

## Sampling strategies for the detection of grapevine fanleaf virus and the grapevine strain of tomato ringspot virus

by

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### Strategien der Probenahme zur Feststellung des Grapevine fanleaf-Virus und des Tomato ringspot-Virus der Rebe

**Zusammenfassung:** Das Ziel der vorliegenden Untersuchung war es, den Einfluß der Jahreszeit, des Wirtsgenotyps, des Virusisolates und des Pflanzengewebes auf den Nachweis zweier Nepoviren durch ELISA zu bestimmen. Das Virus der Reisigkrankheit (GFLV) und der Erreger der Adernvergilbung bei Reben, das Tomatenringfleckenvirus (TomRSV), können durch Enzyme-linked immunosorbent assay (ELISA) leicht nachgewiesen werden. Drei serologisch identische Isolate von GFLV (verantwortlich für Fächerblättrigkeit, Adernaufhellung und Panaschüre) wurden an einer Sorte von *Vitis rupestris* SCHEELE und drei Sorten von *V. vinifera* L., TomRSV wurde an *V. vinifera* cv. Carignane getestet. Hierbei wurden Triebspitzen, ausgewachsene Blätter und Kambiumschabel geprüft. Von dormantem GFLV-infiziertem Rebholz wurden untersucht: Sägemehl, Kambiumschabel, Ruheknospen, sowie aus dem Holz entstandene Triebe, Wurzeln und Kallusgewebe. Die Ergebnisse des ELISA-Tests wurden weder durch die Testrebensorte noch durch das Virusisolat beeinflußt. Mittels ELISA konnten jedoch jahreszeitlich bedingte Unterschiede im GFLV-Titer nachgewiesen werden. Bei den Triebspitzen sanken die Werte von einem Maximum > 4.00 OD<sub>450 nm</sub> im Mai auf ein Minimum von 0.05 OD<sub>450 nm</sub> im September. Auch ausgewachsene Blätter zeigten im Mai die höchsten Werte, die dann rasch auf ein relativ niedriges und gleichbleibendes Niveau zurückgingen. Die ELISA-Werte des Kambiumschabels blieben während der ganzen Vegetationsperiode relativ hoch und stabil. Beim Test von GFLV-infiziertem dormantem Schnittholz zeigten die induzierten, wachsenden Gewebe die höchsten ELISA-Werte. Die ELISA-Werte für TomRSV blieben während der Vegetationsperiode relativ konstant, und es gab kaum Unterschiede zwischen Triebspitzen, ausgewachsenen Blättern und Kambiumschabel.

**Key words:** nepovirus, GFLV, TomRSV, virus isolate, host genotype, sample tissue, season, serology, ELISA, detection, method.

### Introduction

Fanleaf degeneration is an economically important disease that confronts grape growers worldwide. It is caused by grapevine fanleaf virus (GFLV), a member of the nepovirus group, transmitted in the soil by the longidorid nematodes *Xiphinema index* THORNE and ALLEN and *X. italiae* MEYL. (HEWITT *et al.* 1958). GFLV consists of three known isolates that are antigenically uniform, but which produce different types of symptoms (fanleaf deformation, vein-banding and yellow mosaic) on the foliage of different grapevine cultivars (HEWITT *et al.* 1970; MARTELLI and SAVINO 1988). Another important nepovirus is the grapevine strain of tomato ringspot virus (TomRSV), the causal agent of grapevine yellow vein, transmitted by the dagger nematodes, *X. americanum* COBB, *X. californicum* LAMBERTI and BLEVE-ZACHEO and *X. rivesi* DALMASSO (GONSALVES 1988). TomRSV is found throughout the United States and causes serious grapevine disease in the northeastern part of the country.

Detection and identification of GFLV and TomRSV is important, particularly in regards to testing of new selections, propagation stock, nursery materials, vineyards

registered in clean-stock certification programs and grapevines in importation and distribution programs. Various methods exist for detection of these viruses including bioassay on indicator hosts and serological tests (UYEMOTO *et al.* 1976; GONSALVES 1979; SHANMUGANATHAN and FLETCHER 1982). A sensitive serodiagnostic test, enzyme-linked immunosorbent assay (ELISA), has been used to detect a wide variety of different viruses in many crops including GFLV and TomRSV in grapevines. In order to effectively use ELISA, information on virus titer in different host tissues taken over the course of the entire year is needed. Only with this information can routine ELISA testing procedures be developed for the detection of GFLV and TomRSV. Although some preliminary work has been done in this area (WALTER *et al.* 1984; MONETTE 1985; SHANMUGANATHAN and FLETCHER 1982), it is essential to assess sampling strategies and ELISA reliability in each grape growing region. Clean-stock and certification programs and importation and distribution programs would greatly benefit if a year-round testing protocol was established and tailored for use in specific regions. The objectives of this investigation were to develop sampling strategies to optimize ELISA detection of GFLV and TomRSV in growing and dormant plant material.

### Materials and methods

The influence of tissue type and seasonal influence on virus detection were examined using the following isolates (Davis grapevine isolate numbers are included in parentheses): fanleaf deformation in *Vitis rupestris* SCHEELÉ cv. St. George (GFLV 100) and *V. vinifera* L. cv. French Colombard (GFLV 103); vein-banding in St. George (GFLV 101) and *V. vinifera* cv. Gray Riesling (GFLV 104); and yellow mosaic in St. George (GFLV 102) and *V. vinifera* cv. Grand Noir (GFLV 106). The source of TomRSV was an infected vine of *V. vinifera* cv. Carignane (TomRSV 100). All of the above isolates were taken from the University of California, Department of Plant Pathology virus collection. These vines have been virus-infected for 16 years and the infections were therefore considered to be systemic.

Differences in virus titer among seven dormant cane sample tissues were examined in a *V. vinifera* cv. Cabernet Sauvignon grapevine known to be infected with a vein-banding isolate of GFLV (GFLV 108) from the Napa Valley, California for 11 years. Dormant canes from healthy vines were used as controls.

Antiserum for GFLV and TomRSV were prepared at the University of California, Davis. Because the three isolates of GFLV are serologically indistinguishable (HEWITT *et al.* 1970), a single antiserum prepared against fanleaf deformation was used for testing. The above TomRSV was purified from the above infected Carignane vine and antiserum was produced against this isolate. This antiserum reacts poorly against *Prunus* isolates of TomRSV.

### Detection of GFLV and TomRSV during the growing season

The following sample tissues were examined for GFLV and TomRSV during the growing season: shoot tips and young leaves, mature leaves and cambial scrapings from mature canes. A total of 0.5 g of tissue was randomly selected from five places around a vine for each of the sample tissue types. Cambial scrapings were taken by removing any shedding bark, cutting through the phloem, cambium and young xylem and removing tissue strips. Sampling was performed once a month from May until October in 1989 and twice a month in 1990, with the exception of the cambial scrapings which were first taken in July. Tissue samples were ground and prepared for ELISA

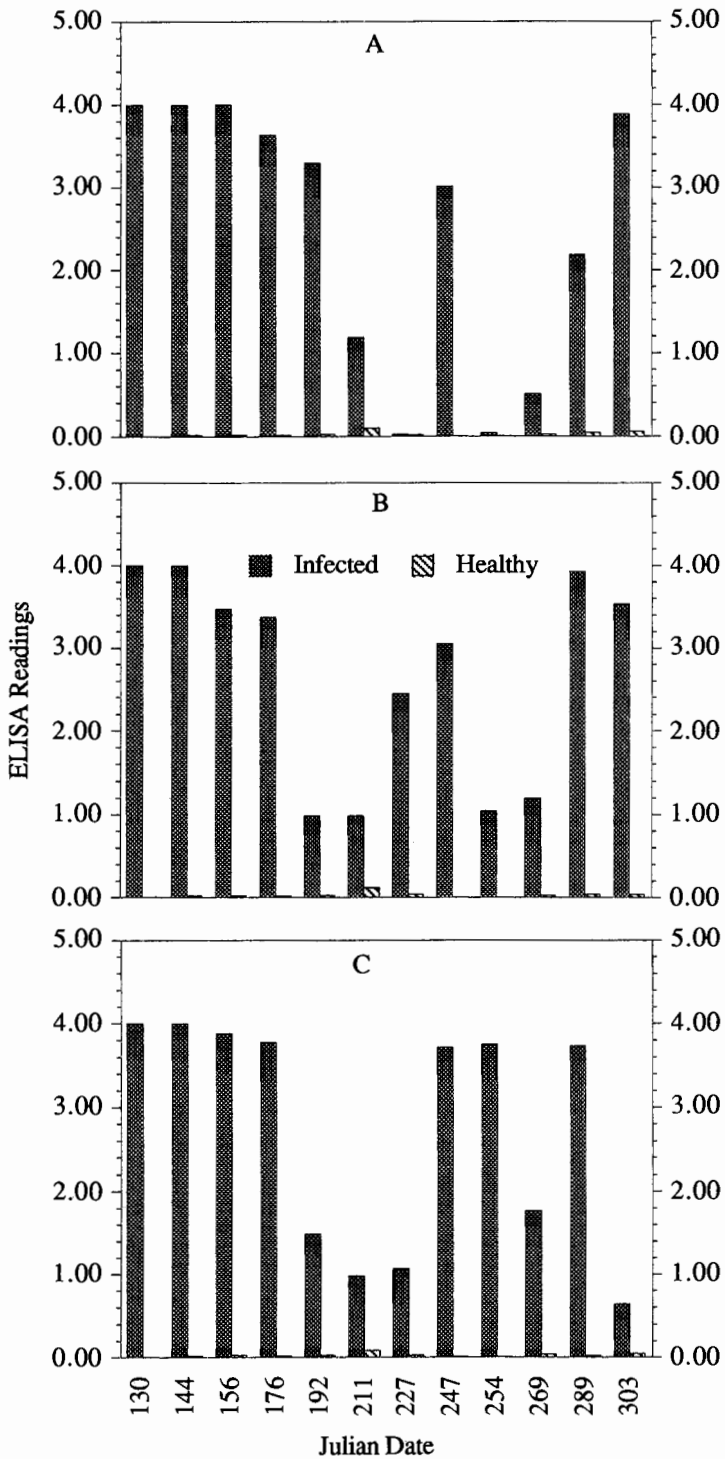
with a Brinkman Polytron homogenizer in a 1/10 w/v dilution of extraction buffer (1.59 g  $\text{Na}_2\text{CO}_3$ , 2.93 g  $\text{NaHCO}_3$  and 0.2 g  $\text{NaN}_3$  per l, pH 9.6 containing 2 % w/v PVP 40, 0.2 % w/v bovine serum albumin (BSA) and 0.05 % Tween 20).

A modification of F(ab')<sub>2</sub> ELISA (ROWHANI 1992) was used to examine the seasonal differences in GFLV and TomRSV titers. Antibody (Ab) was purified for F(ab')<sub>2</sub> ELISA on a protein A-sepharose column (Sigma Chemical Co.). 1–3 ml of antiserum was passed through the bed (previously adjusted to pH 7.3 with 0.02 M sodium phosphate buffer), permitting the binding of Ab onto the sepharose beads. The column was then washed with 200 ml of the 0.02 M sodium phosphate buffer. Elution of Ab was with 0.1 M glycine buffer, pH 3.0. 1 ml fractions were collected, immediately neutralized with 40  $\mu\text{l}$  of 2 M Tris-HCl pH 8.5 and fractions were scanned at 280 nm. The fractions containing purified Ab were pooled, mixed with glycerol (1:1, v/v) and stored at  $-20^\circ\text{C}$ . The F(ab')<sub>2</sub> fragment was prepared by a modification of the method of BARBARA and CLARK (1982). 5 mg of purified Ab in 0.4 ml was adjusted to pH 4.5 with 0.1 ml of 0.5 M sodium acetate buffer, pH 4.5, then 50  $\mu\text{l}$  of 1 mg/ml pepsin in 0.1 M sodium acetate pH 4.5 (Sigma Chemical Co.) was incubated with Ab at  $37^\circ\text{C}$  for 16–20 h. Prior to loading onto a second protein A-sepharose column, the pH of the solution was raised to 8.0–8.4 with a few crystals of Tris. After discarding the initial bed volume, a few ml of 0.02 M sodium phosphate buffer pH 7.3 was layered onto the column and 1 ml fractions were collected. F(ab')<sub>2</sub> fractions were detected by the absorption at 280 nm, as described above. The concentration of F(ab')<sub>2</sub> was adjusted to 1 mg/ml and solutions stored at  $-20^\circ\text{C}$  with glycerol (1:1).

The F(ab')<sub>2</sub> method of BARBARA and CLARK (1982) was adapted for use with GFLV and TomRSV ELISA. The polystyrene microtiter plates (Costar, flat bottom) were coated with 200  $\mu\text{l}$  of virus-specific F(ab')<sub>2</sub> at 0.5  $\mu\text{g}/\text{ml}$  concentration in coating buffer and pre-incubated. The plates were washed in 0.01 M PBST (8.0 g NaCl, 0.2 g  $\text{KH}_2\text{PO}_4$ , 1.15 g  $\text{Na}_2\text{HPO}_4$ , 0.2 g KCl and 0.2 g  $\text{NaN}_3$  per l of  $\text{H}_2\text{O}$ , pH 7.4 containing 0.05 % v/v Tween 20) and 200  $\mu\text{l}$  of sample extract loaded into each well and incubated. Following incubation and washing, the plates were loaded with 200  $\mu\text{l}$  per well of purified Ab (at 1  $\mu\text{g}/\text{ml}$ ) mixed with protein A-peroxidase conjugate (at 0.35  $\mu\text{g}/\text{ml}$ ) in 0.01 M PBST containing 2 % PVP 40 and 0.2 % BSA and then incubated. All incubations were for 1.5 h at  $37^\circ\text{C}$  except for the sample incubation which was overnight at  $4^\circ\text{C}$ . After a final wash, the presence of the immobilized enzyme conjugates was quantified by the hydrolysis of 200  $\mu\text{l}$  per well of o-phenylenediamine, 0.7 mg/ml in substrate buffer (7.3 g  $\text{Na}_2\text{HPO}_4$ , 5.2 g citric acid and 0.4 ml of 30 %  $\text{H}_2\text{O}_2$  per l of  $\text{H}_2\text{O}$ , pH 5.0). The plates were incubated in the dark for 30 min at room temperature and the color intensity measured with an ELISA plate reader (Emax, Molecular Devices) at 450 nm.

#### Detection of GFLV during the dormant season

Variability in GFLV titer was examined in the following dormant cane sample tissues: shoot tips forced from canes inserted in water-filled containers at room temperatures; shoot tips, roots and callus tissue forced from canes under callusing conditions ( $28^\circ\text{C}$ , moist and dark); and sawdust, cambial scrapings and dormant buds taken directly from the canes. Canes were harvested and tested during January of 1990 and 1991 to examine differences in GFLV titers from different dormant cane sampling tissues. Canes were also stored at  $1^\circ\text{C}$  for 1 year beginning in 1990 and compared to freshly harvested canes in 1991. Eight replicates, each with three 15 cm cane sections, were processed for the seven different sample tissues from the known infected canes and from the healthy controls. Each replicate was prepared for ELISA by bulking tissue pieces from the cane sections into a 1.0 g sample. The samples were ground with a Brinkman Polytron homogenizer in 1/10 w/v extraction buffer consisting of 0.2 M



PBST containing 2 % PVP 40 and 0.2 % BSA. The presented ELISA absorbance data are averages of the eight replicates for each sample tissue.

The double-antibody sandwich (DAS) ELISA technique of CLARK and ADAMS (1977) was used to test dormant sampling tissues for the presence of GFLV. Each sample was replicated in three wells on Dynatech Immulon II flat bottomed ELISA plates. The incubation times for the DAS ELISA were 5 h at 37 °C for coating, 18–20 h at 4 °C for sample and 5 h at 33 °C for conjugate. Concentrations of reagents were as follows: 1 µg of GFLV-specific gamma globulin per ml of coating buffer; 1/1000 dilution of alkaline phosphatase bound conjugate in conjugate buffer, and 0.8 mg of p-nitrophenyl phosphate (Sigma Chemical Co.) per ml of substrate buffer. The color intensity was evaluated after 45 min with an ELISA plate reader at 405 nm.

## Results

### Detection of GFLV and TomRSV during the growing season

The data in Fig. 1 represent the average ELISA values of sample replicates (young leaves and shoot tips from St. George) from each GFLV isolate tested in 1990. ELISA absorbance values indicate that the virus titer was high in plant tissue samples when tested early in the growing season (from budbreak until June). It dropped rapidly in the leaf tissue and by July the titer was much lower when compared to prior months. When the sample material consisting of mature leaves was tested similar results were observed. The results also indicate that GFLV titer in leaf tissue fluctuated during the summer and early fall. Similar patterns occurred with all virus isolates, in all tested cultivars and in both 1989 and 1990. ELISA results of the cambial scrapings from lignified canes (Fig. 2) were more consistent over the testing period when compared to leaf and shoot tip samples, although peaks in virus titer were higher from shoot tip and young leaf samples.

The seasonal profile of changes in ELISA absorbance values for TomRSV (Fig. 3) shows that there was little difference between the sample tissues tested. In general TomRSV titer was lower at the beginning of the season, began to increase slightly in July and then remained relatively constant from August until October.

### Detection of GFLV during the dormant season

The results of the experiments to determine the variability of GFLV ELISA readings from dormant cane sampling tissues are shown in Fig. 4, presenting data from 1990, 1991 and from canes stored at 1 °C for 1 year and tested in 1991. Both fresh and stored dormant cane tissue produced ELISA readings that were considered infected. In general, samples from non-active tissue (cambial scrapings, sawdust and dormant buds) had lower levels of GFLV. When tissue was induced to grow from these dormant canes (callus, shoots induced under callusing conditions or shoots forced in water) it

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Fig. 1: 1990 ELISA test results for young leaf and shoot tip samples collected randomly from vines of *V. rupestris* cv. St. George infected with different isolates of GFLV: A) fanleaf deformation, B) vein-banding, C) yellow mosaic. The data represent average absorbance values at OD<sub>450 nm</sub> of duplicate wells.

ELISA-Werte junger Blätter und Triebspitzen von *V. rupestris* St. George (1990). Zufallsproben von Reben, die mit verschiedenen GFLV-Isolaten infiziert worden waren: A) Fächerblättrigkeit, B) Adernaufhellung, C) Panaschüre. Mittelwerte der Extinktion bei OD<sub>450 nm</sub> aus Doppelbestimmungen.

had much higher levels of GFLV, presumably because of the relationship between metabolic activity and virus replication. However, dormant buds taken from the 1990

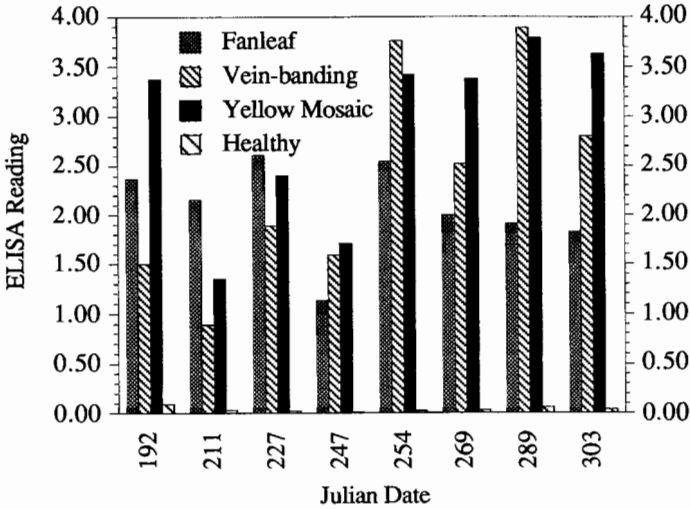


Fig. 2: 1990 ELISA test results for cambial scraping samples collected randomly from vines of *V. rupestris* cv. St. George infected with different isolates of GFLV (fanleaf deformation, vein-banding and yellow mosaic). The data represent average absorbance values at OD<sub>450 nm</sub> of duplicate wells.

ELISA-Werte des Kambiumschabsels von *V. rupestris* St. George (1990). Zufallsproben von Reben, die mit verschiedenen GFLV-Isolaten infiziert worden waren (Fächerblättrigkeit, Adernaufhellung, Panaschüre). Mittelwerte der Extinktion bei OD<sub>450 nm</sub> aus Doppelbestimmungen.

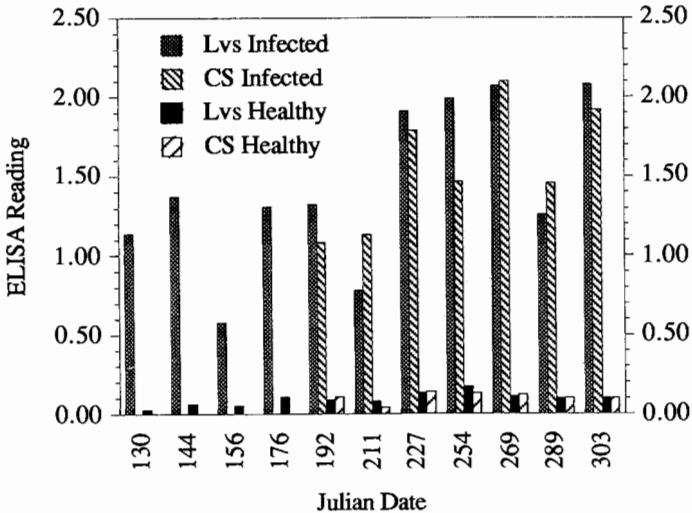


Fig. 3: 1990 ELISA test results for young leaf and shoot tip samples (Lvs) and cambial scrapings (CS) taken at random from *V. vinifera* cv. Carignane infected with TomRSV. The data represent average absorbance values at OD<sub>450 nm</sub> of duplicate wells.

ELISA-Werte junger Blätter und Triebspitzen (Lvs) sowie des Kambiumschabsels (CS) von *V. vinifera* cv. Carignane (1990). Zufallsproben von Reben, die mit TomRSV infiziert worden waren. Mittelwerte der Extinktion bei OD<sub>450 nm</sub> aus Doppelbestimmungen.

GFLV-infected canes produced high ELISA readings, not seen in 1991 from fresh material or from the 1990 material that had been stored for 1 year. There was little variation in ELISA readings from healthy tissues and only the 1991 healthy tissues were included in Fig. 4. None of the ELISA readings for any healthy dormant cane tissue were above 0.025 OD<sub>405 nm</sub>.

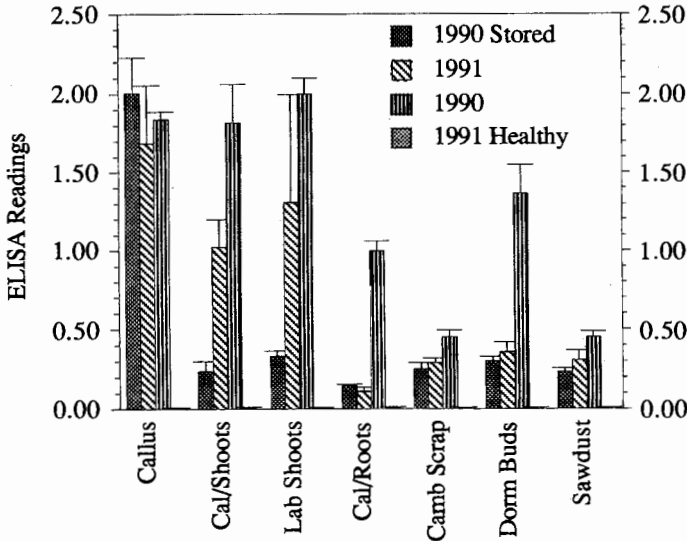


Fig. 4: 1990 and 1991 ELISA test results for various sampling tissues from GFLV-infected dormant canes of *V. vinifera* cv. Cabernet Sauvignon with a vein-banding isolate from the Napa Valley, California. The results include tissue from dormant canes tested in 1990, dormant canes tested in 1991, dormant canes stored at 1 °C for a year and tested in 1991, and dormant canes from healthy vines tested in 1991. Tissues sampled include: callus, shoots, shoots (Cal/Shoots) and roots (Cal/Roots) induced from canes under callusing conditions (28 °C, moist, dark); shoots induced from canes in water at room temperature (Lab Shoots); cambial scrapings (Camp Scrap); dormant buds (Dorm Buds); and sawdust. Each tissue sample type was replicated eight times and each replicate was put in three wells on an ELISA plate; the columns represent the average absorbance values at OD<sub>450 nm</sub>. Standard error bars are included for comparison.

ELISA-Werte verschiedener Gewebeproben aus dormanten Tragruten von *V. vinifera* cv. Cabernet Sauvignon (1990 und 1991); die Reben waren mit einem Adernauffhellung verursachenden Isolat von GFLV infiziert worden. Das Rebholz wurde 1990 sowie 1991 geerntet und getestet, ferner 1 Jahr lang gelagert und 1991 getestet; außerdem wurde 1991 Holz von gesunden Reben getestet. Die Abkürzungen der Gewebeproben bedeuten: Cal/Shoots = Kallus und Triebe, Cal/Roots = Kallus und Wurzeln aus Rebholz bei kallusinduzierenden Bedingungen (28 °C, feucht, dunkel) getrieben; Lab Shoots = Triebe aus Rebholz bei Zimmertemperatur entstanden; Camb Scrap = Kambiumschabe; Dorm Buds = Ruheknospen; Sawdust = Sägemehl. Jede Gewebeart ist mit acht Wiederholungen vertreten, je Wiederholung wurden auf der ELISA-Platte drei Bestimmungen vorgenommen. Die Säulen zeigen die Mittelwerte der Extinktion bei OD<sub>450 nm</sub>. Die Balken geben die Standardabweichungen an.

The GFLV-infected canes that had been in cold storage for 1 year produced high ELISA readings from callus tissue. Roots and shoots induced from the stored wood under callusing conditions were weak and produced relatively low ELISA readings, perhaps due to the relative abundance of callus tissue and its effect on the vigor of roots and shoots. Shoots induced from these dormant canes in the laboratory also had relatively low GFLV levels and appeared weak.

### Discussion

Detection of GFLV by ELISA is variable over the course of the growing season, thus it is important to choose the tissue that will give the optimal reading at a given time of the year. Variability in virus titer in a particular tissue over the course of the season is likely the result of tissue age and/or environmental conditions. Young leaves and shoot tips from GFLV-infected plants gave the highest ELISA absorbance values from bud break until July. Once shoot tips stopped growing GFLV titer dropped, sometimes to barely detectable levels as in the case of the fanleaf deformation isolate in mid-August and mid-September. Since the tested plants were GFLV-infected St. George, a male plant, crop load was not a factor, although inadequate irrigation coupled with high temperatures may have contributed to the lower levels of GFLV. However, none of the other vines experienced such sharp declines in the levels of GFLV. GFLV levels fluctuated in the shoot tip and young leaf samples throughout the summer in each of the isolates, which might be explained by flushes of growth and quiescence brought about by irrigation scheduling and temperatures. GFLV was detected in mature leaves throughout the summer months, although at low, and occasionally at barely detectable levels. The authors have experienced difficulties with ELISA detection of GFLV in a range of cultivars once shoot tips have stopped growing (data not shown).

Detection of GFLV is possible from summer into the dormant season with cambial scrapings from lignified canes. Cambial scrapings gave consistent and reliable results, although GFLV titers were considerably lower than in shoot tips and young leaves during the period of rapid vine growth in the spring.

TomRSV titer in different tissues from an infected vine was quite constant and little fluctuation was observed over the course of the growing season. Therefore, it appears that ELISA testing for TomRSV can be conducted throughout the growing season with any aerial part of the plant and is limited only by tissue availability.

Dormant season sampling for GFLV is convenient since canes can be stored for lengthy periods before processing and a variety of tissues can be used for ELISA. We processed two types of cane tissue: dormant tissue such as the buds, sawdust and cambial scrapings; and active tissue such as the induced shoots, roots and callus. Tissue that is actively growing produces higher ELISA readings probably because it provides a better environment for GFLV replication. In addition, actively growing tissue is succulent and more easily processed in the homogenizer. Tissue that was dormant or less active did not produce high ELISA values. However, all of the tissues produced clearly positive reactions.

GFLV and TomRSV were easily detected with both DAS and F(ab')<sub>2</sub> ELISA. We tested a wide variety of sample tissues and all produced reliably positive reactions with the exception of shoot tips and leaves during the summer on St. George infected with the fanleaf deformation isolate of GFLV. Actively growing tissue sampled from growing vines or induced from dormant canes gave high ELISA readings and was more easily processed. We have also observed that both GFLV and TomRSV titers are very high in flowers and immature berries. Given the variability of the readings during the growing season and the dormant season, those sample tissues which give the highest ELISA readings should be used to allow greater reliability and confidence in the ELISA results when the virus status of a grapevine is unknown.



### Summary

This study was conducted to determine the influence of season, host genotype, virus isolate and sample tissue on ELISA detection of the two nepoviruses. Enzyme-linked immunosorbent assay (ELISA) readily detects grapevine fanleaf virus (GFLV) and the causal agent of grapevine yellow vein, tomato ringspot virus (TomRSV) in infected grapevines. Three serologically identical isolates of GFLV (fanleaf deformation, vein-banding and yellow mosaic) were examined in one cultivar of *Vitis rupestris* SCHEELÉ and in three cultivars of *V. vinifera* L. TomRSV was examined in *V. vinifera* cv. Carignane. The tissues tested included: shoot tips, mature leaves and cambial scrapings. The following tissues taken from GFLV-infected dormant canes were also tested: sawdust, cambial scrapings, dormant buds, and induced shoots, roots and callus. There were no differences in GFLV ELISA results when different cultivars and virus isolates were compared. However, seasonal differences in ELISA detection of GFLV were observed. Shoot tip values went from a high of  $>4.00$  OD<sub>450 nm</sub> in May to a low of 0.05 OD<sub>450 nm</sub> in September. Mature leaves also gave the highest values in May and rapidly decreased to relatively low and constant levels throughout the rest of the season. ELISA values from cambial scrapings were moderately high and relatively constant throughout the season. When GFLV-infected dormant canes were tested, induced actively growing tissues gave the highest ELISA values. TomRSV ELISA values were relatively constant over the season and shoot tips, mature leaves and cambial scrapings produced similar ELISA values.

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