Rapid In Vitro Indexing of Grapevine Viral Diseases: The Effect of Stress-Inducing Agents on the Diagnosis of Leafroll

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ABSTRACT

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The release of new virus-free propagation material and new cultivars of grapevine would be advanced if more rapid diagnostic methods could be developed. A method enabling rapid in vitro indexing of grapevine leafroll is described. Diseased explants poorly express symptoms when placed in culture. However, when placed under mild stress conditions (4% sorbitol), a variety of distinct symptoms appear within 4 to 8 weeks. This method could potentially replace the conventional 18- to 30-month indexing procedure.

Grapevine leafroll is one of the most important and widespread diseases affecting grapevines in all viticultural countries, causing considerable economic damage. The major symptoms consist of downrolling of leaves and interveinal chlorosis. Leafrolling begins at the base of the cane and spreads to younger leaves during midsummer. The major veins in red cultivars remain green, whereas the leaf laminae turn red, and leaves of white cultivars become slightly chlorotic. Leafroll-affected grapevines are less vigorous, producing fewer and smaller clusters. The disease also causes lower sugar levels and color reduction in fruit. Leafroll is typically diagnosed by indexing on woody indicator plants (3,6,8).

Grapevine leafroll is graft-transmissible. Several virus-like particles have been associated with the disease, including closteroviruses, trichoviruses, a potyvirus, and isometric particles (4,7,10,12,17,19,20). However, the etiology of this disease is still undetermined, because disease incidence does not correlate completely with the presence of one of these viruses. Therefore, diagnosis still relies on time- and labor-consuming indexing on woody indicator plants, which may be corroborated by serology, electron microscopy, and molecular methods. The lack of rapid diagnostic methods hampers the production and release of virus-free cultivars.

The availability of grapevine tissue-culture technology prompted attempts to mimic indexing and to enhance symptom development in vitro. We recently described a new indexing method that allows for rapid diagnosis of grapevine corky-bark by micrografting in vitro onto cultured indicators (18). Micrografting reduced the detection time of grapevine corky-bark to 8 to 12 weeks, instead of the usual 2 to 3 years required for conventional indexing.

The current work was initiated to develop a simpler, graftfree method for rapid in vitro indexing of leafroll. Explants from diseased plants had poorly developed symptoms in culture and, then, only inconsistently and at irregular times. In the vineyard, virus-diseased grapevines express symptoms earlier when stressed (e.g., when poorly irrigated). Therefore, we attempted to induce stress in the cultured explants to promote symptoms and develop a reliable and rapid in vitro indexing method for grapevine leafroll. Here we report on a method that takes 4 to 8 weeks.

MATERIALS AND METHODS

Shoots of three healthy grapevine cultivars (Petite Sirah, Gamay, and Mission) were obtained via in vitro apex culturing (2). Shoots were sterilized by immersion for 10 min in 2.5% (wt/vol) sodium hypochlorite containing a drop of Tween 20. The shoots were transferred and maintained on 10 ml of 0.8% agar-Murashige and Skoog (MS) medium containing 3% sucrose (1,11) in 150 × 25-mm tubes. Cultured material was maintained in a growth chamber at 25°C under 16-h of light from cool-white fluorescent tubes (45 $\mu E\ m^{-2}\ s^{-1}$). When plantlets reached 7 to 10 cm in length, each was excised into two to three fragments and transferred to MS medium containing sucrose, lactose, sorbitol, or mannitol. The degree of stress caused by these sugars was assessed by applying two concentrations of each, as specified in Table 1. At least three experiments were carried out, each consisting of 10 plantlets of each cultivar.

The degree of exerted stress was assessed by comparing shoot elongation and development of new nodes to those of control plantlets maintained in a medium with 3% sucrose (standard medium). The differences in elongation and number of new nodes were analyzed by a two-factorial analysis of variance design, with the following parameters as main effects: sugar (sucrose, lactose, sorbitol, or mannitol) and concentration (low or high). The square roots of the numbers of new nodes were used to stabilize variance. Data for mannitol at 4% was excluded from the analysis because the means and standard errors were 0.

In a further experiment, grapevine material was diagnosed as healthy or leafroll-infected prior to culturing by conventional indexing on Cabernet franc and Mission indicator plants (6). Results were corrobor-

Table 1. Effect of various sugars on elongation and new node formation in in vitro-grown healthy Petite Sirah grapevine plantlets (average of 10 replicates)

Sugar	Concentration (%)	Mean elongation (cm ± SE)[z]	Mean of new nodes ± SE[z]
Sucrose	2	3.25 ± 0.55ab	3.3 ± 0.36a
Sucrose	4	4.00 ± 0.38A	4.0 ± 0.49A
Lactose	5	3.95 ± 0.40a	3.7 ± 0.15a
Lactose	10	2.10 ± 0.31B	3.8 ± 0.65A
Sorbitol	2	2.65 ± 0.43b	2.8 ± 0.33a
Sorbitol	4	1.95 ± 0.35B	2.8 ± 0.20A
Mannitol	2	0.20 ± 0.08c	1.7 ± 0.37b
Mannitol	4	0.00	1.0 ± 0.33B

[z] Duncan's multiple range test was used. Means with the same letters are not significantly different. Lower- and uppercase letters correspond to low and high sugar concentrations, respectively.

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ated by enzyme-linked immunosorbent assay (ELISA) with antibodies against grapevine leafroll associated closterovirus type 3 (GLRaV-3) (5,15).

Because sorbitol inflicted mild osmotic stress on the in vitro-grown plantlets, it was selected as a stress-inducing agent in experiments testing symptom development in leafroll-infected plantlets in vitro. Leaf symptoms, internode elongation, node formation and root development of sorbitol-affected, leafroll-infected plantlets were compared to those of noninfected control plantlets.

RESULTS

Sucrose, lactose, sorbitol, and mannitol were tested in vitro as stress-inducing agents for grapevine. Because sugar concentration significantly affected internode elongation, comparisons between low and high concentrations for each sugar and comparisons between sugars at a given concentration level were made. There were no differences between the effects of low and high concentrations, except in the case of lactose, in which the difference between the internode lengths at 5 and 10% sugar was statistically significant ($P < 0.01$).

The effects of various sugars were compared by Duncan's multiple range test, with separate analysis for low and high concentrations. An analysis of effect of sugar concentrations on the number of new nodes indicated that concentration differences were not significant. The number of new nodes obtained in mannitol-containing medium differed significantly from that obtained with other sugars. Internode elongation and number of nodes on plantlets maintained on lactose (even at a relatively high concentration) did not differ from the control values of sucrose-maintained plantlets. Sorbitol exerted moderate stress, and mannitol caused the greatest stress on grapevine cultured plantlets, to the point of near toxicity (Table 1).

Sorbitol was selected as the stress-inducing agent in the in vitro diagnostic assays. Under the mild stress conditions induced in culture by 4% sorbitol, plantlets of leafroll-infected Petite Sirah, Gamay, and Mission exhibited distinct symptoms, including epinasty, leaf-reddening, and inhibition of root development (Figs. 1 and 2). Other symptoms, such as stunting, lack of root development, and death of plantlets, also were observed.

Symptoms appeared 4 weeks after plantlets were placed under stress conditions. Although some of these symptoms also were observed under standard (3% sucrose) conditions, symptom appearance under sorbitol-induced stress was accelerated, symptom severity was accentuated, and incidence of symptom expression in plantlets increased.

Stress factors alone induced disease-like symptoms in noninfected Gamay plantlets as well. However, as the plantlets adapted to the new conditions, symptoms in nondiseased plantlets disappeared, whereas infected material continued to express symptoms. To enable differentiation between disease- and stress-associated symptoms in Gamay, the indexing period was extended to 8 weeks after transfer of plantlets to stress conditions, when nonspecific symptoms had faded. New nodes were counted, and node elongation was measured 4 and 8 weeks after transfer to stress conditions. A Wilcoxon two-sample test was used to analyze differences in length and number of nodes between plantlets under control and stress conditions. The results are summarized in Table 2.

At 8 weeks after stress induction, significantly fewer new nodes were formed in infected than noninfected explants in sucrose. Significantly fewer new nodes were formed on Gamay and Mission grown in sorbitol, but not on Petite Sirah. Noninfected explants of Gamay, Petite Sirah, and Mission maintained on sucrose showed greater elongation than did infected explants. However, the differences between infected and noninfected explants were more pronounced when plantlets were grown in sorbitol.

Another distinct difference between infected and noninfected explants was the

Fig. 1. Gamay grapevine plantlets after 2 months in culture. Samples (from left to right): healthy plantlets on 3% sucrose, healthy plantlets on 4% sorbitol, leafroll-infected plantlets on 3% sucrose, and leafroll-infected plantlets on 4% sorbitol.

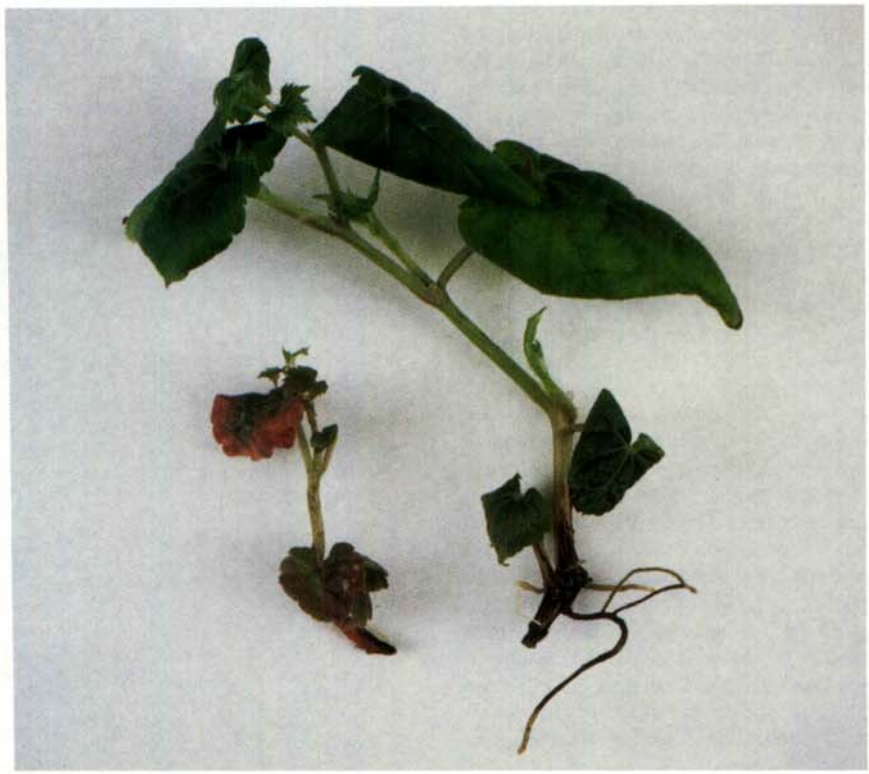

Fig. 2. Mission grapevine plantlets (right: healthy; left: leafroll-infected) 1 month after explants were placed in a culture medium containing 4% sorbitol. The infected plantlet lacks root development.

Table 2. New node formation and elongation in leafroll-infected and noninfected grapevine plantlets under mild stress conditions (4% sorbitol) compared to standard (3% sucrose) conditions

Time[x]	New node formation ± SE				Elongation (cm) ± SE			
	Sorbitol		Sucrose		Sorbitol		Sucrose	
Cultivar	Infected	Noninfected	Infected	Noninfected	Infected	Noninfected	Infected	Noninfected
4 weeks								
Gamay	0.37 ± 0.17[y]	2.00 ± 0.39[y]	1.05 ± 0.49[y]	6.47 ± 0.49[y]	0.07 ± 0.04[y]	0.7 ± 0.14[y]	0.64 ± 0.34[y]	4.29 ± 0.39[y]
Mission	0.27 ± 0.21	0.79 ± 0.30	1.13 ± 0.46	2.29 ± 0.57	0.07 ± 0.06	0.26 ± 0.12	0.36 ± 0.31	1.52 ± 0.40
Petite Sirah	0.69 ± 0.25[z]	1.79 ± 0.35[z]	1.69 ± 0.42[y]	3.60 ± 0.43[y]	0.17 ± 0.05[y]	0.84 ± 0.18[y]	0.89 ± 0.25[y]	2.61 ± 0.38[y]
8 weeks								
Gamay	0.00[y]	2.16 ± 0.27[y]	0.84 ± 0.29[y]	6.16 ± 0.80[y]	0.00[y]	0.82 ± 0.14[y]	0.34 ± 0.15[y]	3.82 ± 0.35[y]
Mission	0.60 ± 0.32[z]	2.93 ± 0.54	0.73 ± 0.61[y]	6.07 ± 0.87[y]	0.21 ± 0.14[y]	1.61 ± 0.31[y]	0.41 ± 0.40[y]	2.96 ± 0.44[y]
Petite Sirah	1.63 ± 0.40	2.07 ± 0.24[z]	2.00 ± 0.42[y]	4.07 ± 0.25[y]	0.46 ± 0.14[z]	1.33 ± 0.22[z]	1.17 ± 0.30[y]	2.75 ± 0.30[y]

[x] Four or eight weeks after placing plantlets under stress conditions.
[y] Difference between values on both sides of the infected/noninfected line is significant at $P < 0.001$.
[z] Difference between values on both sides of the infected/noninfected line is significant at $P < 0.05$.

lack of root development in the latter (Fig. 2). Roots developed normally in all of the control explants. However, root formation was inhibited in 78 to 100% of the virus-infected plantlets.

DISCUSSION

Sugars and other molecules, such as polyethylene glycol (PEG), can cause osmotic shock in plant tissue. Nonmetabolized compounds such as PEG and mannitol (9,13, 14) or the slowly metabolized sorbitol are especially effective in producing shock. Indeed, mannitol was nearly toxic to cultured grapevine shoots and plantlets, whereas the effect of sorbitol was milder but still distinct. Under sorbitol-induced suboptimal conditions, which nevertheless enabled limited growth and development of plantlets, distinctive symptoms appeared in explants from virus-infected plants. Most of the explants remained symptomless under standard (3% sucrose) culture conditions. Symptoms appeared irregularly and late, often requiring up to 6 months for symptom expression.

The phenomenon reported here may serve as an alternative method for indexing grapevine viral diseases. Its usefulness could be studied for other diseases and disorders, and the method could potentially be combined with the recently described micrografting assay (18). The method appears better suited than conventional indexing for large-scale diagnosis and is more rapid. The shortening of symptom-based diagnosis time from years to weeks relieves indexing of its greatest shortcoming and, thus, promotes the rapid release of newly introduced and developed cultivars. The inhibition of root formation in infected explants is pronounced and visible and may serve as an easily observed indication of disease.

The cultivar-specific aspect of the stress response should be taken into account, however. Infected Petite Sirah plantlets responded mildly to stress, and none of the noninfected plantlets developed symptoms. Nineteen of twenty-three infected plantlets developed typical symptoms 4 weeks into the experiment. The response of Gamay to stress was more severe, and temporary symptoms initially developed in noninfected material. However, after adaptation to stress conditions, temporary symptoms disappeared from the noninfected samples, and only infected samples exhibited symptoms. Therefore, an 8-week assay was required for Gamay.

Leafroll syndrome is defined on the basis of symptom development in certain indicator plants. It may be caused by a variety of viruses, singly or in combination (15, 16,20). All the diseased explants used in this study were diagnosed by ELISA as carrying GLRaV-3. A more general application of this procedure is currently being tested with a wider spectrum of leafroll-associated viruses, as well as with grapevine corky-bark and grapevine virus A (10).

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