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Plant Cell Membranes as Biochemical Targets of the Phytotoxin Helminthosporol

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Helminthosporol is one of the natural sesquiterpenoid toxins isolated and identified in the culture medium of the phytopathogenic ascomycete fungus *Cochliobolus sativus*. The effect of this phytotoxin was investigated on enzymatic activities, electron and ion transport in mitochondria, chloroplasts, and microsomes of plant. The results indicate that helminthosporol drastically affects the membrane permeability of these organelles to protons and subtrate anions, inhibiting the mitochondrial oxidative phosphorylation, the photophosphorylation in chloroplasts, and the proton pumping across the cell plasma membrane. The 1,3-β-glucan synthase activity, involved in defense mechanisms of plant cells against stress and damage, e.g., during pathogen attack, was also strongly inhibited by the toxin.

KEY WORDS: Helminthosporol; *Cochliobolus sativus*; phytotoxin; plant; cell membranes.

INTRODUCTION

Helminthosporol is one of the natural sesquiterpenoid toxins isolated and identified in the culture medium of the fungus *Cochliobolus sativus* (teleomorph. Ito & Kurit), also called *Bipolaris sorokiniana* (*Sacc. in Sorok*, Shoemaker), or *Helminthosporium sativum* (Pam King & Bakke), a pathogenic ascomycete responsible for the seedling blight and root rot of cereals (Ludwig, 1957).

Helminthosporol is a monoaldehyde sesquiterpene whose chemical structure (Fig. 1) was initially identified and described as a plant growth regulator which promotes shoot growth of rice seedlings (Tamura *et al.*, 1965) and stimulates the sugar release from deembryonated barley seeds (Briggs, 1966).

It has been shown that this sesquiterpene, together with its dialdehyde analogue, helminthosporal (Fig. 1), were the substances responsible for inducing the plant disease symptoms (De Mayo *et al.*, 1961; White and Taniguchi, 1972). More recently, it has been suggested that the precursor prehelminthosporol is the main substance responsible for the biological phytotoxicity (Carlson *et al.*, 1991; Nilsson *et al.*, 1993).

The biochemical mode of action of these sesquiterpenoid toxins, derivatives or precursors, isolated from various culture media at different stages of growth of the fungus, has been studied for many years but today there is no clear image of their specific mode of action.

It has been reported that the dialdehyde sesquiterpene (helminthosporal) inhibited the succinate and malate oxidation and also the oxidative phosphorylation of plants and mouse liver mitochondria (Taniguchi and White, 1967). The Toxin-enriched fraction was shown to decrease the functional activity of the photosynthetic apparatus in isolated wheat chloroplasts (Fadeev *et al.*, 1988; Adeishvili *et al.*, 1989).

Very early, it was suggested that the primary site of action of many phytotoxins produced by the plant pathogenic fungi was the plasma membrane (Marre, 1980). These results were recently confirmed by the ability of prehelminthosporol to inhibit the activity of the 1,3-β-glucan synthase and proton pumping in barley root plasma membrane vesicles (Olbe *et al.*, (1995).

In this paper we report the effect of the monoaldehyde helminthosporol (which is the major sesquiterpene we have extracted and purified under our

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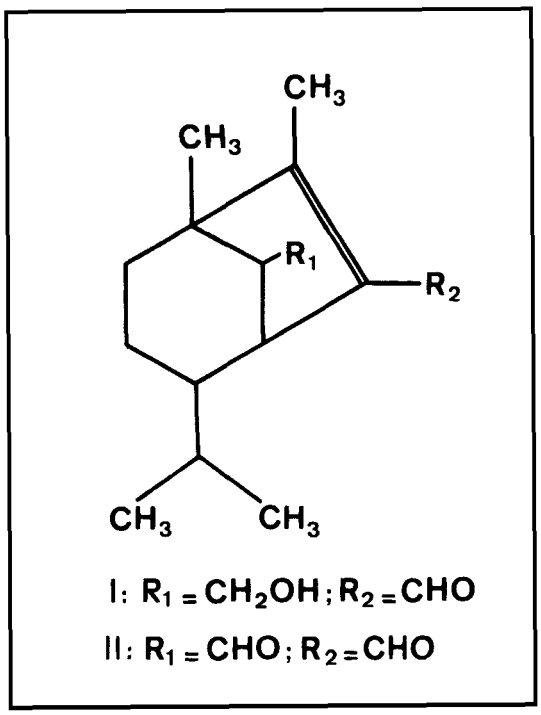

Fig. 1. Chemical structure of helminthosporol (I) and helminthosporal (II).

experimental conditions from the culture medium of a selected highly productive strain of *Cochliobolus sativus*) on a large spectrum of presumed biochemical targets including enzymatic activities and electron and ion transport systems in mitochondria, chloroplasts, and microsomes of plants.

MATERIAL AND METHODS

Fungus Strains and Cultivation

A selected strain of *Cochliobolus sativus* (MUCL 18528) highly productive in sesquiterpenes was precultured in 100 ml of medium containing 3% (w/v) potato dextrose broth (Difco) supplemented by 2 mM $Fe(NO_3)_3 \cdot 9H_2O$, 1.5 mM $ZnSO_4 \cdot 7H_2O$, and 1 mM $MnSO_4 \cdot 4H_2O$ at pH 5.1 and shaken in a 500-ml Erlenmeyer flask at 5°C for five days. A 4-liter Roux bottle containing 1.2 liters of the same medium was inoculated with this preculture and incubated for 28 days at 25°C under constant light. The mycelium developed at the surface of the culture medium was discarded by filtration in a Büchner funnel through two sheets of miracloth.

Isolation and Purification of Helminthosporol

Helminthosporol was extracted from the culture medium of the fungi with diethyl ether, using the procedure described earlier by Sommereyns and Closset (1978), with slight modifications. After evaporation of diethyl ether at 30°C in a rotary evaporator, the residual crude extract was then dissolved in methanol and used for purification by successive preparative thin-layer chromatography on silica gel plates (Merck, Kiesel gel 60, F-254) eluted by a toluene–acetone (4:1, v/v) mixture and by high-performance thin-layer chromatography (Merck, HPTLC plates of Kiesel gel 60, F-254) eluted with the same solvent. The major spot obtained and visualized (R_f = 0.47) under UV light, was scraped and dissolved in methanol.

A further purification can be obtained by HPLC chromatography on a diolated column (Merck, Lichrospher 100 Diol 5μm) eluted with an isochratic mixture of *n*-hexane ethyl acetate (3:1, v/v) with a flow rate of 420 μl/min. The quantification and identification of helminthosporol was carried out successively by UV, infrared, RMN, and mass spectrometry.

Preparation of Plant Mitochondria and Submitochondrial Particles

Mitochondria were isolated and purified from etiolated shoots of faba beans (*Vicia faba L.*) and wheat (*Triticum aestivum L.*) by the procedure described earlier (Boutry and Briquet, 1982).

Submitochondrial particles were prepared by sonication of resuspended purified mitochondria for 30 secs with the cell disrupter (Virsonic) at 0°C in a medium containing 0.4 M mannitol, 10 mM KH_2PO_4, and 0.4% (w/v) bovine serum albumin, pH 7.2 (KOH). The mitochondrial homogenate was centrifuged for 10 min at 10.000 × *g* to eliminate intact mitochondria.

After centrifugation of the supernatant at 105.000 × *g* for 90 min, the submitochondrial pellets were resuspended in 0.25 M sucrose, 1 mM EDTA, and 50 mM Tris at pH 7.4.

Preparation of a Crude Microsomal Fraction

One hundred grams of seven-day-old etiolated shoots of faba bean was homogenized for 5 sec at 4°C in a Braun mixer (Multimix) in the presence of 200 ml grinding medium containing 0.25 mM sucrose, 50

mM Tris, 10 mM KH_2PO_4, 1 mM EGTA, 0.1% bovine serum albumin, 2 mM 2-mercapto-ethanol, and 0.5 mM phenylmethanesulfonyl fluoride, at pH 8. After filtration through two layers of miracloth, the homogenate was centrifuged at 4000 × *g* for 2 min at 4°C. The supernatant was centrifuged at 23.000 × *g* for 15 min to discard the pelleted mitochondrial fraction. The last supernatant was then centrifuged at 105.000 × *g* for 90 min at 4°C. The final pellet obtained is the so-called crude microsomal fraction, which was resuspended in 0.25 M sucrose and 50 mM Hepes at pH 7.25.

Preparation of Plasma Membrane Vesicles

The plasma membrane vesicles were purified from a microsomal fraction of *Nicotiana plumbaginifolia* leaves in an aqueous polymer two-phase system (Palmgren *et al.*, 1990).

Preparation of Crude Chloroplasts

Chloroplasts were isolated from 125 g spinach leaves after homogenization for 5 sec at 0°C in a Braun mixer (Multimix) in the presence of 250 ml grinding medium containing 0.4 M sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 10 mM NaCl, 5 mM 2-mercaptoethanol, 0.4% polyclar, 0.2% (w/v) bovine serum albumin, and 30 mM Tricine, pH 8 (NaOH). After filtration through four layers of miracloth, the homogenate was centrifuged at 4000 × *g* for 3 min at 0°C.

The supernatant was discarded and the pellet of crude chloroplasts was kept at 0°C in the absence of light. Just before use, the pellet was resuspended with a smooth paintbrush in a medium containing 0.4 M sucrose, 2 mM $MgCl_2$, 10 mM NaCl, 0.2% (w/v) bovine serum albumin, 5% (v/v) ethylene glycol, and 30 mM Tricine, pH 8 (NaOH).

ATPase Assays

The plasma-membrane ATPase assays were performed at pH 6, on the crude microsomal fraction (50 μg protein), (Morsomme *et al.*, 1996). The mitochondrial ATPase was measured by the same procedure except that the reaction medium contained 20 mM Tris buffer adjusted to pH 8.9.

1,3-β-Glucan Synthase Activity

This activity was measured on the crude microsomal fraction (10 μg protein) of faba bean by incorporation of UDP-[^{3}H] glucose in the presence of cellobiose (Fredrikson and Larsson, 1989; Olbe *et al.*, 1995).

Acetyl-CoA Stigmasterol Acyltransferase Activity (ACAT)

ACAT activity was determined on 35 μl of the crude microsomal fraction (1 mg protein) by esterification of stigmasterol by [1-^{14}C] oleoyl-CoA as described earlier for cholesterol esterification in rat liver microsomes (Naganuma *et al.*, 1992).

Proton Ejection Measurements Out of Plant Mitochondria

The proton ejection outside mitochondria was measured simultaneously with oxygen uptake in a closed thermostatically controlled chamber of the Rank Brothers oxygen electrode unit, at 25°C, with a Radiometer microcombination electrode coupled to a pH meter. The measurements of proton ejection were performed in anaerobic conditions obtained by flushing nitrogen in the incubation medium containing 0.3 M mannitol, 0.1 M KCl, 5 mM $MgCl_2$ and 1 mM MOPS, pH 6.9, in the presence of 1.5 mM malate + 0.15 mM pyruvate as substrate, and initiated by oxygen pulse (Davy de Virville and Moreau, 1990).

ATP-Dependent Proton Transport Measurements in Plant Plasma Membrane Vesicles

ATP-dependent proton translocation into the plasma membrane vesicles was measured by recording the fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA), checking that the quenching was removed by subsequent destruction of the proton gradient by nigericin as described by Morsomme *et al.*, 1996.

Mitochondrial Respiration Measurements

The mitochondrial oxygen uptake was measured by the oxygen electrode (Rank Brothers, Bottisham, UK) in a closed thermostated cell at 25°C containing 0.4 M mannitol, 10 mM KH_2PO_4, 10 mM $MgCl_2$, and 0.2% lipid-free bovine serum albumin, pH 7.2, as described earlier (Boutry and Briquet, 1982).

Anion Substrate Transport in Plant Mitochondria

The activity of the phosphate, dicarboxylate, and tricarboxylate carriers was measured by the swelling of mitochondria in isosmotic solutions of ammonium salts in the presence of antimycin (Phillips and Williams, 1973). The decrease in light scattering was recorded at 520 nm by an Aminco DW-2 spectrophotometer.

Photosystem Activity Measurements in Chloroplasts

The activities of photosystem I (PSI) and II (PSII) of spinach chloroplasts were measured by observing the oxygen uptake or evolution using the oxygen electrode.

Measurement of PSII

150 μl of chloroplast suspension (± 0.6 mg chlorophyll/ml) was added in the absence of light in the reaction chamber of the oxygen electrode unit (Rank Brothers Limited) containing in a final volume of 2 ml: 0.4 M sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM Tricine, 2 mM KCN, and 2 mM $K_3Fe(CN)_6$, pH 7.8, at 25°C. The reaction was initiated by light. The activity was measured by the rate of oxygen evolution and expressed in μl $O_2 \times h^{-1} \times mg^{-1}$ chlorophyll.

Measurement of PSI

Thirty to 50 μl of chloroplast suspension (± 0.1–0.2 mg chlorophyll/ml) was added in the absence of light in the reaction chamber contained in a final volume of 2 ml, 0.4 M sucrose, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 40 mM Tricine, 2 mM KCN and 20 μM dichlorophenyldimethyl urea (DCMU), 2.5 mM methyl viologen, 5 mM ascorbate, and 1 mM tetramethylphenylenediamine (TMPD), pH = 7.8, at 25°C. The reaction was initiated by light and the activity was measured by the rate of oxygen uptake and expressed in μl $O_2 \times h^{-1} \times mg^{-1}$ chlorophyll.

Photorespiration

150 μl of chloroplast suspension was added to the reaction vessel of the oxygen electrode containing, in a final volume of 2 ml, the same medium used for the measurement of PSII activity. After 7–8 min the light-induced oxygen evolution stopped, or was inhibited by the addition of DCMU. The light-dependent oxygen consumption observed (photorespiration) is the result of the activation of the ribulose-bis-phosphate oxygenase activity of Rubisco in the presence of saturated oxygen concentration in the cuvette. The addition of glycollate stimulating the peroxisomal glycollate oxidase induced a marked increase in the oxygen uptake which can be inhibited by the addition of 2-pyridyl-α-methane sulfonate, the specific inhibitor of glycollate oxidase activity (Zelitch, 1971).

Chlorophyll and Protein Determination

Chlorophyll content was determined by the Arnon spectrophotometric procedure (Arnon, 1949). Protein was measured by the Lowry method (1951).

RESULTS

Effects on Plant Mitochondria

The oxidation of malate in the presence of pyruvate is not inhibited by 0.8 mM helminthosporol in state 4 respiration of *Vicia faba* mitochondria (Fig. 2). The slight stimulation of oxygen uptake observed with methanol (solvent for helminthosporol) is markedly increased in the presence of the phytotoxin, suggesting an uncoupler effect. This is confirmed by the strong inhibition of the ADP-stimulated respiration (state 3) and by the concomitant decrease in respiratory control and the yield of oxidative phosphorylation as shown by the ADP:O ratio and by the loss of effect of the subsequent addition of the uncoupler 2,4-dinitrophe-

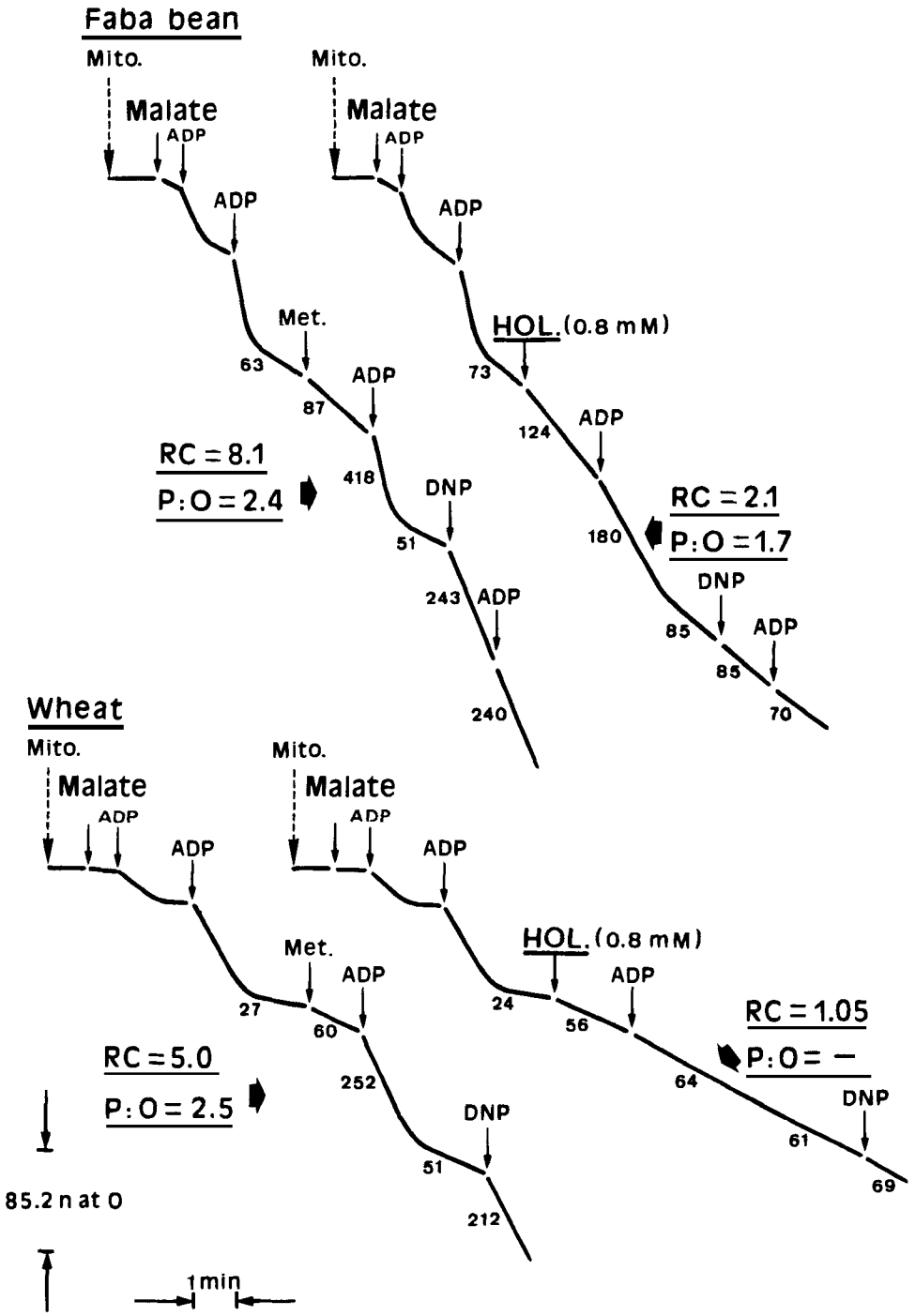

Fig. 2. Effect of helminthosporol on the oxidation of malate by plant mitochondria. To the reaction mixture given in Materials and Methods were added mitochondria (0.3 mg) from faba bean or wheat, 25 mM malate + 2.5 mM pyruvate, 50 μM ADP for the first induced state 3, 100 μM ADP for the following: 5 μM 2,4-dinitrophenol (DNP) and 0.8 mM helminthosporol (HOL) diluted in 20 μl methanol. Methanol (Met) was used in the control assay. The oxygen consumption rates are expressed as nanoatoms × min^{-1} × mg^{-1} of mitochondrial protein.

nol. Similar results have been obtained with succinate as substrate (data not shown).

In Fig. 2, these results were clearly confirmed with mitochondria from wheat, which is one of the favorite target crops for the pathogenic fungus *Cochliobolus sativus*. The respiratory control and the ADP:O ratio are almost completely suppressed by 0.8 mM helminthosporol.

In order to elucidate the precise action site for the toxin in the oxidative phosphorylation mechanism, we measured its effect on the ATPase activity of submitochondrial particles and on the proton-pumping activity of intact mitochondria. The mitochondrial ATPase activity of *Vicia faba* (SMP-ATPase) is not significantly inhibited by 1 mM helminthosporol, compared with the strong inhibition by sodium azide (Table I). On the contrary, Fig. 3 showed that when a pulse of oxygen is introduced as electron acceptor in the anaerobic suspension of mitochondria in the presence of substrate, a very rapid proton ejection occurred, followed by proton re-entry into the mitochondrial matrix. The presence of 1 mM helminthosporol reduced by over 50% this proton-pumping activity, which was completely abolished by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP).

To verify the effect of the toxin on the membrane permeability to other ions, we measured the effect on the phosphate and substrate transport through the mitochondrial inner membrane. As showed in Fig. 4, the transport of phosphate (A), malate (B), and citrate (C), measured by isosmotic swelling experiments, is drastically inhibited by 1 mM helminthosporol. The general inhibition observed in these transport systems suggested that the action site of the phytotoxin was at the level of membrane permeability and not on the functioning of specific protein carriers of the inner mitochondrial membrane.

Table I. Effect of Helminthosporol on Mitochondrial and Plasma Membrane ATPase Activities of Faba Bean[a]

Enzyme	Inhibitors	% activity of control
SMP-ATPase	None	100
	+0.5 mM helminthosporol	97.3
	+1 mM helminthosporol	97.2
	+0.5 mM NaN_3	25.0
	+1 mM NaN_3	22.0
PM-ATPase	None	100
	+0.5 mM helminthosporol	98.0
	+1 mM helminthosporol	98.5
	+0.5 mM vanadate	41.0
	+1 mM vanadate	7.0

[a] Initial specific activity of mitochondrial ATPase in submitochondrial particles (SMP) was 0.47 μmol Pi · min^{-1} · mg^{-1} and in plasma membrane from crude microsomal fraction (PM) was 1.4 μmol Pi · min^{-1} · mg^{-1}. Comparison of helminthosporol was made with the specific inhibitors sodium azide and sodium O-vanadate as control. Values were the average of triplicate experiments.

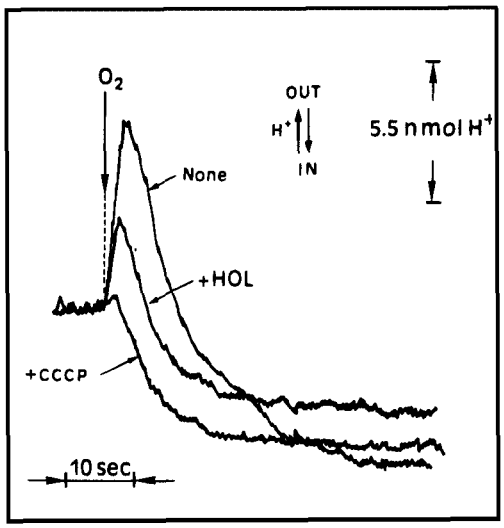

Fig. 3. Effect of helminthosporol on the proton ejection by faba bean mitochondria. The proton ejection was measured in the thermostated chamber of the oxygen electrode on tightly coupled faba bean mitochondria (6 mg) in the reaction medium, described in Materials and Methods, in anaerobic conditions in the presence of 1.5 mM malate + 0.15 mM pyruvate and 80 mg valinomycin/mg protein. Oxygen pulses were carried out by injection of 60 μl of oxygenated water stirred at 25°C (258 μM). 1 mM helminthosporol (HOL) or 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was preincubated for 2 min in the cuvette before the oxygen pulse.

Effect on Microsomal and Plasma Membrane Activities

As described by Olbe *et al.* (1995), for the precursor prehelminthosporol, we also measured the action of helminthosporol on three different plasma membrane activities of vital importance for plant cell survival. Plasma membrane proton ATPase hydrolytic activity (PM-ATPase) is not significantly inhibited by 1 mM helminthosporol. At this concentration vanadate inhibited more than 90% of the enzymatic activity (Table I).

The ATP-dependent H^+ pumping of plasma membrane vesicles isolated from *Nicotiana plumbaginifolia* showed inhibition proportional to the helminthosporol concentration (Fig. 5). Fifty-five to 75% inhibition was obtained at 1 and 2 mM of the toxin, respectively.

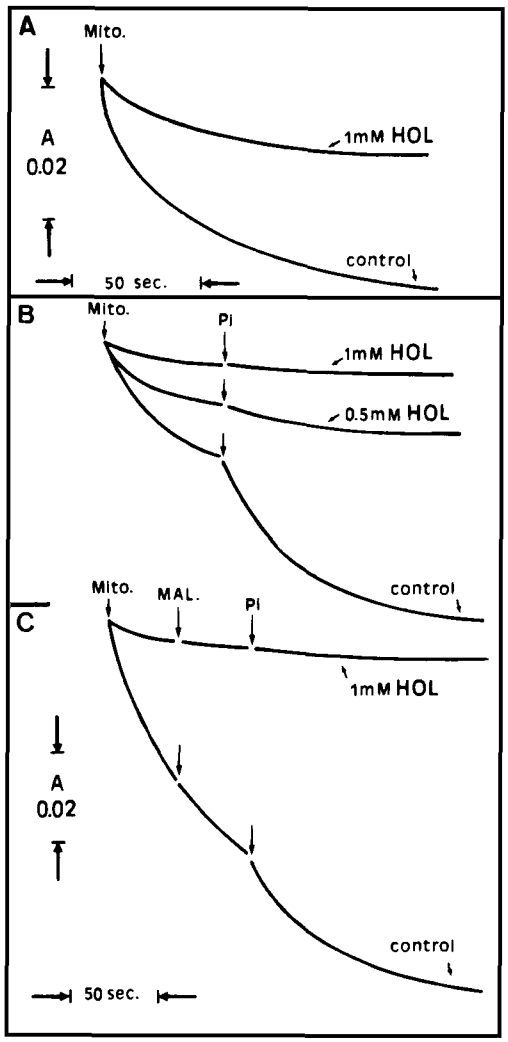

Fig. 4. Inhibition of passive swelling of faba bean mitochondria by helminthosporol. Mitochondria (1 mg) were suspended in the reaction mixture containing 20 mM Tris at pH. 7.4 and 10 μg antimycin A per mg of protein. After a 1 min incubation at 25°C with helminthosporol (HOL) or methanol (Met) in the control trace, 120 mM ammonium phosphate was added in experiment A, 100 mM ammonium malate followed by 5 mM phosphate in experiment B, and 85 mM ammonium citrate followed by 5 mM malate and 5 mM phosphate in experiment C.

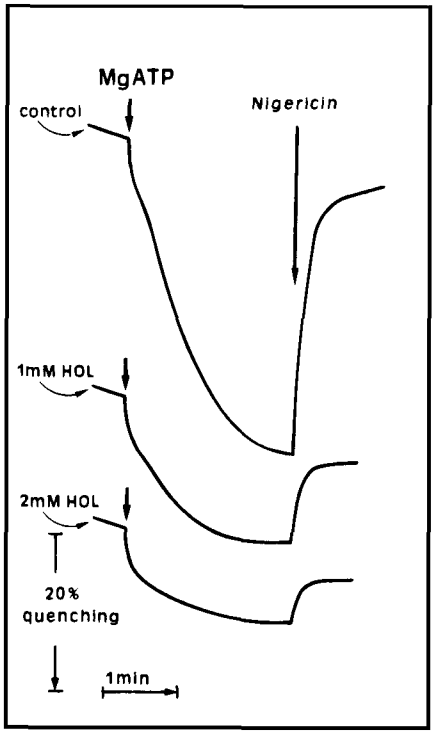

Fig. 5. ATP-dependent fluorescence quenching of ACMA with plasma membrane vesicles from *Nicotiana plumbaginifolia* in the presence of helminthosporol (HOL). The assay contained, in a total volume of 3 ml of the reaction mixture (25 mM K_2SO_4, 5.2% glycerol, 50 mM MES, pH 7.0), 25 μl of sealed plasma membrane suspension (420 μg protein), 10 μl of ACMA solution (0.5 mM in ethanol), and 0.05% Brij 58. After 3 min incubation at 30°C, 100 μl aliquot of MgATP (2 mM final concentration) in 10 mM MES, at pH 7.0, was added to start the reaction. 0.5 ug nigericin was used to check the destruction of proton gradient at the end of the reaction.

The callose-producing enzyme 1,3-β-glucan synthase, which is known to protect the plant cell against damage by depositing callose (Kauss, 1990), was inhibited by increasing concentrations of helminthosporol (Fig. 6). More than 75% inhibition is obtained at 1 mM of the toxin. This slight stimulation of activity observed at concentrations below 100 μM may be the result of the presence of the toxin that cause the microsomal vesicles to leak and expose all 1,3-β-glucan synthase active sites to the substrates, as described earlier for the precursor prehelminthosporol (Olbe *et al.*, 1995).

It has been shown that helminthosporol inhibited the acyl-CoA cholesterol acyltransferase activity in rat liver microsomes (Park, 1993). We observed that the corresponding acyl-CoA stigmasterol acyltransferase activity in plant microsomes from *Vicia faba* shoots is also inhibited by helminthosporol, but a concentration as high as 4 mM is necessary to obtain 45% inhibition (Table II).

Effect on the Photosynthetic Electron Transport Systems, on Photorespiration, and on Photophosphorylation of Chloroplasts

It has been shown by Fadeev *et al.* (1988) that a helminthosporal-enriched ether fraction of toxic sub-

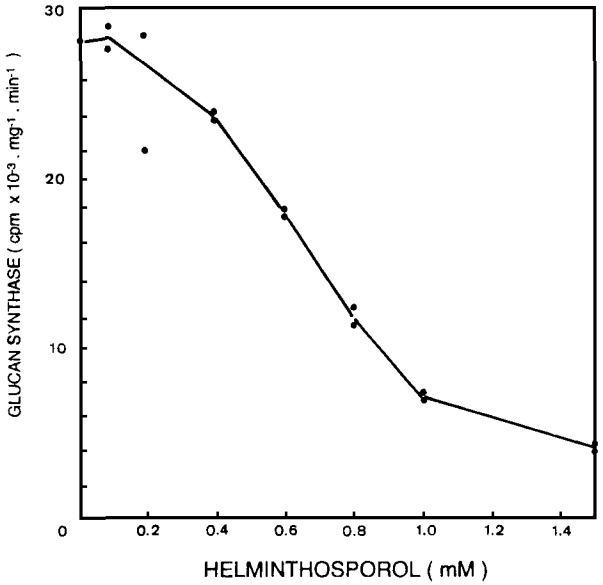

Fig. 6. Effect of helminthosporol on 1,3-β-glucan synthase activity of microsomal fraction from the faba bean. To the assay medium containing 50 mM Hepes, pH 7.2, 2 mM dithiothreitol, 2 mM spermine, 20 mM cellobiose, 0.2 mM $CaCl_2$, 2 mM UDP-[^{3}H] glucose (200,000 cpm), and 0.015% (w/v) of digitonin was added 10 μg of crude microsomal vesicles for 30 min at 25°C.

stances extracted from culture filtrate of the fungus *Bipolaris sorokiniana (Sacc.)* inhibited the photochemical activity of wheat chloroplasts by inhibiting the electron transport and the photophosphorylation coupled to it.

As indicated in Fig. 7 (curves A and B, left side), a slight inhibition (± 10–18%) of photosystem II (PSII), as measured by the oxygen evolution, is observed in the presence of 0.8 mM helminthosporol.

The photorespiration (PH.R) measured by the light-dependent oxygen uptake (Fig. 7, curves A and B, right side) after switching on the ribulose-bis-phosphate oxidase activity, in the presence of high oxygen concentration and after activating the peroxisomal glycollate oxidase by glycollate, was not significantly inhibited by 0.8 mM helminthosporol. A subsequent addition of 2-pyridylmethane sulfonate, a specific inhibitor of glycollate oxidase, suppressed the glycollate activation. To confirm this absence of effect on photorespiration, the electron transport in the thylakoid membranes was first inhibited by DCMU (Fig. 7, curves C and D); the marked activation of photorespiration observed by glycollate addition was not inhibited by the addition of 0.8 mM helminthosporol.

Figure 8 showed that the specific electron transport in photosystem I, as measured by the oxygen uptake in the presence of DCMU to inhibit PSII and of methylviologen as final electron acceptor, was not inhibited.

The photophosphorylation measured by the ADP-stimulated oxygen evolution in intact chloroplasts was inhibited by more than 50% when 1 mM helminthosporol was present in the reaction mixture (Fig.9).

Table II. Effect of Helminthosporol on Acyl-COA Stigmasterol Acyl-Transferase Activity of Faba Bean Microsomes[a]

Helminthosporol (mM)	ACAT specific activity (μmol · min^{-1} · mg^{-1})
0	104
1	88
2	81
4	57

[a] Values were the average of duplicate experiments.

DISCUSSION

The previous works on the mode of action of the toxins excreted by the phytopathogen fungus, *Cochliobolus sativus*, gave a very confusing image of the biochemical targets of the different sesquiterpene derivatives involved in the plant disease symptoms. The results we obtained with the monoaldehyde derivative (helminthosporol) clearly demonstrate that its main site of action was the different membrane systems of the cell. They indicated that helminthosporol inhibited the oxidative phosphorylation mechanism by dissipation of the electrochemical proton gradient of the inner mitochondrial membrane, like a classical uncoupler. The permeabilization effect of the toxin is confirmed by its effect on the transport of phosphate, dicarboxylate, and tricarboxylate substrate through this membrane. The general and nonspecific inhibition observed in these transport systems indicates that this toxin does not affect a specific carrier protein, but the general permeability of the inner mitochondrial membrane to ions.

The inhibition by helminthosporol of the photophosphorylation mechanism in the thylakoid membranes of chloroplasts could also be explained by an uncoupling effect of the toxin. The photorespiration which includes the ribulose-bis-phosphate oxidase activity and the microsomial glycollate oxidase activity is not affected by the phytotoxin.

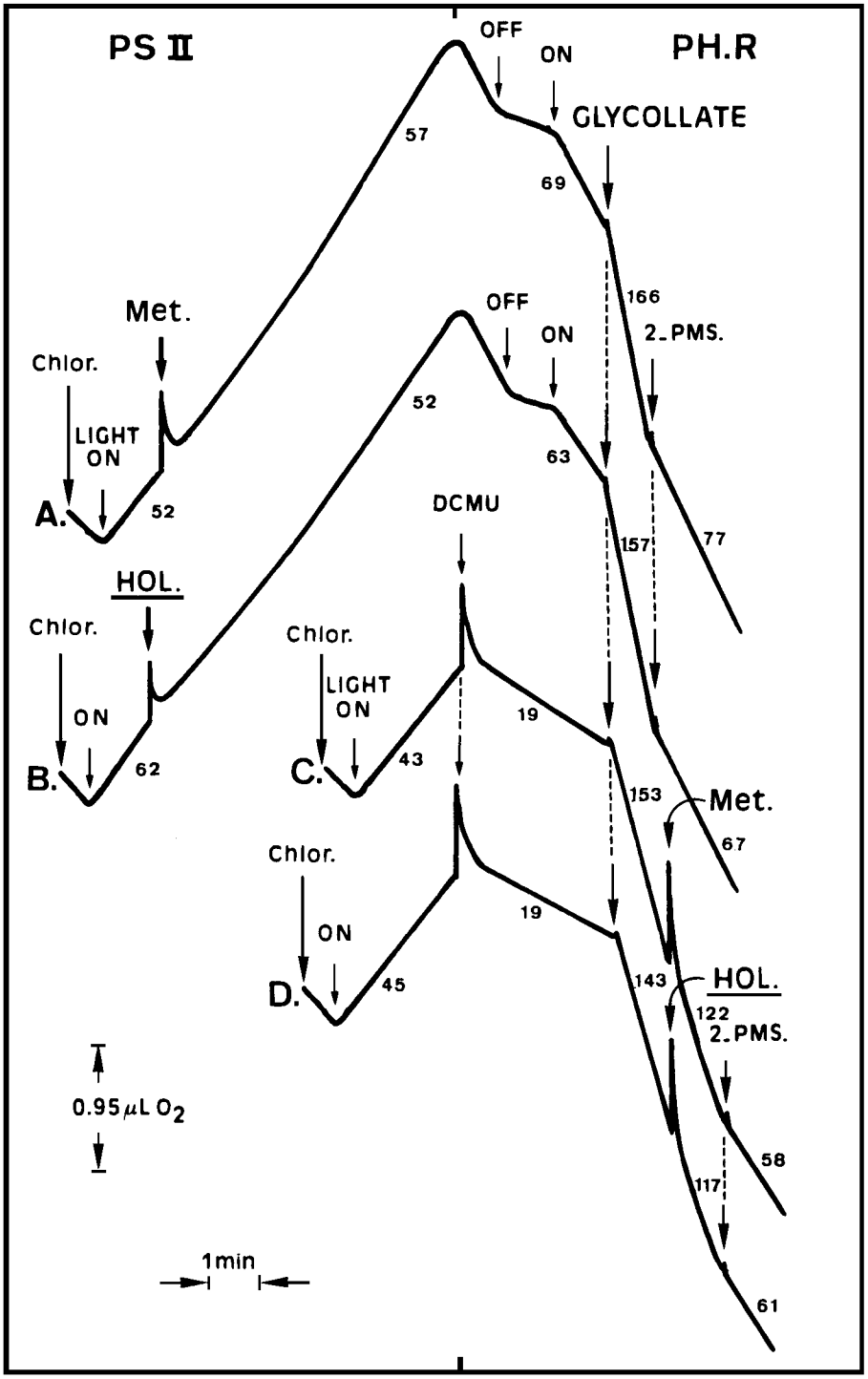

Fig. 7. Effect of helminthosporol on the electron transport in photosystem II and on photorespiration. The activity of photosystem II (PSII) was measured by the rate of oxygen evolution induced by light in chloroplasts of *Vicia faba* in the cuvette of the oxygen electrode containing the reaction mixture, in the absence (curve A) and presence (curve B) of 0.8 mM helminthosporol (HOL) added 2 min after the light came on. Photorespiration (PH.R) was measured in the same graph by following the rate of oxygen consumption in the presence of 10 mM glycollate. 1 mM 2-pyridyl-α-methane sulfonate (2-PMS) was added as a specific inhibitor of photorespiration. The activities are expressed in $\mu l\ O_2 \times h^{-1} \times mg^{-1}$ chlorophyll. Curves C and D measured the effect of helminthosporol on photorespiration when electron transport was inhibited in PSII by the addition of 20 μM dichlorophenyldimethyl urea (DCMU).

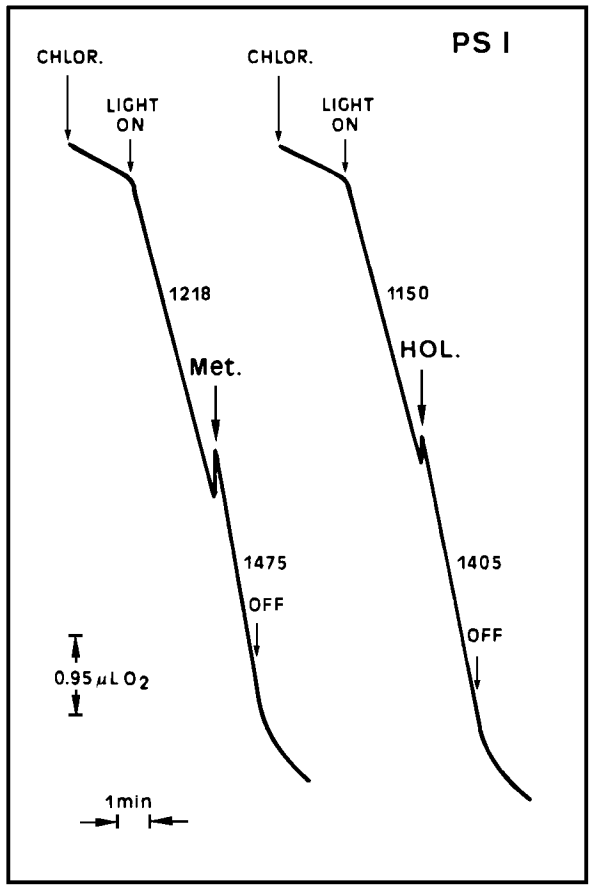

Fig. 8. Effect of helminthosporol on the electron transport in photosystem I. The activity of photosystem I (PSI) was measured by the oxygen uptake in 2 ml of the reaction mixture given in Material and Methods, in the presence of 20 µM DCMU, to inhibit PSII. The activity was started by light and expressed in µl O_2 ts; $h^{-1} \times mg^{-1}$ chlorophyll.

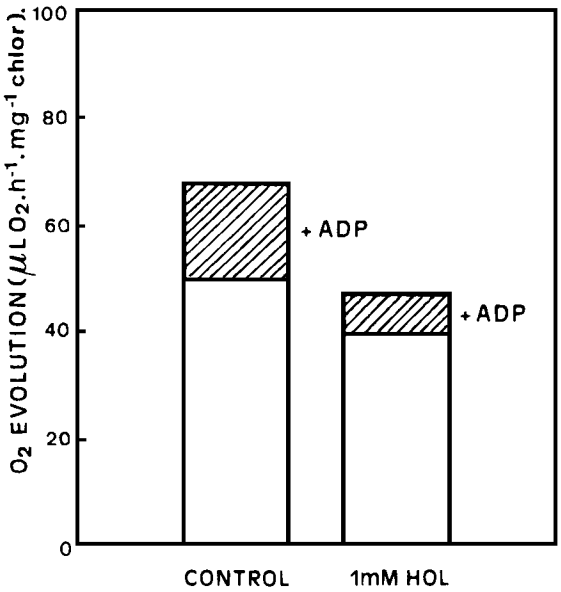

Fig. 9. Effect of helminthosporol on photophosphorylation. To the reaction mixture given in Material and Methods for PSII measurements, 100 µl of chloroplast suspension (0.42 mg chlorophyll) was introduced into the cuvette of the oxygen electrode. After 2 min in the presence of light, 0.5 mM ADP was added and the increased oxygen evolution observed was measured in the presence and absence of 1 mM helminthosporol (dashed area in this figure).

Beside the H^+- gradient built across the mitochondrial and thylakoid membranes by the electron transport systems, another electrochemical gradient of protons of vital importance is created across the cell plasma membrane by an ATP-dependent H^+-ATPase. This H^+-gradient, which is a major driving force for nutrient uptake into cells, was also drastically inhibited by helminthosporol and therefore must be an essential site of action in the pathogeny of the fungus.

When plant cells of sensitive crops are attacked by pathogen or by other external physical stress, the plasma membrane 1,3-β-glucan synthase activity of the sieve cells of conductive tissues is stimulated to produce callose deposit to seal off damaged sieve elements and prevent the loss of phloem sap. Since helminthosporol inhibited this synthase activity, an essential defense mechanism of the plant is neutralized and, moreover, the nonspecific membrane disrupting effect of helminthosporol (possibly caused by the inhibition of sterol esterification of membrane lipids) could facilitate the *in vivo* pathogenic tissue invasion by the fungus.

The important energetic deficit due to the dissipation of the H^+- gradients in the cell plasma membranes, in the inner mitochondrial membranes, and in the thylakoid membranes of chloroplasts could have dramatic effects on the cell metabolism and, obviously, might be largely responsible for the *in vivo* phytotoxicity of this fungal toxin.

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