The Perfect Stage of Pyricularia grisea

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ABSTRACT

Ceratosphaeria grisea, sp. nov., the perfect stage of *Pyricularia grisea,* developed in culture when two isolates of the fungus from crabgrass were mated on Sachs' agar with barley grains and rice straw and incubated at 25 C under weak fluorescent light. Perithecia are long-beaked, with cylindric to subclavate nonpersistent asci containing fusiform hyaline four-celled ascospores. The tip of the ascus does not stain blue with iodine, and has a refractive ring around the pore as found in the *Diaporthaceae.* Crosses of single ascospore isolates indicate that the fungus is heterothallic. Matings of isolates of *Pyricularia* from rice from various countries have so far not produced the perfect stage. Phytopathology 61:83-87.

Additional key words: rice blast, ascomycete.

Blast of rice is one of the most important diseases of this crop, and occurs in most of the rice-producing areas of the world. The causal agent is usually given as *Pyricularia oryzae* Cav.; however, it is morphologically indistinguishable from *P. grisea* (Cke.) Sacc., described earlier from crabgrass (*Digitaria sanguinalis* L.) collected in North America. The specific epithet "*oryzae*" has been retained as a matter of convenience, as most of the literature published on rice blast is found under that name (1). One approach to control of blast has been the development of resistant varieties. The existence of pathogenic races of *Pyricularia* has been demonstrated, and an international set of differential varieties has been established for identification of the races (9). Recent reports (3, 6) that several pathogenic races can be identified in monoconidial cultures from a single lesion has raised new questions concerning the proper method of race identification and the nature of pathogenic variation in this organism.

The perfect stage of *Pyricularia* from rice or grasses is not known. According to Asuyama (1), *Mycosphaerella malinverniana* (Catt) Sacc. has been found in association with rice blast by workers in Italy, India, and Japan, but the connection between *M. malinverniana* and the causal fungus was not established. He recommended more research to determine the relationship between these two fungi, and also suggested mating experiments with isolates of *Pyricularia* from different countries. This paper reports the production of the perfect stage of *P. grisea* in culture.

Production of the perfect stage.—The isolates of *Pyricularia* used are listed below with the investigator's isolate number, origin of the isolate, and the international race number in parentheses. The following 10 isolates were obtained from J. G. Atkins: 67-T-1 Texas (IG-1), 67-T-2 Texas (IG-1), 67-T-3 Texas (IG-1), 67-T-5 Texas (IG-1), 67-T-14 Texas (IG-1), 67T-16 Texas (IG-1), 67-T-17 Texas (IB-1), 67-L-5 Louisiana (IG-1), 66-L-26 Louisiana (IB-4), and 59-L-13 Louisiana (IB-5). The following 19 isolates were obtained from Frances Latterell: 429 Louisiana (IB-54), 449 Costa Rica—X-ray mutant (IB-1), 504 West Pakistan (IC-17), 551 Taiwan (IC-19), 600 Guinea (IB-47), 606 Panama (IB-49), 661 Japan (IE-3), 737 Sierra Leone (ID-5), 760 Guyana (IB-45), 763/802 Guyana (IC-1), 791 Italy (ID-13), 811 El Salvador (ID-6), 872B Louisiana (IB-49), 873/R3 The Philippines (IA-110), 874 Japan (IE-1), 883 Arkansas (IH-1), 888 Arkansas-crabgrass (II-1), 890 Louisiana (IB-49), and 891 Louisiana (IH-1). In addition, isolate No. 11 was obtained from St. Augustine grass (*Stenotaphrum secundatum* [Walt.] Kuntze) from Peru, Nos. 12 to 20 were from crabgrass from North Carolina, and Nos. 45 to 48 from rice from Peru.

Unless otherwise specified, matings were made in petri dishes on barley grains and rice straw partially embedded in modified Sachs' agar (1.0 g Ca[NO$_3$]$_2 \cdot$ 4H$_2$O, 0.25 g K$_2$HPO$_4 \cdot$ 3H$_2$O, 0.25 g KCl, 0.25 g MgSO$_4 \cdot$ 7H$_2$O, 0.85 g CaCO$_3$, a trace of FeCl$_3$, and 20 g agar in 1 liter of water). Small pieces of mycelium of the cultures to be mated were placed on opposite sides of the barley grains and rice straw, or the pieces of mycelium were broken up with a glass rod in 1 to 2 ml of sterile water and poured over the surface of the agar.

In initial experiments, the 10 isolates of *Pyricularia* from Atkins, 9 isolates from crabgrass from North Carolina, and the St. Augustine grass isolate from Peru were mated in all possible combinations and incubated on a laboratory shelf with normal room lighting at 20 to 25 C. These matings were examined periodically for 3 months, and no perithecia were found. Another complete set of matings was made with these 20 isolates; half of the matings were placed near a window to increase the amount of light, the other half placed in brown paper bags to eliminate most of the light. Again, no perithecia were observed.

Sets of 10 to 20 matings were then grown on a variety of media and subjected to different regimes of temp and light. Cholesterol, phenylalanine, or hemp seeds were added to some of the media. Matings were made on woody twigs and herbaceous stems sterilized

by propylene oxide or by autoclaving. Some matings were irradiated with ultraviolet light, some were given high- or low-temp shock treatments, and others were grown in mixed culture with *Pseudoplea trifolii*, which readily produces the perfect stage in culture. In only one of these matings was the perfect stage of *Pyricularia* observed. Perithecia were found in a mating of crabgrass isolates 13 and 20 from central and eastern North Carolina, respectively. The mating was made on Sachs' agar with barley grains and rice straw, to which had been added a drop of a 5% solution of cholesterol in chloroform. The petri dish had been incubated at constant 25 C about 20 cm below a 20 w fluorescent light.

The opt conditions for perithecial production have not been determined. In comparative tests with isolates 13 and 20, however, cholesterol did not seem to increase perithecial production. Perithecia were produced at 20 C as well as at 25 C, but perithecial development was slower at 20 C. The light factor does not seem to be critical, since perithecia were produced in petri dishes incubated in brown paper bags. The perfect stage was also produced when barley or wheat straw was substituted for rice straw.

At 25 C on Sachs' agar plus barley grains and rice straw, perithecia appear about 3 weeks after mating. They generally form on or near the barley grains or rice straw, and are usually wholly or partially embedded, with long beaks protruding from the surface. Sometimes perithecia form in the agar away from the grain and straw, and occasionally they have been observed at the bottom of the petri dish under the layer of agar.

Heterothallism.—Nineteen single ascospore isolates were obtained from the cross of isolate 13 by isolate 20 and mated in all possible combinations. Perithecia were obtained in 51 of the 171 combinations. Each isolate was compatible with at least one of the other isolates. The 19 isolates were divided into two compatibility groups; one designated "plus" contained 10 isolates; the other designated "minus" had 9 isolates. No perithecia were produced when isolates of the plus group were mated with each other, nor when isolates of the minus group were mated with each other. Table 1 shows the variation in fertility obtained in the matings of isolates of the plus group with isolates of the minus

group. Some isolates mated more readily with other isolates and produced more perithecia than others. The fungus appears to be heterothallic.

Matings with isolates from rice.—Additional matings were made under conditions favorable for perithecial production in an attempt to get crosses with isolates from rice. The 10 isolates from Atkins, the 19 isolates from Latterell, and the four isolates from rice from Peru were mated in all possible combinations. Each of these 33 isolates were also mated with isolates 13 and 20 from crabgrass. No perithecia were observed. Each of 33 isolates was then mated with several single ascospore isolates from the cross of isolates 13 × 20. In 930 of these matings, no perithecia were produced.

Some ascospore isolates mated more readily and produced more perithecia than others (Table 1). In an attempt to develop more highly fertile mating lines, the most fertile ascospore cultures were intercrossed, and second generation ascospore isolates were obtained from these crosses. When these second generation ascospore cultures were mated with the 33 isolates mentioned above, a single perithecium was produced in a mating of one of these isolates with isolate 888 from Latterell. Isolate 888 was originally from crabgrass. None of the cultures originating from rice produced perithecia.

Description and classification.—Considerable variation exists in morphological characters of this fungus, some of which probably results from genetic defects in certain isolates. The perithecia occur singly or in groups, without apparent stroma, with the base partially or wholly embedded in the substrate, and with long beaks protruding from the surface. The dark brown to black, globose base of the perithecium is 180 (60-300) μ in diam. The coriaceous peridium is 15-20 μ thick, with an outer layer of short angular cells (Fig. 1-E). The beaks are hyaline, but many become brownish with age, particularly in the basal portions, and measure 90 (60-150) μ × 600 (100-1,200) μ. Long, threadlike, deliquescent periphyses line the inside of the neck (Fig. 1-A). Two or more perithecia occasionally fuse to form a large cavity. The necks are also sometimes fused, and branched perithecial necks have been observed.

Asci arise from the base of the perithecium (Fig. 1-A) and measure 8.5 (7-10) × 70 (55-90) μ. They are cylindrical to subclavate (Fig. 1-B, D), unitunicate,

TABLE 1. Mating reaction of single ascospore isolates from cross of isolates 13 × 20 of *Pyricularia grisea*

Ascospore isolate no. (− group)	Ascospore isolate no. (+ group)									
	26	29	32	33	35	36	41	42	43	44
27	+[a]	−	−	++	++	+	−	−	+	+
28	+	−	−	+	+++	−	−	+	−	−
30	+	−	−	+	+++	+++	−	++	−	++
31	−	−	−	++	++	+++	−	−	−	+
34	+	−	−	−	−	+	−	−	−	−
37	+	−	−	−	++	++	+++	++	−	+
38	++	+	−	++	++	+++	+++	++	−	++
39	−	−	+	+	++	+	++	++	+	+
40	+	++	+++	−	+	+++	+	+++	+	−

[a] Rating based on number of perithecia per petri dish: − = no perithecia; + = 1 to 10 perithecia; ++ = 11 to 50 perithecia; and +++ = more than 50 perithecia.
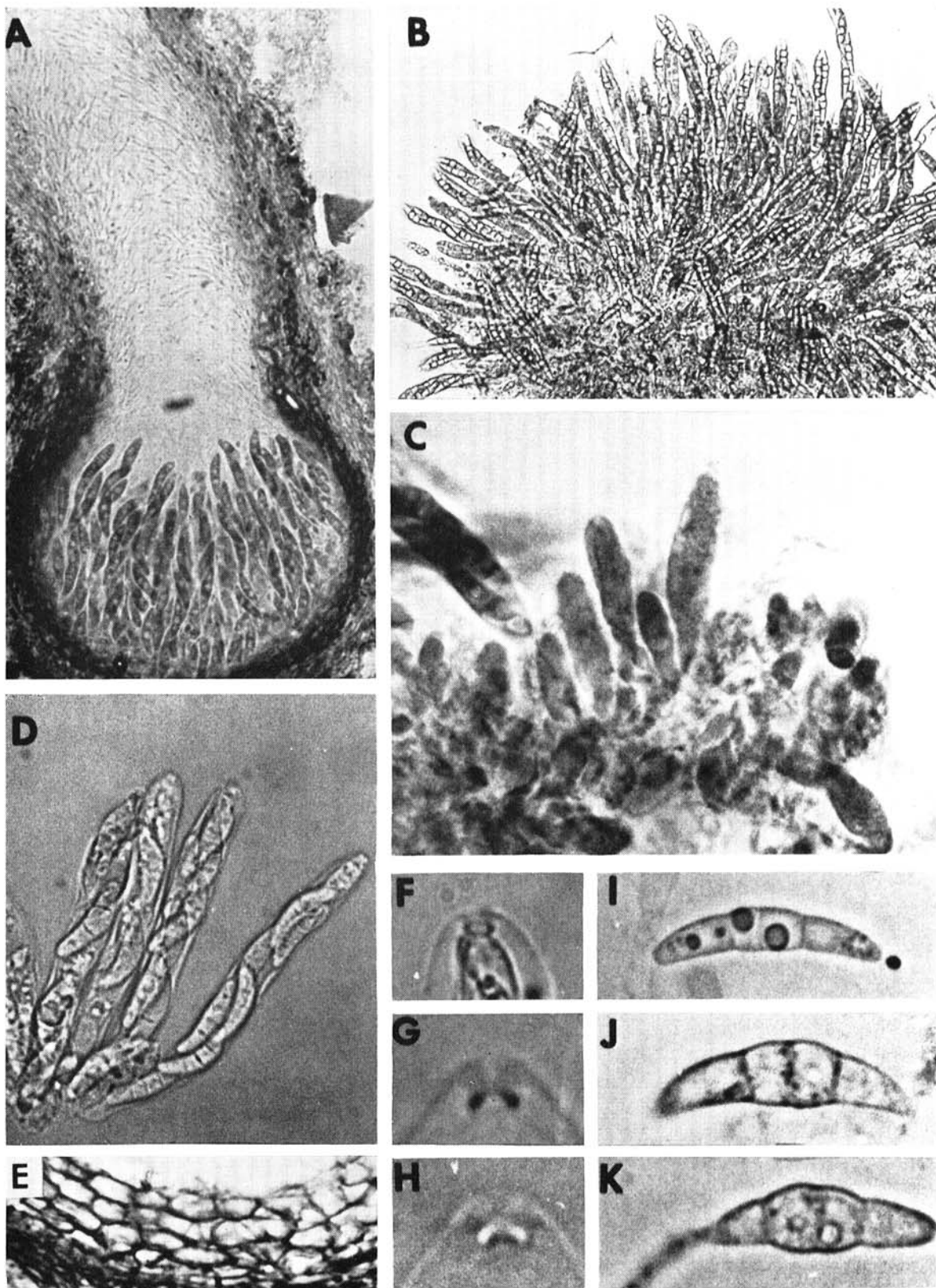
Fig. 1. *Ceratosphaeria grisea.* **A)** Longitudinal section of perithecium mounted in dilute cotton blue in lactophenol. **B)** Fan of asci from crushed perithecium in Melzer's iodine reagent. **C)** Young asci from crushed perithecium in dilute cotton blue in lactophenol. **D)** Asci in dilute acid fuchsin in lactophenol. **E)** Cells of peridium from paraffin section. **F, G, H)** Ascus tip with internal refractive ring. **I, J)** Ascospores. **K)** Germinating ascospore. F, G, H, I, J, K) Mounted in water.

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and thin-walled except at the tip. A refractive ring is present around a pore in the tip of the ascus. With certain orientations of the ascus, a ring can be seen (Fig. 1-F). With transmitted light parallel to the plane of the ring, however, the ascus tip appears to have two symmetric dark spots (Fig. 1-G) or light spots (Fig. 1-H), depending on the focus. The tip of the ascus does not stain blue when treated with Melzer's iodine reagent. In many asci, the tip structure is poorly developed. No paraphyses have been observed. Since in some fungi the paraphyses deliquesce early, a special effort was made to observe young asci. The young asci appear to arise from an ascogenous layer free of paraphyses (Fig. 1-C). A few septate, thin-walled hyphal structures were seen in the contents of some crushed perithecia. They were 6 to 11 μ wide at the base, tapered to a rounded tip 3 to 5 μ wide, and were up to twice as long as the asci. These structures were observed only around the periphery of the ascal fan and never intermingled with the asci, and for this reason are not considered to be paraphyses. Each ascus normally contains eight ascospores, but asci with fewer than eight ascospores are frequently observed.

Ascospores are typically hyaline, fusiform, three-septate with little or no constriction at the septa, usually slightly to moderately curved, without gelatinous sheath or appendages, and measure 5 (4-7) × 21 (17-24) μ (Fig. 1-I, J). Oil globules are usually present in the spores. Misshapen spores with collapsed or enlarged cells are very common. Ascospores germinate by producing germ tubes from one (Fig. 1-K) or both end cells. Spore liberation appears to be by deliquescence of ascal stalks and asci. Both whole asci and free ascospores have been seen in the perithecial neck. Many old perithecia contain only free ascospores and no asci.

The ascal tip structure indicates that this ascomycete is related to the group of fungi that Munk (5) placed in the family Diaporthaceae. Members of this family also have a peridium of short, angular cells. Spore discharge in some members of the family is by deliquescence of ascal stalks or asci and passage of asci or ascospores through an elongated perithecial neck. It is not clear to which members of this family the perfect stage of P. grisea is most closely related. Morphologically, this fungus resembles species of Ceratosphaeria more closely than those of any other genus. Most species of Ceratosphaeria, however, are found on rotten woody stems or twigs, and on an ecological basis one might question whether this species is very closely related to the majority of species now in Ceratosphaeria. The brief descriptions of two ascomycetes, Ceratosphaeria philippinarum Rehm from leaf sheaths of bamboo (8) and Cryptoderis (= Pleuroceras) caricina Rehm from dead leaves of Carex (7), indicate a resemblance to the fungus under study here in both morphology and habitat. The perfect stage of P. grisea is distinguished from the former species by having longer and narrower asci and from the latter by having shorter and wider ascospores.

The perfect stage of P. grisea apparently has not been previously reported, and is described as a new species.

Ceratosphaeria grisea Hebert sp. nov.—Perithecia solitaria vel gregaria, globosa, nigra vel fusca, 180 (60-300) μ diam., rostro longo, hyalino vel subfusco, 90 (60-150) × 600 (100-1,200) μ praedita. Peridium coriaceum, ex cellulis brevibus, angularibus formatum. Asci 8.5 (7-10) × 70 (55-90) μ, unitunicati, cylindrici vel subclavati, octospori, cum annulo refractivo ad apicem. Paraphyses nullae. Ascosporae hyalinae, guttulatae, triseptatae, fusoidae, curvae, 5 (4-7) × 21 (17-24) μ. In cultura agaro cum granis hordei et stramento oryzae. Status conidicus Pyricularia grisea (Cke.) Sacc. est.

Holotype on barley grains and rice straw (obtained in culture by mating two isolates of the fungus from Digitaria sanguinalis L. from North Carolina) deposited in USDA National Fungus Collections (BPI). Isotype deposited in Kew Herbarium.

DISCUSSION.—While the structure of the perfect stage of Pyricularia grisea indicates a relationship to fungi in the Diaporthaceae, the correct placement of the fungus within the family is not clearly indicated. Taxonomically, the group is in a state of flux and there is no consensus yet as to the limits of the family. This is exemplified by the fact that Munk (5) placed both Ceratosphaeria and Zignoella in the Diaporthaceae, whereas Dennis (2) placed Ceratosphaeria in the Ceratostomataceae and Zignoella in the Sphaeriaceae. The limits of the genera also are not clearly drawn, and species are being shifted from one genus to another. Perhaps a new genus is needed to bring together the species under study here with those most closely related to it; however, it seems best to await a revision of this group of fungi before erecting a new genus. The Mycosphaerella observed in association with rice blast by some authors (1) apparently was not the perfect stage of the causal agent. Webster (10) described the perfect stage of Pyricularia aquatica Ingold as Massarina aquatica. Since this fungus has bitunicate asci, it is taxonomically far removed from Ceratosphaeria grisea. Luttrell (4) has suggested that P. aquatica might more properly be classified in the genus Dactylella.

The failure of isolates of Pyricularia from rice to produce the perfect stage when mated in culture is probably due to genetic factors rather than to environmental factors, because compatible isolates of the fungus from crabgrass produced perithecia under the same environmental conditions. It seems unlikely that isolates from rice would have different environmental requirements for perithecial production. The only characteristic that distinguishes "oryzae" isolates from "grisea" isolates is that of pathogenicity, and there is an overlap in host range. Some grisea isolates will infect rice, and some oryzae isolates will infect wild grasses (1).

Although this study makes it possible to study the inheritance of the factors for pathogenicity found in the Pyricularia isolates from crabgrass, it would be highly desirable to have crosses involving the various races of the fungus from rice in order to determine whether the gene-for-gene hypothesis holds true for this disease. Attempts are being made to get crosses with rice isolates by making matings with additional isolates from rice. Also, the most fertile ascospore

cultures from the crosses with crabgrass isolates are being intercrossed in an effort to get more highly fertile mating lines that might cross with rice isolates.

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