Detection and Localization of Grapevine Leafroll Associated Closteroviruses in Greenhouse and Tissue Culture Grown Plants

JUDIT MONIS[1]* and RICHARD K. BESTWICK[2]

The best plant tissue and season for the detection of grapevine leafroll associated viruses (GLRaV) I, II, and III in greenhouse and tissue culture grown infected grapevines were investigated using ELISA. Samples near the bottom portion of actively growing stems and petioles had the highest concentration of virus. Old and symptomatic leaves had higher titers of virus than young leaves. Grapevine leafroll associated viruses I, II, and III antigens were distributed unevenly in infected tissue, although the highest titers of virus were generally found near the lower portion of the plant. The virus associated antigens were detected throughout the year (except early in the growing season) in basal stem and petiole samples, demonstrating that multiple samples should be taken from these tissues to unambiguously detect GLRaV. When explants from individual nodes were propagated *in vitro*, high virus titers were detected in every sample even when the concentration of virus in the original stem or leaf sample was low.

KEY WORDS: grapevine leafroll virus, closteroviruses

Grapevine leafroll disease (LD) is one of the most important diseases in grapevines and occurs wherever vines are grown (7). Grapevine leafroll is associated with undesirable viticultural effects which include reduced yield, delayed ripening, altered fruit pigmentation, and reduced accumulation of sugar (26). Symptoms include downward rolling and interveinal reddening of leaves on varieties with red colored fruit (5).

The etiology of LD is not clear. Although isometric (6) and potyvirus-like particles (25) were reported to be associated with LD, there is an agreement among researchers that only closterovirus-like particles are associated with LD (4,15). Because Koch's postulates have not been completed with the viruses associated with LD, the causal agents are referred to as grapevine leafroll associated viruses (GLRaV) (16). Several laboratories have isolated closterovirus-like particles and prepared monoclonal and polyclonal antibodies (3,10,11,13,14,28,29). The closteroviruses associated with leafroll disease are not serologically related (11,14,28). Furthermore, the particle and coat protein sizes are different for each of the characterized closteroviruses associated with LD (21,28).

Discrepancies found between enzyme linked immunosorbent assay (ELISA) and woody indicator tests have been reported (23). Work in our laboratory has shown that GLRaV I and III were detected in cultivars that had previously been determined to be virus free by biological indexing.

Our work has focused on the diagnosis of leafroll associated antigens in tissue culture and greenhouse grown grapevines using GLRaV I, II, and III monoclonal and polyclonal antibodies. Determination of the best plant tissue for the detection of these viruses was studied using the ELISA. Our results show that the distribution of GLRaV varies in actively growing and dormant material. Preliminary results of our work were previously reported (19).

Materials and Methods

Plant materials: Dormant cuttings from vines infected with GLRaV I, I + II, and III (several plants from the same source) were obtained from Drs. D. Golino and A. Rowhani, University of California at Davis (8). They were rooted and grown in a research greenhouse in Beaverton, Oregon. For *in vitro* propagation, 1-cm nodal sections of vegetatively growing stems were surface-sterilized and grown in the initiation and propagation media (22) under 16-hour light. The healthy plants used as negative controls were provided by Vinifera, Inc. Dormant branches (2 - 3 m) from symptomatic grapevine cultivars were obtained from Oregon and California vineyards. The description of plant material and virus isolates used in this study are found in Table 1.

Antibodies: GLRaV I monoclonal antibodies (10) were purchased from BIOREBA AG (Basel, Switzerland), polyclonal GLRaV II, III, and monoclonal GLRaV III (3,13,14,27) were provided by Dr. D. Gonsalves (Cornell University, New York State Experimental Station, Geneva, NY).

Sample preparation: Leaf and stem samples were collected from actively growing greenhouse vines. Stem samples were collected from dormant vines grown either in the greenhouse or in the field. Tissues were ground with a tissue pulverizer (KLECO, Visalia, CA 93292) or a Polytron (Brinkmann Instruments, Westbury, NY 11590-0207) at a 1:5 and 1:10

[1,2]Research and Development Department, Agritope, Inc., 8505 SW Creekside Place, Beaverton, OR 97008.

*Corresponding author: phone (503) 641-6115, Fax (503) 643-2781, email: jmonis@epitope.com

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Table 1. Virus isolates and ELISA reactivity.

Isolate designation	Grapevine cultivar	ELISA reactivity	Source
GLRaV I + II	Unspecified field selection	GLRaV I, II	FPMS
GLRaV II	Cabernet Sauvignon	GLRaV I, II	FPMS
GLRaV III	Italia	GLRaV III	FPMS
LR 101	Thompson Seedless	GLRaV III	USDA
LR 102	Thompson Seedless	GLRaV I, II	USDA
LR 105	Teroldigo-1	GLRaV I, II, III	USDA
LR 109	Thompson Seedless	GLRaV III	USDA
FC/2	French Colombard	GLRaV I, II	FPMS[1]
CS/8	Cabernet Sauvignon	GLRaV III	FPMS[1]
PNW-2	Unspecified field selection	GLRaV I, II	Oregon Vineyard
Healthy	Couderc 3309	NR[2]	Vinifera, Inc.
Healthy	Riparia Gloire	NR	Vinifera, Inc.
Healthy	Kober 5BB	NR	Vinifera, Inc.

[1]Cultivars tested negative by traditional biological indexing

[2]NR= no reactivity

Table 2. Distribution of GLRaV I antigens in different greenhouse-grown grapevine tissues collected in June.

Sample position[1]	Relative ELISA reactivity[2] Nodes	Internodes	Petioles	Midribs	Leaf blade
1	2+	2+	4+	+	-
2	2+	2+	4+	2+	-
3	4+	4+	4+	+	-
4	3+	3+	4+	-	-
5	2+	2+	4+	ns	-
6	+	+	4+	4+	+
7	+	+	ns	ns	ns
8	+	+	4+	4+	+
9	2+	2+	4+	2+	-
10	+	+	4+	+	+
11	+	+	4+	+	-
12	2+	2+	4+	4+	-
13	3+	3+	4+	+	-
14	+	+	4+	+	-
15	2+	2+	+	+	-
16	+	+	+	+	-
17	+	+	+	+	-
18	-	-	-	-	-
19	-	-	-	-	-
20	+	+	-	-	-
21	+	+	-	-	-
22	4+	4+	-	-	-
23	2+	2+	-	-	-
24	+	+	+	+	+
25	ns	ns	-	-	-

[1]Sample position number one is the bottom-most node, internode, or leaf sample taken from an approximately 2.5 m vine.

[2]The cutoff value for a positive ELISA result (0.438) was determined to be two times the average A_{405} value of the healthy control. The sample readings were scored in relation to the ratio of the A_{405} value of each sample and the positive cutoff value. The following scores, (-), (+), (2+), (3+), (4+), were assigned to each treatment with a ratio of, less than 1 (no reactivity), 1 to 2.4, 2.5 to 3.4, 3.5 to 4.4, and above 4.5, respectively. ns, no sample.

(weight/volume) ratio with 0.5 *M* Tris-HCl pH 8.2, 143 m*M* NaCl, 1% polyethylene glycol (mw 8000), 2% polyvinyl pyrrolidone (mw 40 000), 0.05 % Tween 20. When necessary the ground samples were stored at -20C prior to ELISA.

Enzyme linked immuno sorbent assay (ELISA): The double antibody sandwich (DAS) ELISA was performed as described by Gugerli *et al.* (10) and Hu *et al.* (14). Plates were read after a two hour incubation with substrates with an Anthos 2001 ELISA reader at an optical density of 405 nm. The GLRaV II and III polyclonal antibodies were cross-absorbed with healthy grape leaf extracts prior to immunoglobulin purification (9). Purified immunoglobulins (GLRaV II polyclonal and GLRaV III monoclonal) were conjugated with alkaline phosphatase (12). The cutoff value for a positive ELISA result was determined to be two times the average A_{405} value of the healthy control. In some distribution experiments (Tables 2 and 3), the sample readings were scored in relation to the ratio of the A_{405} value of each sample and the positive cutoff value. The following scores, (-), (+), (2+), (3+), (4+), were assigned to each treatment with a ratio of, less than 1 (no reactivity), 1 to 2.4, 2.5 to 3.4, 3.5 to 4.4, and above 4.5, respectively.

Virus localization during the season - leaves and stems: Experiments were designed to determine if the virus antigen titers vary in different tissues or seasons in greenhouse grown grapevines. Samples from two to four replicate vines of GLRaV-infected vines were collected every month during 1994. Basal (mature) and apical (young) leaf blades, and petioles were analyzed from infected vines. Symptomatic leaf tissue was analyzed when available. Stem samples were collected from the bottom and top of the vines.

Virus titer distribution in sectioned grapevines - greenhouse, field, and tissue culture grown: The difference in virus titer between young and old tissue prompted us to perform a more rigorous sampling analysis of GLRaV-infected grapevine tissues to determine the distribution of GLRaV I, II, and III antigens in different grapevine tissue. The sampling was performed starting at the bottom of the vine (sample position number one). Leaf and stem samples were taken from all positions of replicate vines several times during the 1994 grapevine growing season. Due to the variable distribution of the virus antigens it was not practical to average the results obtained from replicate vines. For greenhouse-grown grapevines, samples were collected from actively growing vines and assayed for the presence of virus antigens using GLRaV I, II, and III antibodies. Stem tissues were cut in 1 to 2 cm consecutive sections including all nodes

Table 3. Distribution of GLRaV III antigens in different greenhouse grown grapevine tissues collected in June.

Sample position[1]	Relative ELISA reactivity[2] Nodes	Internodes	Petioles	Midribs	Leaf blade
1	2+	3+	ns	ns	ns
2	4+	4+	ns	ns	ns
3	2+	3+	ns	ns	ns
4	2+	3+	ns	ns	ns
5	+	4+	ns	ns	ns
6	2+	2+	ns	ns	ns
7	3+	3+	ns	ns	ns
8	3+	3+	ns	ns	ns
9	+	3+	ns	ns	ns
10	+	3+	ns	ns	ns
11	+	3+	ns	ns	ns
12	2+	4+	ns	ns	ns
13	2+	4+	ns	ns	ns
14	+	2+	2+	3+	-
15	+	+	+	+	-
16	+	2+	+	+	-
17	+	2+	+	+	-
18	2+	2+	4+	ns	ns
19	2+	2+	3+	2+	-
20	+	2+	2+	+	-
21	+	+	2+	2+	-
22	2+	+	2+	+	-
23	+	2+	3+	+	-
24	+	+	4+	3+	-
25	+	+	3+	+	-
26	+	ns	-	-	ns

[1]Sample position number one is the bottom-most node, internode, or leaf sample taken from an approximately 2.5 m vine.

[2]The cutoff value for a positive ELISA result (0.438) was determined to be two times the average A_{405} value of the healthy control. The sample readings were scored in relation to the ratio of the A_{405} value of each sample and the positive cutoff value. The following scores, (-), (+), (2+), (3+), (4+), were assigned to each treatment with a ratio of, less than 1 (no reactivity), 1 to 2.4, 2.5 to 3.4, 3.5 to 4.4, and above 4.5, respectively. ns, no sample.+

and internodes. Leaf tissues were dissected into petiole, midrib (area of the blade with primary veins), and leaf blade (area of the leaf blade with secondary and tertiary veins) samples.

The infection status of tissues with low virus titers was determined by propagating *in vitro* axillary bud explants from nodes of the apical portion (top 35 cm, starting at node number 11) of selected greenhouse grown GLRaV III -infected vines. Internode tissues adjacent to these nodes were analyzed by ELISA to compare the distribution of virus antigens from greenhouse and tissue culture grown material. The whole shoots from the first generation of *in vitro* propagated explants were analyzed after subculturing in the appropriate culture media.

The distribution of leafroll associated virus antigens in dormant wood from infected GLRaV I and III vines were analyzed. Dormant field and greenhouse grown vines were completely dissected (nodes and internodes) and tested using the ELISA with GLRaV I and III antibodies.

Results

Cross-reactivity of viral isolates: Initial experiments consisted of ELISA with GLRaV I, II, and III antibodies to determine which antigens were present in source plant material. Several virus isolates reacted to one or more of the antibodies used in this study (Table 1). The French Colombard (FC/2) and Cabernet Sauvignon (CS/8) cultivars that were previously determined to be virus-free by biological indexing reacted to GLRaV I and II, or GLRaV III antibodies, respectively. The rootstock varieties Riparia Gloire (*Vitis riparia* Gloire), Kober 5BB, and Couderc 3309 did not react to the antibodies tested and were used as negative controls. These were confirmed to be free of GLRaV I, II, IIb, III, IV, and corky bark associated antigens (GCBaV) using the Western blot assay (20).

Virus localization during the season - leaves and stems: Figure 1 shows the average ELISA absorbance readings at 405 nm (A_{405}) of samples collected in February of 1994. Older tissue collected from stems and leaves (petioles and veins) located near the bottom of the vines had the highest virus titers. In most samples tested, symptomatic leaves had high virus titers regardless of their position on the vine (samples collected and assayed throughout 1994, not shown). The lowest concentration of viral antigens was found in samples collected from younger tissue located at the top of the plant. Figure 2 shows the virus localization during the season of GLRaV I and III in greenhouse actively growing grapevines. Early in the growing season (February to August) the difference between young (top samples) and older tissue (bottom samples) was evident. In contrast, at the end of the growing season (September to December) the difference between top and bottom samples was less obvious, although the virus titers were lower in top stem samples than in the bottom ones (except December [Fig. 2A]

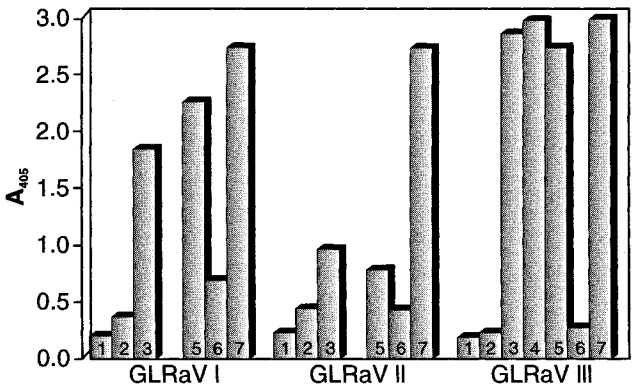

Fig. 1. Localization of grapevine leafroll associated viruses I, II, and III antigens in different grapevine tissues from greenhouse actively growing vines in February of 1994, as determined by DAS ELISA using GLRaV I, II, and III antibodies. The average absorbance (A_{405} nm) of 4 vines for GLRaV I and II, and 2 vines for GLRaV III from the same source plants were analyzed. Bars represent: 1, negative control; 2, leaf (top); 3, leaf (bottom); 4, leaf (symptomatic when available); 5, leaf petiole; 6, stem (top); 7, stem (bottom).

and October [Fig. 2B]). The low titers of GLRaV I (Fig. 2A) and GLRaV II (not shown) recorded in January was probably due to the vegetative stage of plants sampled (young growth, vines were about 0.5 to 0.9 m in length). Longer substrate incubations were necessary to obtain higher ELISA A_{405} readings with an obvious difference between bottom and top samples (not shown). Higher ELISA readings were obtained for GLRaV III than for GLRaV I when vines of the same vegetative stage were sampled (Fig. 2). Stem samples located near the bottom of the vines assayed in September and October had low GLRaV I titers (Fig. 2A). In a more extensive sampling of the same vines performed in September, leaf petioles and mid-veins had high GLRaV I and II titers as compared to stem samples (not shown). The distribution of virus antigens in October (performing multiple sampling using the same vines described in Fig. 2A) showed that stem and petiole samples located near the bottom of the vine had higher A_{405} readings than those shown in Figure 2A, but low or undetectable readings were obtained in leaf blades regardless of their location in the vine (not shown). The average titer of the virus in different tissues from actively growing vines fluctuated throughout the testing season. The highest virus concentrations in the sampled vines were obtained in February (GLRaV I, III; Fig. 2A, B), July (GLRaV III; Fig. 2B), November (GLRaV I, and III; Fig. 2A, B), and December (GLRaV I, Fig. 2A).

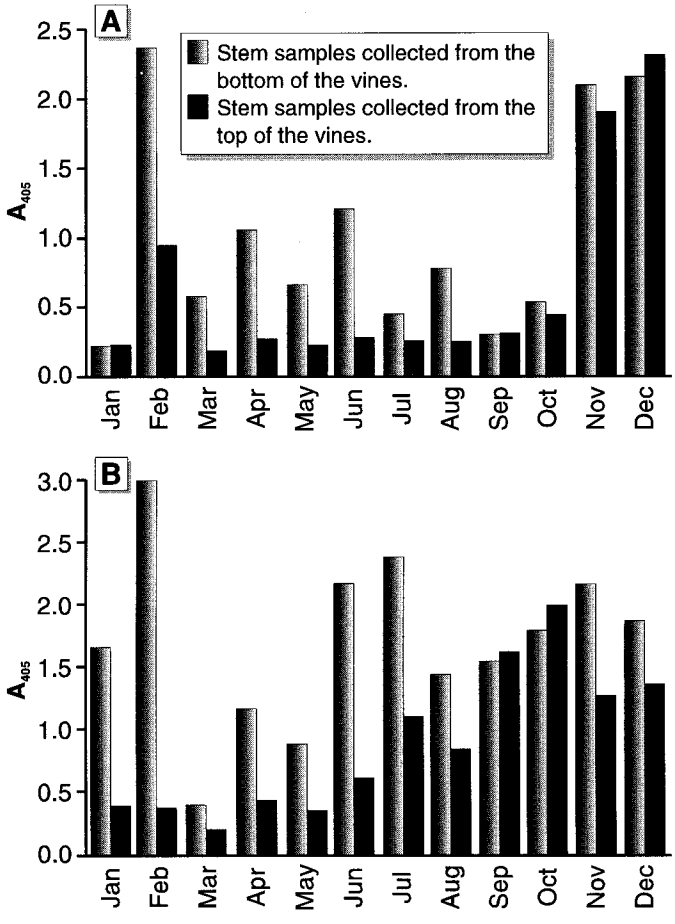

Fig. 2. Virus localization during the season of grapevine leafroll associated viruses I, and III antigens in greenhouse actively growing grapevine stem tissues during 1994 (January - December). Tissues were ground and analyzed by DAS ELISA using GLRaV I (A), and III antibodies (B). The average absorbance (A_{405}) of 4 vines from the same source isolate for GLRaV I and GLRaV III, except February and April (GLRaV III, 2 vines), June (GLRaV I, and III, 2 vines) were analyzed. Filled and open bars represent stem samples collected from the bottom or top of the vines, respectively. The average absorbance readings for the negative controls were 0.196 and 0.200 for GLRaV I and III, respectively.

Virus titer distribution in sectioned grapevines - greenhouse grown: Grapevine leafroll associated viruses I and II antigens were detectable in all stem samples (Table 2) tested except positions 18 and 19. In addition, high virus titers were detected in petiole and midrib samples located near the bottom of the vines. Undetectable or low virus titers were found in samples located near the top of the vine (positions 19 to 26); low virus titers were detected in scattered leaf blade samples near the bottom of the vine, and undetectable titers were found along the vine. Grapevine leafroll associated III antigens were detected in every stem sample (Table 3). The petioles and leaf midribs had detectable virus antigens, while the leaf blades had undetectable GLRaV III virus titers.

The virus titer of nodes or internodes samples collected from two vines of the same plant source infected with GLRaV III were analyzed. Grapevine leafroll associated virus III-antigens were unevenly distributed. Higher concentrations of GLRaV-antigens were found near the bottom of the plant, but no difference in titers between nodes and internodes were detected (not shown).

A distribution study of GLRaV I, II, and III antigens performed in November showed that the titer of the virus antigens fluctuated throughout the vine but all the stem samples analyzed had detectable titers of virus (Fig. 3, A, B, and C).

Virus titer distribution in sectioned grapevines - greenhouse and tissue culture grown: High titers of GLRaV III antigens were detected in all tissue culture grown explants (Fig. 4) The dotted lines between data points indicate two missing data points (*i.e.,* explants 14 and 19 did not survive tissue culture transfers due to bacterial contamination). The tissue culture propagated grapevine material had higher virus titers than the originally tested internodal tissue. The leaf tissue analyzed had low and undetectable titers of virus (Fig. 4).

Virus titer distribution in sectioned grapevines - dormant greenhouse and field grown: The distribution of virus antigens fluctuated in the dormant stems samples tested (not shown). Several samples had low and undetectable GLRaV I titers, and the virus was unevenly distributed throughout the canes analyzed. Higher titers of GLRaV III antigens were found near the bottom of the vines and low or undetectable titers were found in samples from the top of the vines (not shown). In both, GLRaV I- and III-infected material the virus titers were lower in dor-

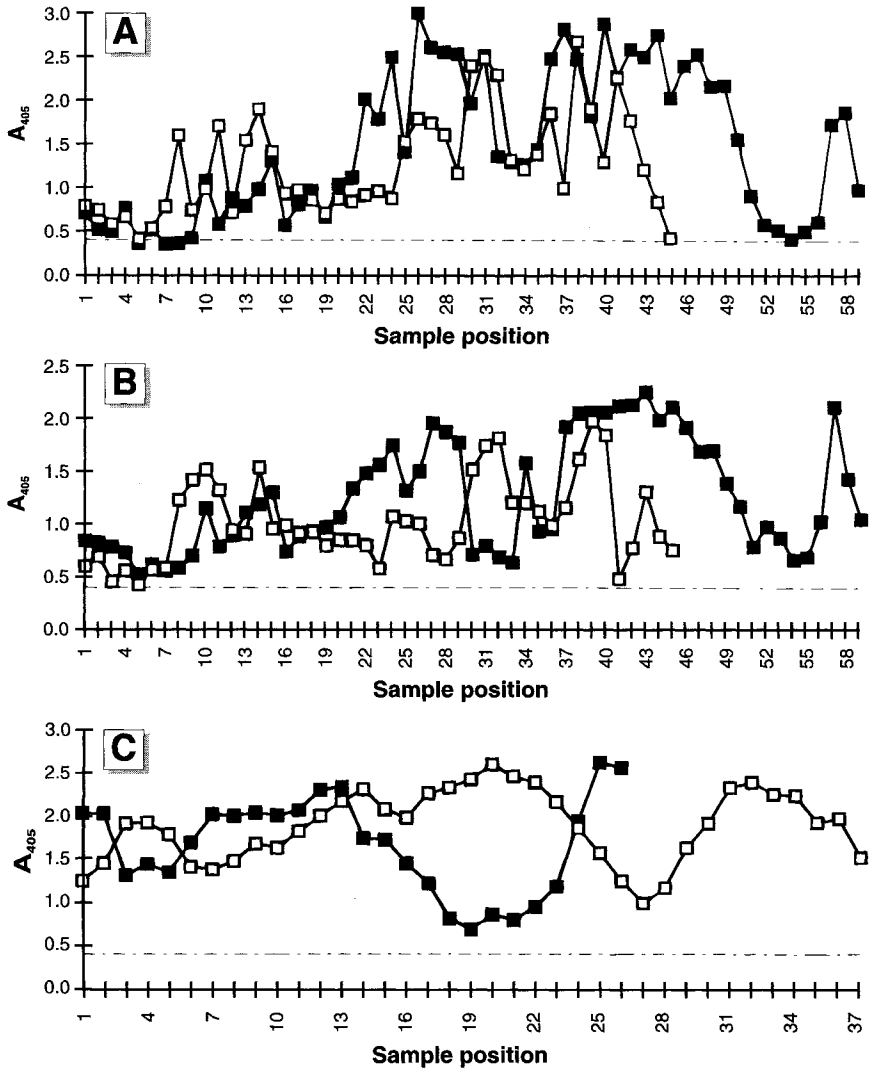

Fig. 3. Virus titer distribution of GLRaV I (A), II (B), and III (C) in sectioned greenhouse actively growing grapevines in November. The stems were completely disected (including node and internode tissues, sample position # 1 corresponds to the sample collected from the bottom of the vine) from vine 1 (closed square) and vine 2 (open square). Vines 1 and 2 for GLRaV I and II testing were, 5.03 m and 2.92 m in length, respectively. The length of vine 1 and 2 for GLRaV III testing were 1.63 m and 2.48 m, respectively. Each data point corresponds to the average absorbance (A_{405}) of duplicate ELISA test wells. The line across the plot represents the cutoff value for a positive sample.

mant canes than in actively growing grapevines of comparable size and vigor (*i.e.*, longer substrate incubations were required in order to detect the virus antigens).

Discussion

The ELISA reactivity with GLRaV I, II, and III antibodies was found to vary in the sampled GLRaV-infected grapevines tested. The highest concentration of virus was detected in samples located near the bottom of the vine and low titers were generally detected in samples located near the top of the vines. Leaf midribs had higher virus concentrations than leaf blades. These results were expected since GLRaVs are phloem limited-closteroviruses.

Grapevine leafroll associated virus-antigens were found to be unevenly distributed in the stem and leaf samples tested. We suggest that the uneven distribution of GLRaV in grapevines might be due to the inefficient movement and replication of viruses in the phloem tissue. The detection of higher virus titers on samples located near the bottom of the vines and at the end of the growing season in all tissues tested (November and December) suggest that the movement of viral antigens in the phloem is slow. These findings were in accordance with those of Teliz *et al.* (24) who reported that the concentration of basal leaf samples from field-grown grapevines had higher GLRaV III titers than apical leaves.

The virus titer of *in vitro* propagated grapevine tissue was higher than in the original actively growing vines tested. High titers of the virus antigens were detected in every test sample. These results suggest that it might be possible to increase the sensitivity of ELISA by propagating questionable and GLRaV-infected material in tissue cul-

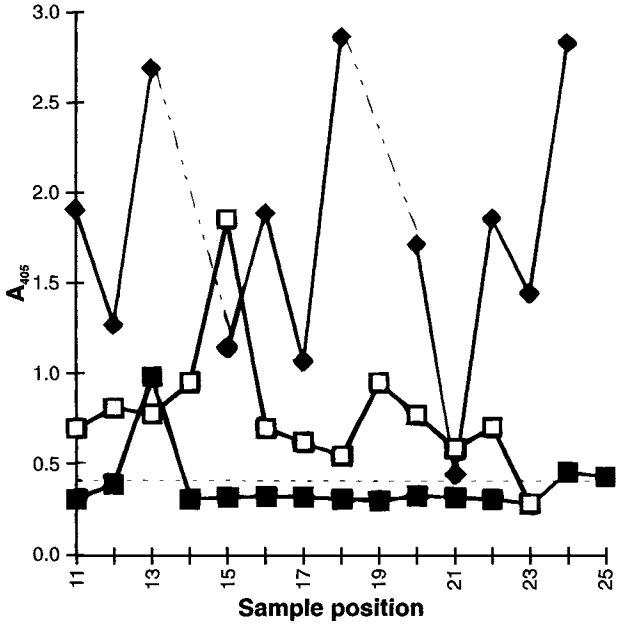

Fig. 4. Virus titer distribution of GLRaV III in sectioned greenhouse actively growing and tissue culture grapevines, leaves (closed square) and stems (open square) from greenhouse plants, and tissue culture grown explants (diamonds) (sample position # 11 corresponds to the bottom-most sample collected or explants initiated from the 35 cm apical portion of the vine). The line across the plot represents the cutoff value for a positive sample.

ture. Experiments performed with *in vitro* propagated grapevine tissue infected with GLRaV III, grapevine fanleaf, and grapevine virus A also indicate that *in vitro* cultures contain high virus titers (1,2,18).

Our work showed that the virus antigen titers were lower in dormant wood than in greenhouse actively growing grapevines. The low virus titers in dormant canes might explain the discrepancies between biological indexing and ELISA. Presently, biological indexing is a widely used technique for the detection of LD (26) and involves the grafting of a bud from a suspicious vine onto an indicator cultivar. Symptom expression is nonspecific and could indicate that the vine is stressed or infected with a virus other than the one associated with LD.

Rowhani and Golino (23) have reported that the Cabernet franc indicator is not completely reliable for indexing LD. In this report, we demonstrated that GLRaV was detected using ELISA in vines that tested negative by traditional indexing methods. Rowhani and Golino (23) concluded that polyclonal antisera specific to GLRaV II and III may react to some undefined component that does not always produce symptoms in Cabernet franc or that environmental factors or variation between virus isolates could be responsible for the discrepancy of the results. These possibilities cannot be ruled out; however, the uneven distribution of virus could be responsible for the lack of correlation between woody indexing and ELISA.

The distribution data showed that the virus titers were low or undetectable in tissue samples taken from different locations on infected vines. Therefore, a grafted bud from an area of low or undetectable virus titers may or may not show typical leafroll symptoms in an indicator host. Furthermore, symptom development will depend on the ability of the virus to move within the indicator host vascular tissues and on environmental growth conditions.

Cross-reactivity studies using ELISA and the Western blot assay indicated mixed infections of GLRaV in the source plant material tested (this report, 21). A Western blot assay developed in our laboratory was shown to be a reliable method for the differentiation and detection of viruses. With the Western blot assay, specific polypeptides can be correlated with the infection of specific viral isolates, instead the ELISA detection is based on a colorimetric reaction (20). In addition, GLRaV III can be specifically detected using immunocapture of viruses and reverse transcription of viral RNA coupled with the polymerase chain reaction (PCR) (17). The development of nucleic acid based techniques and improved immunological reagents will facilitate the unequivocal detection of leafroll associated viruses and complement the ELISA and woody biological indexing techniques presently used in clean stock programs.

Conclusions

Information obtained in this study indicates that multiple samples should be taken to unambiguously detect GLRaVs in actively growing grapevines using the ELISA. In our laboratory we divide the grapevines in quadrants and collect at least one sample from each for ELISA testing. Portions of stems and petioles located near the bottom of the vines are a reliable sample source for the testing of GLRaV-antigens throughout the grapevine growing season in the greenhouse. We recommend the use of older actively growing tissue for efficient virus detection of the antigens associated with leafroll disease.

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