A Serodiagnostic Technique for Detecting Cymbidium Mosaic and Odontoglossum Ringspot Viruses

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ABSTRACT

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A serodiagnostic technique for cymbidium mosaic (CyMV) and odontoglossum ringspot (ORSV) viruses was developed based on an immunodiffusion test. Antisera with titers of 1/8 were obtained with immunogens that were either nondegraded (for CyMV only) or degraded by boiling in 1% sodium dodecyl sulfate (SDS) containing 0.1% 2-mercaptoethanol (for CyMV and ORSV). A relatively high virus incidence in mature, cultivated orchids was found (27% of 1,328 samples representing 17 genera), but no infection was detected in any of the 438 samples collected from their native habitats (11 genera from 21 old- and new-world locations), nor in 231 seedling orchids. Seroassays were used successfully by three orchid growers.

Cymbidium mosaic (CyMV) and odontoglossum ringspot (ORSV) are the most prevalent viruses infecting cultivated orchids (10,15,17). Both are widely distributed through the frequent exchange of plant material by growers and readily transmitted to healthy plants by cutting tools used during propagation and flower harvesting (5,11). No coordinated, industry-wide virus certification program exists for orchids; thus, consumers have little assurance of purchasing virus-free plants. Various techniques have been developed for detecting CyMV and ORSV (3,9,10), but they have certain limitations and are impractical for wide-scale use in the industry.

We describe an agar-gel immunodiffusion technique that can be used effectively by orchid growers.

MATERIALS AND METHODS

Odontoglossum ringspot virus (ORSV) from *Brassocattleya* Poilu and Cymbidium mosaic virus (CyMV) from *Laelia lundii* Rchb. f. & Warm. ex. Rchb.f. were purified from systemically and locally infected leaves of *Nicotiana benthamiana* Domin, respectively. Leaf tissues were homogenized in a chilled mixture of 0.05 M potassium phosphate buffer (pH 7.5, containing 0.125 M Na_2SO_3) and chloroform (1:2:1, w/v/v). The homogenates were centrifuged at 10,000 *g* for 10 min and the pellets were discarded. The viruses were precipitated from the supernatant with 8% (w/v) polyethylene glycol 6000 and subjected to one cycle of differential centrifugation to obtain the final preparations. Purified virus preparations (in 0.02 M phosphate buffer, pH 7.5) were then lyophilized and stored at −4 C.

Several methods of immunogen treatment were evaluated for CyMV. Lyophilized virus (2.5 mg) was resuspended in either 1 ml of water, 1 ml of 0.06% sodium dodecyl sulfate (SDS), or 1 ml of 1.0% SDS containing 0.1% 2-mercaptoethanol (2-ME) and then boiled for 4 min (12). Also, ORSV immunogen was prepared as above and by an additional pyrrolidine degradation (13), in which 1 ml of 5% pyrrolidine was added to 2.5 mg of purified virus and then removed by dialysis. Emulsions were prepared by adding 1 ml of Freund's complete adjuvant for the first injection, Freund's incomplete adjuvant for the two subsequent weekly injections, and injected intramuscularly into the thighs of New Zealand white rabbits (12). Approximately 25 ml of whole blood were collected weekly in glass tubes from nicks in the marginal ear vein and the serum was processed, lyophilized in 2-ml quantities, and stored at −4 C (12).

All antisera were titered against purified, homologous antigens. Both antisera and antigens were tested in a twofold dilution series ranging from 1/2 to 1/2,048. Antisera were diluted in 0.85% NaCl solution, and antigens in 0.02 M potassium phosphate buffer, pH 7.2. Antiserum titers were determined by immunodiffusion and the microprecipitin methods (1,12). Reactions were observed and recorded after 2 and 4 hr with the microprecipitin method and after 24 hr with the immunodiffusion method.

Several immunodiffusion media were evaluated for testing orchid samples. These included: 0.8% Noble agar, 0.5% SDS, 1.0% NaN_3 (6,12); 0.8% Noble agar, 0.2% SDS, 0.7% NaCl, 0.1% NaN_3 (14); 0.8% Noble agar, 0.5% SDS, 1.0% NaCl (2); and 0.8% Noble agar, 0.5% SDS, 1.0% NaCl in 0.05 M tris-HCl (Trizma®) buffer, pH 8.0. Wells in the agar were made with an adjustable gel punch (Grafar Corp., Detroit, MI) consisting of a central well and six peripheral wells (all 7 mm in diameter) spaced 5 mm from the central well. A less expensive gel punch, arranged in three patterns, was made by cementing 7-mm Mauser unprimed cartridge cases (Winchester-Western Division, New Haven, CT) onto Plexiglas disks (100 mm in diameter) with epoxy resin. Leaf disks (6 mm in diameter) were taken from the youngest fully mature leaves with a paper punch and placed into wells with flamed forceps. The punch was dipped into 70% ethanol and flamed after each use. A drop of 1.5% SDS or deionized water was added to each peripheral well before and after addition of the leaf disks to facilitate contact between the disks and the immunodiffusion medium. Loaded plates were incubated at approximately 23 C in a moist chamber, and results were recorded after 24 hr. In some experiments, leaf disks were placed above the surface of a boiling water bath and steam heated for 4 min prior to addition to antigen wells. Leaf disks were heated conveniently by placing them into microtiter plates. Healthy tissue, CyMV-, or ORSV-infected tissue were used as control tests in all experiments.

Cultivated orchids were obtained from private and commercial collections in Florida, Louisiana, and South Carolina. Plants were

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categorized as mature plants or seedlings that had not flowered. In addition, 35 commercially grown cymbidium orchids were obtained from R. I. B. Francki, University of Adelaide, Glen Osmond, Australia.

Noncultivated orchids collected from native habitats were obtained from the following locations (numbers represent the number of samples tested): Brazil, 29; Cayman Islands, 2; Colombia, 10; Costa Rica, 14; Dominican Republic, 12; East Africa/Madagascar, 25; Ecuador, 1; El Salvador, 43; Guatemala, 3; Guyana, 35; Honduras, 56; Jamaica, 12; Panama, 5; Peru, 47; Philippines, 37; Nepal, 13; Nicaragua, 35; Solomon Islands, 18; Thailand, 14; and Venezuela, 27.

RESULTS

The procedure used to purify the viruses yielded 1.14 mg/g of leaf tissue for ORSV, and 0.1 for CyMV. Average $A_{260/280}$ values (not corrected for light scattering) were 0.93 and 1.07 for ORSV and CyMV, respectively.

Antisera prepared to SDS-treated and untreated preparations of CyMV had titers of 1/2,048 in microprecipitin and 1/8 in immunodiffusion tests against 0.025 mg (per antigen well) of untreated purified virus. Antiserum produced to lyophilized ORSV suspended in water or 0.06% SDS gave weak or no reactions (titer of 1/1) with 0.025 mg of purified virus in immunodiffusion tests, but antiserum to 1.0% SDS plus 2-ME (boiled) or pyrrolidine-treated ORSV had titers of 1/8 in gel systems (Fig. 1f) and 1/1,024 in microprecipitin tests.

The most satisfactory immunodiffusion medium for detecting either CyMV or ORSV in leaf tissue of major orchid genera contained 0.8% Noble agar, 0.5% SDS, 1.0% NaCl in 0.05 M tris-HCl buffer at pH 8.0. In most instances, the medium containing 0.8% Noble agar, 0.5% SDS and 1.0% NaN_3 was also sensitive (Fig. 1a and b). The medium of Tolin and Roane (14) was less satisfactory, since precipitin lines were usually fainter and less well defined, and spurious reactions were frequently observed (Fig. 1c). The unbuffered medium of 0.8% Noble agar, 0.5% SDS, and 1.0% NaCl (2) was also considered unsatisfactory for orchid tissues because strong nonspecific precipitin lines and heavy crystalline formations developed (Fig. 1d and e).

Fresh orchid leaf disks gave satisfactory results in immunodiffusion tests. This technique was reliable for CyMV with either boiled or unboiled leaf disks, although reactions for the former were sometimes slightly weaker. Antigen titers in orchid leaf extracts ranged from 1/64 to 1/256. Detection of ORSV was improved by heating leaf disks for 4 min over boiling water (Fig. 1a) prior to testing and, in one trial with this modification, accuracy was increased from 66 to 92% by using plants known to be infected. Maximum antigen titers in orchid leaf extracts for ORSV were 1/64 with heated samples.

Mature, flowering-sized plants infected with CyMV or ORSV were found in all 13 collections surveyed in this and earlier studies (15,17). Virus incidence ranged from 17.5 to 54.4%, and infected plants were detected among all major genera of cultivated orchids (Table 1). Incidence of CyMV exceeded that of ORSV in all 12 collections from the more prevalent virus in the cymbidium samples from Australia (57.1% ORSV and 5.7% CyMV of 35 samples).

None of 231 orchid seedlings was virus infected, including 98 derived from parent plants verified as being infected with either

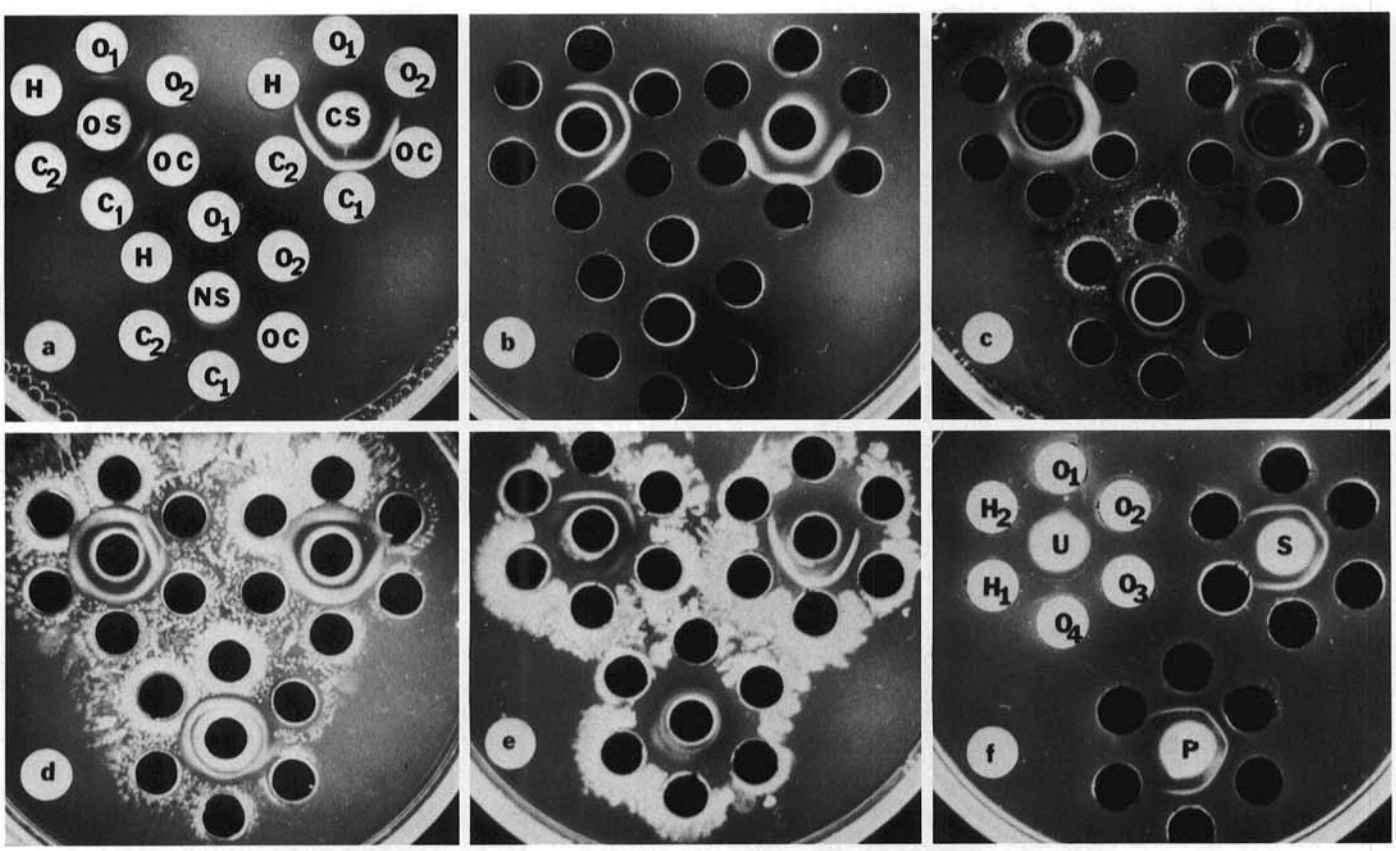

Fig. 1. Efficacy of various immunodiffusion media used to detect CyMV and ORSV. Center wells contain antiserum to pyrrolidine-treated ORSV (OS), untreated CyMV (CS), and normal serum (NS). For all patterns in **a–e,** peripheral wells in their respective positions contain orchid leaf disks (in water) heated (O_1), and unheated (O_2) ORSV-infected tissue; heated doubly infected tissue (OC); heated (C_1) and unheated (C_2) CyMV-infected tissue. Media were: **a,** 0.8% Noble agar, 0.5% SDS, 1.0% NaN_3; **b,** 0.8% Noble agar, 0.5% SDS, 1.0% NaCl in 0.05 M Trizma® buffer, pH 8.0; **c,** 0.8% Noble agar, 0.2% SDS, 0.7% NaCl, 0.1% NaN_3; **d,** 0.8% Noble agar, 0.5% SDS, 1.0% NaCl at 24 hr; and **e,** same medium as **d,** but at 48 hr. **f,** Effects of immunogen treatment on antiserum produced to ORSV: antiserum to untreated ORSV (U), antiserum to ORSV treated with 1.0% SDS and 0.1% 2-ME, boiled (S), and antisera to pyrrolidine-treated ORSV (P). Peripheral wells contain one drop of water and ORSV-infected orchid leaf disks (O_1, O_2, O_3, O_4), and healthy orchid leaf disks (H_1, H_2). Medium contains 0.8% Noble agar, 0.5% SDS, and 1.0% NaCl in 0.05 M Trizma® buffer, pH 8.0.

TABLE 1. Relative incidence of cymbidium mosaic and odontoglossum ringspot viruses in native and cultivated orchids

Genus	Native[a]	Cultivated
Angraecum	0/16	2,0/34[b]
Ascocendra[c]	···	2,0/15
Brassocattleya[c]	···	9,3/35
Brassolaeliocattleya[c]	···	77,4/258
Cattleya	0/84	47,4/160
Cymbidium	0/2	3,21/50
Dendrobium	0/21	10,4/96
Epidendrum	0/94	8,0/31
Laelia	0/19	7,0/21
Laeliocattleya[c]	···	77,2/241
Oncidium	0/66	4,0/17
Paphiopedilum	0/3	1,0/129
Phaius	0/1	2,0/2
Phalaenopsis	0/8	19,2/37
Potinara[c]	···	15,0/36
Sophrolaeliocattleya[c]	···	11,2/54
Vanda	0/30	12,0/36
Miscellaneous[d]	0/94	13,1/60
Totals	0/438	319,43/1312

[a] Plants collected from their native habitats or maintained less than 1 yr in cultivation after being collected. Ratios are the number virus-infected per the number collected.

[b] Ratios are the number of plants infected with CyMV, ORSV/ total number tested; ··· = no samples; all samples were mature, flowering-sized plants.

[c] Polygeneric hybrids that do not occur naturally as species.

[d] In the miscellaneous group 53 genera were represented (numbers represent the number of samples assayed if more than one): *Aerangis*, 8; *Aeranthes*, 8; *Aerides*, 11; *Anota*, 2; *Arundinia*, *Ascocentrum*, 15; *Brassia*, 10; *Brassavola*, 7; *Broughtonia*, 2; *Bulbophyllum, Cadetia, Camarotis, Catesetum, Cattleyatonia*, 3; *Ceratostylus, Chamaergis, Cycnoches, Cyrtopodium*, 2; *Diacreum*, 2; *Diaphananthe, Ephemerantha, Eria, Eulophia, Gastrochilus, Grammatophyllum*, 2; *Jumella*, 3; *Kingiella, Luisia*, 2; *Maxillaria*, 4; *Neobathiea, Odontoglossum*, 5; *Oeniella, Ornithocephalus*, 3; *Peristeria, Pescatoria, Philodota, Phragmopedium*, 6; *Pleurothallis*, 4; *Polystachya*, 3; *Rangaeris*, 3; *Renanthera*, 4; *Rhyncostylus, Rodriguezia*, 3; *Sarochilus, Schaumburgkia*, 11; *Sophronitis*, 3; *Spathoglottis, Spiranthes, Thrixspermum, Tricoglottis, Trigonidium, Vanilla, Vuylsteakeara.*

virus. None of 438 samples collected from their native habitats was infected (Table 1).

These assay procedures were successfully tested at three commercial nurseries. The personnel, who had no prior experience in serodiagnosis, encountered no difficulties in recording results, and two persons could index approximately 100 plants per hour.

DISCUSSION

Immunodiffusion procedures described in this study were suitable for use by orchid growers. Use of a paper punch greatly facilitated sampling. Also, accurate results were obtained with resuspended lyophilized antiserum and the buffered immunodiffusion medium, which contained NaCl instead of NaN_3.

Virus incidence was highest among cultivated, flowering-sized plants, whereas seedling orchids or wild plants were not infected (8,15,16,17). Cymbidium mosaic was much more prevalent among most orchid genera than was ORSV (10,15,17). However, in Australia the cool-growing, large-flowering cymbidiums were more commonly infected with ORSV than with CyMV (4,7).

Immunogen and antigen treatments were critical factors to consider when indexing for ORSV in immunodiffusion tests. Higher-titered ORSV antiserum was produced by degrading the immunogen in pyrrolidine or boiling in 1.0% SDS and 0.1% 2-ME. Antiserum to degraded ORSV reacted much more strongly than antiserum to nondegraded virus in the SDS-agar gel system, and stronger ORSV reactions were noted when antigens were heated over boiling water compared to unheated samples. Heating is presumed to cause capsid degradation, products of which would be expected to react in SDS-immunodiffusion systems against antiserum to degraded virus as previously noted (12).

The prospects of grower control of CyMV and ORSV are excellent if plants are seroindexed, infected ones rogued, and routine sterilization procedures are used, especially since neither virus has a natural vector (5,11).

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