Some Observations on Methods of Scoring Symptom Severity to Papaya Ringspot Virus in Tropical Pumpkin

Linda Wessel-Beaver

University of Puerto Rico, Mayagüez Campus, Mayagüez, Puerto Rico, U.S.A. Email: lindawessel.beaver@upr.edu

Introduction:

A reliable and efficient method of distinguishing between resistant vs. susceptible plants (or sometimes additional categories such as intermediate resistance) is essential when using phenotypic selection for resistance and for inheritance and other genetic studies relating to disease resistance. The development of molecular markers also depends on associating a genotype (the marker) with the correct phenotype. Developing a reliable method of phenotyping for resistance/susceptibility is often a challenging endeavor. It is therefore not surprising how often phenotyping has been a topic of discussion in meetings of breeders and pathologists. Plant breeders and pathologists have developed many ways of phenotyping disease response in plants. An ideal phenotyping method should allow the breeder to study or select for resistance as early as possible in the life cycle of the plant.

Papaya ringspot virus watermelon strain (PRSV-W) and *Zucchini yellow mosaic virus* (ZYMV) are potyviruses that affect cucurbit crops worldwide. Because PRSV and ZYMV produce similar symptoms on squash and pumpkins (*Cucurbita* spp.), researchers often use similar severity scales or scoring systems when carrying out inheritance studies or selecting for resistance to these two viruses. While the details of these phenotyping methods often vary, in my review of the literature I have noted that researchers always seem to consider the entire plant (seedling) when scoring for symptom severity.

Researchers in Brazil (Maluf et al., 1997; Menezes et al., 2015; Oliveira et al., 2003) studied resistance to PRSV in various *Cucurbita* spp. These researchers gave a single severity score to whole plants at anywhere from 10 to 42 days after inoculation (DAI) using a 0-5 scale developed by Maluf et al. (1997) where 0=no symptoms; 1=most leaves with no symptoms, few leaves with mild symptoms usually vein clearing, 2=mild symptoms (mostly vein clearing) on majority of leaves, 3=majority of leaves with mosaic, varied symptoms (vein clearing, sparse chlorotic spots, chlorosis up to 50% of leaf area), 4=almost all leaves with severe mosaic, coalescence of chlorotic spots up to 50% of leaf area, and 5=almost all leaves with severe mosaic, at least one leaf with more than 50% of area affected or severely distorted. These researchers treated symptom severity as a quantitative variable (albeit with only 6 possible values) and used a biometrical approach (generation mean analysis) to study inheritance.

Other researchers have taken a more qualitative approach to scoring resistance/susceptibility in inheritance studies. Paris et al. (1988) evaluated *C. moschata* inoculated with ZYMV. They scored plants at the 3rd to 4th true leaf stage at 19 to 20 days after planting and classified plants as resistant (could have chlorotic spots up to several mm in diameter, usually 1-2 mm) or susceptible (strongly blistered or mosaic).

Paris and Cohen (2000) investigated inheritance of ZYMV resistance in *C. pepo.* They noted that symptoms changed as new leaves developed. Some plants initially showed symptoms but then recovered. When only mild symptoms were observed after the 5th leaf plants were classified as resistant. Plants classified as susceptible sometimes had no symptoms up to the 3rd leaf, but later developed symptoms such as mosaic, blistering and stunting. They concluded that scoring should be done 20-22 days after inoculation (DAI) when *C. pepo* plants had 8 leaves.

Brown et al. (2003) studied resistance to four cucurbit viruses in *C. moschata*, including PRSV and ZYMV. For each of the four viruses, plants were classified into one of two categories: resistant or susceptible. However, they did not describe the criteria used for classification.

Pachner and Lelley (2004) used a 1 to 5 scale to evaluate symptom severity in *C. moschata* populations inoculated with ZYMV. To study inheritance of resistance they then classified plants scored with a 1 or 2 (chlorotic dots or spots; light marbling) as "resistant" and plants rated 3, 4, or 5 (blistered, distorted and/or stunted) as "susceptible."

Pachner et al. (2011) scored plants inoculated with ZYMV as susceptible when symptoms included mosaic, overall yellowing, stunting, blistering, distortions or leaf strapping, and as resistant if plants had only chlorotic dots, spots or streaks. However, they then went on to classify *C. moschata* lines into three categories: highly resistant, resistant and susceptible. In photographs in the paper, two lines classified as highly resistant showed only small spots or streaks while the two lines classified as resistant had much larger chlorotic spots or mosaic. The leaf from the line classified as susceptible ('Waltham Butternut') was distorted and blistered.

Pachner et al. (2015) studied resistance to ZYMV in *C. pepo* using a scale of 0 (symptomless) to 9 (very severely distorted, growth discontinued, plus a score of 10 for stunted plants that later recovered. Plants were evaluated at 10, 17 and 24 DAI (likely up to the 7rd or 8th leaf, although not specified in the paper). Two categories, resistant and susceptible, were formed by grouping plants that scored $\leq$4 (plus certain plants with a score of 10 that later recovered) as resistant and the remaining plants as susceptible. They then used a classical qualitative approach to determine the genetic basis for resistance to ZYMV

Shrestha et al. (2022) identified two KASP markers associated with resistance to PRSV-W in *Cucurbita moschata.* Plants in the F_2 population were scored for symptom severity at 7, 14, 21, and 28 DAI on a 0-3 scale (0=asymptomatic, 1=light yellowing on the leaves, 2=moderate yellowing and mottling and 3=severe leaf yellowing, mottling, stunted plant growth). Four phenotypic classes were evident at 28 DAI. Plants scored 0 or 1 were categorized as resistant while plants scored 2 or 3 were categorized as susceptible.

The studies described above all used a “whole plant” approach to phenotyping resistance, that is, scoring was based on the appearance of the entire seedling. Early on in our tropical pumpkin (*Cucurbita moschata* Duchesne) breeding program at the Agricultural Experiment Station, University of Puerto Rico, we also used that approach. For example, McPhail et al. (2012) and Sierra-Rivera (2012) scored severity of PRSV and ZYMV, respectively, on a scale of 0 to 3 on a whole plant basis, evaluating seedlings with four to five leaves 16 to 20 DAI. However, when seedlings were transplanted to the field or allowed to continue to grow in the greenhouse, plants with few or no symptoms on the 4th leaf would sometimes exhibit symptoms of susceptibility on later leaves. Inheritance studies also proved challenging. When converting a multiple point scale (0 to 3 or 0 to 4, for example) into two classes for an inheritance study, the number of plants in each class can vary widely depending on which severity scores are classified as “susceptible” vs. “resistant”.

Here I describe a severity scoring method for PRSV where each seedling leaf is scored independently from leaf 1 (1st leaf above the mechanically inoculated cotyledons) to leaf 7. Data from leaves 1 to 3 is not presented since I have found that that data is not informative. Data beyond the 7th leaf is difficult to gather in the greenhouse since plants grow too large. My objective is to attempt to answer the following questions: (1) How early can symptom severity for PRSV-W be evaluated on tropical pumpkin seedlings to reliably classify a plant as resistant or susceptible? (2) How should one convert a 0 to 4 severity scale into a two-class system that would be useful for an inheritance study on resistance to PRSV?

Materials and Methods:

The PRSV-W susceptible tropical pumpkin lines ‘Taína Dorada’ and ‘Verde Luz’ were crossed to resistant ‘Nigerian Local’ to produce two F_2 populations segregating for resistance to PRSV-W. Taína Dorada x Nigerian Local (TDxNL) (n=112) and Verde Luz x Nigerian Local (VLxNL), (n=199) were grown in the greenhouse and cotyledons were mechanically inoculated with PRSV-W five days after seeding following the protocol described by Seda-Martínez et al. (2021). A solution with a concentration of 4 g of 20N-8.74P-16.6K (800 ppm N) in 1 L of water was used to fertilize seedlings every 5 to 6 days. Daytime greenhouse temperatures were generally around 35 °C. The study was conducted in the months of January to April in Mayagüez, Puerto Rico, when daylength varies from about 11 hours to 12.5 hours with clear sunny days about 80% of the time.

For each seedling, symptom severity was scored separately on the 4th to the 7th true leaf above the cotyledons. The 4th leaf was scored on 21-day-old seedlings (16 DAI). The 5th and 6th leaves were scored on 26- to 28-day-old seedlings. The final reading on the 7th leaf was taken on 30- to 35-day-old leaves. Symptom severity was scored on a 0 to 4 scale (Figure 1) where 0=absence of virus symptoms, 1=a few small flecks or chlorotic spots, or faint mosaic, 2=numerous small flecks or chlorotic spots or moderate mosaic, 3= a few large chlorotic lesions causing puckering especially in leaf veins or severe mosaic covering most of the leaf area, 4= vein chlorosis, blistering, puckering, and/or leaf distortion.

The analysis involved showing how severity scores on the 4th, 5th and 6th leaf compared to the severity score on the 7th leaf on the same plant. In my experience scoring PRSV, severity scores generally change very little after the 7th leaf. In the analysis some severity scores were combined. A score of 0 or 1 was combined and categorized as “resistant”, a score of 2 was categorized as “intermediate” and a score of 3 or 4 was combined and categorized as “susceptible.” For each resistance category (resistant, intermediate or susceptible) at each of the lower leaf positions (4th, 5th or 6th leaf), the number of plants later categorized as resistant, intermediate or susceptible based on the 7th leaf was counted, then converted to a percentage (Table 1).

Results and Discussion:

PRSV severity score in leaf 4 vs. leaf 7: Plants with a severity score of 0 or 1 (resistant) in the 4th leaf maintained that score in the 7th leaf in only 57% of the cases in the TDxNL F_2 and in an even lower percentage of cases (13%) of the cases in the VLxNL F_2 (Table 1). Likewise, a severity score of 2 (intermediate) in the 4th leaf was followed by that same score in the 7th leaf in only 39% (TDxNL) and 16% (VLxNL) of those plants. By contrast, almost all plants in both F_2 populations

(96% and 99%) with a severity score of 3 or 4 (susceptible) in the 4th leaf also had a severity score of 3 or 4 on the 7th leaf of those same plants. This demonstrates that a very young seedling with only its first four leaves can be reliably classified as susceptible when strong early symptoms (a score of 3 or 4) appear on the 4th leaf. However, the scoring of the 4th leaf cannot reliably be used to classify a plant as resistant or intermediate at this early stage. Scoring PRSV symptoms at the 4th leaf stage will be useful for the breeder who wants to eliminate susceptible plants as early as possible but would not give reliable counts of resistant vs susceptible plants for an inheritance study. Under our tropical conditions in Puerto Rico, the 4th true leaf expands about 21 days after planting, but expansion of the 4th leaf likely occurs later in more temperate climates.

PRSV severity score in leaves 5 and 6 vs. leaf 7: As with the 4th leaf, severity scores of 3 or 4 (susceptible) in the 5th and 6th leaves allowed a plant to be reliably classified as susceptible (that is, with a score of 3 or 4) in the 7th leaf in 90% to 100% of the cases (Table 1). For plants of TDxNL scoring resistant (0 or 1) in the 5th or 6th leaf, the percentage of those plants correctly predicting the score in the 7th leaf was much better than the predictive ability of the 4th leaf. Compared to the 4th leaf, the 5th and 6th leaves of VLxNL also had an improved ability to predict the severity score in the 7th leaf, but in a much lower percentage of the cases compared to the TDxNL population.

Combining severity scores for testing gene models: The testing of gene models often requires categorizing plants into two classes: susceptible and resistant (or sometimes more). Scaling systems with intermediate scores present a challenge in inheritance studies. How symptom severity scores are grouped can dramatically change the ratios of resistant:susceptible (R:S). In a subsample (n=101, Table 2) of plants from the VL x NL population the ratio of R:S was 32:69 when plants with a score of 2 were combined with those scoring 0 or 1. A very different ratio of 7:94 (R:S) resulted when plants with a score of 2 were combined with those scoring 3 or 4. Neither ratio is an obvious fit to simple gene models. Other combinations of the five phenotypic classes for symptom severity are also possible. Considering the data in Table 1, when the 4th, 5th and 6th leaves had an intermediate severity score (=2), those plants were much more likely to maintain that score or receive a higher score in the 7th leaf than they were to have a lower score suggesting that plants with an intermediate score might best be combined with those in the susceptible category.

Conclusions:

Under conditions like those in the greenhouse in Puerto Rico, the presence of strong symptoms (score of 3 or 4) on the 4th leaf of tropical pumpkin seedlings can be used to eliminate plants susceptible to PRSV. Under our conditions this occurs about 16 DAI. Plants exhibiting strong symptoms of PRSV early on very rarely produced later leaves with few or no symptoms. However, few symptoms on the 4th leaf cannot be used to select in favor of resistance at this early stage. The evaluation of the 5th or 6th leaf will give a clearer sense of a plant's resistance although even at this stage a low score will not guarantee that the plant will not show future symptoms. Additional tests such as ELISA or RT-PCR can be helpful beyond visual observation. These techniques would help to minimize the number of lines to be tested in the field. For an inheritance study of PRSV resistance, severity scores of the 5th or 6th leaf should be used. How to simplify a severity scale with more than two categories is still not completely clear, but plants scoring 2, 3 or 4 on a 0-4 scale might best be combined into the susceptible class.

Acknowledgements:

This research was supported by Hatch accession no. 1000526 and Agriculture Specialty Crop Research Initiative grant numbers 2015-51181-24285 and 2020-51181-32139, USDA National Institute of Food and Agriculture.

Literature Cited:

Brown, R.N, A. Bolanos-Herrera, J.R. Myers, and M.M. Jahn. 2003. Inheritance of resistance to four cucurbit viruses in *Cucurbita moschata*. Euphytica 129:253-258. https://doi.org/10.1023/A:1022224327064

Maluf, W.R., J.J. Pereira and A.R. Figueira. 1997. Inheritance of resistance to the papaya ringspot virus-watermelon strain from two different accessions of winter squash *Cucurbita maxima* Duch. Euphytica 94:163-168.

McPhail-Medina, R., L. Wessel-Beaver and J.C.V. Rodrigues. 2012. Inheritance of resistance to *Papaya ringspot virus* in tropical pumpkin is controlled by at least two genes. *In:* (N. Sari, I. Solmaz and V. Aras, eds.) Cucurbitaceae 2012, Proceedings of the Xth EUCARPIA meeting on genetics and breeding of Cucurbitaceae, pp. 697-701.

Menezes, C.B., W.R. Maluf, M.V. Faria, S.M. Azevedo, J.R.V. Resende, A.R. Figueira and L.A.A. Gomes. 2015. Inheritance of resistance to papaya ringspot virus-watermelon strain (PRSV-W) in 'Whitaker' summer squash line. Crop Breeding and Applied Biotechnology 15: 203-209. http://dx.doi.org/10.1590/1984-70332015v15n4a36

Oliveira, A.C.B de, W.R. Maluf, J.E.B.P Pinto and S.M. Azevedo. 2003. Resistance to papaya ringspot virus in summer squash *Cucurbita pepo* L. introgressed from an interspecific *C. pepo* x *C. moschata* cross. Euphytica 132:211–215.

Pachner, M., and T. Lelley. 2004. Different genes for resistance to ZYMV in *Cucurbita moschata*. *In* A. Lebeda and H.S. Paris (eds.), Progress in cucurbit genetics and breeding research, Proceedings of the 8th EUCARPIA Meeting on Cucurbit Genetics and Breeding. Palacký University in Olomouc, Olomouc, Czech Republic, 2004. https://cucurbit.info/wp-content/uploads/2019/04/cuc2004proceedings.pdf

Pachner, M., H.S. Paris, J. Winkler, and T. Lelly. 2015. Phenotypic and marker-assisted pyramiding of genes for resistance to zucchini yellow mosaic virus in oilseed pumpkin (*Cucurbita pepo*). Plant Breeding 134:121–128. doi:10.1111/pbr.12219.

Pachner, M., H.S. Paris, T. Lelley. 2011. Genes for resistance to zucchini yellow mosaic in tropical pumpkin. Journal of Heredity 102:330–335 (online doi:10.1093/jhered/esr006)

Paris, H.S. and S. Cohen. 2000. Oligogenic inheritance for resistance to *Zucchini yellow mosaic virus* in *Cucurbita pepo*. Ann. Appl. Biol 136:209-214. https://doi.org/10.1111/j.1744-7348.2000.tb00027.x

Paris, H.S., S. Cohen, Y. Burger and R. Yoseph. 1988. Single-gene resistance to zucchini yellow mosaic virus in *Cucurbita moschata*. Euphytica 37:27-29. https://doi.org/10.1007/BF00037219

Seda-Martínez, W., L. Wessel-Beaver, A. Linares-Ramírez and J.C.V. Rodrigues. 2021. Virus quantification, flowering, yield, and fruit quality in tropical pumpkin (*Cucurbita moschata* Duchesne) genotypes susceptible or resistant to two potyviruses. HortScience 56:193-203.

Shrestha, S., F. Fu, V.N. Michael and G. Meru. 2022. Whole genome re-sequencing and bulk segregant analysis reveals chromosomal location for Papaya Ringspot Virus W resistance in squash. Front. Plant Sci. 13:848631. doi: 10.3389/fpls.2022.848631

Sierra-Rivera, L.G. 2012. Mejoramiento para resistencia a *Zucchini yellow mosaic virus* en calabaza (*Cucurbita moschata*) en Puerto Rico. M.S. Thesis. University of Puerto Rico, Mayagüez Campus.

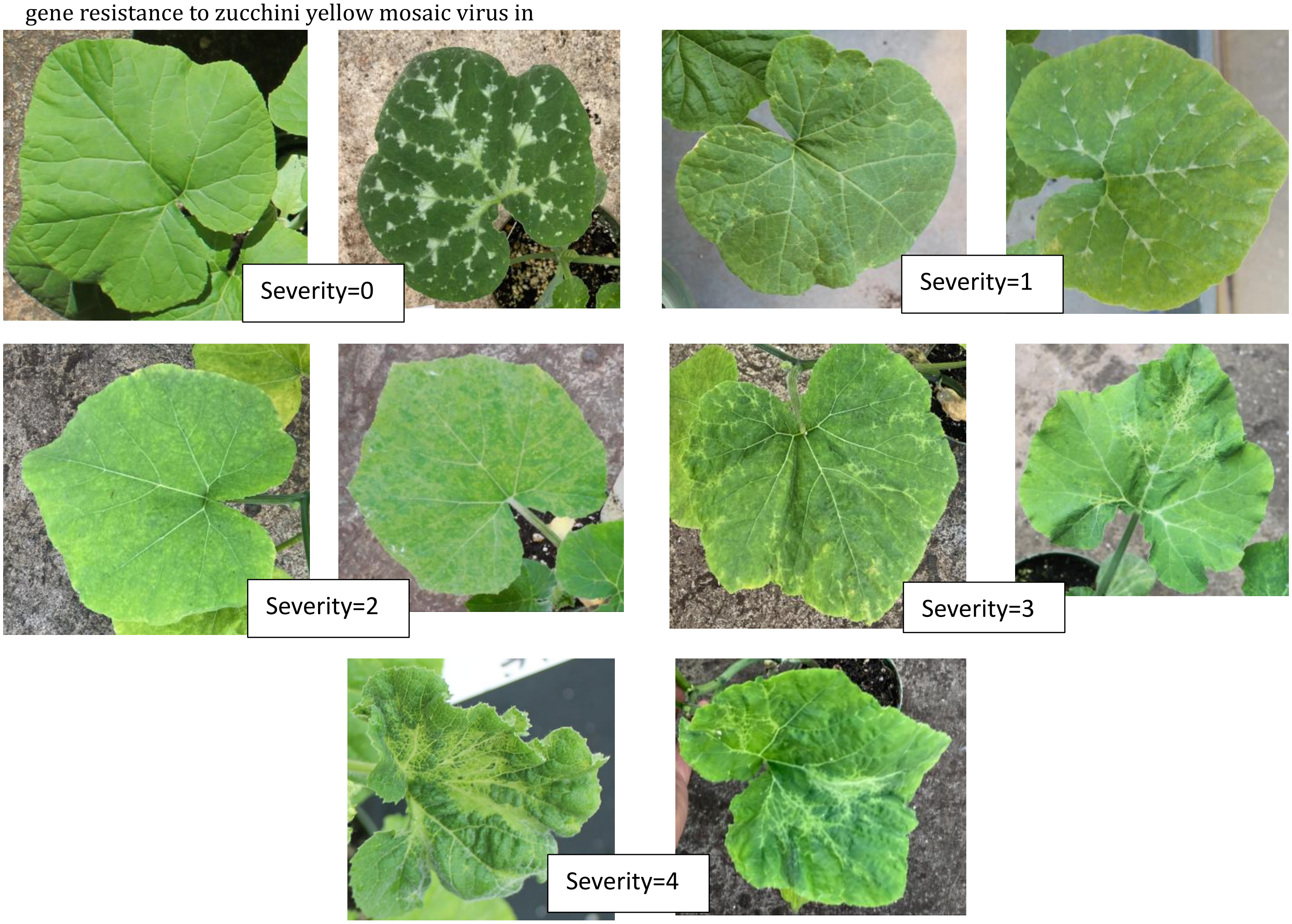

Figure 1. Examples of tropical pumpkin leaves with PRSV-W severity scores from 0 to 4. Note that *Cucurbita moschata*, including tropical pumpkin, can have completely green leaves or varying degrees of silver-grey patches in the axials of leaf veins (the *Mottle-leaf* trait). This type of leaf mottle is not related to PRSV-W virus symptoms.

Table 1. The table presents the percentage of times a symptom severity score in the 4th, 5th or 6th leaf correctly predicts the severity score in the 7th leaf. Data from two F_2 populations of tropical pumpkin (*Cucurbita moschata*) seedlings inoculated with PRSV-W are presented. For each F_2 population, table values within a row correspond to the percentage of plants with a common severity score in early leaves of tropical pumpkin (the 4th, 5th or 6th true leaf above the cotyledons) and the 7th leaf. The diagonals (shaded values) correspond to the percentage of plants were the severity score in the 4th, 5th or 6th leaf matched the severity score observed in the 7th leaf.

Symptom severity score (and resistance category) of 4th, 5th and 6th leaf	Symptom severity score (and resistance category) of 7th true leaf						
	Taína Dorada x Nigerian Local F_2 (n=112)				Verde Luz x Nigerian Local F_2 (n=199)		
	Leaf 7 = 0 or 1 (Resistant)	Leaf 7 = 2 (Intermediate)	Leaf 7 = 3 or 4 (Susceptible)		Leaf 7 = 0 or 1 (Resistant)	Leaf 7 = 2 (Intermediate)	Leaf 7 = 3 or 4 (Susceptible)
Leaf 4 = 0 or 1 (Resistant)	57%	26%	17%		13%	33%	54%
Leaf 4 = 2 (Intermediate)	20%	39%	41%		1%	16%	83%
Leaf 4 = 3 or 4 (Susceptible)	0%	4%	96%		0%	1%	99%
Leaf 5 = 0 or 1 (Resistant)	85%	15%	0%		30.5%	30.5%	39%
Leaf 5 = 2 (Intermediate)	12%	64%	24%		0%	39%	61%
Leaf 5 =3 or 4 (Susceptible)	2%	7%	91%		0%	1%	99%
Leaf 6 = 0 or 1 (Resistant)	96%	4%	0%		60%	40%	0%
Leaf 6 = 2 (Intermediate)	18%	70%	12%		2%	69%	29%
Leaf 6 = 3 or 5 (Susceptible)	0%	10%	90%		0%	0%	100%

Table 2. The distribution of symptom severity in a sample (n=101) of plants from a tropical pumpkin F_2 population (Verde Luz x Nigerian Local) inoculated with PRSV.

Symptom severity score of leaf 5	Number of plants
0 or 1	7
2	25
3 or 4	69