Resistance of *Fusarium fujikuroi* Isolates to Hydrogen Peroxide and Its Application for Fungal Isolation

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The ascomycete fungus *Fusarium fujikuroi* (teleomorph Gibberella fujikuroi) causes a monocyclic bakanae disease in rice and the disease has been recently reemerging in Korea (Lee et al., 2010). Infected or infested seeds play the source of primary inoculum for disease, although ascospores produced on crop residues are reported as the primary inoculum (Leslie and Summerell, 2006; Sunder and Satyavir, 1998). Infected seeds often cause seedling to die at an early tillering stage, but usually result in typical bakanae disease symptom, the formation of elongated stems (Carter et al., 2008; Sunder and Satyavir, 1998).

To control bakanae disease, several fungicides such as prochloraz, tebuconazole, and benomyl have been widely used for seed disinfection in Korea (Kim et al., 2008; Park et al., 2009; Shin et al., 2008b). However, resistant strains against those fungicides have been occurring in field populations, resulting in dramatic increase of bakanae disease incidence in Korea (Kim et al., 2010; Shin et al., 2008a; Yang et al., 2012). Studies on population dynamics of this fungus needs for the development of suitable approaches to control this disease in rice fields. For characterization of the fungal population, it is a prerequisite to collect pure isolates from infected plants, soil, or air, but co-contamination of *F. fujikuroi* with other fungal species including *F. graminearum* and *Magnaporthe oryzae* are often associated with *F. fujikuroi*, hampering pure isolation of *F. fujikuroi* from rice. In this study, we modified a selective medium for *F. fujikuroi* as supplementing both pentachloronitrobenzene and hydrogen peroxide into minimal medium. This medium efficiently suppressed the vegetative growth of *F. graminearum* and *M. oryzae*, but did not significantly reduce *F. fujikuroi* growth, providing an efficient tool for isolating *F. fujikuroi*.

Our working hypothesis was that hydrogen peroxide (H₂O₂) might be used for selective isolation of *F. fujikuroi* because *Fusarium* species showed different levels of resistance to toxoflavin which produces H₂O₂ in eukaryotic cells (Jung et al., 2013;...
Kim et al., 2004). We expected that it is possible to reduce the growth of certain fungal species as supplementing both H$_2$O$_2$ and PCNB into a medium. As a result, this study showed that the vegetative growth of F. graminearum and M. oryzae was completely inhibited but F. fujikuroi can grow on the medium containing both PCNB and H$_2$O$_2$. This result provides an efficient tool for F. fujikuroi isolation as inhibiting the growth of other pathogens including F. graminearum and M. oryzae, and can be applicable for characterizing population dynamics of F. fujikuroi in rice fields.

For this study, 77 F. fujikuroi strains were isolated from rice seeds collected from four provinces, Gangwon, Gyeonggi, Chungnam, and Chungbuk in 2013. Each fungal strain was identified based on morphological characteristics including pigmentation, hyphal growth, and spores on potato dextrose agar (PDA), carnation leaf agar, and carrot agar (Leslie and Summerell, 2006). The morphological characteristics was compared with the phylogenetic characteristics obtained as sequencing partial translation elongation factor 1-alpha (TEF) DNA sequences amplified with the PCR primers, ef1 (forward primer; 5'-ATGGGTAAGGAG(A/G)GACAAGAC-3') and ef2 (reverse primer; 5'-GGAG(A/G)GTACAGT(G/C)ATCATGT-3') (O’Donnell et al., 1998). The TEF sequences were compared to the Fusarium-ID with standard nucleotide BLAST (http://isolate.fusariumdb.org/). F. graminearum strains GJ90 and IS40 previously isolated in Korea (Jung et al., 2013), and GZ3639 and SKX04 strains provided from Center for Fungal Pathogenesis (Seoul, Korea) were used for reference strains of F. graminearum. M. oryzae strains were provided from Center for Fungal Genetics Resources (Seoul, Korea).

To prepare PCNB-H$_2$O$_2$ medium, 0.75 g of PCNB (Sigma-Aldrich) dissolved in 75 ml of ethanol was combined with 1 l of minimal medium (MM) agar (Leslie and Summerell, 2006). The medium was autoclaved and allowed to cool to 50°C before adding 0 to 5 mM H$_2$O$_2$ (Sigma-Aldrich). After inoculating each fungal strain onto the medium, the plates were incubated at 25°C in dark condition and mycelial growth was measured every 24 h until 7 days after inoculation. The experiments were repeated three times with three replicates and Tukey test using SPSS 12.0 software (SPSS Inc., Chicago, USA) was performed to determine if the fungal isolates are significantly different.

On MM supplemented with H$_2$O$_2$, F. graminearum and F. fujikuroi strains were well grown, but F. graminearum produced less carmine red pigment as supplementing PCNB. As supplementing H$_2$O$_2$, while pigmentation of F. fujikuroi was not affected by the concentration of H$_2$O$_2$, tested in this study. On the other hand, M. oryzae strains were more sensitive to H$_2$O$_2$, where its growth was reduced as supplementing H$_2$O$_2$ and completely inhibited at 5 mM H$_2$O$_2$ (Fig. 1). This result showed that fungal pathogens isolated from rice have different sensitivity against H$_2$O$_2$ and F. fujikuroi strains are more tolerant to H$_2$O$_2$ than F. graminearum and M. oryzae strains.

On peptone-PCNB agar (PPA) (Leslie and Summerell, 2006), all F. graminearum strains were well grown as much as peptone agar (PA), but the growth of F. fujikuroi and M. oryzae strains were slightly reduced compared to that on PA (Table 1). Fungal strains tested in this study did not form distinctive colonies on PPA and we tested the fungal colony formation on different media containing PCNB. When F. graminearum and F. fujikuroi species were incubated on MM supplemented with PCNB, they produced similar colony pigmentation patterns as shown on MM without PCNB even though their mycelial growth was decreased responding to the addition of PCNB (Table 1). The growth of M. oryzae was significantly inhibited on MM containing PCNB even though it grew on PPA. This result showed that F. graminearum can be efficiently selected on MM-PCNB rather than PPA because the fungal strains produce carmine red pigment and the growth of other fungal competitors, such as F. fujikuroi and M. oryzae, on rice are inhibited.

We further tested the availability of H$_2$O$_2$ for isolating F. fujikuroi on MM containing PCNB. As increasing H$_2$O$_2$ concentration, the mycelia growth of F. graminearum significantly slowed down and it was completely inhibited at the addition of 5 mM H$_2$O$_2$. In the case of F. fujikuroi, its growth was not inhibited at the presence of H$_2$O$_2$. The growth of M. oryzae was completely inhibited at 3 mM H$_2$O$_2$ (Table 1). This result showed that the addition of H$_2$O$_2$ into MM-PCNB efficiently can inhibit the growth of F. graminearum and M. oryzae except for F. fujikuroi, providing a suitable medium for selecting F. fujikuroi.
Table 1. Vegetative growth of fungal strains on various media supplemented with either PCNB or H₂O₂

|          | PA     | P-PCNB | MM    | MM-PCNB | MM-PCNB-H₂O₂ |
|----------|--------|--------|-------|---------|---------------|
|          | 1 mM   | 3 mM   | 5 mM  |         |               |
| FG       | 50aw   | 50aw   | 50aw  | 41ax    | 14ay          |
| CF242    | 14bw   | 39bx   | 38bx  | 24by    | 26by          |
| CF375    | 44bw   | 37bx   | 41bxw | 26by    | 27by          |
| MO       | 38cw   | 32cx   | 33cx  | 9cy     | 7cy           |

PA, peptone agar; P-PCNB, PA supplemented with PCNB; MM, minimal medium; MM-PCNB, MM supplemented with PCNB; MM-PCNB-H₂O₂, MM supplemented with both PCNB and H₂O₂. 1 mM, 3 mM, and 5 mM indicate the concentration of H₂O₂ supplemented to the medium; FG, *Fusarium graminearum*; CF242 and CF375, *F. fujikuroi* strain CF242 and CF375; MO, *Magnaporthe oryzae*. The growth was measured five days after inoculation.

The same first (a, b, c) and last (w, x, y, z) letters denote no significant differences in a column and row, respectively, according to Tukey’s test (P<0.05).

Fig. 2. Resistance of *Fusarium fujikuroi* strains to different fungicides. The numbers in the parentheses indicate the number of resistant strains to the fungicide.

Out of 77 *F. fujikuroi* strains, 49, 23, and 26 strains were resistant to prochloraz, tebuconazole, and benomyl+thiram, respectively (Fig. 2). The data showed that the resistance against either prochloraz or tebuconazole was not significantly associated with PCNB-H₂O₂ resistance. However, the number of resistant strains against benomyl+thiram might be associated with PCNB-H₂O₂ resistance and especially the number of resistant strains against both prochloraz and benomyl+thiram was highly skewed toward non-resistance against PCNB-H₂O₂ resistance. This result suggested that the application of new fungicide into fields significantly affects the population dynamics of this fungus and the resistance pattern might be one of important characteristics to understand population structure of this fungus.

Although media containing PCNB have been widely used for isolation of strains belonging to the genus *Fusarium*, a selective medium for *F. fujikuroi* isolation has not been developed. In this study, we modified the MM as supplementing both PCNB and H₂O₂, to keep the vegetative growth of *F. fujikuroi* but to inhibit that of *F. graminearum* and *M. oryzae*. Approximately 85% of *F. fujikuroi* strains tested in this study were strongly resistant on the medium developed in this study but 15% were sensitive to the medium, suggesting that this medium might not be used for all field population. However, this medium can be applicable for population study to chase population dynamics related to fungicide resistance and also useful for field study examined with limited number of *F. fujikuroi* strains.

Acknowledgement

This work was supported by Rural Development Administration (PJ0098912015).

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