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Activity of the Natural Algicide, Cyanobacterin, on Angiosperms[1]

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FLORENCE K. GLEASON* AND DEBORAH E. CASE
Gray Freshwater Biological Institute, University of Minnesota, Navarre, Minnesota 55392

ABSTRACT

Cyanobacterin is a secondary metabolite produced by the cyanobacterium (blue-green alga) *Scytonema hofmanni*. The compound had previously been isolated and chemically characterized. It was shown to inhibit the growth of algae at a concentration of approximately 5 micromolar. Cyanobacterin also inhibited the growth of angiosperms, including the aquatic, *Lemna*, and terrestrial species such as corn and peas. In isolated pea chloroplasts, cyanobacterin inhibited the Hill reaction when *p*-benzoquinone, $K_3Fe(CN)_6$, dichlorophenolindophenol, or silicomolybdate were used as electron acceptors. The concentration needed to inhibit the Hill reaction in photosystem II was generally lower than the concentration of the known photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea. Cyanobacterin had no effect on electron transport in photosystem I. The data indicate that cyanobacterin inhibits O_2 evolving photosynthetic electron transport in all plants and that the most probable site of action is in photosystem II.

The cyanobacterium (blue-green alga), *Scytonema hofmanni* (formerly UTEX[2] 1581, now UTEX 2349) was found to inhibit the growth of other algae in two species cultures (9). Inhibition was due to production and excretion of a secondary metabolite by *S. hofmanni*. The active metabolite was isolated and chemically characterized. This compound, called cyanobacterin, is a diaryl-substituted gamma lactone with a chlorine substituent on one of the aromatic rings (Fig. 1). Investigation of the mechanism of action of cyanobacterin showed that the compound inhibited photosynthetic electron transport in the unicellular cyanobacterium, *Synechococcus* sp. (ATCC 27146). In photosynthetically active spheroplasts of *Synechococcus*, cyanobacterin inhibited electron transfer to the Hill acceptors, DCPIP and $K_3Fe(CN)_6$, indicating that the probable site of action is in PSII (4). We report here the activity of the algicide, cyanobacterin, against several species of higher plants and in the pea chloroplast system. The data confirm and extend our previous observations that this natural product disrupts photosynthetic electron transport at a site in PSII.

MATERIALS AND METHODS

Plants. Cultures of *Lemna gibba*, *Lemna* 6746, and *Lemna minor* were obtained from Dr. T. Soulen, University of Minnesota, St. Paul. Stocks were maintained in sterilized Hutner's medium (8) minus sucrose. Peas (*Pisum sativum*, var Little Marvel) were a product of Lofts, Inc., Cambridge, NY. Corn seeds (*Zea mays*, var Goldencross Bantam) were from the Excel Seed Co., Downers Grove, IL. Seeds of Curled Dock (*Rumex crispus*), Wild Buckwheat (*Polygonium convolvulus*), Wild Oats (*Avena fatua*), and Green Foxtail (*Setaria viridis*) were obtained from Seeds for Research, Duane Arneklev, 202 N. Broadmore, Plentywood, MT 59254.

Growth Conditions. *Lemna* cultures were maintained in continuous light, 126 $\mu E/m^2 \cdot s$, from two 40 W Sylvania cool-white lamps. Seeds of terrestrial plants were sown in 100 ml beakers filled with sterilized soil. After germination, the plants were grown under the same lamps at 200 $\mu E/m^2 \cdot s$ on a 16:8 h light:dark regime.

Treatment with Antibiotic. *Lemna.* Cyanobacterin, dissolved in ethyl ether, was added to sterile Hutner's medium at a known concentration. The mixture was allowed to stand at room temperature for 1 h with gentle mixing to evaporate the ether. Control cultures received ether alone. The flasks were then inoculated with 5 to 10 *Lemna* plants from stock cultures. Growth was monitored by counting the plants.

Terrestrial Plants. Cyanobacterin, 25 mg, was dissolved in a small amount (1–3 ml) of ethyl ether. A commercial Spreader-Sticker was then added to the solution. Spreaders used were Ortho X-77 (Chevron Chemical Co., Richmond, CA) or Science Spreader (Science Products Co., Chicago, IL). The Ortho X-77 was then diluted 1:1360 with distilled H_2O and the Science Spreader, 1:100 with distilled H_2O. A stream of air was directed over the mixtures for 30 to 60 min to evaporate the ether. Mixtures were then diluted further to obtain the required concentration of antibiotic, or used directly. The mixtures were sprayed on the plants with a small atomizer. Control plants received an equivalent amount of spreader-ether mixture. Dry weights were obtained 15 d after treatment. Plants were cut off at the soil line and dried for 16 h at 100°C.

Pea Chloroplast Isolation. Pea plants were harvested after 3 weeks (8–10 plants). The leaves were removed and placed in a chilled Waring Blendor. Ice-cold grinding medium, containing 0.33 M sorbitol, 2 mM EDTA, and 25 mM Hepes (pH 7.6) was added. The leaves were processed for 30 to 60 s. The resulting slurry was filtered through 6 layers of cheesecloth. The filtrate was then centrifuged for 5 min at 3,000*g* in a Dupont-Sorvall RC-5 centrifuge at 4°C. The supernatant fraction was discarded and the pelleted chloroplasts resuspended in assay medium containing: 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 5 mM $Na_4P_2O_7$, and 50 mM Hepes (pH 7.6). The chloroplast suspension was stored in the dark, at 0°C under N_2. All preparations were used within 24 h.

Hill Reaction. O_2 evolution by chloroplast preparations was determined with a Clark-type O_2 electrode (Yellow Springs Inst., Yellow Springs, OH) in a 2 ml reaction chamber. The chamber was maintained at 30°C. Full scale was set by adding air-saturated assay medium to the chamber. The O_2 concentration was estimated to be 235 mM (5). The zero point was set by adding dithionite and depleting the chamber of O_2. Chloroplast suspension was added to the chamber and diluted to a final Chl concentration of 30 to 50 μg/reaction. Illumination was provided

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[2] Abbreviations: UTEX, University of Texas Collection, Austin, TX; ATCC, American Type Culture Collection, Rockville, MD; DCPIP, dichlorophenolindophenol.

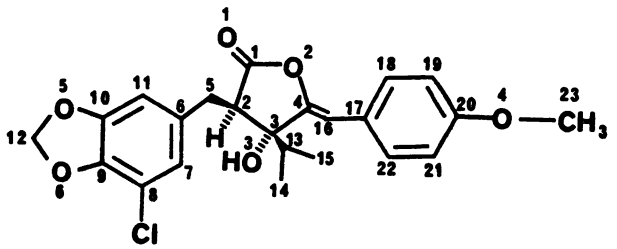

FIG. 1. Structure of cyanobacterin. The basic structure of the molecule was determined by spectroscopic techniques (12). The correct stereochemistry at C-2 and C-3 was resolved by x-ray diffraction (FK Gleason, J Porwoll, J Flippen-Anderson, C George, unpublished data).

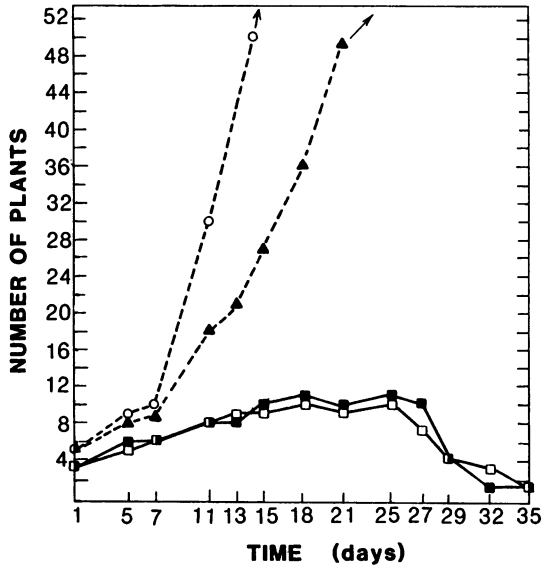

FIG. 2. Effect of cyanobacterin on reproduction of *Lemna gibba*. Modified Hutner's medium containing a known concentration of cyanobacterin was inoculated from a stock culture of *L. gibba* on d 1. (O- - -O), control, ether added; (▲- - -▲), 0.5 μg/ml (1.1 μM) cyanobacterin added; (□——□), 1.0 μg/ml (2.3 μM) cyanobacterin added; (■——■), 2.0 μg/ml (4.6 μM) cyanobacterin added.

from a projector lamp at 150 $\mu E/m^2 \cdot s$. Conditions for measuring chloroplast electron transport were adapted from Izawa (6). The Hill reaction acceptors used for monitoring PSII activity were 0.25 mM DCPIP (Sigma Chemical Co.), 2.5 mM $K_3Fe(CN)6$, and 0.5 mM *p*-benzoquinone (Fisher Scientific) and 0.2 mM silicomolybdic acid (Pfaltz and Bauer, Inc., Stanford, CT). PSI activity was measured by transfer of electrons from ascorbate-reduced DCPIP to 0.1 mM methyl viologen (Aldrich Chemical Co.) as terminal acceptor. Chl concentration of the chloroplasts suspensions was determined spectrophotometrically after extraction with 90% acetone (11). Highly purified cyanobacterin and anhydrocyanobacterin were extracted from *S. hofmanni* as previously described (12). DCMU was obtained from the Sigma Chemical Co., and dissolved in ethyl ether.

RESULTS

Cyanobacterin has been shown to be toxic to most cyanobacteria at a minimum effective dose of 2 μg/ml (4.6 μM) (4). We initially tested the effect of cyanobacterin on the aquatic angiosperm, *Lemna gibba*, by adding similar amounts to the growth medium. The results shown in Figure 2 confirm that cyanobacterin does inhibit plant growth. The antibiotic stopped frond multiplication at a minimum dose of 0.5 μg/ml (1.1 μM). However, at this concentration, the plants recover after approximately 7 to 12 d and numbers increase. A slightly higher concentration of cyanobacterin, 1.0 μg/ml (2.3 μM) is toxic. Plants appear normal for 10 to 15 d after which time, roots fall off and the leaves become chlorotic. After 30 d, most of the plants in these treated cultures had died. The same results were obtained with other *Lemna* species (Table I).

Similar tests were attempted on terrestrial angiosperms. Cyanobacterin, when added to the soil, had no effect. The antibiotic is very hydrophobic and may adhere to the soil particles. Alternatively, it may not be transported through the root vascular system. We then sprayed the antibiotic directly onto the leaves of 1 to 2 week old plants using a commercially available spreading agent to wet the leaf surface. As shown in Table II, cyanobacterin was active against all species tested. Plants grew more slowly after treatment, as reflected in the lower dry weights recorded, and usually died 15 to 20 d later. The concentrations of cyanobacterin

Table I. *Effect of Cyanobacterin on Growth of Lemna Species*

L. gibba data are an average of four separate experiments, *L. minor*, 2 and *Lemna* No. 6746, 3. Increase in plant numbers had generally ceased after 14 d (Fig. 1). Most cultures still appeared normal at this stage. Cultures treated with the lowest concentration (1.1 μM) recovered in all cases. Higher doses were toxic.

Cyanobacterin Added	Number of Plants/Culture 14 d after Addition of Cyanobacterin		
	Lemna gibba	*Lemna minor*	*Lemna 6746*
None	>100	>100	>100
0.5 μg/ml (1.1 μM)	25	56	50
1.0 μg/ml (2.3 μM)	16	34	31
2.0 μg/ml (4.6 μM)	10	46	21

Table II. *Effect of Cyanobacterin on Growth of Terrestrial Angiosperm*

Plant	Cyanobacterin[a]	Dry wt	Comment[b]
	mg sprayed	*mg*	
Setaria viridis (green foxtail)	None	346	
	0.5	42	50% of plants affected
Avena fatua (wild oats)	None	155	
	0.4	116	50% of plants affected
Rumex crispus (curled dock)	None	211	
	0.4	26	65% of plants affected
Polygonium convolvulus (wild buckwheat)	None	194	
	0.6	53	75% of plants affected
Zea mays (var Golden-cross Bantam)	None	116	
	0.9	95	100% killed
Pisum sativum (var Little marvel)	None	88	
	0.5	69	100% killed

[a] Concentrations of cyanobacterin are minimum amounts sprayed on the plants which resulted in a deleterious effect on plant growth. Higher doses were also effective, usually within a shorter time span (data not shown). [b] Plants were sprayed with a known concentration of cyanobacterin in Ortho X-77 or Science surfactant. Plants were allowed to grow for 15 d after treatment, at which time they were cut off at the soil line and dry weight determined. *S. viridis*, *A. fatua*, *R. crispus*, and *P. convolvulus* contained 10 to 20 plants/container. All plants were affected by cyanobacterin but not all were killed, presumably because of variation in the doses received. Data is from 5 separate experiments. *Z. mays* and *P. sativum* contained one plant/container. Plants at 15 d were still alive but smaller and chlorotic. Plants died after 20 to 25 d of cyanobacterin treatment. Data for these species were determined in 14 separate trials.

listed in Table II are the minimum amounts needed to obtain at least 50% lethality.

Cyanobacterin has previously been shown to inhibit photosynthetic electron transport in the cyanobacteria (4). Using photosynthetically active pea chloroplasts, we were able to show inhibition of the Hill reaction by cyanobacterin. Table III shows that cyanobacterin inhibits photosynthetic electron flow to four different acceptors. The I_{50} for cyanobacterin (concentration at which O_2 evolution is reduced to one-half the uninhibited rate) is 16 to 33 nM for all electron acceptors. Under similar conditions, the I_{50} for DCMU is 41 to 64 nM, except for silicomolybdate reduction which is relatively insensitive to DCMU inhibition (1). The cyanobacterin analog, anhydrocyanobacterin, is not an inhibitor of electron transport in any of these systems. PSI activity was monitored using reduced DCPIP (0.2 mM/reaction—reduced with 0.2–0.4 mM ascorbic acid in the presence of 2.8 μM DCMU) as electron donor. Methyl viologen was added as terminal electron acceptor and O_2 consumption was monitored at a light intensity of 700 $\mu E/m^2 \cdot s$. The rate of O_2 evolution was 40 μmol O_2 consumed/h·mg Chl *a* under these conditions and was not affected by cyanobacterin concentrations up to 155 nM.

DISCUSSION

Cyanobacterin is a unique secondary metabolite. It is the only halogenated aromatic compound known to be produced by a freshwater alga. The organism which produces cyanobacterin, *Scytonema hofmanni*, can inhibit the growth of other cyanobacteria in laboratory cultures. Thus, cyanobacterin is believed to be an allelopathic compound which enables *S. hofmanni* to compete with more prolific cyanobacterial species. Initial studies with cyanobacterin suggested that it acted as a general membrane disruptor (9). However, growth assays using highly purified cyanobacterin clearly indicated that the compound is active against both cyanobacteria and eukaryotic algae, but does not inhibit the growth of nonphotosynthetic microorganisms (3, 4). The data implied that cyanobacterin inhibits O_2-evolving photosynthesis. This hypothesis was confirmed in whole cells and spheroplasts of the unicellular cyanobacterium, *Synechococcus* sp. Cyanobacterin inhibited photosynthetic O_2 evolution in these cells and the Hill reaction in spheroplasts. The data suggested that the site of action of cyanobacterin was in PSII (4).

The results reported here on higher plants confirm and extend our data on algae. Cyanobacterin was toxic to all the plants we tested. Although our sample was relatively small, we found that the antibiotic was toxic to both monocotyledonous and dicotyledonous angiosperms. The *Lemna* species (duckweed) were the most sensitive, presumably because cyanobacterin was taken up by the roots from the aqueous medium. Terrestrial plants were affected only after spraying the compound directly on the leaves.

As summarized in Table III, cyanobacterin effectively inhibited the Hill reaction in the pea chloroplast system. The electron acceptors, ferricyanide and DCPIP, are the most convenient and reliable indicators of electron transport activity. Both compounds will accept electrons from plastocyanin in PSII. However, as summarized by Trebst (15), other researchers have found that these compounds will also serve as good electron acceptors from PSI. Our data, using ferricyanide and DCPIP, indicate a definite inhibition of photosynthetic electron transport, but the site of action remains ambiguous. *p*-Benzoquinone, however, is generally believed to accept electrons from PSII, most probably from the plastoquinone pool (6, 14). This electron acceptor has a relatively high redox potential ($E_0' = 0.293$ V) and reoxidation was not a problem in our system since reaction times were kept relatively short (10–20 min). The I_{50} for cyanobacterin in this system was approximately 30 nM which is comparable to the known PSII inhibitor, DCMU, under our assay conditions. These data indicate that cyanobacterin like DCMU acts specifically at a site in PSII. Using DCMU inhibited chloroplast preparations, PSI activity was monitored, using reduced DCPIP as electron donor. Cyanobacterin, at concentrations up to 5-fold higher than those used with other acceptors, had no effect in this system, again confirming that its site of action is in PSII. Cyanobacterin will also inhibit silicomolybdate reduction at a slightly lower concentration ($I_{50} = 16$ nM). Silicomolybdate is believed to accept electrons at the oxidizing side of the primary quinone acceptor in PSII. However, as pointed out by other researchers (1), this compound will also act at other sites depending on the condition of the chloroplasts and concentration of protecting agents such as glycerol and BSA. We attempted to duplicate the conditions these workers used in their tobacco chloroplasts with our pea chloroplast system. Although our rates of O_2 evolution were somewhat lower than theirs, we also found that the reduction of

Table III. *Effect of Cyanobacterin and DCMU on Photosynthetic Electron Transport in Pea Chloroplasts*

O_2 evolution was determined with a Clark-type O_2 electrode as described in "Materials and Methods." Inhibitors were dissolved in ethyl ether and added after the noninhibited rate was established. Rates reported are those which resulted within 5 min of inhibitor addition. Ether, added to controls, did not affect O_2 evolution.

	Electron Acceptor			
	p-Benzoquinone (0.5 mM)	$K_3Fe(CN)_6$ (2.5 mM)	DCPIP (0.24 mM)	Silicomolybdate (0.2 mM)
	μmol O_2 evolved/h·mg Chl a			
Addition				
None	85	70	44	137
Cyanobacterin				
16 nM			20	62
33 nM	26	62	0	0
58 nM	0	35		
110 nM		0		
DCMU				
41 nM	17			
64 nM	0	18	20	
140 nM		0	11	130
290 nM			0	65
Anhydrocyanobacterin				
49 μM	82	66	48	

silicomolybdic acid is relatively insensitive to DCMU up to rather high concentration (approximately 0.3 μM). Since this reaction is much more sensitive to cyanobacterin, it seems reasonable to assume that cyanobacterin acts on the primary quinone but not at the same site as DCMU. To more precisely define the site of inhibition in this reaction, phosphorylation rates should be measured. Presumably no phosphorylation takes place in the presence of silicomolybdate (2). However, if the acceptor acts as multiple sites, some phosphorylation can be measured (1). These experiments are currently in progress to try and more precisely determine the site of inhibition by cyanobacterin.

Both DCMU and atrazine have been reported to bind to a 32 kD or Q_B protein on the reducing side of PSII and prevent electron transfer from a primary quinone acceptor (17). These data indicate that cyanobacterin does not act at the same site. Other proteins in PSII have also been implicated as possible herbicide binding sites (10). Unfortunately, little is currently known about the chemistry of herbicide binding. Shipman (13) has proposed that all PSII inhibitors have, as a minimum requirement, a flat polar component and hydrophobic substituents. The hydrophobic part of the molecule enables it to penetrate the lipid layer of the membrane and fit into a herbicide binding site. The polar substituents provide a site for electrostatic interaction with a membrane protein. Cyanobacterin has some basic resemblance to other known PSII inhibitors. It is certainly a hydrophobic molecule but modeling studies do not show any of the polar substituents in a flat configuration (FK Gleason, JL Carlson, unpublished data). It is obvious that the hydrophobic substituents of the molecule alone are not sufficient for activity. The cyanobacterin analog, anhydrocyanobacterin, which has lost H_2O from positions 2 and 3 of the lactone ring, has no biological activity although it is structurally similar to the parent compound (12). It is quite likely that the hydroxyl group on C-3 is involved in binding cyanobacterin to a site in the thylakoid membrane. It is possible that this substituent provides a weak interaction such as H bonding with a membrane protein (16). One additional unique feature of cyanobacterin is the stereochemistry at carbons 2 and 3. Synthetic cyanobacterin, which is a racemic mixture (7) has half the activity of cyanobacterin in the cyanobacterial spheroplast system. Our preliminary data suggest that only the enantiomer with the R,R configuration (Fig. 1) is biologically active (4). We are currently trying to separate the synthetic isomers to confirm this observation. Unlike conventional herbicides, cyanobacterin can provide more data on the molecular parameters required for activity and can be used to investigate binding at the level of molecular interactions with thylakoid membrane sites.

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