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Cytological studies of grapevine leafroll infected tissue: Further evidence of viroid etiology and improvement of diagnosis

by

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Cytologische Untersuchungen an blattrollkranken Reben: Weitere Beweise für die Viroidnatur des Erregers und Verbesserung der Diagnose

Zusammenfassung: In den Zellen blattrollkranker Reben wurden im wesentlichen zwei Arten von Einschlüssen gefunden, wie sie ähnlich bereits für *Gynura aurantiaca* D. C. bei Befall durch das Citrus-Exocortis-Viroid beschrieben worden sind. Hierbei handelt es sich a) um körniges Cytoplasma (RNA bzw. eine Anhäufung von Viroiden) und b) um einen proteinhaltigen Ring, der den befallenen Zellkern umhüllt.

Der mikroskopische Nachweis dieser Einschlüsse könnte die herkömmliche Technik der Pfropfübertragung auf Indikatorpflanzen ersetzen. Diese einfache cytologische Diagnose nimmt weniger als eine halbe Stunde in Anspruch; sie könnte für die Erzeugung gesunden Rebenpflanzgutes von Nutzen sein.

Die vorliegende Arbeit bestätigt mit Hilfe cytologischer Methoden die auf biochemischem Wege gewonnenen Ergebnisse einer unlängst veröffentlichten Untersuchung und liefert weitere Belege für die viroidbedingte Ätiologie dieser Krankheit.

Key words: leafroll, disease, cytology, symptomatology, selection, plant protection.

Introduction

Grapevine leafroll disease (GLRD), first described in Germany by SCHEU (1936), is one of the most world-wide spread and important grapevine diseases (2). All concerning this graft-transmissible disease has recently been reviewed (10) and, up to now, the virus etiology still remains uncertain.

In 1976, possible viroid etiology was suggested (5). Recently, a viroid and viroid-like RNAs in various GLR-infected varieties have been detected (4, 7, 9). Graft transmission to appropriate indicator plants is still used for GLRD diagnosis. This procedure is, however, extremely expensive and slow.

In infected tissues, histological and cytological changes were found, especially in the phloem fibrous masses (11). In the present paper, we report cytological aspects of several leafroll-diseased grapevine varieties in comparison with healthy plants.

Abbreviations used:

CEV	— citrus exocortis viroid	NII	— nuclear irregular inclusion
CGI	— cytoplasmic granular inclusion	PCI	— protein circular inclusions
GLR/D	— grapevine leafroll/disease	PSTV	— potato spindle tuber viroid
NGI	— nuclear granulated inclusion	RGA	— rounded granular aggregation.

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Material and methods

The GLR-infected varieties used as sources were, together with their origins: 1. Zinfandel (kindly sent by A. C. GOHEEN, University of California, Davis, U. S. A.); 2. Primitivo (kindly sent by V. SAVINO and G. P. MARTELLI, Bari, Italy); 3. Bobal (from Requena, Valencia); 4. Gran negra tinta condado (from Galicia); 5. Monastrell (from Jumilla, Murcia); 6. Ohanes negra (from Pulpi, Almeria); 7. and 8. Parellada and Xarelo (from Cataluña); 9. Perlette (from Jerez, Cádiz); and 10. Valenci negra (from Alicante). The last eight varieties had been field-selected, as severe sources of GLRD, by the senior author.

Healthy LN-33 indicator plants, grown in pots under virus-free conditions, were chip-bud grafted with the above GLRD sources. Ungrafted healthy LN-33 plants were used as controls. All plants were kept in a climatized and insect-proof greenhouse. Those GLR-inoculated plants which showed conspicuous GLR symptoms (practically 100 % infected) served as the GLR sources to be studied.

In a second experiment, the vegetative cycle of similar plants as above was accelerated. In order to confirm our observations under controlled conditions, a phytotron (Ecophyt, Vötsch) was used. On finalizing the dormant stage, healthy LN-33 controls and GLR-infected plants were placed in the climatic chambers under 5 klx and 60 % RH, other environment factors being for the 1st period: constantly 25 °C, 12 h light, 1 month; 2nd period: as above, but at 35 °C; 3rd period: 35 °C day/15 °C night, 14 h light, 2 months; 4th period: 20 °C day/5 °C night, 10 h light, 2 months.

Epidermal strips and sections were detached and stained either by Azure A or by O-G combination, mounted as described (3), and observed in a Zeiss III photomicroscope.

Results and discussion

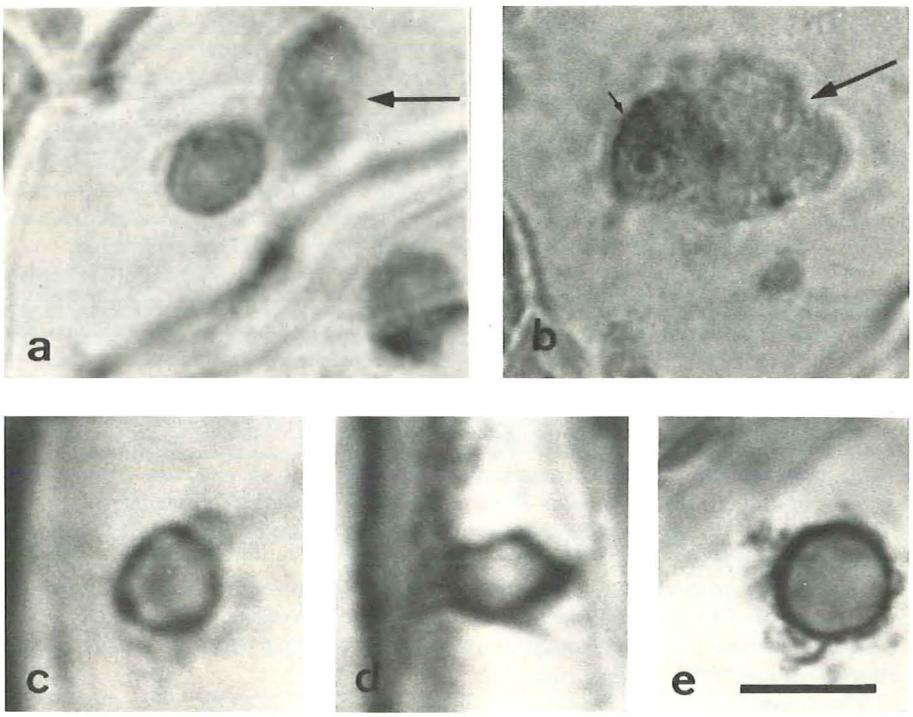
In the cytoplasm of the GLR-infected epidermal tissue stained with Azure A irregular inclusions (Fig. a, b) were observed, being granular vesiculated and stained red violet, thus revealing its RNA composition (3). These inclusions were exactly alike to the cytoplasmic granular inclusions (CGI), as described in the potato spindle tuber (PSTV) and citrus exocortis viroid (CEV)-infected cytoplasm stained with Azure A (6). We are of the opinion that these CGI are viroid derivatives or accumulations.

We observed many interior unstained nuclei of circular shape (Fig. c, d, e) in the Azure A stained GLR-infected cells. These unstained nuclei were surrounded by a dense blue peripheric ring composed of DNA (3).

In the GLR-infected tissue samples stained with the O-G combination (which stains proteins but not nucleic acids) no CGI were seen in the cytoplasms but, on the contrary, many nuclei showed faintly grayish green stained inclusions of circular shape (indicating protein composition). Although not as greenly stained as in the CEV-infected nuclei stained with the O-G combination (6), they were similar to the previous termed protein circular inclusions (PCI), as described for the CEV-infected nuclei (6). A few unstained nucleoplasms surrounded by a dense ring were also observed as in CEV-infected tissue stained either by Azure A or by O-G combination (6).

The healthy control tissue samples stained either by Azure A or by O-G combination did not reveal any of the above mentioned inclusions.

In the first stage of growth, exactly when the leaves expand and before the inflorescences have appeared (corresponding BAGGIOLINI's stage E (1)), the maximum of



GLR-infected cells stained with Azure A: a) Beside the nucleus a cytoplasmic granular inclusion (CGI) can be observed (arrow). This CGI — RNA — could be a viroid aggregation. In the nucleoplasma, a faint round granular aggregation (RGA) can be observed. — b) Large CGI (see large arrow) surrounding part of nucleus. A nuclear granulated inclusion (NGI) can be observed (small arrow). — c, d, e) A blue dense — DNA — peripheric ring enclosing circular inclusion (CI) can be observed on the nucleus. With O-G combination, this stains faint grayish green, indicating protein content (PCI, see text). CI is not stained with Azure A. Similar protein circular inclusions (PCI) can be observed in collenchyma cells: c) from a cryostat midrib section, and in epidermal cells: d) from a freehand petiole section and e) from a leaf strip. — Scale bar 10 μm.

GLR-infizierte, mit Azur A gefärbte Zellen: a) Neben dem Kern ist ein cytoplasmatischer granulärer Einschluß (CGI, Pfeil) zu erkennen. Bei diesem Einschluß — RNA — könnte es sich um eine Ansammlung von Viroiden handeln. Im Nucleoplasma ist eine blaßgefärbte runde granuläre Aggregation (RGA) vorhanden. — b) Ein großer Einschluß (CGI, großer Pfeil) liegt einem Teil des Zellkerns an. Ein nukleärer granulärer Einschluß (NGI, kleiner Pfeil) ist sichtbar. — c, d, e) Der Zellkern ist von einem blaugefärbten dichten peripheren Ring — DNA — umhüllt, der einen ringförmigen Einschluß (CI) umgibt. Dieser Einschluß, der sich mit Azur A nicht anfärbt, wird durch die O-G-Kombination graugrün gefärbt, ein Hinweis auf Protein (PCI, s. Text). Ähnliche proteinhaltige ringförmige Einschlüsse (PCI) können sowohl in Collenchymzellen: c) aus einem Gefrierschnitt durch den medianen Blattnerv, als auch in Epidermiszellen gefunden werden: d) aus einem Handschnitt durch einen Blattstiel, e) aus der abgezogenen Blattepidermis. — Maßstab: 10 μm.

inclusions (CGI and PCI) was observed (in our greenhouse in the middle of March). From March to October, inclusions were extremely seldom to be found. Only during the period corresponding to the apparition of GLR leaf symptoms until leaf fall, the CGI and PCI reappeared in the Azure A strips taken from young and tender infected leaves.

The results of the forced plants in the phytotron, with reference to the greenhouse cultivated plants, confirm the above findings. The maxima of inclusions (CGI and PCI) fell on the 1st and 4th period and their almost absence was noticed during the 2nd and 3rd period.

In the cryostat or freehand sections stained with Azure A, detection of PCI in the nuclei was easier than of CGI in the cytoplasms. PCI were seen in considerable higher quantity in the collenchyma (Fig. c) than in the epidermal cells (Fig. d and e).

Several mounted GLR-infected preparations stained with Azure A, in which we had observed numerous PCI, were demounted. The tissues were rinsed in absolute ethanol (for discolouring) and then restained with O-G combination. These restained and newly mounted preparations revealed faint grayish green circular inclusions, indicating and confirming their correct identification as PCI. No CGI were observed.

While searching for other inclusions, we observed a few nuclear granulated inclusions (NGI) in Azure A preparations (Fig. b) similar to those described for PSTV (6). Also a few rounded granular aggregations (RGA, Fig. a) and fewer small nuclear irregular inclusions (NII) than observed in the CEV-infected nuclei stained with Azure A (6) were found.

Because of the presence of NGI, the infection process as suggested for the PSTV could be also valid for the present viroid.

We detected the viroid-like inclusions (CGI and PCI) in all leaf cells (stained with Azure A) of the 10 GLRD sources. None were found in healthy controls, supporting cytologically the previous biochemical studies (4, 7, 9) which evidence a viroid etiology for GLRD.

The inclusions described in this study suggest that the grapevine viroid discovered is related to the CEV. It is of interest that recently an infectious RNA viroid has been detected in several GLR-infected grapevines (4) resembling by various physical and biological properties the CEV.

The present cytological diagnosis is simple, rapid and economic. Our suggestion is to search for PCI rather than for CGI. The previously mentioned two periods are the best time for their detection.

This report may help to clarify the etiology of GLRD, which cannot be completely defined until KOCH's postulates are fulfilled.

Summary

In the cells of leafroll-infected grapevines mainly two kinds of inclusions were found, similar to those as previously described in citrus exocortis viroid-infected *Gynura aurantiaca* D. C. cells: cytoplasmic granular inclusions (presumably viroid derivatives or aggregations) and protein circular inclusions occupying the infected nucleoplasms. The observation of these inclusions could substitute the traditional leafroll indexing technique. The duration of this simple cytological diagnosis is approximately 30 min; it could be an important aid for the programs of sanitary selection or sanitation of the grapevine. The study confirms cytologically a recent paper based on biochemical techniques, contributing further evidence for the viroid etiology of this disease.

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