Chemical Components of the Lipid of *Helminthosporium oryzae*

Mitsuya Tsuda**, Akinori Ueyama**, Masuo Nakano*** and Yasuhiro Fujino***

Abstract

This paper discusses the lipid of *Helminthosporium oryzae* and its fatty acid components. The total lipid was fractionated on a silicic acid column into nonpolar lipid (NL) and polar lipid (PL), by eluting first with chloroform and then methanol. NL accounted for over 80% of the total lipid. The principal component of NL was triglyceride. PL was principally composed of ceramidemonohexoside (CMH), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and unidentified lipids. The main fatty acid components of *H. oryzae* were C16:0, C18:0, C18:1 and C18:2.

The existence of CMH in the PL fraction has not been reported previously in plant pathogenic fungi.

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Introduction

In the previous papers5,6,7), morphological abnormalities of the mycelium of *Fusarium roseum* and *Helminthosporium oryzae* were reported when the organisms were cultured on Czapek-d-amino acid medium; in the abnormal mycelium, many granules were present which stained with Sudan III and Sudan Black B. Electron microscopy also confirmed an increase of lipid particles in the abnormal mycelium.

From the above-mentioned observations, the authors assumed that abnormal lipid metabolism occurred in the mycelium which was produced by growth on Czapek-d-amino acid medium. Lipid forms one of the principal components of living matter. In the field of plant pathogenic fungi, there are several reports on fatty acid substances and sterols, incorporation of labeled compounds into lipid fraction, and observations of lipid particles by electron microscopy. For *H. oryzae*, Kuroda and Tokuhiro8) reported an increase of fatty acids and ergosterol-like substance in the sterol fraction during spore germination. However, systematic studies using plant phthogenic fungi are scanty.

In the present paper, fundamental studies are described on the lipid constituents of *H. oryzae* which was cultured on Czapek (NaNO₃-sucrose) medium. These studies were under-

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* Studies on the lipid metabolism of plant pathogenic fungi (1).
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Abbreviations; CMH: ceramidemonohexoside, NL: nonpolar lipid, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PL: polar lipid, TL: total lipid.
taken in order to clarify the nature of the lipid and its fatty acid components, prior to carrying out experiments with the abnormal mycelium of *H. oryzae* derived by culture on Czapek-d-amino acid medium.

### Material and methods

**a) Fungus**  *Helminthosporium oryzae* (Perfect stage: *Cochliobolus miyabeanus*) No. 13 (Kyoto Univ.) and No. 13-W (a white mutant of No. 13).

**b) Preparation of mycelium**  One hundred ml portions of Czapek-0.1% yeast extract medium in 500 ml Sakaguchi's shaking flasks were autoclaved at 120°C, inoculated with the fungus and then slowly shaken at 28°C. After ten days, the mycelium was washed thoroughly with deionized water. After filtering with a Nutsche funnel, the mycelium was stored at -20°C in a freezer until further experiments were performed.

**c) Extraction of lipid**  A modification of Folch procedure was employed. As shown in Fig. 1, the mycelium was allowed to stand in about ten times its volume of chloroform-methanol (2:1, v/v) at room temperature. After standing overnight, the mycelium was crushed with a Potter homogenizer using five times the volume of the solvent. After filtration, the residue was again allowed to stand in about ten times the volume of the same solvent, overnight. The chloroform-methanol extracts obtained from these three fractions were combined, and the crude lipid was obtained by evaporation. The crude lipid was dissolved in chloroform-methanol (2:1) mixture. After washing the solution by the Folch procedure, the crude lipid was dissolved in chloroform-methanol (2:1) mixture. After washing the solution by the Folch procedure,

![Fig. 1. Extraction method for lipid from *H. oryzae*.](image1.png)

### Crude lipid

- dissolved in chloroform-methanol (2:1)
- Folch wash
- Chloroform layer
  - evaporated in vacuo
- TL
  - column chromatography (SiO₂, 10 g/g TL)
- Chloroform eluate
- Methanol eluate (NL)

### Fig. 2. Fractionation method for *H. oryzae* lipid.

![Sample lipid 10mg](image2.png)

- dry benzene 1 ml
- 5%HCl-MeOH 8 ml
- refluxed 2 hr
- distilled water 3 ml
- petroleum ether 20 ml × 3

### Petroleum ether layer

- washed with distilled water 100 ml × 7
- dried over anhydrous Na₂SO₄
- evaporated in vacuo

### Fatty acid methylesters

- evaporated in vacuo

![Fig. 3. Preparation method for fatty acid methylesters from *H. oryzae* lipids.](image3.png)
the chloroform layer was recovered and evaporated in vacuo to yield the TL.

d) Fractionation of PL and NL from TL  The TL was chromatographed on a silicic acid column, eluting first with chloroform, then with methanol. The chloroform eluate and methanol eluate were called the NL fraction and the PL fraction, respectively.

e) Thin layer chromatography  Silica gel G (0.35-0.45 mm) glass plates were used. As the solvent systems, unless otherwise noted, petroleum ether-ether-acetic acid (80 : 30 : 1) was employed for the development of the NL fraction, and chloroform-methanol-water (65 : 25 : 4) for the PL fraction. Each lipid component was identified by comparison with the authentic samples. To confirm the identifications, diazomethane treatment, hydrolysis with 0.4 N KOH in methanol (2 hr, 37°C), and spraying with hydroxamic acid were also employed for the NL fraction. For identification of the PL components, comparisons were made with known components from yolk, using the following spray reagents:

1) Zinzadze reagent for phospholipids,
2) Anthrone reagent for choline-containing lipids,
3) Ninhydrin reagent for the lipids having amino group(s).

f) Column chromatography  About 100 mg of the PL fraction was put on the top of a silicic acid column (5 g). Fifty ml portions of chloroform-methanol (100 : 0, 98 : 2, 95 : 5, 90 : 10, 80 : 20, 2 : 1, 1 : 2, 0 : 100) mixtures were used successively as eluents, and 10 ml fractions were collected with a fraction collector. Phosphorus was measured by Bartlett method, sugar was calculated as glucose by the anthrone reagent, and lipid was determined gravimetrically.

g) Preparation of fatty acid methyl esters  One ml of dry benzene and 8 ml of 5% HCl in methanol were added to 10 mg of the sample (Fig. 3), as described by Stoffel et al. The solution was refluxed for 2 hr, cooled, 3 ml of deionized water were added, and then the solution was extracted three times with 20 ml portions of petroleum ether. The petroleum ether layers were combined and washed seven times with 100 ml of deionized water, dried over anhydrous Na₂SO₄, and then evaporated in vacuo.

h) Gas liquid chromatography  Column-DEGS 30%, φ6 mm x 3 m; column temperature-170°C; carrier gas-N₂.

Fatty acid constituents (%) were calculated from the peak areas of the methyl esters on the chromatogram.

Experimental results

a) Lipid contents  Table 1 shows the lipid contents of H. oryzae, indicating about 10% of the dry weight as TL. The NL and PL fractions accounted for 88.6 and 11.4%, respectively, of the TL. The ratio of NL/PL was 7.7. The lipid contents of KU-13 W, a white mutant of KU-13, resembled that of the parent strain, and there was no remarkable difference between them. The mycelium grown on Czapek-β-amino acid medium contained about 20% as TL.

b) NL components  Fig. 4 shows a typical thin layer chromatogram of the NL components. Eight spots were recognized on the chromatogram. By comparison with authentic samples, they were identified as follows: the substance remaining at the origin was PL, nos. 2 and 3 were sterol-analogues, no. 4 was sterol with an overlapping diglyceride, no. 6 was free fatty acid, no. 7 was unknown substance, and no. 8 was triglyceride.

To clarify the chemical nature of these materials, diazomethane treatment, hydrolysis with mild alkali, and spraying with hydroxamic acid were used. Spot no. 6 disappeared on diazomethane treatment, indicating the presence of free fatty acid. By hydrolysis with mild alkali,
three spots, nos. 5, 7 and 8 disappeared, and a part of no. 4 also disappeared. By spraying with hydroxamic acid, a positive result was obtained from four spots, nos. 4, 5, 7 and 8. Spot no. 7 seemed to be still unknown substance. Further experiments are necessary for an exact identification. Spot no. 5 seems to be diglyceride, possibly mixed with overlapping fatty alcohol. To obtain a quantitative picture of the NL components, photodensitometry of the thin layer chromatogram was undertaken. As shown in Fig. 5 spot no. 8 had large areas, indicating that triglyceride is the principal components of the NL fraction.

c) PL components Fig. 6 shows a typical chromatogram of the PL fraction, having at least eight spots. By comparison with the known polar lipids of yolk, spot no. 2 seems to be PC, and spot no. 3 PE. To identify the spots, several reagents were sprayed on the chroma-

<table>
<thead>
<tr>
<th>Table 1. Lipid content of <em>H. oryzae</em></th>
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<tr>
<td>Wet weight (g)</td>
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<td>Dry weight (g)</td>
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<td>TL (mg)</td>
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<td>TL (%)</td>
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<td>TL (applied, mg)</td>
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<td>NL (mg)</td>
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<td>PL (mg)</td>
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<td>NL/PL</td>
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Fig. 4. Thin layer chromatogram of *H. oryzae* NL. Solvent system: petroleum ether-ether-acetic acid (80:30:1).

Visualization: charring with 50% H$_2$SO$_4$.

a. *H. oryzae* NL. b. monoglyceride. c. diglyceride. d. triglyceride. e. fatty alcohol. f. fatty acid. g. ergosterol, the upper spot being the authentic compound and the lower two spots impurities in the commerical sample used.

Fig. 5. Photodensitogram of *H. oryzae* NL. Solvent system: petroleum ether-ether-acetic acid (80:30:1).

Visualization: charring with 50% H$_2$SO$_4$.

1. polar lipid. 2, 3. sterol like substances. 4. sterol and diglyceride. 5. diglyceride. 6. free fatty acid. 7. unknown. 8. triglyceride
Table 2. Color reaction for *H. oryzae* PL

<table>
<thead>
<tr>
<th>Spot</th>
<th>Zinadze reagent</th>
<th>Anthrone reagent</th>
<th>Dragendorff reagent</th>
<th>Ninhydrin reagent</th>
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<tbody>
<tr>
<td>1</td>
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<td>8</td>
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Fig. 6. Thin layer chromatogram of *H. oryzae* PL.
Solvent system: chloroform-methanol-water (65:25:4).
Visualization: charring with 50% H$_2$SO$_4$.

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Fig. 7. Elution pattern of PL of *H. oryzae* on silicic acid column chromatography.
togram (Table 2) with the following results: spot no. 2 was PC, no. 3 PE, and no. 4 (which was composed of two spots) CMH. Spot no. 1 was not identified in the present work. Spot nos. 5-8 showed positive or occasionally positive reactions with the anthrone reagent. These spots seemed to be composed of glycolipids, but further studies are needed to identify the components precisely. The principal components of the PL fraction were thus identified as PC, PE, and CMH; some unknown lipids were also present.

The PL fraction was then chromatographed on the silicic acid column in order to determine the phospholipid and glycolipid contents (Fig. 7). The chloroform-methanol (80:20 and 1:2) eluates contained relatively large amounts of phosphorus, and judging from the thin layer chromatogram these fraction consisted of PE and PC, respectively. The early eluate fraction (90:10) showed positive reaction with anthrone reagent, and this fraction contained CMH. The (98:2) and (95:5) eluates contained large amounts of phosphorus, but the components of these fraction were not elucidated.

From the results of thin layer and column chromatography, it appeared that the major components of the PL fraction of *H. oryzae* were PC, PE and CMH, and in addition the fungus has a significant amount of unknown polar lipids.

d) Fatty acid components To clarify the fatty acid components of *H. oryzae*, the following experiments were done, using the TL, PL and NL fractions. Fig. 8 shows a typical gas chromatogram of the fatty acid methyl esters of the PL fraction: ten peaks were recognized, and on catalytic hydrogenation, peak nos. 4, 8, 9 and 10 disappeared. By comparison with authentic compounds, each peak was identified as follows: C14:0 for 1, unknown for 2, C16:0 for 3, C16:1 for 4, unknown for 5, unknown for 6, C18:0 for 7, C18:1 for 8, C18:2 for 9, and C18:0 for 10. The ratio of the fatty acid components calculated from the area of the methyl esters on the chromatogram is shown in Table 2: the principal fatty acid components were C16:0, C18:0, C18:1, and C18:2, the latter being the major constituent. In the PL fraction, C18:2 accounted for over 60% of the total fatty acid components. The same results were obtained from a white mutant of KU-13, which showed no significant difference in

| Table 3. Fatty acid composition of *H. oryzae* lipids |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | 14 : 0<a)       | 16 : 0          | 16 : 1          | 18 : 0          | 18 : 1          | 18 : 2          | 18 : 3          |
| KU-13 TL       | tr<sup>b)</sup> | 27.5<sup>d)</sup> | 0.7             | tr              | 6.5             | 20.0            | 42.6            | 2.8             |
| NL              | tr              | 28.7            | 0.8             | tr              | 6.1             | 22.4            | 39.7            | 2.3             |
| PL              | tr              | 22.1            | tr              | 4.0             | 8.6             | 60.6            | 4.9             |
| KU-13 W TL     | tr              | 19.0            | tr              | 5.9             | 22.9            | 50.0            | tr              |
| NL              | tr              | 23.7            | tr              | 7.3             | 28.6            | 38.5            | 1.9             |
| PL              | tr              | 16.1            | tr              | 3.4             | 9.8             | 67.7            | 3.1             |

a) Carbon number: double bond number, b) Unknown, c) Trace, d) % recorded.
fatty acid components from the parent strain.

Discussion

These experiments clearly showned that triglyceride is the principal component of the NL fraction of H. oryzae and the amounts of free sterol are very small. Ergosterol analogues are present in the NL fraction. The principal components of the PL fraction were found to be PC, PE, and CMH. The existence of CMH in fungi has not been established up to the present time, except for the reports of Wagner et al.\textsuperscript{14,15} Therefore, details of the chemical constituents of the CMH of H. oryzae will be reported in a subsequent publication.

The principal fatty acid components are C\textsubscript{16:0}, C\textsubscript{18:0}, C\textsubscript{18:1} and C\textsubscript{18:2} with C\textsubscript{18:2} predominating. These four fatty acids accounted for more than 95\% of the total fatty acid components. These results on the fatty acid components agree with those obtained from other plant pathogenic fungi\textsuperscript{3,5,11,13}.

It should be noted that the above data do not exclude the presence of other higher fatty acids and hydroxy fatty acids since these fatty acids having long retention times. A small amount of fatty acids having odd-number of carbon atoms was detected on the gas chromatogram but these acids were not identified.

In the present work, the lipid components and their fatty acid constituents were obtained from the mycelium which was cultured in the laboratory on Czapek (NaNO\textsubscript{3}-sucrose) medium. 

H. oryzae is a pathogen for rice plants. When the fungus invades the rice plants, the utilization pattern of lipids and fatty acids in the host plant is not known. The authors now plan to study changes in the lipids and fatty acid components during sporulation and germination.

Acknowledgement

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Literature cited


和 文 摘 要

イネごま葉枯病菌の脂質について

津田 盛也・上山 昭則・中野 益男・藤野 安彦

イネごま葉枯病菌の脂質および脂肪酸組成を検討した。Folch (1957) 法に準じて本菌をクロロホルム-メタノール（2:1, v/v）で処理して総脂質を得た。総脂質を水に溶かし、色々の脂質を分画し、それぞれの組成を調べた。総脂質の80％以上が非極性脂質であって、おもな構成脂質はトリグリセリドであった。極性脂質画分には、ホスファチジルエタノールアミン、ホスファチジルコリン、セラミドモノヘキソシド、未同定の脂質が認められた。ついで本菌の脂肪酸組成を調べるために Stoffel ら (1965) の方法に従って、総脂質、非極性脂質、極性脂質の各画分を処理し、ガスクロマトグラフィーによって調べた。その結果、本菌の主要構成脂肪酸は C16:0, C18:0, C18:1, および C18:2 であった。中でも C18:2 がもっとも多かった。極性脂質画分に検出されたセラミドモノヘキソシドは植物病原菌においてはいまだ報告されていないものである。