SHORT COMMUNICATION

SURVEY OF GRAPEVINE VIRUSES IN CHILE

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SUMMARY

Grapevines from six Chilean regions were surveyed for virus diseases and tested for the presence of the most important viruses. ELISA testing of 2535 samples and confirmatory RT-PCR of some ELISA-negative samples from symptomatic and symptomless vines gave the following infection rates: 6.36% for *Grapevine fanleaf virus* (GFLV); 4.67% for *Grapevine leafroll associated virus 1* (GLRaV-1); 16.05% for *Grapevine leafroll associated virus 2* (GLRaV-2); 6.41% for *Grapevine leafroll associated virus 3* (GLRaV-3); 0.26% for *Grapevine leafroll associated virus 7* (GLRaV-7); 14.99% for *Grapevine fleck virus* (GFkV); 5.57% for *Grapevine virus A* (GVA) and 0.78% for *Grapevine virus B* (GVB). *Strawberry latent ringspot virus* (SLRSV) *Tomato ringspot virus* (ToRSV) and *Arabis mosaic virus* (ArMV) were not detected. Overall infection in the surveyed Chilean grapevines, considering ELISA and RT-PCR, was 32.35%. Virus infection ratio obtained from ELISA analysis in the six regions, varied between 21.19% (Región Metropolitana) and 74.26% (Coquimbo). RT-PCR was used for detection of the Red Globe strain of GLRaV-2 and *Grapevine rupestris stem pitting associated virus* (GRSPaV), and to confirm and extend ELISA results. GVB, GFkV, GRSPaV, GLRaV-2 RG and GLRaV-7 are new records for Chile.

Key words: Chile, Grapevine viruses, detection, ELISA, RT-PCR.

In Chile, grapevines represent one of the most valuable crops, being extensively grown between Atacama (III) and Maule (VII) regions (Fig. 1). The main wine grape varieties are Cabernet Sauvignon, Merlot, Carménère, Chardonnay, Sauvignon blanc, Syrah and Pinot noir, whereas the prevailing table grape cultivars are Thompson Seedless, Flame Seedless, Red Globe, Crimson Seedless and Superior.

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Between 2002 and 2007, a number of vineyards of different size were repeatedly visited to assess their sanitary status, from austral hemisphere late spring until autumn (October to May) in the following regions: Atacama (III) and Coquimbo (IV) in the north, Valparaiso (V) and Metropolitana de Santiago (RM) in the center, Libertador General Bernardo O'Higgins (VI) and Maule (VII) in the south of the country (Fig. 1).

A total of 2535 wine and table grape vines were sampled during the whole survey period, their geographical position was established by the GPS system and the precise coordinates of sampled plants within the vineyard were recorded to facilitate identification if further sampling were required.

Samples were collected in autumn (April, May and June), primarily from vines that showed virus symptoms but also from apparently symptomless plants. Phloem scrapings from mature dormant canes were used for testing, to bypass the limits posed by the seasonal variation of virus concentration (Kolber *et al*., 1985; Monis and Bestwick, 1997).

Samples were tested for the presence of viruses previously reported from Chile, i.e. *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll associated virus 1, 2 and 3* (GLRaV-1, -2 and -3), *Tomato ringspot virus* (ToRSV), *Grapevine virus A* (GVA), *Arabis mosaic virus* (ArMV), *Strawberry latent ringspot virus* (SLRSV) (Cereceda and Auger, 1979; Auger *et al*., 1994; Herrera and Madariaga, 1994, 2001; Herrera, 1996) and for viruses hitherto unrecorded in the country, i.e. the Red Globe strain of GLRaV-2 , [formerly Grapevine rootstock stem lesion-associated virus (Uyemoto and Rowhani, 2001)], *Grapevine fleck virus* (GFkV), *Grapevine virus B* (GVB), and *Grapevine rupestris stem pitting-associated virus* (GRSPaV). In 2003-2004, *Grapevine leafroll associated virus 7* (GLRaV-7) was included in the testing.

Not all samples were analyzed for all the above listed agents for, in certain cases, the range of viruses to be tested for was suggested by the symptoms shown by the vines.

Detection of GLRaV-1, -2, -3, GFLV, GFkV, GVA, GVB, ToRSV and ArMV was by ELISA (Clark and Adams, 1977) and complemented by RT-PCR, while detection of GRSPaV and GLRaV-2-RG was exclusively

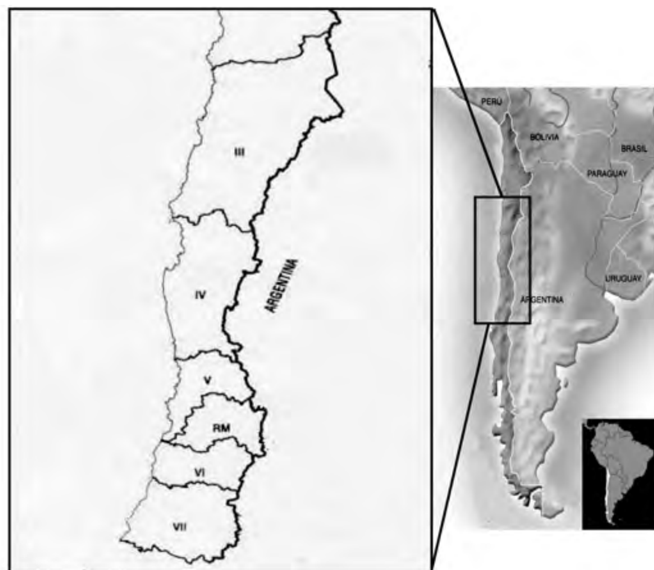

Fig. 1. Map of Chile showing the regions where grapevines were surveyed for the presence of the most economically important viruses. III: Región Atacama; IV: Región Coquimbo; V: Región Valparaíso; VI: Región Libertador General Bernardo O'Higgins; VII: Región Maule; RM: Región Metropolitana.

by RT-PCR. GLRaV-7 was detected only by ELISA. Two ELISA commercial kits were used: (i) Agritest (Valenzano, Bari, Italy), DAS-ELISA for GLRaV-1, -2, -3, -7, GFLV, GVA, ArMV and DASI-ELISA for GFkV and GVB; (ii) Loewe (Munchen, Germany), DAS-ELISA for ToRSV and SLRSV.

Testing was done following the manufacturers' instructions except that the minimal absorbance value considered for a positive reaction was two rather than three times that of the healthy control. This limit was arbitrarily set to increase the chance of detecting mild infections. Questionable and weak reactions were, in any case, verified by RT-PCR.

Total nucleic acids (TNA) extraction was by the silica capture method (MacKenzie *et al.*, 1997; Malinovski, 1997). TNA aliquots were primed with DNA random hexanucleotides (Roche, Basel, Switzerland) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, Madison, Wisconsin, USA). Target-specific primers were C995r/H587f, H28/BB_O and LC1/LC2 for GVA, GVB and GLRaV-3, respectively (Minafra and Hadidi, 1992) ; C3310/H2999 and C867/H428 for GFLV and ArMV (MacKenzie *et al.* 1997) ; V2dCPr1/V2dCPf2 for GLRaV-2 (Bertazzon and Angelini, 2004); GFkV-L630/GFkV-U279 for GFkV (Shi *et al.*, 2000); U1/D1 for ToRSV (Griesbach *et al.*, 1995); 49d/48d for GRSPaV (Zhang *et al.*, 1998) and RGH-SP777-R/RGJSP227-F for GLRaV-2-RG (Rowhani *et al.*, 2000).

To improve detection of GLRaV-1, the primers described by Habili *et al.* (1997) were modified based on the CP sequence of GenBank accession AF195822 (Fazeli and Rezaian, 2000), divergent nucleotides are at positions in bold/underlined: LR-1R - 5' - GTTACG-GCC**T**TTTGTTTA**C**TATGG - 3' antisense and LR-1F - 5' - CGACCCCTTTATTGTTTGAGTA**C**G - 3' sense primers.

Positive controls for ELISA and RT-PCR were fresh grapevine cortical scrapings or lyophilized leaf tissues from infected plants.

During this survey, virus symptoms were frequently seen in most of the vineyards. GFLV-infected vines were less vigorous than normal, showed distorted and mottled leaves, poor fruit setting and irregular ripening and reduced size of the berries. Leafroll symptoms were frequent, especially in red-berried varieties, whereas, despite of the presence of GVA, GVB and GRSPaV, symptoms of rugose wood were rare, likely because most of the old Chilean vineyards are established with self-rooted plants. Typical vein necrosis symptoms, observed on the rootstock 110R, were associated with the presence of GRSPaV.

Graft incompatibility was observed in cv. Red Globe on 1103P infected by GLRaV-2-RG.

Viruses detected by ELISA and their distribution and occurrence in the six regions surveyed are presented in Table 1 and 2, respectively. Of 2535 ELISA-tested vines, 740 were positive for at least one virus (29.19%). Results of RT-PCR assays are in Table 3 which, however, does not include the outcome of a number of tests that confirmed consistently the presence of GFLV, GLRaV-1, -2, -3, GVA, GFkV and GVB in ELISA-positive samples.

Of the three nepoviruses (GFLV, ArMV, ToRSV) looked for, GFLV had an average infection rate of 6.08% (Table 1). This virus occurred in all regions surveyed (Table 2) with a prevalence in the Region Metropolitana

Table 1. Results of virus testing by ELISA.

Virus[a]	Samples tested (No.)	Positive samples (No.)	Positive samples (%)
GFLV	2171	132	6.08
GLRaV-1	2181	89	4.08
GLRaV-2	2479	368	14.84
GLRaV-3	2170	134	6.17
GLRaV-7	387	1	0.26
GFkV	2254	324	14.37
GVA	2261	115	5.08
GVB	2064	15	0.72
ToRSV	908	0	0
ArMV	775	0	0
SLRSV	360	0	0
Overall infection	2535	740	29.19

[a] GFLV = *Grapevine fanleaf virus*; GLRaV-1 to -7 = Grapevine leafroll-associated viruses; GFkV = *Grapevine fleck virus*; GVA = *Grapevine virus A*; GVB = *Grapevine virus B* ; ToRSV = *Tomato ringspot virus*; ArMV = *Arabis mosaic virus*; SLRSV = *Strawberry latent ringspot virus.*

Table 2. Regional distribution of the seven most important viruses detected in Chile based on ELISA results.

Region	Viruses[a]							Overall infection[b]
	GFLV	GLRaV-1	GLRaV-2	GLRaV-3	GFkV	GVA	GVB	
Atacama	2/40 (5.00%)	2/40 (5.00%)	13/40 (32.50%)	1/40 (2.50%)	3/26 (11.54%)	4/40 (10.00%)	0/26 (0%)	19/40 (47.50%)
Coquimbo	10/136 (7.35%)	17/136 (12.50%)	59/136 (43.38%)	19/136 (13.97%)	48/136 (35.29%)	34/136 (25.00%)	6/136 (4.41%)	101/136 (74.26%)
Valparaíso	15/343 (4.37%)	25/358 (6.98%)	104/380 (27.37%)	30/358 (8.38%)	82/380 (21.58%)	37/371 (9.97%)	8/361 (2.21%)	155/384 (40.36%)
Metropolitana	82/884 (9.27%)	14/882 (1.58%)	108/1137 (9.49%)	40/881 (4.54%)	82/1063 (7.91%)	14/1068 (1.31%)	0/991 (0%)	249/1175 (21.19%)
Libertador General Bernardo O'Higgins	22/673 (3.27%)	24/676 (3.55%)	66/697 (9.72%)	36/667 (5.39%)	89/560 (15.89%)	18/558 (3.22%)	1/461 (0,21%)	179/705 (25.39%)
Maule	1/95 (1.05%)	7/89 (7.86%)	18/89 (20.22%)	8/89 (8.99%)	20/89 (22.47%)	8/89 (8.99%)	0/89 (0%)	37/95 (38.95%)

[a] Number and percentage of positives against all analyzed samples for each virus.
[b] Total number of positive samples for at least one virus against all analyzed samples for each region.

de Santiago, where the highest infections rate was detected (9.27%), followed by Coquimbo (7.35%), then, in decreasing order, by Atacama (5.00%), Valparaiso (4.37%), Libertador General Bernardo O'Higgins (3.27%) and Maule (1.05%). The wide distribution of this virus probably reflects both the frequent soil infestation by *Xiphinema index* in all viticultural areas of the country (Aballay *et al.*, 1998) and the high GFLV inoculum reservoir represented by the traditional vineyards from the Central Zone. GFLV was detected in *X. index* by RT-PCR in Región Metropolitana (data not shown).

Because of the previously reported presence in Chile of ToRSV in grapevines and of *X. americanum sensu lato* (Lamberti *et al.*, 1988; Aballay *et al.*, 2001; Insunza *et al.*, 2001) a substantial presence of this virus was expected. This, however, was not the case, for only seven samples gave a weak and questionable ELISA reaction, which was not confirmed by RT-PCR. Furthermore, 213 samples ELISA-negative for ToRSV remained negative in RT-PCR assays (Table 3). Since these results did not confirm those of previous investigations (Herrera and Madariaga, 2001), the seven samples weakly ELISA-positive for ToRSV were re-assayed several times by ELISA and RT-PCR in different seasons, proving consistently free from this virus. This was taken as convincing evidence that no ToRSV was present in any of the 908 vines examined for this virus in our survey.

A comparable situation was encountered with ArMV, for only 2 of 775 vines tested by ELISA gave a reading close to the minimal level of absorbance value considered in this work, but the presence of this virus was not confirmed by RT-PCR, and it was not found in further 156 samples ELISA-negative for ArMV. It is therefore plausible to conclude that ArMV does not represent a problem for the Chilean grapevine industry, also because its nematode vector *Xiphinema diversicaudatum*, does not seem to occur in the country (Gonzales, 1970, 1984; Magunacelaya, 1996).

No evidence was obtained for the presence of the sadwavirus SLRSV, another *X. diversicaudatum*-transmitted pathogen, in any of the 360 vines assayed by ELISA.

Table 3. RT-PCR results of ELISA-negative samples.

Virus	Tested samples (No.)	Positive samples (No.)
GFLV	45	6
GLRaV-1	27	13
GLRaV-2	194	30
GLRaV-3	20	5
GFkV	91	14
GVA	154	11
GVB	129	1
ToRSV	220	0
ArMV	158	0
SLRSV	n.t.	n.t.

n.t.= not tested

ELISA detection of GLRaV-2 was 14.84%. RT-PCR was performed on 194 ELISA-negative samples, using universal GLRaV-2 primer set (Bertazzon and Angelini, 2004) and more than 15% were successfully amplified (Table 3).

As to the distribution of GLRaV-2 in the territory (Table 2), the highest infection level was found in Coquimbo (43.38%) followed by Atacama (32.5%), Valparaiso (27.37%), Maule (20.22%), Libertador General Bernardo O'Higgins (9.72%) and Región Metropolitana (9.49%).

Infections by GFkV accounted for 14.37% (Table 1), with a prevalence in Coquimbo (35.29%), followed by Maule (22.47%), Valparaiso (21.58%), Libertador General Bernardo O'Higgins (15.89%), Atacama (11.54%) and Region Metropolitana (7.91%).

GVA showed a moderate level of infection (5.08%) in the whole territory, its incidence being high in Coquimbo (25%), moderate in Atacama, Valparaiso and Maule (10, 9.97 and 8.99%, respectively) and little significant in Libertador General Bernardo O'Higgins (3.22%) and Región Metropolitana (1.31%).

A relatively low level of global infection by GLRaV-3 (6.17%) and GLRaV-1 (4.08%) was detected by serology. Coquimbo was the region with the highest infection levels (13.97 for GLRaV-3 and 12.50% for GLRaV-1) followed by Maule (8.99 for GLRaV-3 and 7.86% for GLRaV-1). Other regions had lower infection levels, not exceeding 8.38% (GLRaV-3) and 6.98% (GLRaV-1) (Table 2).

The single plant infected by GLRaV-7 out of 387 tested was in Coquimbo. This is not a surprising finding, for this virus is relatively rare being more common in eastern than in western Mediterranean (Digiaro *et al.,* 1999) and was only recently recorded from a few varieties in California (Morales and Monis, 2007).

A low number of plants infected by GVB were detected by ELISA, the global infection level being 0.72%, a figure confirmed by RT-PCR, since only one positive was found out of 129 ELISA-negative samples. GVB highest level of infection (4.41%) was in Coquimbo (Table 2).

As shown in Table 4, GRSPaV was detected in 57 of 236 vines (24.15%). Infections by GRSPaV in Chile most probably originate from propagating material because no natural vector is known, and the experimentally proven presence of the virus in the pollen of *Vitis rupestris* plants and its transmission through seeds (Rowhani *et al*., 2000; Lima *et al*., 2006) may not be significant in *V. vinifera*. GRSPaV alone induces mild or no rugose wood symptoms and some of its strains are latent in *V. rupestris* (Meng *et al*., 1998). Mixed infection with other viruses (e.g. GVA) may be required for rugose wood symptoms to occur (Bonfiglioli *et al*., 1998). This means that vines latently infected by GRSPaV may develop the disease if other viruses are transmitted via grafting or vectors (Gribaudo *et al*., 2006).

Table 4. RT-PCR results of the assay for GLRaV-2-RG and GRSPaV.

Virus [a]	Tested samples (No.)	Positive samples (No.)
GLRaV-2-RG	180	20
GRSPaV	236	57

[a] GRSPaV = *Grapevine rupestris stem pitting associated virus*; GLRaV-2-RG = *Grapevine leafroll-associated virus 2*, Red Globe strain.

To assay for GLRaV-2-RG specific primers were used, since no serological discrimination from GLRaV-2 type strain (GLRaV-2-TS) is possible. Of 72 samples positive for GLRaV-2-TS by ELISA, 13 proved to be GLRaV-2-RG, and of 108 samples ELISA-negative for GLRaV-2-TS, seven were positive for GLRaV-2-RG (Table 4). In four samples, both GLRaV-2 variants were present, indicating that mixed field infections are possible, but not frequent.

GLRaV-2-TS and its variant GLRaV-2-RG were shown to be responsible of graft-incompatibility in grapevines (Greif *et al*., 1995; Uyemoto *et al*., 2001), as determined by the type of rootstock (Pirolo *et al*., 2006). Rootstocks are normally used nowadays in Chile, especially for replants, which makes certification of rootstocks, in addition to scions, of paramount importance to prevent young vine decline problems in new plantings.

A severe disease condition was recently observed in Chilean vineyards of cv. Crimson Seedless, whose bunches were unmarketable because of the pale color and low sugar content. It was ascertained that diseased vines were infected by GLRaV-1, -2 and -3 (Digiaro *et al*., 2006). Further examples of mixed infections were registered in the course of the present survey, the most frequent association being between GLRaV-2 and GFkV. However, combinations of two, three, four and even five different viruses were found, involving practically all detected viruses.

The highest level of infection of Chilean vines was by GLRaV-2, closely followed by GFkV. Since both viruses are disseminated by propagating material (Martelli and Boudon Padieu, 2006), their widespread occurrence is taken as an indication that grape nurseries are not producing plants free from these viruses. A similar conclusion may be drawn with reference to GLRaV-1, GLRaV-3, GVA and GVB, whose primary inoculum originates from commercial stocks, but whose subsequent spread at a site is likely to be mediated by pseudococcid mealybugs like *Pseudococcus viburni*, *Ps. longispinus* and *Planococcus ficus* (Martelli and Boudon Padieu, 2006), which are known to occur in Chile (Gonzales and Volosky, 2006).

In conclusion, this survey has determined that seven viruses, i.e. GFLV, GLRaV-1, -2, -3, GVA, GFkV and GRSPaV, are most frequent in Chilean grapevines. All these viruses should be taken into consideration if a national certification program for the production and marketing of sanitarily improved grapevine propagative material will be implemented, as it appears highly desirable. GVB, although less represented in the field, is no less dangerous than the other viruses. Thus, its addition to the proscription list is recommended.

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