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COMPARATIVE STUDIES ON THE EFFECT OF BENOMYL ON GROWTH AND ULTRASTRUCTURE OF TWO ISOLATES OF *PHYTOPHTHORA INFESTANS* FROM EGYPT

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The effect of benomyl as a fungicide on the growth rate and ultrastructure of two isolates (P623 and P1319) of *Phytophthora infestans* is compared. Using different concentrations (50, 100, 200 and 500 ppm) of benomyl caused an inhibition of the mycelial growth of both isolates depending on the degree of concentration. The isolate P1319 was found to be more sensitive to benomyl than the isolate P623. Ultrastructural studies confirmed these observations. The hyphae of isolate P1319 subjected to 100 and 500 ppm benomyl showed severe changes in the cytoplasm more than isolate P623. The increase of lipid bodies and vacuoles in hyphal cytoplasm of both isolates was the characteristic phenomenon after treatment of benomyl particularly at the concentration of 500 ppm.

Key words: benomyl, *Phytophthora infestans*, radial growth, ultrastructure

INTRODUCTION

Late blight caused by *Phytophthora infestans* (Mont.) De Bary is an important disease of potato (*Solanum tuberosum* L.) in Egypt (Baka 1997) and worldwide (Fry et al. 1993) which causes a tremendous loss in this vital and economic crop every year. The application of systemic fungicides led to the inhibition of zoospore production and enhancement of hyphal lysis (Fenn and Coffey 1984). Various investigators (Gill and Zentmyer 1978, Erselius and de Vallavielle 1984) have studied the intrageneric taxonomy of *Phytophthora*. One of the promising physiological methods for the identification and classification of *Phytophthora* spp. is the gel electrophoresis of their soluble proteins (Faris et al. 1986). It has proved more convenient than serology, although the latter has other advantages (Gallegly 1983). A new approach has been developed for the classification of *Phytophthora* species, which depends on the sensitivity and insensitivity for fungicides. A complete understanding of the action of systemic fungicides requires biochemical studies as well as electron microscopic investigations. The combina-

tion of both approaches aims to an understanding of the mechanisms of action of fungitoxic agents. Electron microscopic studies have yielded detailed information about the mode of action of several systemic fungicides. Electron microscopy complemented biochemical results is so far as a disturbance of the membrane function could be related to changes in morphology and fine structure. In addition to general cytopathological effects, a significant increase in thickness of the cell walls was found after fungicide treatment (Hippe 1985).

Benomyl [methyl 1-(butyl carbamoyl) benzimidazol-2-yl carbamate] is a systemic fungicide effective against most fungi (Johnston and Booth 1983). So far no studies on the effect of this fungicide on *Phytophthora* have been reported. However, the present study was undertaken to compare in vitro between two isolates of *Phytophthora infestans* by using benomyl as a systemic fungicide in terms to its effect on the growth and ultrastructure.

MATERIALS AND METHODS

Organism and media. Two isolates of *Phytophthora infestans* (P623 and P1319) were isolated from diseased potatoes (*Solanum tuberosum* L.) grown in the farm of Faculty of Agriculture at Mansoura University, Egypt. Prof. M. D. Coffey, Department of Plant Pathology, University of California at Riverside, USA was kindly confirmed the identification of these isolates. Stock cultures were stored on potato dextrose agar (PDA: 300 g potato, 20 g dextrose and 15 g Difco purified agar made up to 1 litre with distilled water and the pH was adjusted to 6.1) under sterile mineral oil at 4 °C.

Fungicide testing. Benomyl was tested throughout these experiments as a 50% wettable powder. This fungicide was used at concentrations of 0, 50, 100, 200 and 500 ppm active ingredient.

Growth on solid media. PDA medium was prepared with 15 g/l of Difco purified agar and the pH was adjusted to 6.1. Fifteen ml of autoclaved medium amended with the corresponding concentrations of fungicide was dispensed into 9 cm sterile plastic Petri dishes. A half-cm diameter agar disc, taken from the margins of the active fungal colony, was placed with the fungal side downward in the centre of each plate. Plates were incubated in the dark at 21 °C. Radial growth was determined by measuring colony diameters daily at two points on each Petri dish and taking the average value. Three dishes per each treatment were used per isolate. The experiment was repeated once.

Growth in liquid culture. Isolates of *Phytophthora infestans* were cultured in potato-dextrose liquid medium. Fifty ml of liquid medium amended with the corresponding concentration of fungicide were added to 250 ml Erlenmeyer flasks, stoppered with cotton plugs and autoclaved for 20 min. Inoculum was prepared by culturing the fungus on a solid PDA medium. Two plugs of mycelium (5 mm diameter) were cut from the margins of the fungal colony and transferred to the flasks which containing 50 ml of sterilized PD broth medium. After 6 days of stationary culture in the dark at 21 °C, the culture was minced in a Sorvall Omni-Mixer for 20 s at medium speed. One ml of this minced mycelium was added to each 125 ml flask containing 50 ml of medium. The cultures were harvested at 4, 8 and 12 days and then filtered through 2.5 cm diameter Whatman glass fibre filter discs, oven dried, and weighed. Mycelial dry weights were calculated by subtracting the filter disc dry weight from the dry weight plus mycelium.

Cytological effect of benomyl. The effect of benomyl on *P. infestans* was studied by growing the two isolates on PDA medium amended with 100 and 500 ppm of benomyl. Sterile molten PDA, which amended with these concentrations, dispensed immediately into 9 cm diameter polystyrene Petri dishes. After cooling, each plate was inoculated with a 6 mm diameter mycelial plug from the margin of a 4 day-old culture and the plates were incubated at 21 °C. Four replicate plates were used for each isolate at each concentration of benomyl and from each replicate plate, two mycelial samples from the margin of the cultures were processed for ultrastructural studies.

Tissue processing. This method is based on Hayat (1989) and modified by Baka (1996). Mycelial samples (1.0 mm^2) were incubated for 12 h at 4 °C in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH = 7) and rinsed in the same buffer. Thereafter, samples were postfixed for 1 h at 4 °C (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH = 7) and then dehydrated in graded series of ethanol. The samples then were embedded in Spurr's resin, sectioned using an ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynolds 1963) before examination with Joel 100-S TEM. For each sample, at least 10 ultrathin sections were examined.

RESULTS

Growth on solid media. Table 1 compares the in vitro antifungal activity of benomyl towards mycelial growth of the isolates P623 and P1319 of *P. infestans* cultured on PDA medium. Results showed that both fungicides exerted various degrees of inhibition of linear growth of the two isolates at all concentrations used. The degree of inhibition was decreased with the increase of fungicide concentrations. It was obvious that the two isolates were different in sensitivity to benomyl. Isolate P1319 is more sensitive to benomyl than the isolate P623 (Fig. 1). Increasing the benomyl concentrations to 500 ppm resulted in a reduction of 55 and 51% in the growth of the two isolates P1319 and P623, respectively.

Growth in liquid media. The effect of different concentrations of benomyl on mycelial dry weight of the two isolates of *P. infestans* grown in liquid media is presented in Table 2. As compared to control, all concentrations of benomyl caused a decline in the mycelial dry weight but the degree of this decline was more pronounced at high concentration of fungicide. Isolate P1319 was more inhibited by the fungicide than isolate P623 at all days after inoculation. In the meantime, increasing the benomyl concentration tenfold from 50 to 500 ppm caused a decline in the growth of the two isolates P1319 and P623 from 35 to 78% and 31 to 71%, respectively, at 8 days of incubation period.

Cytological effect of benomyl. In the absence of benomyl, electron microscopy revealed that the subcellular components of both isolates of *P. infestans* are more or less the same. The hyphal cell is enclosed by a distinct

Table 1

Comparison of the inhibitory activity of benomyl on mycelial linear mycelial of two isolates of *Phytophthora infestans* grown on solid potato dextrose medium

Fungicide conc. (ppm)	Isolate P1319		Isolate P623	
	Radial growth rate (mm/h)	% of inhibition	Radial growth rate (mm/h)	% of inhibition
0	0.96 ± 0.08	–	1.05 ± 0.01	–
50	0.92 ± 0.01	4.1	0.98 ± 0.02	6.6
100	0.80 ± 0.02	16.0	0.91 ± 0.03	13.0
200	0.68 ± 0.10	29.0	0.75 ± 0.01	28.0
500	0.43 ± 0.03	55.0	0.51 ± 0.02	51.0

Radial growth was determined by measuring colony diameters daily at two points on each Petri dish for 10 days and taking the average value. Values are means of six samples ± standard error

Table 2

Comparison of the inhibitory activity of benomyl on mycelial biomass growth of two isolates of *Phytophthora infestans* grown on liquid potato dextrose medium

Incubation period (days)	Fungicide conc. (ppm)	Isolate P1319		Isolate P623	
		Dry weight (mg/50 ml)	% of inhibition	Dry weight (mg/50 ml)	% of inhibition
4	0	101.0 ± 2.12	–	110.0 ± 1.19	–
	50	88.0 ± 1.70	28.0	99.0 ± 3.50	10.0
	100	69.0 ± 1.80	39.0	76.0 ± 4.20	30.0
	200	42.0 ± 1.40	58.0	55.0 ± 3.50	50.0
	500	29.0 ± 1.90	71.0	38.0 ± 1.40	65.0
8	0	120.0 ± 3.50	–	123.0 ± 1.41	–
	50	78.0 ± 1.87	35.0	85.0 ± 3.30	31.0
	100	55.0 ± 2.8	54.0	63.0 ± 2.12	49.0
	200	45.0 ± 2.6	62.0	49.0 ± 1.71	60.0
	500	26.0 ± 2.9	78.0	35.0 ± 3.50	71.0
12	0	63.0 ± 4.1	–	65.0 ± 2.5	–
	50	48.0 ± 1.4	23.0	59.0 ± 1.8	10.0
	100	33.0 ± 1.2	77.0	46.0 ± 0.7	29.0
	200	21.0 ± 0.8	66.0	31.0 ± 2.8	52.0
	500	18.0 ± 0.3	71.0	26.0 ± 0.7	60.0

Values are means of five replicates ± standard error

electron opaque cell wall followed by an undulated plasmalemma. Many organelles are observed such as the nucleus which surrounded by a double membrane, endoplasmic reticulum (er) which associated to the nucleus, numerous vacuoles, numerous lipid droplets, Golgi bodies and mitochondria (Figs 1A and 2A). Examination of sections of isolate P623 in the presence of 100 ppm benomyl led to little ultrastructural changes (Fig. 1B) including the appearance of some vesicles adjacent to plasmalemma. The addition of 500 ppm of benomyl to the growth media of the same isolate revealed more conspicuous changes in the hyphal cytoplasm. These changes comprise bigger lipid bodies, more vacuolated cytoplasm, and thickened plasmalemma with electron inclusions on its surface (Fig. 1C).

Cytological alterations are also exhibited in the isolate P623 grown in the presence of 100 ppm benomyl. Remarkable cytoplasmic degeneration, an increase of lipid bodies, an increase of vacuoles was usually noticed (Fig. 2B). On the other hand, the addition of higher concentration of benomyl (500 ppm) to the growth medium of the same isolate led to more

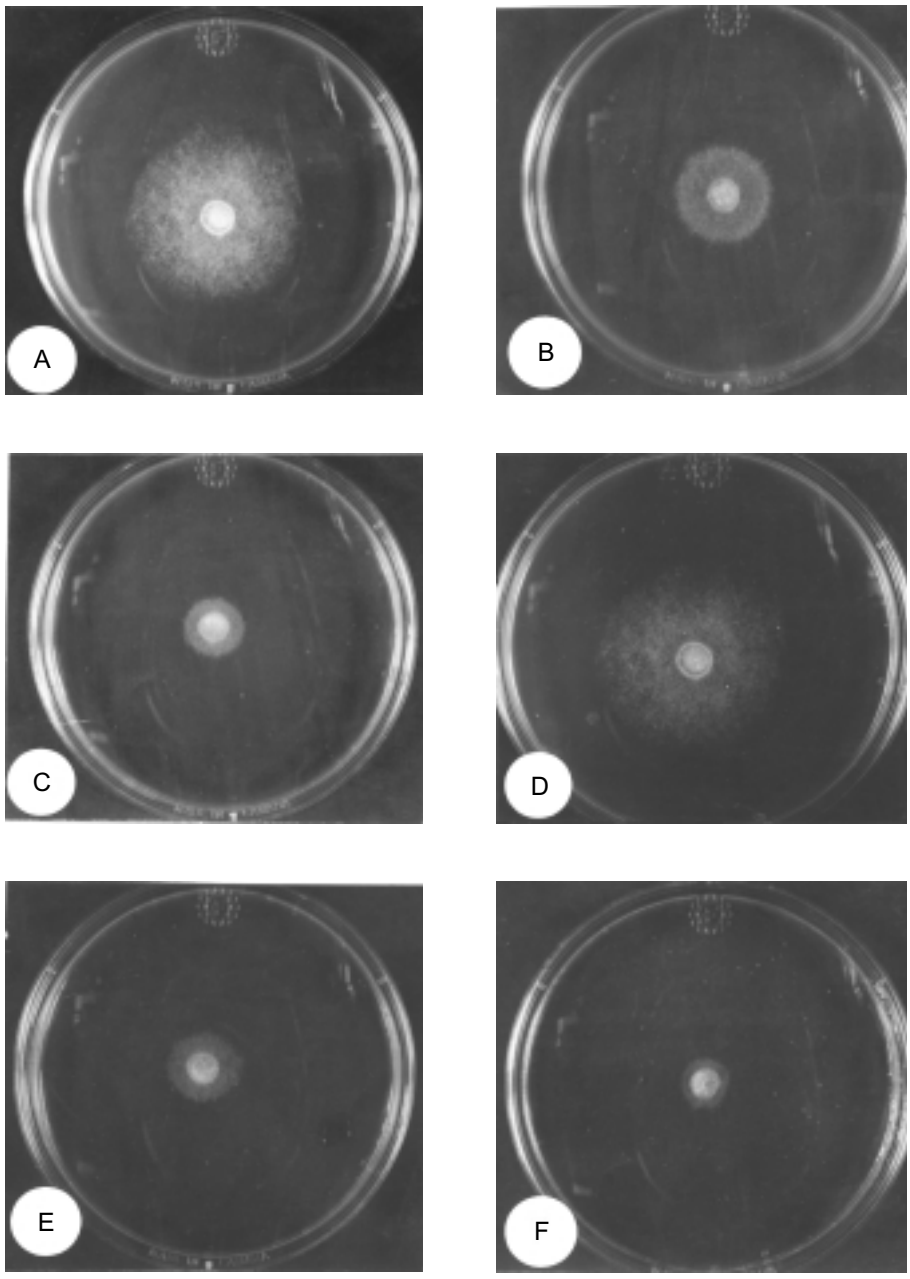

Fig. 1. Colony size of *P. infestans* after 5 days of growth at 21 °C on PDA medium. – A, B and C are representing isolate P623. A: medium without the addition of benomyl (control). B: medium amended with 100 ppm benomyl. C: medium amended with 500 ppm benomyl. – D, E and F are representing isolate P1319. D: control. E: medium amended with 100 ppm. F: medium amended with 500 ppm benomyl

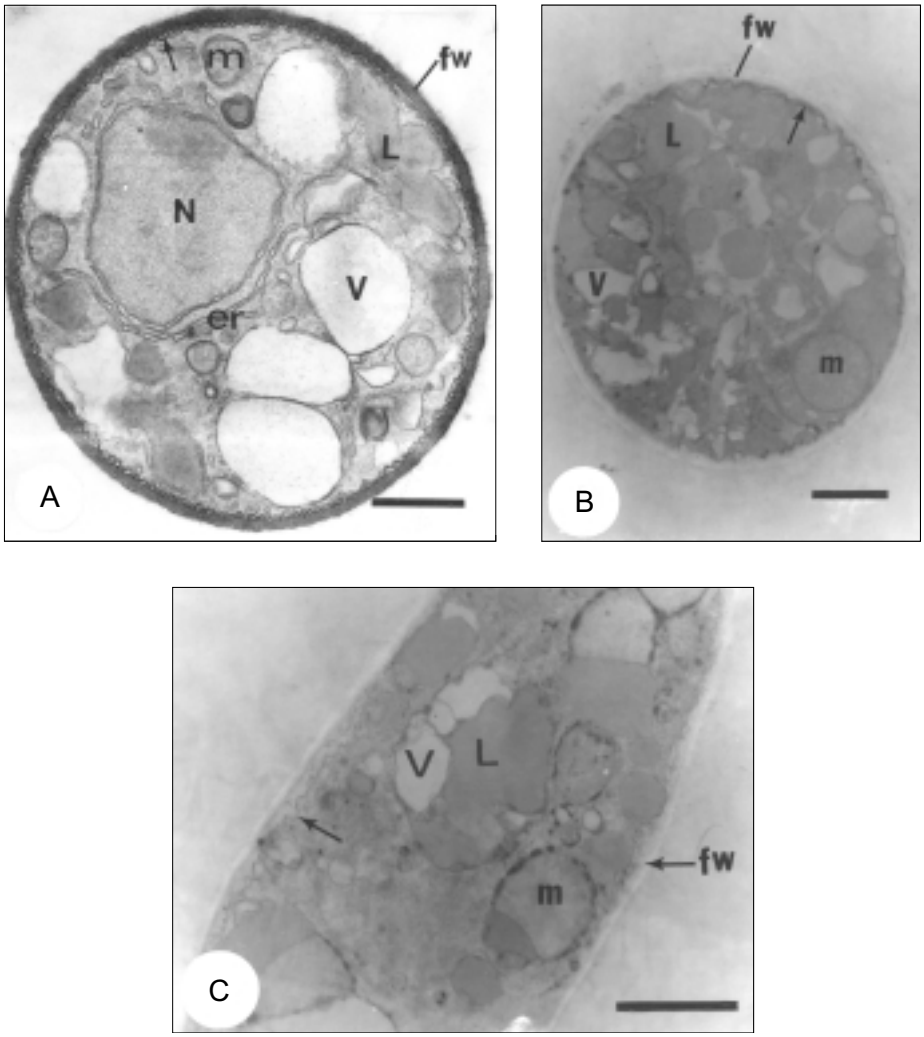

Fig. 2. Effect of benomyl on the ultrastructure of hypha of isolate P1319. – A: a section of hypha from untreated medium (control) showing an electron opaque cell wall (fw), plasmalemma (arrow). The protoplasm contains a nucleus (N) with double-membrane envelope, endoplasmic reticulum (er), vacuoles (V), lipid bodies (L) and mitochondria (m). Bar = 0.5 μm. – B: a section of hypha from medium amended with 100 ppm benomyl. The increase of lipid bodies (L), the collapsed cytoplasm and the increase of vacuoles (V) are observed. The hyphal wall (fw) is electron lucent and the plasmalemma (arrow) is thicker. Note the mitochondria (m). Bar = 1.0 μm. – C: a section of hypha from medium amended with 500 ppm benomyl. The degeneration of cytoplasm, large lipid bodies (L), vacuoles (V) are noticed. Note an electron lucent hyphal wall (fw) and the retraction of plasmalemma (arrow). Note also electron dense materials (arrowheads) are deposited on the envelopes of mitochondria (m). Bar = 1.0 μm

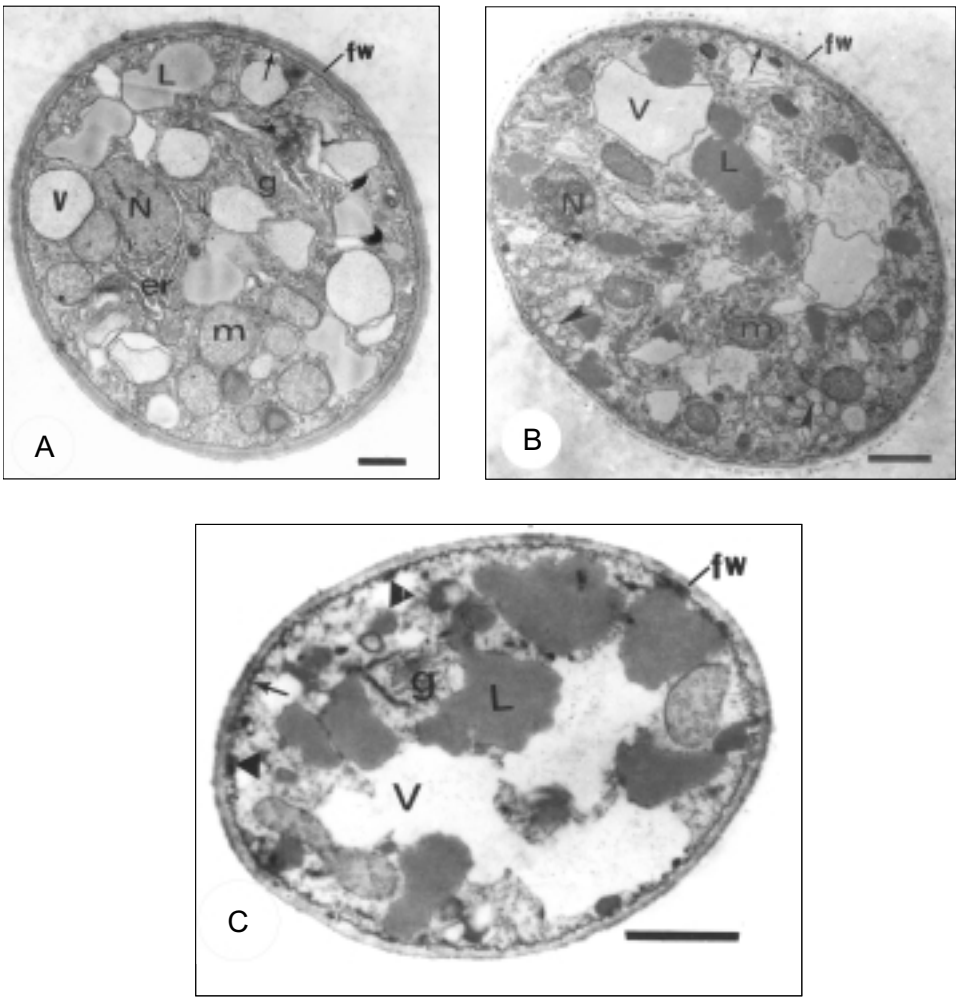

Fig. 3. Effect of benomyl on the ultrastructure of hypha of isolate P623. – A: a section of hypha from untreated medium (control) showing an electron opaque hyphal wall (fw), plasmalemma (arrow). Note the nucleus (N), numerous lipid bodies (L), Golgi bodies (g), endoplasmic reticulum (er), mitochondria (m) and numerous vacuoles (V). Bar = 0.5 µm. – B: a section of hypha from medium amended with 100 ppm benomyl. Note an electron lucent hyphal wall (fw), undulated plasmalemma (arrow), an increase of lipid bodies (L), an increase of vacuoles (V). Note also mitochondria (m), nucleus (N) and the presence of small vesicles (arrowheads) near the hyphal wall. Bar = 0.5 µm. – C: a section of hypha from medium amended with 500 ppm benomyl. Note the hyphal wall (fw), large lipid bodies (L), large vacuoles (V), disorganized Golgi bodies (g). Note also electron dense materials (arrowheads) are deposited on the plasmalemma (arrow). Bar = 1.0 µm

ultrastructural changes. These changes include an increase of big lipid bodies, highly degenerated cytoplasm, and the appearance of electron dense deposits on mitochondrial envelope (Fig. 2C).

Generally, ultrastructural comparison of the hyphae of both isolates treated with benomyl revealed that isolate P1319 is more sensitive than isolate P623. This observation confirmed the results obtained in case of linear growth and mycelial dry weight.

DISCUSSION

Throughout this investigation, benomyl caused a remarkable inhibition on the mycelial growth of two isolates of *P. infestans* when growing on PDA medium. The extent of this inhibition was depending on the concentration of fungicide and also the type of isolate. Our experiments have revealed that benomyl had a high efficacy against the isolate P1319 than isolate P623. The breakdown of benomyl into methyl-2-benzimidazol carbamate (MBC) is known to be rapidly translocated and to have different modes of action. It inhibits fungal growth by inhibiting respiration but its effect seem to be transient (Hammerschlag and Sisler 1973). Quinlan et al. (1980) reported that MBC could disrupt mitosis in fungi by interfering with spindle formation and causing distortion of germ tubes and hyphae.

Benomyl function by binding to fungal B-tubulin and by preventing microtubular polymerisation and thus interferes with a number of cellular processes such as mitosis, meiosis, intracellular transport of molecules and maintenance of cell shape (Orbach et al. 1986, Peterbauer et al. 1992). Our observations are in agreement with the previous works using different strains of *Phytophthora* (Fenn and Coffey 1984, Schinable et al. 1998). Baka (1997) reported that there was an intraspecific isozyme variation between 19 Egyptian isolates of *P. infestans*. These isolates appeared to contain diploid, triploid and tetraploid after measuring the nuclear DNA content.

In the current study, we show that benomyl caused remarkable ultrastructural alterations in both isolates of *P. infestans*. Benomyl caused a severe cellular disorganization in the hyphae of isolate P1319 than those of isolate P623, which ranged from cytoplasmic retardation, fungal wall alteration to complete protoplasm disintegration, thus indicating that it might have impaired cellular metabolism. In addition to causing severe cytoplasmic damage, benomyl also caused pronounced plasmalemma changes in isolate P1319. In general, membrane structure is influenced by the physio-

logical 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 of cell pathological processes. Hippe (1985) reported that the treatment of *Ustilago avenae* by the fungicide triadimenol caused pronounced ultrastructural changes of plasmalemma. The variation in sensitivity to fungicides may also related to the sterol composition in plasmalemma. This theory may fit well our observations, since isolate P1319 is more sensitive to benomyl than isolate P623 and the former is accompanied by more changes in plasmalemma.

More information is required on the extent to which a range of isolates of *P. infestans* may differ in their inherent in vitro and in vivo sensitivity to benomyl. Also, it will be useful to study the effect of benomyl on large number of isolates to detect whether any is resistant to this type of fungicide.

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