Australasian Plant Pathology
https://doi.org/10.1007/s13313-022-00884-7

ORIGINAL RESEARCH ARTICLE

Cowpea mild mottle virus, a sometimes problem for French bean crops

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Received: 20 April 2022 / Accepted: 3 August 2022

Abstract
A carlavirus, closely related to *cowpea mild mottle virus* (CPMMV) and spread by silverleaf whitefly (SLW) was reported affecting fresh market beans in a major Australian growing district in 2016. Further investigations of this virus were completed through regular surveys of crops, weeds and SLW in this district from 2016–2019. Sequencing of the 3'end of the virus genome from a range of samples detected four variants, referred to as CPMMV:A:FB5288 and CPMMV:A:S1 to S3. The distribution of these four variants in survey samples showed the dominant variant in French bean crops as CPMMV:A:FB5288. The surveys also showed disease impacts were limited to autumn and varied over time. This variation is attributed to the influence of rainfall on adult insect vector levels. The experimental host range of CPMMV:A:FB5288 was shown to be limited to the *Phaseoleae* plant tribe and included the Australian native species, *Glycine canescens*. French bean varieties showed a range of susceptibilities to this dominant sequence variant from highly tolerant to very susceptible. The tolerant varieties provide the local industry with some options for disease management where previously there were none. Genetic diversity studies further highlight the need for taxonomic reform of the species referred to as CPMMV.

Keywords Carlavirus · Silverleaf whitefly · *Bemisia argentifolia* · Epidemiology · Disease spread

Introduction

A carlavirus affecting *Fabaceae* in Australia was first detected in 2016 (Persley et al. 2020). The virus was detected in fresh market French beans (*Phaseolus vulgaris*) in the Fassifern Valley, one of Australia's major production areas. The virus disease outbreak was reported by the affected grower to cause losses of up to $AUD 400,000 during a single season. These losses resulted from reduced pod set, abandoned crops and quality defects from virus-affected pods which were distorted and deformed. A second isolate of the virus was detected in a soybean (*Glycine max*) crop about 50 km from the infected bean crop at a similar time (Persley et al. 2020). Both isolates were experimentally transmitted by manual inoculation and by silverleaf whitefly (SLW; *Bemisia argentifolii* previously known as B-biotype and MEAM1-biotype of *B. tabaci*) (Wongnikong et al. 2021) from infected bean plants to uninfected soybean and bean plants (Persley et al. 2020). The virus isolates had some sequence similarity to *cowpea mild mottle virus* (CPMMV) but were not confirmed as belonging to this species. The virus was detected in three other major bean growing districts in Queensland, all at incidences of < 5% (Persley, pers. comm.). To date, only one major disease outbreak from CPMMV is reported outside the Fassifern Valley. This was in a single French bean crop grown in the Bundaberg district in 2019 and had a virus incidence > 50% (Persley, pers. comm.). CPMMV is not reported from other Australian French bean growing districts.

A review of CPMMV including the genome organization, taxonomy, and morphology of CPMMV was published by Zanardo and Carvalho (2017). The virus is a filamentous rod of approximately 10–15 × 610–700 nm in length with a positive single-stranded RNA genome of about 8200 nt. The International Committee for the taxonomy of viruses (ICTV) demarcation threshold for species within the *Carlavirus* genus is less than 72% nt identity between coat protein or polymerase sequences. A comparison of the partial coat protein nucleotide sequences indicated the Australian isolates fall within CPMMV with similar identities to each other (78.9%) and the type species originally reported from Ghana (Brunt and Kenten 1973) (GenBank NC_014730),

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Published online: 21 September 2022

however, partial sequences for the polymerase gene did not meet the ICTV criteria (Persley et al. 2020). There is also good evidence of considerable genetic diversity within the virus species, including the identification of recombinant strains (Zanardo et al. 2014).

CPMMV is one of only a few known species within the genus *Carlavirus*, family *Betaflexiviridae* which is whitefly transmitted, specifically by SLW (Almeida et al. 2005; Marubayashi et al. 2010; Rosario et al. 2014; Persley et al. 2020). SLW is widely distributed in Australian tropical and subtropical horticulture. Virus spread is non-persistent, requiring a 10 min acquisition feeding period and 5 min inoculation feeding without a latent period (Zanardo and Carvalho 2017). Seed transmission of CPMMV is unclear, some studies have reported low rates for some legume species and others were unable to demonstrate seed transmission (Zanardo and Carvalho 2017). It is likely seed transmission is isolate and host species specific.

CPMMV was first reported from Ghana infecting cowpea (Brunt and Kenten 1973) and has a natural host range largely confined to species within *Fabaceae*, however, other isolates are described naturally infecting *Solanaceae* species from Israel and Jordan (Brunt 2016), chia (*Salvia hispanica*) in Argentina (Celli et al. 2016) and *Nyctaginaceae* (*Mirabilis jalapa*), *Cleomaceae* (*Cleome affinis*), and *Asteraceae* (*Blainvillea rhomboidea*) in Brazil (Lamas et al. 2017). The virus also produces local lesions on *Chenopodium amaranticolor* which vary in severity based on the isolate used (Naidu et al. 1998) and is reported to have experimental host species within the plant families *Amaranthaceae*, *Aizoaceae*, *Asteraceae*, *Chenopodiaceae*, *Cucurbitaceae*, *Pedaliaceae*, *Scrophulariaceae*, *Solanaceae* and *Sterculiaceae*. For a full list of host species refer to the CABI website (https://www.cabi.org/isc/datasheet/15735). The virus has a wide geographical range and is reported from at least 29 countries spread across in Africa, Asia, Oceania, and South America (Brunt 2016).

Globally CPMMV causes most impact to soybean crops particularly in Brazil (Zanardo et al. 2014; Zanardo and Carvalho 2017) Argentina (Laguna et al. 2006); Mexico (Chiquito-Almanza et al. 2018), Puerto Rico (Brown and Rodrigues 2017) and China (Wei et al. 2021). Symptoms are variable and include severe stem necrosis, bud blight and dwarfing. Zanardo and Carvalho (2017) reported that CPMMV can be symptomless in some varieties of soybean and the virus also impacts other legume crops such as mung bean and peanut, however, these are less reported than impacts in soybean. CPMMV was reported to cause bean angular mosaic disease in Brazilian bean crops (Costa et al. 1983; Zanardo and Carvalho 2017). The disease was first described in Brazil based on the symptoms observed in the bean cv. Jalo and were a systemic mosaic and leaf distortion. Depending on the bean cultivar, however, symptoms vary, and infection may be asymptomatic (Mink and Keswani 1987; Tavassoli et al. 2008; Brito et al. 2012; Zanardo et al. 2014; Mukoye et al. 2015). The impact of CPMMV was more obvious in Brazilian bean crops following the release of a genetically modified (GM) variety resistant to the whitefly transmitted begomovirus, *Bean golden mosaic virus*, which causes very severe symptoms and masked the presence of CPMMV (de Faria et al. 2016). In addition to Brazil, the virus is also reported to impact fresh bean crops in Australia, Argentina, Taiwan and Tanzania (Mink and Keswani 1987; Pardina et al. 2004; Chang et al. 2013; Rosario et al. 2014; Persley et al. 2020).

This aim of this study was epidemiology discovery to better understand the relationship between virus incidence, SLW vector prevalence and the influence of rainfall on disease outbreaks. It also aimed to evaluate disease management through host genetics. The study describes the temporal spread of CPMMV affecting fresh bean crops in a single growing district in Australia and the presence of four distinct virus variants within the district. The genetic relationships of these four variants are described using the nucleic acid binding protein (NABP) coding region for comparison. Results are presented on the seasonality of disease outbreaks and how this is related to rainfall. It also highlights dominance of a single virus variant impacting commercial crops within a district, that has a diverse population of virus variants. Estimation of SLW populations relative to virus incidence provide an indication of vector population thresholds for disease outbreaks. Virus nomenclature to describe the sequence variants was adopted from (Zanardo et al. 2014) which uses the country and plant host species as descriptors (e.g. CPMMV:A:FB5288 is CPMMV isolate 5288 from Australia detected in French bean). The experimental host range, potential seed transmission and the reaction of bean varieties to the dominant variant were also investigated.

Materials and methods

Virus indexing

For virus surveys, screening for carlavirus was via a generic assay and then used virus-variant specific RT-PCRs. The generic assay was designed to ensure both known Australian sequence variants of CPMMV, CPMMV:A:FB5288 (herbarium isolate Q5288) from French bean and CPMMV:A:S1 (herbarium isolate Q5294) from soybean, (Persley et al. 2020) were detectable. The assay used a forward primer designed with the 3'end of the coat protein (CP) region of the two CPMMV sequence variants, primer CPMMVcpF3 (5'-AAYTTCAATCACGCYAARAAG-3'). This was used with Poty1 reverse primer (Gibbs and Mackenzie 1997) in RT-PCR to amplify approximately 480 bp from the coat

protein through to the 3'end of the genome. Survey samples were initially screened with this assay.

Total nucleic acid extracts (TNAEs) were prepared from leaf samples using the Qiagen Biosprint Kit as per manufacturers' instructions. All primers were fabricated by Macrogen (South Korea). First strand cDNA was generated from 2 µL of TNAE using 10 pmol of Poty1 primer, 0.5 mM dNTPs, 100 U SuperScript® III (ThermoFisher Scientific), 10 U RNaseOUT™ (ThermoFisher Scientific) and 5 mM DTT in a final reaction of 20 µL. Reactions were incubated at 50 °C 1 h then 70 °C for 15 min. PCR used 2 µL of cDNA into a final reaction volume of 50 µL containing 25 pmol of CPMMVCPF3 primer, 5 pmol of Poty1 primer, 0.2 mM dNTPs, 5 X reaction buffer, 1.75 mM MgCl2 and 2.5 U MangoTaq (Bioline). Reactions were incubated at 95 °C 1 min, then through 35 cycles of 95 °C 20 s, 50 °C 20 s, 72 °C 20 s then a final incubation of 72 °C 5 min.

To confirm virus identities, the amplicon from the CPMMVcpF3/Poty1 RT-PCR was sequenced from a selection of samples using direct sanger sequencing of the amplicon. This sequencing identified a variant almost identical to the soybean isolate 5294 and an additional two virus sequence variants not previously known. The variant with high similarity to isolate 5294 is referred to as CPMMV:A:S1 and the two new variants as CPMMV:A:S2 and CPMMV:A:S3 and all three sequences were derived from samples of the weed species *Macroptilium atropurpureum* (siratro). Four new specific forward primers were subsequently developed using these sequences to screen samples for these variants. These primers were CPMP5288F (5'-GCTACG GCGTTTACTCTAAGG-3'; variant CPMMV:A:FB5288), CPMP5294F (5'-CCATCGCTGTTTCCGAGTGAATCC CAAG-3'; variant CPMMV:A:S1), CPMPS2F (5'-TGG CCCCTCTTGTTTAGGCTCAG-3'; variant CPMMV:A:S2) and CPMPS3F (5'-AGATCGGTCTAATCGCAATGAAAT A-3'; variant CPMMV:A:S3) and were used with Poty1 in RT-PCR. First stand cDNA is as described above and PCR reactions were also similar, the exception is only 7 pmol of the forward primers was used in the reaction. Reactions were incubated at 95 °C 1 min, then through 30 cycles of 95 °C 15 s, 60 °C 20 s, 72 °C 30 s then a final incubation of 72 °C 5 min. Survey samples were screened using RT-PCR for all four sequence variants in separate reactions. Amplicon sizes for the four assays were 180 bp CPMP5288F, 298 bp CPMP5294F, 367 bp CPMPS2F and 575 bp for CPMPS3F.

For screening of representative samples from the host range and bean variety studies, the primers Carla4937F (5'-ACIGARTCIGAYTAYGARGCITTYGA-3') was used with Carla5220R (5'-GMRCACATRTCRTC ICCIGCRAA-3') in RT-PCR. These primers were designed to the replicase and modified from those published in 2013 (Chang et al. 2013). The modifications were to expand the specificity of the primer set to amplify more species of carlaviruses. The RT-PCR used the same reagent mix and protocol as listed above except, the PCR was a 25 µL final volume used with 10 pmol of Carla4937F and 10 pmol of Carla5220R. The cycling parameters were 94 °C 1 min, 35 cycles of 94 °C 20 s, 56 °C 30 s, 72 °C 60 s then a final incubation of 72 °C 3 min and the expected product size 283 bp.

CPMMV genetic analyses

A phylogenetic analysis was conducted using the nucleic acid binding protein coding region of the CPMMV genome. This region was amplified using the CPMMVcpF3/Poty1 primer pair and Sanger sequenced for the Australian isolates or extracted from complete CPMMV genome sequences available on Genbank through the National Center for Biotechnology Information. The Australian isolates included in the analyses included CPMMV:A:FB5288, the French bean isolate (Persley et al. 2020) and CPMMV:A:S1 which is identical within this genome region to the soybean isolate 5294 (Persley et al. 2020) and detected from siratro in the Fassifern valley. It also included the two newly detected variants CPMMV:A:S2 and CPMMV:A:S3, both from siratro. Phylogenetic analysis was carried out using the MrBayes program (Huelsenbeck and Ronquist 2001) implemented in Geneious R10.2 with the following parameters: rate matrix GTR + G with four gamma categories; 1.1×10^6 cycles for the MCMC algorithm, sampling one tree per 200 cycles; discarding 10^5 samples as burn-in. Pepper virus A (Genbank: YP009357234) was used as the outgroup.

Virus surveys

Multiple commercial legume crops and weeds were surveyed in the Fassifern valley during the study. This included French bean, soybean, and mung bean (Table 1). Figure 1 shows the spatial spread of survey sites of French bean crops and weeds. All other crops surveyed were within the same areas, adjacent to French bean crops. Regular surveys of crops commenced in early November 2016 and concluded in June 2019. The surveys were done every two to three weeks during production periods. Surveys typically consisted of inspecting 50 m per row of crop extending from the block edge for virus-like symptoms. This was repeated for a total of 16 rows. Using industry standard planting densities, this equates to *ca.* 15,000 plants inspected for French bean, 13,000 for soybean and 19,000 for mung bean. The total number of surveys done for each host crop and the number of crops inspected is provided in Table 1. Crops were surveyed multiple times.

Pumpkin is a highly favoured host of SLW and is present in the district during the summer season. Although pumpkin is not a known host of CPMMV, pumpkin crops were surveyed a total of 36 times during the spring/summer seasons

Table 1 Cowpea mild mottle virus (CPMMV) incidence detected in field samples collected from commercial crops or weeds during the study. The incidence of CPMMV is provided as a percent of the samples tested and used the generic CPMMVcpF3/Poty1 RT-PCR assay and the four specific RT-PCR assays. The proportion of each virus variant is provided as a percent of the positive generic RT-PCR samples determined using specific RT-PCR

Host[a]			Samples tested	CPMMV incidence using generic RT-PCR (%)	CPMMV variant incidence using specific RT-PCR assays[c]			
	Crops surveyed	Total surveys			A:FB5288 (%)	A:S1 (%)	A:S2 (%)	A:S3 (%)
French bean	72	274	312/303 [b]	51.6	44.7	4.9	6.6	8.2
Soybean	19	64	54	0.0	0.0	0.0	0.0	0.0
Mung bean	4	5	3	0.0	0.0	0.0	0.0	0.0
Lucerne	1	1	3	0.0	0.0	0.0	0.0	0.0
Pumpkin	5	36	3	0.0	0.0	0.0	0.0	0.0
Siratro	n/a	n/a	17/16[b]	64.7	43.8	50.0	18.8	62.5
Glycine	n/a	n/a	6	83.3	16.7	0.0	66.7	66.7

[a]for each survey *ca*. 15,000 plants inspected for French bean, 13,000 for soybean and 19,000 for mung bean, multiple crops surveys completed for each host listed

[b]indicates the total samples tested in generic/specific assays where the number varied

[c]Specific RT-PCR assays used the following variant-specific forward primers A:FB = CPMP5288F, A:S1 = CPMP5294F, A:S2 = CPMPS2F and A:S3 = CPMPS3F

to further evaluate its potential as a virus reservoir status. Approximately, 3000 pumpkin plants were inspected per survey. Lucerne is occasionally grown in the district and although not a known host of CPMMV, a single crop was inspected during the 2016/2017 summer season, plant numbers for the survey were not recorded..

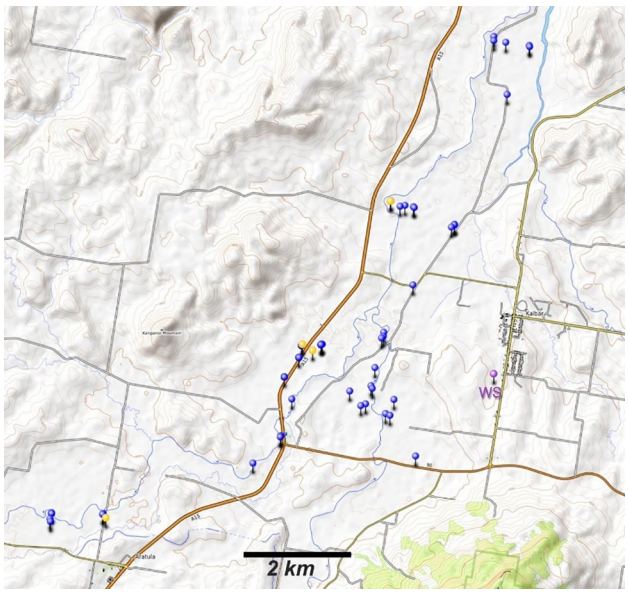

Fig. 1 Map of the Fassifern valley production area showing the locations of positive CPMMV detections in French bean crops (blue markers) and weed hosts (yellow markers). The black line is a scale bar of 2 km is included as a distance reference. The purple marker is the closest weather station

In the same crop survey area, 150 plants were inspected for adult silverleaf whitefly and the number of adults on one leaf per plant recorded. Symptomatic plant samples were collected and indexed for CPMMV by RT-PCR.

In addition to survey of commercial crops, riparian areas and field edges were inspected for potential alterative hosts for CPMMV. This was done multiple times during the study. Whiteflies were also collected using vacuum sampling at various times to evaluate these areas as a reservoir for the vector. A total of 30 vacuum samplings were done from the start of September 2017 through to the end of May 2018. Vegetation in the riparian area vacuum sampled for SLW included volunteer tomato plants, legume weeds including glycine, siratro and phasey bean and various other non-identified plant species. Vacuum collections were from general locations and not made from specific plant species. Representative whitefly samples from the bean crops and riparian areas were morphologically confirmed as SLW.

Weather data and analyses

Weather data was sourced from the Australian Bureau of Meteorology weather station 040,104 located in the township of Kalbar. The station was between 2–9 km from the survey sites (Fig. 1).

Host range and varietal virus tolerance

The host range of CPMMV:A:FB5288, the predominant sequence variant, was evaluated in glasshouse studies using mechanical inoculations. This included testing a range of

plant species as hosts of the virus and French bean varieties for potential virus tolerance (Tables 2 and 3).

The host range study included 23 species from the *Fabaceae* family, all from the subfamily *Faboideae*, one species from *Cucurbitaceae* and two from *Solanaceae* with at least five plants of each species and each variety of soybean tested (Table 2). Host reactions were evaluated under whitefly-free conditions within a glasshouse. Inoculum was prepared from CPMMV:A:FB5288 infected French bean cv. Wyatt or soybean cv P791 by grinding infected leaf tissue in 0.1 M potassium phosphate buffer pH 7 and using celite as the abrasive. Most test species were inoculated when plants were at the first or second trifoliate leaf stage. Presence and types of symptoms were recorded over 3–4 weeks. Test plants were indexed for CPMMV by RT-PCR using the primers Carla4937F/Carla5220R in pooled samples of 10 plants per pool with 100 plants per variety tested.

To evaluate virus tolerance in French bean, a field trial was completed to evaluate 22 varieties (Table 3). Seed was sown as 3 m plots with two replicates per variety. Plants in one metre of both replicates were inoculated with CPMMV:A:FB5288 approximately 14 days after sowing when plants were at the first trifoliate leaf stage. Inoculated plants were rated three times for symptom type and severity using a zero to five scale. The degree of pod damage due to virus was also assessed. Tolerance was defined as asymptomatic to mild symptoms on leaves (rating 0–2) and < 1% deformed pods. Test plants were indexed for CPMMV by RT-PCR using the primers Carla4937F/Carla5220R in pooled samples of 10 plants per pool with 100 plants per variety tested.

Table 2 Host range of variant CPMMV:A:FB5288 as determined from mechanical sap inoculations. Symptom type is indicated as (-) no symptoms, Mo = mottle, Mos = mosaic, Vc = vein clearing. Plants were evaluated in a pot trial under glasshouse conditions

Family	Species	Common name	Symptoms
Cucurbitaceae	*Cucurbita pepo* Squash		–
Fabaceae[1]	*Arachis hypogaea*	peanut	–
	Cicer arietinum	chickpea	–
	Desmodium intortum	greenleaf desmodium	–
	Glycine canescens[b]	silky glycine	Mo
	Glycine cyrtoloba		Mo
	Glycine max[c]	soybean	Mo, Mos, Vc
	Lablab purpureus	dolichos	–
	Medicago sativa	lucerne/alfalfa	–
	Macroptilium atropurpureum	siratro	Mo
	Macroptilium bracteatum	burgundy bean	Mo
	Macroptilium lathyroides	phasey bean	Mo
	Pisum sativum	pea	–
	Phaseolus acutifolius	tepary bean	Mo
	Phaseolus coccineus	runner bean	Mo
	Phaseolus dumosus	year bean	Mo
	Phaseolus filiformis	Slender stem bean	Mo
	Phaseolus lunatus	lima bean	Mo
	Phaseolus vulgaris[d]	French bean	Mo
	Stylosanthes spp.		–
	Vicia faba	broad bean	–
	Vigna luteola	hairy cowpea	Mo
	Vigna radiata	mung bean	Mo
	Vigna unguiculata subsp. *unguiculata*[e]	cowpea	Mo, Mos
Solanaceae	*Capsicum annuum*		–
	Solanum lycopersicum (tomato)		–

[a]All belong to the subfamily *Faboideae*

[b]This species is native to Australia

[c]Six soybean cultivars were assessed. These were A 6785, P 791, Hayman, Fernside, Bunya, Ascot. All showed the same symptom response

[d]The cultivar Wyatt was used as a positive control for the study

[e]The seven cowpea varieties Caloona, Red Caloona, Black Eye, Poona, Black Stallion, Ebony and Calypso were assessed and the first five were susceptible with symptoms as listed. Ebony and Calypso showed no symptoms

Table 3 Reactions of a range of French bean varieties to the variant CPMMV:A:FB5288 as determined from mechanical sap inoculations and evaluated under field conditions. Varieties were given an average rating across both replicate plots based on symptoms. The rating scale was 0 = no symptoms, 1 = very mild, 2 = mild, 3 = leaf mottling/mosaic evident, 4 = severe leaf mottling/mosaic evident and 5 = very severe, plant stunting evident

Variety	Disease rating
Aldrin	5
Cabot	4
Cahill	0–1
Colter	4
Excalibur	4
Greenleaf	0–1
Hickok	4
Jackson	4
Jade	1
Jaguar	0–1
Labrador	1
Messi	0–1
New Pioneer	0–1
Outlaw	0–1
Simba	1
Stanley	1
Sybaris	0–1
Tasman	1
Venice	0–1
Voltage	3
Wyatt	4

Seed transmission

To investigate possible seed transmission of CPMMV, young plants of two bean varieties, Wyatt and Stanley and a soybean variety P791 were grown in 25 m field plots and inoculated with isolate CPMMV:A:FB5288. All plants developed CPMMV-like symptoms. Seed was harvested and stored in a low humidity cool room before being sown and grown in a whitefly-free greenhouse. Germinated seedlings were monitored for four weeks for symptoms. Samples of all test plants were pooled in groups of 10 and screened by DAS-ELISA according to the suppliers' instructions using CPMMV carlavirus antibodies (DSMZ Germany) at the conclusion of experiments.

Additionally, commercial seed batches of French bean (cv. Wyatt), soybean (cvs. ZAM 1, P791, Bunya) and mung bean (cv. Jade) were evaluated for virus transmission. All these varieties were known to be susceptible to at least one Australian variant of CPMMV and most grown in the Fassifern valley. The seed was germinated in a whitefly-free greenhouse and seedlings were monitored for virus symptoms for four weeks and then tested as above by DAS-ELISA.

Results

CPMMV genetic diversity

The complete coding regions of the nucleic acid binding protein was analysed for the collected isolates and showed distinctly different strains, all present within the Fassifern Valley. The GenBank accession references for these are CPMMV:A:FB5288—OM289951, CPMMV:A:S1—OM289950, CPMMV:A:S2—OM289949 and CPMMV:A:S3—OM289948. The Australian isolates clustered into two separate clades with CPMMV:A:FB5288 clustering with the majority of isolates described (Fig. 2) with amino acid identities of 82–87% (Table 4). The other three Australian variants clustered together (Fig. 2) and were 82–88% identical to each other and < 67% identical to CPMMV:A:FB5288 (Table 4). All other relationships of this clade had < 74% amino acid identities. The type species from Ghana (NC_014730) was < 68% identical to all isolates studied, with the highest similarity in the NABP region to CPMMV:A:S3 at 67.6%. The distance matrices for amino acid similarities are available in Table 4.

Surveys

Over 95% of the French bean crops surveyed in the Fassifern Valley were the variety, Wyatt. The other varieties surveyed included Prairie, Aldrin, and Venice. Results of virus and whitefly surveys are shown in Tables 1, 5 and 6. Surveys only detected CPMMV in legume species. These included French bean crops and legume weeds in riparian areas and the virus was not detected from mung bean, soybean, lucerne or pumpkin crops (Table 1). CPMMV:A:FB5288 was found at the highest incidence (44.7%) in French bean crops with the remaining three variants at incidences of 4.9 to 8.2%. This includes CPMMV:A:S1 which is almost identical to the original isolate reported from soybean (Persley et al. 2020) and confirms presence of this variant within the district, although not in soybean crops. All four sequence variants were also found in the legume weed siratro (*Macroptilium atropurpureum*) at similar incidences, except for CPMMV:A:S2 which was only at half the incidence or less than the other variants. Mixed infections of the variants were detected in 12.5% of the CPMMV positive samples including French bean and both legume weeds (results not shown). All four variants were detected from only six samples, three each of French bean and siratro. Spatially, CPMMV was detected throughout the valley (Fig. 1) and highest incidences were detected closest to the riparian areas where infected weeds were detected (results not shown).

Interestingly, during all years surveyed, CPMMV was only found in autumn plantings of French bean crops (Table 5). This was irrespective of the sequence variant. A range of symptom types were seen during surveys in French bean including mosaic, mottle, chlorosis, vein-banding, and leaf distortion (Table 6). The most consistently observed symptoms were of a mottle and/or mosaic. Symptom severity ranged from mild to severe,

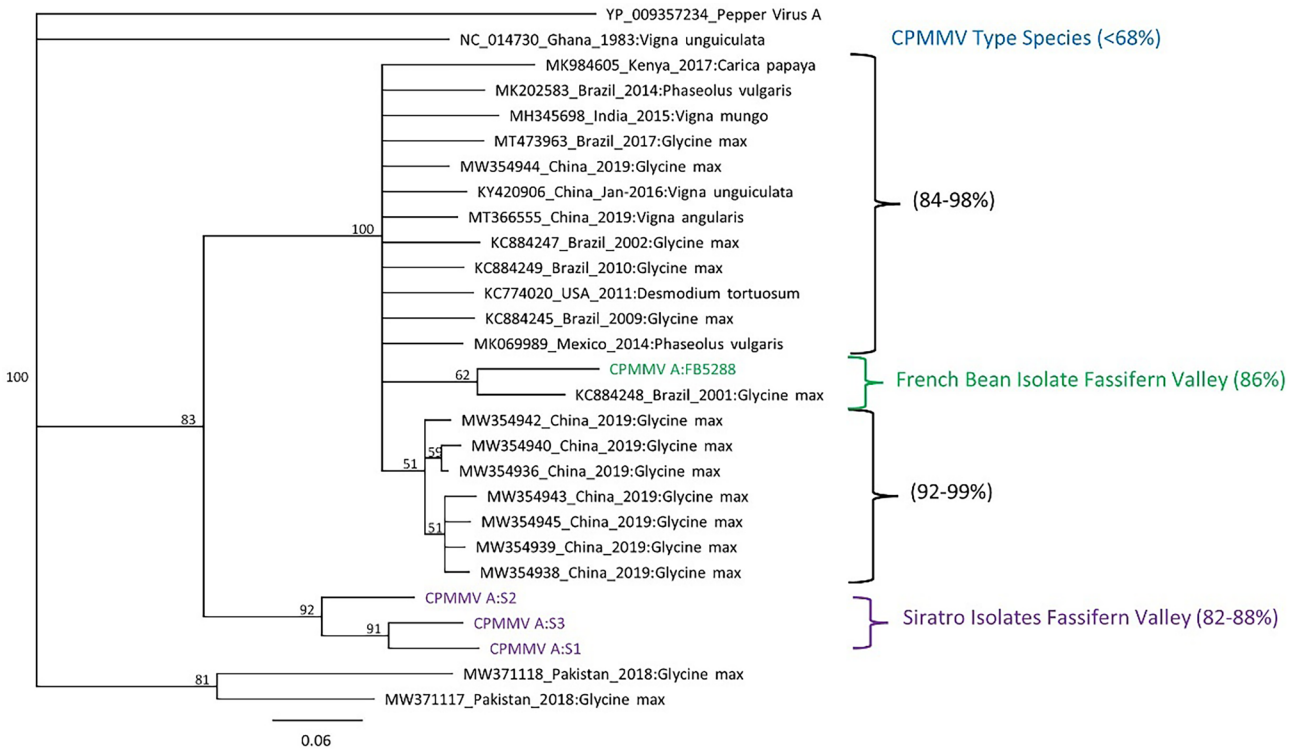

Fig. 2 Genetic relatedness of cowpea mild mottle virus (CPMMV) isolates based on phylogenetic analyses of the amino acid alignment of the nucleic acid binding protein (NABP) using MrBayes. The amino acid diversity as percent identity of selected groups is shown in brackets. The GenBank accession references for the four Australian isolates are CPMMV:A:FB5288 = OM289951, CPMMV:A:S1 = OM289950, CPMMV:A:S2 = OM289949 and CPMMV:A:S3 = OM289948. Pepper virus A was used as an outgroup for the analysis

for all symptom types observed. The observed symptoms are like those reported from disease outbreaks of CPMMV in French bean in other countries (Mink and Keswani 1987; Pardina et al. 2004; Chang et al. 2013; Zanardo and Carvalho 2017).

In general, the average adult SLW numbers across the district tend to reflect incidences of CPMMV within crop (Table 6). Highest numbers of SLW were recorded at the start of autumn for all three years. Very low virus incidence was observed in 2017, low to moderate in 2018 and high in

Table 4 Nucleic acid binding protein coding region alignment of CPMMV isolates. Amino acid sequences were aligned with Muscle in Geneious R10.2 with 10 iterations. The relatedness of isolates is shown as percent amino acid identity

	YP_009357234	NC_014730	MW371118	MW371117	CPMMV A:FB5288	MK984605	KC884248	MH345698	MW354944	MW354942	MW354943	MW354940	MW354936	MW354938	MW354945	MW354939	KY420906	MK202583	MT473963	MT366555	KC774020	KC884245	KC884249	MK069989	KC884247	CPMMV A:S2	CPMMV A:S3	CPMMV A:S1
YP_009357234_Pepper Virus A		53.9	53.3	51.4	51.4	48.2	47.3	52.4	53.3	53.3	53.3	52.4	52.4	51.4	52.4	52.4	52.4	51.4	49.1	51.4	49.1	49.1	49.1	49.1	48.2	48.2	50.9	50
NC_014730_Ghana_1983:Vigna unguiculata	53.9		56.9	57.8	54.9	56.9	54.9	60.8	60.8	60.8	60.8	61.8	61.8	59.8	60.8	60.8	60.8	58.8	57.8	58.8	59.8	58.8	59.8	59.8	57.8	63.7	67.6	61.8
MW371118_Pakistan_2018:Glycine max	53.3	56.9		75.2	55.3	56.3	55.3	59.2	59.2	60.2	60.2	59.2	59.2	58.3	59.2	59.2	58.3	57.3	57.3	57.3	57.3	56.3	57.3	58.3	56.3	58.1	60	61.9
MW371117_Pakistan_2018:Glycine max	51.4	57.8	75.2		60.2	61.2	58.3	62.1	65	65	64.1	64.1	64.1	64.1	63.1	63.1	63.1	63.1	63.1	63.1	63.1	62.1	63.1	62.1	63.1	61.9	65.7	66.7
CPMMV A:FB5288	51.4	54.9	55.3	60.2		85.4	86.4	82.5	85.4	83.5	82.5	82.5	82.5	81.6	82.5	82.5	84.5	84.5	86.4	87.4	85.4	85.4	85.4	84.5	85.4	66.7	63.8	63.8
MK984605_Kenya_2017:Carica papaya	48.2	56.9	56.3	61.2	85.4		87	84.5	89.3	86.4	87.4	88.3	88.3	86.4	87.4	87.4	91.3	89.3	89.8	91.3	90.7	89.8	89.8	89.8	90.7	69.1	66.4	66.4
KC884248_Brazil_2001:Glycine max	47.3	54.9	55.3	58.3	86.4	87		87.4	89.3	87.4	87.4	87.4	88.3	87.4	86.4	87.4	89.3	92.2	86.7	90.3	85	85.8	86.7	85	85.8	70.3	64	64.9
MH345698_India_2015:Vigna mungo	52.4	60.8	59.2	62.1	82.5	84.5	87.4		93.2	91.3	87.4	89.3	90.3	89.3	88.3	89.3	92.2	88.3	90.3	90.3	89.3	89.3	90.3	89.3	88.3	69.5	65.7	66.7
MW354944_China_2019:Glycine max	53.3	60.8	59.2	65	85.4	89.3	89.3	93.2		96.1	92.2	94.2	95.1	94.2	93.2	94.2	95.1	93.2	93.2	95.1	94.2	94.2	95.1	94.2	93.2	70.5	67.6	67.6
MW354942_China_2019:Glycine max	53.3	60.8	60.2	65	83.5	86.4	87.4	91.3	96.1		94.2	96.1	97.1	96.1	95.1	96.1	93.2	91.3	91.3	93.2	92.2	92.2	93.2	94.2	91.3	72.4	68.6	68.6
MW354943_China_2019:Glycine max	53.3	60.8	60.2	64.1	82.5	87.4	87.4	87.4	92.2	94.2		94.2	95.1	96.1	97.1	96.1	91.3	93.2	91.3	92.2	91.3	91.3	92.2	93.2	92.2	70.5	68.6	67.6
MW354940_China_2019:Glycine max	52.4	61.8	59.2	64.1	82.5	88.3	87.4	89.3	94.2	96.1	94.2		99	96.1	97.1	98.1	93.2	91.3	91.3	93.2	93.2	92.2	93.2	94.2	91.3	72.4	68.6	67.6
MW354936_China_2019:Glycine max	52.4	61.8	59.2	64.1	82.5	88.3	88.3	90.3	95.1	97.1	95.1	99		97.1	96.1	97.1	94.2	92.2	92.2	94.2	93.2	93.2	94.2	95.1	92.2	72.4	68.6	67.6
MW354938_China_2019:Glycine max	51.4	59.8	58.3	64.1	81.6	86.4	87.4	89.3	94.2	96.1	96.1	96.1	97.1		97.1	98.1	93.2	91.3	91.3	93.2	92.2	92.2	93.2	94.2	91.3	70.5	66.7	65.7
MW354945_China_2019:Glycine max	52.4	60.8	59.2	63.1	82.5	87.4	86.4	88.3	93.2	95.1	97.1	97.1	96.1	97.1		99	92.2	90.3	91.3	93.2	93.2	92.2	93.2	94.2	91.3	71.4	67.6	66.7
MW354939_China_2019:Glycine max	52.4	60.8	59.2	63.1	82.5	87.4	87.4	89.3	94.2	96.1	96.1	98.1	97.1	98.1	99		93.2	91.3	91.3	93.2	93.2	92.2	93.2	94.2	91.3	71.4	67.6	66.7
KY420906_China_Jan-2016:Vigna unguiculata	52.4	60.8	58.3	63.1	84.5	91.3	89.3	92.2	95.1	93.2	91.3	93.2	94.2	93.2	92.2	93.2		92.2	92.2	95.1	93.2	93.2	94.2	93.2	92.2	69.5	65.7	65.7
MK202583_Brazil_01-Oct-2014:Phaseolus vulgaris	51.4	58.8	57.3	63.1	84.5	89.3	92.2	88.3	93.2	91.3	93.2	91.3	92.2	91.3	90.3	91.3	92.2		95.1	94.2	95.1	95.1	96.1	95.1	96.1	70.5	68.6	66.7
MT473963_Brazil_2017:Glycine max	49.1	57.8	57.3	63.1	86.4	89.8	86.7	90.3	93.2	91.3	91.3	91.3	92.2	91.3	91.3	91.3	92.2	95.1		95.1	96.5	96.5	96.5	96.5	96.5	73	68.5	68.5
MT366555_China_2019:Vigna angularis	51.4	58.8	57.3	63.1	87.4	91.3	90.3	90.3	95.1	93.2	92.2	93.2	94.2	93.2	93.2	93.2	95.1	94.2	95.1		96.1	96.1	97.1	96.1	95.1	71.4	67.6	67.6
KC774020_USA_2011:Desmodium tortuosum	49.1	59.8	57.3	63.1	85.4	90.7	85	89.3	94.2	92.2	91.3	93.2	93.2	92.2	93.2	93.2	93.2	95.1	96.5	96.1		98.2	98.2	98.2	97.3	73	69.4	67.6
KC884245_Brazil_2009:Glycine max	49.1	58.8	56.3	62.1	85.4	89.8	85.8	89.3	94.2	92.2	91.3	92.2	93.2	92.2	92.2	92.2	93.2	95.1	96.5	96.1	98.2		98.2	98.2	97.3	73	68.5	66.7
KC884249_Brazil_2010:Glycine max	49.1	59.8	57.3	63.1	85.4	89.8	86.7	90.3	95.1	93.2	92.2	93.2	94.2	93.2	93.2	93.2	94.2	96.1	96.5	97.1	98.2	98.2		98.2	97.3	72.1	68.5	66.7
MK069989_Mexico_2014:Phaseolus vulgaris	49.1	59.8	58.3	62.1	84.5	89.8	85	89.3	94.2	94.2	93.2	94.2	95.1	94.2	94.2	94.2	93.2	95.1	96.5	96.1	98.2	98.2	98.2		97.3	73.9	70.3	68.5
KC884247_Brazil_2002:Glycine max	48.2	57.8	56.3	63.1	85.4	90.7	85.8	88.3	93.2	91.3	92.2	91.3	92.2	91.3	91.3	91.3	92.2	96.1	96.5	95.1	97.3	97.3	97.3	97.3		73	69.4	67.6
CPMMV A:S2	48.2	63.7	58.1	61.9	66.7	69.1	70.3	69.5	70.5	72.4	70.5	72.4	72.4	70.5	71.4	71.4	69.5	70.5	73	71.4	73	73	72.1	73.9	73		86.5	82
CPMMV A:S3	50.9	67.6	60	65.7	63.8	66.4	64	65.7	67.6	68.6	68.6	68.6	68.6	66.7	67.6	67.6	65.7	68.6	68.5	67.6	69.4	68.5	68.5	70.3	69.4	86.5		88.3
CPMMV A:S1	50	61.8	61.9	66.7	63.8	66.4	64.9	66.7	67.6	68.6	67.6	67.6	67.6	65.7	66.7	66.7	65.7	66.7	68.5	67.6	67.6	66.7	66.7	68.5	67.6	82	88.3	

Table 5 Cowpea mild mottle virus (CPMMV) incidence in French bean crops by season using the generic CPMMVcpF3/Poty1 RT-PCR assay and specific variant assays. The proportion of each virus variant is provided as a percent of the positive generic RT-PCR samples determined using specific RT-PCR

Year-Season	Samples tested	Incidence of CPMMV positive samples using the generic RT-PCR assay (%)	Proportion of CPMMV positive samples using specific RT-PCR assays (%)[a]			
			A:FB5288	A:S1	A:S2	A:S3
2016/2017-Summer	25	0.0	0.0	0.0	0.0	0.0
2017-Autumn	94	48.9	38.3	5.3	1.1	7.4
2017-Spring	37	0.0	0.0	0.0	0.0	0.0
2017/2018-Summer	31	0.0	0.0	0.0	0.0	0.0
2018-Autumn	57	93.0	87.7	10.5	14.0	15.8
2018-Spring	6	0.0	0.0	0.0	0.0	0.0
2018/2019-Summer	0	n/a	n/a	n/a	n/a	n/a
2019-Autumn	54	100.0	92.6	8.0	20.4	16.7

[a]Specific RT-PCR assays used the following variant-specific forward primers A:FB = CPMP5288F, A:S1 = CPMP5294F, A:S2 = CPMPS2F and A:S3 = CPMPS3F

2019. This corresponds to average SLW adult populations per survey site at the start of autumn of about 11, 57 and 144, respectively and virus incidences of < 1%, up to 60% and up to 100%, respectively. This data provides some indication of the threshold of adult SLW needed to trigger an outbreak of CPMMV disease in this district and is about one SLW adult per leaf (2019 data). SLW were regularly detected from the riparian areas at an average of 3.2 adults per sampling and detection occurred from over half the samplings (results not shown). The numbers of adults varied per sampling from none to a maximum of 15 adults. This indicates the riparian areas are a good environmental reservoir for SLW.

Influence of rainfall in whitefly and virus

During the study period there were two years of severe (2016 and 2019) and two of negligible (2017 and 2018) disease outbreaks in the Fassifern Valley bean crops. The autumn crops of 2016 and 2019 had severe disease outbreaks compared to 2017 and 2018. Evaluation of the weather data showed temperature was unlikely to be epidemiologically important as it was relatively uniform across all these years. Rainfall, however, varied considerably in amount and timing (Table 7). The rainfall averages for the four summers were 74.5, 85.8, 153.3, and 34.1 mm, respectively. This was followed by variable average March rainfall. The whitefly population at the start of autumn is unknown for 2016, for 2017 it was relatively low, 2018 it was moderate and 2019 it was high, with 11, 57 and 144 average adult SLW per site, respectively (Table 6).

Both the summer and early autumn rainfall has likely influenced the level of the autumn SLW starting population. Summer averages in themselves, however, are not always a good indicator. The average summer rainfall over the 2015/2016 and 2016/2017 summers were relatively similar, however, virus outbreaks were much worse in the 2016 autumn. This was most likely due to a significant rain event in early March 2017 which reduced the whitefly population whereas in 2016

Table 6 Cowpea mild mottle virus (CPMMV) incidence and adult silverleaf whitefly (SLW) prevalence in autumn crops of French bean crops by visual observations per month. The maximum and minimum range and average across the survey sties for each month are shown

Year	Month	Symptom range[a]	Virus incidence range (% per site)[b]	Average CPMMV across the survey sites (%)	SLW range (total per site)	Average SLW per survey site
2017	March	C, M, Mo, LD, VB	0- < 1%	< 1%	4–54	11.2
	April	C, M, Mo, LD, VB	0- < 1%	< 1%	0–7	3.8
	May	M, Mo, LD, VB	0- < 1%	< 1%	0–5	2.5
2018	March	Not recorded	0- < 1%	< 1%	2–364	56.9
	April	C, M, Mo, LD	0–60%	4.44	3–27	9.3
	May	M, Mo, LD	0–40%	6.88	0–15	2.3
2019	March	M, Mo	0–100	26.57	6–852	143.7
	April	M, Mo, LD, VB	1–100	25.34	2–146	19.4
	May	Mo, VB	0–48	7.64	5–224	33.3

[a] Symptom code: *C* chlorosis, *M* mosaic, *Mo* mottle, *LD* leaf distortion, *VB* vein-banding

[b] *ca*. 15,000 French bean plants inspected per survey

Table 7 Average rainfall data from summer to end of autumn for the study period 2016 to 2019

Month	Average Rainfall (mm)			
	2015/2016	2016/2017	2017/2018	2018/2019
Dec	92.1	100.8	126.7	89.8
Jan	81.5	101.1	86.1	5.6
Feb	50	55.4	247.1	6.8
Av. Summer	74.5	85.8	153.3	34.1
Mar	76.7	320.2	39.2	166.3
Apr	14.8	13.8	16.6	0.8
May	8	7.2	0.6	6

conditions remained relatively dry (Table 7). The more complete data set for 2019 showed the average summer rainfall was very low and whitefly numbers were high at the start of autumn as was virus incidence. The virus incidence was graphed with the average adult SLW detected per survey date (Fig. 3). The graph clearly shows a major peak in SLW numbers approximately one week prior to detection of high incidences of CPMMV within bean crops. Following this major peak there was a sharp drop in SLW numbers. This correlates with a significant rain event, of 117 mm over the 15-16th of March. Although SLW numbers dropped significantly following this rain, average numbers remained higher than previous years as did the virus incidences with high incidences (i.e. > 20%) observed for most of the remaining season. The low virus incidence observed during the 28th of March survey is due to a change in crop age with most surveyed very young, and older crops all harvested. These young crops didn't display virus infections until the following survey on the 5th of April.

Host range and varietal virus tolerance

Of the 23 species tested from the Fabaceae plant family CPMMV:A:FB5288 induced symptoms in 15, including the Australian native *Glycine canescens* (Table 2). All 15 species belong to the *Phaseoleae* tribe of the *Faboideae* subfamily and the only test species from this tribe which was not infected by CPMMV was Lablab purpureus. None of the remaining eight *Fabaceae* species, the single *Cucurbitaceae* or the two *Solanaceae* species tested were infected by CPMMV. Host status for plants with mild symptoms or no symptoms were confirmed as positive or negative by CPMMV RT-PCR. Additionally, representative samples were tested from plants with typical symptoms by RT-PCR and all were confirmed positive for CPMMV. The most common symptom observed was a mottle, with all 15 infected test species showing this symptom. Soybean and cowpea bean also had symptoms of mosaic, and soybean additionally of vein clearing. Although the soybean and mung bean cultivars grown in the Fassifern Valley were shown to be symptomatic hosts of CPMMV:A:FB5288 in this host range study the virus was not found in field crops of either species during the surveys.

In the field trial assessing different French bean varieties a range of host reactions were observed from highly tolerant (rating of 0–1) to highly susceptible (rating of 4–5) (Table 3). Of the 22 varieties evaluated, nine showed high tolerance to CPMMV:A:FB5288, with an average disease rating across the replicate plots of no symptoms or only very mild symptoms (rating 0–1, Table 3). A further five varieties showed only very mild symptoms across the replicate plots (rating 1). The remaining eight varieties were severe or very severely affected by the virus, with only one variety having

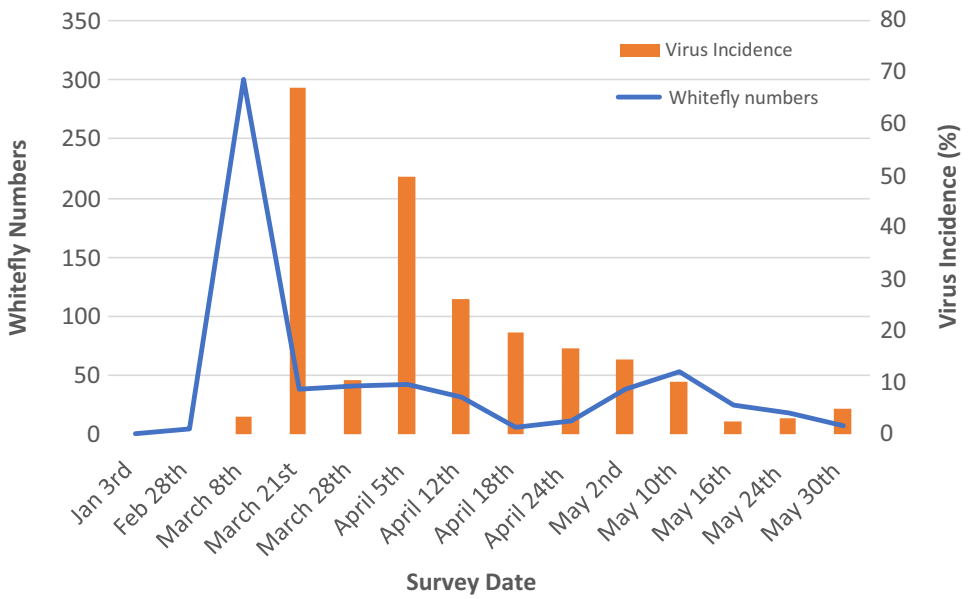

Fig. 3 Graph of virus incidence and adult whitefly numbers for 2019. Average whitefly levels are shown on the left X-axis and virus incidence as a percent on the right Y-axis. Data is an average per survey date

moderate symptoms (Table 3). In addition to these varieties, Borlotti bean and Lawrence were shown to be susceptible but only showed mild symptoms and the varieties Prairie and Voltage showed moderate leaf mottling and mosaic. These were assessed in a preliminary glasshouse study using sap inoculation with CPMMV:A:FB5288 (results not shown). CPMMV was confirmed present in test plants by RT-PCR.

Seed transmission

The seed collected from infected French bean generated 1110 Wyatt and 1080 Stanley seedlings, and from the infected P791 soybean plants 776 seedlings. Symptoms of CPMMV were not seen in any plants over a four-week monitoring period and the virus was not detected from any plants following RT-PCR on pooled samples. The seed germinated from commercial seed lots generated 1864 Wyatt French bean, and 588 ZAM 1, 564 P791 and 930 Bunya soybean seedlings. No virus symptoms were observed in the seedlings over the four weeks of monitoring and the virus was not detected from any plants using the CPMMV DAS-ELISA.

Discussion

This study has shown the variability of impact CPMMV has on French bean crops and gives some insight to why there are these differences. Although four sequence variants of CPMMV were detected within a single growing district, survey results show the predominant variant causing disease in bean crops was CPMMV:A:FB5288. Host range studies indicated this sequence variant is limited to the *Phaseoleae* tribe within subfamily *Faboideae*, family *Fabaceae* and found to be infecting the Australian native species, *Glycine canescens*. Evaluation of French bean varieties indicated a range of susceptibilities to this dominant sequence variant from highly tolerant to very susceptible. The existence of tolerant varieties has provided the local industry with some options for disease management where previously there were none. Prior to this study there was no knowledge on the relative tolerance of French bean varieties to CPMMV.

The survey data highlighted the value of regular crop monitoring to provide insight to high-risk planting windows for pest and disease. In the Fassifern valley, CPMMV was only detected in French bean crops in autumn and economically important incidences were linked to whitefly numbers. This in combination with evaluation of weather patterns allows predictions to be made on high-risk periods for CPMMV in French bean. Genetically tolerant French bean varieties were identified but are not agronomically preferred by growers so knowledge on potentially high-risk planting windows will allow them to better evaluate the cost-benefits of using the different varieties.

The primary spread of CPMMV into bean crops only during autumn is interesting and most likely driven by insect feeding preferences. The dominant crops grown in the district during January and February are pumpkin and soybean. French bean crops are typically not planted until March. Pumpkin often supports very high numbers of SLW but do not overlap with French bean crops and is not a host of CPMMV, thus pumpkin is not the reservoir for primary spread into bean crops. The soybean crops do overlap with French bean plantings, however, CPMMV was not detected in those crops from 64 surveys, including inspections of crops before French bean crops were planted and during the season overlap with the French bean. The soybean crops were thus also not the reservoir for primary spread. The likely explanation for primary spread is SLW moving into riparian areas onto the legume weeds as old pumpkin crops are abandoned or destroyed. During autumn, the major crop in the district is French bean, and thus SLW moves into these crops from weeds in the absence of other preferred hosts. French bean is not considered a favoured host for SLW as little evidence of colonisation was observed during the study and numbers were relatively low compared to populations observed on other hosts such as pumpkin. Spatially CPMMV was distributed throughout the district and the highest recorded incidences of virus was in crops close to the weedy riparian areas (results not shown). Knowledge of the pattern of vector movement and weed host locations also provides an opportunity for disease management. Reduction of adult SLW on pumpkin crops prior to their destruction would confidently reduce risk of CPMMV outbreaks in the subsequent autumn within this district. Similarly, removal of weeds from close to the production blocks would significantly reduce the environmental reservoir of CPMMV and thus lower risk of disease outbreaks. Management of weeds, however, is often difficult, particularly in riparian areas.

Seed transmission of CPMMV is reported for some plant species and some virus isolates (Zanardo and Carvalho 2017). In this study, no evidence of seed transmission of isolate CPMMV:A:FB5288 was obtained from two varieties of French bean and one of soybean. These varieties are commonly grown in the Fassifern Valley and the lack of evidence for seed transmission gives confidence this was not the entry pathway for CPMMV into the district. Furthermore, field crops within the district were regularly surveyed from seedling emergence and virus symptoms were not detected in very young plants, also implying virus introduction was unlikely from French bean or soybean seed.

This study has also provided additional insight into the genetic diversity of CPMMV, a highly variable virus. Four distinct sequence variants were detected in the one growing district and all four were present within the non-cropping

environment in legume weeds. The three siratro isolates appear to form a separate clade from the other CPMMV isolates that were recently described, with the NABP coding sequence more like the type species of CPMMV. Further sequencing of these isolates is required, as the taxonomy of carlaviruses currently described as CPMMV is difficult due to the highly conserved coat protein, and the possibility of recombination in the RNA dependent RNA polymerase coding region (Almeida et al. 2005; Marubayashi et al. 2010; Rosario et al. 2014; Zanardo et al. 2014) and full genome comparisons should be used to clarify taxonomy for this species (Rosario et al. 2014; Zanardo et al. 2014).

Further work is needed to determine the pathogenicity and symptomatology of the remaining sequence variants in French bean or other legumes. The siratro samples they were derived from were co-infected with potyvirus which would mask symptoms if present. Full genome sequencing of all four Australian isolates is underway and will provide additional clarification on the taxonomy of CPMMV.

Acknowledgements We thank the local growers in the Fassifern valley for access to crops for data collection and their generosity for providing agronomic information as needed. We also thank the following companies Australian Grains Genebank, Bayer, Barenbrug Australia, Farmcraft Kalbar, HM Clause, Lefroy Valley Seeds, Sunlands Seeds, and Syngenta Australia for providing legume germplasm for evaluation.

This research was funded by Hort Innovation with co-investment from the Department of Agriculture and Fisheries, and funds from the Australian Government (Project VG15073: Characterisation of a Carlavirus from French bean).

Funding Open Access funding enabled and organized by CAUL and its Member Institutions.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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