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VIRUS-LIKE PARTICLES AND VESICULATED BODIES IN LEAFROLL AND CORKY BARK DISEASED GRAPEVINES

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SUMMARY

Grapevine material of the varieties Rufete and Pinot noir, showing reddening and downrolling of the leaves, gave positive results for leafroll associated closterovirus type III, when tested by direct enzyme-linked immunosorbent assays.

Ultrastructural studies of tissues from these plants revealed the presence of flexuous, rod-shaped virus-like particles, approximately 10-12 nm in diameter and with undetermined lenght, in the sieve elements.

Clusters of vesiculated inclusions, sometimes containing numerous thread-like fibrils of unknown nature were observed in phloen cells. No particular organelle was found to be involved in the origin of these bodies, but the accumulation of small vesicles was detected in the periphery of altered plastids and in some mitochondria.

Closterovirus-like particles and vesiculated inclusions were also observed in LN-33 plants infected with leafroll type III and corky bark. Small isometric particles, possibly of viral nature were also detected in the lumen of mature sieve tubes.

INTRODUCTION

Grapevine leafroll is prabably the most important and world widespread virus disease of grapevine, causing erratic bearing, lowered sugar content of the fruits and delayed ripening of the crop.

Symptoms of the disease on red varieties are reddening and downrolling of the leaves while the veins remain green. Leafroll is transmissible by grafting healthy vines with infected scions or rootstocks, but the etiology of the disease has not been determined yet. Closterovirus-like particles have been

observed by several authors and at least five serotypes have been associated with this disease (Gugerli *et al.*, 1984; Iwanami, 1987; Kim *et al.*, 1989; Zimmerman and Walter, 1990). Poty-virus-like particles (Tanne *et al.*, 1978) and small-isometric-virus-like particles (Castellano and Savini, 1983) have also been reported.

Corky bark (casca encortiçada), although not so common as leafroll, occurs in most grapevine growing countries including Portugal (Pedroso, 1987; Pedroso and Sequeira, 1982, 1983; Pedroso *et al.*, 1985): The vines affected by this disease are stunded and show delayed shoot growth in spring. During summer the leaves roll downwards and become red or bronzish red but unlike leafroll this colour spreads over the major veins. When buds from infected plants are grafted to LN-33 (Couderc 1613 × Thomson seedless) indicator plants, characteristic swelling and cracking of the internodes develop in one year old canes. Although closterovirus-like particles have been associated to corky bark (Fortusini *et al.*, 1989) the etiology of this disease is still uncertain (Tanne and Meir, 1990).

Because of the unsuccess of mechanical transmission of a viral agent to herbaceous hosts, electron microscopy studies of infected grapevine tissues, together with healthy controls, was carried out to investigate the presence of cytopathic effects and virus-like particles.

MATERIALS AND METHODS

Source material

Grapevine of the variety Rufete naturally infected with leafroll associated closterovirus type III.

Grapevine of the variety Pinot noir experimentally infected with leafroll type III by greengrafting two buds from an infected source.

Grapevine of the variety LN-33 (Couderc 1613 × Thomson seedless) greengraft infected with leafroll type III and corky bark.

Healthy plants indexed on woody indicators were found to be free from all known grapevine virus diseases (Pedroso and Sequeira, 1982).

All vines were self rooted in containers, in greenhouse conditions at Estação Agronómica Nacional, Oeiras.

Identification of viruses

For virus identification, plant extracts were tested against antisera to grapevine associated closterovirus types I and III (supplied by Dr. P. Gugerli) using enzyme-linked immunosorbent assay — ELISA (Clark and Adams, 1977).

For transmission electron microscopy, small 1-2 mm pieces of leaf and stem tissues were collected in June and in September/ /October and dipped in 6 % sodium-cacodylate-buffered glutaraldehyde pH 7.2 at 4° C fixed overnight and postfixed in 2 % osmium tetroxide for 2 hours at room temperature. Dehydration was performed in a graded alcohol series (20 %, 50 %, 70 % and 90 % for 10 minute periods and 100 % in three 15 minute periods). The tissues were then embedded in Epon, in special, rubber moulds, which were kept at 70° C during 24 hours for polimerization. Thin sections were cut with an LKB ultrotome IV using glass knives obtained with an LKB 7800 knifemaker. The sections were placed in formvar coated copper grids, stained with 1 % uranyl acetate and 0.3 % lead citrate, and observed in a Philips EM 300 electron microscope at 80 KV. For direct observation of particles, drops of leaf extracts were placed on grids and stained.

RESULTS

In leaf tissue and in one year old stems, collected from plants of the varieties Rufete and Pinot noir infected with leafroll associated closterovirus type III detected by ELISA, rod-shaped filamentous virus-like particles were detected consistently in the phloem cells of the veins and on the secondary phloem.

The particles were approximately 10-12 nm in diameter and their lenght was undetermined. Frequently, the particles looked parallel to each other in a wavy pattern, in large membrane bound aggregates (Fig. 1). These aggregates were detected mainly in the sieve elements, but could also be found in the cytoplasm of other phloem cells including companion cells. Only part of the sieve elements contained virus-like particles, and in most veins only a few showed aggregates.

LN-33 plants infected with the same leafroll type plus corky bark contatined similar rod shaped particles and, in some

sieve elements, a dark, electron dense and granular content that was not detected in healthy controls. At higher magnification, numerous small isometric virus-like particles were observed.

The detailed study of a high number of thin sections of leaf veins from healthy and infected LN-33 tissues led to the conclusion that only in an advanced stage of maturity, the differences between normal and altered sieve elements become apparent.

In the normal sieve elements, during the process of differentiation, the tonoplast was ruptured, the nuclei disappeared, and the sieve plates, multiple in *Vitis vinifera* (Vasconcellos, 1948) and the plasmodesmata were formed. The content of the sieve elements was then limited only by the plasmalemma and became very electron transparent due to the drastic reduction in contrastable elements with absence of ribosomes (Gunning and Steere, 1975). Of the celular organelles, only the plastids with a very reduced membranous system, and some mitochondria were present.

The fibrous structure of P-protein was more obvious (Esau, 1972; 1978). Many electron dense spherical particles with a diameter of about 16-20 nm remained in the cell lumen of some sieve elements when infected tissues were observed. Clusters of similar spherical particles were also detected in the cytoplasm of companion cells and adjacent phloem parenchyma cells (Fig. 2).

The ultrastructural observation of phloem tissues of the leaves and one year old canes of infected plants led to the detection of membranous inclusions with sizes ranging from those of mitochondria to those of chloroplasts. These inclusions were detected in phloem parenchyma cells, sieve tubes and companion cells, generally isolated in the cytoplasm or between the cell wall and plasmalemma (Fig. 3). Sometimes, two superposed membranes enveloped the inclusion. The exterior membrane was then in continuity with the tonoplast and the interior one with the plasmalemma. The observed inclusions consisted of numerous spherical or tubular single-membrane vesicles with a diameter of about 50-200 nm enveloped by a membrane about 13-16 nm thick. Frequently, a vesicle of larger dimensions occupied a more or less central position. Some inclusions con-

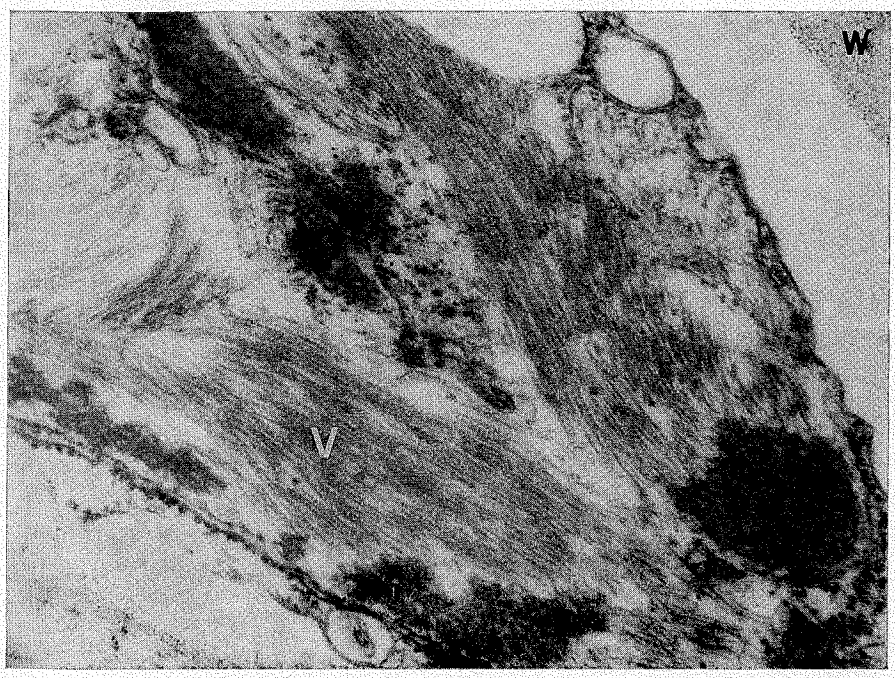

Fig. 1 — Aggregates of flexuous rod-shaped closterovirus-like particles (v) in grapevine thin sections. Leaf midvein phloem cells. Plant of the variety Pinot noir with leafroll type III detected by indexing on woody indicators and enzyme-linked immunosorbent assay (ELISA). Filamentous particles in longitudinal section are arranged parallel to one another in ondulated patterns. W, cell wall. × 107 000.

Agregados de partículas flexuosas em forma de bastonete do tipo closterovírus em secções ultrafinas do floema de nervura principal de folha de videira. Planta da casta Pinot noir infectada com enrolamento foliar do tipo III detectado por indexagem em indicadores lenhosos e através do teste ELISA (enzyme-linked immunosorbent assay). Em secção longitudinal as partículas filamentosas apresentam-se paralelas umas às outras e onduladas. W, parede celular. × 107 000.

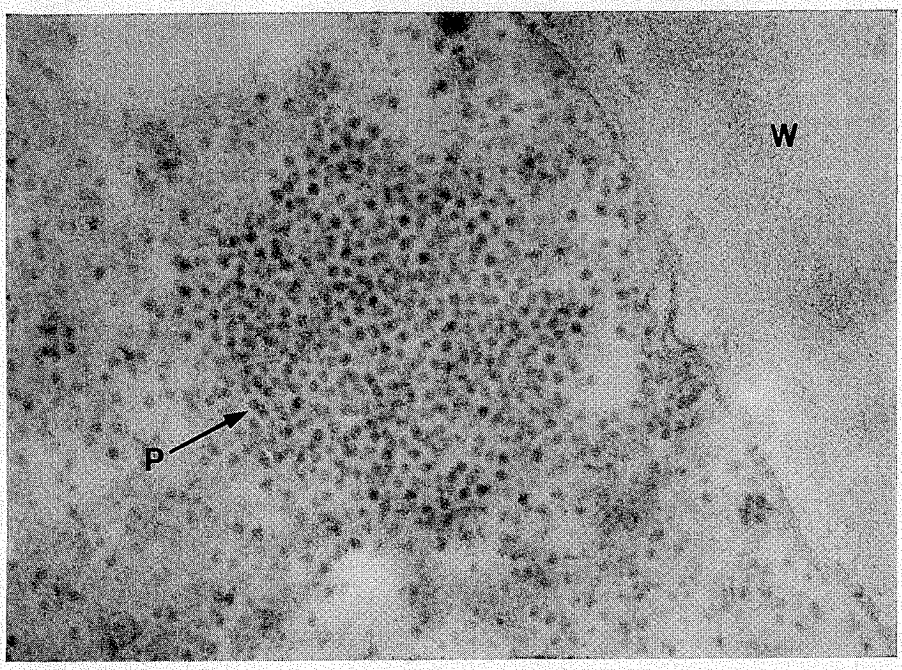

Fig. 2 — Isometric virus-like particles (P) in the cytoplasm of a phloem parenchyma cell of a leaf vein infected with leafroll III plus corky bark. W, cell wall. × 92 000.

Partículas isométricas do tipo viral (P) no citoplasma duma célula do parênquima floémico duma folha infectada com enrolamento foliar do tipo III e com casca encortiçada. W, parede celular. × 92 000.

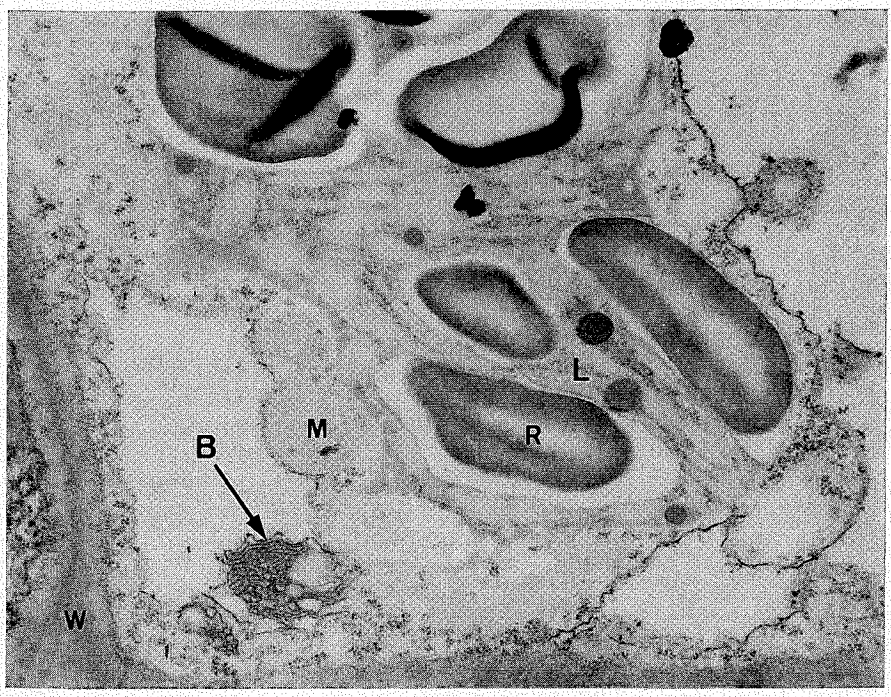

Fig. 3 — Vesiculated body (B) with a membranous envelope and an electron lucent core has the approximate size of a mitochondrion (M). Plastids (L) with large starch grains (R) and distorted thylakoids (T) can be observed. W, cell wall. × 19 000.

Estrutura vesiculada (B) com envólucro membranoso e um anel central electronicamente transparente, com as dimensões aproximadas duma mitocôndria (M). Podem-se observar plastídeos (L) com grandes grãos de amido (R) e tilacóides distorcidos (T). W, parede celular. × 19 000.

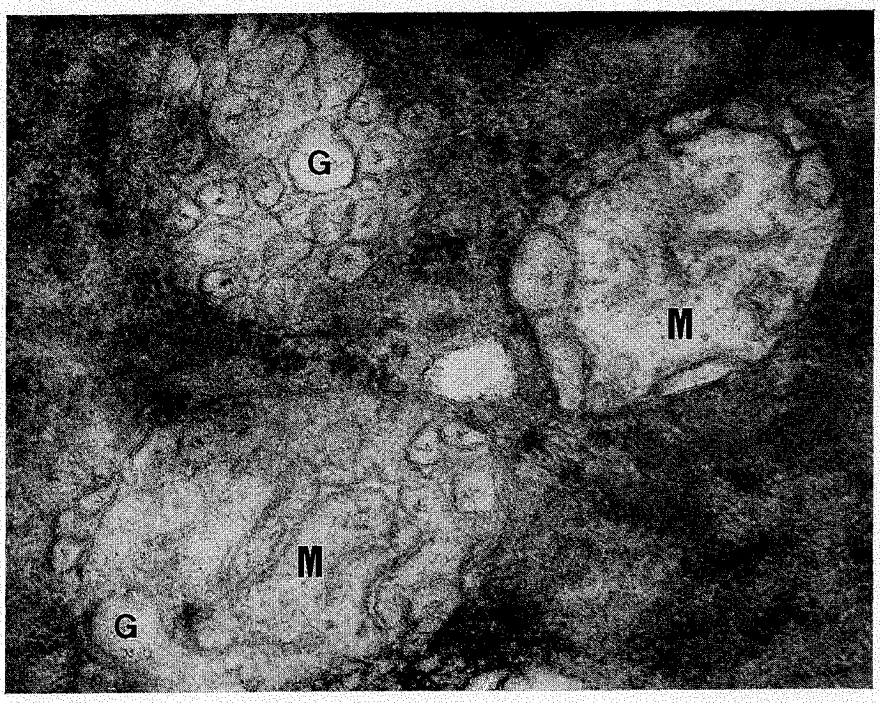

Fig. 4 — Vesiculated organelles in phloem cells of LN-33 leaves infected with leafroll III plus corky bark. M, mitochondria, G, vesicles. × 137 000.

Organitos vesiculados em células floémicas de folhas de LN-33 infectadas com enrolamento do tipo III e com casca encortiçada. M, mitocôndria, G, vesículas. × 137 000.

tained a finely stranded fibrilar material that resembled nucleic acid.

No particular organelle has been recognized to originate these vesicular bodies, but the accumulation of small vesicles in the periphery of much altered plastids and inside mitochondria (Fig. 4) was detected.

DISCUSSION

The occurrence of virus particles in more or less organized aggregates is one of the main aspects for describing the cytopathology of several plant virus groups.

The closteroviruses are economically a very important group of viruses (Lister and Bar-Joseph, 1981). Members of this group have long thin, very flexuous particles containing one single stranded RNA of variable size (Milne, 1988).

Typical closteroviruses are characteristically located in the phloem of their hosts, where large virus particle aggregates are produced, often forming banded inclusions (Hoefert *et al.,* 1970; Esau and Hoefert, 1971).

In recent studies, the cause of leafroll has been associated to the presence of one or more closteroviruses, at least five being presently known. These strain types are morphologically distinct and have particle modal lenghts from 800 nm (Rosciglioni *et al.,* 1983) to 2200 nm (Gugerli *et al.,* 1984) and an approximate diameter of 12 nm (Kim *et al.,* 1989).

In this study, large masses of long flexuous rod-shaped virus-like particles with a diameter of about 12 nm and undetermined lenght in banded aggregates and sometimes inside membrane bound inclusions were observed, mostly in sieve elements, but also in companion cells and other phloem cells, of grapevine varieties with leafroll or leafroll plus corky bark. These particles were not detected in healthy grapevines.

Numerous clusters of small vesicles, frequently envolved by a membrane and containing fine fibrils of unknown nature were also observed, only in the phloem of infected plants, which are thought to be cytopathological effects of virus infection. Although extensively vesiculated plastids were frequent, the origin of these inclusions could not be associated to cell orga-

nelles or other membranous systems such as endoplasmic reticulum or Golgi bodies (Pedroso, 1987).

These results are similar to those reported by Esau and Hoefert (1971) in their study of beet yellows virus (BYV) the type member of closteroviruses. In infected plants, vesicles occur in groups within membrane compartments whose origin is not clear and that are known as «BYV-type vesicles». The authors postulated a *de novo* synthesis of the vesicles, to enclose the characteristic fine fibrils of nucleic-acid material.

Vesiculation has been reported previously in grapevines with leafroll symptoms. In phloem tissues of diseased grapevines, Castellano and Savino (1983) have shown that vesiculated bodies in association with isometric virus-like particles, ordinarily developed from modified mitochondria, sometimes from chloroplasts, and consisted characteristically of double-membrane vesicles.

Closterovirus-like particles were associated to single vesicles of mitochondrial origin by Kim *et al.* (1989), in their study of leafroll affected grapevines. Enzyme-linked immunosorbent assay (ELISA) indicated that the virus was serologically related to a New York isolate (type III) closterovirus. Most of the vesicles contained electron-dense fine fibrils considered characteristic of closterovirus infection.

The vesicles that were observed in this study apparently belong to this second group, except for the absence of a clear relationship to mitochondria or other membranous systems (Pedroso, 1987), which seems to be in agreement with the conclusions of Esau and Hoefert (1971) for BYV above mentioned.

In the present investigation, the possible etiological association of closterovirus type III with the leafroll syndrome studied and the presence of closterovirus-like particles is considered. Nevertheless, the morphological and serological relationship detected needs direct evidence from infectivity assays.

Studying the distribution of beet western yellows virus in beet leaves, Esau and Hoefert (1972) concluded that, in a given leaf, the small spherical particles of the virus appeared initially in the sieve elements, where they become very conspicuous even under low magnification, due to the absence of ribosomes, and moved later to the companion cells. Some times, in appa-

rently normal sieve elements, the particles were detected close to the cell wall. Esau and Hoefert (1972) point out that with enough experience it is possible to distinguish between viral particles and ribosomes, after observing the virus in well developed sieve elements (where ribosomes are absent) and comparing them, afterwards, with the ribosomes of adjacent parenquimatous cells. They concluded that the viral particles were slightly larger and more electron dense than ribosomes, and had better defined shapes.

The small spherical particles, with a diameter of about 16-20 nm, observed in sieve tubes and other phloem cells of LN-33 plants infected with leafroll and corky bark, and not in healthy controls, were in the size range of cytoplasmic ribosomes. Indexing on woody indicators (Pedroso and Sequeira, 1982), as well as mechanical transmission to herbaceous hosts and ELISA tests, gave negative results to grapevine fan leaf (Pedroso, 1987) and bulgarian latent virus infections. However, the generally accepted absence of ribosomes in mature sieve tubes (Milne, 1967; Esau and Hoefert, 1972; Gunning and Steere, 1975; Hatta, 1976) and the improbable arrangement of ribosomes in clusters, in the cytoplasm of parenchyma cells, strenghtens the possibility of a viral nature for the observed structures.

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RESUMO

A presença de partículas virais e corpos multi-vesiculares em videiras infectadas com as doenças do enrolamento e da casca encortiçada

O enrolamento foliar é uma das doenças da videira com maior importância económica e mais disseminadas que se conhece. Vários autores têm referido a existência de partículas em forma de bastonete e isométricas associadas a esta doença. Ainda não foi conseguida a transmissão do patogénio causador desta doença a hospedeiros experimentais e, portanto, a sua etiologia ainda se encontra mal esclarecida.

No presente estudo observaram-se ao microscópio electrónico cortes finos de tecidos de videira das variedades Pinot noir e Rufete onde, através da utilização da técnica ELISA (enzyme-linked immunosorbent

assay) se verificou existir antigénio do closterovirus do tipo III associado ao enrolamento da videira (GLRV III).

Foram observadas partículas semelhantes a closterovírus, por vezes em grandes agregados envolvidos por uma membrana, formando arranjos de partículas paralelas, em tubos crivosos de plantas infectadas e não em plantas sãs.

Detectaram-se, em células do floema, inclusões constituídas por uma membrana, por vezes contendo numerosas fibrilas de natureza desconhecida, com dimensões situadas entre as dos mitocôndrios e as dos plastídeos. Não se verificou o envolvimento de nenhum tipo de organito celular na origem dessas inclusões, embora se tenha observado a acumulação de pequenas vesículas na periferia de plastídeos e de mitocôndrios alterados.

Foram também observadas inclusões vesiculares em plantas com enrolamento foliar e casca encortiçada, outra doença da videira de etiologia desconhecida com dispersão à escala mundial, mas menos frequente do que o enrolamento. Nestas plantas encontraram-se ainda partículas isométricas do tipo viral, no lúmen de elementos crivosos bem diferenciados, e no citoplasma de células parenquimatosas do floema.

RÉSUMÉ

Particules de virus et corps multivesiculaires dans des plantes de vigne atteintes des maladies de l'enroulement et de l'écorce liegeuse

L'enroulement (leafroll) est parmi les maladies de la vigne connues, l'une des plus importantes économiquement et les plus disséminées. Plusieurs auteurs ont referé l'éxistence de particules allongées et isometriques associées à cette maladie. La transmission de l'agent patogenique responsable pour cette maladie à des hôtes experimentels na pas encore eté reussie et en conséquence son etiologie est encore mal éclaircie.

Dans le present étude on a observé au microscope électronique des coupes de tissu de plantes des varietés Pinot noir et Rufete contenant le closterovirus du type III associé au leafroll de la vigne (GLRV III) determiné par la technique ELISA (enzyme-linked immunosorbent assay).

Des particules du type closterovirus ont eté observées, parfois en grandes masses de particules paralleles entourées par une membrane, dans les tubes criblés de plantes infectées et pas dans les plantes saines. Dans les cellules du floéme, on a observé des inclusions constituées par des groupes de vésicules frequemment entourées par une membrane, parfois contenant de nombreuses fibrilles de nature inconnue, avec des dimensions situées entre celles des mitochondries et des plastids. Aucun organite cellulaire n'a pas été associé à l'origine de ces inclusions quoique on aille observé l'accumulation de petites vésicules dans la periférie de plastides et de mitochondries altérés.

Des inclusions vésiculaires ont aussi éte observées dans des plantes avec leafroll et écorce liegeuse (corky bark), une autre maladie de la vigne distribuée à l'échelle mondiale avec une étiologie mal connue. Dans

ces plantes on a observé des particulles isometriques du type viral dans les tubes criblés et dans le cytoplasme de cellules parenquimateuses du floème.

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