The genetics of resistance to grapevine fanleaf virus in *Vitis vinifera*

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Summary: Two wild *Vitis vinifera* accessions from the Middle East previously found to be resistant to grapevine fanleaf virus (GFV) were selfed and also crossed to a GFV-susceptible female cultivar. Five seedling populations of 60 plants each were established. A micrografting procedure was developed for screening the seedlings whereby single-node seedling stem segments were cleft-grafted go GFV-infected stocks *in vitro*. After 8 weeks, scion tissue was scored phenotypically and assayed by ELISA to measure virus titer. Resistance to GFV appears to segregate as a recessive trait controlled by at least two genes.

Key words: fanleaf, GFV, resistance, genetics, crossing, selection, micrografting, serology, ELISA.

Introduction

Fanleaf degeneration, one of the most serious diseases affecting world viticulture, is a disease complex caused by grapevine fanleaf virus (GFV) and the feeding of the vector, *Xiphinema index*. The nematode-vectored nature of the disease was first discovered by Hewitt *et al.* (1958), and this discovery initiated efforts to control the disease. Fumigants and nematicides aimed at eradicating the vector were used at first, but they proved unsuccessful in California (Raski *et al.* 1983). A rootstock breeding program at the University of California, Davis began with a screen of *Vitis* species for *X. index* resistance (Kunde *et al.* 1968). These efforts resulted in the release of two rootstocks with field resistance to fanleaf degeneration (Lider and Goheen 1986), VR O39-16 (United States Patent # 6166) and VR O43-43 (United States Patent # 6319). Since the release of these rootstocks, GFV has been detected in scions on both of them (Walker *et al.* 1989). It appears that, although the two rootstocks have high levels of resistance to *X. index* feeding (Lider and Goheen 1986), chance nematode probing transmits GFV. The next step in the development of fanleaf degeneration-resistant rootstocks is to combine GFV resistance with *X. index* feeding resistance.

The search for GFV resistance began with a screen of the *Vitis* germplasm held at the University of California, Davis and resistance was found in Middle Eastern *V. vinifera* accessions (Walker *et al.* 1985). Resistant and susceptible plants identified in that study have been used to produce hybrid, selfed and open-pollinated seedling populations in an effort to characterize GFV resistance.

Materials and methods

The *V. vinifera* accessions used as parents are as shown in the table.

All of these accessions except Almeria were collected by H. P. Olmo in 1948 in the Middle East - O30-44 in Shirwandah, Iran and the siblings, O30-51 and O30-53, in Adhai, Afghanistan (H. P. Olmo, personal communication). Almeria is a pistillate cultivar and does not set fruit without external pollen (Olmo 1943). O30-53 was originally classified as pistillate, but had functional pollen and was used as a male parent in these crosses. O30-44 was classified as staminate, however it behaved as a hermaphrodite and produced seed each year. O30-51 was staminate and only set seed after chemical hermaphrodization following the techniques of Negi and Olmo (1966) and Srinivasan and Mullins (1979).

Accession number, vineyard location and GFV response of the parents used in the crosses for characterization of GFV resistance

Accession	Location	GFV response
cv. Almeria (UCD clone 1)	Tyree IV R7v22	susceptible
O30-53 (UCD number)	Armstrong M3v20	susceptible
O30-44 (" ")	" " M3v17	resistant
O30-51 (" ")	" " M3v18	resistant

The following seedling populations were produced:
(1) O30-44 open pollinated (resistant plant O. P.)
(2) O30-51 chemically hermaphrodized and selfed (resistant selfed)
(3) Almeria x O30-44 (susceptible x resistant)
(4) Almeria x O30-51 (susceptible x resistant)
(5) Almeria x O30-53 (susceptible x susceptible).

60 seedlings from each cross were randomly selected for each of the populations, except for the O30-51 selfed population, which consisted of all 51 plants produced.

The seedlings were inoculated with GFV by micrografting. Highly GFV-infected (ELISA values > 1.999 OD 405_{nm}) *V. vinifera* cv. Cabernet Sauvignon from a vineyard in the Napa Valley, California, was used as the inoculum source. Shoots were harvested from greenhouse-grown GFV-infected vines and brought into the laboratory for sterilization. Sterilized one-node stem segments (to be used as rootstocks) were trimmed to about 30 mm, their lateral buds removed, and placed in 25 x 150 mm culture tubes, containing 25 ml of rootstock medium, capped and sealed with parafilm. The rootstock medium consisted of 1/2 strength MS (Murashige and Skoog 1962) packaged salts (# 500-1117 EF, Gibco Laboratories, Grand Island, NY), 1/2 strength MS vitamins, no sucrose, 1 mg/l indole-3-acetic acid (# I-1250, Sigma Chemical Co., St. Louis, MO), 300 mg/l cefotaxime (Hoechst-Roussel Pharmaceuticals, Inc. Somerville, NJ), and 6 g/l Sigma plant tissue culture agar (# A-1296). Endophytic bacteria (*Pseudomonas* sp.) were present in the GFV inoculum plants and in the potted seedlings. The cefotaxime was added to control this bacterium and did not appear to have a deleterious effect on GFV spread or graft compatibility. The rootstocks were grown for 3-4 weeks at 27 °C in a growth chamber with 16 h daylength prior to micrografting. Contaminated cultures or those that had not initiated roots were discarded.

Sterilized single-node seedling stem pieces, approximately 30 mm long, were used as scions and the pre-rooted GFV-infected stem pieces as rootstocks. The rootstock and scion were placed on sterile filter paper in a sterile 125 mm glass petri dish. A 10-15 mm longitudinal cut was made at the apical end of the rootstock stem piece through the node towards the base. The basal end of the seedling stem piece was tapered with two slanting cuts and fitted into the rootstock piece with forceps. Care was taken to match the cambium layers of rootstock and scion on at least one side. The completed grafts were placed into 25 x 150 mm culture tubes, containing 25 ml of rootstock medium modified with the addition of 10 g/l sucrose and the omission of growth regulators, capped and wrapped with parafilm. Culture conditions for the micrografts were the same as for rootstock cultures. Four micrografts were made for each seedling.

Samples were collected after 8 weeks. The optimal sample included only scion shoot growth from the lateral bud. Any additional scion stem tissue needed to bring sample weights up to the minimum 100 mg was taken with care to avoid the graft union and union callus tissue to insure that rootstock tissue was not sampled.

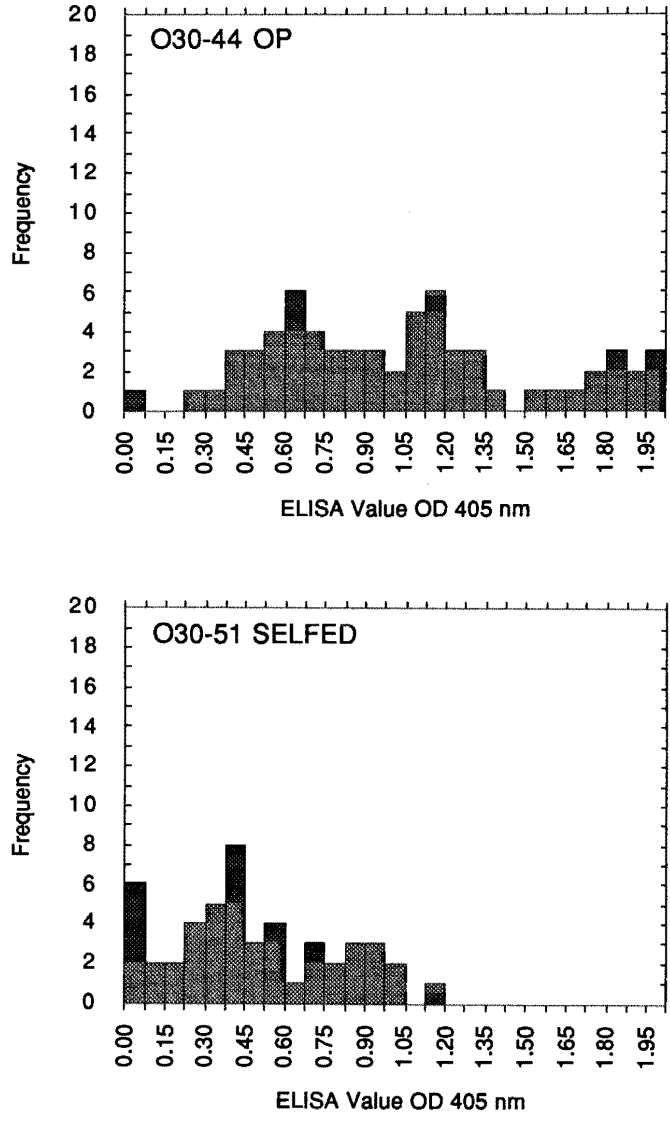
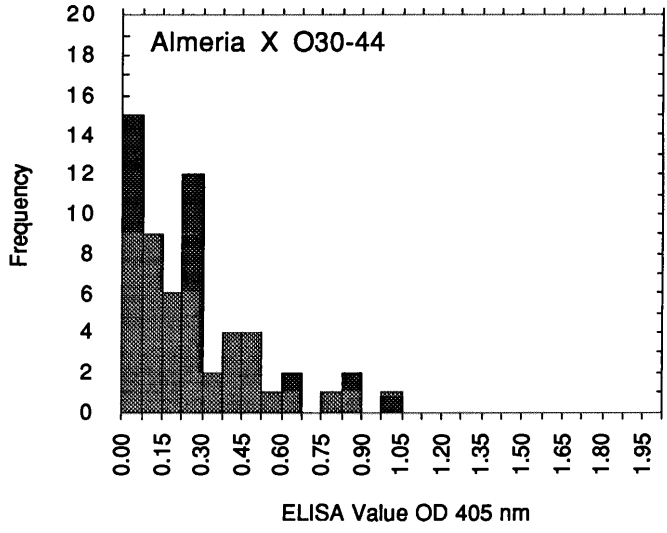


Fig. 1: The highest ELISA values obtained for each seedling within the different seedling populations.

Samples were placed in plastic scintillation vials and GFV extraction buffer was added at a 1/10 (w/v) dilution. Extraction buffer consisted of 0.1 M phosphate buffered saline, 2 % polyvinyl pyrrolidine-40, and 0.5 % Tween 20. The samples were collected, partially frozen to a slurry, then ground with a Brinkman Polytron homogenizer, PT10 probe (Brinkman Instruments, Inc. Westbury, NY), on number 6 setting for 20-25 s, and frozen at -20 °C until used. ELISA (enzyme-linked immunosorbent assay) was used to detect GFV in the samples following the procedures of CLARK and ADAMS (1977). ELISA reactions were read at 405 nm after a 1 h substrate incubation, and values below 0.075 OD 405_{nm} were considered resistant. The inoculated scions were given morphological ratings from 1 to 4 as follows:



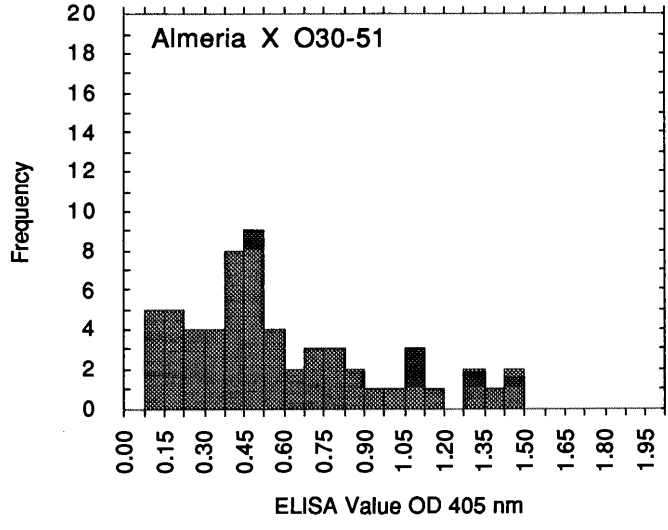


Fig. 1 (continued overleaf).

1 - normal growth
2 - internodes elongated; tall, but not as vigorous as 1; leaves smaller and often vitreous and misshapen
3 - internodes compressed; 3-5 shoots produced from a single lateral bud
4 - internodes greatly compressed with multiple shoots from the lateral bud, producing a moss-like mat of tissue.

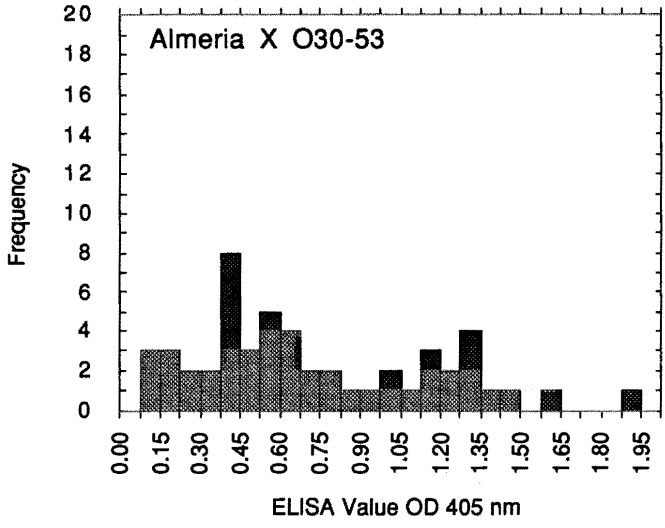


Fig. 1 (continued).

Results

There was a broad distribution of ELISA values within and among the seedlings in each population. In order to reduce some of this variability, the highest ELISA value obtained for each seedling was selected and histograms were constructed (Fig. 1). Histograms were also plotted for the morphological ratings corresponding to the high ELISA values (Fig. 2).

All levels of gene control for one gene and two unlinked genes were evaluated using ELISA and morphological ratings. Only the highest ELISA value obtained for each seedling was used. Morphological ratings of the replications within a seedling were relatively consistent and all of the values were used to evaluate gene models. ELISA values and morphological ratings were classified into various numbers of groups depending on which gene control model was being tested. Fig. 3 presents the four classes that might be expected if a parent heterozygous for two genes with dominance at both loci was selfed.

When ELISA and morphological data were considered as representing either resistant or susceptible classes, without intermediate groups, and seedlings within each population were classified on this basis, the O30-44 OP and the O30-51 selfed seedling populations appear to segregate as though GFV resistance is controlled by two unlinked recessive genes with duplicate dominant epistasis controlling susceptibility. Chi-square analysis supported this hypothesis. There were no good fits of the seedling data with any other gene control model.

Discussion

The ELISA frequency distributions did not appear to fit into discrete classes. This lack of definition could have been due to broad segregation for resistance in the progeny, inconsistencies in the micrografting procedure and ELISA evaluation, or environmentally induced variability in the seedling and stock pieces. Multigene segregation or segregation of an environmentally unstable trait may have been responsible for the broad distribution of ELISA values in each seedling population. Micrografting did produce variable results in many of the inoculated seedlings.

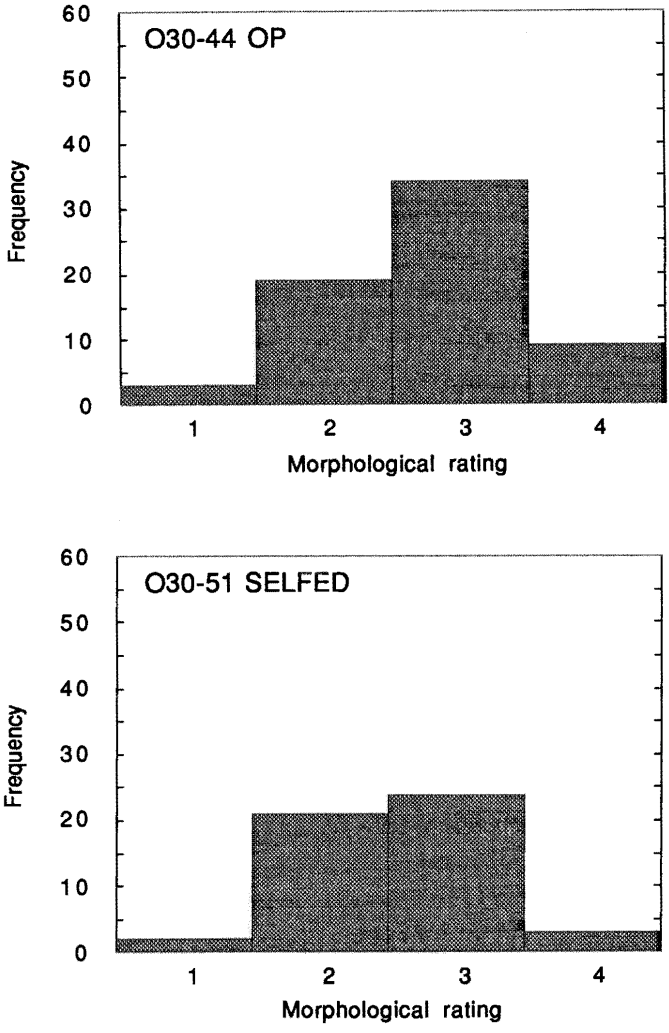


Fig. 2: Morphological rating corresponding to the highest ELISA value of each seedling within the different seedling populations. 1 = normal growth; 2 = internodes elongated, tall, but not as vigorous as 1, leaves small and often vitreous and misshapen; 3 = internodes compressed, 3-5 shoots produced from a single lateral bud; 4 = internodes greatly compressed with multiple shoots from the lateral bud, producing a moss-like mat of tissue.

(continued overleaf.)

Limiting the frequency distributions to each seedling's highest ELISA value and its corresponding phenotype (Figs. 1 and 2) was a means of reducing this variability, but these histograms may not represent the actual resistance reaction, and may impair a quantitative appraisal of resistance.

The stem pieces taken from both seedlings and stock plants for micrografting were used without regard to position on the shoot, or vigor of the seedlings. The physiological state of these donor plants may have contributed to the observed variability within and between the micrografts, both in terms of their stored carbohydrate reserves and their hormone levels.

Another possibility is that the continuous, quantitative nature of ELISA values was not amenable to detection of discrete classes. This last consideration may be important when

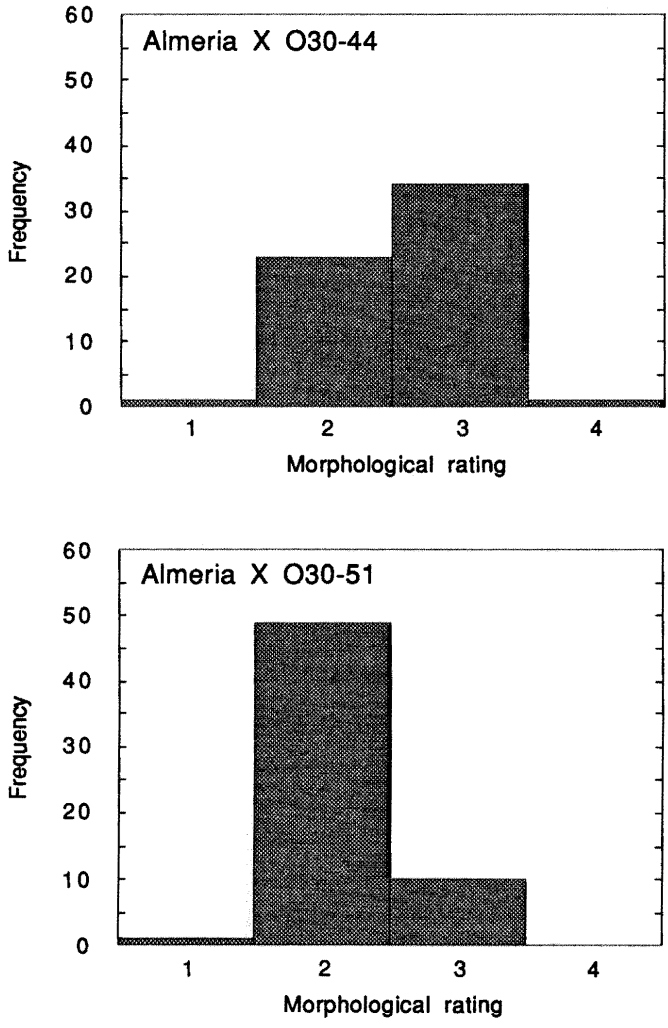


Fig. 2 (continued).

interpreting the results after the ELISA and phenotypic values were reduced to susceptible or resistant classes.

The O30-44 OP and the O30-51 selfed progeny ELISA values seemed to segregate widely, which may have been due to inconsistencies in the micrograft system and its ELISA evaluation, or to the heterozygosity of the parents. If the resistant parents, O30-44 and O30-51, are heterozygous for two unlinked resistance genes, then the susceptibility of Almeria needs to be questioned. If Almeria is considered homozygous susceptible, then crosses between it and the resistant parents should mirror the genotypes of the resistant parent's gametes, and give 1 : 1 : 1 : 1 ratios. These ratios were not detected; there are at least two possible explanations to account for this. Almeria is considered to be ancient Spanish cultivar, but it shares morphological features with Middle Eastern cultivars. It could have originated in the Middle East and been brought to Spain later. Given the hypothesized coevolution of GFV and *V. vinifera* in the Middle East (Hewitt 1970; Vuittenez

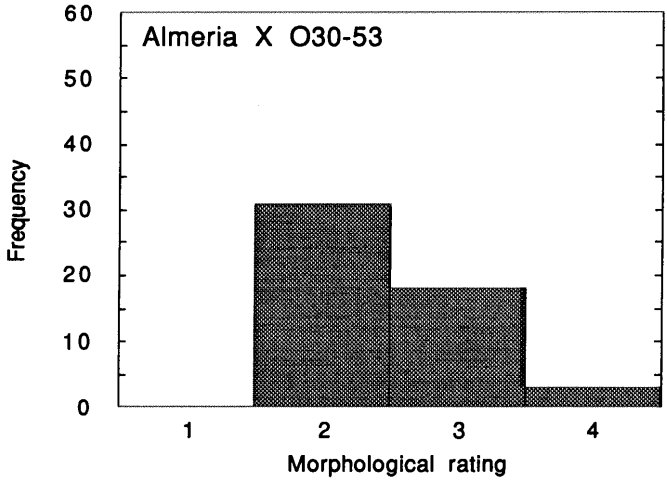


Fig. 2 (continued).

1970), Almeria may share resistance genes with the resistant parents. If resistance is a quantitative trait, then 'test crosses' to Almeria would not be easily resolvable whether it is homozygous or heterozygous.

Conclusions about the inheritance of resistance to GFV require a better understanding of parental reactions to GFV after micrografting and conclusive data to allow quantitative comparisons between parents and progeny. Parental micrograft data were weak and produced more questions than answers. Detection of a quantitative trait is dependent upon accurate parental appraisal, which is necessary for comparisons with progeny populations.

GFV was not detectable in 15 seedlings in the Almeria x O30-44 population and 4 in the O30-51 selfed population, but at the same time these seedlings had morphological ratings suggesting GFV infection. This occurrence was unusual and might be explained as a disease reaction. GFV could be localized at the graft union in these cases and prevented from spreading into scion tissues. This localization might alter the seedling's normal hormonal balance and cause morphological change in the absence of GFV. Seedlings responding in this manner should be reexamined, not only for virus, but also for unusual hormone levels in the scion.

2 seedlings in the O30-44 OP population, 87-7-29 and 87-15-14, and 1 in the Almeria x O30-51 population, 88-9-10, exhibited tolerance, that is, GFV was detectable in their scions, but no corresponding disease symptoms were observed. There were no problems with the grafts or scions that might raise doubts about this response. Tolerance to GFV may be entirely separate from resistance, since GFV was readily detectable in the scions, but there were no corresponding phenotypic reactions. Such tolerance may also be due to environmental interaction, and may not be reproducible in whole plant studies. Tolerance might be expected to be a relatively common occurrence in a coevolving plant/pathogen complex, since tolerance would have a reduced selective impact on the pathogen compared to the selective pressure of resistance. Middle Eastern cultivars and *V. vinifera* populations should be reexamined with GFV tolerance in mind, particularly if *in vitro* tolerance can be correlated with whole plant studies.

In addition to tolerance, GFV resistance seems to be present in 2 seedlings: 87-6-39 in the O30-44 OP population, and 87-5-17 in the O30-51 selfed population. The graft and culture data

O30-44 OP

Morphological Rating

ELISA Value	A	B	C	D	
A	1	0	0	0	1
B	0	1	0	0	1
C	2	4	14	2	22
D	0	3	31	7	41
	3	8	45	9	65

ALMERIA X O30-44

Morphological Rating

ELISA Value	A	B	C	D	
A	0	3	12	0	15
B	0	4	15	0	19
C	0	4	17	0	21
D	0	0	3	1	4
	0	11	47	1	59

O30-51 SELFED

Morphological Rating

ELISA Value	A	B	C	D	
A	0	1	3	1	5
B	0	0	6	0	6
C	0	10	17	1	28
D	0	1	9	0	10
	0	12	35	2	49

ALMERIA X O30-51

Morphological Rating

ELISA Value	A	B	C	D	
A	0	0	0	0	0
B	0	10	3	1	14
C	1	25	6	0	32
D	0	5	9	0	14
	1	40	18	1	60

ALMERIA X O30-53

Morphological Rating

ELISA Value	A	B	C	D	
A	0	0	0	0	0
B	0	3	4	0	7
C	0	9	15	3	27
D	0	12	6	0	18
	0	24	25	3	52

Fig. 3: Number of seedlings in each of 4 classes by both ELISA and morphological rating. Morphological ratings: A = seedlings with replicates having only class 1 ratings (1111), B = 1112 through 2222, C = 2223 through 3333, D = 3334 through 4444. ELISA values: A = readings $\leq$ 0.075 OD 405_{nm}, B = > 0.075-0.250, C = > 0.250-0.800, D = > 0.800.

taken for 87-6-39 did not reveal any reason to doubt its resistant status. ELISA did not detect GFV among the 4 replications of 87-5-17, and 3 of the 4 replications had normal morphology, while the 4th had a rating of 2 (reduced vigor and small leaves). This moderately affected replicate did not graft or grow as well as the other 3 and its abnormal morphology may not have been caused by GFV. These 2 seedlings seem to have a high degree of resistance to GFV. Although they have not yet been screened for resistance in other than a tissue culture environment, they did exhibit much greater resistance to GFV than either of the parents or any of the other seedlings.

GFV resistance seems to be genuine, but further crosses and tests are needed before the number of genes controlling resistance can be accurately determined. Paramount among considerations for the next generation of crosses and selfings is more accurate appraisal of the parental reactions to GFV, both resistant and susceptible, so that a quantitative trait could be assessed. The results suggest that GFV resistance is recessive and controlled by two unlinked genes with duplicate dominant epistasis. However, given the single environment in which the seedlings were evaluated and the seemingly ambiguous parental reactions, this conclusion is tentative at best.

This work has produced seedlings with a wide range of GFV reactions (both resistant and highly susceptible) that can be used to produce a second generation of crosses and selfings. The results from a second generation will better characterize GFV resistance, and should elucidate the heritability of GFV resistance. Tolerance seems to exist in 2 of the seedling populations, and it may or may not be associated with resistance. If *in vitro* tolerance can be verified and shown to persist in whole plant studies it may be more valuable, and in the long term more durable, than resistance. The 2 seedlings that appear to be resistant will be reexamined, by micrografting and whole plant approach grafting. Once they are better understood, they will be crossed to known sources of *X. index* feeding resistance to produce rootstocks that will resist the vector and the virus and provide long term protection against fanleaf degeneration.

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Detection of grapevine nepoviruses in woody canes

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A b s t r a c t : Testing grapevine viruses in woody parts of the plant allows testing of grafting materials just before grafting.

Our results showed that producing a rough sawdust with a chainsaw, blending it 1 : 5 (w/v) with Tris extraction buffer gave a positive signal in the ELISA procedure if only 1 % of the canes in a bundle was infected with nepoviruses (AMV or GFV). Transmission by sawdust from one sample to the next did not occur.

Sawdust samples could be homogenized with an Ultra Turrax or a Tecan Homogenizer just after sawing. The differences in the results between the Ultra Turrax and the Tecan Homogenizer were small.

Rough sawdust samples gave better results than shavings.

Producing small wooden disks before homogenization gave better results than all other methods but this sampling method is relatively time consuming.

Using disks is only possible for small series where high accuracy is needed, rough sawdust is a method for large series with less accuracy, especially for testing grafting materials.