Detection of Sensitivity and Resistance Variation of *Magnaporthe grisea* to Kitazin P, Carbendazim and Tricyclazole

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Abstract: One hundred and twenty-nine isolates of *Magnaporthe grisea* from Guangdong, Guangxi, Anhui and Jiangsu provinces of China were tested for resistance frequency to kitazin P and carbendazim, respectively by the distinctive concentration method. The resistance frequency of the isolates to kitazin P which had not been used in practice for about ten years was as high as 79.1%, and only one carbendazim-resistant isolate was detected in Gaoyao, Guangdong Province (with a frequency of 0.78%). Meanwhile, the minimum inhibition concentration (MIC) of hyphal melanization was adopted to detect the sensitivity of *M. grisea* to tricyclazole. There existed several different degrees of sensitivity to tricyclazole in the melanin biosynthesis of *M. grisea*, but no relationship was found between these MIC values completely inhibiting melanization in hyphae and the EC₅₀ values of tricyclazole against rice blast tested in vivo. After the isolates were induced by chemical taming or UV irradiation in laboratory, kitazin P-resistant and carbendazim-resistant mutants were recovered by both the methods, but none of tricyclazole-resistant mutant was obtained.

Key words: *Magnaporthe grisea*; fungicide resistance; fungicide; mutant; kitazin P; carbendazim; tricyclazole

Rice blast caused by ascomycete fungus *Pyricularia grisea* (teleomorph: *Magnaporthe grisea*) was one of the most economically important diseases of rice in China, for which chemical control by fungicides was adopted as an integrant measure to reduce its damage. In the early 20th century, copper-based fungicides were used in China, but they had not satisfied efficacy against blast disease and often harmed rice plants. During 1980s organophosphorus fungicides such as kitazin (EBP), kitazin P (IBP) and isoprothiolane (FJ-one) with different chemical structures but similar action mechanism were widely used. Melanin biosynthesis inhibitor tricyclazole has been introduced as a substitute till now after severe resistance occurred in field for organophosphorus fungicides. Carbendazim (MBC) was used as an auxiliary chemical against blast disease in practice by mixing with other chemicals such as sulphur.

Evolution and development of fungicide resistance depend on the ‘fungicide-pathogen’ interaction. In the above blasticides, IBP almost had not been used in China for ten years for the resistance problem. But FJ-one which had cross- resistance with organophosphorus fungicides was still sporadic used in some regions. Plant pathogen fungi of many genera have developed resistance to MBC [¹], but none record about sensitivity status of *M. grisea* to this chemical was reported. Control efficiency decrease of tricyclazole, which was suspected to be caused by resistance, was reported in some rice growing regions in China. Because tricyclazole at a concentration less than 20 μg/mL had no direct inhibitory effect on growth or spore germination of *M. grisea* [²], so sensitivity could only be detected through testing median effective concentration (EC₅₀) of the isolates in vivo. However, this sensitivity assay technique was both too much time- and labor-consuming and with large un-avoided errors. Therefore, the objectives of this study were to detect the resistance frequency of *M. grisea* to IBP and MBC by the distinctive concentration method, attempt to detect the sensitivity of *M. grisea* to tricyclazole by adopting the minimum inhibition concentration (MIC) of hyphal melanization (MIC-H), and finally to study the resistance variation of *M. grisea* to the above three chemicals by taming in plates amended with fungicide and UV radiation.

Received: 8 June 2004; Accepted: 5 September 2004
MATERIALS AND METHODS

Fungal isolates and chemicals

MBC (98%, supplied by Shenyang Chemical Research Institute) was dissolved in 0.1 mol/mL HCl to $10^4 \mu$g/mL; 68% IBP was supplied by Shanghai Jiangnan Chemical Company; 90% tricyclazole was supplied by Changzhou Fengdeng Pesticide Factory and dissolved in methanol to $10^4 \mu$g/mL. One hundred and twenty-nine tested single-spored strains were isolated from disease samples collected from Guangdong, Guangxi, Anhui and Jiangsu Provinces in 2000.

Medium and inoculation

Conidia used as inoculums in all experiments were obtained from colonies grown on TPSA medium (tomato, 150 g; potato, 200 g; sucrose, 20 g; agar, 35 g and H$_2$O, 1 L) as described before [3]. The spore suspensions contained 0.1% Tween 20 was adjusted to 10$^5$ spores/mL and inoculated onto rice seedlings at 3- to 5-leaf stage by the foliar spraying method [3]. Rice variety Suyunuo, highly sensitive to blast disease, was grown in a RXZ chamber under dark conditions with a relative humidity (RH) of 100% for 24 h after inoculation, and then under the conditions with a photoperiod of 12 h light/12 h dark and 80% RH. Disease rating was recorded 7 days after inoculation [3]. PSA slants were used to store strains. YPSA medium (yeast extracts, 5.0 g; potato starch, 10.0 g; sucrose, 20 g; agar, 20 g and H$_2$O 1 L) was introduced in all other experiments. All the cultures were conducted at 25°C in a dark growth chamber.

Detection of resistance frequency of $M$. grisea to IBP and MBC

In this experiment, 5 $\mu$g/mL was adopted as a distinctive concentration to detect the resistance frequency of $M$. grisea to MBC [4]. Mycelial plugs (Φ 5 mm) were recovered from the colony margins which had been grown on YPSA medium for 10 days, and then transferred to YPSA plates amended with 5 $\mu$g/mL MBC. After cultured at 25°C for seven days, isolate which could grow on the YPSA plates at 5 $\mu$g/mL MBC was regarded as a MBC-resistant mutant. YPSA plates not amended with MBC were adopted as check.

Resistance frequency of $M$. grisea to IBP was detected as described by Peng et al. [5]. After cultured at 25°C for seven days, isolate which could not grow quickly at YPSA plates amended with 30 $\mu$g/mL IBP was regarded as sensitive (S); which could grow quickly at 30 $\mu$g/mL but could not grow at 50 $\mu$g/mL as low resistant (LR); which could grow quickly at 50 $\mu$g/mL but could not grow at 100 $\mu$g/mL as moderately resistant(MR) and which could grow quickly at 100 $\mu$g/mL as highly resistant(HR). YPSA plates without IBP were adopted as check. Relative growth rate (RGR) was determined for each isolate and every distinctive concentration, respectively and percentage of isolates belonged to different RGR range was calculated as. RGR = (growth rate at the distinctive concentration/growth rate at check) × 100%.

Determination of MIC of hyphal melanization (MIC-H) by tricyclazole

Mycelial plugs (Φ 5 mm), recovered from the colony margins which had been grown on YPSA medium for 10 days, were transferred to YPSA plates amended with 0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 $\mu$g/mL tricyclazole, respectively. YPSA plates not supplemented with MBC were adopted as check. After cultured at 25°C for ten days, the color of medium and hyphae was observed one by one. The minimum dose at which an isolate seemed just like that of buf-mutants with a buff color was the MIC-H of this isolate.

Determination of $EC_{50}$ of tricyclazole against the blast disease

Rice seedlings were sprayed 24 h before inoculation with 20, 10, 5, 2.5, 1.5 and 0 $\mu$g/mL tricyclazole containing $\leq 0.1%$ Tween 20, with two replications for every treatment. Disease severity was investigated 7 days after inoculation [3]. $LD_p$-line and $EC_{50}$ of each isolate were achieved as general.
Resistance variation of *M. grisea* to kitazin P, carbendazim and tricyclazole

The experiment was carried out by using two different methods with isolate DY2 of *M. grisea*.

1. **Taming in plates amended with fungicide.** Mycelial plugs (Φ 2.5 mm), recovered from the colony margins which had been grown on YPSA medium for 10 days, were transferred to YPSA plates containing $5 \, \mu g/mL$ MBC, $100 \, \mu g/mL$ IBP, and $200 \, \mu g/mL$ tricyclazole, respectively. The formation of spontaneous, fast-growing sectors from the restricted colonies on YPSA plates was examined 30 days later. Each sector was then transferred to YPSA plates supplemented with the same fungicide at the same concentration as previous. Seven days after, which could normally grow was regarded as spontaneous resistant mutant. Three repeats for every experiment were carried out to compare the frequency of sectoring and fungicide resistant mutation between different chemicals.

2. **UV mutagenesis.** The spore suspensions ($10^5$ spores/mL) were spilt into YPSA plates amended with $5 \, \mu g/mL$ MBC, $100 \, \mu g/mL$ IBP, and $1 \, \mu g/mL$ or $200 \, \mu g/mL$ tricyclazole (0.2 mL suspensions per plate), respectively. After incubated at 25°C for 2 h, plates were put under a UV lamp (40 W, 254 nm) for 30 s, for which approximately 5% spores survived. Then plates were taken back into a chamber at 25°C in dark. After five to seven days, the colony, which could expand, was considered as a fungicide resistant mutant. For tricyclazole, the colony kept gray to black as that in YPSA plates without tricyclazole also was regarded as a resistant mutant. Then each resistant mutant was respectively transferred to plate supplemented with the same fungicide at the same concentration to determine the purity of resistance.

**Determination of the resistance level of MBC-resistant mutants**

Sensitivity of each MBC-resistant mutant, including mutants from the field and mutants from chemical taming and UV mutagenesis, was tested by growth rate method \[6\].

**RESULTS**

Frequency and distribution of resistance to MBC and IBP in *M. grisea*

In the tested isolates from the different rice cropping regions, one MBC-resistant mutant was detected in the isolates from Gaoyao, Guangdong with a 0.78% resistance frequency. This suggested that low frequency resistance occurred in *M. grisea* to MBC.

The resistance frequency of the tested populations ($n=129$) to IBP was 79.1% (Table 1), with moderately resistant (MR) and high resistant (HR) frequencies 39.5% and 32.6%, respectively. There was significant difference in resistance frequency between different rice cropping areas. Resistance problem in Yuexi, Anhui was the most serious, with a HR frequency 66.7%. HR mutant was detected in all the six tested regions except for Changshu, Jiangsu.

<table>
<thead>
<tr>
<th>City/Province</th>
<th>Population</th>
<th>Host cultivars</th>
<th>No. of isolates</th>
<th>Resistance frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LR</td>
</tr>
<tr>
<td>Gaoyao, Guangdong</td>
<td>GY</td>
<td>Qisanzhan, Boyou 903</td>
<td>15</td>
<td>40.0</td>
</tr>
<tr>
<td>Nanning, Guangxi</td>
<td>NN</td>
<td>Jinyou 99, Shanyou 63</td>
<td>18</td>
<td>0.0</td>
</tr>
<tr>
<td>Changshu, Jiangsu</td>
<td>CS</td>
<td>Wuyujing 1</td>
<td>21</td>
<td>0.0</td>
</tr>
<tr>
<td>Danyang, Jiangsu</td>
<td>DY</td>
<td>Wuyujing 7</td>
<td>27</td>
<td>33.3</td>
</tr>
<tr>
<td>Wangjiang, Anhui</td>
<td>WJ</td>
<td>Liangyoupeijiu</td>
<td>21</td>
<td>28.6</td>
</tr>
<tr>
<td>Yuexi, Anhui</td>
<td>YX</td>
<td>Xieyou 63</td>
<td>27</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>—</td>
<td>129</td>
<td>7.0</td>
</tr>
</tbody>
</table>
At the dose of 30 and 50 μg/mL IBP, most isolates had a large RGR value, with mean RGR of 55.8% and 34.7%, respectively (Fig. 1), implying that IBP at these two doses already had no anti-fungus activity. Although the HR frequency was as high as 32.6%, but mean RGR for the population (n=129) was only 11.7% at the dose of 100 μg/mL. This indicated that 100 μg/mL IBP still had good activity against the growth of *M. grisea*.

**Stability of MIC-H by tricyclazole**

MIC-H of 12 isolates selected randomly was determined in continuous ten generations for each isolate. Results showed that MIC-H was very stable (data in detail not shown), such as 0.1 μg/mL for each generation of isolate WJ6. Some studies reported that the primary action mechanism was inhibiting melanin biosynthesis in fungi \[^{2,6,7}\]. Thus, MIC-H could be used to detect the sensitivity of *M. grisea* to tricyclazole.

**MIC-H distribution of the tested population**

MIC-H distribution curve of the tested population had three peaks at 0.05, 0.2 and 0.8 μg/mL (Fig. 2). But 48.9% of the tested isolates had a MIC-H ≤0.1 μg/mL, and no isolate with a MIC-H>1 μg/mL was detected. Such observations pointed out that melanin biosynthesis of *M. grisea* was still very sensitive to tricyclazole although the tested population already had different sensitivity phenotypes. The primary action target of tricyclazole was tridroxynaphthalene reductase in *M. grisea* \[^7\]. It suggested that variation in tridroxynaphthalene reductase might have occurred already.

**Relationship between MIC-H and EC\textsubscript{50} of tricyclazole against the blast disease tested \textit{in vivo}**

Some studies reported that inhibition of melanin biosynthesis lead to that *M. grisea* has no enough mechanical force to penetrate the cuticular layer of the host \[^{2,6,7}\]. According to this disease control mechanism, less sensitivity of melanin biosynthesis to inhibitor tricyclazole \textit{in vitro}, namely more high MIC-H, then *M. grisea* would be more insensitive to tricyclazole, namely EC\textsubscript{50} be more high. But this correlation between MIC-H and EC\textsubscript{50} tested \textit{in vivo} was not observed as expected in our case of study (Fig. 3). Therefore, MIC-H could not be used to detect the sensitivity of *M. grisea* to tricyclazole.
Resistance variation of *M. grisea* to kitazin P, carbendazim and tricyclazole

Kitazin P-resistant and carbendazim-resistant mutants were recovered both through chemical taming and UV irradiation, but none of tricyclazole-resistant mutant was found (Table 2). This suggested that tricyclazole-resistance mutation had occurred in *M. grisea* at a very low frequency. Highly significant difference was observed in sectoring frequency and resistance-mutation frequency in blast fungus to the three fungicides in the taming study *in vitro*. The highest mutation frequency for sectoring and resistance-mutation was both belonged to kitazin P. Average 2 sectors for kitazin P had one indeed kitazin P-resistant mutant and average 5 sectors for MBC had one indeed MBC-resistant mutant. These suggested that part of the sectors was caused by physiological fitness other than genetic mutation. All the resistance mutants were continuously cultured on YPSA for 5 generations afterwards, and their sensitivities to respective fungicide had not changed, suggesting that the mutants were induced by genetic mutations.

Sensitivity levels to MBC *in vitro* of MBC-resistant mutants from chemical taming and UV irradiation and one MBC-resistance isolate GY5 from the field were shown in Table 3. *EC₅₀* of the field sensitive isolate DY2, which was the parent for induced mutation, was 0.1096 μg/mL. There was no significant difference in sensitivities of the five spontaneous MBC-resistance mutants (DY2-1 to DY2-5) recovered in taming study *in vitro*, with *EC₅₀* ranged from 2.0373 to 2.6385 μg/mL and resistance ratios from 18.5885- to 20.0739-fold. GY5 had the similar sensitivity to MBC with the five spontaneous resistance mutants, with *EC₅₀* 2.6085 μg/mL. Mutants DY2-6 and DY2-7 from UV radiation were the most insensitive to MBC, with resistance ratios 83.5246- and 81.9297-fold, respectively. This suggested that genotypes with different resistance levels existed in *M. grisea* for MBC.

<p>| Table 2. Resistance variation of isolate DY2 to kitazin P, carbendazim and tricyclazole. |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Fungicide</th>
<th>No. of mycelia plugs</th>
<th>Sectoring mutant</th>
<th>Resistance mutant</th>
<th>UV mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitazin P</td>
<td>156</td>
<td>15</td>
<td>9.62 Bbᵇ</td>
<td>8</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>168</td>
<td>26</td>
<td>15.48 Aa</td>
<td>5</td>
</tr>
<tr>
<td>Tricyclazole</td>
<td>420</td>
<td>0</td>
<td>0.00 Cc</td>
<td>0</td>
</tr>
</tbody>
</table>

Within a column, data followed by different uppercase and lowercase letters indicate significant difference at 0.01 and 0.05 levels, respectively.

ᵇResistance mutants from sectors;
ᶜComparing means done in SPSS 11.0;
ᶜNone of resistance mutant recovered.
Fitness always would decrease when a fungus developed resistance to fungicide. Thus, sensitive population would be dominant along with the decreasing resistance frequency if the fungicide was stopped using in practice \[1\]. This study showed that the resistance frequency of \textit{M. grisea} to kitazin P, which has not been used in practice for about ten years, was as high as 79.1%. This might attribute to FJ-one that has developed cross-resistance with kitazin and kitazin P \[5\] also has been used in some regions till now. Results indicated that 100 μg/mL IBP still had high activity against the growth of \textit{M. grisea} according to RGR. However, resistance variation showed that \textit{M. grisea} still expressed a high resistance mutation frequency to IBP at this concentration, suggesting that IBP should not be adopted again in practice to control rice blast disease.

Peng et al \[5\] only used one distinctive concentration (30 μg/mL) to divide \textit{M. grisea} into two phenotypes, i.e. resistance (R) and sensitive (S). Our study showed that \textit{M. grisea} could be divided into four classes, i.e. sensitive (S), low resistant (LR), moderately resistant (MR) and high resistant (HR), according to their growth status on plates amended with 30, 50, and 100 μg/mL IBP, respectively.

In this study, one field resistance isolate to MBC, an important auxiliary chemical against blast disease, was detected with a frequency of 0.78%. \textit{M. grisea} developed resistance to MBC slowly and with a low frequency might be due to the secondary function of MBC in against blast disease and low mutation frequency in blast fungus. This study showed that the resistance frequency to MBC by UV induction in \textit{M. grisea} was 10^{-7}, but as high as 10^{-4} in \textit{Sclerotiorum sclerotiorum} and \textit{Botrytis cinerea} \[8\]. Meanwhile, eight MBC-resistant mutants could be grouped into two phenotypes, i.e. low resistant (LR), including GY5 and DY2-1 to DY2-5, and moderately resistant (MR), DY2-6 and DY2-7 \[9\].

Whether resistance had occurred in \textit{M. grisea} to tricyclazole was an important problem that should be identified at a sudden. Huang et al (1999) detected the resistance to tricyclazole in \textit{M. grisea} through a growth rate inhibition test \[10\], but tricyclazole had no direct inhibitory effect on growth or spore germination of \textit{M. grisea} \[2\]. In our study, attempt was adopted to test the sensitivity of \textit{M. grisea} to tricyclazole \textit{in vitro} according to its disease control mechanism. Results showed there were different sensitivity degrees of melanin biosynthesis in hypha to inhibitor tricyclazole. Further investigation was needed to clarify this attribute to natural difference in isolates or variation in tridroxynaphthalene reductase. However, 48.9% of the tested isolates had a MIC-H ≤0.1 μg/mL, and none isolate with a MIC-H > 1 μg/mL was detected. These suggested that melanin biosynthesis of \textit{M. grisea} was still very sensitive to inhibitor tricyclazole. Meanwhile, none of tricyclazole-resistant mutants was got by chemical

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype^a</th>
<th>LD-p line</th>
<th>Co-efficiency ($R^2$)</th>
<th>EC\text{50} (μg/mL)</th>
<th>Resistance ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>GY5</td>
<td>FR</td>
<td>Y=4.4158X+3.1613</td>
<td>0.9469</td>
<td>2.6085 Bb</td>
<td>23.8002 Bb</td>
</tr>
<tr>
<td>DY2-1</td>
<td>LR</td>
<td>Y=4.4119X+3.6365</td>
<td>0.9845</td>
<td>2.0373 Bb</td>
<td>18.5885 Bb</td>
</tr>
<tr>
<td>DY2-2</td>
<td>LR</td>
<td>Y=3.0612X+3.8912</td>
<td>0.9912</td>
<td>2.3026 Bb</td>
<td>21.0091 Bb</td>
</tr>
<tr>
<td>DY2-3</td>
<td>LR</td>
<td>Y=4.2181X+3.2227</td>
<td>0.9707</td>
<td>2.6385 Bb</td>
<td>24.0739 Bb</td>
</tr>
<tr>
<td>DY2-4</td>
<td>LR</td>
<td>Y=4.5965X+3.3998</td>
<td>0.9874</td>
<td>2.2291 Bb</td>
<td>20.3385 Bb</td>
</tr>
<tr>
<td>DY2-5</td>
<td>LR</td>
<td>Y=3.7979X+3.4265</td>
<td>0.9095</td>
<td>2.5960 Bb</td>
<td>23.6861 Bb</td>
</tr>
<tr>
<td>DY2-6</td>
<td>LR</td>
<td>Y=1.1023X+3.94</td>
<td>0.9522</td>
<td>9.1543 Aa</td>
<td>83.5246 Aa</td>
</tr>
<tr>
<td>DY2-7</td>
<td>LR</td>
<td>Y=1.2364X+3.8214</td>
<td>0.9849</td>
<td>8.7975 Aa</td>
<td>81.9297 Aa</td>
</tr>
<tr>
<td>DY2</td>
<td>S</td>
<td>Y=0.7576X+5.7247</td>
<td>0.9704</td>
<td>0.1096 Cc</td>
<td>—</td>
</tr>
</tbody>
</table>

Within a column, data followed by different uppercase and lowercase letters indicate significant difference at 0.01 and 0.05 levels, respectively.

^aFR, Resistant isolate from field; LR, Resistant isolate in the laboratory; S, Wild sensitive isolate.
taming in vitro or UV irradiation, indicating that tricyclazole-resistant mutation occurred at a very low risk in Magnaporthe grisea, with a frequency significantly lower than that of IBP and MBC. This result might attribute to the action mechanism of tricyclazole. Both IBP and MBC had one target in Magnaporthe grisea, but tricyclazole had two different targets, namely tridroxynaphthalene reductase and tetradroxynaphthalene reductase.\(^\text{[11]}\). But this didn’t mean that resistance would not develop for tricyclazole. Shen et al (1995) reported that sensitivity would decrease when Magnaporthe grisea was continuous tamed in vivo by using tricyclazole as a selection pressure.\(^\text{[12]}\). Meanwhile, as the major chemical against blast disease and without satisfied substitute fungicide, problem would be very serious once Magnaporthe grisea develops resistance to tricyclazole. Therefore, tricyclazole should be used wisely in agriculture according to results of sensitivity monitoring, and it would be necessary to look for a more quick and convenient method for sensitivity detecting.

According to reported disease control mechanism for tricyclazole, the less sensitivity of melanin biosynthesis to inhibitor tricyclazole in vitro\(^\text{[2,6,7,13]}\), the more insensitive reaction of Magnaporthe grisea to tricyclazole. But no correlation between MIC-H values completely inhibiting melanization in hypha and the EC\(_{50}\) values of tricyclazole against rice blast tested in vivo was observed in our study. The reason may involve: 1) tricyclazole has some other action mechanism irrelevant to melanin biosynthesis\(^\text{[13,14]}\); 2) MIC could not correctly reflect the sensitivity of melanin biosynthesis to tricyclazole; 3) Principle of the MIC-H study is that the medium accumulated buff pigment resulted from oxidation product of tridroxynaphthalene when tridroxynaphthalene reductase was inhibited by tricyclazole.\(^\text{[11]}\). Therefore, MIC-H tested in this study could not reflect the sensitivity of tetradoxynaphthalene reductase to tricyclazole. However, succeeding study was needed to clarify this hypothesis.

REFERENCES