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Mating Types and Optimum Culture Conditions for Sexual State Formation of *Fusarium fujikuroi* Isolates

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Twenty-five isolates of *Fusarium fujikuroi* acquired from rice seeds and rice plants evidencing symptoms of Bakanae disease were evaluated to determine their mating types and characterize the formation of their sexual state. The mating types of the isolates were evaluated via multiplex PCR with the diagnostic primers of the mating-type (*MAT*) region: GFmat1a, GFmat1b, GFmat2c, and GFmat2d. Among the 25 isolates, 11 were identified as MAT-1 (male), and 14 as MAT-2 (female). Four MAT-1 isolates and three MAT-2 isolates were mated and cultured to evaluate the optimal culture conditions for the production of their sexual states. Among four tested media, 10% V8 juice agar proved optimal for the perithecial production of the isolates. The isolates also generated the largest numbers of perithecia when incubated at 23°C in alternating cycles of 12 hr fluorescent light and NUV fluorescent light and 12 hr darkness.

KEYWORDS : Bakanae disease, *Fusarium fujikuroi*, *Gibberella fujikuroi*, Mating types, Sexual state formation

The *Gibberella fujikuroi* species complex consists of at least nine mating populations, which represent a variety of biological species (Leslie *et al*., 2004). These are important pathogens of various crops in many world regions (Desjardins, 2003). Bakanae disease in rice is caused by *G. fujikuroi* (Saw.) Ito in Ito and Kimura (anamorph: *Fusarium fujikuroi* Nirenberg). The most typical symptom of this disease is abnormal seedling elongation. Recently, the Korean incidence of this disease has increased steadily.

G. fujikruoi is heterothallic and is known to occur under field conditions (Watanabe and Umehara, 1977). Sexual states are quite important for differentiating species in the *G. fujikuroi* species complex and for genetic analysis (Jurgenson *et al*., 2002; Leslie, 1991; Xu and Leslie, 1996). In particular, *F. fujikuroi* can be distinguished accurately only by sexual cross-fertility tests or via DNA sequencing (Leslie and Summerell, 2006), because they are phylogenetically quite closely related to several *Fusarium* species, e.g. *F. proliferatum* and *F. verticillioides*. Accordingly, the ability to mate isolates of this fungus in the laboratory may prove to be a very valuable research tool. This study was conducted to identify the mating types of *F. fujikuroi* isolates associated with bakanae disease in Korea and to clarify the optimal culture conditions for the production and formation of the sexual states of *F. fujikuroi* isolates.

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Materials and Methods

Isolation. *Fusarium fujikuroi* was isolated from seeds, infected seedlings, and stems of rice. The seeds and fragments of infected plants were plated on water agar (WA) and incubated for 5 to 7 days at 22~25°C. These were then purified via single-spore isolation on WA and maintained on Synthetic Low Nutrient Agar (SNA) at 10°C.

DNA extract and investigation of mating types. In order to extract genomic DNA, *Fusarium* isolates were cultured in potato dextrose broth (PDB) without shaking at 25°C for 5 days. Harvested mycelial cultures were suspended in CTAB extraction buffer and extracted with PCI (25 : 24 : 1) and chloroform/isoamylalcohol (24 : 1). DNA pellets were washed in 70% EtOH, dried, and resuspended in 1mM TE buffer. Mating types of the isolates were evaluated using multiplex PCR with the diagnostic primers of the mating-type (*MAT*) region, as described previously (Steenkamp *et al*., 2000).

Formation of perithecia. Generally, crosses of the *G. fujikuroi* species complex were conducted via the method developed by Klittich and Leslie (1988). Put another way, strains serving as female parents were inoculated on plates containing carrot agar, and male parent strains were inoculated on slants containing complete medium. After seven days, the spore suspensions of male parent strains in 2.5% Tween 60 solution were spread onto the surfaces of the

female cultures. Fertilized plates were incubated at the appropriate temperature and light conditions. In order to evaluate the effects of media, female isolates were grown on four types of media: V8 juice agar, carrot agar, water agar, and synthesized low-nutrient agar. In the case of V8 juice agar, various concentrations of V8 juice were tested. To assess the effects of temperature, fertilized plates were cultured at different temperatures--18, 23, 26, and 28°C. In order to determine the optimal light conditions, the fertilized plates were incubated under a variety of different light conditions. The tested light periods and types were as follows: continuous light or dark and 12 hr light/12 hr dark cycle using fluorescent (FL) and near ultra violet (NUV) light. All crosses were examined weekly for the presence of perithecia, and positive scores were assigned to samples in which ascospore-oozing perithecia were noted.

Results and Discussion

Mating types of isolates. A total of 25 *Fusarium fujikuroi* isolates obtained from rice seeds and diseased rice plants were evaluated for the identification of mating types via multiplex PCR amplification. Among the 25 isolates, 11 were identified as MAT-1 and 14 as MAT-2 (Fig. 1). Although few isolates were utilized in this study, the results were similar to those obtained in other *Gibberella* species--that is, among the 64 fertile isolates of *G. circinata*, MAT-2 (+) and MAT-1 (−) mating types were segregated at a ratio of 35 : 29 (Britz *et al*., 1998). In the case of *G. coronicola*, however, the proportions of MAT-1 and MAT-2 isolates differed depending on the region (Bentley *et al.*, 2008). Thus, it was also necessary to evaluate the ratio of MAT-1 and MAT-2 idiomorphs of the Korean *F. fujikuroi* population.

Medium conditions for perithecial production. Based on their pathogenicity, DNA sequencing of the elongation factor 1α gene, responses to fungicide, and morphological characteristics of the isolates, four MAT-1 isolates and three MAT-2 isolates were selected to crosses (data not shown). Among the tested media, V8 juice agar was the best for the production of sexual states (Table 1), and the concentration of V8 juice was 10% v/v (Fig. 2). Klittich and Leslie (1988) reported that the fertility of this fungus is generally higher on carrot agar than on the more commonly used V8 juice agar. By way of contrast, our results demonstrated that V8 juice agar was more effective than carrot agar. This result indicates that perithecial production is associated with the differences in ingredients

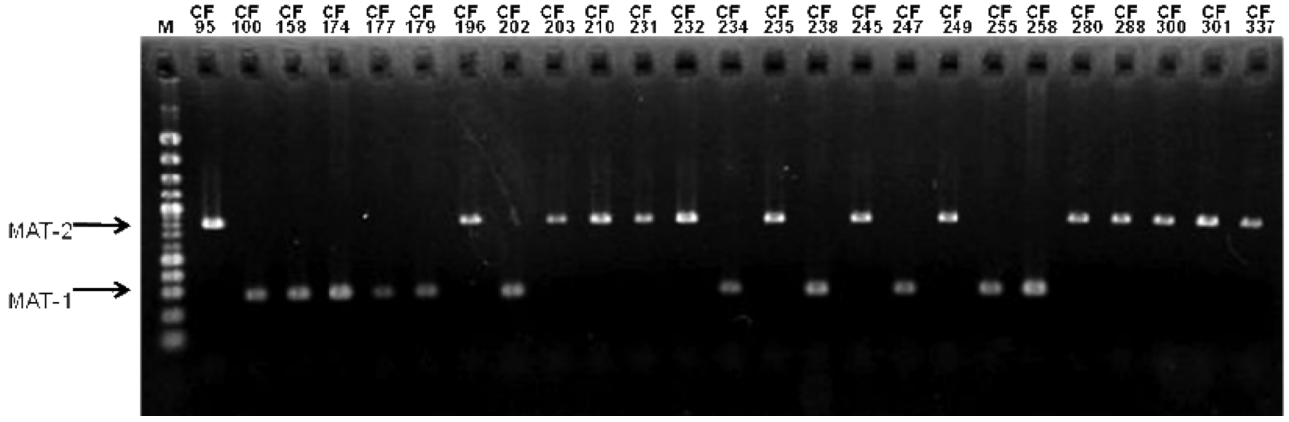

Fig. 1. PCR amplification patterns of mating types from *Fusarium fujikuroi* isolates. M, 100 bp marker; MAT-1 idiomorph, 200~300 bp; MAT-2 idiomorph, 800~900 bp.

Table 1. Effect of various media on production of perithecia by *Fusarium fujikuroi* isolates

Isolates mated	Production of perithecia on media at weeks after mating[a]															
	V8 juice agar				Carrot agar				Water agar				Synthetic nutrient low agar			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
CF210 × CF202	−	−	+	++	−	−	−	+	−	−	−	−	−	−	−	−
CF232 × CF202	−	−	+	++	−	−	−	++	−	−	−	−	−	−	−	−
CF232 × CF234	−	+	+	+++	−	−	+	++	−	−	−	−	−	−	−	−
CF337 × CF179	−	+	++	+++	−	−	+	++	−	−	−	−	−	−	−	−
CF337 × CF100	−	+	+	+++	−	−	+	++	−	−	−	−	−	−	−	−

[a]Production of perithecia; −, no perithecia; +, less than 5 perithecia per 1cm-diameter circle on media; ++, 5~10 perithecia per 1cm-diameter circle on media; +++, more than 10 perithecia per 1cm-diameter circle on media.

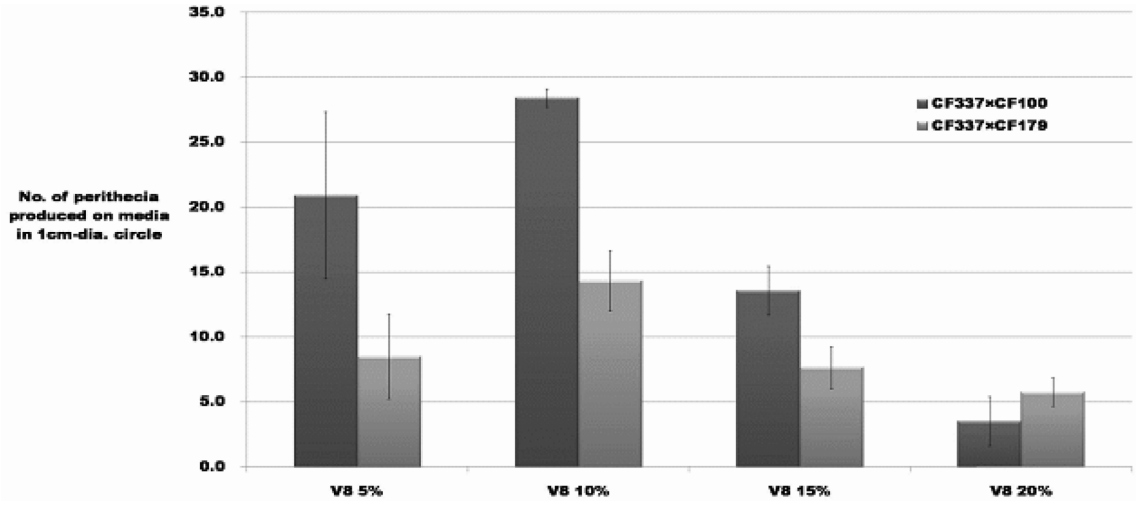

Fig. 2. Effect of different concentrations of V8 juice on perithecial production by *Fusarium fujikuroi* isolates.

among materials such as carrot and V8 juice.

Temperature conditions for perithecial production. As a result of the temperature experiments, the perithecia of *G. fujikuroi* were formed only at 23°C, and not at other temperatures (Table 2). The effects of temperature on sexual reproduction have been evaluated in several *Gibberella* species. In *G. fujikuroi*, more perithecia were generated at 20°C than at 25°C, but none at 15 or 30°C (Hsieh *et al.*, 1977). The optimal temperature for the perithecial formation of *G. zeae* was 28.5°C (Tschanz *et al.*, 1976). In *G. circinata*, the causal agent of the pitch canker disease of pines, when fertilized plates were incubated at 20°C rather than 25°C, many perithecia were formed within two weeks (Covert *et al.*, 1999). In *G. baccata*, temperature was not shown to affect perithecial formation (Afanide *et al.*, 1976). These results demonstrate that temperature conditions perform a very important

Table 2. Effect of different temperatures on production of perithecia by *Fusarium fujikuroi* isolates

Isolates mated	Production of perithecia on media at weeks after mating [a]															
	18°C				23°C				26°C				28°C			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
CF210 × CF202	–	–	–	–	–	–	+	++	–	–	–	–	–	–	–	–
CF232 × CF202	–	–	–	–	–	–	+	++	–	–	–	–	–	–	–	–
CF232 × CF234	–	–	–	–	–	–	++	+++	–	–	–	–	–	–	–	–
CF337 × CF179	–	–	–	–	–	–	++	+++	–	–	–	–	–	–	–	–
CF337 × CF100	–	–	–	–	–	+	++	+++	–	–	–	–	–	–	–	–

[a]Production of perithecia; –, no perithecia; +, less than 5 perithecia per 1cm-diameter circle on media; ++, 5~10 perithecia per 1cm-diameter circle on media; +++, more than 10 perithecia per 1cm-diameter circle on media.

Table 3. Effect of various light conditions on production of perithecia by *Fusarium fujikuroi* isolates

Isolates mated	Production of perithecia on media at weeks after mating[a]																							
	Continuous dark				12 hr/FL + NUV & 12 hr/dark				12 hr/FL & 12 hr/dark				12 hr/NUV & 12 hr/dark				Continuous FL				Continuous NUV				Continuous FL+NUV			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
CF210 × CF202	–	–	–	–	–	–	+	++	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
CF232 × CF202	–	–	–	–	–	–	+	++	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
CF232 × CF234	–	–	–	–	–	+	++	+++	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
CF337 × CF179	–	–	–	–	–	+	++	+++	–	–	–	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	–	–
CF337 × CF100	–	–	–	–	–	+	++	+++	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a]Production of perithecia; –, no perithecia; +, less than 5 perithecia per 1cm-diameter circle on media; ++, 5~10 perithecia per 1cm-diameter circle on media; +++, more than 10 perithecia per 1cm-diameter circle on media. FL, fluorescent light; NUV, near ultra violet.

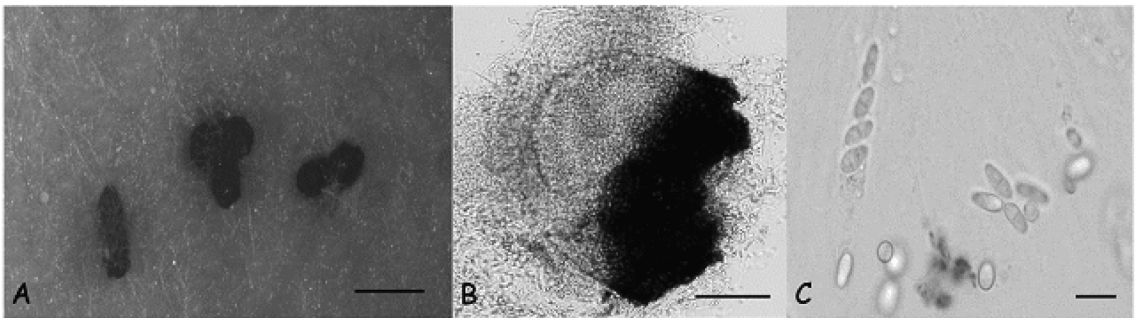

Fig. 3. Sexual states of *Gibberella fujikuroi*. A, perithecia formation on V8 juice agar medium (scale bar = 500 μm); B, asci and ascospores release from perithecia (scale bar = 100 μm); C, ascospores enclosed in asci, and released ascospores (scale bar = 10 μm).

function in the fertility of crosses, depending on *Fusarium* species.

Light conditions for perithecial production. In the light condition experiments, perithecia were abundantly formed under alternating 12hr/light and 12 hr/dark cycles with both FL/NUV and NUV light (Table 3). Moreover, a mix of FL and NUV light was shown to be superior to only NUV light with regard to perithecial and ascospore production. However, light conditions of 12hr/light and 12 hr/dark using only FL light proved unsuitable for the development of sexual states. Perithecia were only formed under light conditions, including NUV light. It has been reported previously that conditions of 12 hr light and darkness per day are excellent for the production of fungal perithecia (Bentley *et al*., 2008; Lim *et al*., 2001).

In conclusion, in order to generate the greatest number of *G. fujikuroi* perithecia, the female strains should be cultured on 10% V8 juice agar and fertilized plates should be incubated at 23°C in alternating cycles of 12 hr FL and NUV light and 12 hr of darkness (Fig. 3). Further studies will be necessary to assess more accurately the effects of other factors, such as gas exchange, for perithecial development.

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