Stability, Host Range, and Distribution of Kalanchoë Mosaic Potyvirus in *Kalanchoë blossfeldiana*

Karen Husted, Senior Scientist, and **Karen Bech,** Scientist, Department of Plant Pathology and Pest Management, Danish Institute of Plant and Soil Science, DK-2800 Lyngby, Denmark

ABSTRACT

Husted, K., and Bech, K. 1996. Stability, host range, and distribution of *Kalanchoë* mosaic potyvirus in *Kalanchoë blossfeldiana*. Plant Dis. 80:211-214.

The distribution of Kalanchoë mosaic potyvirus (KMV) coat protein in *Kalanchoë blossfeldiana* was found to be very uneven, both in individual leaves and among leaves from different parts of the plant. Amounts of viral coat protein were highest in well-developed leaves in the middle of the plants and lowest in the older bottom leaves and in the small top leaves just appearing. For routine detection it is recommended to test leaf pairs number 3 to 5 from the top and to include at least two repetitions per plant. There was a high variation among different cultivars of *K. blossfeldiana* both in expression of symptoms and in coat protein concentration as measured by enzyme-linked immunosorbent assay (ELISA). Expression of symptoms did not correlate with ELISA values either in naturally infected or in mechanically inoculated plants, and many latent infections were noted. Symptoms were most pronounced in spring coinciding with vigorous plant growth. None of 17 other *Kalanchoë* spp. mechanically inoculated developed symptoms even though half were latently infected. KMV was infective in a 10^{-5} to 10^{-6} dilution of plant sap, and remained infectious after heating to 55°C, and after storage in sap at room temperature for 16 days.

The European *Kalanchoë blossfeldiana* Poelln. production in 1994 was estimated to amount to 120 million potted plants; of these, 35 million plants were produced in Denmark.

Kalanchoë mosaic potyvirus (KMV) was identified in *K. blossfeldiana* with growth reduction and a varying degree of green island mosaic (6), severity depending on the cultivar. KMV is widely distributed among cultivars. The Danish certification system for production of improved horticultural crops includes a testing program for *K. blossfeldiana*. KMV is not yet included as a testing requirement; however, several KMV-free cultivars are produced in specialized firms selling cuttings for growers. *Kalanchoë blossfeldiana* is propagated by cuttings from stock plants.

Spread and transmission of KMV takes place by cuttings and by aphids. Other possibilities for transmission are under investigation. The number of diagnostic species was very limited, in that only *Chenopodium quinoa* Willd. and *C. amaranticolor* Coste et Reyn. showed local lesions when inoculated with KMV, whereas 12 other common diagnostic species were symptomless (6).

Corresponding author: K. Husted
E-mail: dipsly@inet.uni-c.dk

Accepted for publication 20 November 1995.

Publication no. D-1995-1228-04R

Recommendations for sampling procedures are valuable in indexing programs. To determine which leaves to test in routine diagnosis, distribution of KMV coat protein in different parts of plants and differences in susceptibility and symptom expression among cultivars of *K. blossfeldiana* during different seasons have been studied. The objectives of our investigations were also to extend the host range studies to include a number of other *Kalanchoë* species. Finally, some physical properties of the virus were measured.

MATERIALS AND METHODS

The virus strain was previously characterized and was purified as described earlier (6). Purified virus was stored at –20°C after the addition of 1 vol of glycerol.

Thermal inactivation point, dilution end point, and longevity in vitro were determined by procedures described by Király (7). Expressed sap from KMV-infected *C. quinoa* was diluted 1:1 with 0.01 M phosphate buffer pH 7.0 prior to the treatments. Samples from each heat treatment (10 min at each temperature from 40 to 80°C with 5°C intervals), dilution (10^{-3} to 10^{-8} in 0.01 M phosphate buffer, pH 7.0), and time-interval tests (samples at room temperature from 1 to 40 days) were assayed by inoculation onto *C. quinoa*. Tests were done three times.

Double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA) was performed by a standard procedure (4) using the KMV-antiserum with a coating IgG concentration of 0.5 µg/ml and alkaline phosphatase–IgG conjugate dilution of 1:2,000 (6). Samples were prepared by homogenizing 0.5 g of leaf material pooled from two separate leaves in 5 ml of buffer. The association of viral coat protein with specific leaf area was investigated by tests of small leaf pieces (1.5 mm × 1.5 mm) taken from dark and light yellowish green areas from cvs. Pink Star, Charme, and Sally, showing distinct green

Table 1. Level of Kalanchoë mosaic potyvirus (KMV) coat protein in eight cultivars of *Kalanchoë blossfeldiana* as measured by enzyme-linked immunosorbent assay after mechanical inoculation of three young plants per cultivar

	Months post inocution (p.i.)				
Cultivar	**1 (n = 3)[x]**	**2 (n = 3)**	**2.5 (n = 9)**	**3 (n = 9)**	**5 (n = 9)**
TF Goldstrike[y]		1.89 ± 0.19 a[z]	0.82 ± 0.37 ab	1.03 ± 0.35 b	
PM Goldstrike	0.14 ± 0.04 c	1.42 ± 0.73 abc	1.37 ± 0.25 a	1.39 ± 0.42 a	0.95 ± 0.54 bc
PM Charme	1.70 ± 0.35 a	1.91 ± 0.12 a	0.42 ± 0.16 ef	0.51 ± 0.26 c	1.45 ± 0.24 a
TF Charme		1.75 ± 0.37 abc	0.70 ± 0.39 bcd	0.78 ± 0.39 bc	
Attraction		1.27 ± 0.02 abcd	0.72 ± 0.22 bc	1.02 ± 0.21 b	1.16 ± 0.21 ab
PM Rarakoe	0.65 ± 0.03 b	0.70 ± 0.15 d	0.63 ± 0.11 bcde	0.98 ± 0.27 b	1.22 ± 0.43 ab
TF Rarakoe	0.41 ± 0.09 bc	1.21 ± 0.29 bcd	0.46 ± 0.31 def	0.76 ± 0.29 bc	
PM Muna	0.15 ± 0.05 c	1.84 ± 0.26 ab	0.65 ± 0.28 bcde	0.50 ± 0.28 c	0.98 ± 0.32 bc
PM Beta	0.29 ± 0.07 bc	1.13 ± 0.50 cd	0.57 ± 0.19 cdef	1.04 ± 0.38 b	
PM Fame	0.16 ± 0.18 c	1.39 ± 0.17 abc	0.33 ± 0.19 f	0.88 ± 0.35 b	0.97 ± 0.32 bc
PM Isabella	0.57 ± 0.49 b	1.15 ± 0.74 cd	0.33 ± 0.19 f	0.52 ± 0.29 c	0.78 ± 0.17 C
LSD	0.39	0.67	0.24	0.30	0.32

[x] Number of samples per cultivar; n = 3, one sample from each of 3 plants. n = 9, three samples from each of 3 plants.
[y] Cuttings were taken from plants originating from two nurseries, PM and TF.
[z] Average absorbance readings ± SD. Values followed by the same letter within a column are not significantly different at $P = 0.05$. Due to differences in the duration of substrate incubation only values corresponding to the same time should be compared.

island symptoms. Virus distribution within leaves was investigated by individually testing 36 small pieces cut out from an area of about 1 cm^2.

Natural distribution in plants. Infected plants of the cvs. Splendor, Orange Kie, Keep Sake, Rarakoe, Charme, Camilla, and Isabella obtained from growers were tested. These plants were at the same stage of development and were grown vegetatively in a greenhouse. Samples were taken from bottom leaves, well-developed middle leaves (the fourth to fifth leaf pairs from the top), and newly developed top leaves (0.5 to 1 cm) four times during a year: March, July, October, and January. For each combination of cultivar and leaf position, two samples in March and four during the other seasons were tested individually by ELISA. At each time the amounts of coat protein at different leaf position were compared within each cultivar. Data analysis was done by a *t* test with the Proc GLM procedure of PC SAS 604 (SAS Institute, Cary, NC). Symptoms were noted in connection with leaf sampling.

Cultivar differences. Cuttings were established from eight KMV-free cultivars (Table 1). For the most common cultivars (Rarakoe, Charme, and Goldstrike) cuttings were taken from two mother plants, originating from different nurseries, referred to as TF and PM. At the three- to five-leaf-pairs stage, three plants of each cultivar were inoculated with 10 µg of purified KMV diluted in 0.5 ml of 0.03 M phosphate buffer pH 7.6 containing 4% polyethylene glycol (PEG 6000) with Carborundum added. Inoculated plants were observed at 20 to 30°C for 3 to 5 months. Symptoms were registered and ELISA performed 1, 2, 2.5, 3, and 5 months post inoculation (p.i.). In the first two tests, one sample from each of the three plants was taken and mean as well as standard deviation of these calculated. In the last three tests, three samples were taken from each plant giving a total of nine samples per cultivar. The mean value and the standard deviation of these nine samples were calculated. At each time the amounts of coat protein in the different cultivars were compared. Data were analyzed by a *t* test with the Proc GLM procedure of PC SAS 604.

Transmission to other *Kalanchoë* species. Cuttings were established from 18 different *Kalanchoë* species, including *K. blossfeldiana* cv. Attraction, which was used as reference. The mother plants were first tested and found to be free of KMV. Four plants of each species were inoculated as described above, and observed in a greenhouse for 3 months. Virus coat protein was monitored by ELISA tests 1, 2, and 3 months p.i., covering both local and systemic infections.

RESULTS

The highest temperature to which samples could be heated and remain infectious was 55°C. Samples remained infectious for 16 days at room temperature, but had no infectivity at 32 days. The dilution end point was 10^{-5} to 10^{-6}.

Natural distribution in plants. KMV coat protein was found to be distributed in all parts of the plants that were tested but the concentration showed large variations, even among leaves at the same position on the plant (Fig. 1). Healthy *K. blossfeldiana* plants gave absorbance values of 0.01 to 0.05, and are not included in figures and

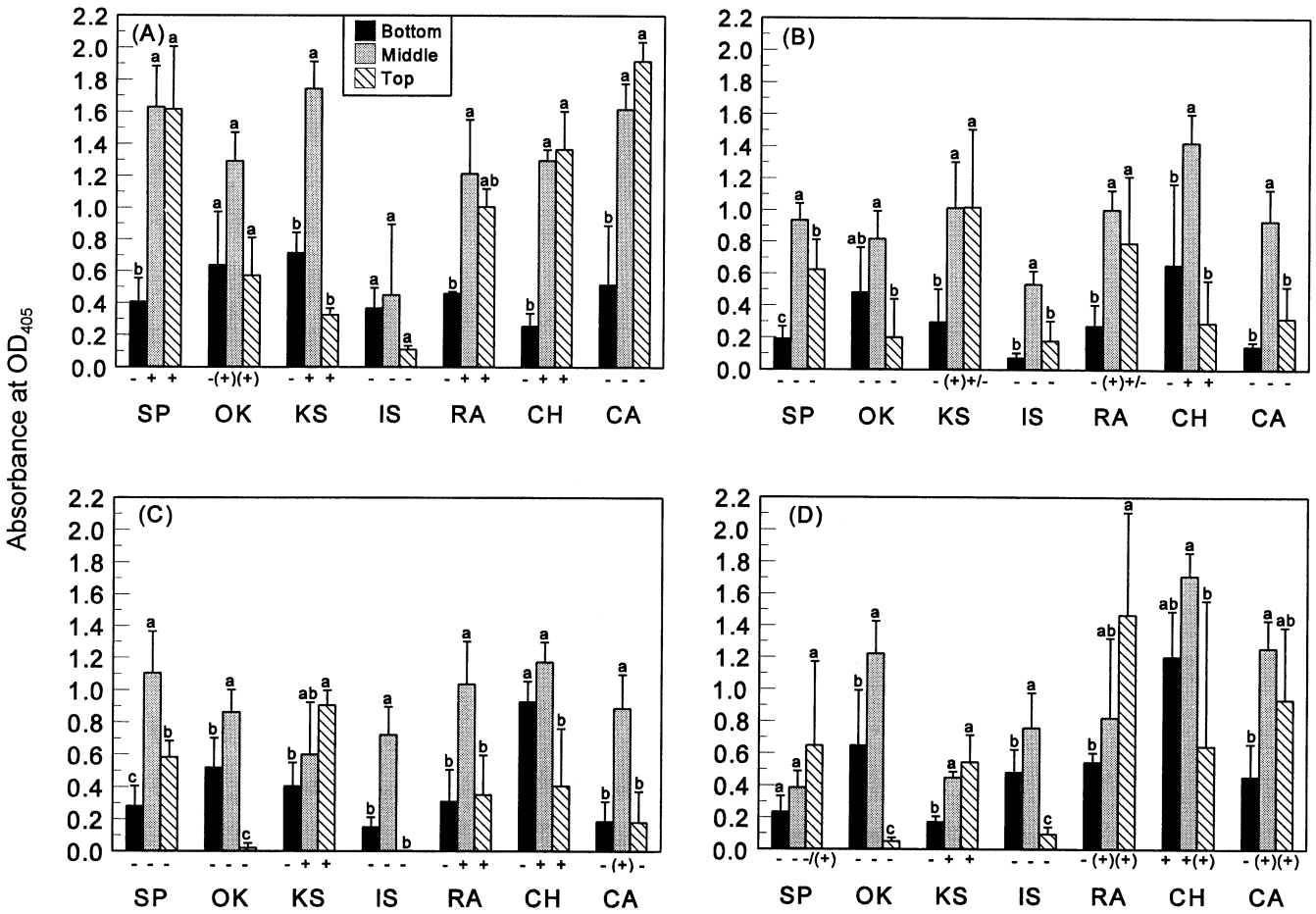

Fig. 1. Distribution of Kalanchoë mosaic potyvirus (KMV) coat protein in three parts (bottom, middle, top, shown by different types of bars) of different naturally KMV infected cultivars of *Kalanchoë blossfeldiana* plants measured by enzyme-linked immunosorbent assay four times during the year. SP = cv. Splendor; OK = cv. Orange Kie; KS = cv. Keep Sake; IS = cv. Isabella; RA = cv. Rarakoe; CH = cv. Charme; CA = cv. Camilla. Bars show mean absorbance values at 405 nm ± standard deviation. Bars capped with the same letter for each cultivar are not significantly different at $P = 0.05$. Symptoms in the various plant parts are indicated as + (positive), – (negative), (+) (weak and uneven). n = number of samples at each position for each cultivar. Due to differences in incubation times comparisons should only be done at each separate time. **(A)** 3/31/93, n = 2. **(B)** 7/13/93, n = 4. **(C)** 10/13/93, n = 4. **(D)** 1/14/94, n = 4.

tables. There was a tendency for the highest concentration to be found in the well-developed leaves taken from the middle of the plants and the lowest concentration in the older leaves. The concentration in the small top leaves varied considerably, but some cultivars had a high concentration in these leaves (e.g., Keep Sake, Rarakoe, and Splendor), whereas others usually had a low concentration (e.g., Orange Kie and Isabella). The differences in absorbance values between the top leaves and the middle leaves were lower in March than in July and October.

Symptom expression also varied considerably among cultivars. There was no correlation between severity of symptoms and amounts of KMV coat protein as measured by ELISA (Fig. 1). Cultivars Orange Kie, Isabella, and Camilla were latently infected in most tests. Symptoms were distinct in Charme, which has light green leaves, whereas they were difficult to see in cultivars with thick dark leaves, such as Splendor and Orange Kie. It was also difficult to distinguish viral symptoms in Isabella, which naturally has mosaic-like leaves. Symptoms were generally most pronounced during spring, milder during the summer, and were again very distinct in some cultivars during autumn and winter. Symptoms were most clearly seen in the uppermost three to four leaf pairs, mainly because these leaves are lighter green than the older leaves. Viral coat protein was also detected in flower parts and roots, but in lower concentration than in leaves of the same plants (data not shown).

In the test of small leaf pieces taken from dark green or yellowish areas, from leaves with distinct green islands, all samples from the yellowish areas were positive, whereas most samples from the dark green areas were negative. The few positive samples from the green areas had absorbance values much lower than values from the yellowish areas. The yellowish areas had a nonhomogeneous distribution of the viral coat protein. When tissue selected randomly from asymptomatic Fame or Goldstrike leaves was tested in a similar way, the standard deviation among the absorbance values for the small pieces was low compared with the deviation found among pieces from clearly symptomatic Charme leaves (data not shown). These results suggest a more uniform distribution of KMV in the cultivars with sparse symptoms.

Cultivar differences. Symptom expression in infected plants from growers varied much among cultivars. To perform more controlled investigations on cultivar differences, artificial inoculations of healthy cuttings from eight cultivars of *K. blossfeldiana* were made. The plants were mechanically inoculated in spring, which normally is the optimum time for symptom expression. Resulting symptom intensity was highly variable, mainly due to differences in color and leaf morphology (Fig. 2). The earliest green island symptoms were observed in Charme, Attraction, and Rarakoe after only 14 to 16 days. Less distinct symptoms were seen in Muna and Goldstrike, for the last-mentioned the grayish, dark-colored leaves tended to mask the symptoms. Finally, Beta, Fame, and Isabella showed no or weak and indistinct symptoms. For all cultivars the symptoms were most distinct in younger leaves. A pronounced growth reduction and reduced leaf size were observed in Goldstrike and less pronounced in Rarakoe, Charme, and Isabella, compared with non-inoculated plants of the same cultivar.

The amounts of viral coat protein as measured by ELISA are summarized in Table 1. Since the absorbance readings were made after varying duration of substrate incubation, it is unfortunately not possible to follow the disease development as a function of time. A significant difference among the readings from the three plants representing each cultivar was found only in a few cases. All the samples taken from each cultivar were accordingly pooled for analysis of the variation among cultivars. The rating of the cultivars based on the amounts of viral coat protein is not exactly the same at the different sampling times. It is, however, reasonable to propose three groups, with Goldstrike, Charme, and Attraction as the cultivars with the highest amounts of KMV coat protein, Rarakoe, Muna, Beta, and Fame intermediate, and Isabella with the lowest amounts.

Transmission to other *Kalanchoë* species. Purified KMV was inoculated onto 18 species of *Kalanchoë*, including *K. blossfeldiana* cv. Attraction. No symptoms, either local or systemic, developed in any of the species except for the mosaic in *K. blossfeldiana*. Half of the species were, however, latently infected (Table 2). Natural infection has only been tested in two to three plants per species and all plants were found to be uninfected.

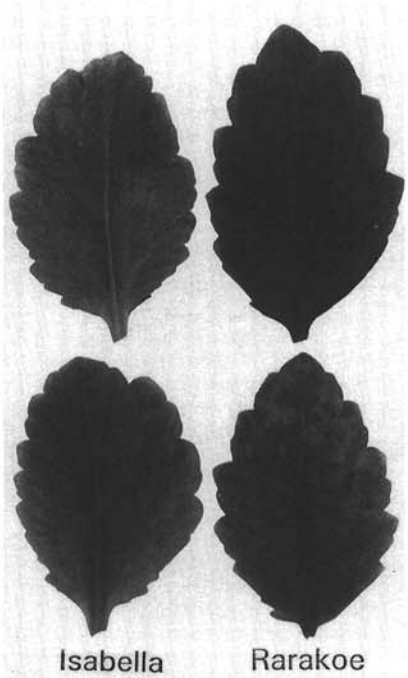

Fig. 2. Two cultivars of *Kalanchoë blossfeldiana* 1.5 months after mechanical inoculation with purified Kalanchoë mosaic potyvirus (KMV): Isabella (showing no symptoms) and Rarakoe (showing distinct green island mosaic). Top: healthy leaves. Bottom: KMV-infected leaves, both tested positive in enzyme-linked immunosorbent assay.

Table 2. Infection by Kalanchoë mosaic potyvirus (KMV) in *Kalanchoë* species (four plants per species) inoculated with purified KMV as determined by symptoms and enzyme-linked immunosorbent assay

		Relative absorbance readings[x]	
Species	**Symptoms**	**Local[y]**	**Systemic[z]**
K. blossfeldiana cv. Attraction	+	+++	+++
K. tubiflora	–	+++	+++
K. daigremontiana	–	+	++
K. mariko	–	+	++
K. blossfeldiana × *manginii*	–	+	+
K. fedtschenkoi	–	+	+
K. pinnata	–	+	+
K. multi	–	–/+	+
K. manginii	–	+	+
K. gracilipes	–	+	–/+
K. tomentosa	–	+	–/(+)
K. hybrida Tessa	–	+	–
K. hildebrandtii	–	–	–
K. laciniata	–	–	–
K. flammea	–	–	–
K. scapigera	–	–	–
K. kewensis	–	–	–
K. marmorata	–	–	–

[x] Absorbance values compared with the value of *K. blossfeldiana* cv. Attraction.
[y] Local infections measured 1 and 2 months post inoculation (p.i.). One sample per plant.
[z] Systemic infections measured 1, 2, and 3 months p.i. One sample per plant 1 and 2 months p.i., 2 samples per plant 3 months p.i.

DISCUSSION

The natural distribution of KMV coat protein in *K. blossfeldiana* plants was found to be very uneven. This agrees with the general observation for systemically infecting plant viruses (9), and is shown clearly for pelargonium line pattern virus (2).

The thermal inactivation point (55°C) for KMV resembles that found for other potyviruses (5). The longevity in vitro was rather long and the dilution end point was high (10^{-6}) compared with other potyviruses (5). Both findings suggest that KMV could spread easily in nurseries, either in connection with the vegetative propagation, by the watering system or through soil. These potential mechanisms for spread are being investigated.

During summer and autumn the level of KMV coat protein was lower in the top leaves than in mature leaves, whereas the level at the two positions was about the same in March. The higher level seen in March could be a result of the vigorously growing plants and thereby an intensive transport, including also KMV, to the top part of the plants. In contrast, the lower level found later on could be explained by rather high temperatures (25 to 30°C) during spring and early summer suppressing the virus and also the growth of the plants. Preliminary results from controlled heat treatment in combination with mini cuttings and meristem tip culture have confirmed that KMV is eliminated by high temperature.

Corresponding to results reported for cucumber mosaic virus (CMV) in tobacco leaves (8), preliminary trials showed that *K. blossfeldiana* leaves that developed during a 3-week period at a constant temperature of 30°C showed no symptoms although a high level of viral coat protein was detected, whereas leaves that developed after the plant was returned to 22°C showed typical green island symptoms. A similar temperature influence might explain the weakening of distinct KMV symptoms during summer.

Cultivars of *K. blossfeldiana* show very different and often seasonally dependent symptoms. Due to the aphid transmissibility of KMV, latent infections obviously are a possible hidden infection source in the nurseries.

Expression of symptoms seems to depend on at least two factors: the distribution of virus within the individual leaves, and the natural color and leaf morphology of the plants. In cultivars of *K. blossfeldiana* with distinct green island symptoms, viral coat protein is found primarily in the light yellowish areas of the leaves: the virus concentration in the dark green islands is very low. This corresponds to observations made for another potyvirus, watermelon mosaic virus in pumpkin leaves (11), and for other viruses, e.g., CMV (8) and tobacco mosaic virus (TMV) (1,10), both in tobacco leaves. The nonuniform distribution even within yellowish areas supports observations made for TMV (10). Uneven distribution of coat protein within individual leaves of some cultivars can explain the variation among ELISA values for leaves at the same plant position, since these values depend strongly on the ratio of dark to light area in the part of the leaf used for ELISA. The natural appearance of the cultivars differs in leaf color (light and dark green) and in the thickness of leaves. Lighter green colors favor observation of green islands.

Corresponding to this, younger light green leaves show the most typical symptoms, whereas symptoms are only very seldom seen in the old, darker green, thicker leaves, although viral coat protein is detected by ELISA. The explanation could again be either the different leaf appearance or differences in the distribution of the virus within the leaves. When older leaves without symptoms are compared with younger leaves with symptoms taken from the same plant, the distribution within the old leaves is more even.

The observation that half of the 18 *Kalanchoë* species investigated became latently infected points to the possibility of a hidden source of infection in nurseries where species other than *K. blossfeldiana* are produced. In this study infections were made by mechanical inoculations only. Aphid and graft transmission could give different results.

Two earlier reports describe a mosaic-like disease in *K. flammea* (3), *K. laciniata, K. flammea,* and *K. daigremontiana* (12). In all cases virus is proposed as the cause. These putative viruses are, however, almost certainly not KMV because the diseases could not be transmitted by mechanical means (mechanical transmission to *K. blossfeldiana* was also tested and found unsuccessful). Secondly, the symptoms described are different from those caused by KMV.

In connection with an indexing program, a recommendation for routine tests for KMV can be made. We recommend taking samples from the third to fifth leaf pairs from the top, and testing one to three separate samples from each plant, depending on plant size. These leaves seem to have the highest KMV content throughout the year. Candidate material for stock plants should be tested twice with a 3-month interval in between to allow for renewal by top cuttings. Since symptoms are most pronounced in spring coinciding with vigorous plant growth, this is the optimum season for growers to observe symptoms and select breeding material, if the plants are not tested by ELISA. With the help of such an indexing and certification program, a high number of KMV-free cultivars have been established in Denmark.

LITERATURE CITED

1. Atkinson, T. G., and Matthews, R. E. F. 1970. On the origin of dark green tissue in tobacco leaves infected with tobacco mosaic virus. Virology 40:344-356.
2. Bouwen, I., and Maat, D. Z. 1992. Pelargonium flower-break and pelargonium line pattern viruses in the Netherlands; purification, antiserum preparation, serological identification, and detection in pelargonium by ELISA. Neth. J. Plant Pathol. 98:141-156.
3. Burnett, H. C., and Long, R. A. 1962. Mosaic, a new virus disease of *Kalanchoë flammea*. Plant Dis. Rep. 46:692-693.
4. Clark, M. F., and Adams, N. A. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for detection of plant viruses. J. Gen. Virol. 34:475-483.
5. Hollings, M., and Brunt, A. A. 1981. Potyvirus group. No. 245 in: Descriptions of Plant Viruses. Set 15. B. D. Harrison and A. F. Murrant, eds. CAB, The Holywell Press Ltd., Oxford, England.
6. Husted, K., Bech, K., Albrechtsen, M., and Borkhardt, B. 1994. Identification, partial sequencing, and detection of a potyvirus from *Kalanchoë blossfeldiana*. Phytopathology 84: 161-166.
7. Király, Z., ed. 1970. Methods in Plant Pathology, with Special Reference to Breeding for Disease Resistance. Academy Kiadó, Budapest.
8. Loebenstein, G., Cohen, J., Shabtai, S., Coutts, R. H. A., and Wood, K. R. 1977. Distribution of cucumber mosaic virus in systemically infected tobacco leaves. Virology 81:117-125.
9. Matthews, R. E. F. 1991. Plant Virology. 3rd ed. Academic Press, San Diego, CA.
10. Rezende, J. A. M., and Sherwood, J. L. 1991. Breakdown of cross protection between strains of tobacco mosaic virus due to susceptibility of dark green areas to superinfection. Phytopathology 81:1490-1496.
11. Suzuki, N., Kudo, T. Shirako, Y., Ehara, Y., and Tachibana, T. 1989. Distribution of cylindrical inclusion, amorphous inclusion, and capsid proteins of watermelon mosaic virus 2 in systemically infected pumpkin leaves. J. Gen. Virol. 70:1085-1091.
12. Uschdraweit, H. A. 1963. Viruskrankheiten in der Gattung *Kalanchoë (Crassulaceae)*. Nachrichtenbl. Pflanzenschutzdienst in DDR. 15:182-184.