Inhibition of photosynthetic electron transport by metabolites produced by *Phialophora gregata*

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In soybeans, adzuki beans, and mung beans the disease brown stem rot, which is caused by *Phialophora gregata*, is characterized by vascular browning of the stem and, in some cases, by chlorosis and necrosis of the leaves. We investigated the effect of compounds produced by *P. gregata* on light-driven electron transport in thylakoid membranes. The compounds investigated were contained in three different extracts isolated from the fungal culture: crude extract, gregatin A and the mother liquor from the crystallization of gregatin A. Electron transport rates were measured potentiometrically using a Clark-type oxygen electrode and spectrophotometrically by monitoring light-induced absorbance changes due to cytochrome f. The data show that at least one metabolite produced by *P. gregata* can completely inhibit photosynthetic electron transport.

The most potent inhibitor was the mother liquor which inhibited the reaction from *H₂O* to methylviologen by 50% at a concentration of 20 μg ml⁻¹. Comparison of the efficacy of the different extracts indicates that gregatin A is a less effective inhibitor. The effect of the extracts on electron transport reactions that include limited segments of the photosynthetic apparatus indicates that there are at least two sites of inhibition, the Photosystem II complex and the cytochrome *b/f* complex. The results raise the possibility that the leaf symptoms observed in brown rot may be due to inhibition of photosynthetic electron transport by a metabolite produced by *P. gregata*.

INTRODUCTION

The disease brown stem rot in soybeans, adzuki beans, and mung beans [2, 12] is caused by the fungus *Phialophora gregata* [7], which attacks through the roots invading...
the vascular system. Brown stem rot is characterized by vascular browning of the stem, and in some cases, by interveinal chlorosis and necrosis of the leaves [1, 9]. The impact of infection by P. gregata on soybean yield can be quite significant; losses ranging from 20 to 44% have been reported [6, 8, 10]. The cause of the yield reduction in infected plants is unknown. Using isolates of P. gregata that produce differential symptoms in soybeans, Gray [8] showed that the yield losses correlate with the development of leaf symptoms. In controlled field experiments, no significant yield losses were observed in plants infected with P. gregata isolates that caused only stem browning, while infection with isolates that, in addition to stem browning, caused interveinal chlorosis and necrosis of the leaves, resulted in a yield reduction of nearly 40%.

Gray & Chamberlain suggested that both the stem browning and leaf symptoms are caused by a metabolite produced by P. gregata. This suggestion is based on the observation that the disease symptoms could be induced in soybeans by autoclaved extracts from infected plants [9]. Support for the idea that a toxin is a key element in the development of brown stem rot is provided by the work of Kobayashi & Ui who isolated several metabolites from P. gregata, gregatin A, B, C, D, and E [12]. Three of these, gregatin A, C and E, produced both stem and leaf symptoms in adzuki beans [12, 13].

In the work described here we investigated the effect of metabolites produced by P. gregata on photosynthetic electron transport. We show that at least one of the metabolites is an effective inhibitor of light-driven electron transport in photosynthetic membranes. It has been demonstrated that inhibition of photosynthetic electron transport can lead to leaf chlorosis and necrosis [3, 14]. These results raise the possibility that the leaf symptoms observed in plants infected with P. gregata are due partially, or totally, to inhibition of photosynthetic electron transport by a toxin produced by the fungus.

MATERIALS AND METHODS

Fungal cultures
The S-1 isolate of P. gregata (NRRL 13198, USDA/ARS, Peoria, IL) used in this study causes foliar chlorosis and necrosis, as well as vascular browning in infected soybeans [15]. The isolate was maintained in the dark on soybean stem agar plates at 18°C [2]. Spore transfers were made at 30-day intervals by placing plugs from the old plate in 2–10 ml of sterile H₂O, swirling and adding the spore-containing liquid to the new plates.

Crude extract
Crude toxin was obtained from P. gregata cultured on soybean stem medium, prepared by the method of Kobayashi & Ui [13] except that soybean stems were used in place of adzuki bean stems. Ten 250-ml autoclaved flasks were inoculated with two 2 × 5 mm agar plugs of P. gregata and were incubated in the dark at 23°C. After 35 days the mycelial mat was removed by vacuum filtration. An extract was obtained by extraction of the filtrate (about 900 ml) with ethyl acetate (1 part to 5 parts filtrate). The volume of the ethyl acetate extraction was reduced to approximately 1 ml using a rotary evaporator at 38–40°C. The extract was further dried in a tared vial under a dry air stream until the ethyl acetate odor was absent and then redissolved in 100% ethanol.
Electron transport inhibition by *P. gregata* metabolites

**Gregatin A**

Purified gregatin A (mol. wt = 276) was prepared by the method of Taylor et al. [15]. The gregatin A exhibited a single spot on thin-layer chromatographs developed in methylene chloride:methanol (99:1, v/v). The nuclear magnetic resonance and mass spectral data, and the melting point were consistent with reported values [12].

**MLA**

The mother liquor from gregatin A crystallization (MLA) was obtained during a preliminary gregatin A purification procedure. Flasks of rice culture (16 g of converted white rice, 60 ml of H₂O, autoclaved for 20 min) were inoculated with S-l fungal spores and incubated at 25 °C. After 4 weeks the cultures were extracted three times with 100 ml ethyl acetate/flask. The ethyl acetate was decanted from the rice slurry and vacuum filtered, and the resulting filtrate was dried. This crude extract was redissolved in methanol:H₂O (9:1, v/v) and mixed with hexane to remove excess oil. The hexane layer was discarded and the methanol:H₂O layer was vacuum dried. The mixture was redissolved in methylene chloride and passed through a Kieselgel 60 silica column (75 g silica per gram of sample mixture). Compounds which did not stick to the column were eluted with approximately 50 ml methylene chloride. The column was eluted with methylene chloride:methanol (98:2, v/v). The first 100 ml fraction was discarded. A yellow band containing gregatin A was then eluted and collected as a 30 ml fraction and dried. The sample was redissolved in methylene chloride and was further fractionated on a second silica column using hexane as the solvent. The sample was eluted with hexane:ethyl acetate (9:1, v/v). After the first 20 ml fraction, four 15 ml fractions were collected all of which were found to contain gregatin A by high performance liquid chromatography. The fractions containing gregatin A were combined, dried down, and redissolved in a small amount of hexane. Gregatin A crystals were collected by vacuum filtration. The MLA was collected and dried.

**Chloroplast isolation**

Thylakoid membranes were isolated from spinach leaves obtained from local markets or from soybean leaves (Century). The procedure for isolating the thylakoid membranes is described elsewhere [18]. The chlorophyll concentration was determined as described previously [18].

In the majority of the experiments described below we chose to use spinach rather than soybean thylakoids because spinach thylakoids provide a better characterized system than that of soybeans. In addition, we found that spinach thylakoids are not as labile as soybean thylakoids.

**Electron transport measurements**

Electron transport rates were measured polarographically using a Clark-type O₂ electrode fitted in a water-jacketed 1.9 ml cell. The actinic light was provided by a 250 W tungsten–halogen lamp filtered by heat filters and a Corning CS 2-61 red filter. All experiments were done using saturating light and illumination times of 20–40 s. Thylakoids were suspended in a reaction medium consisting of 30 mM Tricine/KOH, 100 mM sorbitol, 20 mM KCl, and 2 mM MgCl₂. Further additions are given in the
The percentage inhibition shown in the figures is defined as the ratio of the rate of electron transport measured in the presence of the indicated concentration of extract divided by the rate of electron transport in the absence of the extract multiplied by 100. Each point is the average of at least two samples. Control samples were measured before and after the inhibited samples and the average value of the rate was used. Addition to control samples of ethanol volumes equivalent to those added with the extracts did not significantly alter the inhibition curves shown here.

Diaminodurene (DAD) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) were obtained from Aldrich Chemical Company. Diaminodurene was recrystallized to form DAD-2 HCl [20]. All other chemicals purchased from Sigma.

**RESULTS**

In thylakoid membranes electron transport from H$_2$O to NADP$^+$ involves three integral protein complexes operating in series. These are the Photosystem II (PS II), cytochrome $b/f$, and Photosystem I (PS I) complexes. Electrons are transferred from PS II to the cytochrome $b/f$ complex by plastocyanin, while plastocyanin transfers electrons from the cytochrome $b/f$ complex to PS I. In this study we have determined the effect on light-driven electron transport of three samples isolated from cultures of *P. gregata*: crude extract, gregatin A and MLA. We found that each of these extracts inhibited electron transport from H$_2$O to methylviologen (MV), a reaction that includes electron transfer from PS II through PS I (H$_2$O$\rightarrow$PS II$\rightarrow$cytochrome $b/f$$\rightarrow$PS I$\rightarrow$MV). In order to localize the sites of inhibition within this sequence, we investigated the effect of the extracts on three partial electron transport reactions, each of which requires a different segment of the electron transport series [11].

**Effect of crude extract on electron transport**

In spinach thylakoids electron transport from H$_2$O to methylviologen could be completely inhibited by the crude extract. Electron flow was inhibited 50% in the presence of 190 µg ml$^{-1}$ of crude extract, and 90% in the presence of 490 µg ml$^{-1}$ (Fig. 1). In thylakoids isolated from soybeans the reaction from H$_2$O to MV was inhibited approximately 50% in the presence of 260 µg ml$^{-1}$ of crude extract (data not shown). The results shown below reveal that this inhibition involves at least two different sites.

In the light-driven reaction from H$_2$O to DAD$_{ox}$ (H$_2$O$\rightarrow$PS II$\rightarrow$DAD$_{ox}$) electron transport requires PS II but not PS I or the cytochrome $b/f$ complex. This reaction was inhibited by 50% in the presence of 130 µg ml$^{-1}$ crude extract, and 90% in the
Electron transport inhibition by *P. gregata* metabolites

**Fig. 1.** Effect of the crude extract from *Phialophora gregata* on light-driven electron transport from H$_2$O to methylviologen (MV) in spinach thylakoid membranes. In addition to the standard reactions medium, the mixture containing 0.1 mM MV, 5 μg gramicidin, chloroplasts equivalent to 15 μg ml$^{-1}$ chlorophyll and crude extract as indicated. The average control rate of electron transport was 1340 μequiv mg$^{-1}$ chlorophyll h$^{-1}$.

presence of 380 μg ml$^{-1}$ (Fig. 2). In view of the large number of photosynthetic electron transport inhibitors that produce similar results by binding to PS II [5, 16] the most straightforward interpretation is to assume that the crude extract inhibits the turnover of PS II. While this assumption is sufficient to account for the above data, the effect of the crude extract on an electron transport reaction that does not require PS II indicates a second site of inhibition.

The reaction from duroquinol (DQH$_2$) to MV (DQH$_2$→cytochrome b/f→PS I→MV) requires the cytochrome b/f complex and photosystem I but not photosystem II. In this reaction electron flow was inhibited by 50% in the presence of 230 μg ml$^{-1}$ crude extract. However, the inhibition was incomplete up to the highest levels of crude extract that we added (Fig. 2).

The electron transport reaction from DAD$_{red}$ to methylviologen (DAD$_{red}$→PS I→MV), which requires PS I, was relatively insensitive to the crude extract (Fig. 2), suggesting that the second site of inhibition involves the cytochrome b/f complex.

Support for inhibition at the cytochrome b/f complex is provided by measurements of the effect of the crude extract on the rate of cytochrome f rereduction following illumination. Cytochrome f is normally reduced in the dark and is rapidly oxidized upon illumination. When the actinic light is turned off cytochrome f is rereduced as the cytochrome b/f complex oxidizes plastoquinol [4, 17, 19]. We measured the absorbance change at 554 nm due to the α-band of cytochrome f as a function of time after the actinic light was turned off. In the absence of crude extract cytochrome f was rereduced by electrons from quinol with a half-time of 27 ms. In the presence of 833 μg ml$^{-1}$ crude extract, the half-time was increased to 59 ms (Table 1). In addition to slowing the rate of cytochrome f rereduction the presence of the crude extract caused the extent of cytochrome f turnover to be decreased by 20%.

**Effect of gregatin A on electron transport**

In contrast to the crude extract, we found that the gregatin A extract could only partially inhibit electron transport. In spinach thylakoids the reaction H$_2$O to MV was
The crude extract from *Phialophora gregata* on light-driven electron transport in spinach thylakoid membranes. 

**Figure 2.** Effect of the crude extract from *Phialophora gregata* on light-driven electron transport in spinach thylakoid membranes. H$_2$O to DAD$_{ox}$ (○): in addition to the standard reaction medium, the reaction mixture contained 0.5 mM DAD, 1.5 mM K$_2$Fe(CN)$_6$, chloroplasts equivalent to 15 μg ml$^{-1}$ chlorophyll and crude extract as indicated; the average control rate of electron transport was 1540 μequiv mg$^{-1}$ chlorophyll h$^{-1}$. DQH$_2$ to MV (□): in addition to the standard reaction medium (pH 7.5), the mixture contained 0.5 mM DQH$_2$, 0.1 mM MV, 25 μM DCMU, 300 units ml$^{-1}$ SOD, chloroplasts equivalent to 15 μg ml$^{-1}$ chlorophyll and crude extract as indicated; the average control rate of electron transport was 1090 μequiv mg$^{-1}$ chlorophyll h$^{-1}$. DAD$_{ox}$ to MV (△): in addition to the standard reaction medium, the mixture contained 1.5 mM DAD, 0.1 mM ascorbate, 0.1 mM MV, 5 μM gramicidin, 25 μM DCMU, 900 units SOD/ml, chloroplasts equivalent to 15 μg/ml chlorophyll and crude extract as indicated; the average control rate of electron transport was 5500 μequiv mg$^{-1}$ chlorophyll h$^{-1}$. DAD, diaminodurene; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DQH$_2$, duroquinol; MV, methylviologen; SOD, superoxide dismutase.

**Table 1.**

**Half-life of cytochrome f reduction following a 100 ms actinic flash in the reaction of duroquinol (DQH$_2$) to methylviologen (MV).** The half-life was monitored by the absorbance change at 554–540 nm. For details, see the text. In addition to the standard reaction medium at pH 7.5, the mixture contained 0.5 mM DQH$_2$, 0.1 mM MV, 3 μM gramicidin, 20 μM DCMU, spinach chloroplasts equivalent to 20 μg ml$^{-1}$ chlorophyll and 833 μg ml$^{-1}$ crude extract as indicated.

<table>
<thead>
<tr>
<th>Control</th>
<th>27</th>
<th>0.00056</th>
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<tbody>
<tr>
<td>Plus crude extract</td>
<td>59</td>
<td>0.00047</td>
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inhibited by 50% in the presence of 63 μg ml$^{-1}$ gregatin A (230 μM) (Fig. 3). The difference between the efficacy of gregatin A and the crude extract is clearly demonstrated by the fact that of the highest concentrations of gregatin A that we could attain without observing a precipitate (approximately 100 μg ml$^{-1}$) the reaction H$_2$O to MV maintained 30–40% of control activity, whereas the crude extract was able to produce total inhibition (Fig. 1). We found that the gregatin A extract inhibited the reactions H$_2$O to DAD$_{ox}$ and DQH$_2$ to MV to a similar extent (Fig. 4). In neither case was complete inhibition observed. The reaction DAD$_{reg}$ to MV was insensitive to gregatin A (Fig. 4).

Unless the solubility and/or efficacy of gregatin A is significantly altered in the crude extract these observations lead us to conclude that inhibition of electron transport by the crude extract cannot be accounted for by gregatin A content alone. In fact,
Electron transport inhibition by *P. gregata* metabolites

**Fig. 3.** Effect of gregatin A on light-driven electron transport from H$_2$O to methylviologen in spinach thylakoid membranes. The conditions were exactly as described in Fig. 1, except that the control rate was 1400 μequiv mg$^{-1}$ chlorophyll h$^{-1}$.

**Fig. 4.** Effect of gregatin A on light-driven electron transport in spinach thylakoid membranes. The conditions were exactly as described in Fig. 2 except that (a) the H$_2$O to DAD$_4$ (●) control rate was 1670; (b) the DQH$_2$ to MV (□) control rate was 1030 μequiv mg$^{-1}$ chlorophyll h$^{-1}$; (c) the DAD$_{red}$ to MV (▲) control rate was 3800 μequiv mg$^{-1}$ chlorophyll h$^{-1}$.

we cannot conclude unequivocally from these data that gregatin A inhibits electron transport. The possibility that a contaminant present in the 50% inhibited sample at 2 μg ml$^{-1}$ was responsible for the limited inhibition cannot be discounted.

**Effect of the MLA on electron transport**

The MLA was found to be the most potent inhibitor of electron transport on a microgramme per millilitre basis. In spinach thylakoids, electron transport from H$_2$O to MV was inhibited 50% by 20 μg ml$^{-1}$, and 90% by 35 μg ml$^{-1}$ MLA (Fig. 5). Inhibition was complete at 72 μg ml$^{-1}$. While a fraction of the MLA is gregatin A, the fact that the efficacy of MLA inhibition is significantly higher than that of purified gregatin A indicates that the most active inhibitor in the MLA is a compound other than gregatin A. The MLA also inhibited the reaction H$_2$O to DAD$_{red}$ (50% inhibition at 9 μg ml$^{-1}$) and DQH$_2$ to MV (50% inhibition at 28 μg ml$^{-1}$) (Fig. 6). To our surprise, at higher
concentrations, MLA partially inhibited the reaction DAD$_{red}$ to MV, a reaction that is insensitive to most inhibitors [16].

In soybean thylakoids the reaction from H$_2$O to MV was inhibited approximately 60% by 21 µg ml$^{-1}$ MLA (data not shown).

DISCUSSION

These data demonstrate that P. gregata produces one or more metabolites that inhibit photosynthetic electron transport in thylakoid membranes. The effect of the extracts on electron transport reactions that involve different segments of the photosynthetic apparatus show that there are at least two sites of inhibition, one at PS II and the other at the cytochrome b/f complex. Although it was not the purpose of this study to characterize the mechanism of inhibition, it is worth noting that there are numerous compounds that inhibit one or both of these complexes [5, 16]. The most common
Electron transport inhibition by \( P.\ gregata \) metabolites

A mechanism of inhibition is thought to be due to competition between the endogenous electron carrier plastoquinone and the inhibitor for the catalytic site on the complex [5].

Although inhibition of photosynthetic electron transport is sufficient to account for the leaf symptoms observed in brown stem rot, other mechanisms are known. In order to determine if the inhibition described here is a significant element in the development of the disease several questions must be addressed. Does \( P.\ gregata \) produce a photosynthetic inhibitor when growing in a plant? If so, does the inhibitor reach a high enough concentration in the chloroplast to lower photosynthetic activity? One approach to answering these questions is to select strains of \( P.\ gregata \) that do not produce metabolites that inhibit photosynthetic electron transport. Should these strains infect plants without producing leaf symptoms then inhibition of photosynthesis would appear to be a factor in the development of the disease brown stem rot.

REFERENCES