Squash Mosaic Virus Detection in Individual Seeds and Seed Lots of Cucurbits by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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Enzyme-linked immunosorbent assay (ELISA) done in polystyrene plates readily detected squash mosaic virus antigen in extracts from seed coats, papery layers, and distal halves of embryos from individual cucurbit seeds. Antigen presence in the embryo was independent of that in the seed coat or papery layer. Plants produced from the germinative halves of the same embryos were observed for embryonic transmission. No embryos judged virus-free by ELISA produced virus-infected plants but several embryos that contained antigen detectable by ELISA produced virus-free plants. A 90% probability of detecting the equivalent of one virus-infected melon seed in 400 was obtained when ELISA was done with polystyrene beads as the solid phase. Virus antigen was detected on unwashed pollen of several cucurbits but not on washed or washed and broken pollen.

Squash mosaic virus (SqMV) is a multicomponent, seedborne, beetle-transmitted virus with a narrow host range. The earliest reports of a seedborne virus in cucurbits, probably SqMV, were those of McClintock (19) and Kendrick (13). Since Freitag (8) characterized SqMV in detail, it has been reported worldwide in cucurbits (14,15,18,23), probably carried in infected seed. Infection by SqMV reduces the size and weight of melon fruits (20) and the number of melon fruits per plant and delays maturity (1).

Serological and infectivity tests have been used in studies of the mechanism of seed transmission and virus distribution within the seed. Cucurbit seeds offer an advantage in such tests because their size and shape allow them to be divided easily into a distal half and a germinative half. The distal half can be tested for virus and the germinative half can produce a plant, providing an excellent check of the validity of the test. Radioautography (21), immunofluorescence, and immunodiffusion (2) were not sensitive enough to detect virus in seeds until they had germinated for 3–6 days. Assays on systemic and local lesion hosts detected virus in imbibed seed, but a small percentage of virus infection was missed (2). Enzyme-linked immunosorbent assay (ELISA) is a sensitive technique for studying the nature of seed infection and provides quantification of viral antigen. The ease and rapidity with which large numbers of samples can be tested by this procedure has led to the screening of bulk seed (7,11,12,16,17).

The objectives of this study were to develop ELISA for SqMV detection, to study the virus-embryo relationship, and to test for virus in pollen and bulk seed.

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MATERIALS AND METHODS

Virus source and purification. The virus isolate was that used by Alvarez and Campbell (2). Virus was increased in and purified from 1-mo-old pumpkin plants (*Cucurbita pepo* var. *pepo* L. 'Small Sugar') by the procedure of Alvarez and Campbell (2), except leaves were frozen overnight before grinding rather than freezing the crude extract.

Production of infected seed lots. Seeds of the following hosts were planted in experimental plots at the University of California at Davis: Small Sugar pumpkin, cantaloup (*Cucumis melo* L. var. *reticulatus* Ser. 'PMR-45' and 'Rocky Ford'), honeydew melon (*C. melo* L. var. *inodorus* Naud.), acorn squash (*Cucurbita pepo* var. *pepo* L. 'Table Ace'), yellow crookneck squash (*C. pepo* var. *melopepo* (L.) Alef. 'Cracker'), butternut squash (*Cucurbita moschata* (Duchesne) Poir.), and buttercup squash (*C. maxima* Duchesne). Seedlings were inoculated mechanically when 2 wk old and again when 4 wk old if they were symptomless. One month later, all plants were tested for SqMV infection by agar double diffusion, and uninfected plants were removed. Seeds were collected as the fruits matured and combined into one lot for each host. Two months after harvest, five replicates of 100 seeds of each cultivar were planted in flats of pasteurized potting mix in the greenhouse to determine the percentage of embryonic transmission. Infected plants were determined visually by symptoms in the first three leaves. In questionable cases, agar double-diffusion assays were used to confirm embryonic transmission. This procedure is referred to as the "grow-out test."

Fourteen melon seed lots including cantaloup, honeydew, and Persian cultivars were obtained from seed companies. Lots were coded and the results of the grow-out tests that had been done previously at the companies were not provided until after the lots had been tested by ELISA.

Seed preparation for ELISA. All seeds used for ELISA were soaked overnight in distilled water. For assays of individual seed parts, seeds were separated with sterilized forceps and scalpel into seed coat, papery layer (remnants of the nucellus and endosperm), and embryo. Whole seeds or dissected embryos were cut in half transversely. The distal half was used for ELISA or local lesion assays. The germinative half was either stored in a gelatin capsule at 4 C until needed for other tests or planted in vermiculite in a 100-ml plastic pot. Pots were kept in a growth chamber at 27 C and watered with dilute nutrient solution. Plants were observed for virus infection until the three- to four-leaf stage.

Half seeds or dissected parts were placed in individual wells in a Plexiglas board and triturated with a clean glass rod in 600 µl of extraction buffer that contained phosphate-buffered saline (PBS) (0.02 M phosphate + 0.15 M sodium chloride, pH 7.4), 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate), 2% polyvinylpyrrolidone (mol wt 40,000), 0.2% bovine serum albumin (BSA), 0.02% sodium azide, 5% (w/v) mannose, and 5% (w/v) glucosamine. Mannose and glucosamine added to the extraction buffer after preliminary tests without the sugars gave lower ELISA absorbance values for infected half embryos than for infected seed coats and papery layers.

Seed samples were prepared by weight after determining the average seed weight in each lot. After seeds were soaked overnight, the water was removed and replaced by 10 volumes of extraction buffer per gram of dry seed. Samples were ground in a Polytron homogenizer with a PT20ST generator (Brinkmann Instruments, Westbury, NY) and centrifuged at 4,000 *g* for 5 min. The supernatant was poured into a centrifuge bottle that had been coated with 2% BSA in PBS-Tween (PBS + 0.05% [v/v] Tween 20) by incubation for at least 1 hr at 25 C to prevent antigen binding to the container.

Plate ELISA procedure. The antiserum (precipitin titer 1:1,024) was prepared by Alvarez and Campbell (2). Before use, antibodies to leaf proteins were absorbed by mixing the antiserum with an extract of virus-free Small Sugar pumpkin leaves (1:10 [w/v] in 0.05 M phosphate buffer, pH 7.5) and incubating for 1 hr at 37 C and 1 hr at 4 C. The supernatant from centrifugation at 3,000 *g* for 10 min was treated by a modified procedure of Clark and Adams (5). Immunoglobulin (Ig) was purified by ammonium sulfate precipitation and chromatography on diethylaminoethyl cellulose (Whatman DE 52). Coating Ig was stored at 1 mg/ml in PBS. Conjugate with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) was prepared by the method of Wilson and Nakane (24), except the mixture was not chromatographed. Both coating and conjugate Ig were tested at 0.5–2 µg/ml. Coating Ig was diluted in carbonate buffer, pH 9.6, and 200 µl/well was added to MicroELISA plates (Immulon I, Dynatech Laboratories, Inc., Alexandria, VA). After incubation for 1 hr at 37 C, plates were washed three times with PBS-Tween with 3–5 min between each wash. Test samples were then added, with each sample duplicated at least once. Each plate had a virus-free control consisting of a part of a Small Sugar pumpkin seed from a virus-free lot and a positive control of purified virus or leaf extract from an infected plant. Incubation was for 1 hr at 37 C and the plates were washed as before. Conjugate diluted in conjugate buffer (the same as the extraction buffer but without sodium azide and sugars) was added and the plates incubated for 1.5 hr at 37 C. The substrate was *o*-phenylenediamine (Sigma) at 0.7 mg/ml in citrate-phosphate buffer, pH 5.0, containing 0.05% (v/v) H_2O_2. After 5 min, absorbances ($A_{450\ nm}$) were read with a Titertek Multiskan plate reader (Flow Laboratories, Inc., Inglewood, CA) and averaged for each sample.

Bead ELISA procedure for seed lots. In a preliminary plate ELISA, SqMV antigen was detected in ≤50% of the samples of seed lots with 5% or less embryonic transmission. The large volume of buffer needed to grind the large, tough seeds coupled with the small sample applied to the ELISA plate presumably reduced the probability of detecting virus. To overcome this problem, a bead ELISA system, modified from that of Chen et al (3), was developed so that an Ig-coated, polystyrene bead could bind small amounts of antigen distributed throughout the seed extract.

The buffers, temperatures, washing procedure, and coating and conjugate concentrations were the same as for the plate assay. After the beads (6.4 mm in diameter, Precision Plastic Ball Co., Chicago, IL) were coated and washed, one was placed in each seed extract and incubated without agitation at 37 C. Each group of samples always included two controls: an extract of 100 virus-free honeydew melon seeds and an extract of virus-infected leaves (1:1,000 [w/v] in extraction buffer). After 2 hr, the extracts were removed by aspiration and the beads transferred to BSA-coated glass test tubes, where they were washed and mixed with conjugate for 1.5 hr. The conjugate was removed, the beads were washed and transferred to clean test tubes to which substrate was added for 10–15 min. Duplicate samples of the reactant mixture were transferred to a clean ELISA plate and absorbances measured in the plate reader.

In initial tests with beads, healthy seed extracts often produced high absorbance values. These were eliminated by mixing 0.5 ml of the extract of 100 virus-free seeds ground in conjugate buffer with 25 ml of conjugate. The mixture was incubated for 2 hr at 37 C. The supernatant recovered after centrifugation at 12,000 *g* for 5 min was used in the bead ELISA.

Pollen. Pollen was collected from flowers of virus-infected Rocky Ford cantaloup, honeydew melon, Small Sugar pumpkin, Table Ace squash, buttercup squash, and butternut squash. Pollen was also collected from virus-free cucumber (*Cucumis sativus* L. 'Beit Alpha'), honeydew melon, and *C. metuliferus* E. H. Mey. ex Schrad. Three treatments were compared: unwashed pollen, washed pollen, and washed, broken pollen. Pollen was suspended in buffer (0.1 M phosphate, pH 7.5) and tested directly in plate ELISA for the unwashed treatment. Washing was done by either one of two methods: 1) pollen suspended in buffer was centrifuged (150 *g* for 5 min), the pellet resuspended in fresh buffer, and the process repeated three times; or 2) the pollen suspended in buffer was collected on a cellulose acetate filter (5-µm pore diameter). The pollen was washed off the filter with fresh buffer and the process was repeated twice. Pollen grains were broken by placing drops with washed pollen on glass slides and freezing to liberate any internal viral antigen. Breakage was confirmed by light microscopy and the drops were transferred to ELISA plates.

RESULTS

Embryonic transmission. The following average percentage of embryonic transmission and standard deviations were obtained in the grow-out tests with seed produced at Davis: Small Sugar pumpkin 12.1 ± 7.1%, PMR-45 contaloup 4.8 ± 1.8%, Rocky Ford cantaloup 16.8 ± 8.0%, honeydew melon 2.6 ± 0.8%, acorn squash 4.2 ± 1.9%, cracker squash 0.4 ± 0.9%, buttercup squash 5.3 ± 4.2%, and butternut squash 11.0 ± 5.3%.

Plate ELISA characteristics. The optimum concentration for economy and sensitivity for both coating and conjugate

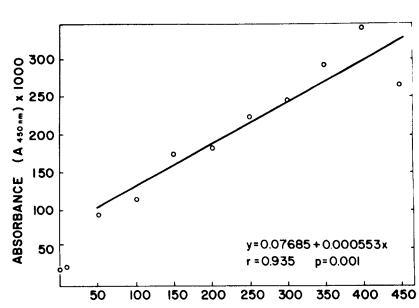

Fig. 1. Relationship between squash mosaic virus concentration and absorbance in plate enzyme-linked immunosorbent assay. Each data point is the average of three duplicate samples. Regression line is calculated for 50–450 ng/ml.

was 1 μg Ig/ml. With these concentrations, purified virus was always detected at ≥50 ng/ml but not at 10 ng/ml (Fig. 1). This was consistent for each of three preparations of coating Ig and conjugate.

The absorbance values of half seeds or dissected seed parts formed a continuous distribution. Determination of whether a value was positive or negative was made by a combination of methods. The average of the virus-free control (background) plus three standard deviations as suggested by Clark (4) was used initially. Often, however, virus-free seeds gave readings of 0, so the method was not applicable. Visual examination of the ranked samples was used as an alternative method (Fig. 2). There were very small differences, less than 0.005 O.D., between the low values. As the slope of the curve started to increase, there was a point, usually around 0.030 O.D., where differences between values also increased. This value and the one determined by Clark's method were often close. The higher of the two values was used as the cutoff point. A sample less than that value was considered negative by ELISA and a sample greater than or equal to that value was considered positive by ELISA.

Detection and distribution in individual seeds. Seed coats, papery layers, and distal half embryos from 51 individual seeds of each of the cultivars Rocky Ford, Small Sugar, Table Ace, and Cracker were tested by ELISA and the germinative half embryos by the grow-out assay. Data for Rocky Ford, ranked by absorbance values of the half embryos, were typical of all cultivars tested (Fig. 3). Thirteen (25.5%) half embryos were positive by

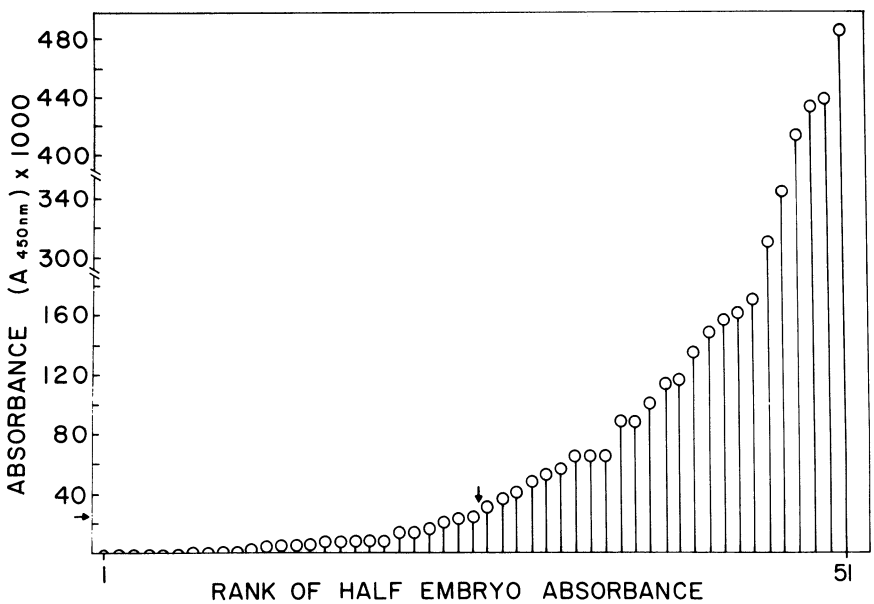

Fig. 2. Ranked enzyme-linked immunosorbent assay absorbance values of Small Sugar pumpkin half embryos for determination of positive and negative values. Each data point is the average of three duplicate samples from one half embryo. Horizontal arrow denotes cutoff using the average plus three standard deviations. Vertical arrow denotes cutoff from the visual examination of the ranked results.

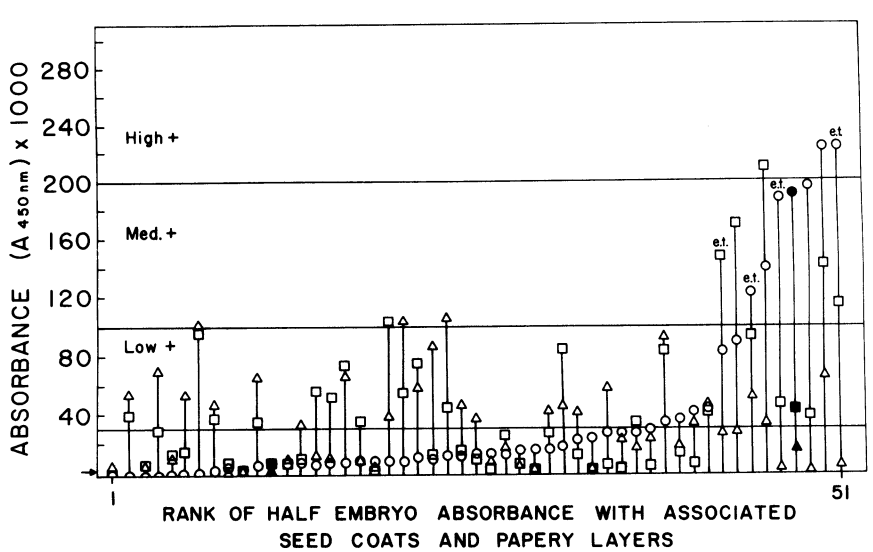

Fig. 3. Relationship between enzyme-linked immunosorbent assay values of the seed coat, papery layer, and half embryo of individual Rocky Ford cantaloup seeds, ranked by half embryo values. ○ = Half embryo, □ = papery layers, and △ = seed coat. Solid symbols indicate the plant died in the grow-out test. e.t. = Embryonic transmission of squash mosaic virus in the grow-out test. Each data point is the average of three duplicate samples. Arrow denotes average value of virus-free controls.

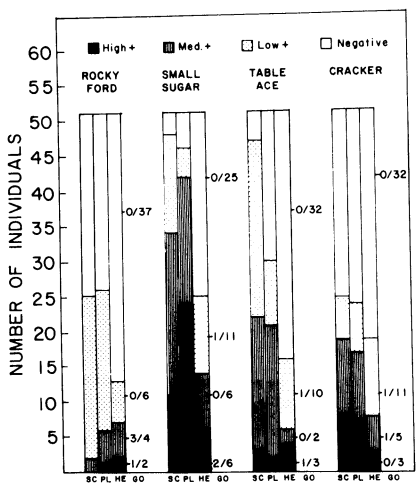

Fig. 4. Relationship between enzyme-linked immunosorbent assay absorbance values of individual seed parts and grow-out tests of Rocky Ford cantaloup, Small Sugar pumpkin, Table Ace squash, and Cracker squash. SC = seed coat, PL = papery layer, HE = half embryo, and GO = grow-out test. Numbers in the GO column are the number of plants showing embryonic transmission of squash mosaic virus over the number of half embryos in the ELISA value group, excluding embryos that died.

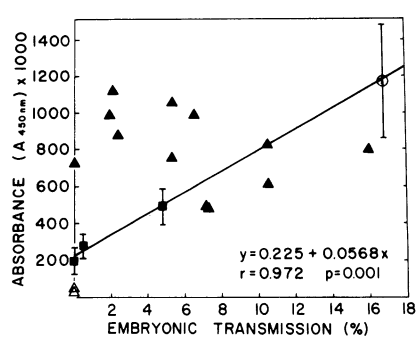

Fig. 5. Relationship between bead enzyme-linked immunosorbent assay (ELISA) values and percentage of embryonic transmission in melon seed lots. All replicate samples consisted of 400 seeds each. The averages of four replicates of each of three experimental PMR-45 cantaloup seed lots (■) and two replicates of a Rocky Ford cantaloup experimental seed lot (○) were used to calculate the regression line. Vertical bars indicate standard deviations. The averages of four replicates of commercial seed lots scored as virus-infected by bead ELISA (▲) are plotted against the grow-out test results provided by the supplier. Commercial seed lots scored as virus-free by bead ELISA and the grow-out test (△) are the averages of 13 replicates.

ELISA ($A_{450\ nm} \geq 0.030$). Forty-nine germinative halves produced seedlings, of which only four were infected. Furthermore, there was poor correspondence among absorbance values of the half embryo, papery layer, or seed coat.

Results of all four cultivars, including those for the Rocky Ford in Figure 3, are summarized in Figure 4. No embryo that was negative by ELISA produced a virus-infected plant in the grow-out test, ie, there were no false negatives. Although a slightly higher percentage of the embryos with high ($A_{450\ nm} \geq 0.201$) absorbance values produced virus-infected seedlings, some embryos with medium ($A_{450\ nm} = 0.101–0.200$) and low ($A_{450\ nm} = 0.030–0.100$) values also produced virus-infected seedlings. Plants derived from seeds with high values did not have a higher mortality rate than those with low values. Symptomless infection of seedlings has not been reported for SqMV. Nevertheless, several symptomless seedlings derived from half embryos with high positive values were virus-free when tested by both agar double diffusion and ELISA. Overall, more individuals were rated positive by ELISA than by the grow-out test, ie, there were false positives by ELISA.

Results of ELISA and local lesion assays of the distal half embryo were compared with results of the grow-out assay for 50 Rocky Ford cantaloup embryos and 51 Small Sugar pumpkin embryos. The local lesion assay was similar to that of Alvarez and Campbell (2). After the samples for ELISA were taken, the remainder of the extract was inoculated to half-leaves of *Cucumis metuliferus* with a cotton swab. The opposite half-leaf was inoculated with a standard extract from virus-infected leaves. Extracts from 12 Rocky Ford cantaloup embryos were positive by ELISA, four of which corresponded to virus-infected plants in the grow-out test, but only one of them, a high ELISA positive, was infectious (three lesions). The inoculum standard had an average of 15 lesions per half-leaf and an $A_{450\ nm} = 0.340$ by ELISA. Among the Small Sugar pumpkin samples, extracts from 12 were positive by ELISA, one was positive in the grow-out test, but none of the extracts produced local lesions. The standard in this case had an $A_{450\ nm} = 0.130$ and an average of 2.5 lesions per half-leaf.

In another trial, both the distal and germinative halves of 13 Small Sugar pumpkin embryos were tested by ELISA. No consistent differences were found between the two halves; the average $A_{450\ nm}$ was 0.056 ± 0.10 for the distal half and 0.067 ± 0.11 for the germinative half.

Detection of virus in seed lots. Because assay of dissected seeds showed that embryonic transmission occurred in embryos with low positive ELISA values, it was decided that test sensitivity should be measured by the ability to detect such seeds in mixtures with virus-free seed. Single stored halves of Rocky Ford cantaloup seeds with the low positive range of ELISA values were mixed with samples of 200 or 250 virus-free seeds and the samples assayed. Antigen was detected 8 of 10 times with 250 seeds and 9 of 10 times with 200 seeds by bead ELISA, indicating a 90% probability of detecting one low positive seed in 400. For a 90% probability of a seed lot having ≤ 1 seed with SqMV antigen in 1,000, 13 samples of 200 seeds each must be scored negative by bead ELISA (9).

The relationship between $A_{450\ nm}$ values of 400 seed samples and embryonic transmission was examined. PMR-45 cantaloup seeds were used directly or diluted with healthy seeds to produce infection levels of 0.48 and 0.048%. The bead ELISA values of four replicates of each of three PMR-45 cantaloup lots and two replicates of the Rocky Ford cantaloup were analyzed by regression (Fig. 5). A virtually identical line ($Y = 0.226 + 0.0561X$) was calculated for the three PMR-45 lots alone. Fourteen commercial seed lots were tested with a modified sequential sampling approach. Four 400-seed replicates of each lot were tested. If no positives were found, the remaining nine replicates were done. The bead ELISA results were consistent. Each of the first four replicates of 12 seed lots were positive but all 13 replicates of two lots were negative. The averages from the ELISA are plotted using the percentage of embryonic transmission reported by the supplier (Fig. 5).

Pollen. The A_{450} values of 11 collections of unwashed pollen from infected plants ranged from 0.118–0.644. Eight of these samples after washing or washing and breaking had $A_{450\ nm} = 0–0.006$ except for one sample at 0.013. Unwashed pollen from virus-free plants had $A_{450\ nm} = 0.001–0.005$.

DISCUSSION

Plate ELISA was useful for detecting SqMV in plants or individual seeds, whereas bead ELISA provided an adaptation for testing seed lots in which sample volumes were large and virus concentration was low. With plate ELISA, we have not encountered the problem of false negatives (assay results negative but infected seedlings developing from the germinative half of the seed) that occurred with previous techniques (2). On the other hand, false positives (assay results positive but healthy seedlings developing from the germinative half of the seed) occurred frequently. Tests of embryo sections showed good agreement between absorbance values of distal and germinative halves so there was no bias in using distal halves to select and rank seeds. The positive values must derive from viral antigen because virus-free seeds used as controls had uniformly low values. It is possible that ELISA detected viral antigens such as unassembled coat protein, degraded virus, or virus that did not infect seedlings systemically during the grow-out test. The present results may support the hypothesis (21) that SqMV can increase in localized parts of the embryo but not spread systemically. This problem of false positives may exist in other hosts such as barley (17) but not be recognized because those embryos cannot be divided and subjected to two tests. The possible presence of non-infectious antigen also makes determination of the virus content of the seed by ELISA uncertain.

The occurrence of false positives when individual seeds are assayed is reason to believe that assays of seed lots by bead ELISA may give false positives as well. False positives that would result in abandonment of clean seed lots would be as detrimental as false negatives that would result in planting of contaminated seed lots. Although there was a significant linear relationship between embryonic transmission in experimental seed lots and bead ELISA values, commercial seed lots varied widely from this regression line. There are several possible explanations for this. The variable amount of antigen in seed parts and its unknown role in seed transmission could affect ELISA values. The level of embryonic transmission in a seed lot is known to decline with time (20) but perhaps antigen detectable by ELISA does not. The regression line was based on experimental seed lots produced and tested in 1982, but the length of storage and dates of the grow-out tests of the commercial seed lots were not known. Some of the seed lots had been damaged by insects and contained numerous empty seed coats, which could have increased the ELISA values if they were virus-contaminated. In addition, the grow-out tests done at seed companies with the commercial lots used variable numbers of plants, greenhouse procedures, and observation times. A larger number of seed lots of known ages and known levels of seed transmission derived from recent, uniform grow-out tests needs to be tested by ELISA. Nevertheless, bead ELISA is quick and reliable in determining whether a seed lot came from plants, some of which were SqMV-infected.

Absorption of antiserum with leaf extract removed antibodies that reacted with virus-free leaf tissue in plate ELISA but did not eliminate nonspecific reactions in the bead ELISA of virus-free seed lots. This may be due to high concentrations of seed proteins in the extracts because a second absorption of the conjugate with an extract of virus-free seeds eliminated the nonspecific reactions. Mannose and glucosamine in the extraction buffer increased ELISA absorbance values when virus antigen was present in seed extracts. The sugars may bind competitively with seed

proteins, which prevent viral antigen binding to the Ig-coated surface. Ghabrial et al (10) also used sugars, but for reduction of nonspecific reactions. The interfering seed proteins were not lectins because lectins were not found in the seeds of *Cucurbita maxima, Cucumis sativus*, or *C. melo* until germinating seeds were at least 5 days old (22) and extracts of seed lots used in this study did not cause hemagglutination (M. Etzler, *personal communication*).

The lack of correspondence among ELISA absorbance values of the various seed parts supports previous findings that the seed coat and papery layer may contain SqMV independently of the embryo (2). Although possible mechanical inoculation of seedlings from virus in the seed coat and papery layer cannot be ruled out, the virus most likely to be involved in seed transmission is that in the shoot and radicle.

The viral antigen apparently occurred on pollen as a superficial contaminant that was removed by washing and was not within the pollen. Similar results have been reported for Prunus necrotic ringspot virus (PNRV) in almond and cherry where viral antigen occurred on, but not in, pollen (6). Nevertheless, PNRV was pollen transmitted to the progeny, whereas SqMV was not (2).

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