Isolation of Fatty Acid Metabolites in Oat Leaves Responding with Resistance to *Puccinia coronata avenae*

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**Key words:** *Puccinia coronata*, oat, resistance, fatty acid metabolites, antifungal activity.

It has been already found that avenalumins (Avls) are accumulated in oat leaves responding to infection with incompatible races of the crown rust fungus*4,5,13). These substances are benzoxazin and are considered to be phytoalexin to contribute to the expression of the cultivar-race specific resistance. A possible biosynthetic route for Avls, based on their chemical structures, is the phenylpropanoide pathway.

Recently, however, we have shown that lipoxygenase (LOX) activity increases in leaves of six oat cultivars with an inoculation of incompatible race of *Puccinia coronata*12,15). This suggests that oat plants metabolize fatty acids to produce defensive substances against the rust infection as in the case of the rice-blast resistance, in which the production of antifungal unsaturated fatty acids is indicated in association with increased LOX activity2,3,8,11).

In this study, therefore, we attempted to isolate possible new antifungal substances in the incompatible oat-crown rust system.

Oat cultivars Shokan-1, Pc 47, 48, 50, 51 and 61 (*Avena sativa* L.) and races 226 and 203 of *Puccinia coronata* f. sp. *avenae* Fraser et Led. were used. Shokan-1, Pc 48, 50, 51 and 61 are highly resistant to race 226, responding with a vigorous hypersensitive reaction. Shokan-1 and Pc 47 are susceptible to races 203 and 226, respectively14,15).

Oat leaves (fresh weight 100 g) were harvested 48 hr after inoculation and stored at -80°C until use. Samples were dipped in 1,000 ml of Et2O, kept at 5°C for 72 hr, and homogenized with a Physcotron. The homogenate was filtered with Miracloth and the filtrate was shaken twice with 2% aqueous NaHCO3 and then with H2O, repeatedly. The Et2O phase was added to 0.1 N HCl and then shaken 3 times with H2O. The Et2O was evaporated to dryness in vacuo and the residue was dissolved in H2O. The solution was partitioned with n-hexane and the aqueous phase was shaken with Et2O. The Et2O phase was evaporated to dryness in vacuo and the residue was dissolved in MeOH (neutral fraction: NEF).

On the other hand, the aqueous phase containing NaHCO3 was adjusted to pH 2-3 with HCl and shaken twice with Et2O. The Et2O phase washed twice with H2O was evaporated to dryness in vacuo and the residue was dissolved in MeOH (acidic fraction: AEF).

The neutral and acidic fractions were subjected to isolation of antifungal substances by preparative HPLC using an Asahipak ODP-50 column for NEF and a Finepak SIL C18 T-5 column for AEF, respectively. The former column was first eluted with 30% CH3CN containing 1% HCOOH, and then the linear gradient was established with 30-100% CH3CN containing 1% HCOOH at 1 ml/min for 20 min, and finally washed with CH3CN. The latter column was eluted with 60% MeOH containing 0.1% AcOH at 1 ml/min.

Each fraction taken was dried in vacuo to remove HCOOH or AcOH prior to bioassay, and made to original volume with MeOH. Inhibitory activity was bioassayed by two methods and active fractions were collected.

Each fraction (25 µl) was added onto paper disks (Whatmann 3 MM; 0.5 cm) and vacuumed overnight to remove organic solvents. The paper disks, on which uredospores were uniformly dispersed,
Fig. 1. HPLC elution profiles of Et₂O extracts from Shokan-1 oat leaves 48 hr after inoculation with incompatible race 226. (1) Neutral Et₂O fraction from a column of an Asahipak ODP-50, flow rate 1 ml/min, OD 230 nm. (2) Acidic Et₂O fraction from a column of a Finepak SIL C₁₈-T-5, flow rate 1 ml/min, OD 210 nm. Blacked peaks A, B and C contained the antifungal substances A, B and C, respectively.

were put onto 0.8% water agar and then incubated at 20°C for 6 hr (paper disk method). Each fraction (25 µl) was spotted onto silica gel TLC plates (Merck GF₂₅₄, 60; 0.25 mm thickness). The plates were developed with a solvent system of CHCl₃: MeOH: H₂O (65:25:4, v/v) for 1.5 hr, and dried in vacuo to remove the solvents, and then well moistened by spraying with H₂O. Uredospores were dispersed heavily on plates and brushed to make a uniformly dense layer. The plates were again sprayed with a mist of H₂O and incubated in a moist chamber at 20°C for 16 hr (TLC method). Germination and germ tubes were observed under a light microscope.

ED₅₀ values of substances A, B and C were estimated by dissolving dried samples to appropriate concentrations with MeOH and examined inhibitory activity by the paper disk method.

NEF was separated by HPLC into three major peaks and four minor peaks, as measured at 230 nm (Fig. 1-1). The peak at $R_t=13.6$ min showed antifungal activity (substance A) and revealed a single spot on the TLC plate at $R_f=0.50$ under ultraviolet light after spraying with a solution of Rhodamine 6G (0.005%) in 95% EtOH, or by the naked eye after spraying with H₂O. This spot coincided with the antifungal spot shown by the TLC plate bioassay. Substance A was a yellowish oil and the UV spectrum in MeOH solution revealed a maximum absorption at 205 nm with three shoulders at 230, 285 and 325 nm. IR spectrum of A exhibited broad peaks at 1,400 and 1,010 cm⁻¹ and sharp peaks at 2,820 cm⁻¹.

AEF subjected to Finepak SIL C₁₈-T-5 exhibited three major peaks and six minor peaks, as measured at 210 nm (Fig. 1-2). Antifungal activity was found at $R_t=10$ (substance B) and 15 (substance C) min, respectively. Both B and C formed yellowish needle crystals in MeOH. The UV spectrum of substance B showed a maximum at 320 nm with shoulders at 220 and 340 nm, and that of C showed a maximum at 345 nm with a shoulder at 210 nm. The IR spectrum of B exhibited broad peaks at 2,520, 2,230, 2,050, 1,670 and 1,020 cm⁻¹ and a sharp peak at 2,800 cm⁻¹, while the spectrum of C showed broad peaks at 2,520, 2,050 and 1,030 cm⁻¹ and a sharp peak at 2,850 cm⁻¹. The spots of B ($R_f=0.31$) and C ($R_f=0.37$) developed on TLC plates were positive (yellow) to the spraying of anisaldehyde reagent and vanillin reagent. Each spot coincided with a strong inhibition area shown in the TLC plate bioassay. These qualitative results indicate that A, B and C are antifungal metabolites of fatty acids. These three substances seemed to be a distinct single peak at the retention time on GLC (unpublished data), respectively.

Each blacked peak area in Fig. 1 was collected in order to ensure purity. The yields of substances A, B and C were 0.45 mg, 5 mg and 2 mg from 100 g of Shokan-1 leaves 48 hr after inoculation with incompatible race 226, respectively. These substances could hardly isolate from Shokan-1 leaves which
Table 1. Content of antifungal substances A, B and C produced in primary leaves of oat cultivars inoculated with races 226 or 203 of *Puccinia coronata avenae*

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Race</th>
<th>Host response</th>
<th>Content* (µg/g fresh wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td><em>Pc</em> 47</td>
<td>226</td>
<td>Susceptible</td>
<td>0.4</td>
</tr>
<tr>
<td><em>Pc</em> 48</td>
<td>226</td>
<td>Resistant</td>
<td>1.4</td>
</tr>
<tr>
<td><em>Pc</em> 50</td>
<td>226</td>
<td>Resistant</td>
<td>2.4</td>
</tr>
<tr>
<td><em>Pc</em> 51</td>
<td>226</td>
<td>Resistant</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Pc</em> 61</td>
<td>226</td>
<td>Resistant</td>
<td>4.5</td>
</tr>
<tr>
<td>Shokan-1</td>
<td>226</td>
<td>Resistant</td>
<td>4.8</td>
</tr>
<tr>
<td>Shokan-1</td>
<td>203</td>
<td>Susceptible</td>
<td>0.1</td>
</tr>
</tbody>
</table>

a) Samples were extracted from oat leaves 48 hr after inoculation. The amount of antifungal substances was estimated by analytical HPLC. Peak areas were measured by a computing integrator.

were either non-inoculated or inoculated with compatible race 203. Subsequent experiments with oats compatible (*Pc* 47) and incompatible (*Pc* 48, 50, 51 and 61) with race 226 showed that substances A, B and C markedly accumulated only in the incompatible combinations (Table 1). The germination assay with A, B and C showed more than 50% inhibition at concentrations of about 7 µg/disk, 17 µg/disk, and 7 µg/disk, respectively (Fig. 2). Thus, the level of antifungal activity of these substances was about equal or rather higher than that of Avls and oxygenated fatty acids in rice plant.*

The results obtained in this study strongly suggest that the metabolites of fatty acid, A, B and C are newly found antifungal substances in oat leaves and may contribute to the specific resistance expression in the interactions between oat cultivars and rust races.

Recently, Namai et al.* suggested that hydroperoxides of unsaturated fatty acids may cause hypersensitive cell death of blast-infected rice leaves. Substances A, B and C isolated in the present study were not cytotoxic to oat protoplasts which had been prepared by the previous method.

The structure of substances A, B and C will be the subject of a following study. Further the localization and the function as antifungal agents should be investigated in future studies.

We are grateful to Dr. F. Tsao of Hillsdale College, Michigan, and Dr. H. Tamura of Kagawa University for proofreading of the manuscript.

**Literature cited**


和 文 摘 要

田中明美・山本弘幸・谷 利一：冠さび菌感染の抵抗性エンパク素からの脂質酸化代謝物の単離

冠さび菌不親和性レースに感染した品種豚冠1号のエンパク素から3種の抗菌性物質(A, B および C)を単離した。一方、健全葉および親和性レース感染葉からは3物質はほとんど検出されなかった。物質Aはエーテル中性画分から、物質BおよびCは同酸性画分からそれぞれHPLCを用いて精製した。UVおよびIR吸収特性ならびにTLCによる定性反応から、これら3種は脂質酸化代謝物と考えられた。3物質の発芽および発芽管伸長に対するED50は7〜17μg/g玉片(20mm2)の範囲にあった。新規に単離した3種の脂質は抵抗性を示す他の5品種も検出されることとより、これらの脂質もアパレルミンとともに、エンパク素冠さび病の抵抗反応発現に関与していると思われる。

(Received December 14, 1992)