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ULTRASTRUCTURAL STUDIES ON *VICIA FABA* AND ITS PATHOGEN *BOTRYTIS FABAE* IN RESPONSE TO LITHIUM CHLORIDE

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Ultrastructural investigations of the effect of lithium chloride on *Botrytis fabae* and its host, *Vicia faba* are described. Five concentrations (1, 3, 5, 7, 9 mM) of lithium chloride are chosen in the study. The results indicate that the chlorophyll content of host leaves is not affected at lower concentration (3 mM), whereas the higher concentration (9 mM) decreased the chlorophyll content. These two concentrations caused a pronounced cellular disorganization of *B. fabae* that ranged from disruption of the wall to marked cytoplasmic degeneration. Inoculated host leaves with *B. fabae* led to the appearance of more vacuolated cytoplasm, a disorganized membrane system of chloroplast and an increase in number of plastoglobuli. These observations are similar to those of host cells treated with 9 mM of lithium chloride. Host cells are not affected by the concentration of 3 mM. Ultarstructural studies indicate that the lower concentration of lithium chloride can be used as a safe fungicide to control *B. fabae* without a harmful effect on the host (*V. faba*).

Key words: *Botrytis fabae*, chlorophyll, chloroplast, fungicide, lithium chloride, photosynthetic pigments, *Vicia faba*, ultrastructure

INTRODUCTION

Chocolate spot disease caused by *Botrytis fabae* is an important disease of broad bean (*Vicia faba*) worldwide occurring almost in all regions where broad beans are grown. According to Gaunt (1983), symptoms of chocolate spot are varied, and ranges from minor necrosis to complete destruction of large areas of host tissues. Leaves are the main tissues infected, but under favourable condition stems, pods and flowers may also be infected.

The chemicals, which are used to control plant pathogens, should have a high degree of preferential toxicity for the pathogens. Their selective toxicity depends on differences in their toxicity to the host and parasite, brought about by their differing metabolism (Abood 1990).

Earlier studies (Pendias and Pendias 1984) suggested that the severity of fungal infection be markedly reduced when lithium salts were applied via the root system. Lithium is a normal soil constituent, readily mobile in plants and at certain concentrations it may enhance plant growth. Application of lithium

chloride and nitrate to wheat seedlings stimulated growth at low concentrations and at higher concentrations inhibited growth (Kent 1941). Powdery mildew attacked wheat plants and cucumber was reduced after the application of lithium chloride (Kent 1941, Abood 1990).

A complete understanding of the action of lithium chloride requires biochemical studies as well as electron microscopic investigations. Electron microscopic studies have yielded detailed information about the mode of action of several systemic fungicides.

So far no studies on the control of *Botrytis fabae* by lithium chloride have been reported. However, the objective of this study is to use ultrastructural methods to study the effect of lithium chloride on *Botrytis fabae* and also its effect on the host to determine the suitable and effective concentration for controlling the pathogen.

MATERIAL AND METHODS

Treatment of Botrytis fabae *by lithium chloride*

The effect of lithium chloride (purchased from Sigma Company, UK) on *Botrytis fabae* Sard. was studied by growing the fungus on potato dextrose agar (PDA) medium amended by 1, 3, 5, 7 and 9 mM of lithium chloride. Sterile molten PDA amended with these concentrations then dispersed immediately into 9 cm diameter polystyrene Petri plates. After cooling, each plate was inoculated with a 6 mm diameter mycelial plug from the margin of a day old culture of *Botrytis fabae.* Then, the plates were incubated at 25 °C.

Plant growth conditions

Seeds of *Vicia faba* were obtained from Agricultural Research Center, Giza, Egypt and surface sterilized in 7% calcium hypochlorite for 15 min., and washed with sterilized D. W. Seeds were planted then in plastic pots containing sterilized soil. Each treatment was composed of 20 replicate plants, five per pot. Pots were completely randomized in glasshouse under natural environmental conditions of day length and light intensity in winter and watered regularly.

Treatment of the host by lithium chloride

The effect of recommended doses of lithium chloride on plant growth in pot experiment was investigated. After 3 weeks from planting, lithium chloride at different concentrations (1, 3, 5, 7 and 9 mM) was applied as a drench.

Inoculation of the host by Botrytis fabae

After 5 weeks from planting, *Vicia faba* plants were inoculated with conidial suspension of *B. fabae*. The one to two weeks old culture of *Botrytis fabae* has grown on PDA medium was used. Conidial suspension at concentration ranging from 10,000–50,000/ml was used for inoculation. The suspension was sprayed over leaves with a sprayer until the leaves were uniformly wet. Then, the plants were covered by plastic bags and placed in greenhouse. Untreated plants (control) were drenched with water only at the same time.

Determination of photosynthetic pigments

Plant photosynthetic pigments (chlorophyll *a* and *b*, and carotenoids) were determined according to the spectrophotometric method recommended by Metzner *et al.* (1965). A known fresh weight of leaves was homogenized in 85% acetone for 5 min. The homogenate was filtered and made up to volume with 85% acetone. The filtered extract was measured against a blank of pure 85% acetone at 3 wavelengths for 452.5, 644 and 663 nm using Acta Beckmann spectrophotometer.

Electron microscopy

Mycelial samples of *Botrytis fabae* as previously prepared, infected leaves of *Vicia faba* by *Botrytis fabae* and comparable healthy leaves and *Vicia faba* leaves treated with lithium chloride only were processed for transmission electron microscopy (TEM) by a method based on Woods and Gay (1987) and modified by Baka (1987). All samples were prefixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH = 7 for 24 h at room temperature. Then, the samples were rinsed in fresh buffer and postfixed in 1% osmium tetroxide in the same buffer for 1–2 h at room temperature. After postfixation, the samples were washed in fresh buffer, dehydrated in a graded ethanol series and embedded in Spurr's resin (Spurr 1969). Ultrathin sections were cut using JUM-S ultramicrotome, stained by 2% aqueous uranyl acetate (Juniper *et al.* 1978) followed by lead citrate (Reynolds 1963). The ultrathin sections were examined and photographed by JUN-5 G electron microscope.

Table 1
Effect of different concentrations of lithium chloride on photosynthetic pigments (mg/gm f.wt.) of *Vicia faba*. Each value is the mean of 3 replicate±standard error

Concentration (mM)	Chlorophyll *a*	Chlorophyll *b*	Carotenoids
Control	36.39±0.75	18.38±5.87	22.44±0.99
1	36.53±2.58 NS	27.40±3.62 S	13.93±1.42 S
3	34.28±3.42 NS	17.32±6.42 NS	16.68±1.35 S
5	41.36±0.62 HS	9.20±1.77 S	17.32±2.34 S
7	35.67±2.04 NS	8.06±3.53 S	17.73±1.38 S
9	15.99±4.64 HS	8.48±4.64 HS	14.50±1.87 S

Statistical analysis

Statistical analysis of the data was performed using *t*-test with significance level of entry = 0.05%. Each data represented through this work at least the mean of 3 replicates.

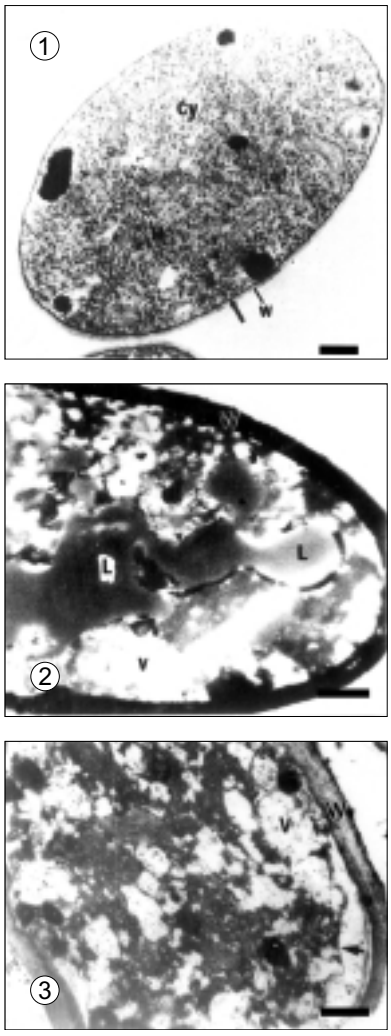
RESULTS

*Effect of lithium chloride on photosynthetic pigments of the host (*Vicia faba*)*

At the concentration of 1 mM, chlorophyll *a* was found to be a non-significant increase than control, whereas chlorophyll *b* showed a significant increase. The carotenoids at this concentration showed a significant decrease. At the concentration of 3 mM, the results showed a non-significant decrease of both chlorophyll *a* and *b* and a significant decrease of carotenoids. At the concentration of 5 mM, chlorophyll *a* showed a highly significant increase, while chlorophyll *b* and carotenoids showed a significant decrease. At the concentration of 7 mM, chlorophyll *a* exhibited a non-significant decrease, whereas chlorophyll *b* and carotenoids showed a significant decrease. Finally, at the concentration of 9 mM, chlorophyll *a* and *b* showed a highly significant decrease and carotenoids showed a significant decrease (Table 1). In conclusion, it seems that chlorophyll content was not affected at lower concentration of lithium chloride, while the higher concentration decrease the chlorophyll content.

Effect of lithium chloride on the ultrastructure of Botrytis fabae

Two concentrations (3 and 9 mM) of lithium chloride were selected for electron microscopic work. TEM examination revealed that, in the absence of



Figs 1–3. Botrytis fabae hypha grown on medium: 1 = lacking lithium chloride. The cell is surrounded by a thin wall (W) and the cytoplasm (Cy) contains mitochondria (M). Note an electron-dense plasmalemma (arrow); 2 = containing 3 mM lithium chloride. Note the thickening of cell wall (W) and disintegration of cytoplasm. Note also large lipid bodies (L) and vacuoles (V). 3 = containing 9 mM lithium chloride. Note the thickening of cell wall (W), disintegration of cytoplasm, and vacuoles (V). Note also the retraction of plasmalemma (arrowhead). Bar = 0.5 µm

lithium chloride, a thin, electron-lucent wall against electron-dense plasmalemma delimited the hyphae of *B. fabae*, which was closely appressed to the cell wall. The hyphal cell contained polysome-rich cytoplasm with numerous organelles such as mitochondria and nuclei indicating high level of metabolic activity (Fig. 1). Examination of sections from *B. fabae* grown in the presence of 3 mM lithium chloride showed damage to this fungus. A pronounced cellular disorganization that ranged from disruption of the wall to marked cytoplasmic degeneration was detected. The affected *Botrytis* cells were generally surrounded by swollen, electron-dense, distorted cell walls, and the cytoplasm appeared highly altered with no discernible organelles (Fig. 1). In many cases, an increase in lipid bodies and vacuoles was observed (Fig. 2). Examination of sections from *B. fabae* grown in the presence of 9 mM lithium chloride showed severe alterations in the hyphal cell. The cell wall was thicker than normal and followed by a retracted plasmalemma. The retraction and disintegration of the cytoplasm and increasing of vacuoles are common characteristic features of hyphal cells at this concentration (Fig. 3).

Effect of B. fabae *on the ultrastructure of host cell*

Uninfected host cell showed a normal elongated chloroplast with an organized membrane system. The chloroplast was also contained few plastoglobuli, small starch grains and enclosed by a normal chloroplast envelope (Figs 4 and 5). On the other hand, the nucleus of uninfected cell appeared spherical in shape and contained a nucleolus, patches of electron-dense heterochromatin, and electron-lucent euchromatin (Fig. 6). Infected cell from host leaf by *B. fabae* showed a spherical chloroplast, which was not closely associated to the cell wall. Disorganized membrane system and increasing in number of plastoglobuli were also observed (Fig. 7). The nucleus was not affected as a result of infection except the absence of nucleolus. The appearance of large lipid bodies was a characteristic phenomenon during the infection (Fig. 8). In addition, the more vacuolated cytoplasm and disorganized cristae of mitochondria were also detected (Fig. 9).

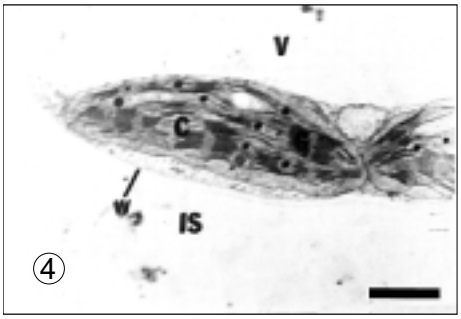
Effect of lithium chloride on the ultrastructure of host cell

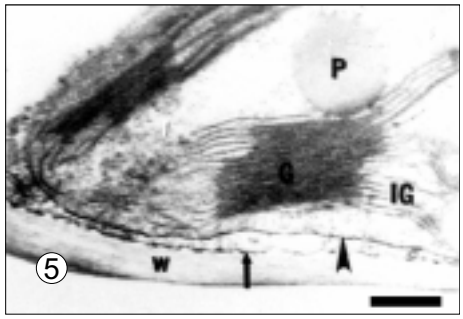
Untreated host cell with lithium chloride gave a cell containing a normal ellipsoidal chloroplast with an organized membrane system and few number of plastoglobuli similar to uninfected host cell (Figs 4 and 5). The treated host cell with 3 mM lithium chloride indicated the presence of more or less normal chloroplast with an organized membrane system and few number of plastoglobuli. The other organelles such as mitochondria, endoplasmic reticulum and microbodies were not affected at this concentration (Fig. 10). The concentration of 9 mM caused severe alterations in the chloroplast, such as the disorganization of membrane system and increasing in number of plastoglobuli. The chloroplast envelope was more thick than normal (Figs 11 and 12).

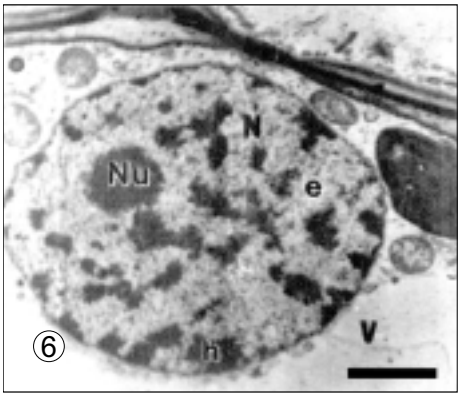
Figs 4–9. 4 = Uninfected host cell showing normal ellipsoidal chloroplast (C) with grana (G). The chloroplast is closely associated to the cell wall (W). Note intercellular space (IS) and cell vacuole (V). Bar = 2.0 μm. 5 = A magnified part of chloroplast from uninfected host cell. Not organized granum (G), intergranal lamellae (IG), plastoglobuli (P) and chloroplast envelop (arrowhead). Note also host plasmalemma (arrow) and cell wall (W). Bar = 0.5 μm. 6 = A spherical nucleus (N) from uninfected host cell with nucleolus (Nu). Note the patches of an electron-dense heterochromatin (h) and an electron-lucent euchromatin (e). Note also the cell vacuole (V). Bar = 2.0 μm. 7 = Infected host cell showing a spherical chloroplast (C) with disorganized membrane system. Note the large number of plastoglobuli (arrowhead). The chloroplast is not closely associated to the cell wall (W). Note the host cell vacuole (V). Bar = 2.0 μm. 8 = Infected host cell showing a nucleus (N) without nucleolus. Note cell vacuole (V) and large lipid body (L). Bar = 2.0 μm. 9 = Infected host cell showing the vacuolation (V) of cytoplasm. Note the affected chloroplast (C) and the disorganized cristae of mitochondria (M). Bar = 2.0 μm

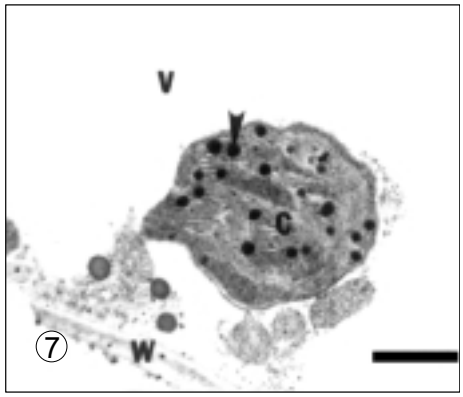
DISCUSSION

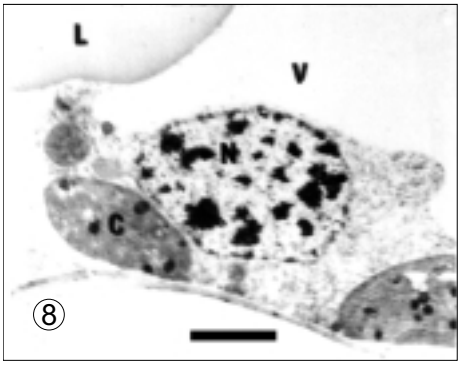
TEM examination of sections from *B. fabae* grown in the presence of 3 and 9 mM lithium chloride showed a pronounced cellular disorganization of the fungus. Abood (1990) reported that low and high concentration of lithium chloride inhibited the growth of the powdery mildew fungus, *Sphaerotheca fuliginea*. Most of these changes are in agreement with the findings of

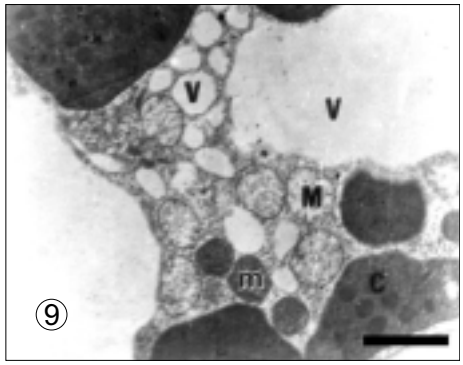


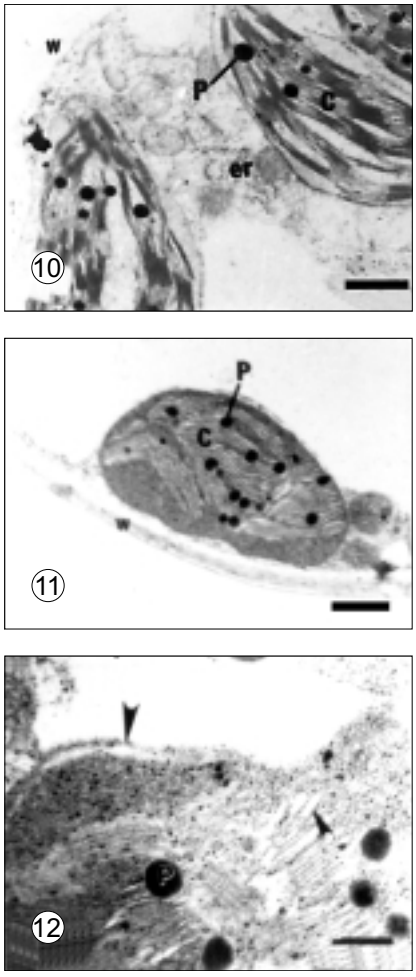












Figs 10–12. Treated host cell with lithium chloride: 10 = at the concentration of 3 mM showing organized membrane system of chloroplast (C). Note the cell wall (W), plastoglobuli (P) and endoplasmic reticulum (er). Bar = 1.0 µm. 11 = at 9 mM showing degenerated chloroplast (C) with an increase of plastoglobuli number. Note the cell wall (W). Bar = 1.0 µm. 12 = A magnified part of the degenerated chloroplast from host cell treated with lithium chloride at the concentration of 9 mM. Note a thickened chloroplast envelope (large arrowhead) and swollen thylakoids (small arrowhead). Bar = 0.5 µm

Khashaba (2000) who treated *Cephalosporium acremonium*, *Penicillium chrysogenum* and *Aspergillus flavus* with the fungicide PCNB at the concentration of 100 ppm. These changes also in coincidence with the observations of Abdel-Fattah and Baka (2000) in case of *Phytophthora infestans* treated with 100 ppm benomyl. The alterations in the fungus ranged from cytoplasmic retardation and changes in fungal wall thickness to complete protoplasm disintegration, thus indicating impaired cellular metabolism. In addition to causing severe cytoplasmic damage, lithium chloride also caused pronounced plasmalemma changes in the fungus. In general, membrane structure is influenced by the physiological state of the cell (growth stage, supply of nourishment, etc.) (Takeo *et al.* 1976). On the other hand, metabolic processes depend on the consistency of the membranes, particularly the lipid/protein ratio (Gennis and Jonas 1977). Changes in membrane structure are therefore important hints for cell pathological processes ultrastructural changes of the plasmalemma. The variation in sensitivity to fungicides may also be related to the sterol composition of the plasmalemma (Hippe 1985).

The treatment of host cells with 3 mM lithium chloride indicated the presence of more or less normal chloroplasts with organized membrane system. In contrast, the concentration of 9 mM led to severe alterations in the chloroplasts. These

results are in support of the data of chlorophyll. The concentration of 3 mM showed a non-significant decrease in both chlorophyll *a* and *b* while a significant decrease in case of 9 mM was observed. These results in agreement with those obtained by Abood (1990) when he found that low concentration of lithium chloride could stimulate the chlorophyll content, but high concentration inhibited the chlorophyll content of cucumber leaves.

Infection of *V. faba* leaves by *B. fabae* led to disorganization of chloroplast membrane system, breakdown of the chloroplast envelope, an increase in number and size of plastoglobuli and disappearance of starch grains. These results agree in part with the findings of Shabana *et al.* (1997) who reported that an increase of plastoglobuli in water hyacinth leaves infected with *Alternaria eichhorniae*. The function of plastoglobuli is not fully understood, but they are believed to be reservoirs of excess lipids (Greenwood *et al.* 1963) and may be products of senescence since they increased in both size and number during aging (Baka and Aldesuquy 1991). The disappearance of starch from chloroplasts due to infection coincided with the observation of Baka (1987) on different infected hosts. Decrease in starch is common in many foliar diseases (Wheeler 1975).

The dramatic ultrastructural changes in host cell organelles after infection by *B. fabae* may be due to the fact that the pathogen secretes specific enzymes to dissolve cell wall and affect organelles. This is in agreement with the observation of Baka and Krzywinski (1996). Fungal pathogens also produce hydrolytic enzymes such as cellulase, hemicellulase and cylanase that can degrade cell wall polysaccharides and utilize the conrides as a source of carbon for their growth and development (Campbell *et al.* 1980, Cooper *et al.* 1980, Langsdorf *et al.* 1991). Hancock and Miller (1965) have also demonstrated the importance of these enzymes in leaf spot diseases. The disruption of cell plasma membrane as a result of fungal toxins after infection was also reported by Park *et al.* (1977) in case of susceptible apple leaves infected with *Alternaria mali*, and Langsdorf *et al.* (1991) in case of tomato leaves treated with alternaric acid.

Remarkable changes in nuclei of *V. faba* leaves infected by *B. fabae* were also demonstrated. These changes include a decrease in heterochromatin amount and disappearance of the nucleolus, which coincided, with the findings of Baka (1987) on infected hosts by rust fungi. The decline of heterochromatin led to a decrease of nuclear DNA (Al-Khesraji 1981). The later phenomenon suggested a specific effect of the pathogen on host metabolism (Whitney *et al.* 1962).

In conclusion, this study revealed that the lower concentration of lithium chloride (3 mM) caused less harmful effect on the host (*V. faba*). In contrast, lower and higher concentrations (3 and 9 mM) inhibited the growth of the pathogen. However, the lower concentration of lithium chloride can be used

as a safe fungicide to control *B. fabae*. This conclusion in support to the results of chlorophyll obtained in the present investigation and also from morphological, physiological and cytological data (unpublished).

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