Five carboxin-resistant mutants from *Aspergillus oryzae* were characterized by the sensitivities of their mycelial growth and succinate dehydrogenase (SDH) activity to carboxin and three related fungicides. Despite a significant resistance to carboxin, exhibited by all the mutants, their patterns of sensitivity to the other fungicides was distinct. This provides clues to the molecular interaction between SDH and these fungicides.

Key words: carboxin; carboxamide fungicide; succinate dehydrogenase

Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) is a well-established fungicide that inhibits basidiozymecete fungi in particular. To date, many carboxin-related fungicides with carboxanilide as the base structure have been developed. Although the oxathiin ring is the specific structure of carboxin, it is replaced by a benzene ring in the derivative fungicides, flutolanil (α,α,α-trifluoro-3′-isopropoxy-o-toluaniilde) and o-toluaniilde, and by a pyridine ring in boscalid (2-chloro-N-(4′-chlorobiphenyl-2-yl)nicotinamide) (Fig. 1A–D). While most members of this group of fungicides have specific inhibitory activity on basidiomycete fungi in particular. To date, many carboxin-related fungicides with carboxanilide as the base structure have been developed. Although the oxathiin ring is the specific structure of carboxin, it is replaced by a benzene ring in the derivative fungicides, flutolanil (α,α,α-trifluoro-3′-isopropoxy-o-toluaniilde) and o-toluaniilde, and by a pyridine ring in boscalid (2-chloro-N-(4′-chlorobiphenyl-2-yl)nicotinamide) (Fig. 1A–D). While most members of this group of fungicides have specific inhibitory activity on basidiomycete fungi, boscalid is effective on a broader spectrum of filamentous fungi, including the *Ascomycetes* and *Deuteromycetes*. Carboxin and related chemicals prevent mitochondrial respiration by interfering with electron transfer from succinate to quinone on succinate dehydrogenase (SDH). The SDH–carboxin binding model indicates that carboxin binds to the site where quinone normally binds to SDH. The model also indicates that the carboxin- and quinone-binding sites are comprised of residues in subunits of SdhB, SdhC, and SdhD. Resistance to carboxin and related fungicides is reportedly conferred by mutations in three genes encoding the subunits of SdhB, SdhC, and SdhD. The amino acid positions replaced in these three different types of mutants are located around the carboxin–binding site in SDH, but are not adjacent to each other. The physiological differences among the carboxin-resistant mutations in the *SdhB*, *SdhC*, and *SdhD* genes have not been clarified, because until recently no species had been found in which all three types of mutants were available. In a previous study, however, we screened carboxin-resistant mutants from *Aspergillus oryzae* and identified five different types of mutants carrying a mutation in either *SdhB*, or *SdhC*, or *SdhD* (Table 1). In this study, the sensitivity of the five types of carboxin-resistant mutants of *A. oryzae* to carboxin and to three carboxin-related fungicides, o-toluaniilde, flutolanil, and boscalid, was analyzed in terms of mycelial growth and SDH activity.

Carboxin and related fungicides were examined to clarify the inhibitory effect on mycelial growth of wild type strain RIB40 and the five carboxin-resistant mutants of *A. oryzae*, shown in Table 1, that were isolated previously. To examine the sensitivity of mycelial growth to fungicides, AY plate medium (144 mM acetate-acetic acid buffer at pH 6.5, 5 g/L of yeast extract and 10 g/L of agar) with carboxin (Wako, Osaka, Japan), o-toluaniilde (Sigma-Aldrich, St. Louis, MO), flutolanil (Wako), or boscalid (Sigma-Aldrich) added at various concentrations was used, and the mycelial growth rate was evaluated by measuring the colony diameter after 3 d of incubation at 28 °C. Without fungicide, the mycelial growth rates of the wild type and the mutants were almost equal (Fig. 1A). The mycelial growth rate of the wild type strain was completely inhibited by carboxin even at a concentration of 200 μM, whereas the three *sdhB*-type mutants showed strong resistance to carboxin, and the *sdhC* and *sdhD* mutants showed weak resistance (Fig. 1A). The mycelial growth rate of the wild type strain was only somewhat limited by o-toluaniilde, although the chemical structure highly resembles that of carboxin (Fig. 1B). No mutants showed more vigorous growth on the o-toluaniilde-containing medium (Fig. 1B). Because flutolanil was insoluble in the AY medium at 200 μM and higher concentrations, cultures with added flutolanil only at 50 μM and 100 μM concentrations were examined in the mycelial growth test. Flutolanil did not show a significant inhibitory effect on the mycelial growth of the wild type or the mutants (Fig. 1C), although an equivalent concentration of carboxin significantly inhibited the growth of the wild type. Boscalid inhibited the mycelial growth of the wild type and of the *sdhB-N*, *sdhC*, and *sdhD* mutants even at a concentration of 20 μM, while the *sdhB-Y* and *sdhB-L* mutants showed clear resistance to boscalid (Fig. 1D). The strong inhibitory effect of boscalid and growth restoration by the *sdhB*-type mutations should make possible a more efficient gene
transformation system than the system we have established using carboxin and the mutant sdhB genes.\textsuperscript{15)

The inhibitory effects of carboxin, \textit{o}-toluanilide, flutolanil, and boscalid on the SDH activity of the five carboxin-resistant mutants was investigated by a previously described method.\textsuperscript{15) The electron transfer activity of SDH was evaluated as SDH activity by measuring the succinate-cytochrome \textit{c} reductase activity of the crude mitochondrial membrane fraction. To determine the sensitivity of the SDH to the various fungicides, SDH activity was measured and expressed as a percentage of the basal SDH activity (Fig. 2A–D). Boscalid exhibited the highest inhibitory effect among the fungicides examined, inhibiting the SDH activity of the wild type at the remarkably low concentration of 2 µM. A carboxin or flutolanil concentration of 200 µM reduced the SDH activity of the wild type to less than 30%, indicating that the SDH of the wild type was sensitive to carboxin and flutolanil. The activity of the wild type was also reduced by \textit{o}-toluanilide, but retained 67% of basal level even at 200 µM. The three sdhB-type mutants showed different responses in terms of SDH activity to these fungicides. The SDH of the sdhB-L mutant was clearly resistant to carboxin, flutolanil, and boscalid, that of the sdhB-Y mutant was resistant to carboxin and boscalid but sensitive to flutolanil, and that of the sdhB-N was resistant to carboxin and flutolanil, but, unlike the other two sdhB-type mutants, was sensitive to boscalid. The SDH of both the sdhC and the sdhD mutant showed low-level resistance to carboxin, but they were highly sensitive to boscalid. Sensitivity to \textit{o}-toluanilide and to flutolanil was, however, quite different between these two mutants. The SDH activity of the sdhC mutant was less sensitive to \textit{o}-toluanilide and to flutolanil, while that of the sdhD mutant was as sensitive or more sensitive than that of the wild type.

These experiments revealed the specific sensitivities of the different types of carboxin-resistant mutants to carboxin-related fungicides. Interestingly, the carboxin sensitivity of the SDH of the various mutants was not correlated with their sensitivities to the other fungicides examined. These differences in fungicide-specific sensi-

Table 1. Carboxin-Resistant Mutants of A. oryzae

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>Strain name in previous study\textsuperscript{15)</th>
<th>Subunit</th>
<th>Amino acid position of mutation</th>
<th>Residue in the wild type (codon)</th>
<th>Mutated residues (codon)</th>
<th>Accession no. of encoding mutant gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>sdhB-Y</td>
<td>CR4</td>
<td>SdhB</td>
<td>249</td>
<td>His (CAC)</td>
<td>Tyr (TAC)</td>
<td>AB435538</td>
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<tr>
<td>sdhB-L</td>
<td>CR9</td>
<td>SdhB</td>
<td>249</td>
<td>Leu (CTC)</td>
<td>Asn (AAC)</td>
<td>AB435540</td>
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<tr>
<td>sdhB-N</td>
<td>CR14</td>
<td>SdhB</td>
<td>249</td>
<td>Asn (AAC)</td>
<td>Tyr (TAC)</td>
<td>AB435541</td>
</tr>
<tr>
<td>sdhC</td>
<td>CR6</td>
<td>SdhC</td>
<td>90</td>
<td>Thr (ACC)</td>
<td>Ile (ATC)</td>
<td>AB449813</td>
</tr>
<tr>
<td>sdhD</td>
<td>CR7</td>
<td>SdhD</td>
<td>124</td>
<td>Asp (GAC)</td>
<td>Glu (GAG)</td>
<td>AB449815</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of Carboxamide Fungicides on the Mycelial Growth of A. oryzae Wild Type Strain and Carboxin-Resistant Mutants.

Conidia of the A. oryzae wild type strain and carboxin-resistant mutants were incubated on AY medium with added carboxin (A), \textit{o}-toluanilide (B), flutolanil (C), and boscalid (D) at the indicated concentrations. All values are averages for three independent determinations.

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activity among the mutants are probably related to the structural differences between the fungicides. In spite of the high structural similarity between o-toluanilide and carboxin, o-toluanilide exhibited remarkably weaker inhibitory activity than carboxin, suggesting that replacement of the methyl-oxathiin ring of carboxin with the benzene ring of o-toluanilide is responsible for the difference in activity. Structural analysis has revealed that the oxygen in the methyl-oxathiin ring of carboxin is localized close to SdhB-His207 of E. coli, which corresponds to the amino acid substitution in the sdhB-type carboxin-resistant mutants of A. oryzae and other organisms.5,10,15,16 This indicates that the position corresponding to the oxygen in the methyl-oxathiin ring is important to the specific inhibition effect of carboxanilides. In boscalid, the corresponding position is occupied by the nitrogen of the 2-chloropyridine ring, and the sensitivity of boscalid was affected by the mutation in SdhB-His249 of A. oryzae, indicating that the nitrogen is highly effective in the interaction between carboxanilide compounds and SdhB-His249. Although resistance to both carboxin and boscalid is conferred by mutations at the conserved His residue in SdhB in various organisms, the amino acid replacements for resistance to these fungicides are different. Carboxin resistance is conferred by replacements by Leu, Asn, and Tyr, while boscalid resistance was conferred by amino acid replacement by Arg, Leu and Tyr, but not Asn (Figs. 1 and 2).12,15 Flutolanil, as well as carboxin, exhibited the potential to inhibit the SDH activity of wild type A. oryzae, and was more effective in this regard than o-toluanilide (Fig. 2). The replacement by the CF3 group on the benzene ring of flutolanil may be responsible for the higher inhibitory activity of flutolanil than of o-toluanilide, as described by Ohsumi et al. (1988).17 As with boscalid, flutolanil resistance to SDH activity was conferred by particular mutations within the mutations conferring carboxin resistance. Two of the three sdhB-type carboxin resistant mutations also conferred flutolanil resistance on their SDH activities, suggesting that the chemical structure that corresponds to the CF3 group in flutolanil and to the methyl group in the oxathiin ring of carboxin is recognized by SdhB-His249 when SDH interacts with carboxanilide compounds. The sdhC mutation also conferred significant resistance to flutolanil and o-toluanilide on SDH, although no direct interaction between carboxin and the amino-acid residue of SdhC-Thr90, which is mutated in the sdhC mutant, has been found.6,15 Replacement by the benzene ring in carboxanilide compounds may increase the relative importance of the amino acid residue at position 90 of SdhC to recognize the compound. In boscalid, the position corresponding to the methyl group of carboxin is occupied by the chloro group, but the effect of the substitution could not be evaluated in our experiments. The replacement by the chloro group on the benzene ring confers lower fungicidal activity on carboxanilide chemicals than by methyl- or the CF3-group,17 suggesting that more effective fungicides can be developed by replacements of this position in boscalid.

In conclusion, our study suggests that among carboxin-related fungicides, the chemical structures that correspond to the oxathiin ring of carboxin are important in determining the sensitivity of carboxin-resistant mutants to carboxin-related fungicides. The development of fungicide-resistant populations is a serious risk in the management of plant pathogens in agriculture. By considering the chemical structure-specific sensitivity of various types of mutants as investigated here, new fungicides that are less likely to engender resistant mutants might be developed.

**Fig. 2.** Effects of Carboxamide Fungicides on the SDH Activity of the A. oryzae Wild Type Strain and Carboxin-Resistant Mutants.

Mitochondrial membrane fractions were prepared from the A. oryzae wild type strain and carboxin-resistant mutants, and the electron transfer activities of SDH were evaluated by measuring succinate-cytochrome c reductase activity. A–D. SDH activity with added carboxin, o-toluanilide, flutolanil, and boscalid at the indicated concentrations. Activity without fungicides was defined as 100%.
References