Biochemical and Ultrastructural Characterization of the Modes of Action of the Soybean Phytoalexin Glyceollin to Fungal Hyphae*

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Abstract

Glyceollin, a phytoalexin produced by soybeans, partially and completely inhibited growth of young hyphae of Phytophthora capsici in liquid culture at 20 and 50 μg/ml, respectively, within 4 hr of incubation. Glyceollin at the same concentrations induced electrolyte leakage from hyphae immediately following the treatment and inhibited incorporation of an amino acid or nucleoside into an acid soluble "pool" fraction of hyphae. Oxygen uptake by hyphae was also inhibited by glyceollin but only after a lag period of several minutes. These results suggested that the possible primary mechanism of glyceollin action on the fungal hyphae was to interfere with the functions of plasma membrane. Ultrastructural study showed that glyceollin at the inhibitory concentrations induced characteristic alterations in the structure of fungal plasma membranes at early stages (30 to 60 min) following treatment, while membrane systems of intracellular organelles remained relatively undamaged. Plasma membranes of the glyceollin-treated hyphae were flattened compared to those of the untreated hyphae and separated from cell walls at some places, and vesicle-like structures were observed between the plasma membranes and cell walls. Furthermore, plasma membranes appeared to be absent from some portions of the cell periphery. In contrast to glyceollin, some toxicants such as cycloheximide and 2, 4-dinitrophenol at the doses that completely inhibited hyphal growth did not induce significant distortion in the structure of plasma membranes, although the latter inhibitor induced electrolyte leakage as rapidly as did glyceollin. These results suggest that the observed distortion in plasma membrane structure is not a nonspecific general response of fungal hyphae due to growth inhibition caused by a variety of toxicants, but it appears to be a characteristic morphological symptom induced by glyceollin. Possible use of this morphological parameter in evaluating the role of glyceollin in soybean disease resistance is suggested.

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Key words: Phytophthora capsici, glyceollin, mode of action.

Introduction

Inducible production of antibiotic molecules, phytoalexins, has been suggested to confer natural disease resistance in higher plants. More than 70 chemically-defined phytoalexins have been isolated from nearly 100 plant species10). Despite their extensive

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occurrence in higher plants, the precise function of phytoalexins in disease resistance as well as their modes of antibiotic action are not fully established.

The early work by VanEtten and Bateman\(^{19}\) on the mechanism of action of phaseollin demonstrated that the phytoalexin drastically affected the function of fungal and plant membranes, resulting in rapid inhibition of solute uptake into cells or leakage of cell constituents into the media. They also showed that phaseollin rapidly lysed human erythrocytes. Since then, similar effects on membranes of fungal, bacterial, plant, and animal cells have been shown with different phytoalexins including pisatin\(^{12,16}\), glycinol\(^{20}\), rishitin\(^{5,6,11}\), phytuberin\(^{5,6}\), and capsidiol\(^{18}\) as well as phaseollin\(^{3,4,17}\). These studies indicate that the phytoalexins rapidly alter the structural integrity of various cell membranes and dysfunction a variety of membrane-associated processes. Thus, the phytoalexins appear to be relatively nonspecific membrane antagonists.

Specific ultrastructural effects of phytoalexins on cellular membranes and other structures of fungal pathogens have not been characterized except the case of phytuberin\(^{6}\). In addition to assisting the understanding of modes of phytoalexin action on fungal cell, such ultrastructural studies may clarify the presumed role of phytoalexins in disease resistance. If a phytoalexin induces certain characteristic morphological alterations in fungal ultrastructure \textit{in vitro} and similar alterations are observed \textit{in vivo} on the fungus in resistant responding plant tissues, this would provide additional independent evidence that the phytoalexin is indeed active \textit{in situ} in inhibiting the invading fungus during resistance expression.

Glyceollin is an isomeric 6\(a\)-hydroxy pterocarpan phytoalexin produced by soybean. It has been suggested that glyceollin is involved in active defence mechanism of soybean in both cultivar-race specific and general resistance\(^{10,21,23,26,27}\). The main purpose of the present experiments was to characterize specific morphological alterations in fungal ultrastructure induced by glyceollin \textit{in vitro}. Some biochemical evidence relating to the mode of action of glyceollin is also presented. \textit{Phytophthora capsici} was used as a glyceollin-sensitive test fungus since a synchronous growth system, a necessary system for these \textit{in vitro} studies, has been developed with this fungal species in our laboratory\(^{24}\). The results show that glyceollin induces drastic changes in ultrastructure and function of fungal plasma membrane.

**Materials and Methods**

\textbf{Preparation of glyceollin.} Glyceollin was isolated from germinating soybean \textit{[Glycine max (L.) Merr., cv. Harosoy 63]} seeds challenged with \textit{Phytophthora capsici} Leonian according to the method previously described\(^{26}\) except that Sephadex LH-20 column chromatography was included in the purification steps. The partially purified ethyl acetate fraction was evaporated to dryness, dissolved in 10 ml of 80\% ethanol, and then applied to a column (2.5×60 cm) of Sephadex LH-20 (Pharmacia). The column was eluted with 80\% ethanol, and 10 ml fractions were collected. After the fractions containing glyceollin were pooled, evaporated to dryness, and then re-dissolved in 95\% ethanol, they were applied to preparative thin-layer plates and developed as described previously\(^{26}\). Glyceollin thus purified was composed of a mixture of glyceollin
isomers, and this mixture was used for the present experiments without further separation.

Cultural condition and glyceollin treatment. Synchronized cultures of young hyphae of *P. capsici* were induced by the method previously described\(^24\) with a slight modification. Zoosporangia suspended in deionized water were germinated at 20°C for 1 hr. The resulting zoospore suspension was encysted by vigorous shaking, and then the suspension was diluted with deionized water to \(2 \times 10^6\) encysted zoospores per millilitre. Twenty millilitres of the suspension were introduced into 200 ml of an asparagine-glucose liquid medium\(^24\) supplemented with 20 mg \(\beta\)-sitosterol per litre medium, and incubated at 28°C for 15 hr with reciprocal shaking at 120 strokes/min. To measure the effects of glyceollin on fungal growth, the resulting young and uniform hyphal suspension was divided into 50 ml portions to which glyceollin was added as ethanol solution. Cycloheximide and 2, 4-dinitrophenol were also used as control inhibitors as a comparison to the biochemical and ultrastructural effects of glyceollin, and the fungus was treated with these inhibitors as the case with glyceollin. The final ethanol concentration in the cultures was adjusted to 0.5% for all treatments. Rates of fungal growth were followed by filtering 10 ml cultures through pre-weighed fiberglass filters (Whatman GF/A, 2.4 cm diameter) after appropriate periods of incubation and hyphal mats on the filters were washed with 5 ml of deionized water. After drying at 90°C for 24 hr, dry weight was measured.

Transmission electron microscopy. A hyphal suspension of 15 hr-old-culture was treated with glyceollin or other inhibitors as described above. After appropriate periods of incubation, 2 ml samples of hyphal suspension were mixed with the same volume of 4% glutaraldehyde buffered with 0.2 M sodium cacodylate at pH 7.2 according to Sabatini *et al.*\(^15\). Hyphae were collected on Miracloth (Calbiochem) by filtration and embedded in small agar blocks. After washing with the same buffer and dehydration in ethanol and propylene oxide, the samples were embedded in epoxy resin (Epon 812). Sections were cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome and stained with 1% aqueous uranyl acetate for 20 min followed by Reynolds\(^13\) lead citrate for 3 to 5 min. Sections were also stained with a periodate-chromate-phosphotungstate procedure which accentuates plasma membranes of various sources\(^14\). Stained sections were viewed under a Hitachi HU-12A electron microscope operating at an accelerating voltage of 100 kV.

Determination of electrolyte leakage. Twenty millilitre of 15 hr-old-culture were filtered through Miracloth without suction. Young hyphae collected on Miracloth were washed with 1% glucose and suspended in 10 ml of 1% glucose in a 50 ml beaker. The beakers were shaken at 28°C at 120 strokes/min. Conductivity (\(\mu\)mhos) of the bathing solution was measured at various intervals using a dip type electrode cell-equipped conductivity meter (CD-M2, M & S Instr. Inc.) after addition of glyceollin or other inhibitors at the indicated concentrations. The results were expressed as per cent electrolyte loss of total electrolytes originally contained in hyphae. Total electrolytes were obtained by measuring conductivity of the bathing solution after heating the incubation contents at 100°C for 10 min at the end of experiment.

Incorporation of labelled amino acid and nucleoside into acid soluble and
insoluble fractions. Two μCi of ¹⁴C-phenylalanine or 10 μCi of ³H-uridine in 0.1 ml deionized water was placed in a 125 ml flask containing appropriate amounts of glyceollin or other inhibitors. Specific radioactivity of ¹⁴C-phenylalanine and ³H-uridine was adjusted to be 5 and 25 mCi/mmmole, respectively, by addition of the unlabelled amino acid or nucleoside. Incubation was initiated by the addition of 20 ml of 15 hr-old-culture, and the flasks were incubated at 28°C on a reciprocal shaker. After the indicated periods of incubation, 5 ml cultures were filtered through fiberglass filters and hyphal mats on the filters were washed with 1% glucose. The washed hyphal mats were placed and dispersed in 5 ml of ice-cold 5% trichloroacetic acid. After keeping for 30 min on ice, hyphal suspensions were filtered through the fiber-glass filters and hyphal mats on the filters were washed with 5 ml of 5% trichloroacetic acid. Radioactivity of combined filtrates (acid soluble fraction) and hyphal mats (acid insoluble fraction) was determined as described previously22).

Measurement of respiration. Oxygen uptake was determined with an oxygen electrode (YSI, Model 58) at 28°C, using 10 ml of 15 hr-old-culture. The rate of oxygen usage by the hyphal suspension was measured for 10 min following glyceollin addition.

Results

Determination of the minimum inhibitory concentration of glyceollin for fungal growth

Hyphal growth of P. capsici in the liquid medium was partially and completely inhi-
bited by 20 and 50 µg/ml glyceollin, respectively (Fig. 1). Higher concentrations of glyceollin decreased the initial dry weight of the fungus. The growth inhibition was evident by 1 hr after incubation of the fungus with glyceollin at 50 µg/ml or higher concentrations. The fungal growth thus appeared to be more sensitive to glyceollin in the liquid medium as used in the present study than in the corresponding solid medium on which radial hyphal growth of either P. capsici or P. megasperma f. sp. glycinea was only partially inhibited by glyceollin at 50 or 100 µg/ml.

To assess whether glyceollin effects on fungal ultrastructure are specific for glyceollin action or represent nonspecific fungal responses to a variety of toxicants, some other growth inhibitors were also used as control inhibitors. Of several inhibitors tested, 2, 4-dinitrophenol and cycloheximide were selected as the control inhibitors, mainly because of their contrasting effects on fungal membrane permeability. 2, 4-Dinitrophenol induced rapid electrolyte leakage from fungal hyphae as did glyceollin, but cycloheximide did not induce leakage as described later. As shown in Fig. 1, cycloheximide at 5 µg/ml and 2, 4-dinitrophenol at 20 µg/ml almost completely inhibited hyphal growth, and these concentrations of the inhibitors were accordingly used in the subsequent studies.

**Effect of glyceollin on fungal ultrastructure**

A typical longitudinal section of a young hypha of P. capsici that was not treated with glyceollin is shown in Plate I (a), in which apparently intact cytoplasmic organelles such as nucleus, mitochondrion, and Golgi apparatus as well as plasma membrane are observed. When the fungus was treated with glyceollin, a characteristic alteration at the early stage (0.5 hr after incubation) was observed on plasma membrane structure while the membrane systems of intracellular organelles remained relatively undamaged [Plate II (a)]. Plasma membrane of control hyphae were of highly waved appearance, as observed in other Phytophthora species, but continuous around the cytoplasm [Plate I (b)]. In contrast, plasma membranes of glyceollin-treated hyphae were devoid of such finely-waved structure and appeared to be flattened or at some places protruded. In addition, they were separated from cell walls, and small vesicle-like structures were usually observed between plasma membranes and cell walls [Plate II (a) and (b)]. Furthermore, plasma membranes appeared to be absent from some parts of cytoplasmic periphery [Plate II (a) and (c)].

In addition to plasma membranes, structures of various intracellular organelles appeared to be seriously damaged in hyphae treated with glyceollin for prolonged periods of time such as 1 to 2 hr, as shown in cross sections [Plate III (a) and (b)]. It might be emphasized, however, that there were no cases in glyceollin-treated hyphae in which intracellular structures were visibly damaged without serious distortion of plasma membranes. Furthermore, the distortion of the plasma membranes was confirmed in sections stained with a periodate-chromate-phosphotungstate procedure, which was reported to be specific for plasma membranes of various sources [Plate III (c) and (d)].

The specificity of the plasma membrane distortion was evaluated by assessing whether other growth inhibitors induced plasma membrane alterations similar to those induced by glyceollin. In contrast to glyceollin, 2, 4-dinitrophenol and cycloheximide at the doses that completely inhibited hyphal growth did not induce serious plasma mem-
brane distortion. 2, 4-Dinitrophenol had little effect on either intracellular structures or plasma membranes at the early stage (1 hr) after treatment [Plate IV(a) and (b)]. Although cytoplasmic structures were significantly altered at prolonged periods of incubation (4 hr) with 2, 4-dinitrophenol, plasma membranes still appeared to exist around cytoplasmic periphery [Plate IV(c)]. Similarly, typical plasma membrane damages as induced by glyceollin were not observed in hyphae treated with cycloheximide at either the early [1 hr, Plate V(a) and (b)] or late [4 hr, Plate V(c)] stage after the inhibitor treatment, although intracellular structures were considerably distorted even at the early stage. Furthermore, the structures as shown in Plate V(c) were frequently observed at the late stage, in which cytoplasmic structures were almost entirely destroyed but fragmentation or dissolution of plasma membranes rarely occurred.

The frequency of plasma membrane distortion was examined in various preparations of thin sections (Table 1). When the fungus was treated with glyceollin at 50 or 100 µg/ml for 0.5 to 2 hr, almost all the observed hyphal sections (85 to 100%) revealed distorted plasma membranes at some locations of cytoplasmic periphery and more than 75% of the total cytoplasmic periphery observed was associated with distorted plasma membranes. In contrast, the frequency of plasma membrane distortion in hyphae treated with either 2, 4-dinitrophenol or cycloheximide at 20 or 5 µg/ml, respectively, was low, although a slightly higher frequency of membrane damage was induced by the latter inhibitor. These results suggested that plasma membrane distortion visualized in glyceollin-treated hyphae was not a nonspecific general response of fungal hyphae due to growth inhibition caused by a variety of toxicants. Instead, such appearance of

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µg/ml)</th>
<th>Incubation period (min)</th>
<th>Fungal cell with damaged plasma membranea) (% of total cells observed)</th>
<th>Fungal cytoplasmic periphery with damaged plasma membranea) (% of total length of periphery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>60 – 240</td>
<td>3 – 6</td>
<td>1 – 2</td>
</tr>
<tr>
<td>Glyceollin</td>
<td>20</td>
<td>60</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>50</td>
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<td>87</td>
<td>76</td>
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<tr>
<td></td>
<td>60</td>
<td>120</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>20</td>
<td>60</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>240</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5</td>
<td>60</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>240</td>
<td>28</td>
<td>12</td>
</tr>
</tbody>
</table>

a) Plasma membranes that were flattened or protruded with vesicle-like structures between plasma membranes and cell walls, or that were absent from cytoplasmic periphery, were referred to as damaged plasma membranes. Twenty to thirty hyphal sections were observed for each treatment.
the damaged plasma membranes might be a characteristic morphological symptom induced by glyceollin.

**Effect of glyceollin on membrane permeability of fungal hyphae**

Glyceollin at 20 to 100 μg/ml rapidly induced electrolyte leakage from fungal hyphae (Fig. 2). The leakage began almost immediately following the addition of glyceollin and its degree was dependent on glyceollin concentrations. These rapid effects on electrolyte leakage suggest that glyceollin directly interacts with fungal plasma membranes. In contrast, cycloheximide had only negligible effects on electrolyte leakage during the incubation periods. 2, 4-Dinitrophenol, however, induced electrolyte leakage similar to glyceollin, although it did not induce visible plasma membrane distortion as described above. It therefore appears that structural distortion of plasma membranes as observed in glyceollin-treated hyphae is not caused by all chemicals that are capable of inducing electrolyte leakage.

Plasma membrane function was further examined by testing the effects of glyceollin on uptake of a labelled amino acid and nucleoside by fungal hyphae. Glyceollin at 20 to 50 μg/ml strongly inhibited incorporations of both 14C-phenylalanine and 3H-uridine into the acid soluble (“pool”) fraction (Table 2). However, incorporations of 14C-phenylalanine and 3H-uridine into the acid insoluble fraction, possibly representing protein and RNA fractions, respectively, were much less inhibited. The slight inhibition of the macromolecule synthesis by glyceollin probably resulted from the reduced uptake of the labelled precursors. As might be predicted, cycloheximide did not significantly inhibit the incorporation of both the labels into the acid soluble fractions but prominently
Table 2. Effects of glyceollin and cycloheximide on incorporation of \(^{14}\text{C}\)-phenylalanine or \(^{3}\text{H}\)-uridine into the acid-soluble (pool) and acid-insoluble (protein or RNA) fractions of *Phytophthora capsici* hyphae

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (µg/ml)</th>
<th>(^{14}\text{C})-Phenylalanine</th>
<th>(^{3}\text{H})-Uridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>Pool(^{b}) 20778(0) (^{d})</td>
<td>Pool(^{b}) 28158(0)</td>
</tr>
<tr>
<td>Glyceollin</td>
<td>20</td>
<td>Protein(^{c}) 27507(0)</td>
<td>Protein(^{c}) 23039(0)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>5</td>
<td>1217(94)</td>
<td>1223(96)</td>
</tr>
</tbody>
</table>

\(^{a}\) Fungal hyphae were incubated with one of the labelled precursors for 20 min and then fractionated as described in Materials and Methods. Data are from a typical experiment representative of four separate experiments.

\(^{b}\) The fraction soluble in 5% trichloroacetic acid.

\(^{c}\) The fraction insoluble in the same acid.

\(^{d}\) Values in parentheses are per cent inhibition relative to the control.

Inhibited the incorporation of the labelled amino acid into the acid insoluble fraction. These results also support the contention that glyceollin directly interferes with the function and morphology of fungal plasma membranes but has little effects on other metabolic processes such as macromolecule synthesis at the early stage.

Fig. 3. Effect of glyceollin on oxygen uptake by hyphae of *Phytophthora capsici*. Oxygen consumption was measured by an oxygen electrode. Arrows indicate the time of glyceollin addition at 20 (○) and 50 (●) µg/ml, and dotted lines represent the initial rates of oxygen consumption before glyceollin addition. Data are from a typical experiment representative of three separate experiments.
Effect of glyceollin on fungal respiration

Glyceollin at 20 or 50 µg/ml inhibited oxygen uptake by fungal hyphae (Fig. 3). It was noteworthy, however, that the inhibition of oxygen uptake was initiated only after a lag period of several minutes when the hyphae were treated with 20 µg/ml of glyceollin, at which concentration the leakage was induced almost immediately following the glyceollin addition as described above. This difference in the inhibition kinetics strongly suggests that the primary site of glyceollin action to fungal hyphae is on plasma membranes and that the observed respiratory inhibition by glyceollin probably arises from dysfunction of plasma membranes.

Discussion

The present experiments showed that glyceollin induced rapid alterations in fungal ultrastructure. Among various structures, plasma membranes appeared to be the most sensitive to glyceollin. Plasma membranes in control hyphae were finely waved but was continuous around the cytoplasmic periphery. In glyceollin–treated hyphae, plasma membranes were flattened or at some places protruded and separated from cell walls, and not detectable in some portions of the cytoplasmic periphery. These plasma membrane alterations appeared to precede other structural distortions of intracellular organelles. Furthermore, plasma membrane distortion did not appear to be a nonspecific fungal response to a variety of toxicants, since other fungal growth inhibitors tested, such as 2, 4-dinitrophenol and cycloheximide, did not induce typical plasma membrane abnormalities as observed in glyceollin–treated fungal hyphae. It may therefore be considered that the observed plasma membrane abnormalities represent a characteristic morphological symptom induced by glyceollin.

The observed effects on the ultrastructure of fungal plasma membranes may be related to the primary mode of action of glyceollin. Glyceollin, at the concentrations that induced structural distortion and inhibited hyphal growth, rapidly interfered with biochemical functions of fungal plasma membranes, resulting in electrolyte leakage almost immediately following its treatment and inhibition of uptake of labelled amino acid and nucleoside into the acid soluble "pool" fraction. Glyceollin, however, did not appear to directly inhibit either fungal respiration or synthesis of macromolecules such as nucleic acid and protein. These biochemical studies suggested that the possible primary action of glyceollin on fungal hyphae is to interact with plasma membranes. It is noteworthy, however, that membrane antagonists do not necessarily induce morphological abnormality in plasma membranes, since 2, 4-dinitrophenol, which was recently reported to increase plant membrane permeability⁹, induced rapid electrolyte leakage from fungal hyphae as did glyceollin but did not incite prominent alterations in ultrastructure of fungal plasma membranes.

In contrast to our study, Kaplan et al.⁹ reported that glyceollin was a potent inhibitor of respiration by isolated soybean mitochondria. They showed that glyceollin inhibited the electron transport system of mitochondria but did not inhibit oxidative phosphorylation. Boydston et al.¹¹ further showed that glyceollin was a site-specific inhibitor of electron transport in soybean mitochondria, mainly acting as a site I inhibi-
itor in a manner similar to rotenone. These studies implicate mitochondrial electron transport system as a possible primary site of glyceollin action. However, it has not been demonstrated that glyceollin can penetrate into the cytoplasm and directly interact with mitochondria in a whole cell system. Instead, our present study indicated that glyceollin induced electrolyte leakage more rapidly than respiratory inhibition, suggesting that the primary site of glyceollin action to fungal hyphae was on plasma membranes and that the observed respiratory inhibition by glyceollin probably arose from dysfunction of plasma membranes.

Several phytoalexins including phaseollin, pisatin, and rishitin have been suggested to affect plasma membranes of various organisms, resulting in either electrolyte leakage or cell lysis. Although detailed molecular mechanisms of the interaction between phytoalexins and cell membranes are not currently known, recent studies showed that certain phytoalexins such as glyceollin, phaseollin, and pisatin (Yoshikawa, unpublished work) as well as rishitin can interact with artificial lipid membranes (liposomes), resulting in rapid leakage of liposome-sequestered substances. These results therefore suggest that such phytoalexins may primarily interact with lipid moieties of various biological membranes.

Although more different toxicants must be tested, the observed plasma membrane structure in glyceollin-treated fungal hyphae was conspicuous and might be unique for glyceollin action. In addition, the abnormal plasma membrane structures were detected in almost all sections of fungal hyphae treated with glyceollin. It is therefore possible that such structures may be used as a morphological parameter to assess the possible in situ role of glyceollin in disease resistance expression. Fungal ultrastructure in resistant-responding soybean hypocotyls will be described in the subsequent paper.

**Literature cited**


Explanation of plates

Plate I
Ultrastructure of control hyphae of Phytophthora capsici.
(a). A longitudinally-sectioned hypha, showing apparently intact intracellular structures as well as plasma membrane. ×29,400.
(b). A magnified structure of cytoplasmic periphery. Note that plasma membrane is of highly-waved appearance but continuous along with cell wall. ×48,000.

Plate II
Ultrastructure of glyceollin-treated hyphae of Phytophthora capsici.
(a). A longitudinally-sectioned hypha treated with glyceollin at 50 µg/ml for 30 min. Note that plasma membrane structures are flattened or at some places protruded in comparison with those of the control hyphae and are absent from some portions of cytoplasmic periphery. ×18,600.
(b) and (c). Magnified appearance of distorted cytoplasmic periphery of hyphae treated with glyceollin similar to (a). (b) Note that plasma membrane is fattened and separated from cell wall with small vesicle-like structures in the intervening space. ×60,000. (c) Note that plasma membrane is absent from cytoplasmic periphery. ×63,000.

Plate III
Ultrastructure of cross-sectioned hyphae of Phytophthora capsici treated or untreated with glyceollin.
(a). Control hypha. ×31,500.
(b). Hypha treated with glyceollin at 50 µg/ml for 60 min. Note that various intracellular structures as well as plasma membranes are largely distorted at this stage. ×27,600.
(c) and (d). Sections stained with a periodate–chromate–phosphotungstate procedure\textsuperscript{14).} (c) Control hypha, showing densely-stained plasma membrane along with cell wall. $\times 20,500$. (d) Hypha treated with glyceollin at 100 $\mu$g/ml for 60 min. $\times 19,600$.

Plate IV

Ultrastructure of \textit{Phytophthora capsici} hyphae treated with 2,4-dinitrophenol.

(a). Hypha treated with the inhibitor at 20 $\mu$g/ml for 60 min. Note that plasma membrane as well as various intracellular structures are not seriously damaged at this stage. $\times 18,900$.

(b). A magnified structure of cytoplasmic periphery of hypha treated similarly to (a), showing highly waved but continuous plasma membrane indistinguishable from that of control hyphae. $\times 38,400$.

(c). Hypha treated with the inhibitor at 20 $\mu$g/ml for 240 min. Note that intracellular structures are largely distorted at this late stage but plasma membrane still exists continuously around cytoplasmic periphery. $\times 19,600$.

Plate V

Ultrastructure of \textit{Phytophthora capsici} hyphae treated with cycloheximide.

(a). Hypha treated with the inhibitor at 5 $\mu$g/ml for 60 min. Note that intracellular structures are largely altered but plasma membrane is not seriously damaged. $\times 24,000$.

(b). A magnified structure of cytoplasmic periphery of hypha treated similarly to (a), showing waved but continuous plasma membrane similar to that of control hyphae. $\times 60,000$.

(c). Hypha treated with the inhibitor at 5 $\mu$g/ml for 240 min. Note that intracellular structures are almost entirely destroyed but plasma membrane still exists continuously around cytoplasmic periphery. $\times 16,900$.

Abbreviations:

Plate I

(a) Diagram showing various cellular components labeled:
- CW (Cell Wall)
- PM (Plasma Membrane)
- N (Nucleus)
- M (Mitochondria)

(b) Diagram showing:
- PM (Plasma Membrane)
- CW (Cell Wall)

Legend:
- G: [Identify the corresponding structure]
Plate III
Plate V