato ringspot nepovirus in *Ponthieva racemosa*, a terrestrial orchid, collected in the Guajataca Forest Reserve in Puerto Rico. This virus was also detected by Goff and Corbett (8) in several cultivated *Cymbidium* Snowbird 'Jayhurst' plants. The vector of this virus is probably a dorylaim nematode such as a *Xiphinema* sp. Such nematodes are known to transmit nepoviruses including tomato ringspot virus, which can in turn infect a wide variety of monocotyledonous and dicotyledonous plants (2). Inasmuch as nematodes are soil-inhabiting organisms, nepoviruses are unlikely to be encountered naturally in epiphytic orchids (26). *Xiphinema* species were not detected in soil samples collected from this area a year later, however (O. W. Barnett, Jr., *unpublished data*).

Unlike the case with the nepoviruses, natural vectors of ORSV and CymMV have not been identified, and neither of these viruses was detected in the wild-collected orchids by SDS immunodiffusion. Furthermore, TMV-U_1, TMGMV, and CMV were not detected with this technique in the wild-collected or cultivated orchids. Cucumber mosaic virus, which is known to infect orchids (7,12,26) apparently occurs infrequently in cultivated orchids despite being transmitted readily by aphids and occurring naturally in such weeds as *Commelina* spp. in Puerto Rico (1).

While some strains of TMV have been reported to infect orchids (6,11) and while both TMV-U_1 and TMGMV are known to infect members of Gesneriaceae (27) that are frequently cultivated with orchids, tobamoviruses other than ORSV do not appear to be serious pathogens of orchids. For example, Corbett (4) inoculated *Cattleya* seedlings with tobacco mosaic virus, but the virus did not appear to spread systemically beyond the inoculated leaf.

While highly sensitive and widely recommended for virus detection, Western blot tests and ELISAs are subject to a high degree of cross-reactivity between members within a virus group (18). For example, A_{405} values three times higher than those of the healthy controls were observed for several of the cultivated orchid samples tested by ELISA with TMV-U_1, TMGMV, and ORSV polyclonal antisera. Similar ELISA results were observed in which A_{405} values of both heterologous and homologous comparisons of these three antisera were more than three times higher than the healthy controls. Homologous A_{405} values, however, were typically much higher than heterologous values. Heterologous reactions could explain some reports of TMV in orchids. Such distinctions between tobamoviruses are important to orchid growers since they help to identify potential sources of primary inoculum. Whereas TMV-U_1 and TMGMV are commonly found in tobacco products (27), ORSV appears to be exclusively a pathogen of orchids, and to date we have not detected TMV-U_1 or TMGMV in orchids.

Twenty cultivated and 60 wild-collected orchid samples were indexed for CymRSV with antigen-trapped D-ELISA. While A_{490} values two or more times greater than the healthy controls were observed for some samples, no conclusive evidence for virus infection was obtained. The absence of virus was confirmed by manual inoculations to host plants reported by Hollings et al. (9) to be suscepts and by further indexing with antigen-trapped D-ELISA. While Hollings et al. (9) reported CymRSV in cultivated *Cymbidium* plants in England in 1962, there are no subsequent reports of this virus infecting orchids. Likewise, CymRSV was not detected in recent surveys of older orchid collections (7). Most of the orchids that gave elevated A_{490} values belonged to genera of subtribe Stanhopeinae and *Vanilla*. The noncultivated plants were, however, collected from many different locations throughout Ecuador.

The necessity of using more than one technique to identify viruses is particularly apparent when indexing orchids. While earlier diagnostic techniques may have their limitations, other more recently developed ones may lead to misleading conclusions if results are interpreted narrowly. For ELISAs, the commonly accepted practice of considering any absorbance value positive that is two to four times greater than that of the healthy control (16) can be misleading in certain plant systems, particularly if the plants are as genetically heterogeneous as Orchidaceae.

In contrast to orchids, most field crops such as potato are, on a genus and species level, relatively homogeneous genetically. Thus, fewer healthy and diseased controls are sufficient for accurate virus assessment. The heterogeneity of orchid collections, however, makes the availability of healthy and diseased controls virtually impossible. Indexing a breeder's germ plasm collection or a typical collection of orchids may require healthy controls for each genus or species tested. In this study, we found that several orchid species within *Vanilla*, *Gongora*, and *Stanhopea* produced nonspecific reactions with virus antisera as well as some of the preimmune sera used as controls. Thus, while the use of ELISA for virus detection in orchids has been recommended for its sensitivity (13), caution is warranted in interpreting the results.

Since many orchid species have become extinct or very rare in the wild due to habitat loss and/or over-collecting, some tissue culture companies and environmental organizations are multiplying native orchid species with the intent of reintroducing them to their native habitats. In doing so, they may maintain plants under cultivation, thereby exposing them to virus. Except for tomato ringspot, none of the known orchid viruses has as yet been detected in wild-collected orchid plants. For this reason, these organizations should also assume the responsibility of producing virus-free plantlets and indexing them prior to reintroduction into the wild, preferably with one or more techniques with appropriate controls.

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