Factors affecting *Cucumber green mottle mosaic virus* incidence and control in California cucurbits

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Cucumber green mottle mosaic virus (CGMMV) was first reported from the U. S. in 2013, from Yolo County California cucurbit fields of melons, watermelons and cucumbers (Kim et al., 2014). This CGMMV originated from melon seed from Sutter County. It is not known for sure from where the original CGMMV was introduced, but attempts were made to contain the original introduction, the seed fields were quarantined, plants destroyed and all seed was quarantined to be destroyed. This past year (2014) CGMMV was again found in California. It was found in commercial seedless watermelon fields in San Joaquin County, then in Fresno and Kern counties. Initial analyses by us, funded by a grant from the Farm Bill, allowed for us to sequence the 2013 and 2014 isolates and compare them. Our analysis showed that the 2014 CGMMV was not identical to that found in 2013, suggesting a new introduction. This suggests that CGMMV introductions may occur again. Thus, we must have the ability to detect the diversity of CGMMV isolates that may be encountered, and because California imposes strict rules regarding replanting in fields where CGMMV is found, we need to understand CGMMV survival to help make informed decisions regarding land use. Our efforts here addressed both of these points.

CGMMV has a wide host range among both wild and cultivated cucurbits, including cucumber, squash, melons and watermelon (Ainsworth, 1935; Nozu, 1971). It has been reported in many regions of the world including Asia, Europe and the Middle East, but its recent finds in California, Canada (2013) and this year in Australia (2014) are of concern. CGMMV is a Tobamovirus (like *Tobacco mosaic virus* [TMV]). Tobamoviruses are very stable viruses. Unlike most plant viruses, tobamoviruses are not spread by insect vectors but by mechanical wounding of plants, most often by human handling. Simple touching, transplanting, grafting and general plant handling are the primary means for spread. Tobamoviruses such as CGMMV are highly infectious, and plant handling introduces very small wounds that are sufficient for subsequent infection (Nozu, 1971).

CGMMV also is seed borne in several cucurbits. This most likely serves as its main means of spread to new locations and serves as the primary inoculum in cucurbit production. Other tobamoviruses such as TMV and *Pepper mild mottle virus* (PMMV) also are seed borne in tomatoes and peppers. However, TMV and PMMV are carried external on the seedcoat and seed treatments (e.g. tri-sodium phosphate) are used to inactivate the seed borne virus allowing for planting clean seed. CGMMV can be carried externally, but also internally within the seed coat. The latter characteristic makes it such that treatments such as with tri-sodium phosphate are not sufficient to remove CGMMV from cucurbit seeds.

Because CGMMV is seed borne and cannot be effectively eliminated by seed treatments, seed indexing is recommended as a strategy to prevent CGMMV introduction via contaminated seeds. The International Seed Testing Association (ISTA) recommends testing 2000 seeds and then rejecting a seed lot if even one CGMMV-positive seed is detected. This recommendation is really without biological validation. Indirect tests such as ELISA (enzyme-linked immunosorbent assay) are used to test seeds. ELISA, which is commonly used for seed testing, is very sensitive and accurate but cannot discriminate between infectious or inactivated CGMMV, thus a positive result does not mean that the detected CGMMV is infectious. Furthermore, the 1 in 2,000 threshold is just a number, it has not been tested to determine if such a level relates to subsequent field infection. Finally, CGMMV can be carried in the seed, but because it is not in the embryo, but in the seedcoat, even if it is infectious it may or may not

infect the developing seedling. Thus, detection by indirect methods such as ELISA does not mean that the CGMMV will infect the plant resulting from a given seed. Research is needed to understand CGMMV-cucurbit seed interactions and the resulting biology. We currently are researching this area with a grant from the Farm Bill and the American Seed Trade Association.

The fact that CGMMV has now been found twice in California, most likely from distinct introductions in different cucurbit producing areas in California, strongly suggests that it will happen again. The new reports of CGMMV from Canada and Australia support that CGMMV has the potential to be a widespread problem in commercial production worldwide. Why is CGMMV now showing up in California cucurbits? What factors allowed for it now to appear? Can steps be taken to prevent CGMMV from becoming a consistent problem for California cucurbits? These questions are all important but all cannot be answered immediately. We took efforts below to help answer questions regarding CGMMV detection, and attempts to assess survival in California soils. This report shows our progress, and our work will continue using additional sources of funds.

Our primary objective was to determine how long CGMMV survives in soil, thus helping to make good decisions regarding planting of CGMMV susceptible crops in areas found to previously have CGMMV. However, based on accumulating anecdotal data (and our data, see below) suggesting that existing serological approaches did not readily detect all CGMMV isolates, and because we proposed to use antibodies as part of our soil analysis, we first compared CGMMV isolates using existing commercially available antibodies. CGMMV immunostrips, for example, failed to consistently detect the 2014 San Joaquin CGMMV isolate from symptomatic leaf tissues. Therefore, we prepared purified CGMMV antigens for the 2014 San Joaquin CGMMV isolate, and used these for polyclonal antibody production in rabbits, which was done at the Comparative Pathology Laboratory at UC Davis. To check the purity of the virion preparations before immunization we analyzed proteins by SDS-polyacrylamide gel electrophoresis (Figure 1).

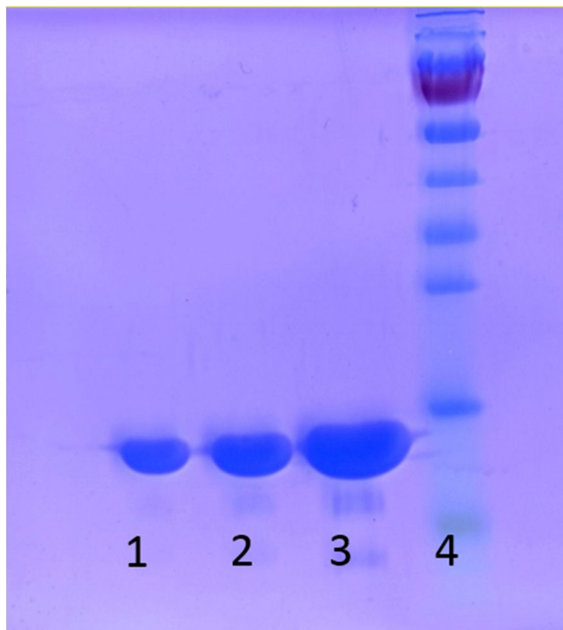

Figure 1. SDS-polyacrylamide gel of purified CGMMV virions and stained with Comassie Brilliant Blue. Lane 1: 2uL virion sample. Lane 2: 5uL virion sample. Lane 3: 10uL virion sample. Lane 4: 8uL Page ruler (Thermo Scientific). CGMMV capsid proteins are approximately 17 kDa.

The resulting antisera have now been evaluated and work very well and gave strong, specific reactions with the homologous CGMMV antigen Image 1). We then purified IgG from whole serum and

conjugated a portion of the IgG to alkaline phosphatase, creating an in-house DAS-ELISA enzyme pairing. We tested them with our CGMMV isolates that we have in hand so far, and compared the absorbance to the commercially available CGMMV ELISA kit from Agdia. Our antibodies are as good as those from Agdia and we have hundreds of ml of crude antisera. We then tested and compared our antibodies and the commercially available Agdia antibodies against the ATCC isolates 391 (Japan CGMMV) and 1067 (ZGMMV), and neither antibody set gave positive reactions with these antigens. We confirmed by RT-PCR and sequence analysis that ATCC 391 is CGMMV, but its failure to be detected by ELISA is noteworthy. We have now obtained 10 additional CGMMV isolates from collaborators around the world (all under USDA APHIS PPQ permit), and have another 10-20 being prepared for shipment. We will continue testing to assess CGMMV variability.

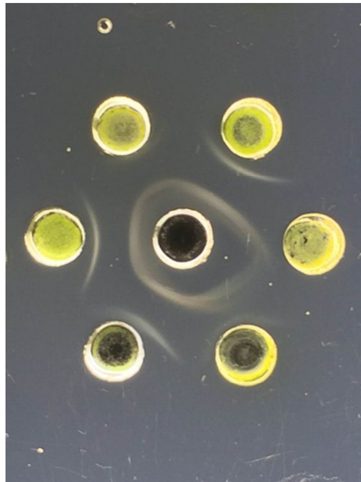

Image 1. Immunodiffusion slide of rabbit anti-CGMMV against the California isolates of CGMMV. Serum was pipetted into the central well to diffuse towards the sample well. Sample wells clockwise from, starting from the top left: healthy, SJ14, Kern14, Fresno14, Yolo13, and SJ14. All plant tissue used in immunodiffusion were *Nicotiana benthamiana*. Antibodies in the serum binding to virus antigen forms a white precipitate in the agar, forming bands close to the central well due to coat protein subunits, and in crescents around wells caused by intact virions. The serum reacted strongly to SJ14 and Yolo13. There was a weaker reaction to Fresno14, and undetectable reaction to Kern14.

Given that Tobamoviruses have infectious particles that are very stable in the environment, and that CGMMV has been described in soil, surface water, and hydroponic water, there is the potential for the virus to be stable in the soil environment as a source of infection in subsequent crops (Metcalf, 1995). To determine if CGMMV is detectable from soils, we collected soil samples from two fields in San Joaquin County that had CGMMV outbreaks in 2014 in watermelons. In the first field, after the crop was destroyed the grower completed a soil fumigation application of methyl bromide. The plan for this field, in association with CDFA, was to monitor the field for CGMMV using plots of susceptible host plants and collect soil from each plot to test for the virus. CDFA completed monitoring and testing of the susceptible host plants and we completed soil testing. The second field was not treated after crop destruction, and the grower farmed a non-host crop in the field in accordance with the abatement protocol. At both locations we took random samples from every 4-5 acres. In the first field we collected 40 soil samples, and in the second field we collected 15 soil samples. Although we have attempted CGMMV detection from these samples and had some encouraging results, we are not yet ready to be confident with testing field soils for CGMMV. Therefore, we focused efforts on soils that we artificially infested with CGMMV and attempted to develop methods for consistent and sensitive CGMMV detection.

Although the primary literature on CGMMV states that the virus is detectable in soil and water, the methods used in those studies were not applicable for our research. The primary goal in those studies was environmental detection involving large sample volumes and filtration or ultracentrifugation to concentrate virus particles for testing, usually with very few samples per study or location. In contrast, if we are to sample and test fields for CGMMV, multiple smaller samples are likely to be required.

Therefore, the majority of our work was testing protocol validation using spiked soil samples. For protocol validation and to determine if we could detect CGMMV in the soil with some degree of sensitivity we incorporated CGMMV infected plant debris into clean field soil. We used both fresh and dried plant parts as starting material for incorporation. Clean soil was collected from a fallow field of Yolo Loam series, which is compositionally similar to the soil found at the San Joaquin County sites. Contaminated material was incorporated (g/g) at the following rates: 1/20, 1/50, 1/100, 1/250, 1/500, 1/1000, 1/2500, 1/5000, and 1/10000. Clean soil samples were also retained as negative controls. Three protocol methods were used and evaluated for sensitivity, reproducibility, and feasibility. They were: buffer suspensions followed by ultracentrifugation for virion concentration, polystyrene ball based DAS-ELISA in large sample volumes, and large sample volumes mixed with extraction buffer and tested with traditional DAS-ELISA. Because CGMMV is quarantined in California, all of our research with infectious CGMMV, including debris and soils, was done inside the UC Davis Biosafety 3P Contained Research Facility.

Using the artificially infested soils, we started with ultracentrifugation to concentrate virus particles. Our belief was this method would incorporate a large initial sample size and yield a small final sample that could be tested by antibody and/or molecular techniques such as RT-PCR. This overall method was the most sensitive assay, but had technical issues. The initial sample of 5 grams was homogenized in 25mL of extraction buffer, filtered through 40um filter paper, then sequentially centrifuged to remove larger particles, and finally ultracentrifuged to concentrate virion particles, and the final pellet resuspended in 500uL buffer. This final sample was then used for ELISA and for RT-PCR. Although this gave good results for sensitivity, we chose not to use this protocol as it is too impractical. The issues with this assay were length of time and labor inputs. We were limited by the number of samples that could be processed per run due to centrifuge time and space. We were limited to eight samples per run, with each run taking approximately five days to complete. Given the number of samples to test, this was not a feasible option.

The second method we tested was using polystyrene balls coated with capture antibody. The idea is that these antibody-coated balls could be circulated through soil extract samples, capture CGMMV which could then be detected using conventional serological or RT-PCR-based assays. We used our newly made antibodies as well as those from Agdia for some tests. Soil samples of 1g were homogenized in 5mL general extraction buffer, and 2-4 antibody-coated balls were added to the sample. The samples were then gently agitated for 2 hours at room temperature, and then the polystyrene balls removed and transferred to 2mL microcentrifuge tubes to complete the steps for DAS-ELISA. The results were less sensitive than the ultracentrifuge protocol, and the more critical issue was contamination of the beads due to handling for the washing steps between removal of beads from the sample homogenate and adding the conjugate antibody. There were also issues with negative controls having relatively high OD readings, invalidating sample runs. Overall, the quality of assay repetitions led us to abandon this protocol.

The final assay, which we chose to use, was a standard DAS-ELISA format with specific soil sample handling. We added approximately 500mg soil to 2mL general extraction buffer, vortexed the samples for 30s, then let the samples sit overnight at 4°C before adding the samples to ELISA plates coated with CGMMV antibodies. This gave sensitive detection of the virus down to 1/500 (Figures 2 and 3).

These results indicate that CGMMV can be detected in sandy loam soils. However, it is unknown whether this analysis reflects infectious virus particles. Also sampling only 500 mg at a time may seem

like a small amount of soil sample, but the fact that this assay can be done in 96 well plates suggests that we might be able to achieve testing a large number of small samples as a way to gain accuracy.

Soil Dilution Agdia ELISA results

sample	OD 1	OD 2	Average OD
Soil -	0.067	0.069	0.068
1/20	1.013	1.008	1.0105
1/50	1.033	1.029	1.031
1/250	0.552	0.581	0.5665
1/500	0.795	0.764	0.7795
1/1000	0.137	0.139	0.138
1/2500	0.1	0.101	0.1005
1/5000	0.163	0.167	0.165
1/10000	0.092	0.008	0.05
Buffer	0.071	0.076	0.0735
neg. control	0.135	0.137	0.136
positive cont.	2.913	2.836	2.8745

Figure 2. Agdia DAS-ELISA results of soil samples spiked with fresh homogenized CGMMV infected melon tissue, then allowed to dry. Then, 0.5g soil was homogenized in 1mL general extraction buffer, incubated overnight at 4°C, and the supernatant used for ELISA testing. The spike amounts of tissue per gram of soil correspond to 50mg, 20mg, 4mg, 2mg, 1mg, 0.4mg, 0.2mg, and 0.1mg. Positive results were dilutions down to 1/500, or 2mg infected tissue per gram of soil.

Rabbit polyclonal Antibody ELISA

Sample	OD 1	OD 2	Average
Soil -	0.063	0.065	0.064
1/20	0.673	0.652	0.6625
1/50	0.608	0.566	0.587
1/250	0.225	0.268	0.2465
1/500	0.429	0.395	0.412
1/1000	0.112	0.104	0.108
1/2500	0.115	0.114	0.1145
1/5000	0.13	0.116	0.123
1/10000	0.089	0.088	0.0885
Buffer	0.059	0.06	0.0595
neg. control	0.066	0.072	0.069
positive cont.	1.07	1.104	1.087

Figure 3. Rabbit polyclonal antibody DAS-ELISA results of soil samples spiked with fresh homogenized CGMMV infected melon tissue, then allowed to dry. The results agreed with those obtained by the Agdia DAS-ELISA (above), though the OD readings were lower. The same soil samples were used for both assays.

Protocols

RT-PCR was performed using previously described protocols with primers amplifying a 452bp amplicon from the coat protein region (Shang et al., 2011). All reaction volumes were 25uL, and enzymes used were Go Taq Flexi and Superscript II following manufacturers' protocols (Invitrogen, Carlsbad, CA).

DAS-ELISA was performed with reagents from Agdia Inc. (Elkhart, IN), following the manufacturer's protocol for plate based assays. Polystyrene beads (Polysciences Inc., Warrington, PA) were used with equivalent reagent volumes as for the plate based detection assays. Sampling handling diverged from the manufacturer's protocol as follows. For the bead assay, we added the coated bead directly to 0.25g soil samples in 2mL general extraction buffer. For the plate based assays, we added approximately 500mg soil to 2mL general extraction buffer, vortexed the samples for 30s, then let the samples sit overnight at 4°C before adding the samples to ELISA plates coated with CGMMV antibodies and continued as the manufacturer recommended. In addition to the Agdia ELISA kit, we also tested the soil samples using the antibodies we prepared against the San Joaquin isolate of CGMMV. We used the same buffers and ELISA protocol as Agdia recommends.

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