and percent marketable curds for cauliflower cultivars grown in all 3 plantings is shown in Table 5. Due to the variability of cultivar performance among planting times, each cultivar should be selected based on performance during particular phases of the season. Growers in west-central Florida may consider planting 'White Summer' throughout the season, 'White Rock' when temperatures at transplanting are cooler, and 'Igloo' and 'Snowflower' only during the cool winter months. In comparison to the 1981-82 season, 'White Summer' continues to be a favorable option (5).

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USE OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY IN FLORIDA'S LETTUCE MOSAIC VIRUS SEED INDEXING PROGRAM

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Abstract. **Optimal conditions are described here for performing the enzyme-linked immunosorbent assay (ELISA) for detection of lettuce mosaic virus (LMV) in lettuce** (Lactuca sativa **L.) seeds. Several factors such as immunoglobulin concentration, sample incubation conditions, and sample preparation affect the reliability of the test for detecting LMV. Therefore, strigent adherence to the conditions described here are necessary. Also, results are given where in 1982-1983 seeds of 21 Florida breeding lines were tested for LMV by ELISA and 19 LMV free lines subsequently were planted in commercial fields where no LMV was observed. In 1982-1983 six commercial seedlots were tested for LMV by the standard** Chenopodium quinoa **Willd. test and by ELISA and both tests showed the same seedlots to contain LMV.**

Lettuce mosaic virus (LMV) is a potentially serious pathogen wherever lettuce is grown (3). LMV severely threatened Florida's lettuce industry until the 1970's when seed indexing for LMV was implemented. This indexing program insures that only commercial seed free of LMV will be planted, and thus no seedborne LMV will be introduced into the field (Rules of the Department of Agriculture and Consumer Services, Division of Plant Industry, Chapter 5B-38). This program has maintained control of LMV in Florida. This year the Florida Lettuce Mosaic Committee will be changing from the *Chenopodium quinoa* test to the enzyme-linked immunosorbent assay (ELISA) to index seedlots.

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The LMV ELISA test is very fast and has many advantages over the *C. quinoa* test. However, many variables can affect the reliability of ELISA tests (1, 2). Because of the tremendous potential of LMV to cause destruction to Florida's lettuce industry, the ELISA LMV indexing method must be done carefully and the appropriate controls must be included in the test so that the results will be interpreted correctly. We have tested a variety of conditions for their effects on the detection of LMV in lettuce seeds by ELISA, and we report here the optimal conditions for testing lettuce seeds for LMV.

Materials and Methods

ELISA tests were done mostly as described (1, 2), using the LMV antisera produced in Florida. Immunoglobulins (IgG) always were purified using ammonium sulfate fractionation and diethylaminoethyl cellulose (DEAE) column chromatography. Siliconized glass and plasticware were used for IgG purification and storage. The enzyme conjugate was prepared using alkaline phosphatase (SIGMA, Type VII-S) at 2,000 units per mg purified IgG. The standard buffers used in all cases were:

Coating buffer: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3, per liter distilled water, pH 9.6.

Phosphate buffered saline (PBS): 8.0 g NaCl, 0.2 g KH_2PO_4, 2.17 g $Na_2HPO_4 \cdot 7H_2O$, 0.2 g KCl, 0.2 g NaN_3, per liter distilled water, pH 7.4. PBST is PBS containing 0.5 ml Tween 20 per liter. PBST-PVP is PBST containing 20 g polyvinyl pyrrolidone 40 per liter.

Substrate buffer: 97 ml diethanolamine, 800 ml distilled water, 0.2 g NaN_3, adjust pH to 9.8 and bring up to one litter with distilled water. Substrate always was prepared fresh and the optimal concentration was 0.6 mg/ml.

All buffers were stored at 4°C until used.

Results were assessed photometrically at 405 nm using a spectrophotometer or EIA reader. In the latter case, only

200 μl of substrate was used for the enzyme reaction instead of 250 μl as was done previously (2). All tests were done using Dynatech Immulon II flat bottom microtiter plates (Dynatech Laboratories, Inc. Alexandria VA, Cat. #011-010-3450).

In all cases a seed sample refers to 500 lettuce seeds ground in 10 ml PBST-PVP, and a seedlot is 30,000 lettuce seeds. Seeds used as healthy controls were those that had passed *C. quinoa* tests in the preceding year. LMV-infected seeds were grown in the greenhouse and mixed with healthy seeds as described (2), to simulate low levels of LMV for LMV-infected control seed samples.

To compare various conditions such as immunoglobulin concentration or time of sample incubation, aliquots from the same healthy and test samples were used in different plates to test different conditions. The seeds of various lettuce breeding lines that were found to be free of LMV by ELISA were subsequently planted in commercial lettuce fields and observed throughout the growing season for LMV.

Results and Discussion

Several factors such as plate type, immunoglobulin concentration and in some cases sample preparation affected the ELISA test for LMV. However some factors were much more important than others. Of the microtiter plates tested, the Dynatech Immulon II gave the most reproduceable results and therefore was used in all seed tests. Also, seeds from several lettuce varieties have been tested using ELISA. No differences have been detected between varieties that might affect the reliability of the ELISA test.

Three of the most critical factors affecting the reliability of the test were sample preparation, immunoglobulin concentration, and time of sample incubation. To be able to detect 1 LMV-infected seed in a 500 seed sample, all seeds must be ground thoroughly. This was accomplished easily by soaking the seeds in PBST-PVP for 1 to 2 days at room temperature to soften them before grinding and then thoroughly grinding for 10 to 15 sec using the Tissuemizer. The thoroughness of seed grinding can be checked by centrifuging samples for 1000 x *g* for 3 min. The bottoms of the polypropylene centrifuge tubes can then be examined visually to insure that no whole seeds are present. Any whole seeds that were not ground up will easily be visible in the bottom of the tube. If whole seeds are present the samples must be ground longer, but care must be taken to insure that seed samples do not heat up significantly during the grinding as this can inhibit the detection of LMV. At most, samples should be only slightly warm when touched to the palm of the hand. Therefore the grinding probe is rinsed in ice water between samples to help keep it cool and to wash it between samples. However, samples should be soaked at room temperature and not chilled before grinding, as keeping them too cool also decreases the intensity of the positive reactions. It also is necessary to insure that only the clear supernatants of the ground and centrifuged samples are placed in the microtiter plates. In several cases where we have added seed samples to plates that were not sufficiently clarified but that contained visible particulate seed pieces, the non-specific reactions of these samples was increased significantly, and therefore affected the differentiation of healthy and infected samples.

Using low concentrations of coating immunoglobulins are necessary to insure detection of LMV in seeds and maintain a low background. In all cases when IgG concentrations were higher than 0.25 μg/ml, background reactions increased and in 2 of 3 experiments shown here, some of the LMV-infected samples were not detected (Table 1). Concentrations below 0.25 μg/ml were not as effective for detecting LMV-infected seeds when using our antisera.

Table 1. Effects of coating immunoglobulin concentration on detection of LMV in lettuce seeds.

Concn.[z]	Avg.[y]	Avg. + 3SD[x]	No. of LMV +[w]	Avg. of LMV[v]
0.50	0.10	0.21	11/24	0.38
0.25	0.03	0.10	14/24	0.27
0.10	0.05	0.14	14/24	0.24
0.50	0.17	0.32	4/12	0.47
0.25	0.14	0.28	5/12	0.40
0.10	0.12	0.27	3/12	0.32
0.50	0.13	0.24	5/12	0.62
0.25	0.10	0.21	5/12	0.55
0.10	0.09	0.20	5/12	0.52

[z]Concntration of LMV immunoglobulin (μg/ml) used to coat plates.
[y]The mean absorbance value at 405 nm of 12-500 seed healthy lettuce seed samples.
[x]The baseline value [3 times the SD plus the mean of the healthy samples] used to differentiate positive and negative samples.
[w]The number of infected samples over the number tested (500 seeds per sample). The same samples were tested at each immunoglobulin concentration for a given experiment.
[v]The mean absorbance value at 405 nm for the LMV positive samples.

We also tried to decrease the amount of time needed to assay seedlots so that more seedlots could be tested per week. However, soaking seeds for 1 to 2 days made them much easier to grind and incubating samples overnight at room temperature after grinding and before placing them in plates was necessary to inhibit non-specific reactions (2). We also tried to decrease sample incubation times in microtiter plates from 18 hr to 1 hr. In some cases this did not affect LMV detection, but in some instances LMV-positive samples, as determined by our standard method, were not detected by 1 hr sample incubation (Table 2). Therefore, the conditions given here are necessary to insure accurate detection of LMV. Even when these conditions are used we routinely tested 6 to 9 seedlots per week.

We have simultaneously tested the commercial Florida lettuce seedlots for the last 2 yr by ELISA while the Florida Lettuce Mosaic Committee tested these seedlots using the *C. quinoa* test. In 1983 only 6 seedlots were tested yet this still required ca. 2 months using the *C. quinoa* test. Both ELISA and the *C. quinoa* test detected LMV in 2 of 6 seedlots (Table 3), but the ELISA tests were done in 1 wk.

Besides being much faster than the *C. quinoa* test, the ELISA test has another advantage for Florida's lettuce industry. The lettuce breeding material developed by Florida's cultivar development program must be planted in commercial lettuce fields to insure accurate comparsion of new lines with established varieties. This seed can carry LMV, and therefore could introduce it into commercial fields. However, this seed can be indexed using ELISA before planting in commercial fields. In the 1982-83 growing season we tested seed of 21 Florida breeding lines for LMV using ELISA (Table 4). In cases where large amounts of seed were not available only 3500 to 4500 seeds (7-9 samples) were tested. Of the 21 breeding lines tested in 1982-83, 2 were found to have LMV and only 4500 seeds were tested for one of these lines. The remaining 19 lines that were LMV free were planted in commercial fields and observed for LMV. None showed any LMV (V. L. Guzman, personal communication).

One factor that has not yet been sufficiently investigated is what effects the age of lettuce seed has on LMV survival and its detection using ELISA. In general we tested only seeds that were harvested the same year we tested them. We

Table 2. Effects of sample incubation time and immunoglobulin concentration on LMV detection in lettuce seeds.

Expt. no.	1 hr IgG[z]	1 hr Avg. + 3SD[y]	1 hr No. LMV +[x]	1 hr Avg. LMV[w]	18 hr Avg. + 3SD[y]	18 hr No. LMV +[x]	18 hr Avg. LMV[w]
1	1.00	0.15	0	—	NT[v]	—	—
1	0.50	0.17	0	—	NT	—	—
1	0.25	0.17	0	—	0.15	3	0.50
2	1.00	0.19	0	—	NT	—	—
2	0.50	0.27	2	0.30	NT	—	—
2	0.25	0.13	5	0.19	0.15	5	0.20
3	0.25	0.17	3	0.25	0.22	4	0.28
4	0.25	0.16	5	0.26	0.19	5	0.41
5	0.25	0.21	3	0.42	0.19	3	0.38

[z]Concentration of coating immunoglobulin used.
[y]Three times the SD plus the mean for 12-500 seed samples of healthy lettuce seeds.
[x]The number of samples scored 'positive' for LMV.
[w]The mean absorbance value for the positive samples.
[v]Not tested.

Table 3. Results of testing 1982-1983 Florida lettuce seedlots by the *Chenopodium quinoa*[z] and ELISA tests.

Seedlot	*C. quinoa*[y]	ELISA[x]
83-01	—	0/60
02	—	0/60
03	—	0/60
04	+	3/60
05	—	0/60
06	+	0/60, 1/60[w]

[z]The *C. quinoa* tests were performed by the Florida Lettuce Mosaic Committee.
[y]A plus (+) indicates the seedlot contained LMV and a minus (–) indicates no LMV.
[x]Indicates the number of samples containing LMV over the number tested.
[w]The first test, 0/60, indicated this seedlot contained no LMV, however, it was re-tested after the *C. quinoa* results were obtained.

have tested one LMV-infected seedlot 6 months after harvest and again 18 months after harvest. In this single test there was no difference in the amount of LMV detected by ELISA at the 2 test dates. However, when we tested seeds by ELISA in 1983 that were from 1978 and 1979 and had failed the Florida LMV *C. quinoa* test at those times, we detected no LMV in them. They may have contained very low levels of LMV when tested in 1978 and 1979, or perhaps LMV declines upon storage of lettuce seeds and these seeds no longer contained LMV when we tested them by ELISA.

We believe that the ELISA method of indexing lettuce seeds for LMV will be very beneficial to Florida's lettuce industry. The test will be much faster than the *C. quinoa* test, it will be less expensive to perform and indexing can be done on entire seedlots or small samples of breeding material at anytime of the year. The conditions given here for the test (Table 5) are optimal when using our antiserum and minor deviations from the exact method can result in incorrect interpretation of test results. We anticipate that future improvements will be made for the ELISA test for LMV in lettuce seeds, but until these have been adequately tested the procedure as described here should be followed closely.

Table 4. Results of testing lettuce breeding lines for LMV using ELISA.

Sample	Date	No. infected/no. tested[z]
FL-24069	9-7-82	0/60
49069	9-7-82	0/60
49563	10-5-82	0/8
49736	10-5-82	2/9
49714	10-5-82	0/9
49703	10-5-82	0/8
49712	10-5-82	0/9
49702	10-5-82	0/7
48076	10-5-82	0/7
49526	10-11-82	0/60
49528	10-11-82	0/60
49529	10-18-82	0/60
49533	10-18-82	0/60
49532	10-26-82	0/60
49530	10-26-82	0/60
43008	11-3-82	0/60
48060-B	11-3-82	0/60
48060-B[y]	11-16-82	0/60
49659	11-16-82	0/60
49532	11-16-82	0/60
49667	1-18-83	0/18
5413	1-18-83	3/18
49667[y]	2-1-83	0/18
5413[y]	2-1-83	3/18

[z]The number of infected seed samples over the number tested.
[y]Indicates the sample was a re-test to confirm earlier results.

Table 5. Optimal conditions for performing the LMV-ELISA seed test.

1. Divide seeds into samples of 500 each (60 samples per seedlot) in polypropylene centrifuge tubes and add 10 ml PBST-PVP. Leave seeds at ambient room temperature (16-25°C) overnight to soften before grinding.
2. Homogenize samples for 10-15 sec using a Tissuemizer (SDT 1810 motor and SDT-182E shaft), wash shaft in ice water between samples. Leave samples at ambient room temperature (16-25°C) overnight.
3. Coat microtiter plates using LMV antiserum immunoglobulins, 200 µl per well at 0.25 µg/ml in coating buffer. Seal with tape and incubate 3 hr at 25°C.
4. Wash plates 3 times for 3 min each using PBST. (Coated and washed plates can be stored in plastic bags at 4°C for at least 2 wk).
5. Centrifuge samples at 1000 x *g* for 3 min to clarify and place 200 µl of the clear sample supernatants in plates, with each sample replicated twice. For each test use 12 samples of healthy seeds and 12 samples of LMV infected seeds (containing ca. 1 infected seed per 2000 seeds) as controls. Seal with tape. Let samples incubate overnight (ca. 18 hr) in plates at 4°C. [The controls can be saved in the refrigerator (4°C) and used again if more seeds are to be tested the following day. However, they should be saved only for 24 hr and then discarded.]
6. Wash 3 times with PBST.
7. Add 200 µl of a 1/1500 dilution (in PBST-PVP) alkaline phosphatase conjugated LMV immunoglobulins per well. Seal with tape. Incubate 4 hr at 25°C.
8. Wash 3 times with PBST 3 min each.
9. Add 200 µl/well *P*-nitrophenylphosphate substrate (0.6 mg/ml in substrate buffer) and incubate at room temperature for 3 hr.
10. Add 40 µl 3M NaOH per well to stop hydrolysis. Assess results at 405 nm.
11. Calculate the mean ($\bar{X}$) and standard deviation (SD) of the healthy control samples. Use 3SD + $\bar{X}$ as the baseline and if both replicates of any sample are above the baseline the sample is positive for LMV.

Whenever new immunoglobulins or enzyme-conjugate are prepared it is necessary to test them against standards to insure that the concentrations used are optimal for seed testing. We have prepared new immunoglobulins and enzyme-conjugate more than 10 times and the conditions given here have been optimal in all cases except where the enzyme conjugate was sometimes used at 1/2000 instead of 1/1500.

Although to index lettuce seeds for LMV by ELISA requires precision and attention to the details of the test method, the benefits that can be gained by using ELISA are worth this effort.

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STATUS AND POTENTIAL OF PESTICIDE RESISTANCE AMONG FUNGAL AND BACTERIAL PATHOGENS OF VEGETABLE CROPS IN FLORIDA[1]

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Abstract. **Pathogen-host relationships in Florida vegetable crops where instances of resistance of fungi or bacteria to fungicides or bactericides have occurred are reviewed. The potential for development of resistance problems in other pathogen-pesticide combinations are discussed.**

Resistance of insects to insecticides became an important problem in agriculture in the early 1950's (2) following the introduction of chlorinated hydrocarbons and organophosphate compounds. Although organic compounds have also been used as fungicides and bactericides since about the same time, resistance to them has become a significant problem only in recent years (14). The rapid increase in number of reported instances of fungicide-resistance and bactericide-resistance (hereafter referred to as fungicide-resistance) has been mainly due to the development and introduction for agricultural use of highly effective fungicides with limited specific metabolic action sites. These new fungicides were developed in part to provide a high degree of control of a narrow spectrum of organisms.

Although bioenvironmental methods of disease control play a part in management of many vegetable diseases in Florida (16), fungicides have been, and continue to be a primary element in the management of many diseases. Indeed, fungicides are often the only practical means of controlling some diseases (16). There is no indication that fungicide use in Florida, on tomato, for instance, has declined because of development of host resistance or other bioenvironmental means of disease management (16). Metabolic site-selective fungicides have been used and are used for several important vegetable diseases in Florida. Several site-selective fungicides are also in various stages of development for management of vegetable diseases.

The purpose of this paper is to review fungicide-resistance in vegetable crop diseases in Florida, indicate potential resistance problems, discuss techniques of delaying the development of resistance, and discuss coping with resistance once it has developed.

Instances of documented fungicide-resistance among Florida vegetable pathogens

Resistance of fungicides or bactericides has become a problem only among a few vegetable crop pathogens in Florida. Table 1 lists the documented cases of resistance following initial successful use of a fungicide or bactericide on vegetables in Florida.

Bacterial spot of tomato and pepper incited by various races of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye is regarded by some as the most important pathogen of tomatoes and peppers presently in south Florida (7). In the early 1950's there were reports of successful control of *X. c.* pv. *vesicatoria* with streptomycin sprays (4). However, within the same decade, failures to control the pathogen

Table 1. Documented cases of fungicide or bactericide resistance in vegetable crops in Florida.

Disease (pathogen)	Host	Chemical
Bacterial spot (*Xanthomonas vesicatoria*)	Tomato, pepper	streptomycin
Bacterial spot (*Xanthomonas vesicatoria*)	Tomato, pepper	copper
Bacterial blight (*Pseudomonas apii*)	celery	streptomycin
Early blight (*Cercospora apii*)	celery	benomyl

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