Collaboration for Plant Pathogen Strain Identification

Guidelines for Cucumber mosaic virus inoculation and variety resistance evaluation on pepper differentials

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Host: *Capsicum annuum*, *Capsicum spp*.
Pathogen: Cucumber mosaic virus

Background:
Cucumber mosaic virus (CMV) is one of the most prevalent plant viruses worldwide. It was first discovered in cucumbers and muskmelons in 1916 and can infect over 1,200 different plant species (Doolittle 1916, Murphy 2003, Li et al. 2020). This includes many commercially relevant crops such as cucurbits, lettuce, pepper, tomato, legumes and ornamentals. Severe CMV outbreaks on pepper can result in high yield loss (Li et al. 2020). CMV is transmitted both mechanically and by aphid vectors. Over 80 aphid species are able to transmit the virus contributing to its abundance and widespread distribution. Additionally, CMV can be transmitted through infected seed, other host plants including weeds, and soil or plant debris containing the virus can contribute to transmission (Ali and Kobayashi 2010, Pares and Gunn 1989).

Symptoms of CMV in pepper include, mosaic, mottle, vein clearing, yellowing, narrowing or shoe-stringing (**Figure 1**). Infected pepper plants can also be stunted and produce less flowers, while fruits can be bumpy, small or contain necrotic lesions (**Figure 2**). Symptoms vary based on viral strain, pepper genotype, and environmental conditions (Murphy 2003). Younger plants tend to exhibit more classic symptoms, while older plants may be asymptomatic or develop oak leaf or ringspot patterns (**Figure 3**; Zitter and Murphy 2009).

Figure 1. CMV symptoms on pepper plants in the field. *Photo: Ed Sikora, Auburn University*

Figure 2. Necrotic lesions on pepper fruit. *Photo: DPIRD Government of Western Australia*.

Figure 3. Oak leaf or ringspot patterns on pepper leaves. *Photo: Zitter and Murphy 2009.*

CMV is a member of the genus *Cucumovirus* in the family *Bromoviridae*. Its genome consists of three single-stranded, positive-sense RNAs, RNA1, RNA2, and RNA3, which are encapsidated separately (Palukaitis et al. 1992). Encoded proteins 1a, 2a, and 3a contribute to viral replication and cell-to-cell movement in the plant host. RNA2 and RNA3 contain subgenomic RNAs, RNA4A and RNA4, respectively. Notably, RNA4A encodes the 2b protein which contributes to CMV virulence in the plant host through gene silencing in systemic tissues (Ding et al. 1994). RNA4 encodes the coat protein which is the determinant for aphid transmission of the virus particles (Li et al. 2020).

CMV strains and resistance:
Many sources of resistance to different CMV isolates have been identified in pepper, although, some are multifaceted and likely mediated by several loci (Li et al. 2020). This is noted by the various genetic control mechanisms predicted for CMV resistance in the pepper variety, Perennial (Pochard 1982, Lapidot et al. 1997). Perennial provides moderate resistance to several CMV isolates and exhibits restricted CMV multiplication and movement (Caranta et al. 1997). Analysis of CMV-resistance in Perennial found three QTLs that accounted for more than half of the phenotypic resistance observed.

Strain-specific CMV resistance in pepper has been reported. For example, single gene dominant resistance (*Cmr1*) in the variety Bukang provides resistance to Korean CMV strains (Kang et al. 2010). Choi et al. 2016, found that Korean CMV isolates, termed CMV-P1 strains, could break *Cmr1* resistance as opposed to CMV-P0 strains. Single recessive gene (*cmr2)* resistance was later identified in Lam32 (Choi et al. 2018) and is thought to contribute to resistance against several Korean CMV-P1 strains. Recessive resistance can result from the absence or impaired functions of essential host factors necessary for viral infection, *cmr2* is thought to restrict viral movement but the exact function is unclear.

CMV strains have been classified based on symptoms observed in different host species with early examples of this for crops including sweet potato, spinach, and tobacco (Fulton 1950, Kaper and Waterworth 1981, Li 1995). For pepper, a set of hosts was identified that results in five separate pepper CMV strain groups based on testing 59 CMV isolates from China (Yang et al. 1992). However, this specific classification system has not been globally adopted. Serology and phylogenetics of the CMV coat protein (CP) gene classify CMV isolates into subgroups I and II with pepper CMV isolates falling mainly into subgroup I due to their higher temperature tolerance (Moury and Verdin 2012, Li et al. 2020).

Additionally, the World Vegetable Center has published a catalog of resistance sources to CMV and other viruses of pepper (Green and Kim 1994).

Table 1. Expected responses of the *C. annuum* differentials to the pepper CMV isolate 144-I

		CMV isolate
Differential	**Gene(s)**	**144-I****
Early Cal Wonder	-	S
Perennial	*	IR
Lam32	*cmr2*	HR

S = Susceptible, IR = Intermediately Resistant, HR = Highly Resistant
*Likely multiple loci contributing to resistance in Perennial either through recessive or incompletely dominant means.
**Based on Choi et al. 2018 the 144-I CMV isolate could be classified as a CMV-P0 or P1 strain/race as it cannot overcome *cmr2*-mediated resistance. *Cmr1*-mediated resistance to differentiate P0 from P1 was not compared. Thus, the information provided is specific to the given isolate rather than a reported CMV strain/race group. The 144-I isolate was selected as it is commonly used by the vegetable seed industry in the United States.

Preparation of the host plants and inoculum:
For the host plants, pepper seed are sown in 32- or 60-cell trays filled with potting mix. If seed quantities allow, seed can be double planted and thinned post germination so one seedling per cell remains. Ideally, 25 plants of each differential variety will be used for the test with 20 plants designated for inoculation and 5 designated as mock-inoculated controls. Plants are grown in either a greenhouse or growth chamber with 16 hours of light and temperatures between 21-26°C during the day and 18-21°C at night. Temperatures towards the higher end of these windows will facilitate faster pepper growth.

To prepare the inoculum, the CMV isolate should be propagated in a susceptible host plant to ensure sufficient inoculum quantities. For our evaluation the CMV isolate 114-I (**Table 1**) was propagated in squash leaves. Alternative susceptible hosts include tobacco or periwinkle.

Inoculation, incubation and evaluation for resistance and susceptibility:
Once plants are at the "3-leaf stage" with the first true leaf emerging but not fully expanded they are ready for inoculation. Inoculum should be prepared by grinding 1g of infected leaf tissue (fresh or frozen from squash or other susceptible host used for propagation) in chilled phosphate buffer at 1:10 or 1:5 ratio. The solution can be filtered through cheesecloth to remove any larger leaf debris. If starting from frozen material, it is recommended to increase in the pathogen on squash plants first and use fresh material for the inoculation.

An abrasive agent, such as carborundum or diatomatious earth should be added to the inoculum solution or dusted on leaves prior to inoculation. Mock-inoculations should include buffer and the abrasive agent. With a gloved finger, cotyledons and first true leaves are rub-inoculated using the freshly prepared inoculum solution. An optional rinsing of the inoculated leaves can be performed to remove excess inoculum and abrasive.

Post-inoculation, plants are kept in the dark for 24hrs and then returned to the greenhouse or growth chamber (although with preference for the greenhouse at this stage if conditions allow and temperatures are not too hot). At the time of moving plants from the dark to the greenhouse, fertilizer can be added. From then on fertilizer can be added and at a rate of once a week. If there is too much fertilizer post-inoculation symptom development on susceptible pants may be limited.

Symptoms can be evaluated 2 – 3 weeks post inoculation. Optimally, two ratings are performed with, one earlier (10-14 dpi) and one later (21 dpi). As noted above, symptoms can vary with viral isolate, pepper genotype, environmental conditions, and plant age. Plants inoculated at a younger age tend to have more symptoms than plants inoculated at a more mature stage.

Disease rating scale:
1 = Healthy plants, no symptoms
3 = Less than 20% mild mottling or mosaic and/or slight leaf distortion
5 = Significant mottling or mosaic and/or leaf distortion, systemic to emerging leaves
7 = Significant mosaic and/or leaf distortion on most of the plant, stunting may occur
9 = Severe mosaic and/or leaf distortion/shoestring on the whole plant, stunting may occur
Note: necrosis can also be seen for certain CMV isolates.

Plants scored 1 to 3 are considered highly resistant, 4-6 intermediately resistant, and 7-9 are susceptible. ELISA can also be used to detect the presence of CMV and quantity of viral titers, which may help evaluate resistance (Lapidot et al. 1997). Based on our ELISA testing viral titers were higher in Perennial when compared to Lam32.

Figure 4. Pepper plants inoculated with CMV 114-I. Top: Susceptible Early Cal Wonder plants showing CMV symptoms. Bottom left: Perennial plants with intermediate resistance, plants are slight stunted compared to mock. Bottom right: Highly resistant Lam32. *Photos: HM Clause.*

Figure 5. Susceptible Early Cal Wonder plants showing CMV symptoms ranging from, leaf mosaic and distortion (left) to mild leaf mottle (right). *Photos: HM Clause.*

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Ordering seed of the pepper differentials and the CMV type isolate:
The pepper differential seed and CMV isolate shown in **Table 1** are available through CPPSI.

Seed:
Early Cal Wonder is currently available as CPPSI accessions via the USDA-ARS National Plant Germplasm System. Visit the USDA-GRIN website (https://www.ars-grin.gov/) and search "CPPSI*" to see a list of all accessions validated and deposited by CPPSI.

Perennial and Lam32 are available through CPPSI directly. Please contact the CPPSI Director, Kelley Clark, at kjclark@ucdavis.edu for assistance. These differentials will also be available via USDA-GRIN once the inventory process is complete.

Pathogen isolate:
The CMV isolate 144-I is also available through CPPSI directly by request. This isolate was originally collected from pepper plants in Ventura Co. California in 1990 by Bryce Falk's group (Han-Xin Lin et al. 2003). It has been used as an industry standard for several years.

CMV is considered a native or naturalized plant pest by USDA APHIS and can be moved within the continental US without a permit. See the APHIS Native and Naturalized Plant Pest List here.

Did you find this information useful?
If so, please consider giving back. CPPSI is a science-based initiative based at the UC Davis Seed Biotechnology Center to standardize the identification of plant pathogen strains or races and the determination of variety resistance. These resources are made available due to continued support from the vegetable seed industry, USDA germplasm network, and other collaborators.

Donte to CPPSI today: https://give.ucdavis.edu/APLS/APSVPPS

Or consider CPPSI membership. All individual firms or corporations engaged in the distribution, breeding, production, testing, and enhancement of seeds are eligible for membership in CPPSI. Please send membership inquiries to Kelley Clark at kjclark@ucdavis.edu

Liability waiver
CPPSI and all other associated members and participating organizations have done their best to provide information that is up-to-date and published in refereed journals and, therefore, no liability for the use of this information is accepted. The inoculation protocol described in this document has been demonstrated to be effective for CMV characterization on pepper.

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