

CYTOPATHOLOGY OF CALLUS CELLS INFECTED WITH GRAPEVINE LEAFROLL-ASSOCIATED VIRUS 3*

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ABSTRACT

The cytopathology of grapevine (*Vitis* spp.) callus tissue infected with *Grapevine leafroll-associated virus 3* (GLRaV-3), genus *Vitivirus* was studied in order to investigate the usefulness of callus cultures to study grapevine leafroll-associated viruses. Ultrathin sections were made from *in vitro* callus obtained from stems and shoots of GLRaV-3 infected grapevine plants. Callus was composed of two types of tissue. Translucent, soft callus was formed and composed of large loosely arranged cells, containing big vacuoles and a thin layer of cytoplasm. Other parts of the callus were brown-coloured and composed of small compactly arranged cells, which showed flexuous and rod-shaped closterovirus-like particles, with 10-12 nm in diameter, at higher

magnifications. Groups of vesicles formed by a single membrane were also observed, with sizes ranging from 50-200 nm, containing fine fibrillar material, also typical of closterovirus infections. Virus concentration was monitored by Immunosorbent electron microscopy (ISEM) tests, which showed that *in vitro* culture of callus tissue from grapevine infected plants, could be used to study the GLRaV viruses through many successive generations, despite the decline in virus concentration after repeated transfers. No virus particles were observed in callus tissue obtained from healthy grapevines.

Additional keywords: *Closterovirus*, *Vitis* spp., transmission electron microscopy.

RESUMO

Citopatologia de tecido caloso infetado pelo vírus do enrolamento da folha da videira 3

Análises citopatológicas do vírus do enrolamento da folha da videira 3 (*Grapevine leafroll-associated virus 3*, GLRaV-3), gênero *Vitivirus* foram feitas utilizando-se tecido caloso obtido *in vitro*, para investigar seu uso no estudo do vírus associado ao enrolamento da folha da videira (EFV). Seções ultrafinas foram feitas a partir de cultura de calos obtidos de hastes e brotos de videiras (*Vitis* spp.) infetadas pelo GLRaV-3. Dois tipos de calos foram observados. Um tipo transparente composto de células grandes e altamente vacuoladas contendo uma fina camada de citoplasma. Um outro tipo, de coloração marrom, composto de um grupo de células pequenas, compactamente arrançadas, cujo citoplasma era bem mais abundante. Nestas células foram observadas, em maior aumento, massas

formadas por fibras flexuosas com 10-12 nm de diâmetro, cujo aspecto corresponde ao das partículas de closterovírus em cortes de tecido. Grupos de vesículas também foram observados, com tamanhos variando entre 50-200 nm, formadas por uma única membrana e contendo material fibrilar muito fino, característico de infecções causadas por closterovírus. A concentração do vírus foi monitorada por testes de Immunosorbent electron microscopy (ISEM), os quais mostraram que a cultura de calos obtidos de videiras infetadas pôde ser usada para estudar os vírus associados ao EFV durante várias gerações, apesar da diminuição da concentração de vírus após repetidas transferências. Não foram observadas partículas de vírus em tecido caloso obtidos de videiras sadias.

INTRODUCTION

Grapevine Leafroll Disease (GLR) is one of the most important graft-transmissible diseases affecting grapevines (*Vitis* spp.) and occurs world-wide (Goheen, 1988). Symptoms include downward rolling and interveinal reddening or

yellowing of leaves in dark and white fruit-bearing varieties, respectively, resulting in reduced yield, delayed ripening and reduced accumulation of sugar (Weber *et al.*, 1993).

The etiology of GLR has not been completely clarified. Although spherical (Castellano *et al.*, 1983) and potyvirus-like particles (Tanne *et al.*, 1977) were reported to be associated with GLR, it is apparently caused by several serologically distinct phloem-limited closteroviruses, first described by Namba *et al.* (1979). Because Koch's postulates have not been completed with the viruses associated with

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GLR, they are referred to as Grapevine Leafroll-Associated Viruses (GLRaVs). So far, eight closteroviruses have been reported from GLR affected plants and designated as GLRaV-1 to 8 (Hu *et al.*, 1990; Boscia *et al.*, 1995; Choueiri *et al.*, 1996; Monis, 2000). Several antisera have been produced against the different GLR-associated closteroviruses (Zimmermann *et al.*, 1990b) and mixed infections were frequently detected (Zimmermann *et al.*, 1990a). More recently several of these closteroviruses have been partially sequenced, and many primers are available for use in RT-PCR tests (Saldarelli *et al.*, 1998; Zhu *et al.*, 1998; Martin *et al.*, 2000).

The family *Closteroviridae* consists of two genera, a monopartite genus named *Closterovirus* with *Beet yellow virus* (BYV) as a type member and a bipartite genus named *Crinivirus* with *Lettuce infectious yellows virus* (LIYV) as a type member. In a recent review on the genetic diversity of closterovirus, Karasev (2000) suggests a modification on the current closterovirus classification based on the type of vector transmission. This classification would consist of three genera, as follows: *Closterovirus* (aphids), *Vitivirus* (mealy bugs) and *Crinivirus* (whiteflies). According to this review, the grapevine closteroviruses would be part of the *Vitivirus* genus, having *Grapevine leafroll-associated virus 3* (GLRaV-3) as a type member. Grapevine closteroviruses are difficult to study, mainly because of their low concentration and uneven distribution of virus particles and also because of the presence of high concentration of phenolic compounds and tannins in *Vitis* spp. plants. These compounds are highly reactive with proteins, interfering in serological reactions and in virus purification. One way to overcome this difficulty is to use *in vitro* grown plants, which are regarded as having higher virus titer and lower tannin content (Barba *et al.*, 1989; Monis & Bestwick, 1996). On the other hand, the culture of callus tissue, also used for its low phenolic content, has been generally associated with a decrease in virus concentration (Mori & Hosokawa, 1977; Omura & Wakimoto, 1978) with possible application for obtaining virus-free plants.

In this study, we present evidence that *Grapevine leafroll-associated virus 3* (GLRaV-3) multiplies in the callus tissue obtained from infected grapevines, through several generations, showing the usefulness of callus tissue cultures for the study of grapevine viruses. A preliminary communication of these results was previously presented (Vega *et al.*, 1989).

MATERIAL AND METHODS

Plant materials and virus source

Symptomatic grapevine plants of the varieties Pinot Noir, Seibel 2 and LN 33, which tested positively for GLRaV-3 by indexing and serological procedures, were kept in the greenhouse and used as virus source for the experiments. Grapevine plants from the same varieties, tested negatively for GLRaV by the same procedures, were used as negative controls. Healthy plants obtained from seeds were also used

as negative controls.

In vitro culture of callus tissue

Vegetatively growing stems and shoots (1-2 cm) from infected and healthy vines were harvested from the above-mentioned plants and were surface-sterilized by 70% alcohol and commercial sodium hypochlorite (0.4%). After surface sterilization, each end of the stems was trimmed to eliminate the bleach damaged tissue and rinsed in sterile distilled water. The stems and shoots were cultured in vials in propagation media, according to Murashige & Skoog (1962), containing 20.0 ml l⁻¹ of MS inorganic salts, 30.0 g l⁻¹ sucrose, 8.0 g l⁻¹ agar, 0.1 g l⁻¹ myo-inositol, 1.0 g l⁻¹ casein, 0.3 mg l⁻¹ IAA, 1.0 mg l⁻¹ 2,4-D and 0.3 mg l⁻¹ kinetin. The cultures were maintained at 25 °C in the dark and transferred to fresh medium every month. After transferring to fresh medium, a new generation of callus tissue was formed.

In situ Electron Microscopy

Only callus tissue was used for cytopathological studies, avoiding explant tissue present in the initial callus generation. Samples were fixed with 4% paraformaldehyde and 1.25% glutaraldehyde, postfixed in 1% OsO₄, both in cacodylate buffer (0.05 M, pH 7.0), then contrasted *en bloc* with 2% uranyl acetate, dehydrated in acetone and dimethoxypropane and embedded in Epoxy medium. Sections were stained with lead citrate (1%) and observed with a ZEISS EM 10C transmission electron microscope.

Serological methods

Plants were tested by F(ab')₂ Enzyme linked immunosorbent assay (ELISA) according to Rowhani (1992) using polyclonal antisera provided by Dr. A. Rowhani and Dr. D. Golino (University of California-Davis, USA). Immuno Sorbent Electron Microscopy (ISEM) tests were used to detect GLRaV through the callus generations. Tests were done according to Milne & Lesemann (1984) with some modifications, using GLRaV-3 antiserum provided by Dr. Gugerli (Nyon, Switzerland). Copper grids covered with Parlodion and carbon films were coated using [1:1000] dilutions of antisera, during 60 min at room temperature. Grapevine callus tissue was extracted in 0.06 M phosphate buffer, pH 7.3, containing 1% PEG (Polyethylene Glycol MW 6,000) and 2.5% PVP (Polyvinyl Pyrrolidone MW 40,000). Samples were prepared using 2.0 ml of buffer per gram of tissue and were incubated on the grids overnight at 4 °C. After each step the grids were rinsed with PBS buffer and distilled water and prior to observation they were negatively stained with 2% uranyl acetate.

RESULTS

Cytopathology of grapevine callus

Callus proliferation from stems and shoots of all three grapevine varieties was mainly translucent and soft, composed of large loosely arranged cells containing big vacuoles and a thin layer of cytoplasm next to the cell wall.

Portions of this callus tissue were brown-coloured, composed of small compactly arranged cells with more abundant cytoplasm (Figure 1A). Cells of this compact callus showed, at higher magnification, that a great part of the cytoplasm was occupied by large masses of flexuous closterovirus-like particles, ca. 10-12 nm in diameter (Figure 1B). Regular cell organelles, such as mitochondria in groups or isolated (Figure 1C), and lipid droplets (Figure 1B) were seen embedded in the virus-particle masses. Vesicular bodies were frequently seen within the virus particles masses. These bodies are formed by single membrane limited vesicles with sizes ranging from 50-200 nm, containing very fine fibrillar material (Figure 1D). The above-described alterations were not observed in callus tissue obtained from healthy grapevine plants.

Monitoring virus concentration from callus tissue by ISEM

Closterovirus-like particles were detected by ISEM

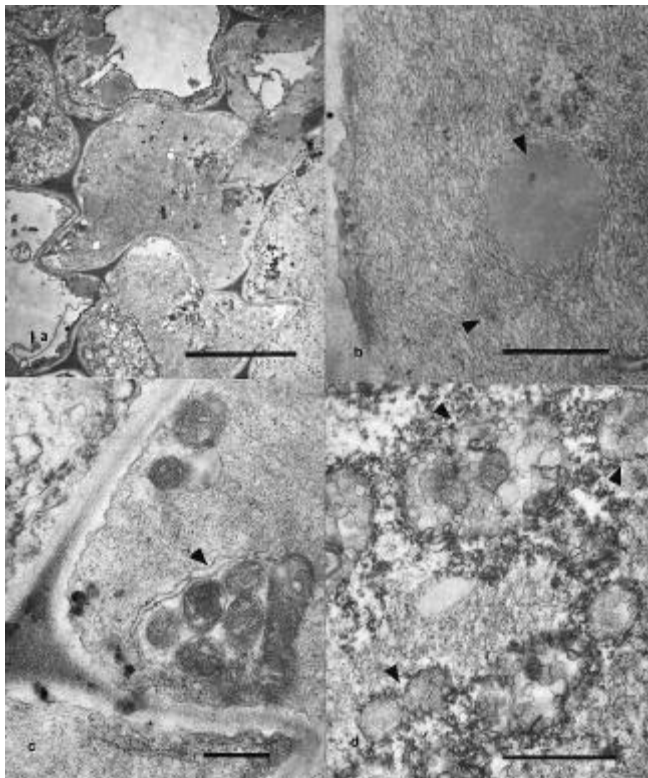


FIG. 1 - Transmission electron micrographs from infected grapevine (*Vitis* spp.) callus tissue. **A)** Groups of small cells of callus tissue from Grapevine leafroll disease (GLR) infected grapevines exhibiting a more abundant cytoplasm (Bar= 5 μm); **B)** Higher magnification of the cytoplasm from the cells in A, showing large masses of closterovirus-like particles (arrowhead) (Bar = 500 nm); **C)** Cells showing mitochondria in groups (arrowheads) and isolated embedded in the virus-particle masses (Bar= 500 nm). **D)** Groups of vesicles (arrowheads) showing some granular material surrounded by masses of virus particles (Bar= 1 μm).

using antibodies prepared for GLRaV-3 (Figure 2). The virus was detected by ISEM over approximately 15 generations and beyond that no virus particles were detected.

DISCUSSION

The cytopathology observed in the callus tissue obtained from GLRaV-3 infected grapevine plants is similar to that described for infected leaf-tissue (Faoro *et al.*, 1992). The masses of long and flexuous virus particles, next to vesicles containing fibrillar material are characteristics of closterovirus infected tissue (Francki *et al.*, 1985; Lesemann, 1988). The formation of characteristic vesicular bodies in grapevine plants infected by GLRaV-3 was described as starting from the vesiculation of modified mitochondria (Kim *et al.*, 1989; Faoro & Carzaniga, 1995). Our results do not show mitochondria in the vesiculation process, but the vesicular bodies and the fibrillar material seen in the interior of some group of vesicles could correspond to the final stage of mitochondria vesiculation. The finely fibrillar content of these vesicles has been shown to be RNA, which are probably replication sites of the virus (Faoro & Carzaniga, 1995).

This study shows that GLRaV-3 is present in callus tissue growing *in vitro* and that the virus persists through many successive generations. The distribution of the virus particles in the callus tissue appeared to be uneven, since high concentrations occurred only in some groups of cells. These results are in contrast with the predominant idea in the literature, where it is generally assumed that callus tissue is not appropriate for virus replication, and in fact is a possible application for obtaining virus-free plants (Omura & Wakimoto, 1978; Xu *et al.*, 2000). Our results indicate that

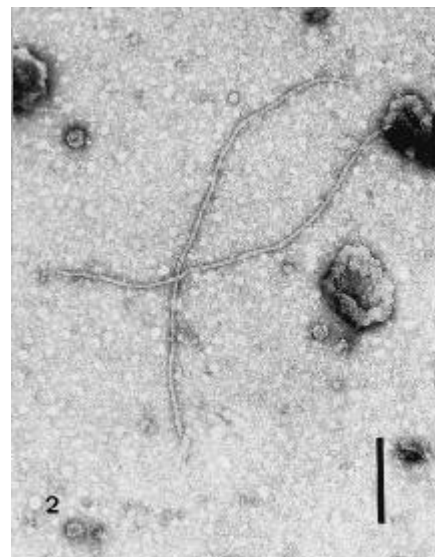


FIG. 2 - Electron micrograph of *Grapevine leafroll-associated virus-3* (GLRaV-3) particles trapped in ISEM test using homologous antibody (AS-GLRaV-3) (Bar = 250 nm).

callus may be useful for studying viruses in plants that have high contents of phenolic compounds, as in grapevine. Apparently, phenolics are in lower concentration in callus tissue, as suggested by the slower darkening of callus extracts, compared to leaf extracts. It is well known that phenolics readily oxidize and turn brown, generating products that form complexes with proteins, interfering in the recognition of viral proteins by antibodies (Croteau *et al.*, 2000).

Another possibility for the easier detection of GLRaV-3 in callus is that during callus formation, cell proliferation occurs from some tissues more than others. Michaux-Ferrière & Carron (1989) observed in *Hevea brasiliensis* (Muell-Arg.) that a compact type of callus was formed from perivascular cells, indicating the possibility that this type of callus contains a higher proportion of vascular-parenchymatous cells. Since closterovirus are limited to the phloem parenchyma, the resulting callus could have a higher proportion of virus-containing cells.

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