Field Transmission of Grapevine Leafroll Associated Virus 3 (GLRaV-3) by the Mealybug *Planococcus citri*

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ABSTRACT

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The spatial distributions of grape plants with symptoms of grapevine leafroll in nine vineyards in the Galician wine-growing region Rías Baixas (northwestern Spain) were analyzed. In five, significant aggregations of diseased plants and/or disease gradients toward one of the borders of the plots constituted indirect evidence of vector transmission by a nonflying vector. From 1991 to 1995, vines from two vineyards were tested for grapevine leafroll associated virus 3 (GLRaV-3) by enzyme-linked immunosorbent assay. In one of these vineyards, which had been virus-free when planted, GLRaV-3 was detected 3 years after planting. In the second, the incidence of GLRaV-3 increased from 33 to 83% between 1991 and 1995. The mealybug *Planococcus citri* was associated with the infected plants in this vineyard. GLRaV-3 was present in *P. citri*, and under controlled conditions this mealybug transmitted GLRaV-3 to five of five previously healthy grape plants.

Grapevine leafroll disease has been described in all grape-growing countries as one of the most serious viral diseases of grapevine (30). The etiology of the disease is not well established, but several filamentous viral particles have been associated with it (23,25,31,38), including seven serologically unrelated closterovirus-like particles referred to as grapevine leafroll associated viruses 1 to 7 (GLRaV 1 to 7) (4,5,11,20). Grapevine leafroll has also been associated with grapevine virus A (GVA), a trichovirus included in the rugose wood complex (6,13).

Until the 1980s, the spread of grapevine leafroll disease was assumed to occur only through infected plant material, mainly asymptomatic American grapevine rootstocks (14,22). In 1983, however, it was found that some mealybugs (*Pseudococcidae*) are vectors of grapevine leafroll associated viruses (33). Since then, several reports on field transmission of grapevine leafroll disease have confirmed the role of mealybugs as vectors of GVA and GLRaV-3 (17-19, 26,32); although, in other studies, no vectors were identified (10,15,24, 27,37). The four mealybug species mentioned to date as vectors, in the field or in

controlled conditions, of viruses associated with grapevine leafroll, are *Pseudococcus longispinus* Targioni Tozzetti (33), *Planococcus ficus* Signoret (16,17), *Planococcus citri* Risso (1), and *Pseudococcus affinis* (21). The only *Pseudococcidae* reported to transmit GLRaV-3 are *Planococcus ficus* and *Pseudococcus longispinus* (16,34,36) but the scale insect *Pulvinaria vitis* L. may also be a vector (3).

The incidence of grapevine leafroll is high in all the grapevine-growing areas in northwestern Spain. Both GLRaV-3 and GLRaV-1 are present but only GLRaV-3 is widely distributed, with an incidence of about 40% (35). Most new plantings in Spain are grown on virus-free, certified rootstocks, and a great effort is being made to select virus-free plant material from the traditional cultivars of each grape wine-growing area, especially those producing high quality wines, such as Rías Baixas. The spread of grapevine leafroll disease to the new, healthy vineyards is of great concern for sanitation programs. The aim of this work was to evaluate the spread of grapevine leafroll disease on the basis of the spatial distribution of diseased plants in several GLRaV-3-infected vineyards and the time-dependence of incidence, and to relate any observed spread to the presence of possible vectors of grapevine closteroviruses.

MATERIALS AND METHODS

Field sampling design and vineyard selection. A study was carried out in the wine-growing area of Rías Baixas in northwestern Spain. Nine *Vitis vinifera* L. 'Albariño' vineyards, from 6 to 15 years

old and between 1,000 and 30,000 m² in size, with vines planted in a rectangular pattern (3 × 4 m) were sampled for the presence of leafroll disease. In this region this cultivar is traditionally trained on a horizontal trellis to form a continuous canopy at a height of about 2 m. Growth is vigorous, and the trellis is covered 3 to 4 years after planting. The vineyards were selected on the basis of a preliminary survey carried out following the methodology recommended by Barnett (2): mature leaves were picked randomly from 10 plants per ha on one or both diagonals of each vineyard and were analyzed by enzyme-linked immunosorbent assay (ELISA). Nine vineyards where GLRaV-3 had been found were selected for the spatial or temporal studies reported here (Table 1).

Diagnostic method. Mature leaves with their petioles were picked in late summer and tested for GLRaV-1, GLRaV-3, and GFLV by double antibody sandwich (DAS)-ELISA (12). Antibodies and their conjugates were obtained from Bioreba AG, (Basel, Switzerland). Dilutions and tissue extracts were prepared following the supplied instructions. The extraction buffer was 0.5 M Tris HCl (pH 8.2) with 1% polyethyleneglycol (MW 6000), 0.8% NaCl, 2% polyvinylpyrrolidone (MW 24000) and 0.05% Tween 20 (23). Two replicates of each sample were analyzed. Absorbance at 405 nm was recorded with a microplate reader (Titertek Uniskan, Helsinki, Finland). Samples with absorbance readings more than three times that of the healthy control and higher than 0.100 were considered positive.

Analyses of the spatial distribution of diseased plants. In previous work (7) we obtained a good correspondence between leafroll symptoms and the presence of GLRaV in the grape white cultivar Albariño when symptoms were observed in August. Therefore, in this work, only in one vineyard (Meaño) was every vine in the orchard tested for GLRaV-3 by ELISA. In the other eight, vines were evaluated only for visual leafroll symptoms. Vines were recorded as diseased, healthy, or missing, in a matrix representing the planting lattice (28).

Symptom incidence data were subjected to regression analysis with row (or column) number as independent variable, and the incidence of disease in that row (or

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column) as the dependent variable. In additional regression, the linearized forms of exponential and inverse power law models were evaluated. Only the results for the exponential model are reported here. Determination coefficients were calculated with Maixner's PATCHY program (28), and significant deviation of the regression line slope from zero was taken as indicating the existence of a disease gradient in the direction corresponding to the independent variable.

For vineyards with more than 20 vines in each row and/or column, unidimensional aggregation analysis (ordinary runs) was performed (8). Bidimensional analysis was performed by successively dividing a whole vineyard into identical subplots of increasing size, calculating for each size, mean number of infected vines per subplot, and using these data to calculate Morisita's index of dispersion (8). The statistical significance of deviations of Morisita's index from unity was estimated. All calculations were performed with the program PATCHY (28).

The average sizes of aggregations (mean focus sizes) were estimated by means of $I\delta$, which has maxima when quadrat size equals focus size.

Analysis of time dependence. Increase of grapevine leafroll disease was investigated in two vineyards, Beluso 1 and Meaño. Leaf samples taken each summer were tested for GLRaV-3 by DAS-ELISA as described above.

In the Beluso 1 vineyard, 1,668 Albariño vines grafted on rootstock 161.49C occupy about 2 ha. The vineyard was planted in

1979 with plant material that had not been certified as virus-free. Forty-two plants taken on two diagonals were tested every year from 1991 to 1995.

The Meaño vineyard is an experimental plot for evaluation of four Albariño clones grafted on two rootstocks (196.17C and SO4), with 20 replicates per clone and rootstock. Virus-free, certified rootstocks were planted in 1988, and scions taken from four controlled, leafroll-free Albariño plants were grafted in 1989. Plants with leafroll symptoms were observed during the summer of 1991, and GLRaV-3 was detected by ELISA. Since 1992, all the plants in the plot have been analyzed every year.

Potential vectors of GLRaV-3. Nine vineyards were inspected for potential insect vectors. Observation of mealybugs in a plot adjoining Beluso 1 in 1993 led to the 42 ELISA-monitored vines being examined more thoroughly in 1994 and 1995 by lifting the bark on the upper branches.

ELISA detection of GLRaV-3 in mealybugs. Mealybugs collected in autumn 1994 and spring 1995 from Beluso 1 grapevines infected with GLRaV-3 were tested for GLRaV-3 by ELISA with the method and buffers described above for plant material. Twenty mealybugs feeding in one potted, GLRaV-3-infected "Cabernet franc" kept in a growth chamber were also tested individually for GLRaV-3 by ELISA. Mealybugs reared on potato sprouts, and *Coccus hesperidum* L. from an orange tree and from leafroll-infected grapevines, were used as controls in the serological tests.

Transmission test in controlled conditions. Mealybugs identified as *Planococcus citri* Risso were taken from field grapevines and reared on potato sprouts for several generations. Leaf fragments bearing females and eggs were transferred to a GLRaV-3-infected, potted grapevine. The nymphs were allowed to feed and develop, and groups of 20 to 30 nymphs were transferred to each of five healthy, potted Albariño plants. After 1 month, mealybugs were eliminated by repeated spraying of dimethoate (1.2 ml of the 40% commercial product Diluq per liter [LUQSA, Lérida, Spain]). Mature leaves were tested for GLRaV-3 by ELISA after 2, 4, 6, 8, and 10 months. In all stages of this experiment, plants were kept in a growth chamber at 25/16°C day/night, with a 16-h photoperiod. Healthy plants were kept in a growth chamber and greenhouse at the same conditions and tested as controls.

RESULTS

Spatial analysis. The results of the regression analyses of the data for the 9 vineyards are presented in Table 1; in each case, the land use of the neighboring plots is also indicated. For five of the vineyards, the significant deviation of regression line slopes from 1 indicates a disease gradient toward one of the borders of the plot. In four of these cases the land adjoining that border was planted with grapevines; at the Beluso 1 site the gradient pointed toward a stand of a *Eucalyptus* sp.

The aggregation analysis results are listed in Table 2. Where unidimensional analysis was possible, between 11 and 38% of rows or columns had significant aggregations of diseased plants. The bidimensional analysis of quadrats of various sizes showed significant aggregations of diseased vines in 5 vineyards with maximum $I\delta$ values of 1.113 to 1.891. The estimated mean focus size varied between 4×2 and 4×9 plants, but in four of the five plots aggregation was also significant on a smaller scale (3×2 for San Anton). In the other four plots the distribution was trendless: for no subplot size was $I\delta$ statistically different from 1, and for some subplot sizes it was significantly less than 1. In all four of these plots the incidence of leafroll was high (range 22.7 to 46.4%), whereas in four of the five plots exhibiting significant aggregation incidence was lower (11.8 to 25.3%).

Time dependence. Figure 1 shows the increase in grapevine leafroll disease during the study period in the Meaño vineyard. The number of GLRaV-3-infected plants was 34 (21.3%) in 1992 and increased to 35 in 1993, 38 in 1994, and 40 (25.3%) in 1995; both rootstocks and all four scion clones were affected. At Beluso 1, the incidence of leafroll was 33% in 1991 (14 plants), 40.5% in 1992 (17 plants), 64.3% in 1993 (27 plants), 76.2% in 1994 (32 plants), and reached 83% in

Table 1. Slopes and intercepts of the disease gradient regression lines correlating the incidence of grapevine leafroll with row or column position, in 9 Albariño vineyards, after linearization of the exponential model^a

Vineyard	n ^b	Plot outline ^c	s ^d	int
Meaño	8 r		0.01 NS	3.1
	20 c		-0.18 **	4.1
Cornazo	7 r		-0.28 NS	4.6
	6 c		-0.37 *	6.0
Montes	9 r		0.24 NS	2.17
	3 c		-0.1 NS	4.6
San Antón	18		0.02 NS	2.22
	26		-0.06 *	3.3
Sisan	15 r		-0.005 NS	2.7
	19 c		0.19 ***	0.33
Leira Nova	12 r		0.01 NS	3.4
	11 c		0.02 NS	3.2
As Pedras	10 r		-0.07 NS	3.1
	11 c		0.14 NS	0.9
Beluso 2	28 r		-0.003 NS	3.7
	18 c		-0.004 NS	3.8
Beluso 1	45 r		-0.01 *	3.9
	47 c		0.06 NS	3.43

^a Significant deviations of the slopes from zero and crop at each border of the vineyard are indicated. $\ln(I) = \text{int} - s \cdot D$; s, slope; int, intercept; D, position of rows or columns.

^b Number of vines in rows (r) or columns (c).

^c Arrows show direction of disease gradient. Vineyard borders: ——— grapevine; - - - - - eucalyptus wood; = = = garden; vegetable garden.

^d NS = not significant; *, **, *** significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

1995 (36 plants); the greatest increment (10 newly infected plants) occurred between 1992 and 1993.

Search for vectors. Likely vectors were found only in Beluso 1. In 1993, colonies of mealybugs were observed in a vineyard about 200 m from this vineyard. In September 1994, after an exhaustive search (lifting the bark on the upper side of canopy branches) we found small numbers of mealybugs (*Planococcus citri* Risso) in Beluso 1 feeding on 13 of the 42 monitored plants; all those plants had been found by ELISA to be infected with GLRaV-3. In spring 1995, colonies were found on four infected plants.

Detection of GLRaV-3 in mealybugs by ELISA. The virus was detected in groups of 10 or 30 mealybugs collected in September 1994, in most of those collected in June 1995, and also in five of the 20 tested mealybugs reared on the potted, infected vine used in the transmission experiment. Levels of GLRaV-3 were generally high enough in the mealybugs for detection by ELISA with high absorbances at 405 nm when analyzed in groups of five insects, but also in individual specimens.

Transmission test in controlled conditions. ELISA of mature leaves of the five previously healthy grapevine plants showed them to be positive for GLRaV-3 8 months after elimination of mealybugs. The presence of GLRaV-3 was confirmed 2 months later. Previous analyses (at 2, 4, and 6 months) had been negative and healthy controls remained negative after 10 months.

DISCUSSION

The high incidence of grapevine leafroll in the Rías Baixas region had been thought to be exclusively due to the use of pre-infected plant material, because use of cer-

tified rootstocks is recent and many of the century-old Albariño plants used traditionally as sources of scions are also leafroll infected (35). However, infected plant material would give a random distribution of diseased plants in the vineyards, whereas in this work several Rías Baixas vineyards, including a vineyard established from virus-free stocks and scions, showed significant aggregations of diseased vines, which is suggestive of field transmission. Furthermore, several of the vineyards studied exhibited a disease gradient toward one of their borders (usually an adjacent vineyard) besides significant aggregations of diseased vines, suggesting that infection had spread into the vineyard from a neighboring vineyard and then inside the plot. We interpret these findings as a good indication of vector transmission, as Maixner did (28) on the basis of a similar spatial analysis of German vineyards with a yellows disease ("Vergilbungskrankheit") suspected to be transmitted by *Cicadellidae*.

In the fields that did not show a disease gradient, usually the diseased plants were randomly distributed and GLRaV-3 incidence was high, suggesting the use of pre-infected plant material mixed with healthy material.

The natural spread of grapevine leafroll disease was confirmed in the two vineyards monitored over 4 to 5 years, even though they had different patterns of spread and possible vectors were only found in one. At Meaño, the incidence of disease in an originally healthy vineyard reached 21.3% within 3 years of planting, after which only a few newly infected plants were detected in subsequent years in spite of all the plants in the plot being tested each year. While mealybugs or other possible vectors were not observed, vector transmission from infected neighboring vineyards is the best hypothesis. At Beluso 1 the number of infected plants increased steadily during the 5-year study period, and backward extrapolation suggests that in-

Table 2. Aggregation analysis of leafroll symptoms in 9 Albariño vineyards^a

Vineyard	Year	Age (years)	n ^b	Incidence of leafroll (%)	(% r/c) ^c	Iδ max ^d	Focus size	
							1st Iδ>1	max Iδ
Meaño	1995	6	159	25.3	25% r	1.397 **	2 × 5	3 × 5
Cornazo	1994	7	129	28.7		1.351 NS	—	—
Montes	1994	8	151	46.4		1.000 NS	—	—
San Antón	1994	9	459	11.8	13% c	1.440 **	3 × 2	4 × 2
Sisan	1994	10	284	17.3	16% c	1.765 ***	2 × 4	4 × 9
Leira Nova	1994	10	148	30.4		1.051 NS	—	—
As Pedras	1994	10	145	15.2		1.891 *	4 × 3	4 × 3
Beluso 2	1994	14	475	44.6	17% c	1.005 NS	—	—
Beluso 1	1994	15	1,668	41.7	11% r/ 38% c	1.113 ***	2 × 8	4 × 4

^a Ordinary runs analysis with percentages (%) of rows (r) and columns (c) with significant aggregation of symptomatic vines, and quadrat analysis with Morisita's index of dispersion (Iδ).

^b Number of vines.

^c Ordinary runs analysis not performed when number of vines in rows or columns is <20.

^d NS, not significantly >1; *, **, *** significant at $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.

Fig. 1 Incidence of grapevine leafroll associated virus 3 at the Meaño vineyard. Four clones of cv. Albariño (1 to 4) grafted on two rootstocks (A, B) with 20 replicates/rootstock and clone. Closed circles (●) indicate infected grapevines in 1992; infections along the successive years are shown as ⌘ (1993), ◆ (1994), and ■ (1995). Open circles (○) show healthy vines.

festation may have occurred in 1988, although no possible vectors were found until 1994. GLRaV-3 could be detected by ELISA in the mealybugs found at Beluso 1 in 1994 and 1995, and this prompted us to carry out a controlled assay of grapevine leafroll transmission by *Planococcus citri*. The results of the laboratory transmission test show that this mealybug can indeed be a vector of GLRaV-3; however, the time elapsed between transmission of the virus to the test plants and its detection by ELISA was longer than reported by other authors for *Planococcus ficus* and *Pseudococcus longispinus* (34,36). This is the first reported evidence that *Planococcus citri* can act as a vector of GLRaV-3 both in the field and in controlled conditions; previously, it was found to be a vector of GVA in controlled conditions (1). In Australia and New Zealand vineyards with vines trained on vertical trellises, the transmission of GLRaV-3 by slow-moving vectors has been suggested, due to observed aggregation of affected vines within rows (24, 26). In contrast, in Albariño there is no reason for the virus to be transmitted preferentially along either rows or columns, because the vines are trained in a continuous, horizontal trellis; the shapes and sizes of the foci in the vineyards examined in this work suggest no such preference.

The known and suspected *Pseudococcidae* vectors of GLRaV-3 have a wide range of herbaceous and woody crops hosts, are common in Mediterranean countries, and have been reported in most regions of Spain (9). As in the colder grapevine-growing regions of Central Europe, mealybugs are not common in the humid north-western coastal region of Spain (Galicia), where observation of their presence in vineyards is furthermore hindered by the height of the canopy and the elimination of sooty molds (which generally indicate the presence of mealybugs) by systematic spraying for downy mildew (*Plasmopara viticola* (Berk. & M. A. Curtis) Berl. & de Toni in Sacc.) and botrytis (*Botrytis cinerea* Pers.:Fr.). They have not been considered to be an important pest and have not been subjected to any pest control measures (29). In the New Zealand vineyard where the only previous well-documented case of fast field spread of GLRaV-3 over several years occurred (26), there was heavy infestation by mealybugs. However, our findings suggest that in Galicia large populations of *Planococcus citri* are not necessary for field transmission and this may be the case for other reports on the natural spread of grapevine leafroll where no vectors have been identified (10,15,24, 27,37). Therefore, measures must be taken against mealybugs as soon as initial foci are detected, even in regions where they have not hitherto been regarded as a problem.

In conclusion, we have carried out a comprehensive study of the spread of

grapevine leafroll disease in Galician vineyards. In addition to detecting field contamination of vines, as reported in wine-growing regions of Italy (34), South Africa (18,19), and Australia (24), we also found that in several successive years the disease spread in the field at a rate similar to that reported by Jordan et al. in New Zealand (26), and we identified *Planococcus citri* GLRaV-3 carriers that proved to be vectors of GLRaV-3 in controlled conditions. Aggregation analysis of the spatial distribution of infected plants in vineyards showed that field transmission of grapevine leafroll may be common. We conclude that, besides the use of virus-free propagation material, control strategies that target mealybugs should be recommended.

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