Morphological and Cytologic Studies of Two Isolates of *Pyricularia oryzae* in Relation to Their Pathogenic Variability on Rice

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ABSTRACT

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The morphological, cytologic, and pathogenic behavior of isolates T63 and P2017 of *Pyricularia oryzae* on rice (*Oryza sativa*) showed the former as stable and the latter as an unstable isolate. Studies showed that it was feasible to select a stable isolate on the basis of morphologic-like colony characters, absence or presence of intertwining hyphae, anastomosis, and nuclear condition of hyphae that provide complementary information in addition to pathogenic patterns on test cultivars.

Pyricularia oryzae Cav., the incitant of rice (*Oryza sativa* L.) blast, is a variable fungus. Variability in both pathogenicity and physiologic characters has been observed even in isolates originating from single conidia (2,13,15). Although breeding for resistance to blast started as early as 1920 in India, 1927 in Japan, and subsequently in several other countries, stable varieties resistant to *P. oryzae* are not available because of the evolution of new virulent races in a short time that attack such varieties. During routine screening of germ plasm sources at the International Rice Research Institute, isolate T63 (race ID 14) gave a consistent reaction from repeated subcultures on known host genotypes, whereas P2017 (race IA 65) gave a variable reaction. Therefore, the morphology, cytology, and pathogenic patterns of the two isolates were studied to determine reasons for the differences.

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MATERIALS AND METHODS

For morphological and cytologic studies, the cultures were grown on plates of prune agar (prune extract of two fruits, yeast extract 1.0 g, lactose 5.0 g, agar 20.0 g, and distilled water to make 1,000 ml). Sterilized 1-cm^2 pieces of cellophane were placed at the periphery of the colony. As the fungus grew over the cellophane, it could be removed easily in preparation for fixing, staining, and observation. Using the HCl-Giemsa technique (1,4), the culture was fixed in glacial acetic acid-alcohol (1:3) for 10 min and then transferred for 5 min each to 35% ethanol and distilled water. The culture was hydrolyzed for 7 min in 1 *N* HCl at room temperature (25–27 C) and for 7 min at 60 C. The excess acid was removed from the culture with distilled water. The culture was then rinsed in phosphate buffer (pH 6.9) and stained in Giemsa for 30 min (two drops of stain per milliliter of buffer). The culture on the cellophane square was dipped in the phosphate buffer to remove excess stain, then mounted in stain on a microscope slide. A cover glass (22 × 22 mm, no. 1) was placed over the culture, excess stain was blotted, and the cover glass was sealed with paraffin.

Acid fuchsin staining was done to differentiate the nucleolus. Cultures on cellophane pieces were fixed in modified Helly's fixative for 10 min, washed in 70 and 35% ethanol, stained in acid fuchsin (1:60,000 in 1% acetic acid) for 30–60 sec, and mounted in 1% acetic acid (14).

Five cultivars giving either resistant or susceptible reactions to *P. oryzae* isolates were selected as parents (Table 1), and the crosses Tetep × Sensho, Tetep × IR 36, Sensho × IR 36, Carreon × KTH 17, and Carreon × Sensho were made (Table 2). Five kilograms of pulverized soil was used to fill rectangular plastic trays (30 × 23.5 × 11.5 cm), to which 50 g of ammonium sulphate was added to each tray of soil and mixed. Seeds of F_1 and F_2 crosses were sown in such trays and were watered once daily. The greenhouse-grown seedlings were inoculated at the 3.5- to 4.5-leaf stage by being injected through the leaf sheath with a conidial suspension (3×10^4 conidia per milliliter) that reached the center of the youngest folded leaf (5,8). Plants were incubated on a greenhouse bench (24–30 C) for 7 days. Reactions were measured and scored as resistant or susceptible to *P. oryzae* depending on the absence or presence of blast lesions. The ratios of the plants producing resistant and susceptible reactions to *P. oryzae* were tested using the chi square (χ^2) goodness-of-fit test (3).

Table 1. Blast reaction of the rice cultivar parents to isolates T63 and P2017 of *Pyricularia oryzae*

Parent	Disease reaction	
	T63	P2017
Carreon	R[a]	R
KTH	R	S
Tetep	R	S
IR 36	S	R
Sensho	S	S

[a] R = resistant to *P. oryzae*, no development of blast lesion; S = susceptible to *P. oryzae*, blast lesions developed after inoculation with the pathogen.

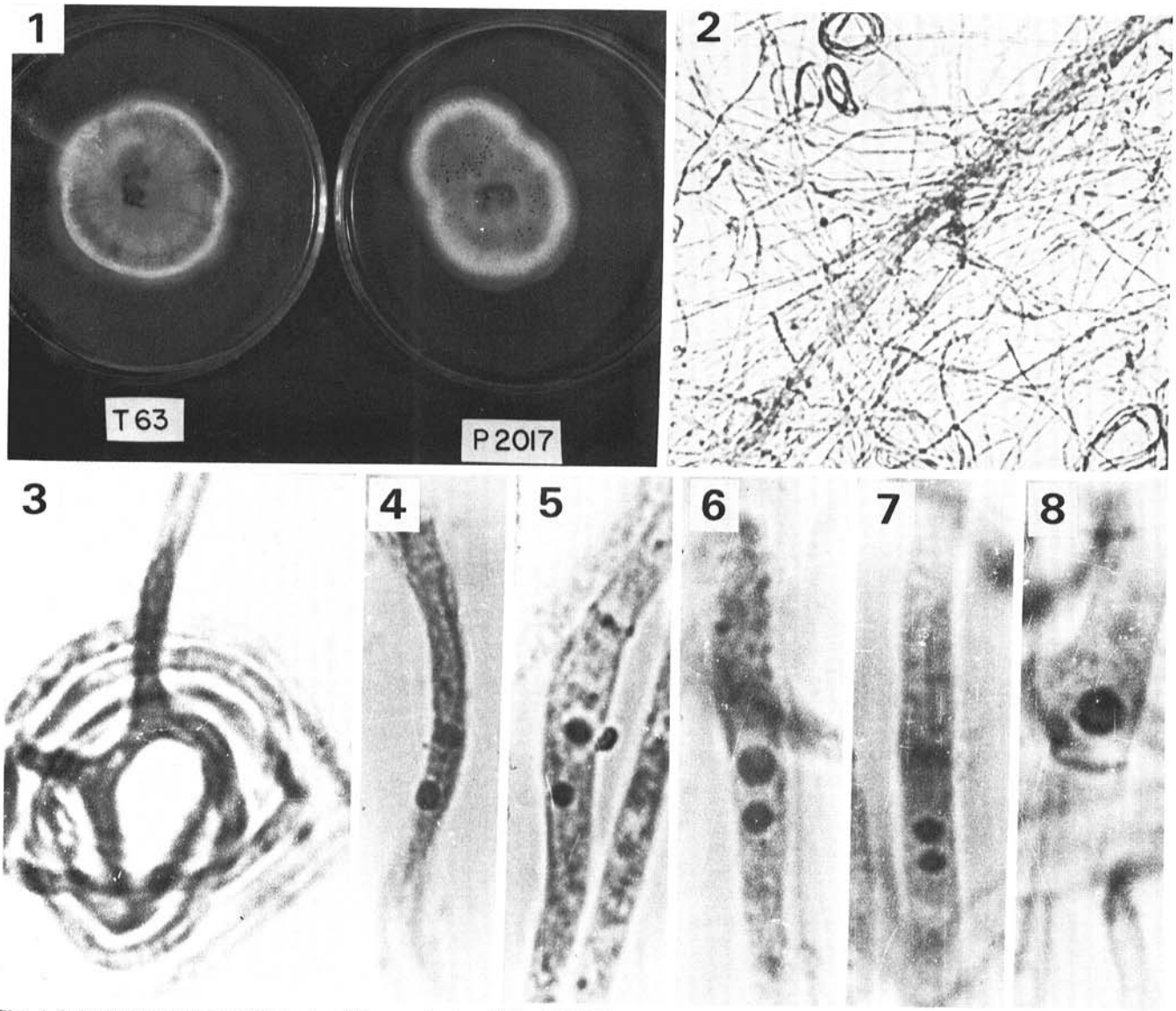
Table 2. Blast reaction on the F_1 and F_2 populations of different rice cultivar crosses to isolate T63 and P2017 of *Pyricularia oryzae*

Cross	Generation	Isolate	Number of plants showing disease reaction		χ^2 Test (3:1)
			R[a]	S	
Tetep × Sensho	F_1	T63	12	0	
Tetep × Sensho	F_2	T63	126	29	3.27
Tetep × IR 36	F_1	T63	8	0	
Tetep × IR 36	F_2	T63	116	52	3.17
Tetep × IR 36	F_1	P2017	4	7	
Tetep × IR 36	F_2	P2017	107	136	124.28**[b]
Sensho × IR 36	F_1	P2017	3	25	
Sensho × IR 36	F_2	P2017	231	127	20.95**
Carreon × KTH	F_1	P2017	2	9	
Carreon × KTH	F_2	P2017	482	188	3.34
Carreon × Sensho	F_1	P2017	14	5	
Carreon × Sensho	F_2	P2017	110	58	7.38**

[a] R = resistant to *P. oryzae*, no development of blast lesion; S = susceptible to *P. oryzae*, blast lesions developed after inoculation with the pathogen.
[b] ** = Significant at the 1% level ($P = 0.01$).

RESULTS AND DISCUSSION

The morphological and cytologic observations revealed that the two isolates differed in several characters. Isolate T63 produced flat, light to dark olivaceous colonies that turned gray with sporulation (Fig. 1). Isolate P2017 produced raised, whitish colonies with holelike depressions that became conspicuous in older colonies (Fig. 1). The color of P2017 colonies became identical to that of T63 colonies upon sporulation. Mycelia of T63 did not anastomose or intertwine, and the mycelial cells were never binucleate. In isolate P2017, several hyphae intertwined to form a ropelike structure (Fig. 2). Sometimes a loop was formed by encirclement of either one (Fig. 3) or several hyphae. Although not frequent, self-anastomosis was observed in isolate P2017. Normally the mycelial



Figs. 1–8. *Pyricularia oryzae*: **(1)** Colonies of *P. oryzae* isolates T63 and P2017 on prune agar. Dark pinhead dots are holelike depressions in the colony of P2017. **(2)** Intertwining of hyphae of isolate P2017 and the formation of loops by encircling hyphae (×300). **(3)** Single hypha of isolate P2017 encircled several times to make a heavier loop (×1,800). **(4 and 5)** Hyphal cells showing binucleate condition (×1,800). **(6 and 7)** Hyphal cells of isolate P2017 showing binucleolar nucleus enclosed in the nuclear wall. Note the lighter zone around nucleoli (×1,800). **(8)** Nucleus in hypha of isolate P2017 with a large nucleolus that is almost double the size of an average nucleolus (×1,800). (Structures in Figures 2–5 stained by HCl-Giemsa and in Figures 6–8 by acid fuchsin.)

cells were uninucleate, but occasionally binucleate cells (Figs. 4 and 5), binucleolar nucleus (Figs. 6 and 7), and nuclei with a single, larger nucleolus were observed (Fig. 8). These observations indicated that in isolate P2017 there is a likelihood of nuclear transfer through anastomosis.

The significance of hyphal loop formation and the intertwining of hyphae was not ascertained. The presence of binucleate cells, binucleolar nuclei, and the larger nucleolus indicated that a parasexual cycle probably occurs in this isolate. Yamasaki and Niizeki (17) found that 13–20% of the cells of *P. oryzae* are multinucleate. Anastomosis and migration of the nucleus were observed and indicated the formation of heterodiploids. Wu and Tsai (16) suggested that parasexuality might be the mechanism of variation, but in none of these cases has cytologic evidence for fusion of the nuclei been shown.

The blast reactions on F_1 and F_2 of different crosses with isolates T63 and P2017 were compared (Tables 1 and 2). The F_1 plants of Tetep × Sensho and Tetep × IR 36 showed a resistant reaction to isolate T63, suggesting that resistance to *P. oryzae* is dominant over susceptibility. In the F_2, these crosses gave a Mendelian segregation ratio of 3:1 as resistant and susceptible to *P. oryzae* (Table 2). This confirmed that the resistance of Tetep against isolate T63 is governed by a single dominant gene. Against isolate P2017, the reaction of F_1 plants of four crosses was variable. Segregation for resistance and susceptibility to *P. oryzae* in all the F_2 plants, except of Carreon × KTH, did not conform to the 3:1 pattern, χ^2 being significant at the 0.01 level (Table 2). Because the original reactions of the parents to isolate P2017 in these crosses are known, the nature of the isolate becomes questionable. These observations indicated that isolate T63 was stable and that isolate P2017 was unstable.

There is controversy regarding the occurrence of variation in different isolates and races of the pathogen (9). A very high rate of change in the virulence pattern has been reported in the fungus; even daughter conidia from a monoconidial culture have been separated into several pathogenic races (2,10–13). In contrast, Latterell (9) observed several cultural variants, but she considered the ratio of variants to stable conidia as low. Once the type cultures of most races were selected for sporulating capacity, they retained their characteristic growth habit through 10–20 yr of periodic transfer as well as pathogenic patterns on different varieties (9). One Japanese worker (7) has also found similar situations where some isolates maintained their original virulence. Some isolates, however, lost their virulence, but in others it has increased. Some isolates have maintained their original virulence on cultivars with blast resistance genes *Pi-k* and *Pi-m* since isolation in 1953 and 1955 (7).

Apparent use of either variable or stable isolates of the pathogen by different workers may be one major reason for the controversial results. Variable isolates do not provide a satisfactory explanation of resistance to *P. oryzae* operating in a cultivar (6,15). In studies like gene analysis and race identification, stable isolates must be used for reliable results. Kozaka (7) has indicated that stable isolates of the pathogen can be obtained by careful selection. The present study indicates that morphological and cytologic observations provide additional information that may be useful for selecting a stable isolate of the pathogen for fundamental studies.

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