Lack of Seed Coat Contamination with *Cucumber mosaic virus* in Lupin Permits Reliable, Large-Scale Detection of Seed Transmission in Seed Samples

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ABSTRACT

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Sowing seed stocks with minimal virus content provides a key control measure in preventing damaging epidemics of *Cucumber mosaic virus* (CMV) in crops of narrow-leaved lupin (*Lupinus angustifolius*). A seed testing service provides an estimate of percent CMV infection based on a dry seed test in which bulked subsamples of ungerminated seed are ground to a fine powder for testing. When enzyme-linked immunosorbent assay (ELISA) was used, CMV antiserum that gave low background optical density (A_{405}) values with extracts of powder from subsamples of healthy seed provided greatest accuracy, readily detecting one infected seed in subsamples of 100 seeds. In comparative ELISAs on duplicate subsamples from eight different seed stocks, germination and dry seed tests always gave similar percent infection values. When seed coats were separated from the embryos of CMV-infected and healthy lupin seeds before testing by ELISA, the virus was only detected in embryos from infected seeds and never in their seed coats. Treatment with trisodium phosphate did not alter the low ELISA optical density (A_{405}) values obtained with seed coats separated from infected seeds. Therefore, seed coat contamination with CMV is lacking in lupin, justifying large-scale routine use of a dry seed test to estimate percent virus infection in commercial seed samples.

Additional keywords: antigen, embryo infection

When serological tests done directly on ungerminated seed samples are used in attempts to determine the incidence of virus transmission through seed to progeny seedlings, such testing generally provides considerable overestimates due to viral contamination of seed coats in the absence of embryo infection (1,16,26). With legume seeds, soaking in water, removing the seed coat, and rinsing the exposed embryos can help overcome the problem (26). However, this procedure is far too labor intensive for routine large-scale use. Albrechtsen (1) located only four published records of seed-borne virus pathosystems where detectable viral antigen is absent from the seed coat. The viruses concerned were all *Potyviridae* belonging

to the genus *Potyvirus*: *Lettuce mosaic virus* in lettuce (*Lactuca sativa*), *Peanut mottle virus* (PeMV) and the peanut stripe strain of *Bean common mosaic virus* (BCMV) in peanut (*Arachis hypogaea*), and *Soybean mosaic virus* (SMV) in soybean (*Glycine max*) (2,8,25,32). In such instances, direct testing of whole seed samples can be used routinely to establish virus seed transmission rates to seedlings (1).

Narrow-leaved lupin (*Lupinus angustifolius*) is an important cool season grain legume crop in parts of eastern Europe, Russia, southern Africa, and Australia. "Sweet" (i.e. low alkaloid content) cultivars are used for this purpose, while "bitter" forms (i.e. ones with high alkaloid contents) are used for other purposes in many additional countries, e.g., to provide green manure, groundcover, and forage, or as medicinal plants (11-13). *Cucumber mosaic virus* (CMV; family, *Bromoviridae*; genus, *Cucumovirus*) causes a serious seed-borne disease of narrow-leaved lupin worldwide (23). CMV epidemics pose a major constraint to production in south-

western Australia, where the crop is widely grown in rotation with wheat (*Triticum aestivum*) on nutrient-deficient, sandy soils, and the grain is exported mostly for stock feed. An integrated disease management approach is used to minimize yield losses, and sowing lupin seed stocks with minimal virus contents provides a key control measure within this strategy (4-7,17-20,24,30). Since 1988, a commercial testing service for CMV in lupin seed samples has provided an estimate of percent CMV infection based on a 1,000-seed test. This helps farmers to avoid sowing seed with infection levels likely to result in serious yield losses. For lupin-growing areas at lower risk from CMV, a threshold of <0.5% seed infection suffices to avoid serious yield losses in most years, but for high-risk areas a threshold level of <0.1% is used for lupin grain crops (i.e. a zero test result on a 1,000-seed sample) (4,19,20). A model forecasting CMV epidemics in lupins was incorporated into a decision support system by the Department of Agriculture and Food, Western Australia, for use by farmers and agricultural consultants in planning CMV management (30) and is accessed via the department's website.

A dry seed test on whole ungerminated seeds using enzyme-linked immunosorbent assay (ELISA) was employed initially to test 1,000-seed samples of lupin. For this, the seed samples were divided into subsamples, which were ground to a fine powder and extracts from the powder tested (24). Subsequently, a reverse transcription-polymerase chain reaction (RT-PCR) procedure that was sufficiently sensitive to detect one infected seed in an extract from a small amount of powder from 1,000 seeds was substituted to provide an initial indication of CMV presence or absence in seed samples (31). If infection was found, subsamples from a further 1,000 seeds were ground and tested by ELISA to provide a percent CMV infection value. More recently, a semi-quantitative test using TaqMan technology has been used instead. It gives an approximate CMV infection value from a single test on a

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small amount of powder from a sample of 1,000 seeds (28). Using samples of whole, ungerminated lupin seed in tests to determine percent CMV seed transmission was possible because of lack of detectable CMV in the seed coat, but evidence demonstrating its absence from seed coats was left unpublished. This article provides the proof that CMV antigens are absent from lupin seed coats. It also demonstrates the reliability of using a dry seed test routinely for a legume-virus pathosystem where viral seed coat contamination is lacking.

MATERIALS AND METHODS

Plants, seed sources, and antisera. CMV-infected cv. Wandoo and healthy cv. Gungurru lupin plants were grown from

infected and healthy seed stocks in insect-proof, air-conditioned glasshouses maintained at 15 to 20°C. In ELISA, CMV-infected cv. Wandoo and healthy cv. Gungurru leaf material provided positive and negative controls, respectively, for all germination tests, while dry powder samples from healthy cv. Merrit and CMV-infected cv. Gungurru seed stocks were always included as negative and positive controls in dry seed tests, respectively. Unless otherwise stated, the CMV-specific polyclonal antiserum used was from Loewe Biochemica, Sauerlach, Germany. Initially, antisera obtained from Bio-Rad Phyto-Diagnostics Laboratories, Marnes-La-Coquette, France, and Agdia Inc., Elkhart, IN, USA, were also employed. Nine seed stocks of four different

lupin cultivars from different locations in two Australian states were used in the tests (Table 1, Fig. 1).

ELISA. Leaf tissue or seed powder were extracted (1 g/20 ml) in citrate buffer (0.5 M sodium citrate), pH 6.8, containing ethylenediaminetetra acetic acid di-sodium salt (EDTA) at 5 mM, polyvinyl pyrrolidone at 20 g/liter, Tween 20 at 5 ml/liter and thioglycolic acid at 28.9 mM, using a leaf press (Pollahne, Hanover, Germany). Double antibody sandwich ELISA (DAS-ELISA) was used as described previously (7). Duplicate wells on microtiter plates were used for each subsample. Sample optical density values (absorbance at 405 nm [A_{405}], 40 min incubation) more than twice those recorded for healthy leaf or powder samples were considered positive. Percent infection was estimated from the results of the ELISA tests on grouped seed subsamples using the formula of Gibbs and Gower (10).

Germination tests. Lupin seeds were germinated in rolled moist paper towels, and the radicles were used for subsequent testing by ELISA as described previously (21,22).

Dry seed tests. The dry seed test procedure followed was described previously (20), except for two amendments: (i) Nunc "Maxisorp" microtiter plates (Nunc, Roskilde, Denmark) were used throughout, and (ii) the 10-min soaking step after overnight sample incubation was omitted. Samples of 1,000 seeds were grouped into 10 subsamples of 100 seeds each and

Table 1. The *Cucumber mosaic virus*-infected and healthy seed stocks used: identification, cultivars, origins, and year(s) harvested

Stock no.	Cultivar	Origin	Year harvested
Individual seed stocks			
1 ^a	Merrit	Naracoorte, SA ^b	1997
2	Gungurru	Bordertown, SA	1997
3	Gungurru	Strathalbyn, SA	1997
4	Kayla	Merredin, WA ^b	1996
5	Gungurru	Merredin, WA	1998
6 ^a	Gungurru	Manjimup, WA	1996
7	Wandoo	South Perth, WA	1999
Combined seed stocks			
8	Gungurru	Manjimup + Mt. Barker, WA	1996/1999
9	Gungurru	Manjimup + Avondale, WA	1996/1999

^a Healthy seed stock.

^b SA = South Australia, WA = Western Australia.

Fig. 1. Map of Australia showing locations where the lupin seed stocks originated. Australian states: WA = Western Australia; NT = Northern Territory; QLD = Queensland; SA = South Australia; NSW = New South Wales; VIC = Victoria; TAS = Tasmania.

ground to a fine powder using a seed crusher (Glen Creston, Stanmore, UK). The powder from each 100-seed subsample was tested separately.

Antiserum comparison. Powder from the same ground samples from lupin seed stocks 3, 4, and 9 (Table 1) was used in a series of tests that compared the magnitude of the positive and negative optical density values obtained in dry seed ELISA using three different CMV antisera. The antisera were used at conjugate and globulin dilutions of 1:750 (Loewe Biochemica), 1:100 (Bio-Rad Laboratories), and 1:200 (Agdia Inc.). Then, five healthy seeds from each of seed stocks 4, 6, and 7 were tested using the Loewe and Bio-Rad antisera to investigate background effects due to antiserum source. The seeds were left to imbibe for 2 days in rolled moist paper towels, cut open, and the seed coat and embryo components separated. Each seed component was ground individually, and the extracts were tested by ELISA.

Comparison of germination and dry seed tests. Comparisons of germination and dry seed tests were done using duplicate samples from eight different lupin seed stocks. Samples of seeds were tested by each procedure using Loewe antiserum. Percent CMV transmission efficiencies were calculated for each group of subsamples.

Comparison of virus detection in seed coats and embryos. Thirty seeds each from seed stock 7 and an additional infected seed stock of cv. Kayla from an unknown location (seed stock 10, not shown in Table 1) were left to imbibe for 2 days in rolled moist paper towels. Then, each seed was cut in half and the half seed coats separated from the half embryos. Each individual component was put into a separate 5-ml tube, which was labeled to identify the individual seed concerned. For each seed coat and embryo component from an individual seed, 2 ml of distilled water was added to one half and 2 ml 10% aqueous trisodium

phosphate to the other half. The tubes were then left to stand for 2 h at room temperature. The seed components were then washed individually six times for 1 min in distilled water before being dried using absorbent paper towel. They were then extracted individually and tested by ELISA using the Loewe antiserum.

RESULTS

Antiserum comparison. The Loewe antiserum always produced very low mean optical density (A_{405}) background values with healthy subsamples of powdered dry seed, making it easy to distinguish between the healthy and infected ones (Table 2). The average negative background values were higher using the Bio-Rad antiserum, but distinguishing between infected and healthy subsamples was still easy, and the infected subsamples identified were the same as the ones found with the Loewe antiserum. With the Agdia antiserum, the difference between optical density values (A_{405}) was insufficient to distinguish readily between infected and healthy subsamples, such that six "false positives" arose with seed stock 4 and one "false negative" with seed stock 3. Thus, the tests with Loewe and Bio-Rad antibodies detected 0.7 and 0.1% CMV infection in seed stocks 3 and 4, but the Agdia antiserum suggested infection levels of 0.5 and 1.0%, respectively. Overall, because of the low optical density values it gave with healthy seed, unless otherwise stated, Loewe antiserum was used in later tests.

In the tests involving separated individual seed coats and embryos from healthy seeds from infected seed stocks, the five seeds from stock 4 gave mean optical density values (A_{405}) of 0.03 (Loewe) and 0.01 (Bio-Rad) for seed coats, and 0.03 (Loewe) and 0.05 (Bio-Rad) for embryos. With stock 6, the corresponding mean values were 0.01 (Loewe) and 0.02 (Bio-Rad) for seed coats, and 0.02 (Loewe) and 0.04 (Bio-Rad) for embryos; and with

stock 7, 0.02 (Loewe) and 0.03 (Bio-Rad) for seed coats and 0.02 (Loewe) and 0.08 (Bio-Rad) for embryos. Overall, the Loewe antiserum usually gave the lowest absorbency values for both seed coats and embryos.

Comparison of germination and dry seed tests. The germination and dry seed tests gave similar results for the different sets of samples tested from each of the eight seed stocks (Table 3). Identical results would not have been expected for each seed stock because of small random variations in the numbers of infected seeds present within each separate sample of seeds tested. For example, with seed stock 4, by chance one infected seed was detected in the sample used for the dry seed test but none in the sample used for the germination test. For the dry seed test, distinguishing optical density (A_{405}) values for healthy and infected subsamples was always easy with the Loewe antiserum with all seed stocks.

Comparison of virus detection in seed coats and embryos. No virus was detected in any seed coats from seed stocks 7 and 10, regardless of presence or absence of embryo infection within the same seed (Table 4). Also, there was no difference between the number of embryos found infected regardless of whether a soak treatment with or without trisodium phosphate was used. Trisodium phosphate treatment had no effect on the optical density (A_{405}) values obtained with separated seed coats from seeds regardless of whether they had healthy or infected embryos. Thus, although within infected seeds the outer surface of the embryo was in contact with the inside surface of the seed coat before separation, no virus was detected in the seed coat even when this chemical was not used. In contrast, for infected embryos the optical density values were somewhat lower when the trisodium phosphate soak was used than when it was not. Presumably this difference reflects

Table 2. Dry seed test for *Cucumber mosaic virus* in lupin seeds: magnitude of positive and negative enzyme-linked immunosorbent assay optical density values (absorbance at 405 nm [A_{405}]) and effect of using different antisera^a

Seed stock no.	No. positive/10 subsamples	Positive values		Negative values		% CMV	Negative controls ^c mean	Positive controls ^c mean
		Mean	Range ^b	Mean	Range ^b			
Loewe								
3	5	0.25	0.12–0.44	0.02	0.02–0.02	0.7	0.02	0.3
4	1	0.17	0.17	0.03	0.03–0.04	0.1	0.04	0.4
9	3	0.20	0.16–0.27	0.02	0.02–0.03	0.4	0.02	0.3
Agdia								
3	4	0.62	0.38–0.77	0.22	0.17–0.27	0.5	0.15	1.8
4	7	0.25	0.21–0.29	0.14	0.14–0.15	1.0	0.10	1.8
9	3	0.28	0.22–0.38	0.16	0.1–0.19	0.4	0.11	1.8
Bio-Rad								
3	5	1.10	0.58–1.72	0.15	0.14–0.19	0.7	0.10	2.8
4	1	0.58	0.58	0.19	0.15–0.26	0.1	0.15	3.0
9	3	0.66	0.25–1.32	0.07	0.05–0.09	0.4	0.10	2.8

^a For each seed stock sample, 1,000 seeds were divided into 10 subsamples of 100 seeds each for testing. Percent infection was estimated from the results using the formula of Gibbs and Gower (10).

^b Ranges shown are means of optical density (A_{405}) values for paired subsample wells on microtiter plates.

^c Negative and positive control samples consisted of powder from healthy and infected seed stocks 1 and 5, respectively.

some limited disruption of virus particles by the remaining trisodium phosphate during embryo extraction since the virus was inside the infected embryo.

DISCUSSION

In comparative ELISAs on duplicate seed samples from different seed stocks belonging to different lupin cultivars and originating in geographically distant locations, germination and dry seed ELISAs gave similar percent CMV infection values. The virus was only detected in the embryo and never in the seed coat. Also, treatment to remove viral antigen that might be contaminating the seed coat did not reveal that any was present in untreated seeds. Lack of detectable virus contaminating the seed coat was therefore confirmed, justifying large-scale routine use of a dry seed test involving bulked samples of whole seeds to estimate percent CMV infection in commercial lupin seed samples. This appears to be the first example of a pathosystem where detectable viral antigen is absent from the seed coat but the virus concerned is not a member of the

Potyviridae. Moreover, it is only the third example of nondetection in the seed coat for a legume species, the other such examples being for peanut and soybean (1).

In general, the immunogenicity of CMV is relatively poor compared with that of many other viruses, and so production of high-quality polyclonal CMV antiserum was often difficult in the past (9). When we used three different CMV antisera to test extracts of powder from the same subsamples from three different infected seed stocks, only two of these antisera gave optical density (A_{405}) values that provided a clear distinction between positive and negative results in bulked dry seed tests involving infected and healthy seeds. When one of these antisera was used to test eight different lupin seed samples, germination and dry seed tests always gave comparable results.

Treatment of seeds with trisodium phosphate has traditionally been used to remove virus particles that contaminate seed coats, for example, when such treatment removed *Tobacco mosaic virus* (Family, Unassigned; Genus, *Tobamovirus*) from

tomato seeds (14,15) or *Subterranean clover mottle virus* (Family, Unassigned; Genus, *Sobemovirus*) from clover seeds (27). CMV is relatively unstable and its infectivity is lost rapidly outside the cell at room temperature (29). If a virus is unstable and does not persist readily outside the living cell, it may not survive on seed coats. This seems the most plausible explanation for our failure to detect CMV in detached seed coats with or without trisodium phosphate treatment, and for the close agreement between germination and dry seed tests with the CMV–lupin pathosystem. We report here the results of tests done in 1999–2000 on seed stocks some of which were recently harvested so lack of viral seed coat contamination could not be attributed to its gradual decay during long-term storage of seeds.

Following the major epidemics of the late 1980s (17,20), the success in combating CMV-induced epidemics in lupin crops in southwestern Australia resulted in large part from the wide-scale adoption of the commercial dry seed test on samples sent in for testing (19,20). Use of the seed test-

Table 3. Testing lupin seeds for presence of *Cucumber mosaic virus* by enzyme-linked immunosorbent assay (ELISA): comparison of germination and dry seed tests

Seed stock no.	Germination test		Dry seed test							
	No. seeds tested	% CMV	No. seeds tested	% CMV	Negative values		Positive values		Negative control mean ^b	Positive control mean ^b
					Mean	Range ^a	Mean	Range		
Individual seed samples										
1	3,000 ^c	0	1,000 ^d	0	0.03	0.03–0.04	...	...	0.04	0.28
2	3,000	0.3	1,000	0.3	0.02	0.02–0.03	0.47	0.46–0.48	0.05	0.52
3	3,000	0.5	1,000	0.7	0.04	0.04–0.04	0.28	0.16–0.45	0.04	0.61
4	3,000	0	1,000	0.1	0.03	0.03–0.04	0.14	0.14–0.14	0.04	0.61
5	3,000	0.2	1,000	0.4	0.05	0.05–0.06	0.54	0.37–0.63	0.06	0.56
6	3,000	0	1,000	0	0.01	0.01–0.02	...	...	0.06	0.56
Combined seed samples										
8	3,000	0.3	1,000	0.3	0.01	0.01–0.02	0.32	0.3–0.34	0.06	0.56
9	3,000	0.5	1,000	0.4	0.03	0.03–0.04	0.37	0.36–0.39	0.03	0.49

^a Ranges shown are means of optical density (A_{405}) values for paired subsample wells on microtiter plates.

^b Negative and positive control samples used consisted of powder from healthy and infected seed stocks 1 and 5, respectively.

^c Germination tests were done by ELISA on groupings of 3 or 5 radicles. Percent infection was estimated from the results using the formula of Gibbs and Gower (10).

^d Dry seed tests involved subsamples consisting of 10 lots of 100 seeds. Percent infection was estimated as with germination tests.

Table 4. Magnitude of enzyme-linked immunosorbent assay optical density values (absorbance at 405 nm [A_{405}]) in separated seed coat and embryo tissue extracts from two *Cucumber mosaic virus*-infected lupin seed stocks

Soak treatment	Seed component	No. of seed components with virus detected/30	Positive values		Negative values		Negative controls mean ^b	Positive controls mean ^b
			Mean ^a	Range ^a	Mean ^a	Range ^a		
Seed stock 7 ^c								
Distilled water	Seed coat	0	...	...	0.01	0.003–0.05	0.05	0.2
	Embryo	9	0.89	0.56–1.22	0.04	0.01–0.07	0.07	
Trisodium phosphate	Seed coat	0	...	...	0.01	0.002–0.01	0.06	0.2
	Embryo	9	0.51	0.19–0.82	0.01	0.004–0.02	0.07	
Seed stock 10 ^c								
Distilled water	Seed coat	0	...	...	0.01	0.001–0.02	0.06	0.26
	Embryo	1	0.73	0.70–0.75	0.03	0.006–0.06	0.08	
Trisodium phosphate	Seed coat	0	...	...	0.01	0.001–0.01	0.05	0.35
	Embryo	1	0.44	0.43–0.45	0.01	0.002–0.01	0.07	

^a Means and ranges shown are for individual optical density values within the paired wells tested on each microtiter plate.

^b Mean negative control values are from healthy cv. Merrit seed. Mean positive control values are from CMV-infected lupin cv. Wandoo leaf tissue.

^c Thirty seeds from each seed stock were separated into seed coat and embryo components, and each component was tested individually for virus presence.

ing service has diminished since the introduction of the first two lupin cultivars with inherently low CMV seed transmission rates in 1996 (cv. Wonga) and 1998 (cv. Tanjil) (21,23,24), but commercial seed samples from southwestern Australia are still being tested 18 years after the service was first introduced. Thus, following the 1995 growing season, 961 seed samples were tested, whereas following the 2005 growing season (i.e., 10 years later) only 149 samples were tested. With the former, CMV was found in 21% of seed samples, 2% of these having >0.2 to 1% infection and 2% having >1% infection, while with the latter the corresponding figures were 26, 0, and 0%, respectively. Limited use by southeastern Australian farmers of seed testing and lupin cultivars with inherently low seed transmission rates is reflected in the higher CMV incidences still being found in their seed. Thus, of the 25 seed samples from that region sent to Western Australia for testing following the 2005 growing season, four of the eight CMV-positive samples had >1% infection. Sowing seed with such high infection levels is likely to result in substantial grain yield losses in years when aphids arrive early in the lupin crop, resulting in high final CMV infection incidences (3,6,19,20,24).

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