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Ultrastructural Features of *Catharanthus roseus* Leaves Infected with Cucumber Mosaic Virus

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ABSTRACT

Catharanthus roseus var. *rosea*, infected with Malaysian isolate of cucumber mosaic virus (CMV-MP), exhibited leaf mosaic, leaf deformation and malformed flowers. Electron microscopic examination of the infected leaf cells revealed significant alteration of the chloroplasts in the mesophyll cells. Large starch grains in necrotic zones and disorganized thylakoid system were the most prominent modifications observed within the chloroplasts of the infected tissues. Meanwhile, membrane-bound vesicles were detected in the vacuoles of the CMV-MP-infected leaf cells. A crystalline array of phytoferritin macromolecules was detected in the chloroplast at 40 days post-inoculation. However, neither single nor aggregate of CMV-MP particles was detected in the cytoplasm due to difficulties in differentiating them from the ribosomes. Nonetheless, structure resembling the inclusion bodies, commonly produced after virus infection, could not be found in the infected leaf cells. Similarly, structure abnormality in the nucleus or mitochondria was also not observed.

Keywords: Catharanthus roseus, cucumber mosaic virus, leaf cells, chloroplast abnormalities

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INTRODUCTION

The cucumber mosaic virus (CMV) has isometric particles about 28-30 nm in diameter and is composed of a capsid (coat) protein shell that encapsidates a single-stranded, positive-sense RNA genome. The virus carries a tripartite genome containing four single stranded positive – sense RNAs

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and has a very wide host range (Palukaitis *et al.,* 1992).

Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don (which is also known as 'kemunting cina' in Malaysia), is an ornamental plant that produces very important anticancer drugs such as vincristine and vinblastine (Manganey *et al*., 1979; Svoboda, 1983; Cragg & Newman, 2005), as well as antihypertensive compounds, ajmalicine and serpentine (van de Heijden *et al*., 2004). The plant has been recorded as one of the hosts for CMV infection (Ong & Ting, 1977; Inon *et al*., 1999). Recently, studies on the aetiology of mosaic symptoms in *C. roseus*, grown wild or cultivated in pots, revealed that an isolate of CMV was the causal agent of the disease (Mazidah *et al*., 2012). The diseased leaves exhibited light and dark green patches and were deformed in shape. The flowers were malformed with slight colour-breaking on the petals. The CMV isolate was characterized at molecular level (Mazidah *et al*., 2012) and its complete coat protein gene was cloned and sequenced (Accession number EU726631). This isolate was identified and designated as Malaysian periwinkle isolate (CMV-MP).

Cytopathological data can reflect viral characteristics which stand independent from particle morphology, particle serology and possibly host reactions in the sense of symptoms, transmission in plants and resistance phenomena (Lesemann, 1988). Studies on the cytopathic effects of CMV infection in leaf cells of host plants with mosaic symptoms (Misawa & Ehara 1966; Honda & Matsui, 1973) and local lesions (Ehara, 1979; Ishihara *et al*., 2002) have been reported. However, no ultrastructural study has been conducted on the leaves of virus-infected *C. roseus.* Thus, this paper reports on the ultrastructural changes in *C. roseus* leaf cells systemically infected with CMV-MP.

MATERIALS AND METHODS

Source of the Virus Isolate

CMV-MP was isolated from the leaves of *C. roseus* var. *rosea,* which exhibited leaf mosaic, leaf deformation and malformed flowers grown in a field in Serdang, Selangor. The inoculum was prepared by grinding symptomatic leaves in 0.01 M phosphate buffer, pH 7.0, containing 0.25% DIECA and carborundum (600 mesh). The extract was mechanically inoculated onto the leaves of healthy *C. roseus* seedlings at four-leave stage. The plants were then kept in an insect proof glasshouse. Ultrastructural observations were made on the second leaves and these showed distinct mosaic symptoms at 12, 25 and 40 days post-inoculation (dpi). The leaf tissues from mock-inoculated plants (inoculated with buffer only) were used as uninfected controls.

Tissue Processing and Electron Microscopic Examination

Small tissue sections (about 2 mm x 2 mm) were excised from the mosaic area of the CMV-MP-infected leaves. Symptomless leaf from the mock-inoculated plant was used as an uninfected control. The selected

tissue samples were fixed in buffered 4% glutaraldehyde solution (pH 7.0) for 48 hours. The fixed specimens were washed three times (at 30 min intervals) with 0.1 M sodium cacodylate buffer, followed by two hours of post-fixing in 1% osmium tetroxide at 4°C. The samples were washed again three times (at 30 min intervals) before they were dehydrated in ascending concentrations of acetone solution, sequentially followed by acetone: resin (1: 1 and 1: 3 v/v) to facilitate the resin entry into the tissues, and finally embedded in epoxy resin. The resin was polymerized at 60°C for 48 hours. Ultrathin sections were made using a diamond knife in an ultramicrotome mounted in copper grids. These sections were then stained with uranyl acetate, followed by lead citrate. The sections were observed under a Hitachi H-7100 transmission electron microscope.

RESULTS AND DISCUSSION

The development of the mosaic symptoms, caused by viral infection, is related to drastic metabolic changes that occur in the host tissue (Kato & Misawa, 1974). Distinct mosaic symptoms were evident on the leaves of CMV-MP-inoculated *C. roseus* as compared to the mock-inoculated ones (Fig.1).

The ultrastructural changes in the *C. roseus* leaf cells were observed in this study following inoculation with CMV-MP. At 12 dpi, the chloroplasts in the infected leaf cells became swollen (Fig.2C and Fig.2D) compared with those in the uninfected leaf cells (Fig.2A and Fig.2B). At 25 dpi, alteration of the chloroplasts was detected (Fig.3A and Fig.3B) compared to those in the uninfected mesophyll cells (Fig.3C and Fig.3D). The alterations were in the form of large starch grain formation in the necrotic

Fig.1: Appearance of mosaic symptom on the CMV-MP-infected leaf (left) compared with the mock-inoculated one (right).

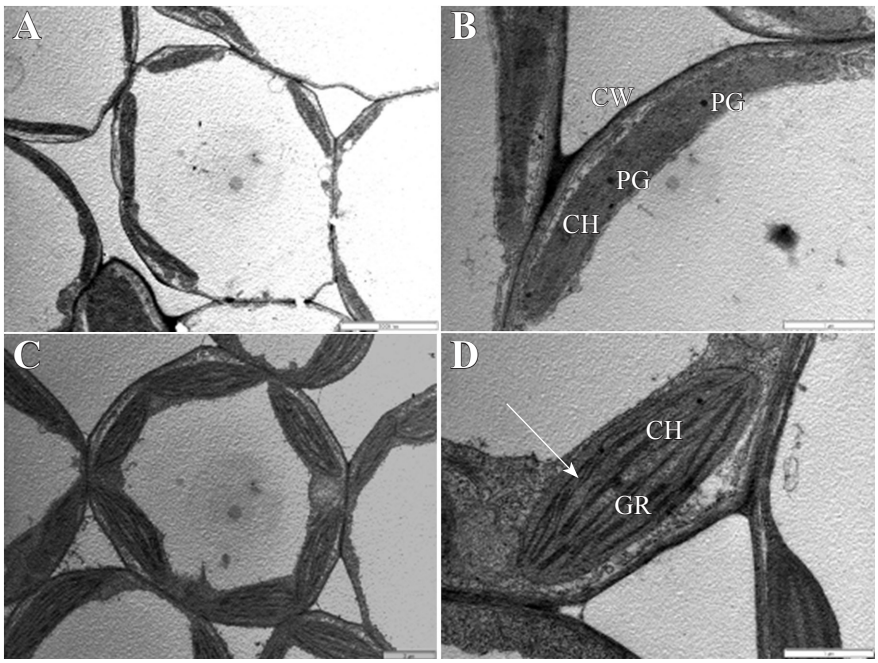

Fig.2: Electron micrographs of the leaf cells from uninfected and CMV-MP-infected *C. roseus* at 12 days post-inoculation. (A and B) Chloroplasts in the mesophyll cells of uninfected leaf tissues at X4,600 and X21,500, respectively. (C and D) Chloroplasts in the mesophyll cells of CMV-MP-infected leaf tissues at X7,700 and X21,500, respectively. A white arrow shows stroma lamellae. PG=plastoglobuli, GR=grana, CW=cell wall, CH=chloroplast

zones and disorganized thylakoid system within some of the chloroplasts in the infected leaves. The stromatic lamellae and grana were disintegrated by the presence of the large starch grains. Similar chloroplast alterations, induced by CMV infection, have been reported by Poolpol and Inouye (1986) who found that the mesophyll cells of a cucumber leaf infected with CMV alone showed deformed chloroplasts with an abnormal thylakoid system and large starch grains.

Vesicular bodies were detected in the vacuoles of the CMV-MP-infected leaf cells (Fig.3E) which were absent in the uninfected leaf tissues. Similar structures have been observed in the vacuoles in the cells surrounding necrotic local lesions of CMV-infected cowpea leaves (Ehara, 1979) and *Nicotiana glutinosa* leaves (Ishihara *et al*., 2002). These membrane-bound vesicles are associated with tonoplasts and may be the sites of viral RNA synthesis (Hatta & Francki, 1981).

Arrays of phytoferritin macromolecules (iron-containing molecules) were detected in the chloroplast stroma of CMV-MP-infected leaf cells at 40 dpi (Fig.4A and Fig.4B). These macromolecules were not detected in the uninfected leaf tissue sections. Wildman and Hunt (1976) detected phytoferritin particles in the chloroplasts of yellow leaf pinnae from coconut palm (*Cocos nucifera* L.). They concluded that these particles

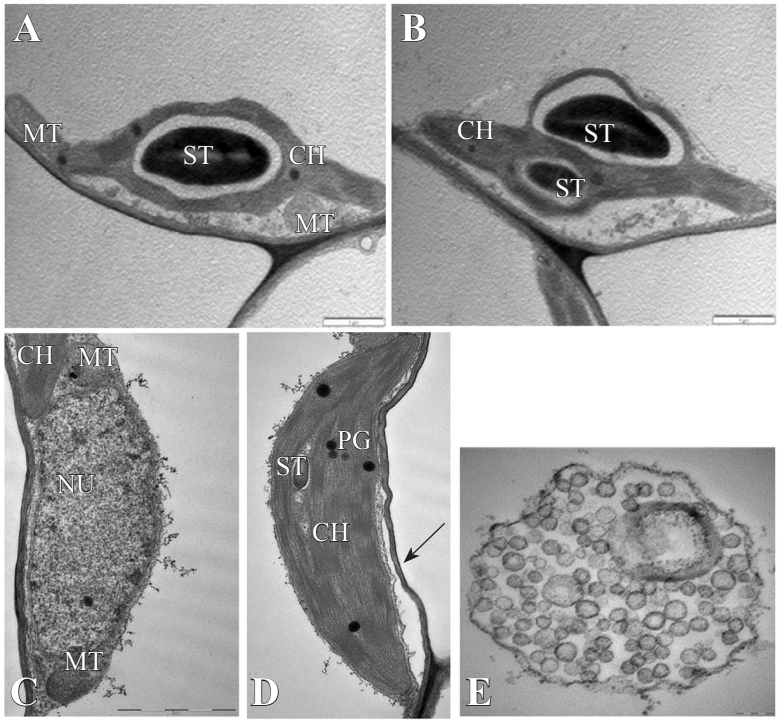

Fig.3: Electron micrographs of the leaf cells from uninfected and CMV-MP-infected *C. roseus* at 25 days post-inoculation. (A) and (B) show the altered chloroplasts with large starch grains and abnormal thylakoid systems in the CMV-MP-infected leaf cells of *C. roseus*. (C) Mitochondria and nucleus in the uninfected leaf cells of *C. roseus*. (D) A structure of chloroplast with a starch grain and a distinct cell wall (arrow) in the uninfected leaf cells of *C. roseus*. (E) Vesicular bodies were detected in the cytoplasm of CMV-MP-infected *C. roseus* leaf cells. (A) and (B) X16,500; (C) X70,000; (D) X17,000; (E) X70,000. CH=chloroplast, ST=starch grain, MT=mitochondrion, NU=nucleus, CW=cell wall, PG=plastoglobuli

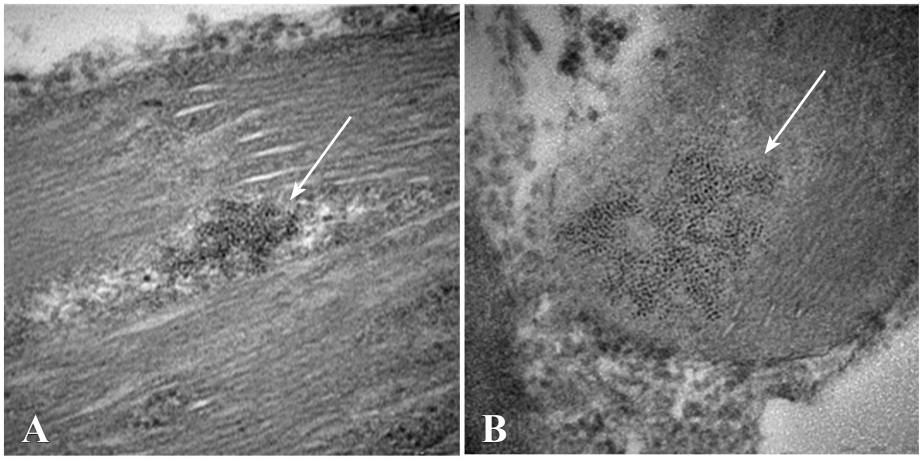

Fig.4: Arrays of phytoferritin macromolecules (white arrows) were detected in the chloroplast stroma of CMV-MP-infected leaf cells at 40 days post-inoculation. (A) X50,000;(B) X100,000.

were a breakdown product associated with the disruption of the chloroplast structure and photosynthetic activity in *C. nucifera,* but were not specifically associated with disease. Tomenius and Oxelfelt (1982) also discovered phytoferritin in the chloroplasts stroma in the chlorotic regions of pea leaf cells which had been infected with red clover mottle virus (strains N and S). They suggested that the phytoferritin molecules were associated with viral infection in the plant.

Starch and phytoferritin are the main energy and iron reserves in plant, respectively. Accumulation of starch and phytoferritin in the CMV-MP-infected *C. roseus* chloroplasts suggested that the metabolism of carbohydrate and iron-containing compounds were affected by viral infection. Meanwhile, alteration of the chloroplasts may have contributed to the mosaic symptom development in CMV-MP-infected *C. roseus.* Nonetheless, no clear evidence of structure abnormality was found in the nuclei or mitochondria. Infection by CMV-MP may not affect these organelles. Inclusion bodies are intracellular structures produced as a result of viral infection, which may contain virus particles, virus-related materials or ordinary cell constituents in a normal or degenerating condition (Sofy *et al.,* 2007). In this study, the inoculated plants were detected positive to CMV infection by DAS-ELISA analysis but no inclusion body was detected in the organelles and in the cytoplasm of the CMV-MP-infected leaf cells under TEM examination. CMV-MP appeared as densely stained particles with the sizes around 20 to 23 nm in diameter and was difficult to be distinguished from cytoplasmic ribosomes (Hatta & Francki, 1979).

CONCLUSION

The present investigation showed that CMV-MP infection in *C. roseus* had induced ultrastructural changes in the leaf cells which were parallel to the morphological alteration of the infected leaves. These ultrastructural changes seemed to be restricted in the chloroplasts. Meanwhile, enlargement of starch granules and accumulation of phytoferritin macromolecules in the infected leaf cells suggest that CMV-MP infection may have interfered the metabolism of carbohydrate and iron-containing compounds in *C. roseus*. Modification of the chloroplast structure following CMV-MP infection may have attributed to mosaic symptoms development.

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