Radioimmunosorbent Assay for Detection of Lettuce Mosaic Virus in Lettuce Seed

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ABSTRACT

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Reliability of radioimmunosorbent assay (RISA) or enzyme-linked immunosorbent assay (ELISA) for detection of lettuce mosaic virus in lettuce seed was improved by modification of the extraction medium. Addition of mannose and glucosamine to the conventionally used phosphate-buffered saline in the case of RISA, or the use of lower pH citrate buffer with diethyldithiocarbamate as an additive in the case of ELISA, reduced the nonspecific background that sometimes obscures specific reactions in these tests. Individual seed assayed by RISA showed a 15-fold variation in lettuce mosaic virus content. Blind testing of lettuce seed lots of known content of the virus showed that three infected seeds per 30,000 could be consistently detected by RISA when five to 10 sublots per seed lot were assayed.

Lettuce mosaic virus (LMV) has been controlled effectively for a number of years in the lettuce growing areas in California through the implementation of a high-standard seed-indexing program (4). Under this program, only seed lots with an index of zero infected seed in 30,000 are acceptable for planting. Currently, the seedling grow-out method (4) and the Chenopodium method (7) are used for seed indexing. Although effective, these procedures are laborious, time-consuming, and require extensive greenhouse space. The highly sensitive enzyme immunoassay and radioimmunoassay methods, recently adopted in plant virus diagnosis (1,3), promise to be well suited for seed indexing purposes. Jafarpour et al (6) reported that the enzyme-linked immunosorbent assay (ELISA) method (1) detected LMV in artificially mixed lettuce seed samples with one infected seed homogenized with as many as 1,400 healthy seeds.

Recently, we described a highly sensitive radioimmunosorbent assay (RISA) that detected LMV in purified preparations at a concentration as low as 2 ng/ml (3). The RISA procedure, which is a microplate method based on the principle of double-antibody sandwich (9), follows essentially the protocol of the ELISA procedure (1) except that ^{125}I-labeled γ-globulin is substituted for the γ-globulin enzyme conjugate. The bound ^{125}I γ-globulin is dissociated by acidification, and the released radioactivity is proportional to virus concentration (3). This paper describes the application of the RISA procedure for detection of LMV in lettuce seed samples.

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MATERIALS AND METHODS

Preparation of seed extracts. Extracts from individual lettuce seeds were prepared in a Plexiglass board with multidepressions. Seeds, or half seeds, were placed individually in the depressions, soaked for 30 min in the extraction buffer, and ground with glass rods. Bulk seed samples were soaked for 1 hr in the extraction buffer and homogenized with a Polytron homogenizer using a PT-10 generator (Brinkmann Instruments Inc., Westbury, NY 11590). The homogenates were clarified by passage through a glass-wool pad packed at the bottom of a disposable, 10-ml syringe. The extraction buffer that was added to the seed samples at a ratio of 20:1 (v/w) was comprised of 0.02 M phosphate-buffered saline (pH 7.4), containing 2.0% polyvinylpyrrolidone, 0.2% ovalbumin, 0.05% Tween 20, and 0.02% sodium azide (PBS-PVP-OA-T). Unless otherwise stated, mannose and glucosamine were routinely added to PBS-PVP-OA-T, each at the ratio of 5% (w/v).

Serology. An antiserum to LMV was produced as described earlier (3). Procedures for preparation of γ-globulin, labeling of γ-globulin with the enzyme alkaline phosphatase or with iodine-125, and procedures for the ELISA and RISA methods were previously described (3). Unless otherwise stated, the ELISA and RISA plates were coated with γ-globulin at 1.0 μg/ml. The enzyme γ-globulin conjugate or the ^{125}I-labeled γ-globulin were routinely used at a dilution of 1/400; this corresponds to 2.5 μg/ml, assuming no change in γ-globulin concentration during the labeling process. In the RISA procedures, the final radioactivity of ^{125}I γ-globulin was 40,000 to 120,000 counts per minute (cpm) per well.

Seed extracts from a healthy seed lot of the cultivar Climax served as the healthy control in the serological assays. A seed lot with about 10% LMV infection was used as a source of infected seeds.

Table 1. Effects of certain additives to the extraction buffer on background obtained with lettuce seed extracts in the radioimmunosorbent assay (RISA) method for detection of lettuce mosaic virus (LMV)

Experiment	Additive	RISA[a] values, ^{125}I cpm, with extract from		
		Healthy seed[b]	Healthy leaf	Infected leaf[c]
1	None	594[d]	57	558
	2,Mercaptoethanol (0.5%, v/v) plus dithiothreitol (1 mM)	60	55	287
2	None	177	22	392
	Mannose and glucosamine (5% w/v of each)	47	20	386
3	None	508	73	6,370
	Mannose and glucosamine (5% w/v of each)	277	63	6,175

[a] Coating γ-globulin (1 μg/ml, 220 μl/well), ^{125}I-labeled γ-globulin (1:400 dilution, 200 μl/well); final radioactivity per well of 80,000, 40,000, and 90,000 cpm for experiments 1, 2, and 3, respectively.
[b] 500-seed samples from a healthy seed lot homogenized in 10 ml of extraction buffer (PBS-PVP-OA-T) with or without additives.
[c] Healthy or LMV-infected lettuce leaves homogenized in PBS-PVP-OA-T (1:10 w/v) with or without additives.
[d] Values are averages for duplicate or triplicate wells.

Reference samples simulating seed lots with different contents of infected seeds were prepared by mixing at varying proportions extracts from the seed lots with 0 and 10% seed infection.

RESULTS

Nonspecific interference of lettuce seed extracts. Extracts from healthy seed samples, but not from healthy lettuce leaf, exhibited some nonspecific interference in the RISA test for LMV. Several attempts were made to reduce the background obtained with lettuce seed extracts, including the addition of reducing agents or simple sugars to the extraction buffer. The reducing agents mercaptoethanol and dithiothreitol, added in combination to the extraction buffer, were very efficient in reducing the background. However, the RISA values (released radioactivity, ^{125}I cpm) for extracts from LMV-infected lettuce leaves were reduced by half (Table 1). Mannose and glucosamine, on the other hand, had no deleterious effects on the specific reaction, and their incorporation in the extraction buffer reduced the background values by 50–70%.

Similar trials of varying the extraction conditions to reduce background with healthy seed were done with the ELISA method. Many trials with different buffer salts, additives of various sorts (mainly glycosides, reducing agents, and chelating agents), or variations in the amounts of PVP or ovalbumin added to a PBS extraction medium were essentially negative. Only a lower pH and addition of a mild reducing agent that also chelates copper ions consistently reduced the ELISA background. Homogenization of seed in 0.1 M citrate, pH 6.2, containing 0.2 M diethyldithiocarbamate (but without PVP or ovalbumin) consistently gave lower background reactions for healthy seed. It was determined, however, that the citrate-diethyldithiocarbamate extraction medium must be prepared fresh on each occasion. Table 2 shows a sample ELISA trial with fresh and aged citrate-diethyldithiocarbamate extraction with infected seed.

Table 2. Effect of fresh or aged citrate-diethyldithiocarbamate extraction medium[a] on enzyme-linked immunosorbent assay reactions with healthy seed or seed infected with lettuce mosaic virus (LMV)

LMV in seed (%)[b]	Absorbance at 405 nm	
	Fresh medium	Aged medium[c]
0.0	0.008	0.113
0.05	0.065	0.127
0.1	0.094	0.258
1.0	0.591	0.731
10.0	1.547	1.720

[a] 0.1 M citrate, pH 6.2, containing 0.02 M diethyldithiocarbamate.
[b] Percentage of LMV was simulated by diluting an extract of seed with 10% LMV infection with an extract of healthy seed prepared in the same manner. In each case, 500 seeds were homogenized in 5 ml of buffer.
[c] Prepared 1 wk before use.

Sample size. A sample size of 500 seeds or 0.5 g of seeds (average weight of lettuce seed is 1 mg) homogenized with 10 ml of extraction buffer was best for thorough extraction and efficient processing of the homogenates. Thus, the RISA method must be sensitive enough to detect one infected seed in 500. The sensitivity of the assay at this low level was evaluated as follows: Extracts of 500-seed samples from a seed lot with 10% LMV infection were diluted 50-fold, using seed extracts from healthy seed samples of equivalent size as the diluent. The resultant mixtures thus simulated extracts from seed lots with 0.2% infection, or one infected seed in 500. Samples simulating seed lots with two, three, four, and five infected seeds were also prepared. Samples from both seed lots were homogenized in PBS-PVP-OA-T either with or without mannose and glucosamine.

The results indicated that supplementing the extraction buffer with simple sugars was essential for definitive detection of LMV in extracts simulating seed samples with one infected seed in 500 (Table 3). In the absence of sugars, definitive detection of LMV was possible only with simulated samples of three or more infected seeds. In some other experiments, remaining half seeds from previously identified infected seeds were individually homogenized with 250 seeds, and the extracts were tested by the RISA method. Average RISA values for three such tests were 390, 354, and 278 ^{125}I cpm; the mean value (± standard deviation) for extracts from equivalent size samples of healthy seeds in the same experiment was 133 ± 12.

Table 3. Radioimmunosorbent assay (RISA) values for lettuce seed extracts from a seed lot with 10% lettuce mosaic virus (LMV) infection diluted with seed extracts from a healthy seed lot

Dilution of original extracts[a]	RISA values, ^{125}I cpm[b]			
	Exp. 1: Extraction buffer		Exp. 2: Extraction buffer	
	With sugars	Without sugars	With sugars	Without sugars
1:10	2,518[c]	2,051	571	434
1:12.5	1,408	1,440	475	411
1:16.6	1,079	965	360	350
1:25	668	631	257	215
1:50	505	615	196	173
Healthy seed extract[d]	277	508	84	172

[a] 500-seed samples from a seed lot with 10% LMV infection were homogenized in 10 ml of extraction buffer (0.02 M phosphate-buffered saline [pH 7.4], containing 2.0% polyvinylpyrrolidone, 0.2% ovalbumin, 0.05% Tween 20, and 0.02% sodium azide) with or without the sugars mannose and glucosamine (5% w/v of each).
[b] Radioactivity of ^{125}I-labeled γ-globulin (2.5 μg/ml, 200 μl/well) applied per well was 90,000 and 40,000 cpm, respectively, for experiments 1 and 2.
[c] Values are averages for duplicate wells.
[d] 500-seed samples from a healthy seed lot were homogenized in 10 ml of extraction buffer with or without sugars.

Virus titer in infected lettuce seeds. RISA test values for extracts from individual infected seeds indicated a considerable variation in LMV titer among infected seeds (Table 4). Assuming that RISA values higher than twice the healthy control constituted positive results, differences of as much as 15-fold in RISA values were detected between infected seeds. The variation in virus titer among infected seeds was also demonstrated in an ELISA test with individual half seeds (Table 5). The volume of extract applied per well corresponded to 200 and 330 μg of seed tissues in the RISA and ELISA tests, respectively. In a 500-seed sample with one infected seed, homogenized in 10 ml of extraction buffer, the contribution by weight of such a seed to the test extract (200 μl) is 20 μg (calculated on the basis of an average weight of 1 mg per seed). Thus, the assay methods should be sensitive enough to

Table 4. Frequency distribution of radioimmunosorbent assay (RISA) values for extracts of individual lettuce seeds[a] from seed lots with 0 and 10% infection with lettuce mosiac virus

Infection in seed lot (%)	Seeds tested (no.)	Distribution of seeds based on RISA[b] values of extracts (^{125}I cpm)					
		30–55	56–75	100–199	200–299	300–399	1,500–1,800
0	12	12 (40 ± 8)[c]	0	0	0	0	0
10	84	64 (42 ± 6)	12 (65 ± 7)	3 (133 ± 43)	2 (230)	1 (377)	2 (1,648)

[a] Individual seeds were homogenized with 1 ml of extraction buffer (1:1,000, w/v), and 200-μl samples were applied per well.
[b] Plate was coated with γ-globulin (1 μg/ml, 200 μl/well); ^{125}I-labeled γ-globulin was used at a dilution of 1:400 (final radioactivity of 60,000 cpm/well).
[c] Values are means ± standard deviation.

detect LMV in as little as 20 μg of infected seed tissue.

The sensitivity of RISA and ELISA in detecting LMV in small portions of infected seeds was compared in the following experiment. Remaining half seeds of previously identified infected seeds were homogenized with 200 μl of extraction buffer, and three samples of 50 μl each were tested as follows: one sample was applied to a RISA plate; the second to an ELISA plate; and the third was diluted sixfold with extraction buffer, and duplicate samples (50 μl) were tested in each of the RISA and ELISA plates. The volume of original and diluted extracts applied per well would thus correspond to 125 and 21 μg of infected seed tissues, respectively. Definitive positive results were obtained by both assay methods in tests with original extracts from all infected seeds regardless of virus titer (Table 6). The RISA procedure also detected LMV in the diluted extracts of all infected seeds. On the other hand, definitive detection of LMV in the diluted extracts by the ELISA procedure was dependent on virus titer (Table 6).

Testing of lettuce seed lots. The reliability of the RISA procedure in detecting LMV in seed lots with very low proportions of seed infection was evaluated in a blind comparative test with the seedling grow-out method of 13 seed lots that had previously been indexed at 0–8 infected seedlings in 30,000. Five to 15 samples (500 seeds each) from each seed lot were tested by the RISA method. Seed samples from a healthy seed lot were tested in the same plates with the unknown samples. Results of four separate experiments, run in four consecutive days, are compiled in Table 7. Results were considered positive for test samples if the average RISA value was twice that of healthy control samples in the same plate. Four of six seed lots that indexed no infected seedlings in 30,000 in the seedling grow-out method were also negative by the RISA test. The RISA values of as many as 15 samples from each of these four seed lots did not exceed that of healthy control samples. Some of the samples from the remaining two seed lots that indexed no infected seedlings in 30,000 had RISA values higher than those of the healthy control, but not twice as high. Samples with RISA values higher than the healthy control but not high enough to qualify as positive were also obtained from the seed lots that indexed 1 or 2 infected seedlings in 30,000. Because the presence of infected seeds with uniformly low virus titer in such seed lots cannot be ruled out, these seed lots were tentatively considered negative. Testing of more samples from these lots will be required before their suitability for planting can be determined.

Definitive positive RISA results were obtained with seed lots that indexed three

Table 5. Frequency distribution of enzyme-linked immunosorbent assay (ELISA) absorbance values for extracts of individual lettuce seeds[a] from seed lots with 0 and 10% infection with lettuce mosaic virus

Infection in seed lot (%)	Seeds tested (no.)	Distribution of seeds based on ELISA[b] absorbance values at A_{405}					
		0.015–0.05	0.051–0.085	0.1–0.12	0.15–0.2	0.25–0.4	0.55–0.7
0	5	5 (0.028 ± 0.012)[c]	0	0	0	0	0
10	75	40 (0.03 ± 0.01)	23 (0.066 ± 0.012)	5 (0.111 ± 0.012)	2 (0.172)	2 (0.327)	3 (0.585 ± 0.031

[a] Half seeds were individually homogenized with 75 μl of extraction buffer (1:150, w/v), and 50-μl samples were tested per well.
[b] Plate was coated with γ-globulin (4 μg/ml, 50 μl/well) and conjugated γ-globulin was used at a dilution of 1/100 (50 μl/well); substrate was added at 200 μl/well, and enzyme-substrate reaction time was 1.5 hr.
[c] Values are means of fivefold dilutions of reacted substrate ± standard deviation.

Table 6. Sensitivity of the radioimmunosorbent assay (RISA) and enzyme-linked immunosorbent assay (ELISA) procedures for detection of lettuce mosaic virus in portions of infected individual seeds[a]

Seed no.	ELISA[b] absorbance values ($A_{405\times1/5}$)		RISA[d] values (^{125}I cpm)	
	Original extract	Sixfold dilution[c]	Original extract	Sixfold dilution[c]
Infected				
1	0.042	0.018	285	122
2	0.044	0.022	366	152
3	0.05	0.023	396	170
4	0.069	0.032	346	132
5	0.074	0.024	425	173
6	0.2	0.07	1,076	320
7	0.483	0.217	4,136	1,156
Healthy[e]	0.019 ± 0.04[f]	0.014 ± 0.003	104 ± 21	66 ± 14

[a] Remaining half seeds from previously identified infected seeds were individually homogenized with 200 μl of extraction buffer, and three 50-μl samples were tested as follows: one was applied to a RISA plate, the second to an ELISA plate, and the third was diluted sixfold with extraction buffer and tested in duplicate in both plates (50 μl/well).
[b] Coating γ-globulin (4 μg/ml, 50 μl/well); enzyme γ-globulin conjugate (1:100 dilution, 50 μl/well); substrate (200 μl/well); substrate-enzyme reaction time 1.5 hr.
[c] Values are averages for duplicate wells.
[d] Coating γ-globulin (4 μg/ml, 50 μl/well); ^{125}I γ-globulin (1:100 dilution, 120,000 cpm/well).
[e] Three half seeds from a healthy seed lot were treated similarly to the infected half and tested on the same plates.
[f] Values are means ± standard deviation.

Table 7. Detection of lettuce mosaic virus (LMV) in lettuce seed lots with very low proportions of seed infection by radioimmunosorbent assay (RISA)[a] and the seedling grow-out method

Seed lot[b]	Seedling method index (no. infected/30,000)	Distribution of samples based on RISA values[c] (^{125}I cpm)				No. positive samples/total tested[d]
		220–280	325–499	500–750	>750	
15-78	0	15	0	0	0	0/15
21-78	0	15	0	0	0	0/15
29-78	0	15	0	0	0	0/15
30-78	0	5	0	0	0	0/5
09-78	0	0	5	0	0	0/5*[e]
11-78	0	3	12	0	0	0/15*
01-78	1	5	5	0	0	0/5*
02-78	1	1	9	0	0	0/5*
28-78	1	4	1	0	0	0/10*
25-78	2	4	1	0	0	0/10*
16-78	3	8	1	1	0	1/10
19-78	3	9	0	0	1	1/10
22-78	8	2	1	1	1	2/5

[a] Coating γ-globulin (1 μg/ml, 200 μl/well); ^{125}I-labeled γ-globulin (1/400 dilution, 200 μl/well); radioactivity 120,000 cpm/well.
[b] Seed lots were indexed and submitted by the Cooperative Extension Service in Salinas, CA.
[c] Average RISA values for four samples from a healthy seed lot tested in the same experiments with the unknowns were 228, 252, 263, and 267 cpm.
[d] Results were considered positive for a test sample when its average RISA value was twice that of healthy control samples.
[e] * Seed lot is tentatively negative.

or more infected seedlings in 30,000. Detection of LMV in these seed lots was possible in RISA tests of five to 10 samples.

DISCUSSION

The incorporation of mannose and glucosamine in the extraction buffer reduced the background by 50–70% and made it possible to readily detect LMV in lettuce samples with the equivalent of one infected seed in 500. The nonspecific interference of lettuce seed extracts may be caused by the presence of lectins in such extracts. Lectins, through binding to the carbohydrate moiety of γ-globulins, may cross-link the coating and labeled γ-globulins. This binding may be competitively inhibited in the presence of simple sugars. Although there is no evidence for the presence of lectins in lettuce seed extracts and no attempt was made to demonstrate hemagglutination activity in lettuce seed extracts, lectins are known to occur in high concentration in seeds from certain plant species (2,5) and seed lectins have been reported to bind to serum globulins (5).

A sample size of 500 lettuce seeds homogenized with 10 ml of extraction buffer is recommended for testing lettuce seed lots by the RISA method. Homogenization of the seed samples with the Polytron homogenizer provided an efficient and standardized procedure for preparation of seed extracts. Furthermore, it alleviated the need to germinate the seed samples prior to processing (6).

The variation in LMV titer among infected individual lettuce seeds may reflect the time at which the parent plants became infected. The length of time between virus infection and flowering of the parent plants may be critical not only to the rate of seed transmission (8) but also to the virus titer in infected seeds. The variation in LMV titer among infected seeds should be an important consideration in tests with artificial mixtures of seeds in which one infected seed is homogenized with a large number of healthy seeds (6). The virus titer of the infected seed in such artificial mixtures may be more critical to the outcome of the tests than the size of the sample.

In the comparative tests of RISA and the seedling grow-out method, some samples from seed lots that indexed 0, 1, or 2 infected seedlings in 30,000 had higher RISA values than average healthy control samples, but not twice as high. Although these samples did not qualify as positives, their frequency suggests that such seed lots may have a high incidence of seed coat infection with LMV but very low or no embryonic infection. Another possibility is that the infected seeds in these seed lots had a uniformly low virus titer and that the majority of the infected seedlings produced from such seeds were symptomless. At any rate, these seed lots are suspect, and RISA tests of more samples will be required before they are acceptable for planting.

Definitive, positive RISA results were obtained with samples from seed lots that indexed three to eight infected seedlings by the seedling method. These seed lots thus could be rejected based on RISA tests of only 2,500–5,000 seeds, as compared with the 30,000 seeds required in the seedling method. Large-scale screening of lettuce seed lots by the RISA method may be performed more efficiently if small numbers of samples (five to 10) from several seed lots are simultaneously tested. Extensive testing (60 samples or 30,000 seeds) will thus be reserved for these seed lots that are most likely acceptable for planting.

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