DOI: 10.1590/1808-1657v71p4572004

CYTOPATHIC FEATURES INDUCED BY CALADIUM VIRUS X (*POTEXVIRUS*) IN *CALADIUM BICOLOR* AND *GOMPHRENA GLOBOSA*

E.B. Rivas[1], S.R.Galleti[1], L.M.L. Duarte[1], M.A.V. Alexandre[1], M.E.M. Estelita[2]

[1]Instituto Biológico, Centro de Pesquisa e Desenvolvimento de Sanidade Vegetal, Av. Cons. Rodrigues Alves, 1252, 04014-002, São Paulo, SP, Brasil. E-mail: rivas@biologico.sp.gov.br

ABSTRACT

Dasheen mosaic virus (DsMV), a *Potyvirus*, and a *Potexvirus* (herein tentatively named Caladium virus X, CalVX) were found naturally infecting *Caladium bicolor* (Araceae) with chlorotic and necrotic spots and rings. Electron microscopy observations from infected leaves revealed cytoplasmic cylindrical inclusions, induced by DsMV, as well as masses of elongated particles and virus-like particles scattered in the cytoplasm. In *Gomphrena globosa* foliar cells experimentally infected by CalVX, electron-dense crystalline inclusions, without elongated particles, were also present in the cytoplasm and nucleus. Complex inclusions which consisted of a central, stained faint yellow area surrounded by groups of dense, olive-green stained, bodies were frequently observed in epidermal cells when stained with calcomine orange-luxol brilliant green combination. These inclusions correspond to a cytoplasm region rich in vesicles, ribosomes, scattered virus-like particles and large virus aggregates.

KEY WORDS: *Caladium bicolor*, Araceae, *Potexvirus*, cytopathic alterations, electron microscopy, light microscopy.

RESUMO

ASPECTOS CITOPÁTICOS INDUZIDOS POR CALADIUM X (*POTEXVIRUS*) EM *CALADIUM BICOLOR* E *GOMPHRENA GLOBOSA*. *Caladium bicolor*, com manchas e anéis cloróticos e necróticos nas folhas apresentou-se, naturalmente, infectado por 2 vírus de partículas alongado-flexuosas: um *Potyvirus* (*Dasheen mosaic virus*, DsMV) e um *Potexvirus* (tentativamente, denominado Caladium virus X, CalVX). Observações ao microscópio eletrônico de transmissão revelaram, em tecidos foliares naturalmente infectados, a presença de inclusões cilíndricas, induzidas pelo DsMV, assim como partículas dispersas no citoplasma ou formando massas, induzidas pelo CalVX. Em células de tecido foliar de *Gomphrena globosa*, experimentalmente infectada com CalVX, também a presença de inclusões cristalinas elétron-densas, sem patículas virais, no citoplasma e no núcleo foram observadas. Inclusões complexas, as quais consistiam de uma área central corada de amarelo claro circundada por grupos de corpos densos corados de verde-oliva foram, freqüentemente, observadas em células epidérmicas quando coradas com a combinação de corantes "calcomine orange-luxol brilliant green". Tais inclusões correspondiam, provavelmente, a regiões do citoplasma ricas em vesículas, ribossomos, partículas dispersas no citoplasma e grandes agregados de partículas virais.

PALAVRAS-CHAVE: *Caladium bicolor*, Araceae, *Potexvirus*, alterações citopáticas, microscopia eletrônica, microscopia de luz.

INTRODUCTION

The Araceae comprises a large family of herbaceous monocots that occurs in all continents, except the Antarctic, and has two main centers of distribution – tropical America and tropical Asia (CROAT, 2000).

Caladium, an aroid native to South America, is grown as an ornamental due to its large, colorful, conspicuously veined and generally heart-shaped leaves (MILLER, 1997). It is used as a foliage plant, indoors and out, and as cut leaves due to its longevity in arrangements (MILLER, 1997). In Brazil, the German Adolpho Lietze has been recognized as the great hybridist of caladiums, who in the late 19th and early XX centuries produced more than 600 varieties that were spread over Brazil and the whole world (FIGUEIREDO, 1936). This hybridist used

[2]Universidade de São Paulo, Instituto de Biociências, São Paulo, SP, Brasil.

Caladium bicolor (Ait.) Vent, and its 38 natural varieties, as the main crossing ancestral base (FIGUEIREDO, 1936). Despite the wide range of the foliar color patterns from Brazil, presently 95% of commercialized caladiums in the world come from Florida, USA (ZETTLER & HARTMAN, 1987).

Viral diseases have been reported as limiting factors for caladium production (ZETTLER & HARTMAN, 1995). The only virus naturally occurring in *Caladium* is *Dasheen mosaic virus* (DsMV) (ZETTLER & HARTMAN, 1995). Indeed, DsMV is considered the most prevalent and widespread virus among cultivated aroids (ZETTLER & HARTMAN, 1995).

From a house garden, a plant of *C. bicolor* with conspicuous virus-like symptoms was submitted to lab assays, which revealed the presence of DsMV and a putative novel *Potexvirus* species, tentatively named 'Caladium virus X' (CalVX) (RIVAS et al., 2004).

In this paper, we describe the inclusion types induced by CalVX in foliar cells from natural and experimentally infected plants.

MATERIAL AND METHODS

The cytopathic effects induced by CalVX were observed in leaf tissues from naturally infected *Caladium bicolor*, showing foliar chlorotic spots and rings and necrotic rings (Fig. 1), as well as necrotic, red halo-surrounded spots from 14-day inoculated *Gomphrena globosa* (Fig. 2). Since healthy, naturally growing *C. bicolor* could not be found, no cytopathic comparison was possible.

Fragments (2 mm x 2 mm) from infected or healthy *G. globosa* leaves, and also from naturally infected *C. bicolor*, were fixed at 4° C for 12h in 2.5% glutaraldehyde in 0.1 M phosphate (pH 7.0), post-fixed at the same temperature for 2h in 0.5% OsO_4 in the same buffer, stained / fixed "en bloc" overnight at 4° C in 2% uranyl acetate and dehydrated in graded acetone dilutions before embedding in Spurr medium (BOZZOLA & RUSSEL, 1999). Thin sections were post-stained in uranyl acetate and lead citrate, prior to observation under a Philips EM 208 transmission electron microscope.

For light microscopy observations, freehand razor-cut paradermal sections of the lower foliar epidermis from infected and healthy *G. globosa* were used as fresh material. The epidermal strips were stained in azure A stock solution mixed with 0.2y M dibasic sodium phosphate, in a ratio of 9:1, or in a mix of Luxol brilliant green BL, calcomine orange 2 RS and distilled water in a ratio of 8:1:1, respectively. The stain stock solutions were prepared in 2-methoxyethanol, according to CHRISTIE & EDWARDSON (1986). After 10-15 min, the excess of stain was removed by three quick changes of 95% ethanol, the strips mounted in a drop of 60% glicerine, and the slides examined with a Zeiss Jenaval microscope equipped with a differential interference contrast (DIC) apparatus.

RESULTS

Ultrastructural observations of *C. bicolor* infected leaf tissues revealed the presence of the so-called cytoplasmic cylindrical inclusions, typical of *Potyviridae* infection, sectioned at different angles. The various shapes of the cylindrical inclusions, namely scrolls and laminated aggregates, were interspersed among other cytoplasmic organelles such as mitochondria, endoplasmic reticulum, peroxisome, and chloroplasts (Fig. 3A); virus-like particles were observed in parallel array to the axis of transversally sectioned scrolls (Fig. 3A). These inclusions were more frequently found in palisade than in spongy parenchyma cells.

In addition to these cylindrical inclusions, fibrous masses (Fig. 3B) and cylindrical inclusions were seen in distinct cytoplasmic regions of the same cell or, more frequently, they were detected separately in different cells. Membranous structures (Fig. 3B) and the so-called fibrous masses which represent ordered virus aggregates (Fig. 3B) were found in cytoplasm regions containing large numbers of double membrane vesicles with electron-dense contents. Virus-like particles were scattered in the whole cytoplasm, closer to (Fig. 3A) or far from (Fig. 3C) cylindrical inclusions.

In naturally infected cells, some chloroplasts showed grana and thylacoidal stroma indistinguishable from each other, while others showed localized swelling of the thylacoids (Fig. 3A); the rough and smooth endoplasmic reticulum were also swollen (Fig. 3A, C); primary and secondary plasmodesmata appear to be altered (Fig. 4) and some of them were close to groups of double membrane vesicles (Fig. 4A), which could represent transversal sections of plasmatubule or paramural bodies. Three features could be observed in naturally infected leaf cells from *C. bicolor*: osmiophilic bodies, with or without crystals (Fig. 5A); amorphous X-bodies (complex inclusion) composed by globular and fibrous osmiophilic bodies (Fig. 5B); and an elongate structure surrounded by a unit membrane, which extends a long distance into the cytoplasm and contains reticulate and parallel internal organization (Figs. 5C, D). Although these structures are present in infected cells, they may not be definitively associated with virus infection.

Fig. 1 - *Caladium bicolor*, naturally infected by *Dasheen mosaic virus* and Caladium virus X, showing chlorotic spots and rings, and necrotic rings.

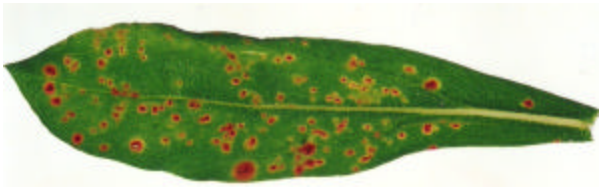

Fig 2 - *Gomphrena globosa*, experimentally infected by Caladium virus X, showing necrotic, red halo-surrounded spots 14 day after inoculation.

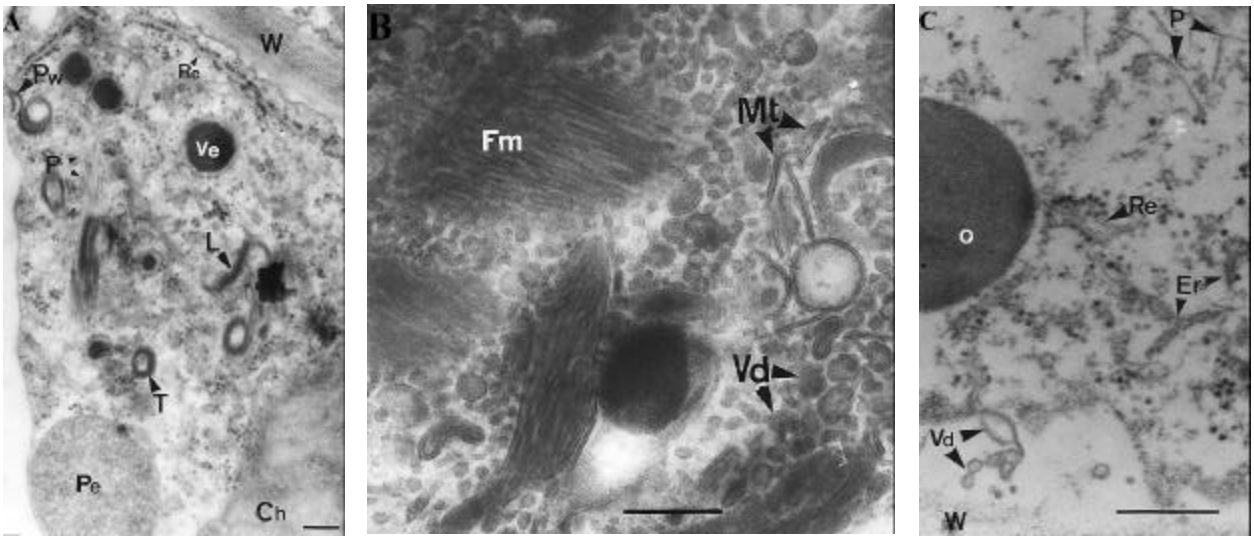

Fig. 3 - Thin sections of palisade cells of *Caladium bicolor* naturally infected by DsMV and CalVX. A– Transversally (T) and longitudinally (L) cylindrical inclusions, close to organelles and virus-like particles (P) in the cytoplasm containing, in sections. B–Fibrous mass (Fm) in a cytoplasmic region rich in double membrane vesicles (Vd) and membranous tubules (Mt). C– Virus-like particles scattered in the cytoplasm showing intumescent endoplasmic reticulum (Er); vesicles with double membrane (Vd) can be observed between cytoplasmatic membrane and cell wall. Ch– chloroplast, O– osmiophilic globule, Pe– peroxisome, Re- rough endoplasmic reticulum, Ve– vesicles with electron-dense contents, W– cell wall. Bars = 200 nm.

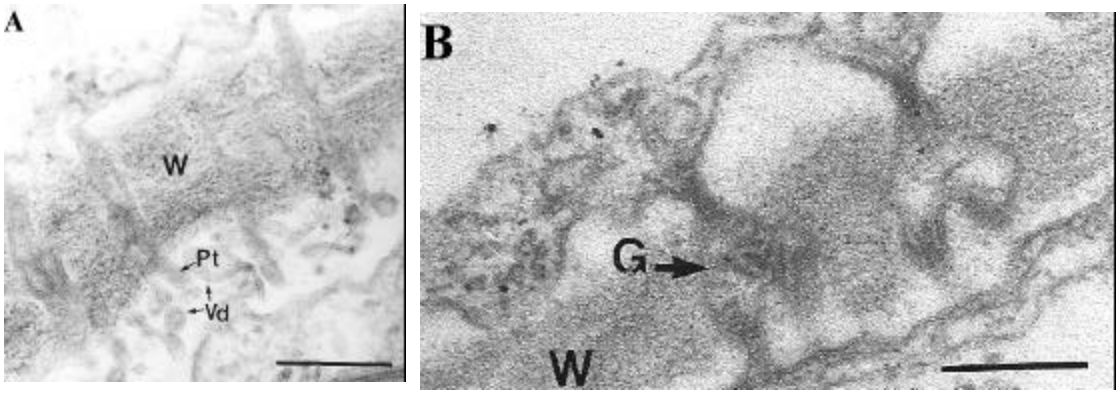

Fig 4 - Plasmodesmata between *Caladium bicolor* infected mesophyll cells. A– Plasmodesmata with protruding plasmatubules (Pt); vesicles with double membrane or desmotubules in transversal sections (Vd). B– Dilated plasmadesmata containing electron-dense globules (G) in which desmotubules could be occasionally observed. W– cell wall. Bars = 200 nm.

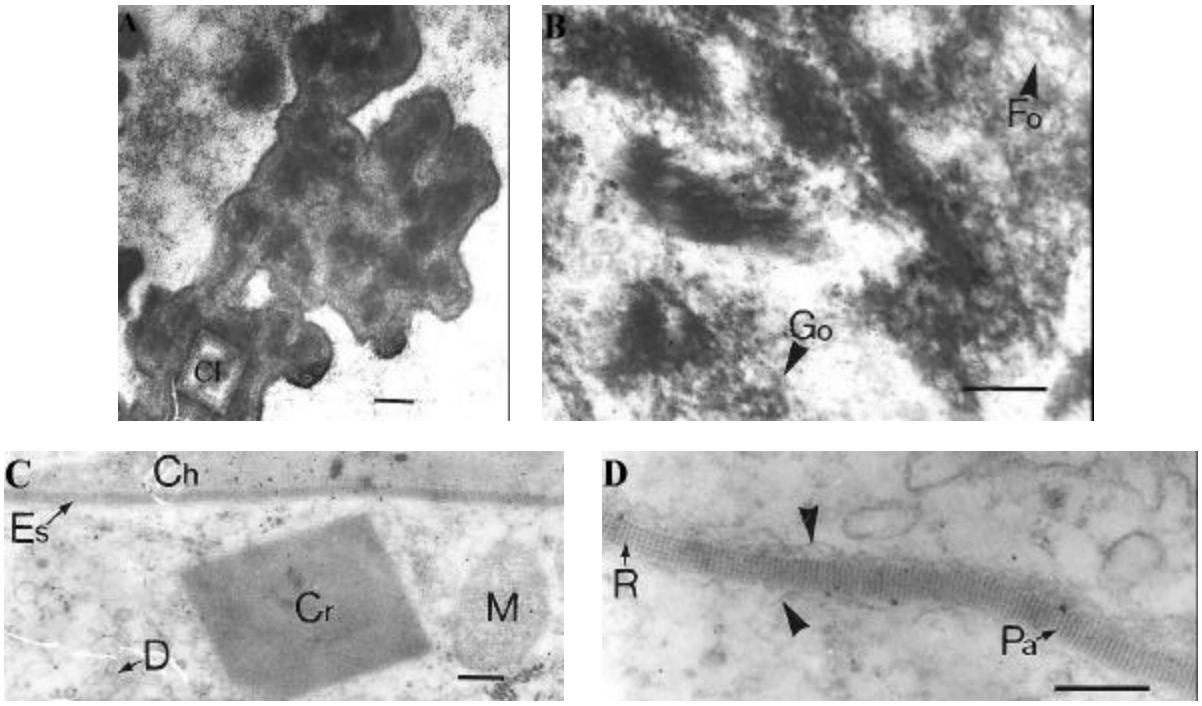

Fig. 5 – Unusual aspects observed in *Caladium bicolor* cytoplasm mesophyll cells. A– Rounded osmiophilic bodies imersed in electron-dense mass, containing or not crystalline inclusion (CI). Bar = 1470 nm. B– Amorphous X-bodies composed by granular (Go) and fibrous (Fo) osmiophilic bodies. C– Elongated structure (Es) close to organelles: chloroplast (Ch), dictyosome (D), mitochondrion (M), and peroxisome containing a crystal (Cr). D– Detail of the elongated structure showing its regular internal organization with reticulate (R) and parallel (Pa) array; note a membrane surrounding the structure (arrow-head). Bars = 200 nm.

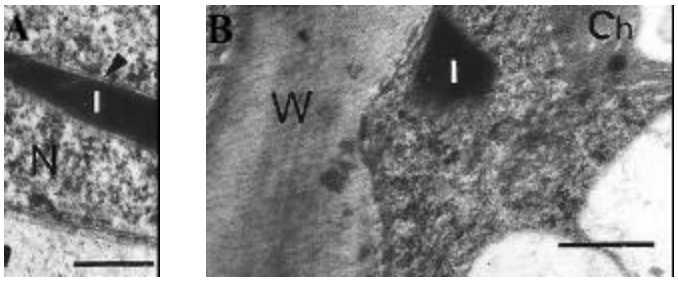

Fig. 6 – Electron-dense, crystalline inclusions (I), apparently involved by a membrane (arrowhead) in mesophyll cells of *Gomphrena globosa* infected by CalVX. A– Inclusion probably located in a protruding cytoplasm in the nucleus (N). B– Inclusion in the cytoplasm. Ch– chloroplast. W– cell wall. Bars = 500 nm.

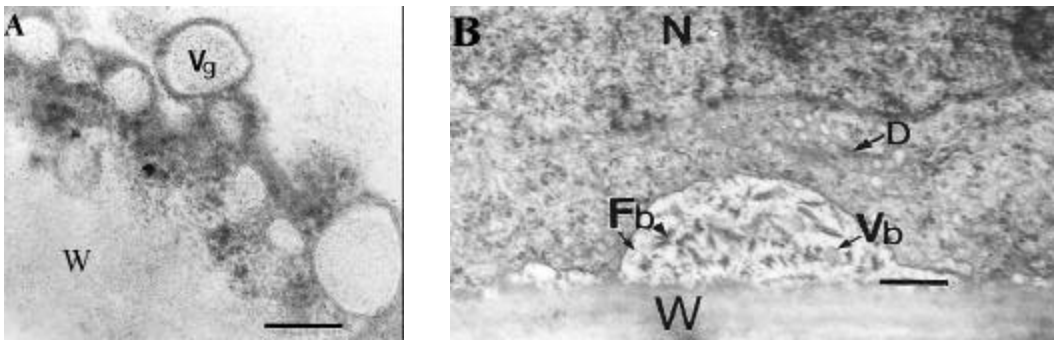

Fig. 7 – Mesophyll cells of CalVX infected *Gomphrena globosa*. A– Vesicles (Vg) with granular contents in the cytoplasm close to cell wall. B– Vesicular bodies (Vb) and filamentous bodies (Fb) between cytoplasmic membrane and cell wall. D– dictyosome, N– nucleus, W– cell wall. Bars = 600 nm.

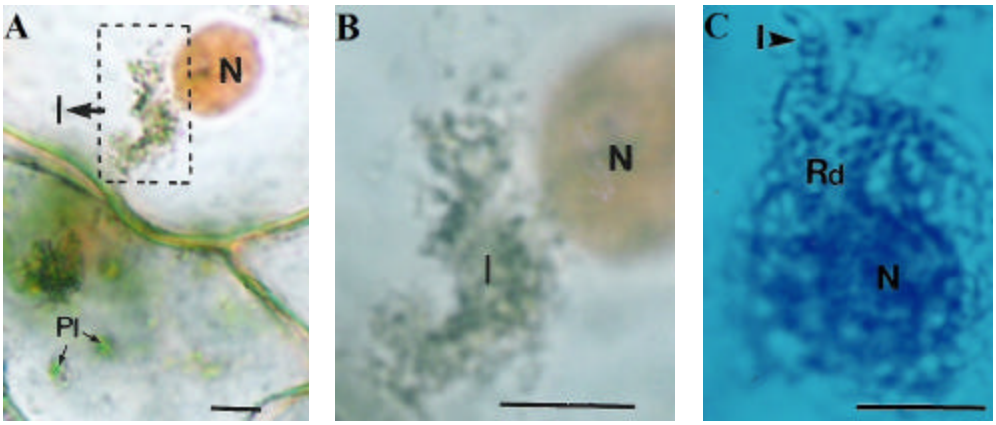

Fig. 8 – Light microscope micrography of paradermal sections of *Gomphrena globosa* infected by CalVX. A– Granular and dense region (I) of cytoplasm stained with Calcomine orange – Luxol brilliant green. B– Detail of delimited area in A. C– Banded inclusion (I) imersed in granular and dense region (Rd) of cytoplasm stained with Azure A. N– nucleus, Pl– plastids. Bars = 10 µm.

In electron microscopy observations from local lesions of experimentally infected *G. globosa*, mesophyll cells exhibited virus-like particles scattered in the cytoplasm and crystalline, electron-dense inclusions in the nucleus and cytoplasm (Fig. 6). These angular-shaped inclusions were not composed by virus particles and appeared to be surrounded by a membrane (Fig. 6). In the heavily infected cells, vesicles with double membrane and granular contents were present in the cytoplasm close to cell walls (Fig. 7A); paramural bodies and filamentous structures located between cytoplasmatic membrane and cell walls may also be present (Fig. 7B).

However, in paradermal sections stained with luxol-calcomine combination (O-G), cytoplasmic complex inclusions consisting of a central, faint-yellow stained area, surrounded by groups of dense, olive-green stained granular bodies were frequently observed (Fig. 8A, B). When sections were stained with azure A, dense and granular bodies were visualized with difficulty because they stained blue like the cytoplasm, although banded inclusion bodies, virus particles ordered in two different axes, could be observed (Fig. 8C). These features were never found within cells from healthy leaf tissues.

DISCUSSION

Higher plants commonly experience infection with several different viruses at a same time (mixed infection), and a number of plant diseases are attributed to a synergistic interaction between two unrelated viruses in the same plant (Vance et al., 1995).

Mixed infections could be detected by electron microscopy of ultrathin sections from infected tissues. Thus, in *C. bicolor* displaying virus-like symptoms we were able to detected the presence of two flexuous viruses. The former, a *Potyviridae*, was identified through its typical and exclusive cytoplasmic cylindrical inclusions. The virus species was previously identified by immuno electron microscopy as DsMV (*Potyvirus*)(Rivas et al., 1994). The latter, due to flexuous filamentous virions length, was identified as *Flexiviridae*; later, using primers directed to *Potexvirus*, Rivas et al. (2004) identified it as a probably novel species in the genus.

Members of the family *Flexiviridae* are flexuous virions that include the genera *Allexi-*, *Capillo-*, *Carla-*, *Fovea-*, *Mandari-*, *Potex-*, *Tricho-* and *Vitivirus* (Adams et al., 2004).

Potexviruses are highly infectious, positive single-stranded RNA viruses, whose virions have 470-580 nm in length (Adams et al., 2004). There is no typical cytopathic alteration for diagnosing potexviruses inclusions, except for *Potato virus X* (PVX) which induces the exclusive laminate inclusion component (LIC) (Allison & Shalla, 1974). Then, virions are found in cytoplasm and occasionally in nuclei and / or cell vacuoles; inclusions induced by potexviruses, harboring or not virions, could be found as crystals in the cytoplasm and / or nucleus as amorphous X-bodies, viroplasm or as unusual in shape (Brunt et al., 1996). Complex inclusions, present in long duration infections, are a combination of virions, material attributed to viral infection and host organelles, which are found in epidermal and mesophyll cells from sectioned leaves under electron or light microscopy (Christie & Edwardson, 1977; Brunt et al., 1996).

The fibrous masses observed in cells infected by CalVX were composed of flexuous, filamentous particles, similar to those found in potexvirus infections, as well as in carlavirus and closterovirus infections (Kikumoto & Matsui, 1961; Purcifull &

EDWARDSON, 1981; BRUNT et al., 1996). In the fibrous mass induced by PVX, the individual particles are loosely interwoven with each other (KIKUMOTO & MATSUI, 1961) as in the case of CalVX-infected cells.

Under light microscopy, the banded bodies similar to those produced by other potexviruses were rarely observed in fresh tissue of CalVX-infected gomphrena, probably due to the fact that integrity of the inclusions could be affected by ethanol, 2-methoxyethanol or simply by tearing and cutting the tissues (CHRISTIE & EDWARDSON, 1977). However, this kind of inclusion was not observed in ultra thin sections from infected tissue, probably due to its sensitivity to the fixatives used (CHRISTIE & EDWARDSON, 1977). Then, the frequent presence of the CalVX-like particles aggregates (fibrous mass) could represent disaggregated banded bodies, whereas the most frequent type of inclusion found in *Potexvirus* infection is a disarranged virus mass that occasionally shows traces of orientation (CHRISTIE & EDWARDSON, 1977). Indeed, the dense and granular region present in cell of fresh tissue from infected gomphrena, corresponding to amorphous X-bodies or complex inclusion of the potexviruses (CHRISTIE & EDWARDSON, 1977), could represent amorphous bodies, composed by granular and fibrous osmiophilic bodies, found in the same plant species when observed in the electron microscope.

The most striking feature of CalVX-infected cells from gomphrena leaves was the presence of electron-dense crystalline inclusions. In some potexvirus infections, crystalline inclusions are formed by virus aggregates (BRUNT et al., 1996), but the crystalline ones induced by CalVX are not formed by viral particles. This kind of inclusion was found in *Bamboo mosaic virus* (BaMV) infections in both naturally and experimentally infected *Bambusa* and *G. globosa*, respectively (KITAJIMA et al., 1977), but a conspicuous feature in the case of CalVX is the presence of crystalline inclusions with a surrounding membrane. On the other hand, crystalline inclusions were not observed in fresh cells from gomphrena, perhaps due to failure in the staining process or to the stage of virus infection.

As might be expected, CalVX infection also induced light senescence aspects in organelles, mainly in *C. bicolor*, as a consequence of the chlorotic and necrotic symptoms. The alterations in the organelles, such as chloroplasts and endoplasmic reticulum, are similar to those induced by fungus infection, mineral deficiencies, water imbalance, day length, as well as programmed senescence (BUTLER & SIMON, 1971; THOMAS & STODDART, 1980).

Plasmatubules, tubular and spherical evaginations of the plasmalemma (HARRIS & CHAFFEY, 1985), or plasmalemmasomes, vesicles and membranous structures from plasmalemma, were frequent in mesophyll cells of the infected *C. bicolor*. It is known that virus infected plants, kinetin-treated leaves and dwarf plants have a high frequency of the paramural bodies in their cells (MARCHANT & ROBARDS, 1968; GRUNER & SATORI, 1991).

Some structures observed in CalVX infected cells were also found in potexvirus infections. Vesicles with double membrane interposed between the plasmalemma and the primary cell wall, for example, as observed in CalVX infection, were also detected in local lesions of gomphrena infected by PVX (ALLISON & SHALLA, 1974).

As for the plasmodesmata, those from *C. bicolor* appeared generally branched and with plasmatubules extending away from the primary cell walls, in a region populated with vesicles, as observed in local lesions induced by PVX in gomphrena (ALLISON & SHALLA, 1974). Indeed, in some dilated central plasmodesmata cavities granular bodies could be observed. ALLISON & SHALLA (1974), on the other hand, observed the presence of PVX particles in the central cavities of complex plasmodesmata.

G. globosa has been used as a local host for many potexviruses (BRUNT et al., 1996), and also as a model for studying ultrastructural and cytopathological events (ALLISON & SHALLA, 1974; KITAJIMA et al., 1977).

CONCLUSIONS

The general structure of the cytoplasmic inclusions found in this study are similar to those reported in potexvirus infections. The microscopic analysis corroborates the classification of the CalVX as a species in the *Potexvirus* genus. However, unusual and unreported structures were present in infected gomphrena cells, and they remain to be clarified.

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Received on 27 / 9 / 04
Accepted on 27 / 12 / 04