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Mechanical sap transmission of a closterovirus from *in vitro* shoot tip cultures of a leafroll-affected grapevine to *Nicotiana benthamiana*

by

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Transmission manuelle d'un clostérovirus au *Nicotiana benthamiana* à partir de cultures *in vitro* d'une vigne atteinte de l'enroulement

Résumé : Des cultures *in vitro* d'une vigne de *Vitis vinifera* Limberger atteinte de l'enroulement ont été broyées dans une solution tampon contenant de la nicotine. Les extraits ont été inoculés sur des feuilles de *Nicotiana benthamiana* et de 6 autres espèces de plantes herbacées. 3 semaines plus tard, seuls les plants de *N. benthamiana* ont démontré des symptômes. Ceux-ci consistaient d'un nanissement systémique accompagné d'un éclaircissement des nervures qui se transformait en chlorose interveinale. Les feuilles de ces *N. benthamiana* contenaient le virus A de la vigne (GVA), démontré par la méthode ISEM.

Key words: virosis, leaf roll, closterovirus, GVA, tissue culture, test plant, transmission, analysis, serology, ISEM, ELISA, etiology.

Introduction

Grapevine leafroll (GLR) disease occurs worldwide and is of great economic importance. Several viruses and viruslike particles have been associated with the disease, including a potyvirus (TANNE *et al.* 1974), isometric viruses (NAMBA *et al.* 1979 a; CASTELLANO *et al.* 1983) and long (1400—2200 nm) or short (800 nm) closteroviruses (NAMBA *et al.* 1979 b; FAORO *et al.* 1981; CASTELLANO *et al.* 1983; GUGERLI *et al.* 1984; MILNE *et al.* 1984; MOSSOP *et al.* 1985; ENGELBRECHT and KASDORF 1985, 1987; ROSCIGLIONE and GUGERLI 1986; ZEE *et al.* 1987; ZIMMERMANN *et al.;* 1988); but definitive results on the etiology of GLR are still lacking.

Research on the etiology of GLR has been hampered by the lack of a suitable herbaceous host for the disease agent(s). GLR has been transmitted from grapevine to grapevine using dodder, but this method was unsuccessful for transmitting GLR to herbaceous plants (WOODHAM and KRAKE 1983). Mechanical sap transmission of viruslike particles from leafroll-affected grapevines to herbaceous plants has only rarely been reported. TANNE *et al.* (1974), using phenol extracts from leaves of leafroll-affected vines, succeeded in transmitting a potyvirus (GPV) to *Nicotiana glutinosa*, *N. tabacum* and *N. debneyi*. This virus was purified and characterized in 1977 (TANNE *et al.* 1977) and was detected in 1985 (TANNE and GIVONY 1985) in all GLR-affected vines tested. A closterovirus, grapevine virus A (GVA), was also detected in all vines tested concurrently with anti-GVA serum (TANNE and GIVONY 1985). This closterovirus, GVA, has been transmitted mechanically from a grapevine to a herbaceous host on only one occasion. CONTI *et al.* (1980) succeeded in transmitting GVA from the roots of a Pigato vine which was showing symptoms of stem pitting to *N. clevelandii* GRAY. No clear evidence has been published linking grapevine stem pitting disease and GVA. Some reports suggest that GVA may be associated with grapevine leafroll disease (TANNE and GIVONY 1985; ENGELBRECHT and KASDORF 1985, 1987). ROSCIGLIONE *et al.* (1983) have

transmitted GVA from grapevines to *N. clevelandii* and *N. benthamiana* by using mealybugs, but could not transmit it to herbaceous plants by grapevine sap inoculation. This communication reports on the mechanical sap transmission of a closterovirus, GVA, from *in vitro* shoot tip cultures of GLR-affected *V. vinifera* Limberger to a herbaceous host, *N. benthamiana*.

Materials and methods

Plant material

The parent vine for the *in vitro* shoot tip cultures used in this study was a GLR-affected vine of *V. vinifera* Limberger. It was GLR-affected, corky bark-free and rupestris stem pitting-free, based on indexing results obtained with the woody indicators *V. vinifera* Pinot noir, LN-33 (Couderc 1613 × Thompson Seedless) and *V. rupestris* St. George. The herbaceous test plants were seedlings of *Nicotiana benthamiana, N. clevelandii* GRAY, *N. glutinosa, Gomphrena globosa, Chenopodium amaranticolor* and *C. quinoa* ssp. *milleanum* and *quinoa*.

Grapevine *in vitro* shoot tip culture

In vitro shoot tip cultures were used as inoculum because they have proved useful for the detection of both viral antigens (MONETTE 1985) and disease-associated nucleic acid (MONETTE *et al.* 1989). Media and procedures for the initiation and maintenance of grapevine shoot tip cultures *in vitro* have been reported elsewhere (MONETTE 1988).

Transmission tests

In vitro shoot tip cultures (0.5—1.0 g F.W.) of Limberger were collected at the shoot proliferation stage and ground with a chilled mortar and pestle in 5 volumes (w : v) of 0.01 M potassium phosphate buffer (pH 7.0) containing 2.5 % nicotine, final pH 9.5 (CADMAN *et al.* 1960). The extract was then rub-inoculated onto corundum-dusted leaves of the herbaceous indicators. Control plants were buffer-inoculated. The *N. benthamiana, N. glutinosa* and *Gomphrena* plants were inoculated at the 6-leaf stage and the *Chenopodium* and *N. clevelandii* plants at the 7 to 9-leaf stage. Two herbaceous plants of each species were used per trial and all were maintained in the same greenhouse.

Immunosorbent electron microscopy (ISEM) and enzyme-linked immunosorbent assay (ELISA)

Anti-GVA goat serum was generously provided by D. J. ENGELBRECHT. ISEM, similar to that described elsewhere (VAN REGENMORTEL 1982), was used to identify GVA. Extracts for this procedure were prepared by grinding plant tissue (a shoot tip culture of Limberger or selected leaves from systemically infected *N. benthamiana*) in a mortar with 10 volumes (w : v) of 0.06 M potassium phosphate buffer, pH 7.0 (6-PB). Antiserum coating of grids (backed with Formvar-carbon films) and subsequent virus particle trapping and decoration on appropriate droplets of GVA antiserum (dilution 1 : 1000 and 1 : 100 in 6-PB) or extract were for 3, 1, and 1/2 h, respectively. Grids were washed with a stream of buffer (about 1 ml) between treatments and in a stream of distilled water after the last step, then stained with 2 % uranyl acetate. Grids were examined with a JEOL JEM-100C electron microscope.

For ELISA, *in vitro* cultures of Limberger were ground with a chilled mortar and pestle in 10 volumes (w : v) of 0.05 M sodium carbonate buffer, pH 9.6. 200 µl was pipet-

ted into each well of a microtitration plate. The plate was incubated at 37 °C for 3 h, then placed at 8 °C overnight. The wells were washed 3 times with 0.01 M phosphate buffered saline, pH 7.4, containing 0.5 ml/l of Tween 20 (PBS-Tween). 200 µl of a 1 : 500 dilution of goat anti-GVA serum in PBS-Tween containing 2 % polyvinyl pyrrolidone (PVP) and 0.2 % bovine serum albumin (BSA) was placed in each well. The plates were incubated at 37 °C for 3 h and placed at 8 °C overnight. The wells were washed 3 times with PBS-Tween. Rabbit anti-goat alkaline phosphatase conjugate (Bio-Rad) was diluted 1 : 1000 in PBS-Tween containing 2 % PVP and 0.2 % BSA, and 200 µl was placed in each well. After a 3 h incubation at 37 °C, the wells were washed 3 times in PBS-Tween. 200 µl of p-nitrophenyl phosphate at a concentration of 0.6 mg/ml in 9.7 % diethanolamine, pH 9.8, was then added to each well and color development was allowed to proceed for 1½—2 h, after which time A_{405} was measured using a Titertek Multiscan (Flow Laboratories) microplate reader.

Results and discussion

Of the 7 herbaceous indicators tested, only the *N. benthamiana* plants developed disease symptoms. These consisted of a systemic dwarfing with vein clearing of the tertiary and smaller veins which progressed to interveinal chlorosis with darker main vein banding (Figs. 1 and 2). These symptoms appeared after 3 weeks. The inoculations with homogenized Limberger shoot tip cultures were repeated 5 times. Symptom expression in *N. benthamiana* was obtained in 4 of the 5 trials. In 2 trials both *N. benthamiana* plants became infected; in 2 other trials 1 of the 2 *N. benthamiana* plants became infected and in the fifth trial no disease symptom developed.

GVA was reproducibly detected in the Limberger shoot tip cultures by both ELISA and ISEM. Shoot tip cultures of virus-free Limberger had an A_{405} (± SE) value of 0.093 ± 0.009 (n = 3), while shoot tip cultures of the leafroll-affected Limberger had a value of 0.486 ± 0.025 (n = 4). In ISEM of shoot tip cultures of leafroll-affected Limberger, closterovirus-like particles were both trapped and decorated with goat anti-GVA serum. No such particles were detected in shoot tip cultures of virus-free Limberger. Symptomatic leaves of *N. benthamiana* contained closterovirus-like particles, which were trapped and decorated with anti-GVA serum (Fig. 3). These particles were not detected in leaves from uninfected control plants.

Our successful and reproducible transmission of a closterovirus directly from grapevine tissues to *N. benthamiana* may be due to the fact that we were using *in vitro* shoot tip cultures as inoculum. MONETTE (1985) reported that grapevine fanleaf virus (GFLV) could consistently be detected by ELISA in shoot tip cultures of fanleaf-affected grapevines. GFLV cannot reliably be detected by ELISA in the leaves of field- or greenhouse-maintained vines at certain times of the year, due to seasonal variations in virus content (WALTER *et al.* 1984) and to the uneven distribution of the virus within the vine. By analogy, the lack of a consistently high concentration of virions in the inoculum may account for the fact that CONTI *et al.* (1980) succeeded only once in transmitting GVA from Pigato roots to a herbaceous host plant. The failure of ROSCIGLIONE *et al.* (1983) to transmit GVA to *N. benthamiana* by mechanical sap inoculation might also be explained on this basis. The mechanical transmission reported here was reproducible 4 out of 5 times, using only 2 indicator plants per trial. A higher percentage transmission might have been obtained, had we used a greater number of test plants.

The literature contains conflicting reports as to whether GVA is more closely associated with GLR or with grapevine stem pitting. The results reported here are consistent with the view (TANNE and GIVONY 1985; ENGELBRECHT and KASDORF 1985, 1987) that GVA is GLR-associated, as the Limberger grapevine used in this study was free of

detectable stem pitting. We do not know if the virus detected in this study is a causative agent of leafroll, and we cannot eliminate the possibility that our Limberger vine may harbor other viruses associated with the disease. TANNE (1985) has reported that a few

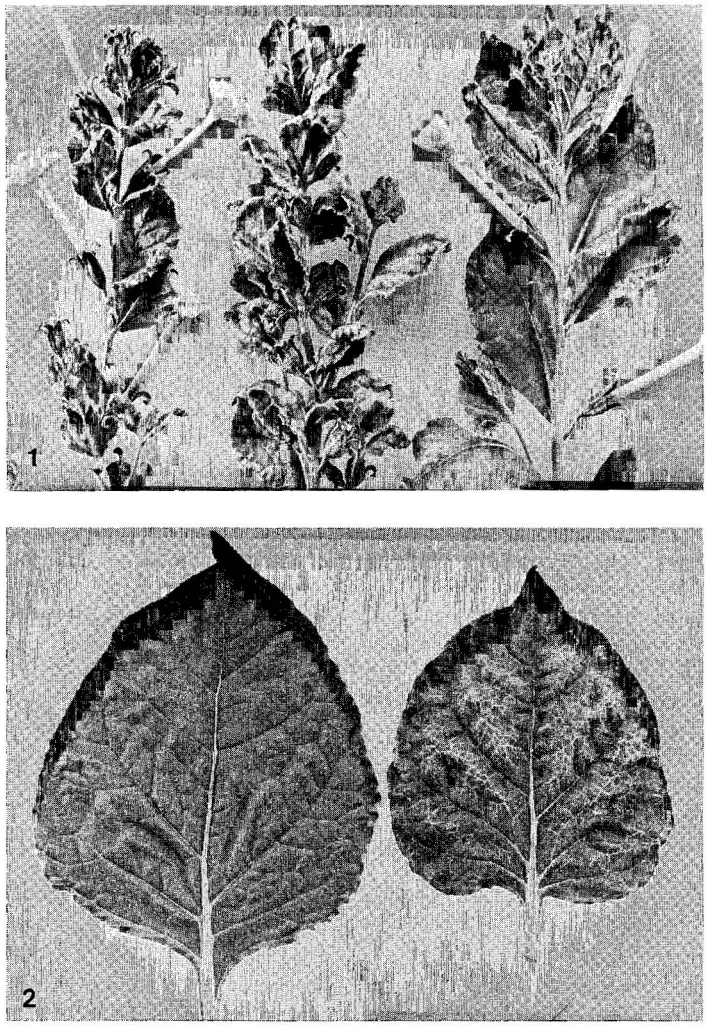

Fig. 1: Shoots of *N. benthamiana* plants inoculated with extract from shoot tip cultures of leafroll-affected Limberger (left and centre), showing a systemic dwarfing and interveinal chlorosis in comparison to a buffer-inoculated plant (right).

Fig. 2: Leaves from *N. benthamiana* plants inoculated with buffer (left) or extract from a shoot tip culture of leafroll-affected Limberger (right), the latter showing dwarfing and vein clearing.

Fig. 1: Deux pousses de *N. benthamiana* inoculées avec un extrait obtenu de cultures *in vitro* de Limberger atteinte de l'enroulement (à gauche et au centre). Ces plants démontrent un nanissement, en comparaison avec un plant inoculé avec une solution tampon (à droite).

Fig. 2: Des feuilles de *N. benthamiana* inoculées avec une solution tampon (à gauche) ou avec un extrait obtenu de cultures *in vitro* de Limberger atteinte de l'enroulement (à droite). La feuille à droite, nanisée, a les nervures éclaircies.

grapevines inoculated with phenol extracts from GPV-infected *N. glutinosa* developed symptoms of leafroll. We have not yet attempted to inoculate grapevines with the virus transmitted to *N. benthamiana.*

The observations that several distinct virus-like particles have been detected in leafroll-affected grapevines is consistent with the view that GLR may have a complex etiology. It is possible that leafroll-like symptoms can result when a grapevine is infected with different viruses, either singly or in various combinations. In order to resolve this question, the purified viruses must be transmitted to grapevine and must be re-isolated from inoculated vines showing leafroll symptoms. The use of grapevine *in vitro* shoot tip cultures may prove a useful adjunct to serological and electron microscopical analyses in determining the etiology of grapevine leafroll disease.

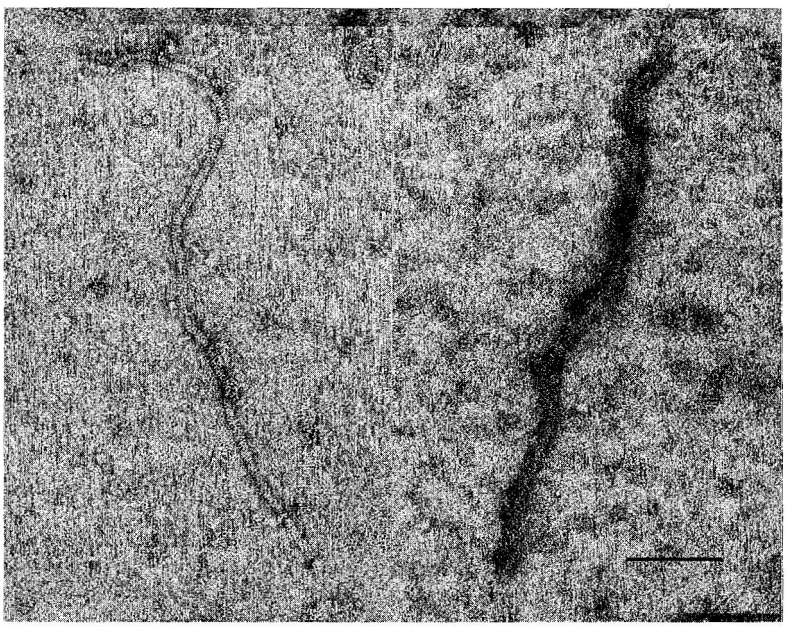

Fig. 3: Anti-GVA serum trapped (left) and trapped and decorated (right) virus particles from a leaf of *N. benthamiana* showing vein clearing. Uranyl acetate stain. Bar = 100 nm.

Particules virales présentes dans les feuilles symptomatiques de *N. benthamiana.* Les particules ont été adsorbées (à gauche) et décorées (à droite) à l'aide de serum anti-GVA. Coloration à l'acétate d'uranyle. La barre représente 100 nm.

Summary

In vitro shoot tip cultures of a *V. vinifera* Limberger vine affected with grapevine leafroll disease were ground in a nicotine-containing buffer and the extract was rub-inoculated into *Nicotiana benthamiana* and 6 other herbaceous species. 3 weeks after inoculation the *N. benthamiana* plants, but none of the others, developed a systemic dwarfing with an auxiliary vein clearing which progressed into an interveinal chlorosis with darker primary vein banding. Symptomatic leaves contained GVA, as shown by ISEM.

Acknowledgement

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