Transmission, Movement, and Inactivation of Cymbidium Mosaic and Odontoglossum Ringspot Viruses

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ABSTRACT

Hu, J. S., Ferreira, S., Xu, M. Q., Lu, M., Iha, M., Pflum, E., and Wang, M. 1994. Transmission, movement, and inactivation of cymbidium mosaic and odontoglossum ringspot viruses. Plant Dis. 78:633-636.

Transmission and movement studies were conducted on orchids mechanically inoculated with cymbidium mosaic virus (CyMV) or odontoglossum ringspot virus (ORSV). Transmission of CyMV and ORSV to University of Hawaii (UH) *Dendrobium* hybrids was efficient; both viruses were detected in inoculated leaves a minimum of 3 days after inoculation. Cymbidium mosaic virus moved systemically from inoculated leaves to roots (minimum 10 days after inoculation) and then to other leaves (minimum 20 days after inoculation). Thirty-two of 33 CyMV-inoculated orchid plants were systemically infected. Systemic movement of ORSV took about 7 mo and occurred in only one of 38 inoculated orchid plants. Seven chemicals were evaluated for inactivation of CyMV on pruning tools for disease control. Skim milk was found to be effective, noncaustic, and inexpensive for the inactivation of CyMV inoculated on local lesion indicator host plants. However, when systemic host plants (orchids) were used in evaluation, skim milk and other chemicals were ineffective for inactivation of CyMV. A 1% concentration of NaOH inactivated both CyMV and ORSV, but 10 and 20% concentrations were phytotoxic.

Cymbidium mosaic potexvirus (CyMV) and odontoglossum ringspot tobamovirus (ORSV) are the most prevalent and economically important viruses infecting orchids worldwide (6,7,9). CyMV causes chlorotic to necrotic sunken patches on leaves and necrosis on flowers; ORSV induces necrotic rings on leaves and color breaking and distortion on flowers (6,8,9). Both viruses reduce plant vigor and growth rate, and reduce flower quality (8,9). They are not transmitted by natural vectors but are spread by contaminated tools and pots during division of plants and harvest of flowers (6). A recent survey (3) showed CyMV and ORSV are widespread in many orchid genera in Hawaii. The incidence of CyMV-infected seed-propagated *Dendrobium* hybrids produced by the University of Hawaii (UH) averaged 94% on some farms. ORSV was not detected in any of the more than 4,000 UH *Dendrobium* hybrid samples tested, but it was found in other orchid genera on the same farms (3). A possible reason for this difference is that UH *Dendrobium* hybrids may be resistant to ORSV infection.

Current recommended control methods for CyMV and ORSV involve sanitation practices and the use of chemicals to sterilize pruning tools (6,8). Sodium hypochlorite, skim milk, ethanol, Agribrom, and Physan have been used by orchid growers in Hawaii to inactivate the viruses on cutting tools, but their safety and effectiveness have not been determined.

To understand the epidemiology and potential management of these viruses, we studied the effectiveness of different virus inoculation techniques, measured the rate and extent of movement of CyMV and ORSV through UH *Dendrobium* hybrid orchids, and evaluated the effectiveness of several chemicals in inactivating the viruses. A preliminary report of this work was published (4).

MATERIALS AND METHODS

Plant materials and virus isolates. Mature plants of the University of Hawaii *Dendrobium* hybrid cultivars *D.* × Jaquelyn Thomas 'Uniwai Supreme' (UH232) and *D.* × Jaquelyn Thomas 'Uniwai Prince' (UH503) were used in the experiments. The plants were about 1 m tall with seven leaves per pseudobulb. Only one pseudobulb per orchid was used; the others were removed with sterilized tools. The orchids were tested for CyMV and ORSV by enzyme-linked immunosorbent assay (ELISA) prior to inoculation to be sure they were free of these viruses.

The CyMV and ORSV isolates were obtained from individual infected *D.* × Jaquelyn Thomas and *Cattleya* plants, respectively. The stock plants were tested by ELISA using specific antibodies to be sure that they were not mixedly infected by the two viruses. Inoculum was prepared by grinding 1 g of CyMV- or ORSV-infected orchid leaves in 10 ml of potassium phosphate-buffered saline (PBS, 0.05 M $NaHPO_4$, pH 7.2; 0.15 M NaCl) using mortars and pestles.

Virus transmission and movement. Three inoculation methods were used. In "rub" inoculation, the third leaf from the bottom of each pseudobulb was inoculated with CyMV or ORSV extracts. Carborundum was sprinkled on the surface of each inoculated leaf. Prepared virus extracts were rubbed on the surface of the leaves with a pestle, and the inoculated leaves were rinsed immediately with tap water for about 15 sec. In "slash" inoculation, the surface of the third leaf was slashed with a razor blade contaminated with leaf extract which contained virus. In "cut" inoculation, flower spikes (sprays) were excised using a razor blade contaminated with leaf extract which contained virus. Inoculated plants were kept in a greenhouse at 25–30 C and tested every other day (in one experiment every day) for virus infection by ELISA. In each test, leaf samples were collected from all the inoculated leaves with a sterilized razor blade. The transmission experiments were repeated three times.

When the inoculated leaves of the rub-inoculated orchid plants tested positive for CyMV or ORSV, movement of viruses was monitored. Samples from the roots and all leaves were collected at 5-day intervals and were tested individually by ELISA for the presence of virus.

Virus inactivation. Dilution end point tests were conducted to examine the effectiveness of different chemicals in inactivating CyMV and ORSV. Virus-infected leaves were ground and serially diluted 10-fold (up to 10^{-7}) in each of the following seven chemicals: skim milk (undiluted), liquid detergent (0.01, 0.1, or 1%), NaOH (0.1, 1, or 5%), commercial bleach (0.1, 1, or 5%), Physan (Consan Pacific Inc., Whittier, CA; 0.1, 1, or 5%), Agribrom (Great Lakes Chemical Co., West Lafayette, IN; 0.1, 1, or 5%), and ethanol (70, 80, or 90%). PBS was used as a control in inactivation tests. Diluted samples were inoculated on local lesion indicator plants (*Senna occidentalis* (L.) H. Irwin & Barneby for CyMV and *Chenopodium amaranticolor* Coste & Reyn. for ORSV) or orchid hybrids (UH232 and UH503). Leaves were lightly dusted with Carborundum and inoculated by rubbing with a pestle dipped in virus extracts. Inoculated leaves were rinsed immediately with tap water for about 15 sec. Plants were kept in a greenhouse at 25–30 C and checked

This is Journal Series 3894 of the Hawaii Institute of Tropical Agriculture and Human Resources.

Accepted for publication 2 March 1994.

daily for local symptoms from 7 to 14 days after inoculation. The orchid plants were tested by ELISA for virus infection at 7 and 14 days after inoculation. NaOH, commercial bleach, Physan, Agribrom, and liquid detergent at 10 or 20% concentrations were used in later experiments.

ELISA. Double antibody sandwich ELISA was done as described (2,4). Microtiter plates were coated with specific antibody in a sodium bicarbonate coating buffer at a concentration of 1 μg/ml, 100 μl per well. The plates were then incubated overnight at 4 C and washed with a mixture of PBS plus Tween 20 (PBS-Tween) three times, with the plates sitting for at least 3 min each time. The plant samples were prepared with extraction buffer at a dilution of approximately 1:10 and added to the plate at 100 μl per well. The plates were again incubated overnight at 4 C and later washed with the PBS-Tween three times for 3 min. Alkaline phosphatase-labeled specific antibody in enzyme conjugate buffer at a dilution of 1:2,000 was added and incubated for 4 hr at 30 C. The plates were washed again with PBS-Tween three times for 3 min. Substrate (*p*-nitrophenyl phosphate at 1 mg/ml) in 100 μl of substrate buffer was added to each well and incubated 1 hr at room temperature. Absorbance at 405 nm was measured with a Model 450 Microplate Reader (Bio-Rad Laboratories, Richmond, CA). Controls with virus extraction buffers, healthy samples, and virus-infected samples were included in all tests. A reaction was considered positive only if the absorbance was >0.1, which was at least three times the background mean of the healthy control.

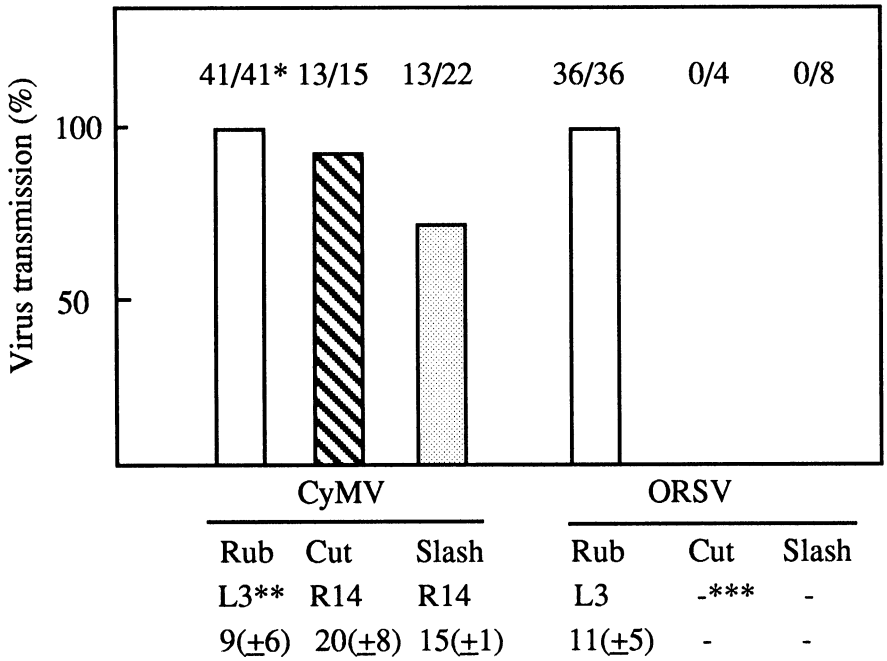

Fig. 1. Transmission of cymbidium mosaic virus (CyMV) and odontoglossum ringspot virus (ORSV) to UH *Dendrobium* hybrid orchids by three inoculation methods. * = Number of plants infected over number of plants inoculated. ** = Values indicate the minimum and average days (±SD) after inoculation for the first detection of the viruses in either leaf (L) or root (R) tissues. *** = Plants were not infected by ORSV when they were inoculated by cut or slash methods.

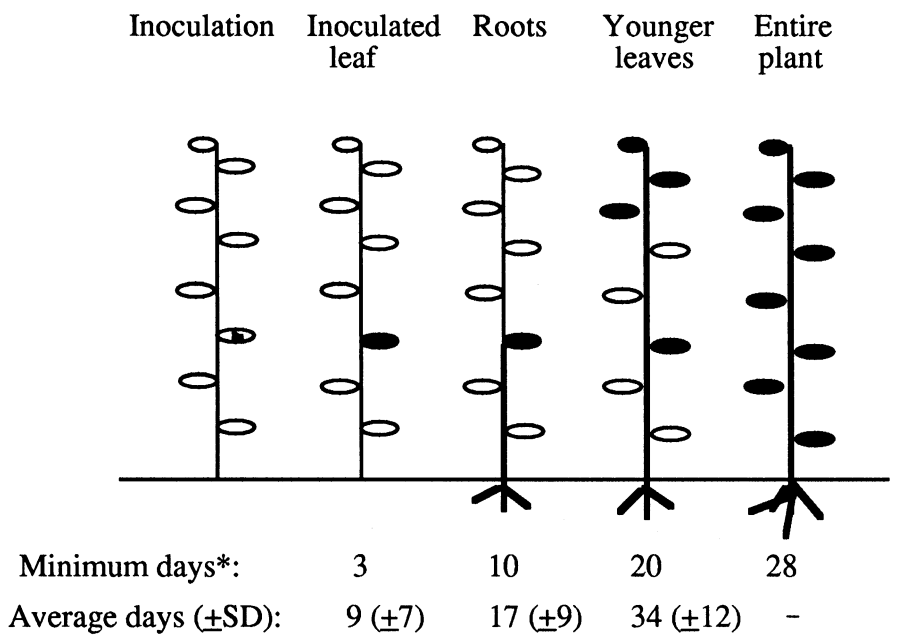

Fig. 2. Movement of cymbidium mosaic virus (CyMV) in infected UH *Dendrobium* hybrid orchids. Virus was rub inoculated on the third leaf of the plants, and samples from all leaves and roots were tested by enzyme-linked immunosorbent assay (ELISA) every 5 days. The bold parts represent positive reactions in ELISA. * = Values indicate the minimum number of days (from one of the tests) after inoculation and the average days and standard deviation (from all tests) needed for the detection of the virus.

Electron microscopy (EM) and Western blot. Electron microscopy and Western blot analysis were done as described previously (5).

RESULTS

Virus transmission and movement. Transmission of CyMV and ORSV by the rub inoculation method to UH *Dendrobium* hybrids was 41 of 41 and 36 of 36, respectively. Efficiency for CyMV was 13 of 15 and 13 of 22 for slash and cut inoculation methods, respectively (Fig. 1). It was first detected from inoculated leaves in the rub inoculation after 3 days, and was first detected from root tissues in the slash and cut inoculations after 14 days (Fig. 1). No ORSV was transmitted by the slash or cut inoculation methods (Fig. 1).

CyMV moved rapidly from rub-inoculated leaves to roots and other leaves of UH *Dendrobium* hybrid orchids. It was detected in roots 10 days after inoculation and in younger noninoculated leaves 20 days after inoculation (Fig. 2). It moved systemically in 32 of 33 inoculated UH *Dendrobium* hybrid plants. Although all inoculated leaves were ELISA-positive for ORSV, systemic movement of ORSV to noninoculated leaves was detected from only 1 of 38 inoculated UH *Dendrobium* hybrid plants 7 mo after inoculation.

Virus inactivation. Skim milk and ethanol were the most effective chemical treatments in preventing CyMV infection on local lesion indicator plants (Table 1). When CyMV suspension was mixed 1:1 with undiluted skim milk or ground in ethanol (70, 80, or 90%), no infections occurred. The 1 and 5% concentrations of NaOH, commercial bleach, and Physan also prevented infection, but the 0.1% concentrations did not (Table 1). Liquid

Table 1. Inactivation of cymbidium mosaic virus (CyMV) with different chemicals as evaluated on local lesion host, *Senna occidentalis*[a]

Virus dilutions	No. local lesions/no. inoculated leaves																			
	PBS[b]	Skim milk	Ethanol (%)			Commer. bleach (%)			NaOH (%)			Physan (%)			Liquid detergent (%)			Agribrom (%)		
			70	80	90	0.1	1	5	0.1	1	5	0.1	1	5	0.01	0.1	1	0.5	1	5
1:10	289/12	0/12	0/14	0/14	0/12	50/21	0/26	0/16	134/8	0/10	0/10	100/9	0/16	0/10	289/12	201/11	130/16	98/6	75/4	37/4
1:100	319/10	0/10	0/13	0/14	0/12	10/26	0/22	0/16	22/8	0/10	0/10	16/10	0/16	0/9	319/10	66/12	44/10	40/4	60/4	25/6
1:500	93/12	0/12	0/8	0/14	0/8	4/24	0/24	0/18	4/12	0/12	0/12	0/15	0/18	0/12	93/12	53/10	9/14	4/4	0/6	2/6
1:1,000	85/12	0/12	0/14	0/14	0/14	4/22	0/24	0/18	0/12	0/12	0/14	0/9	0/16	0/10	85/12	23/10	7/12	0/6	9/4	0/6
1:2,000	27/10	0/11	0/8	0/14	0/8	0/14	0/24	0/18	0/12	0/12	0/12	0/8	0/9	0/12	27/10	55/12	0/11	4/6	9/6	0/6
1:4,000	47/7	0/12	0/8	0/14	0/8	0/18	0/16	0/18	0/12	0/14	0/12	0/5	0/12	0/14	47/7	16/8	0/12	22/6	0/6	0/6
1:8,000	11/14	0/12	0/8	0/14	0/8	0/16	0/18	0/16	0/12	0/12	0/12	0/7	0/10	0/12	11/14	26/10	1/12	12/4	9/6	0/6
1:16,000	8/14	0/14	0/10	0/14	0/8	0/20	0/18	0/16	0/12	0/12	0/12	0/9	0/10	0/16	8/14	21/14	1/12	12/6	1/6	4/6

[a] Orchid leaves infected with CyMV were ground and diluted in each of the chemicals, and the mixtures were then inoculated on local lesion indicator plants (*S. occidentalis*). The plants were kept in the greenhouse at 25 C, and symptoms on local lesion host plants were recorded from 7 to 14 days after inoculation. Experiments were repeated 2–5 times.
[b] Phosphate-buffered saline.

detergent at 1% and Agribrom at 5% did not inactivate CyMV (Table 1).

Because skim milk was effective, safe, and inexpensive, it was selected for further evaluation. Crude leaf extracts of CyMV (1 g of leaf tissue in 10 ml of PBS) were mixed 1:1 with undiluted skim milk and incubated for different times (10, 5, or 1 min, or 30 or 15 sec, or mixed and used immediately) and used for inoculation. Virus infectivity was lost immediately in all combinations. Different concentrations of skim milk (10, 20, 30, 40, 50, 60, 70, 80, and 90%) were tested, and the minimum concentration of skim milk needed for virus inactivation was 30%. Heating skim milk at 90 C for 10 min did not reduce its ability to inactivate virus transmission. After skim milk treatment, only virus bundle aggregates were observed in electron microscopy, ELISA reactions were strongly positive, and Western blot analysis showed an intact coat protein band.

Although both skim milk and ethanol effectively inactivated CyMV when tested on local lesion hosts, they were not effective when tested on UH *Dendrobium* hybrid orchids (Table 2). Neither Physan, Agribrom, nor liquid detergent inactivated CyMV, even at the highest concentration tested (20%) (Table 3). Commercial bleach and NaOH inactivated CyMV, but they also caused injury to orchids at 10 and 20% concentrations (Table 3). Because commercial bleach and NaOH showed promising results, they were selected for further evaluation with more replications and dilutions on both CyMV and ORSV. A 1% concentration of NaOH inactivated both CyMV and ORSV, but commercial bleach at 2% concentration did not inactivate ORSV (Table 4). Results from further experiments showed that bleach at 5% concentration did not inactivate ORSV; 11 of 16 inoculated plants were infected.

Table 2. Inactivation of cymbidium mosaic virus (CyMV) with skim milk and ethanol, as evaluated on a systemic host, UH *Dendrobium* hybrid orchids[a]

Experiment	No. infected leaves/no. inoculated leaves		
	Skim milk (undiluted)	Ethanol (70%)	PBS
1	9/10	3/9	9/9
2	2/10	6/12	6/6
3	10/10	11/16	5/5
4	10/10	...	10/10
Total	31/40	20/37	30/30
	(77.5%)	(54.1%)	(100%)

[a] One gram of CyMV-infected orchid leaves was ground in 10 ml of skim milk, 70% ethanol, or phosphate-buffered saline (PBS), and the homogenate used to inoculate UH *Dendrobium* hybrid orchids. The plants were kept in the greenhouse at 25–30 C, and the inoculated leaves were tested for virus infection by enzyme-linked immunosorbent assay 7–14 days after inoculation.

Table 3. Inactivation of cymbidium mosaic virus (CyMV) with chemicals, as evaluated on UH *Dendrobium* hybrid orchids[a]

Chemicals	Concentrations of chemicals					
	2%		10%		20%	
	Infect.[b]	Injury[c]	Infect.	Injury	Infect.	Injury
Commercial bleach	0/12	−	0/12	+	0/12	++
NaOH	0/12	−	0/12	++	0/12	+++
Physan	5/12	−	4/12	++	5/12	+++
Agribrom	12/12	−	12/12	−	12/12	−
Liquid detergent	12/12	−	12/12	+	12/12	++

[a] Two grams of CyMV-infected orchid leaves were ground in 10 ml PBS and diluted 1:1 with 4, 20, or 40% concentration of test chemicals. The mixtures were used to inoculate UH *Dendrobium* hybrid orchids. The plants were kept in the greenhouse at 25–30 C, and the inoculated leaves were tested for virus infection by enzyme-linked immunosorbent assay 7 to 14 days after inoculation.
[b] Infection = number of leaves infected over number of leaves inoculated.
[c] − = No injury, + = slight injury (<5% leaf area burned), ++ = moderate injury (5–60% leaf area burned), and +++ = severe injury (>60% leaf area burned).

DISCUSSION

All three inoculation methods readily transmitted CyMV. These results demonstrate that CyMV can be transmitted when plants are repotted or flower sprays are harvested. Therefore, sanitation during handling of plants and harvesting of flowers is critical for preventing virus spread. The main difference between the rub, slash, and cut inoculation methods was the pattern of virus spread. Virus was first detected in the inoculated leaves of rub-inoculated plants; however, it was first detected in roots of plants inoculated by slash or cut, presumably because virus was delivered into the vascular tissue directly in slash and cut inoculations. Virus was found in the roots within weeks, and within a month it was found throughout the orchid plants. The CyMV is a stable virus that maintains a high concentration in plants (6). The ease of transmission, high concentration, rapid movement, and stability contribute to the widespread occurrence of CyMV in Hawaii's orchid nurseries.

In a previous survey, CyMV was detected in 90–100% of UH *Dendrobium* hybrids, but ORSV was never detected in these hybrids (3). In this study, we demonstrated that the UH *Dendrobium* hybrids are not resistant to infection by

Table 4. Inactivation of cymbidium mosaic virus (CyMV) and odontoglossum ringspot virus (ORSV) with commercial bleach and NaOH as evaluated on UH *Dendrobium* hybrid orchids[a]

Concentration (%)	Commercial bleach			NaOH		
	CyMV	ORSV	Injury	CyMV	ORSV	Injury
20	0/34[b]	0/33	++[c]	0/32	0/33	+++
10	0/34	0/31	+	0/32	0/33	++
2	0/33	15/30	−	0/36	0/33	+
1	3/32	18/31	−	0/33	0/32	−
0.5	16/29	17/20	−	4/32	1/23	−

[a] Virus-infected orchid leaves (2 g) were ground in 10 ml of phosphate-buffered saline and diluted 1:1 with 40, 20, 4, 2, or 1% bleach or NaOH. The mixtures were then inoculated on UH *Dendrobium* hybrid orchids. The plants were kept in the greenhouse at 25–30 C, and the inoculated leaves were tested by enzyme-linked immunosorbent assay for virus infection from 7 to 14 days after inoculation.

[b] Number of leaves infected over number of leaves inoculated.

[c] − = No injury, + = slight injury (<5% leaf area burned), ++ = moderate injury (5–60% leaf area burned), and +++ = severe injury (>60% leaf area burned).

ORSV. In fact, both CyMV and ORSV were easily transmitted to the inoculated leaves of UH *Dendrobium* hybrids. However, we were unable to consistently demonstrate systemic movement of ORSV in UH hybrids, although our ELISA results showed that ORSV did move systematically in other non-UH hybrid *Dendrobium* species. The results suggest that the UH hybrids are resistant to systemic movement of ORSV, which may be the reason why ORSV was not detected from UH hybrids in the earlier survey (3).

Skim milk was safe and inexpensive for inactivating CyMV inoculated to local lesion hosts. Based on results from biological, serological (ELISA and Western blot), and EM tests, we conclude that the inactivation of CyMV by skim milk is due to aggregation rather than to enzymatic digestion by proteinases in the milk. However, when orchid plants, a systemic host, were used in the evaluation experiments, skim milk was not effective for inactivation of CyMV. Previously, skim milk was reported to be useful for cleaning cutting tools to inactivate paprika mosaic virus (1). In our study, skim milk was not effective at preventing systemic infection of CyMV in orchids, and thus we do not recommend its use. We found that plants in which virus can move systemically are more sensitive than local lesion indicator hosts for evaluation of the chemicals. Our results show that although local lesion indicator plants are useful for the initial screening of chemicals for virus inactivation, it is important to confirm their effectiveness on orchid plants, in which the virus can move systemically.

Control of CyMV and ORSV by sanitation practices has been recommended for years (6,8). Various chemicals have been suggested for inactivation of plant viruses on cutting tools. Commercial bleach, skim milk, ethanol, Agribrom, and Physan have been tried by orchid growers in Hawaii; but the effects on CyMV were not verified. Bleach and NaOH have been advised for sanitation treatment for control of CyMV and ORSV (6), and their effectiveness was confirmed in this study. NaOH at 1% concentration inactivated both CyMV and ORSV and did not cause phytotoxic damage on orchid plants. Therefore, we recommend that growers use NaOH at 1% concentration to sterilize pruning tools. Our previous survey results showed that CyMV was not found in two nurseries employing strict sanitation practices (3), demonstrating that successful CyMV control can be obtained with strict application of sanitation practices.

ACKNOWLEDGMENTS

This study was supported in part by a grant from USDA Federal Floriculture Project 3894.

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