Action of an algicide from a cyanobacterium, *Oscillatoria laelevirens*, on photosystem II

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*Oscillatoria laelevirens* produces an algicide, named oscillarin (OS), which inhibits growth of higher plants. Effect of purified oscillarin and some 'urea-triazine type' herbicides was studied on photosystem II activity and composition of pigment protein complex in spinach thylakoid membrane. For oscillarin the I₅₀ at 10 µg chlorophyll concentration, inhibitor constant (Ki), specific binding sites and Hill coefficient were calculated to be 1.45, 0.15, 2.3 and 0.2 µM respectively. Metribuzin and oscillarin affected towards the donor side and brought about identical changes in polypeptide composition of PSII complex. Further, metribuzin and atrazine exerted antagonistic and synergistic responses on oscillarin action. Some of these parameters were also studied on weed plants to assess upon the weedical potential of oscillarin.

Cyanobacteria are photosynthetic prokaryotes, and propagate in two dimensions. They form biofilms on solid surfaces, known as cyanobacterial mats, and the planktonic forms usually grow as aquatic surface scums. Competitors for available surface are related cyanobacteria rather than other organisms. It is not surprising that cyanobacteria have developed mechanisms to combat competing cyanobacteria through chemical means by secreting algicidal allelochemicals. Frequently, these secondary metabolites in interaction with photosynthetic organisms inhibit electron transport in PSII, and consequently weaken thylakoid membrane integrity by modifying the architecture of antenna and light harvesting complex. Broadly, their mechanism of action resembles to that of 'urea-triazine type' herbicides, which inhibit electron transport between QA and QB, the primary and secondary quinone acceptors on the acceptor side of PSII. Herbicides of this family and quinone Qb bind to the same region of Di protein in PSII complex and they displace each other. Although precise site of action of algicides of cyanobacterial origin is not completely understood, various results suggest that these compounds may have binding niche distinct from that of diuron and atrazine.

We have isolated, quantified and characterized an active compound, called oscillarin (OS) from a planktonic filamentous cyanobacterium, *Oscillatoria laelevirens*. It exhibits a minimum inhibitory concentration of 0.9 µM against other cyanobacteria, and an interesting herbicidal activity. The molecule has a mass of 444 Da and a novel structure not found so far in natural products. Preliminary experiments have shown that OS has action site in the PSII. Its binding domain may overlap with that of 'urea-triazine type' herbicides or may share with them a common mechanism of action. In this situation, they should show additive, cumulative or concerted pattern of inhibition. In this study, we elucidated this possibility upon interacting OS with some herbicides.

**Materials and Methods**

*Isolation of OS*—The crude OS was extracted in ether from 100 g (f. wt.) of *O. laelevirens* cells and the algicidal activity was tested according to Bagchi on a test cyanobacterium, *Synechococcus*. For further purification, the procedure of Bagchi and Ray and Bagchi was adopted. In brief, ether-extracted material was resolved on silica gel column with 65% methanol in distilled water (v/v), the fraction showing algicidal activity was refluxed with methanolic-KOH and the condensed fraction was acid-hydrolyzed and partitioned in ether. Further purification was achieved by thin-layer chromatography and extraction with RP₈ ODS-reverse phase cartridges. The eluted algicide-active fraction gave single peak upon HPLC and was considered to be pure OS, which was weighed and dissolved in methanol so as to reach a concentration of 15 mg ml⁻¹.

*Plants*—Spinach (*Spinacia oleracea*) plants were grown for 30 days in open plots (0.75-m² area) under natural light. Weed plants *Cassia tora*, *Tephrosia
purpuria, Alternanthera sessiles, Amaranthus viridis and Parthenium hysterophorous were collected from field.

Herbicide treatment—Twenty mg of herbicides and OS were dissolved in 1 ml of methanol and diluted to 150 ml with 0.1% (w/v) polyethylene 40 stearate in distilled water. Directing a stream of air cut within 7-14 d as the leaves began to bleach/wilt in amount of surfactant-methanol mixture. Plants were cut within 7-14 d as the leaves began to bleach/wilt in the treated sets.

Preparation of thylakoid membrane according to Leegood and Malkin—Freshly cut leaves (500 g) were de-ribbed and suspended in 50 ml ice-cold medium A containing 0.3 M sucrose, 2 mM Na2EDTA and 25 mM Tricine/NaOH buffer (pH 7.6). Leaves of weed plants were frozen and thawed before adding medium A, which in addition contained polyvinylpyrrolidone (PVP-40) to a final concentration of 4% (w/v) for overcoming the interference of phenolics. These were ground with a mortar and pestle and if necessary blended. The slurry was filtered through six layers of cheesecloth and filtrate was centrifuged at 800 g for 1 min followed by 3000 g for 10 min at 4°C to collect chloroplasts. Chloroplasts were ruptured by homogenizing in medium B containing 0.08 M sucrose, 10 mM NaCl, 5 mM MgCl2 and 10 mM Tricine/NaOH buffer (pH 7.8). After spinning for 5 min at 3000 g, crude thylakoid membrane was pelleted at 42,000 g for 20 min at 4°C, washed once and suspended in medium B and was used within 6 h of preparation.

Hill reaction—This was measured as light-dependent reduction of dichlorophenol indophenol (DCPIP) with water or diphenylcarbazide (DPC) as electron donor, and as O₂ evolution using a Clark-type oxygen electrode (Hansatech, U.K.) in the presence of 0.25 mM DCPIP or 0.5 mM parabenzoquinone (PBQ) as electron acceptor as described earlier. The suspension of thylakoid membrane was diluted to desired chlorophyll a density and herbicides/OS or methanol was added in dark 2-3 min before assay was initiated.

Pigment-protein complex (PPC)—Procedure of Martinson and Plumley was adopted for resolution of PPC's. Pelleted thylakoid equivalent to 60-80 μg chlorophyll a was suspended in 100 μl of medium C containing 100 mM dithiothreitol and 100 mM Tris/HCl buffer (pH 8.5). n-Dodecyl, β-D-maltoside to a final concentration of 2% (w/v) was added and mixture was incubated for 15 min in ice-bath, after which time SDS (0.17%) was added and preparation was centrifuged for 30 sec at 12,000 g. The green supernatant was resolved by nondenaturing polyacrylamide gel electrophoresis (9% acrylamide, 10% glycerol) at 4°C for 18 hr. The upper reservoir buffer contained 0.1% (w/v) of SDS.

The PPC bands from ten identical slots were excised with a razor blade and equilibrated at 60°C for 1 hr in 3 ml of 125 mM Tris/HCl buffer (pH 6.8) containing 1 mM dithiothreitol and 2% SDS. The mixture was freeze-dried, resuspended in 10 μl of Laemmli's extraction buffer and subjected to SDS-polyacrylamide gel electrophoresis with pre-casted 8-15% gradient gel using a PhastSystem apparatus (Pharmacia, Sweden). Gels were stained with silver nitrate.

Chemicals—All chemicals were purchased at their purest grades from Sigma Chemical Co., U.S.A. and Qualigens and E. Merck, India.

Results and Discussion

Incubation of spinach thylakoid membrane with purified OS or herbicides impaired photosynthetic reactions. As shown in Table I, with H₂O-DCPIP and H₂O-PBQ, all these compounds rapidly inhibited electron flow and concomitant O₂ evolution. Whilst, diuron and atrazine were also able to block electron flow between DPC and DCPIP, bentazon exerted a partial inhibition, whereas metribuzin and OS were ineffective even at concentration up to 5 μM (data not shown). DCPIP is believed to be an electron donor to PS II at a site prior to P₆₈₀. Apparently the action site(s) of metribuzin and OS lies on the donor side of PS II. Several factors, viz. UV-B irradiation and incubation with low concentrations of herbicides, have resulted in restoration of DCPIP reduction in the presence of DPC, as they mediated the inhibition at the donor side. Our results however differ from those of Carpentier et al [7], with regard to diuron and atrazine effects at low concentrations, as we found persistence of inhibition of DPC-dependent DCPIP reduction at similar concentrations of herbicides. The reason for this inconsistency is that, in unresolved thylakoid membrane similar to the one used in present work the inhibitory sites are weakly accessible to these herbicides.

In an attempt to elucidate the interaction between action of OS and herbicides, spinach thylakoid membrane was simultaneously incubated with the
contain a lipophilic alkane chain and a polar phenol. The combined in a bove fashion, neither of them could affect the re s idue thre$h_{4}$xybenzamide derivative s, which were shown structurally, (data not shown). Apparently, the activity domain of extent of total inhibition exceeded the sum of independent inhibitions. The $I_{50}$ for OS was reduced to 0.6 µM. Therefore, the influence of metribuzin and atrazine on OS action was respectively antagonistic and synergistic. When atrazine and metribuzin were combined in above fashion, neither of them could affect the respective $I_{50}$ value of the other herbicide (data not shown). Apparently, the activity domain of OS overlapped with that of metribuzin and atrazine, although it interacted with them in opposite manner.

The PSII inhibitory activity depends on overall lipophilicity of the molecule. Oscillatorin, which contains a lipophilic alkane chain and a polar phenol residue, seems to function similar to trihydroxybenzamide derivatives, which were shown to affect both on acceptor and donor sides of PSII. Structurally, these inhibitors exhibit dual polarity.

The binding characteristic of OS to thylakoid was studied as described by Tischer and Strotmann. The calculated $I_{50}$ values for OS, whether alone or in presence of partial inhibitory concentrations of atrazine and metribuzin, were plotted against chlorophyll concentration of membrane (Fig. 2). With every chlorophyll concentration, the partial inhibitory concentration of herbicides as above was calibrated. The inhibitor constants (Ki) for OS, OS + metribuzin

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**Table 1—Effect of OS and herbicides on photosynthetic reactions in spinach thylakoid membrane**

<table>
<thead>
<tr>
<th>Photochemical activity</th>
<th>None (equivalent methanol added)</th>
<th>Addition</th>
<th>Atrazine (184 nM)</th>
<th>Bentazon (138 nM)</th>
<th>Diuron (46 nM)</th>
<th>Metribuzin (46 nM)</th>
<th>Oscillatorin (1.5 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O-DCPIP</td>
<td>836</td>
<td>20</td>
<td>160</td>
<td>210</td>
<td>475 (150)</td>
<td>410 (120)</td>
<td></td>
</tr>
<tr>
<td>DPC-DCPIP</td>
<td>545</td>
<td>nd</td>
<td>193</td>
<td>nd</td>
<td>572 (550)</td>
<td>523 (520)</td>
<td></td>
</tr>
<tr>
<td><strong>O₂ evolution (µmol O₂ evolved/hr/mg chlorophyll a)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with DCPIP</td>
<td>200</td>
<td>nd</td>
<td>nd</td>
<td>140</td>
<td>90 (50)</td>
<td>120 (50)</td>
<td></td>
</tr>
<tr>
<td>with PBQ</td>
<td>460</td>
<td>210</td>
<td>230</td>
<td>240</td>
<td>260 (nd)</td>
<td>210 (nd)</td>
<td></td>
</tr>
</tbody>
</table>

Inhibitors were added 2-3 min prior to assay.
Values in parentheses show activity at 2-fold high concentration of inhibitors.
nd—not detectable.
and OS + atrazine were extrapolated from respective \( I_{50} \) values at zero chlorophyll concentration. These were 0.15, 2 and -0.35 \( \mu M \) respectively. This suggests that metribuzin substantially lowered efficiency of OS as PSII inhibitor while atrazine enhanced this.

The concentration of specific binding sites was calculated by using an established formula
\[
I_{50} = K_i + \frac{1}{2xt},
\]
where \( xt \) is concentration of specific binding sites for unit chlorophyll concentration. Regardless of presence or absence of metribuzin or atrazine, specific binding sites for OS per chlorophyll molecule was calculated to be 2.3 ± 0.2, which shows that OS-binding niche is unaffected by more strongly binding herbicides. The presence of such a low number of binding sites suggest a weak affinity of OS as compared to metribuzin, which has thirty times more binding sites.

A Hill co-efficient for OS was calculated from the data of Fig. 1 according to Borse et al. The slope of a plot between logarithm [inhibition/(1 - inhibition)] and logarithm concentration of OS is Hill co-efficient, which provides information about the acceptor molecule binding to the inhibitor. For OS, it was 0.2 (plot not shown), suggesting that the acceptor molecule in the thylakoid membrane has estimated one binding site per five molecule of OS. However, it may be difficult to distinguish the specific and non-specific binding sites.

The effect of a prolong exposure of plants to OS and herbicides on composition of spinach thylakoid-pigment protein composition was studied. The result of non-denaturing electrophoretic analysis of dodecyl maltoside-solubilized membrane is shown in Fig. 3. Overall, six PPC bands were resolved in every sample. In control plants, both solubilized membrane and the extracted material from the indicated band (Fig. 3) exhibited partial PSII reaction as DPC-dependent DCPIP reduction (data not presented). Although a band in the corresponding position was visible even in the treated plants, it was without such activity. This suggests that, even though several

![Fig. 3—Nondenaturing mild SDS-PAGE resolution of thylakoid pigment protein complex in control plants (C) and in plants subjected to atrazine (A), bentazon (B), diuron (D), metribuzin (M) and OS (N) treatments. Arrow indicates the 'PPC' band showing PSII activity (DPC-DCPIP).](image1)

![Fig. 2—Changes in \( I_{50} \) values of OS for PSII activity (\( \text{H}_2\text{O} \)-DCPIP) at different chlorophyll \( \alpha \) concentration without (-O), and with addition of atrazine (-\( \Delta \)) and metribuzin (-\( \Delta \)). The Ki is an intercept calculated statistically.](image2)

![Fig. 4—Polypeptide composition of excised material from 'PPC' bands (second from the bottom, Fig. 3) in control plants (C) and in plants subjected to atrazine (A), bentazon (B), diuron (D), metribuzin (M) and OS (N) treatments.](image3)
herbicides may affect on the donor side (refer Table 1), the long-term manifestation of herbicide action invariably is inactivation of PSII towards the acceptor side.

The excised material from this band from all the lanes could be resolved in minimum four distinct polypeptide bands (Fig. 4). The molecular weights correspond to CP47 (47 kD), CP43 (43kD), CP 26 (±26 kD) and cytochrome b$_{59}$ (10.5 kD). In addition, a 17-kD band and several bands in high molecular weight range were discernable in mitribuzin- and OS-treated plants. We propose that PSII complex-intrinsic polypeptides are unaffected by given treatments. It has been shown that UV-B inhibitory action on PSII activity does not affect the polypeptide composition of PSII core complex. However, as shown with dimethylsulfoxide treatments, numerous stress-induced extrinsic polypeptides may be synthesized in mitribuzin- and OS-treated plants.

Efficiency of PSII inhibitors was assessed upon on some weed plants. Table 2 lists the Ki values for herbicides, which shows a marginal range of values within the plants tested (18-31 nM). Such index for OS was much higher and was more broadly ranged. Therefore, a direct application of OS as a weedicidal agent can not be recommended, although such natural algicides may provide novel structures enabling synthesis of more effective phytotoxic compounds.

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References


