Development and Application of an Enzyme-Linked Immunosorbent Assay (ELISA) Test to Index Lettuce Seeds for Lettuce Mosaic Virus in Florida

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ABSTRACT

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Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed for indexing lettuce seed lots for lettuce mosaic virus (LMV). ELISA tests showed no differences for six geographically different LMV isolates. In addition, the percentage of LMV in four seed lots was found to be similar when tested by seedling growout, *Chenopodium quinoa* assay, and ELISA, indicating that detectable LMV antigens in seeds are probably only associated with infectious LMV. When samples containing large numbers of seeds were tested by ELISA, significant nonspecific reactions were obtained in healthy samples. These were overcome by incubating samples at room temperature for 24 hr after grinding and before adding them to test plates. Simultaneous indexing of commercial Florida lettuce seed lots by *C. quinoa* and ELISA methods showed no differences in detectability of LMV in the seed lots. The ELISA test is much faster and easier than the *C. quinoa* test.

Additional key word: serology

Lettuce mosaic virus (LMV) has been reported in at least 14 countries (10) and causes one of the most important lettuce diseases in the world. Because the usual source of primary inoculum for LMV is seed-transmitted virus, the elimination of seed lots carrying LMV has adequately controlled this disease where seed indexing programs have been implemented (8,9).

Control of LMV was first achieved in California when a seed indexing program was implemented to ensure that only lettuce seed indexed to contain zero infected seeds in 30,000 was planted commercially (8). The seedling growout method was used originally, and the *Chenopodium quinoa* Willd. test was implemented later as a more practical alternative (13). The *C. quinoa* test has been used to index lettuce seeds for LMV in southern Florida since 1974 (19). This test, however, is time-consuming and because of southern Florida's subtropical climate, the test must be done in an air-conditioned greenhouse and can only be done during the cooler months of the year (December through April). Florida state law dictates that all commercially plated seed lots pass the zero in 30,000 LMV seed test before they can be planted.

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Recently, it has been demonstrated that LMV (6,7,12) and some other seedborne viruses (eg, soybean mosaic virus [3], barley stripe mosaic virus [15], and bean common mosaic virus [12]) can be detected in seeds, using a variety of sensitive serological techniques such as serologically specific electron microscopy and enzyme-linked immunosorbent assay (ELISA). Because of the high sensitivity, small space requirements, ease with which large numbers of samples can be processed, and quantitative nature of the test (4), ELISA is a desirable alternative to current LMV indexing methods, particularly in Florida. ELISA has shown promise for detecting LMV in seeds (6,7,12). Jafarpour et al (12) reported the detection of one LMV-infected seed in a sample of 1,400; however, ELISA has not yet been developed sufficiently for practical indexing of commercial lettuce seed for LMV. A method similar to ELISA, radioimmunosorbent assay (RISA), has been suggested as a practical LMV test whereby three infected seeds in 30,000 could be detected (6,7). RISA, however, requires expensive sophisticated equipment and use of the moderately dangerous isotope ^{125}I. We have attempted to refine the ELISA test and make it useful as a practical substitute for the *C. quinoa* test. This paper reports the characteristics of this method.

MATERIALS AND METHODS

Virus sources. Six LMV isolates were maintained and used throughout the tests. Isolates G and 344 were obtained from naturally infected lettuce (*Lactuca sativa* L.) in Florida. Isolates NY-75 and NY-79 were from New York. Isolate P was obtained from lettuce seed produced in France and isolate R was from lettuce seed produced in California. All six virus isolates were maintained in *C. quinoa* by mechanical transfer using 0.03 M phosphate buffer, pH 7.0, and Carborundum as an abrasive.

Antiserum. LMV isolate 344 was used as the viral antigen for production of antisera to LMV. The virus was purified from infected pea plants (*Pisum sativum* L. 'Little Marvel'), using a modification of a procedure developed by Huttinga (11). Four hundred grams of infected tissue collected 1–2 mo after inoculation was homogenized in 1,200 ml of 0.1 M Tris buffer, pH 9.0, containing 0.1 M 2-mercaptoethanol. Three hundred twenty milliliters of CCl_4 and 320 ml of $CHCl_3$ were added and the slurry was centrifuged at 10,400 *g* for 10 min. The supernatant was adjusted to 6% polyethylene glycol 6000 (PEG) and 0.1 M NaCl and stirred for 30 min. After centrifugation at 10,400 *g* for 10 min, the pellets were resuspended in 200 ml of extraction buffer containing 1% Triton X-100. The suspension was stirred for 1.5 hr and centrifuged at 3,000 *g* for 10 min; the supernatant was adjusted again to 6% PEG and 0.1 M NaCl, stirred for 30 min, and centrifuged at 17,300 *g* for 10 min. The pellets were resuspended in extraction buffer and centrifuged to equilibrium in cesium sulfate gradients prepared in extraction buffer (36,000 rpm for 18 hr with a Beckman SW 50.1 rotor). Virus-containing zones were recovered from the gradients by droplet fractionation, diluted 1:1 with extraction buffer, and clarified by centrifugation at 12,000 *g* for 10 min. The virus was recovered from the supernatant by PEG precipitation followed by centrifugation at 12,000 *g* for 10 min and the final pellets were suspended in 1–2 ml of 0.05 M Tris-HCl, pH 7.5.

Antisera were produced in four rabbits, using a combination of toe-pad and intramuscular injections with virus preparations emulsified with adjuvant. Antisera were collected, processed as described previously (16), and stored frozen. All antisera used had micro-precipitin titers of 1,024.

ELISA. Double-antibody sandwich ELISA as described by Clark and Adams (5) was used in all cases. Microtiter plates (1-223-29, Dynatech Laboratories, Inc.,

Alexandria, VA) were coated with purified IgG at 0.25 μg/ml for 3 hr at 25 C. Samples were extracted in phosphate-buffered saline containing 0.05% Tween 20 and 2% polyvinyl pyrrolidone (PVP) (mol wt 40,000) (PBST-PVP) and incubated as two replicate 200-μl samples in plates overnight at 4 C. Alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO) was conjugated to immunoglobulins (2:1) with glutaraldehyde. The enzyme-IgG conjugate (0.5 μg/ml in PBST-PVP) was incubated in plates for 2 hr at 25 C. Substrate (*P*-nitrophenyl phosphate, 0.6 mg/ml) in diethanolamine buffer was added to plates, and reactions were allowed to progress for 2 hr at room temperature. Reactions were stopped by adding 50 μl of 3 M NaOH, and absorbance values for undiluted samples (350 μl) were determined photometrically at 405 nm.

Seed assays. The amount of LMV in seed samples was determined by three different methods. Seedling growout was used to determine the amount of seedborne infectious LMV in two seed lots. Individual seeds were sown in 6-cm-square plastic pots containing Metro Mix 300. Seedlings were tested individually for LMV by ELISA 25 days after sowing.

The *C. quinoa* test was also used to test individual seeds as well as seed lots for LMV. Individual seeds were tested by grinding one seed in 0.5 ml of inoculation buffer (0.05 M phosphate, pH 7.7, containing 0.1% 2-mercaptoethanol) followed by inoculation to two *C. quinoa* plants in the five- to six-leaf stage. Seed lot assays were done with both commercial seed lots and mixtures of healthy and LMV infected seeds prepared to simulate various levels of LMV in the seed lots. Seed lots (30,000 seeds) were tested for LMV by the method of Kimble et al (13). Sixty subsamples of 500 seeds each were triturated in 8 ml of inoculation buffer for 8 min with a ball mill. Each subsample was inoculated to five *C. quinoa* plants. Leaves were washed with water 10 min after inoculation. Plants were examined for symptoms of LMV infection 14–21 days after inoculation. All *C. quinoa* plants showing symptoms of LMV infection were tested by ELISA.

Individual seeds as well as samples of seed lots were tested for LMV by ELISA. Individual seeds were ground in 0.5 ml of PBST-PVP with a mortar and pestle. Two replicate 200-μl aliquots of extract were tested for each seed. Seed lots were tested by homogenizing seed samples (250, 500, or 750 seeds) in 7.5 ml of PBST-PVP with a Tissuemizer (Tekmar, SDT-1810 motor and SDT-182 E shaft). Samples were clarified by centrifugation for 3 min at 3,000 rpm. Two 200-μl replicate aliquots from the supernatants of each sample were used for ELISA tests. Seeds from seed lots that had passed the Florida *C. quinoa* zero in 30,000 LMV test in 1979 and 1980 were used as healthy controls.

RESULTS

Characteristics of ELISA. Initial experiments to determine optimal conditions for ELISA tests showed that the greatest sensitivity and the lowest nonspecific reactions (background) of healthy extracts or buffer alone were obtained by using 0.25 μg/ml IgG to coat microtiter plates and enzyme-antibody conjugate at 0.5 μg/ml. Because minimal background reactions were important when testing seed samples, these relatively low concentrations were used for all further experiments. Experiments to test LMV isolates of different geographic origins showed that all six isolates reacted similarly when dilutions of leaf sap from infected plants were tested by ELISA (Fig. 1). Although no attempts were made to check for differences in detectability using exact quantities of purified virus, the fact that all six isolates were always positive at 1/10,000 sap dilution and sometimes at 1/100,000 indicates these isolates are closely related serologically.

The relative efficiency of ELISA for detecting LMV in single lettuce seeds was compared with the *C. quinoa* and seedling growout methods. The *C. quinoa* and seedling growout tests determine the actual amount of infectious LMV in lettuce seeds; ELISA detects viral antigens irrespective of infectivity. Results of the seedling growout and *C. quinoa* assays were similar to those of ELISA for the four seed lots tested. ELISA results for seed lots 1 and 2 showed 104/906 (11.5%) and 3/225 (1.3%) seeds to be infected, whereas *C. quinoa* tests of the same seed lots showed 12/100 (12%) and 41/1,075 (1.02%), respectively. Seed lots 3 and 4 were found to have 24/324 (7.4%) and 6/378 (1.6%) infected seeds by ELISA and 10/126 (7.9%) and 7/270 (2.6%) infected seeds by seedling growout, respectively. These data suggest that positive results obtained by ELISA also represented infectious LMV and that potential false positives that might be obtained if significant amounts of noninfectious viral antigens were present in seeds are unlikely.

Initial experiments for detecting LMV-infected seeds among a large number of lettuce seeds demonstrated significant variability in the reactions of seed samples. High background reactions (A_{405} value of 0.2) were obtained occasionally with healthy seed extracts, often making known infected samples indistinguishable from known healthy samples. To reduce nonspecific background reactions, various procedures were attempted, including heating extracts to 50 C, changing extraction buffers, treating seed extracts with various enzymes, or clarifying extracts with organic solvents before putting samples in the test plates. Heating of samples and clarification of seed extracts by mixing with chloroform resulted in no LMV detection when testing known infected seed samples. Enzyme treatments of extracts did not increase detection of LMV. Pectinase (100 μg/ml) and cellulase (100 μg/ml) had no effect, but Driselase at 0.1% increased background reactions compared with untreated controls. The buffer described by Ghabrial et al (6) decreased background reactions, but incubating the samples for 24 hr at room temperature after grinding and before placing them in test plates was the best treatment for reducing the nonspecific background

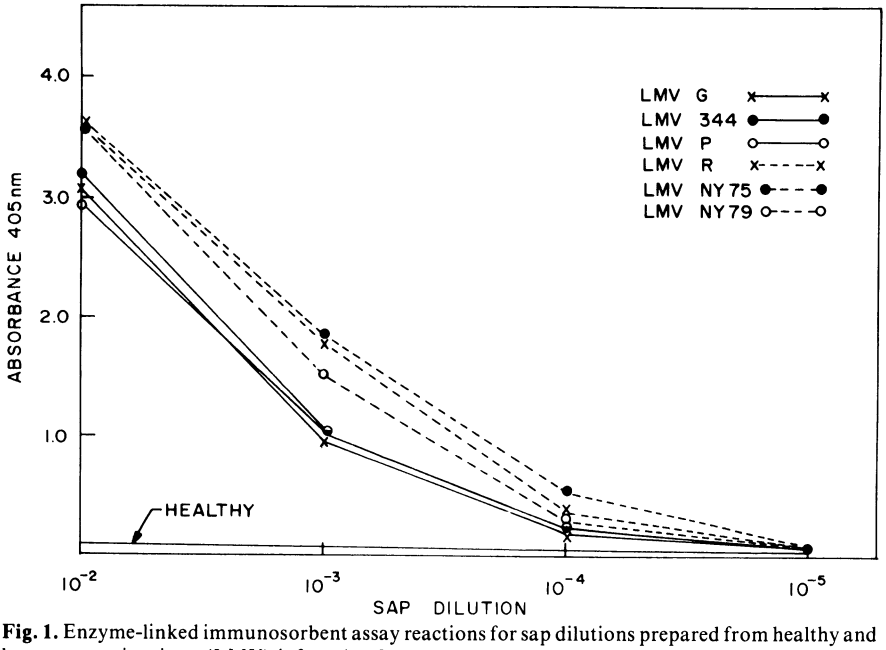

Fig. 1. Enzyme-linked immunosorbent assay reactions for sap dilutions prepared from healthy and lettuce mosaic virus (LMV)-infected *Chenopodium quinoa* leaves. Six LMV isolates were compared.

reactions and it did not affect the reactions of positive samples (Table 1). Therefore, this method was used for further testing of seed samples containing more than 100 seeds.

The A_{405} values of seed samples positive for LMV were variable, eg, samples 10 + 11 of the infected controls (4 + 0 after 24 hr) (Table 1). Because ELISA results are quantitative, they can be analyzed statistically (4). Three times the standard deviation plus the mean A_{405} value of healthy control samples has been suggested as the baseline to separate healthy and infected samples statistically (4). This was used to analyze seed samples in further tests. In one experiment, we tested, the infectivity of seed samples (four seeds each from a seed lot containing 11.5% LMV) which gave low A_{405} values barely above the baseline. The baseline was 0.21, and one of the test samples with an A_{405} value of 0.24 was infectious on *C. quinoa*. In all further tests, if both replicates of a sample were above the baseline, the sample was scored positive.

Experiments conducted to determine the sensitivity of ELISA for detecting one LMV infected seed in a given sample were done by mixing four seeds from a seed lot containing 11.5% LMV with a given number of healthy seeds. This was easier and more accurate for our purposes than previously used methods of adding known LMV positive half-seeds to healthy seeds (6,7,12). The probability distribution for the binomial experiment is given by the following formula:

$$C_x^z (u)^x (1-u)^{z-x} = P,$$

where z = number of seeds from a known infected seed lot that were added to each sample, x = number of LMV-infected seeds expected in the added sample, u = percentage of LMV infected seeds in the infected seed source, and P = probability of the test sample containing an LMV-infected seed. Therefore, when four seeds from a seed lot containing 11.5% LMV are tested, we expect 38% of the samples to contain LMV. Although we did not achieve 38% positive samples in our tests, these data may suggest that the original seed source may have contained less than the expected 11.5% LMV-infected seeds. There were no significant differences between control samples containing only four seeds or the seed mixtures containing 250 or 500 seeds (Table 2). One infected seed was consistently detected in samples containing 250 or 500 seeds but not in samples of 750 seeds. The A_{405} values for positive samples decreased by about 20–30% when samples contained 500 seeds as compared with 250 seeds (Table 3). Positive samples, however, were easily differentiated visibly from healthy checks.

Seed lot assays. Commercial lettuce seed lots from 1980, 1981, and 1982 were indexed by ELISA to compare results with those obtained by the Florida Lettuce Mosaic Committee with the *C. quinoa* test. Two seed lots were rejected by the committee in 1980 because of LMV; however, when these seed lots were tested by ELISA in 1982, no LMV was detected. One of the seed lots was tested again by the *C. quinoa* test and found to be free of LMV. All 1981 seed lots passed both the ELISA and *C. quinoa* tests. Testing of 16 commercial seed lots in 1982 by ELISA and by *C. quinoa* again showed that none contained LMV. ELISA tests thus did not indicate that any seed lots that had passed the *C. quinoa* test contained LMV. Nevertheless, it was still desirable to test commercial seed lots containing very low amounts of LMV by ELISA. Therefore, two replicates each of four seed lots containing low amounts of LMV were prepared by mixing healthy seed with infected seed. One replicate was tested by

Table 1. Enzyme-linked immunosorbent assay absorbance values for lettuce seed samples tested immediately after grinding and 24 hr later

	Number infected + number healthy seeds[a]					
	0 + 250		4 + 0		4 + 250	
Sample no.	0[b]	24	0	24	0	24
1	0.06[c]	0.05	0.32(−)[d]	0.28(+)[d]	0.26(−)	0.05(−)
2	0.18	0.05	0.36(−)	0.26(+)	0.22(−)	0.1 (−)
3	0.14	0.06	0.25(−)	0.23(+)	0.39(−)	0.1 (−)
4	0.13	0.06	0.16(−)	0.06(−)	0.5 (+)	0.49(+)
5	0.14	0.06	0.12(−)	0.03(−)	0.18(−)	0.31(+)
6	0.15	0.06	0.14(−)	0.04(−)	0.21(−)	0.29(+)
7	0.16	0.04	0.2 (−)	0.12(−)	0.27(−)	0.11(−)
8	0.16	0.12	0.27(−)	0.06(−)	0.63(+)	0.52(+)
9	0.4	0.07	0.30(−)	0.22(+)	0.38(−)	0.18(+)
10	0.22	0.10	0.72(+)	0.67(+)	0.09(−)	0.05(−)
11	0.24	0.05	0.18(−)	0.14(+)	0.17(−)	0.04(−)
12	0.09	0.05	0.08(−)	0.05(−)	0.28(−)	0.15(+)

[a] Number of lettuce seeds from a seed lot containing 11.5% LMV plus the given number of healthy seeds.
[b] Samples were tested by placing them in microtiter plates immediately after grinding (0) and after 24 hr at room temperature (24).
[c] Absorbance values for undiluted samples determined photometrically at 405 nm.
[d] (−) Indicates the sample did not contain LMV; (+) indicates the sample was rated positive for LMV. Samples were considered positive if absorbance values for both replicates were greater than three times the standard deviation plus the mean ($3s = \bar{x}$) of healthy control samples; $3s + \bar{x}$ at 0 hr = 0.41, $3s + \bar{x}$ at 24 hr = 0.13.

Table 2. Sensitivity of the enzyme-linked immunosorbent assay for detecting lettuce mosaic virus (LMV) in lettuce seed samples

Infected + healthy[a]	Experiment[b]								Total	Expected[c]
	1	2	3	4	5	6	7	8		
0 + 250	0	0	0	0	0	0	0	0	0 a[d]	0
4 + 0	6	3	2	3	4	4	6	2	30 b	36
4 + 250	3	5	4	5	7	2	2	5	33 b	36
4 + 500	0[e]	0	6	8	2	5	3	5	29 b	36
4 + 750	NT[f]	0	NT	0	0	0	0	0	0 a	27

[a] Number of lettuce seeds from an infected seed lot containing 11.5% LMV plus the given number of healthy seeds.
[b] Twelve samples of each mixture of infected and healthy seeds were tested in each experiment.
[c] Total number of expected positive samples (see text) from all experiments when samples contained the given number of seeds from a seed lot containing 11.5% LMV.
[d] Numbers followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[e] In experiments 1 and 2, a smaller shaft was used with the Tissuemizer. This shaft did not efficiently grind samples containing 500 seeds or more.
[f] Not tested.

Table 3. Effects of seed number on the absorbance values (A_{405}) of lettuce mosaic virus (LMV)-infected lettuce seed samples[a]

Infected + healthy[b]	A_{405}[c]	
	Experiment 1	Experiment 2
4 + 0	0.5	0.59
4 + 250	0.25	0.30
4 + 500	0.22	0.19
4 + 750	0.08	0.06
0 + 500	0.12	0.10

[a] Samples were homogenized in 7.5 ml of PBST-PVP, using the Tissuemizer as described in text.
[b] Number of lettuce seeds from an infected seed lot containing 11.5% LMV plus the given number of healthy seeds.
[c] Numbers are average absorbance values (A_{405}) for the positive samples at the given treatment. There were no positive samples for the 4 + 750 treatment; the A_{405} value is the mean of 12 samples in each experiment. For healthy controls (0 + 500), numbers are three times the standard deviation plus the mean for 12 healthy samples in each experiment.

Table 4. Results of testing lettuce seed lots (30,000 seeds) for lettuce mosaic virus (LMV) by the *Chenopodium quinoa* and enzyme-linked immunosorbent assay (ELISA) methods

Infected + healthy[a]	ELISA	*C. quinoa*
500 + 29,500 (10)	5[b]	4
1,000 + 29,000 (20)	5	10
2,000 + 28,000 (40)	14	10
5,000 + 25,000 (100)	24	32

[a] Number of seeds from an infected seed lot containing 2% LMV plus the given number of healthy seeds. Numbers in parentheses indicate the expected number of LMV seeds in the given sample.

[b] The number of positive samples (60 samples of 500 seeds each per seed lot) detected at each level of LMV.

ELISA and the other by the *C. quinoa* assay. LMV was detected in the four simulated seed lots by both methods (Table 4). The ELISA results for the four seed lots, however, were obtained in 5 days compared with the 6 wk required to perform the *C. quinoa* tests.

DISCUSSION

The ELISA test described here differs only slightly from originally described methods for detecting LMV in lettuce seeds (6,12), but it does demonstrate that manipulation of test conditions permits practical application of ELISA for indexing lettuce seeds for LMV.

Nonspecific background reactions of healthy seed samples initially was the main problem encountered. The reasons for the high background reactions are not known, but similar results have been obtained by others with lettuce seed samples (6,7) and when testing aphid vectors for potato leaf roll virus (PLRV) (17). Samples of 500 lettuce seeds ground in 7.5 ml of PBST-PVP approximate a 1:15 (w/w) sample dilution very rich in seed proteins. This high concentration of proteins could possibly have some effect on background reactions. In any case, because of the very low quantitative estimates for the amount of LMV per infected seed (6), it was necessary to use conditions that minimized background reactions and still allowed detection of low amounts of LMV. Background reactions were overcome by incubating samples for 24 hr at room temperature after grinding, as done for detecting PLRV. We also used relatively low coating and conjugating immunoglobulin concentrations. Thus, detection of one infected seed in samples containing as many as 500 seeds was consistently achieved.

Other workers have demonstrated that double-antibody sandwich ELISA is often not the best method for detecting a wide array of serologically related strains of a virus (1,2,14,18). In some cases, conjugation of the antibody and enzyme alters the ability of the antibody to react with heterologous serologically related virus strains. This obviously is a potential problem in an indexing system such as for LMV. We are aware of no data demonstrating serologically different strains of LMV, however, and the data presented here showing detection of six geographically different LMV isolates by double-antibody sandwich ELISA indicate this is not a critical problem. Nevertheless, we are investigating the application of various indirect ELISA methods to the LMV system to circumvent this potential problem.

We have also demonstrated that detection of LMV in a lettuce seed by ELISA corresponds with other methods for detecting infectious LMV. This question had not been addressed previously for the LMV-lettuce seed system, but it certainly has important implications for an indexing system. If noninfectious virus or viral antigens were present in seeds, as may be the case with barley stripe mosaic virus and barley seeds (15), detection of noninfectious LMV by ELISA could result in rejection of the particular seed lot in question. Our data indicate this is an unlikely possibility for LMV.

Although all of our sample reactions were analyzed by measuring absorbances with a spectrophotometer, this is probably not necessary for routine indexing of seed lots. In all cases, positive samples, as determined by their A_{405} values, were visibly yellow. It is important, however, to include adequate control samples to ensure that nonspecific reactions have been eliminated and that the test consistently detects LMV in known infected seed samples. We routinely included 18 samples of 500 healthy seeds as controls and 12 samples of which 38% contained one infected seed in 500. Therefore, absorbance values of any questionable seed lot samples could be subjected to statistical evaluation. Samples were also refrigerated and retested if there was any question with the original test.

The objectives of this work were to develop a practical, rapid, efficient, and sensitive technique for indexing lettuce seeds for LMV, particularly one that would be useful under conditions in southern Florida. Our data demonstrate that ELISA is as sensitive as the *C. quinoa* test currently employed in Florida and is much easier and more practical. Seed lots can be indexed at any time of the year, which is important in Florida. We easily indexed six seed lots per week. For the 16 commercial seed lots that were tested in Florida in 1982, the ELISA tests were completed in 3 wk, whereas the *C. quinoa* testing took 4.5 mo.

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