Screening Method for Cellulose Biosynthesis Inhibitors with Herbicidal Activity

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A new screening method for inhibitors of cellulose biosynthesis is described. This method utilized three microbial strains; a cellulose-containing fungus Phytophthora, and a cellulose non-containing fungus Candida, and a bacterial strain of Acetobacter, a cellulose-producing acetic acid bacterium. The primary screen examined microbial cultures for selective growth inhibition against Phytophthora with no inhibition against Candida. The secondary screen tested for herbicidal activity. Thirdly, the active cultures were examined for their inhibition of cellulose biosynthesis by an Acetobacter strain. A screening trial with this new method led to the discovery of two microbial metabolites named phthoxazolin A and phthoramycin as new inhibitors of cellulose biosynthesis with herbicidal activity.

Herbicides are useful in agriculture for crop production. Today they comprise the largest portion of agricultural pesticides. They are required to possess potent activity and safety to animals and humans as well as to the ecosystems.1) Microbial metabolites are biodegraded in nature, giving less stress to the ecosystems, and are more likely to meet the above requirements than synthetic pesticides. Although many microbial metabolites show herbicidal activity, few of them, e.g. bialaphos, have found practical usefulness as herbicides, largely because of insufficient cost-effectiveness and of instability under agricultural weathering conditions.

Cellulose is contained in the cell envelopes of all the plant and alga systems, but is not at all in any animal systems. Therefore cellulose biosynthesis provides a promising target site for safe and non-selective herbicides. No reports have described trials of finding cellulose biosynthesis inhibitors from microbial metabolites, although coumarin2) and synthetic chemicals 2,6-dichlorobenzonitrile2) and isoxaben3) have been suggested to have this type of activity in vivo.

The present paper describes an attempt at the construction of a screening method for inhibitors of cellulose biosynthesis of microbial origin. The discovery of two new compounds phthoxazolin A4~6) and phthoramycin7) (Fig. 1) by the use of this screening method will also be presented.

![Fig. 1. Structure of phthoxazolin A and phthoramycin.](image-url)
Materials and Methods

Microorganisms

Microorganisms used in the present work were: Phytophthora parasitica subsp. nicotianae IFO-4873, P. palmivora IFO-30285, P. capsici KF-278, P. cactorum KF-279, Candida albicans KF-1, and Acetobacter aceti subsp. xilinum IFO-3288. Other fungi described in Table 1 are laboratory strains of The Kitasato Institute.

Growth of Microorganisms

P. parasitica subsp. nicotianae IFO-4873 and other Phytophthora strains were grown at 27°C for two days with shaking (110 rpm) in a liquid V8 medium9 (10 ml) which contained V8 vegetable juice (Suntory Co., Japan) 14% (v/v), glucose 1%, and agar 0.1% (pH 6.0). The vegetative cultures were poured into a teflon homogenizer. Mycelial pellets were homogenized softly, and a portion of the mycelial suspension was used to seed at 1% (v/v) to prepare V8 agar plates. Leucomycin (50 μg/ml) was supplemented to V8 agar. The plates were incubated at 27°C for two days under lightening with a fluorescent lamp.

C. albicans KF-1 was grown in GY medium (glucose 1%, yeast extract 0.5%, pH 6.0) at 27°C for two days. This culture was used to seed into GY agar medium. Other fungi were grown in conventional manners.

Acetobacter aceti subsp. xilinum IFO-3288 was grown at 27°C on a Bouillon agar slant, and stored in the cold. This culture was used to inoculate into a 500-ml vol. conical flask containing 100 ml of a growth medium (Bouillon supplemented with sterile ethanol 2% and KH₂PO₄ 0.3%), incubated statically at 27°C for 4 days. A portion (10 ml) of this vegetative seed culture was transferred to another conical flasks containing 100 ml of the same growth medium, and incubated statically at 27°C for 3 to 4 days. This culture was used for resting cell experiments to measure cellulose biosynthesis.

Determination of Cellulose Biosynthesis in Resting Cells of A. aceti subsp. xilinum IFO-3288

Cellulose biosynthesis was determined by measuring the incorporation of [14C]glucose into the cellulose fraction of resting cells of strain IFO-3288. The method described by Swissa et al.9 was used with modifications as follows.

A. aceti subsp. xilinum IFO-3288 was grown at 27°C statically in a phosphate-buffered ethanol-Bouillon medium. Under these conditions, IFO-3288 grew to 3~5 cm in height. The reaction mixture contained in a total volume of 0.1 ml, and [14C] glucose (10 μCi/ml) 0.06 ml. The reaction was carried out at 27°C for an hour with shaking, stopped by adding 1 ml of 1 N NaOH solution, then heated for 20 minutes in a boiling water bath. Labeled cellulose that was synthesized was collected, together with disrupted cellular particles, on a glass filter (GF/C, pore size of 1.2 μm, Whatman). After washing with water, the filter was used to count for radioactivity.

Herbicidal Activity

Herbicidal effect was estimated by two methods. In one method the inhibitory effect on germination and proliferation was measured as follows. Cultured broths or solutions of known compounds (each at 0.5 ml) were put dropwise onto pieces (1.0 cm x 1.0 cm) of defatted cotton. The cotton pieces were allowed to stand overnight at room temperature. The dried cotton pieces were then put into the bottom of test tubes (2 cm x 10 cm). Tap water (0.5 ml) was dripped onto the cotton pieces. Each five seeds of radish (Raphanus sativus L.) and sorghum (Sorghum bicolor) were layed on the wet cotton. The test tubes, the top of which was covered with a steel cap, were incubated for 4~5 days at 27°C under fluorescent lamps. Under these conditions both radish and sorghum in control tubes grew to 3~5 cm in height. Herbicidal activity was determined by comparison of plant heights with those of no drug control. Cycloheximide (10 μg/ml) was used as a positive control drug.

The second method estimated post-emergence effect. Active cultures were applied onto leaves of 4 day-grown plants by using a small brush. After a further several days of incubation, plant heights or damages on leaves were scored against no treatment control.

Results and Discussion

Growth of Phytophthora spp. and Anti-Phytophthora Activity of Known Antibiotics

Phytophthora spp. are plant-pathogenic fungi whose control is one of the most challenging problems in today’s agriculture. This group of fungi is unique in that the cell walls contain cellulose as one of the essential polysaccharide components.10 We reasoned that, by taking advantage of this physiology, it would be possible to detect inhibitors of cellulose biosynthesis by conventional growth inhibition assays with a Phytophthora sp. as an indicator strain.

Because no report has described the use of Phytophthora spp. in antibiotic screening, we started with
testing antibiotic sensitivity and the growth of Phytophthora spp. under laboratory conditions. \textit{P. parasitica} subsp. \textit{nicotianae} IFO-4873 and three other \textit{Phytophthora} strains, \textit{P. palmivora} IFO-30285 and \textit{P. capsici} KF-278, and \textit{P. cactorum} KF-279 were compared for their growth on V8 agar at 27°C. \textit{P. parasitica} subsp. \textit{nicotianae} IFO-4873 grew best and most steadily under the conditions employed. It grew better under lightening with fluorescent lamps than in the dark. V8 agar was a good growth medium for \textit{P. parasitica} IFO-4873, although other complex media containing other types of vegetable juice than V8 juice, or a chemically defined medium composed of glucose, ammonium sulfate and mineral salts also supported growth of this fungus to lesser extents. Its growth increased gradually on successive transfers on the latter media.

A supplement of leucomycin (50~100 μg/ml) or other antibacterial agents to the V8 agar was effective to suppress occasional bacterial contamination, particularly in a screening run.

Anti-\textit{Phytophthora} activity of known antibiotics shown in Table 1 was determined with \textit{P. parasitica} subsp. \textit{nicotianae} IFO-4873 grown on V8 agar. Table 1 also reports antifungal activities of the same antibiotics against \textit{Candida}, \textit{Saccharomyces}, \textit{Mucor}, and \textit{Pyricularia}. \textit{P. parasitica} subsp. \textit{nicotianae} IFO-4873 was sensitive to many antifungal agents. These include cycloheximide, antimycin, nanaomycin, rapamycin, but was insensitive to polyenes. This insensitiveness is because \textit{Phytophthora} and other oomycetes group of fungi lack squalene epoxidase, and do not synthesize de novo cholesterol and ergosterol, which are the target molecules of polyeone antifungals.

**Herbicidal Activity of Known Antibiotics against Radish**

Table 1 shows the herbicidal activity of known antibiotics, mostly antifungals, as estimated by germination and proliferation method with radish (\textit{Raphanus sativus}) as test plant. Among 30 antibiotics tested, several compounds showed herbicidal activity. The herbicidal activity did not correlate with antifungal potency.

<table>
<thead>
<tr>
<th>Structural group</th>
<th>Antibiotic</th>
<th>Conc. (µg/ml)</th>
<th>Antimicrobial activity (mm)</th>
<th>Herbicidal activity against radish*</th>
<th>Inhibition (%) of cellulose biosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quinone</td>
<td>Antimycin</td>
<td>100</td>
<td>49</td>
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<td>13</td>
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<td></td>
<td></td>
<td>10</td>
<td>41</td>
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<td>10</td>
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<td></td>
<td>Nanaomycin A</td>
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<td>40</td>
<td>17</td>
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<td></td>
<td>10</td>
<td>20</td>
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<td></td>
<td>Oligomycin</td>
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<td>30</td>
<td>14</td>
<td>43</td>
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<td>Disulphide</td>
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<td>Coumarin</td>
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<td>19</td>
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</table>

* The following compounds (each at 1 mg/ml) were inactive against \textit{Phytophthora parasitica} IFO-4873 on a paper disc assay: (Cell wall-actives) polyoxins, exomycin, globopeptin, aculeacin A; (polyenes and polyols) amorphinicin B, nystatin, eurocidin, fungichrome, copiamycin, azalomycin F; (polyethers) valinomycin, momenpin A, danamycins, (miscellaneous) cerulenin, pyrrolnitrin, stecamin, griseofulvin, irumamycin, and chatreusin.


* – 0~30% inhibition of plant growth; +, 30~80% inhibition; + +, 80~100% inhibition.
Inhibition of Cellulose Biosynthesis by Known Antibiotics

Inhibition of cellulose biosynthesis by known antibiotics was determined by the method described in Materials and Methods, using the resting cells of A. aceti subsp. xylinum IFO-3288.

The incorporation of [14C] glucose into the cellulose fraction of IFO-3288 proceeded linearly to the reaction period of time (Fig. 2-A) at its optimal pH of 6 (Fig. 2-B). The effect of known antibiotics on cellulose biosynthesis is shown in Table 1. None of the antibiotics tested, except for antimycin A, inhibited cellulose biosynthesis by more than 50% at 100 μg/ml. Whereas respiratory inhibitors such as antimycin A inhibited it, possibly because ATP generation was inhibited by these antibiotics in Acetobacter cells.

Noteworthy is that coumarin, which is postulated as a cellulose biosynthesis inhibitor in plant systems, showed moderate inhibition in all the three assays; growth inhibition against Phytophthora and Candida, herbicidal activity, and cellulose biosynthesis inhibition. Unfortunately, 2,6-dichlorobenzonitrile and isoxaben, proposed cellulose biosynthesis inhibitors, were not active in these assays. All the other compounds tested that are not proved to be cellulose biosynthesis inhibitors were inactive, or active either in one or two of the three assays. It is suggested that microbial cultures showing positive responses in all the three assays can be assumed to contain cellulose biosynthesis inhibitors.

Screening of Microbial Cultures in Search for Inhibitors of Cellulose Biosynthesis

A screening trial was carried out with the three screens established as above in which active microbial cultures were selected in the order and with criteria as follows:

1. Positive anti-Phytophthora activity but negative (or weak) anti-Candida activity with a difference of 10 mm or more in diameter of inhibition zones.
2. Herbicidal activity of more than 80% as determined by the reduction of plant heights in either one or both of radish and sorghum.
3. More than 10% of inhibition of cellulose biosynthesis by 10-fold concentrated culture extracts (final concentration in resting cell reaction mixture was the same as the cultured broth).

About 30,000 cultures of actinomycetes and fungi were subjected to this screening system. This trial led to the discovery of two new compounds, phthoxazolin A and phthoramycin (Fig. 1). Phthoxazolin A was produced by Streptomyces sp. OM-57144~6) and three other unidentified actinomycete strains, OM-5739, K93-615 and K93-5455. Phthoxazolin A was active in vitro against Phytophthora spp., but was inactive against other fungi and bacteria tested, none of which contain cellulose in the cells. Phthoramycin was produced by Streptomyces sp. WK-1875.7) It showed in vitro antifungal activity against Phytophthora, as well as Mucor and Pyricularia.

The two compounds inhibited cellulose biosynthesis in a resting cell system of A. aceti subsp. xylinum IFO-3288 (Table 2). They were potently herbicidal. These results demonstrate that the screening system described in this paper is useful in search for new cellulose biosynthesis inhibitors of both microbial and synthetic origins. This screening method is characterized by the use of microorganisms for herbicidal screening. This is the first example of the use of Phytophthora in antibiotic screening on a mass scale. Other fungi and bacteria containing or producing cellulose would also be utilized.

During the course of the above screening run, several known compounds were found to exhibit anti-Phytophthora activity with or without herbicidal activity. These include toyocamycin, sangivamycin, nocardamide, saignemycin, SS-8201D, and BQ-180B as metabo-
lites of actinomycetes, and penicillic acid, fusaric acid, dehydrofusaric acid, patulin, gliotoxin, gliovirin and N-methyl-gliovirin as fungal metabolites. The anti-
Phytophthora activity of these compounds were detected in the present study for the first time. Their herbicidal activity has been described by other research groups, except for nocardamine, SS-8201D and BQ-180B, which were not herbicidal.

Among these, saigenmycin, a lactone bonded to phthoxazolin moiety, inhibited cellulose biosynthesis in the Acetobacter system employed here, probably due to the phthoxazolin moiety (data not shown).

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