Formation in Culture of *Cochliobolus miyabeanus*, the Perfect State of *Helminthosporium oryzae*

Akinori Ueyama** and Mitsuya Tsuda**

Abstract

*Cochliobolus miyabeanus*, perfect state of *Helminthosporium oryzae*, has been produced in culture by certain crosses. Media and methods for formation of the perfect state are described. In routine experiments, the authors employed Sachs agar medium plus rice straw at the constant temperature of 24°C for 25-30 days. Morphological data have concurred with the original data of Ito and Kuribayashi (1927, 1931), with the one exception of the length of the ostiolar beak. (Received November 29, 1974)

Introduction

The authors have been interested in the chemical biology of fungi in two areas: a) the role of sexual factors in the perfect state cycle, and b) metabolism during the appearance of sexual characters or ability to change the cycle to the perfect state from the imperfect state. Ueyama has already reported the former case. In the latter case, the authors report here on changing the cycle between *Helminthosporium* and *Cochliobolus*.

The leaf blight disease caused by *Helminthosporium oryzae* Breda de Haan is one of the most important diseases of rice plants. Symptoms include leaf spot in the incipient stage of rice cultivation in the paddy field. The discoloration of rice grains known as the autumn fall phenomenon (Akiochi-decline) appears in the harvest stages. Ear blight ("Hogare") is also principally caused by the same fungus in the later stages.

In Japan, the over-wintering form of the fungus has been considered to be conidia and mycelia of the imperfect state present on or in rice debris.

Up to 1927, the perfect state of the fungus had not been reported. Ito and Kuribayashi tentatively identified the ascigerous state in culture as a species of *Ophiobolus, O. miyabeanus*. They commented on the peculiar helicoid ascospores in the ascus. In 1934, Drechsler erected the genus *Cochliobolus* based on the morphological characteristics of *C. heterostrophus* (Drechsl.) Drechsl. (*Ophiobolus heterostrophus*) which was the type species. The name of *Ophiobolus miyabeanus* was also changed to

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* Chemical biology of the sexual factors in the fungi (3). This paper was presented at the Annual Meeting of the Kansai Branch, Phytopathological Society of Japan held at Okayama-city, Nov. 1-2, 1973. This study has been supported in part by a Grant in Aid for Fundamental Scientific Research from the Ministry of Education, Japan.

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Cochliobolus miyabeanus. Nelson, Luttrell and others\textsuperscript{8,9} found perfect states of Helminthosporium and Curvularia spp. which belonged to Cochliobolus. However the perfect state was difficult to bring maturity in laboratory and was uncommon in nature. Some species of the genus Cochliobolus, such as C. heterostrophus\textsuperscript{15} and C. sativus (Ito et Kurib.) Drechsler ex Dastur\textsuperscript{12} have been carefully studied to produce the perfect state in culture and to clarify genetical and cytological problems.

In H. oryzae, as already pointed out by Honda\textsuperscript{4}, the formation of the perfect state has been considered to be difficult. Sakamoto has also mentioned problems in this regard\textsuperscript{11}.

The present study re-investigates the life cycle of C. miyabeanus and clarifies methods of forming the perfect state in culture. As a second objective, the authors hope to characterize the sexual factors of this fungus in view of the chemical biology of the fungi.

Recently the authors obtained the perfect state of H. oryzae in culture and reported this as a preliminary note\textsuperscript{14}. In the present paper, media and methods for formation of the perfect state are described and then morphological characteristics are compared with the original data of Ito and Kuribayashi\textsuperscript{6,7}.

**Materials and methods**

**Isolation of the fungus**: the isolates of H. oryzae used in this study were mononidial isolates. Diseased glumes and leaves of rice plants were collected from several sites in the suburbs of Kyoto city, 1972. The samples were surface-sterilized with sodium hypochlorite (five times dilution of Antiformin), 10–60 sec, and kept at room temperature. Several days later, each conidium that had formed on the diseased rice tissue was picked off with a needle under a binocular microscope, and was transplanted directly into a slant culture tube. Isolation sources and collection sites of the isolates are listed in Table 1.

**Media for perfect state formation**: a) rice straw decoction agar medium; 100 g of dried rice straw was boiled in one liter as shown in the usual manner, b) Sachs agar medium; this medium was prepared by the procedure described in the Laboratory guide for plant pathologists in Japan\textsuperscript{1}. And it contains KNO\textsubscript{3}, 1.0 g; NaCl, 0.5 g; MgSO\textsubscript{4}, 0.5 g; Ca(NO\textsubscript{3})\textsubscript{2}, 0.5 g; Ca\textsubscript{3}(PO\textsubscript{4})\textsubscript{2}, 0.5 g; FeCl\textsubscript{3}, trace; agar, 10.0 g; de-ionized water, 1 liter.

| Table 1. Sources of isolates of H. oryzae and their localities |
|---------------------|---------------------|---------------------|
| Isolates | Sources | Localities |
| A | leaf | Iwakura, Kyoto |
| B | glume | Daigo, Kyoto |
| C | glume | Ichijoji, Kyoto |
| D | glume | Iwakura, Kyoto |

Fig. 1. Inoculation method for the perfect state formation of Helminthosporium oryzae.
Ten to 15 ml of the medium was poured into a 9 cm Petri dish. After solidifying, a 3-4 cm piece of rice straw, which was previously sterilized by autoclaving at 120°C, was placed on the surface of the medium in the centre of the dish.

As shown in Fig. 1, two monoconidial isolates were inoculated some distance apart on opposite sides of a straw. The Petri dish cultures were kept in an incubator at a constant temperature of 24°C for 20-30 days.

**Results**

**Formation of the perfect state on agar medium**: as shown in Table 2, the perfect state was observed on Sachs medium plus a rice straw, but not on Sachs medium without a rice straw. The perfect state did not form on the rice straw decoction (100 g/l) agar medium.

<table>
<thead>
<tr>
<th>Media</th>
<th>Pseudothecia formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sachs agar</td>
<td>−</td>
</tr>
<tr>
<td>Sachs agar + rice straw</td>
<td>+</td>
</tr>
<tr>
<td>Rice straw decoction agar (100 g/l)</td>
<td>−</td>
</tr>
<tr>
<td>Rice straw decoction agar (100 g/l) + rice straw</td>
<td>−</td>
</tr>
</tbody>
</table>

**Time course observations of the formation of the perfect state and maturation on Sachs medium plus a rice straw**: A and C isolates* were inoculated in pairs on this medium. About one week later, a bordered mycelial zone between the two isolates formed around the rice straw or in the centre of the agar plate. Gray or grayish-white mycelial clumps were recognized on the bordered line with the naked eye, and then small black spots appeared. After ten days, the small black spots filled with pseudoparaphyses and became one fourth to one third as large as normal matured pseudothecia. About two weeks after inoculation, the spots developed to the same extent as normal mature pseudothecia, some of which began to show elongation of the ostiolar beaks. At the stage, ascus initials were observed among the pseudoparaphyses in the ascocarp(s).

Following the development of the ostiolar beaks, asci were gradually formed, and ascospores ripen in the asci. In the incipient stage of ascus development, the walls are thin. Asci with partially mature ascospores have a thick wall as seen under a light microscope. In the early stages of ascospore maturation, an ascus is filled with unclarified and unorganized cytoplasm. Then peculiar helicoid ascospores, which had like worms in the intestine of mammals, are gradually observed to form.

After 20 days, ascospores are clearly recognized in almost all of the asci, while pseudoparaphyses have disappeared gradually from the ascocarps. Normally, when the ascus is mature, the ascus wall ruptures, and ascospores with several septa become segregated. In some cases, ascospores are discharged from the ostiolar beak. Gelatinous masses of ascospores were also observed around the ostiolar beaks of ascocarps.

* Will be discussed in later papers.
Morphological data on newly formed *C. miyabeanus* (Table 3 and Plate I & II)

a) Pseudothecium; fully developed, mature pseudothecium 377 μm × 380 μm (mean value). Generally, pseudothecia formed on or near the rice straw are superficial or inbedded with long beaks protruding from the surface. Ascocarp bodies are almost spherical and have clearly distinguished ostiolar beaks. They measure 125–325 (233) μm length × 50–113 (76) μm width.

b) The ascus is bitunicate and cylindrical or clavate with a short stipe. It contains one to eight ascospores arranged in a tight helix. Ascospores are arranged spirally in the ascus. They are slightly coloured or hyaline.

c) Ascospores are filiform or flagelliform with several septa. The ascospore can be distinguished clearly from pseudoparaphyses and mycelium by the difference in reflection index under a light microscope.

Ascospores germinate readily in water. Germination occurs from each cell of the ascospore. When the ascus was placed in water, germination occurred directly in the ascus. Germination tubes develop abundantly and penetrate ascus wall.

Table 3. Morphological characteristics of *Cochliobolus miyabeanus*

<table>
<thead>
<tr>
<th>Parts of pseudothecia</th>
<th>I&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>II&lt;sup&gt;b)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (μm)</td>
<td>238–688×213–750 (376.6&lt;sup&gt;f&lt;/sup&gt;)</td>
<td>370–760×370–780 (379.8&lt;sup&gt;f&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Ostiolar beak (μm)</td>
<td>125–325×50–113 (233.3)</td>
<td>95–200×55–110 (76.8)</td>
</tr>
<tr>
<td>Ascus (μm)</td>
<td>133–250×17.5–35.0 (183.3)</td>
<td>142–235×21–36 (24.4)</td>
</tr>
<tr>
<td>Ascospore (μm)</td>
<td>116–391×5.2–13.9 (242.8)</td>
<td>235–468×6–9 (7.8)</td>
</tr>
<tr>
<td>Septa of ascospore</td>
<td>4–19 (7–12)</td>
<td>6–16 (8–12)</td>
</tr>
</tbody>
</table>

a) Ueyama and Tsuda.
b) Ito and Kuribayashi<sup>7)</sup>.c) Mean value (μm).

Comparison with the original data of Ito and Kuribayashi<sup>6,7)</sup>: the present authors compared newly formed propagula with the observations reported by Ito and Kuribayashi. Almost all of our values agreed with those given in the original description. However, we found that the size of the ostiolar beak was longer about two times that described by Ito and Kuribayashi.

Discussion

Ito and Kuribayashi observed the perfect state of *H. oryzae* on rice straw decoction agar medium (100 g/l), but we could not produce ascospores on the same medium. Some of the reasons given included the difference between rice varieties used and their chemical components due to the agronomic practice used for rice growing at that time.

The difference of the length of the ostiolar beak seems related to the difference in maturity. The maturation of ascospores in the ascarp is related closely to the elongation of the ostiolar beak. In this context, it is supposed that the ascocarps obtained by the original authors contained many unripened protothecia.
From the above-mentioned results, we concluded that we have re-confirmed formation of the perfect state of *H. oryzae*, *C. miyabeanus*, in culture.

In our discussion, sexuality and related cytological problems such as heterothallism and ascospore development, were not fully covered. These subjects will be discussed in a later paper.

Acknowledgement: We are indebted to Dr. G. Gooday, University of Aberdeen, Scotland, and to Dr. M. M. Kulik, U.S.D.A.-Beltsville, for reviewing the draft.

Literature cited

Explanations of Plate 1

1. Pseudothecia on rice straw.
2. Pseudothecia on Sachs agar-rice straw medium.
3. Primordia of pseudothecia in the agar.
4. A pseudothecium on a rice straw.
5. Several asci with helicoid ascospores.
Explanations of Plate II

6. A bitunicate ascus with partially mature ascospores.
7. An ascus with packed helicoid ascospores.
8. Eight ascospores from an ascus.
9. Ascospores germinating through an ascus wall.
10. Ascospore masses emerging from an ostiolar beak.