ULTRASTRUCTURAL CHANGES
IN ASTER YELLOWS PHYTOPLASMA
AFFECTED *LIMONIUM SINUATUM* MILL. PLANTS.
II. PATHOLOGY OF CORTEX PARENCHYMA CELLS

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ABSTRACT

In *Limonium sinuatum* Mill. plants with severe symptoms of aster yellows infection phytoplasmas were present not only in the phloem but also in some cortex parenchymas cells. These parenchyma cells were situated at some distance from the conducting bundles. The phytoplasmas were observed directly in parenchyma cells cytoplasm. The number of phytoplasmas present in each selected cell varies. The cells with a small number of phytoplasmas show little pathological changes compared with the unaffected cells of the same zone of the stem as well with the cells of healthy plants. The cells filled with a number of phytoplasmas had their protoplast very much changed. The vacuole was reduced and in the cytoplasm a reduction of the number of ribosomes was noted and regions of homogenous structure appeared. Mitochondria were moved in the direction of the tonoplast and plasma membrane. Compared to the cells unaffected by phytoplasma, the mitochondria were smaller and had an enlarged cristae internal space. The chloroplasts from affected cells had a very significant reduction in size and the tylacoids system had disappeared. The role of these changes for creating phytoplasma friendly enviroment is discussed.

KEY WORDS: phytoplasma, aster yellows, parenchyma ultrastructure.

INTRODUCTION

Phytoplasmas are the prokaryotes belonging to the Mollicutes class. Most of Mollicutes like Mycoplasma, Ureoplasma and Spiroplasma are facultative anaerobes and many grow best in microaerophylic or anaerobic conditions. Phytoplasmas have not been cultivated in any cell-free medium and their physiological characteristics have been limited to in situ studies. Generally phytoplasmas were confined to the sieve tubes of phloem, but it has been reported that phytoplasmas can also infect companion cells (Lombardo et al. 1970; Worley 1970), phloem parenchyma cells (Esau et al. 1976) and parenchyma cells adjacent to phloem (Hirumi and Maramoroch 1973; Siller et al. 1987; Worley 1970). Occasionally phytoplasmas were reported in other plant tissues such as xylem vessels (Padmanabha et al. 1973; Doi et al. 1967) mesophyl parenchyma (Semancik and Peterson 1971) and cortical parenchyma cells (Cousin et al. 1970). Phytoplasma was also reported in pericyclic cells (Gourret 1970). The localisation in cells outside the phloem

was exceptional and the changes observed in the infected cells could lead us to conclusions about conditions necessary for phytoplasma growth.

In some cortex parenchyma cells of diseased *Limonium* plants phytoplasmas were found. A comparison of the structure of cortex parenchyma cells from diseased plants with and without phytoplasmas and cortex parenchyma cells from healthy plants was the aim of this work.

MATERIAL AND METHODS

Diseased and asymptomatic plants of annual statice *Limonium sinuatum* Mill. were collected in field plantations, potted and maintained in an insect-proof greenhouse. The diseased plants show leaves and flowers discoloration and malformation, shoots dieback, proliferation and flowers virescence. Our investigations showed that plants were affected by phytoplasma from the aster yellows group (Kamińska et al. 1999).

Specimens for electronmicroscopical examination were fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer pH = 7.2, for 4 h. Postfixation in 1% osmium tetroxide for 2 h at 4°C was applied. The material was dehydrated in ethanol (10-70%), acetone (70-100%) and embedded in Epon 812. The ultra-thin sections on copper grids were stained with uranyl acetate and lead citrate. Observations were taken on a JEM 100C and JEM 1220 electron microscope in the Laboratory of Electron Microscopy Warsaw Academy of Agriculture.

Profiles of mitochondria from parenchyma cells were selected and photographed under magnification 20 000×. The measurement of mitochondria was done on electronmicroscopical photographs with the use of computer program Analysis 2.1 Soft-imaging Software GmbH. Twenty five profiles of mitochondria from infected parenchyma cells were selected. Similar number of mitochondria from not containing phytoplasma parenchyma cells of diseased plants and healthy ones were randomly selected and photographed. All parenchyma cells belonged to the same region of stem cortex.

RESULTS

The stems of infected plants showing witch's broom symptoms appear pale yellow compared to the bright green of control plants and a complete reduction of chlorenchyma situated in control plant stems under the epidermis was noted. In cortex parenchyma many cells had not a regular shape but cells from diseased and control plants showed no difference in their structure. They were cells with a central vacuole and a thin layer of cytoplasm. Few mitochondria and not many endoplasmic reticulum cisternae were distributed in the cytoplasm. Chloroplasts from the cortex parenchyma of the stems of control and infected plants show no structural differences. They had grana with few tylakoids and little starch deposits in the stroma (Fig. 12).

In the phloem of infected plants phytoplasmas were present in the sieve tubes, which is typical for phytoplasma infections. But in plant stems there were observed also cortex parenchyma cells showing the presence of phytoplasmas in their protoplast. Some of this parenchyma were separated from the phloem region at least by one or a few other parenchyma cells not showing the presence of phytoplasmas (Figs 1, 2, 3). The phytoplasmas appeared in the cytoplasm of the affected cell with their outer membrane surrounded

directly by hialoplasm. These phytoplasmas looked the same as phytoplasmas from the sieve tubes of *L. sinuatum* plants. A distinct membrane surrounded them and their cell structure was comparable to that of the sieve tubes. No phytoplasmas were observed in the vacuole, neither in plastids or mitochondria, nor in the space between the cell wall and the plasma membrane. The nucleus of the cell seems not to be changed by the presence of phytoplasma in the cell. No phytoplasmas were observed on the nucleus territory nor was it in contact of outer membrane of the nucleus.

The observed parenchyma cells affected by phytoplasma show differences in the number of phytoplasmas present in their protoplast. In some of these parenchyma cells there were present only a few phytoplasma cell observed on the cross section of the cell (Fig. 4) while in others there were masses of phytoplasmas filling almost the whole lumen of the cell (Fig. 2).

Changes in the *L. sinuatum* parenchyma cell structure vary depending on the number of phytoplasmas present. The cells with a small amount of phytoplasmas had a protoplast structure comparable to the cells from the infected plants not possessing phytoplasma, as well as to the parenchyma cells of control plants (Figs 4, 5). There was the thin layer of cytoplasm with a big vacuole. Chloroplasts present in cytoplasm were comparable to the chloroplasts of other cortex parenchyma cells. In these cells the phytoplasmas were situated some distance one from another and near different organelles of the cell and also mitochondria. Some of the phytoplasmas had an elongated shape and a few separate regions of nucleoids which indicate the dividing form (Fig. 5).

Parenchyma cells with more phytoplasmas show more changes. The phytoplasma were situated in groups gathering several of them (Fig. 3). In the cytoplasm appeared regions with a fine homogenous structure very poor in rybosomes (Figs 6, 7). These regions usually were placed close to phytoplasma grouped together (Fig. 7). The cells had rounded chloroplasts with less grana observed (Fig. 9).

Parenchyma cells filled with masses of phytoplasmas (Figs 1, 8) were usually enlarged and their protoplast was much changed. The vacuole of the cell was very much reduced, but still present in the cell (Fig. 2). In the cytoplasm of the cells there were observed, but rarely Golgi apparatuses, a few endoplasmatic reticulum cisternae and almost no rybosomes. Golgi apparatuses and endoplasmatic reticulum cisternae were situated among the phytoplasma cells. The chloroplasts were changed very much com-

TABLE 1. Comparison of mitochondria present in parenchyma cells from the healthy and infected plants.

Stem cortex parenchyma cells of plant:	Number of mitochondria measured	Mean area (μm^2) of a mitochondrion	Mean no. of cristae in a single mitochondrion	Sum of crist perimeter (μm) in a single mitochondrion	Sum of crist area in a single mitochondrion (μm^2)
AY-affected, cell containing phytoplasmas	25	0.0955 SE 0.0065	4.4 SE 1.8	1.10 SE 0.10	0.0171 SE 0.0016
AY-affected, cell not containing phytoplasmas	27	0.1968 SE 0.0198	13.1 SE 5.3	3.51 SE 0.35	0.0429 SE 0.0061
Healthy	27	0.1869 SE 0.0134	12.8 SE 3.8	3.27 SE 0.22	0.0379 SE 0.0030

Fig. 1. Two cortex parenchyma cells filled with phytoplasmas (arrow) separated from the phloem (asterisk) by a few other parenchyma cells not showing the presence of phytoplasmas. Magn. 300 $\times$, bar represents 50 μ m.

Fig. 2. Two cortex parenchyma cells with numerous phytoplasmas filling almost the whole lumen of the cell except the vacuole region (asterisks). Magn. 2500 $\times$, bar represents 5 μ m.

Fig. 3. The cortex parenchyma cell with a group of phytoplasmas (P) in cell cytoplasm. Magn. 8000 $\times$, bar represents 2 μ m.

Fig. 4. A part of a cortex parenchyma cell with phytoplasmas (P) situated in cytoplasm near the cell nucleus (n). Magn. 11 000 $\times$, bar represents 2 μ m.

pared to the other cortex parenchyma cells (Fig. 12). Neither grana nor tylakoids were seen, only several vesicles were present in the stroma (Figs 9, 10, 11).

Compared to chloroplasts from cortex parenchyma cells without phytoplasmas and to chloroplasts from cortex parenchyma cells from healthy plants not only reduction of tylakoids was noted but also the size of these plastids was reduced (Figs 9, 10, 11, 12). No changes between chloroplasts from cortex parenchyma cells from diseased plants,

but without phytoplasmas and chloroplasts from cortex parenchyma cells from healthy plants were observed.

Mitochondria present in these cells were moved to the part of the cytoplasm near the cell wall as well as to the tonoplast or to the region near the nucleus of the cell. Usually there were no mitochondria present in the territory occupied by phytoplasma.

To compare the changes in the mitochondria structure (Fig. 13) cells were selected filled with phytoplasmas

Fig. 5. Dividing phytoplasma in the cytoplasm of a cortex parenchyma cell. Magn. 47 500 $\times$, bar represents 300 nm.

Fig. 6. Changed cytoplasm with reduced number of ribosomes (arrows) and fuzzy material from the cell of a cortex parenchyma cell possessing phytoplasmas (P). Magn. 26 000 $\times$, bar represents 500 nm.

Fig. 7. A group of phytoplasmas in cytoplasm showing lack of ribosomes and membrane structures from a cortex parenchyma cell. Magn. 22 000 $\times$, bar represents 500 nm.

Fig. 8. A part of a cortex parenchyma cell showing cytoplasm filled with tightly packed phytoplasma cells. Mitochondrion (M) and Golgi apparatus (arrow) is seen. Magn. 17 500 $\times$, bar represents 1 μ m.

and showing the biggest changes in their structure. Three such cells were found. Both the control plant cells and cells without phytoplasma from infected plants were chosen from the same region of the stem. The results of measurements of these mitochondria are shown in Table 1. The measured area of the cross section of these mitochondria show the reduction in size of mitochondria present in the cells containing phytoplasma compared with the mitochon-

dria from other parenchyma cells. No difference in size of mitochondria from the cortex parenchyma cells of control and diseased plants was observed (Fig. 14). The mitochondria from the phytoplasma containing cells show a reduction in the number of cristae and cristis perimeter per mitochondrion. Cristae from mitochondria present in the cells containing phytoplasma were inflated. The perimitochondrial space of mitochondria was also enlarged. In one of

Fig. 9. Chloroplast from phytoplasma containing parenchyma cell. Magn. 30 000 $\times$, bar represents 400 nm.

Fig. 10. Chloroplast from phytoplasma containing parenchyma cell. Magn. 30 000 $\times$, bar represents 400 nm.

Fig. 11. A normal shape mitochondrion situated in invagination of chloroplast from phytoplasma containing a parenchyma cell. Magn. 30 000 $\times$, bar represents 400 nm.

Fig. 12. Chloroplast from parenchyma cells of a healthy plant. Magn. 30 000 $\times$, bar represents 400 nm.

Fig. 13. Mitochondria from a phytoplasma infected parenchyma cell. Magn. 57 000 $\times$, bar represents 200 nm.

Fig. 14. Mitochondria from parenchyma cells of a healthy plant. Magn. 57 000 $\times$, bar represents 200 nm.

the infected cells a normal shape mitochondrion was seen. This mitochondrion was separated from the rest of the cytoplasm, because it was situated in invagination of the chloroplast (Fig. 11).

DISCUSSION

Examination of *Limonium sinuatum* Mill. tissues showing proliferation and virescence symptoms revealed phyto-

plasma resembling those found in other countries (Muller et al. 1973; Baker et al. 1983; Chang et al. 1996). Muller et al. (1973), Baker et al. (1983), Chang et al. (1996) reported that phytolasmas were present only in the sieve tubes of affected plants. Contrary to them, our studies showed the presence of phytoplasmas not only in phloem sieve tubes but also in parenchyma outside the phloem.

Although the presence of phytoplasmas in cells other than sieve elements were questioned and interpreted as a misinterpretation (Mc Coy 1979; Davis and Lee 1982) in

some cases seems to be undoubtful. It does not change the fact that a really hostile environment for phytoplasmas in the plant body is the phloem, especially the sieve tubes. This may be connected with the fact that phytoplasmas like the other Mollicutes are anaerobes and may be sensitive to oxygen. The cells of plants usually have chloroplasts with a II fotosystem that increase the oxygen concentration in the cell protoplast. Although Sears et al. (1997) failed to show the influence of different levels of O_2 in the air on phytoplasma abundance in *Oenothera* leaf tips cultures, the level of O_2 in the cells in which the phytoplasma lives and multiplies may vary in the plant body and be quite different than the level in the surrounding air. The phloem tissue chloroplasts have little photosystems enzymes compared to the other tissues, especially parenchyma cells. The reduction of chloroplasts in the infected parenchyma tissues may play an important role in the presence of phytoplasma. In *L. sinuatum* examined shoots a complete reduction of chlorenchyma, situated in control plant stems under the epidermis, was observed. Also changes in the chloroplast structure especially a reduction of the tylacoids system in affected parenchyma cells took place. Besides the lack of fotosyntetically active chloroplasts may be the reason that phytoplasmas were found in parenchyma cells of *Cuscuta odorata* (Siller et al. 1987). The dodder is a parasitising plant and their plastids have a very little amount of chlorophyll and no photosynthesis occurs in these chloroplasts. Also the presence of phytoplasma in root phloem *Vicia faba* plants is significant (Lherminier et al. 1994). The cells of the root having no chloroplasts can be much more hostile for phytoplasma because of lower oxygen in them and this could be a reason for the bigger concentration of phytoplasma in this part of the plant.

The presence of phytoplasmas in parenchyma cells had an influence also on the condition of mitochondria. They changed their shape and were smaller, but the inflated cristae and enlarged perimitochondrial space seems to express their energised condition. Bertaccini and Marani (1980) also observed changes in the mitochondria structure of *Gladiolus* phloem parenchyma cells in cases where phytoplasmas were found in these parenchyma cells.

The phytoplasmas heterotrophic way of life as well as the anaerobic conditions of their metabolism change the conditions in parenchyma protoplasts which influence the parenchyma cell organelles, on the other hand the changes in the parenchyma cell organelles such as changes in chloroplasts and mitochondria make the condition more hostile for phytoplasma. These changes, especially in the structure of chloroplasts show that the phytoplasma require the condition of a reduced oxygen level in the cells of their host.

Different toxins were found in other Mollicutes infections. Putative toxin produced by *Spiroplasma citri* have been shown to activate polyphenol oxidases in detached bean leaves (Daniels and Meddins 1974). Mycoplasma sp. produce H_2O_2 as a major metabolic by-product that can impair host cell metabolism and result in red blood cell lysis (Cole et al. 1968). A proteinaceous exotoxin was postulated as a pathogenicity factor of *Mycoplasma neorolyticum* (Thomas 1967). On the other hand no defence reactions, degeneration and necrotic reactions were seen in parenchyma cells affected by phytoplasma.

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ZMIANY CYTOPATOLOGICZNE
ZACHODZĄCE W ROŚLINACH *LIMONIUM SINUATUM* MILL.
PORAŻONEGO ŻÓŁTACZKĄ ASTRA.

II. ZMIANY PATOLOGICZNE W KOMÓRKACH MIĘKISZU KORY PIERWOTNEJ ŁODYG

STRESZCZENIE

W roślinach *Limonium sinuatum* Mill. wykazujących ostre objawy choroby charakterystyczne dla żółtaczki astrów i wykazujących występowanie fitoplazm w rurkach sitowych, obserwowano również komórki miękiszowe występujące na terenie kory pierwotnej które posiadały w swym wnętrzu komórki fitoplazm. Zaobserwowano komórki o niewielkiej liczbie fitoplazm występujących w cytoplazmie. Komórki te nie wykazywały większych zmian cytologicznych. Natomiast komórki wypełnione dużą ilością fitoplazm były powiększone a ich protoplast ulegał dużym modyfikacjom cytologicznym w porównaniu do analogicznych komórek nieporażonych. Przestrzeń wakuoli ulegała redukcji a w protoplaście zanikały rybosomy. Pojawiały się wydzielone przestrzenie o homogennej naturze. Mitochondria tych komórek ulegały przesunięciu do tonoplastu i plazmolemmy. W stosunku do mitochondriów innych komórek miękiszu kory pierwotnej zarówno z roślin porażonych jak i kontrolnych mitochondria te były mniejsze i charakteryzowały się mniejszą ilością bardziej rozdętych grzebieni. W chloroplastach komórek zawierających fitoplazmy uległ redukcji cały system tylakoidów, tak że nie przypominały one chloroplastów z innych komórek miękiszu kory łodygi *Limonium sinuatum* Mill. Znaczenie tych zmian dla wytwarzania się warunków sprzyjających życiu fitoplazm jest dyskutowane.

SŁOWA KLUCZOWE: fitoplazma, żółtaczka astrów, ultrastruktura miękiszu.