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1 Title: Characterization of mutants with single and combined Q<sub>i</sub> and Q<sub>o</sub> site mutations in *Saccharomyces*  
2 *cerevisiae* reveals interactions between the picolinamide fungicide CAS-649 and azoxystrobin

3

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8

9 ABSTRACT

10 Picolinamide and strobilurin fungicides bind to the Q<sub>i</sub> and Q<sub>o</sub> sites on cytochrome *b*, respectively, and  
11 target many of the same plant pathogens. Using *Saccharomyces cerevisiae* as a model system, we explore  
12 effects of amino acid changes at each site on sensitivity to a fungicide acting at the opposite site and  
13 examine the relationship between altered sensitivity and growth penalty. In addition, double mutants  
14 containing the G143A or F129L mutations responsible for strobilurin resistance in combination with Q<sub>i</sub>  
15 site mutations that confer resistance to picolinamides are characterized in terms of their sensitivity to QiI  
16 and QoI fungicides and growth rate. Mutants containing amino acid changes at the Q<sub>o</sub> site varied in their  
17 growth rate and sensitivity to the picolinamide CAS-649, and increased sensitivity was associated with a  
18 greater growth penalty. Conversely, changes at the Q<sub>i</sub> site affected sensitivity to azoxystrobin and also  
19 showed a correlation between increased sensitivity and reduced growth. There was no overall correlation  
20 between resistance to azoxystrobin and CAS-649 among mutants, however negative cross-resistance  
21 occurred in the case of mutations which conferred resistance to either compound and also carried a  
22 growth penalty. These results suggest the use of QoI fungicides to delay the emergence of pathogen  
23 resistance to QiIs, and vice versa. Double mutants containing G143A or F129L in combination with Q<sub>i</sub>  
24 site changes N31K, G37C/V or L198F that cause resistance to picolinamides generally exhibited lower  
25 resistance factors for both azoxystrobin and CAS-649 than corresponding resistant strains with a single  
26 mutation. Reduced growth was observed for all F129L-containing double mutants, whereas the growth

27 rate of double mutants containing G143A was significantly reduced only by the Q<sub>i</sub> site mutations N31K  
28 and G37V that confer a larger growth penalty. Our results suggest that resistance to picolinamides in  
29 pathogens could emerge more readily in a strobilurin-sensitive genetic background than in a strobilurin-  
30 resistant one.

31

32 Keywords:

33 Picolinamide fungicide

34 CAS-649

35 Florylpicoxamid

36 Strobilurin

37 Azoxystrobin

38 Resistance mutation

39

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## 44 **1. Introduction**

45 The cytochrome  $bc_1$  complex (complex III) of the mitochondrial electron transport chain represents  
46 one of the most successful biochemical targets for commercial fungicides and contains two distinct  
47 binding sites, known as the  $Q_o$  and  $Q_i$  sites, which are located on cytochrome  $b$  (cyt  $b$ ) on opposite sides  
48 of the inner mitochondrial membrane and correspond to the quinol oxidation and reduction sites,  
49 respectively. Compounds are classified as QoI (Quinone outside Inhibitor) or QiI (Quinone inside  
50 Inhibitor) fungicides based on their site of action (Sierotzki, 2015). QoIs originated from the strobilurin  
51 class of natural products and are commonly referred to as “strobilurin” fungicides. First introduced in  
52 1996, the strobilurin fungicides represent a large class of active ingredients and control a broad spectrum  
53 of diseases caused by ascomycete, basidiomycete and oomycete pathogens. Resistance to QoIs is now  
54 widespread and associated primarily with two cyt  $b$  mutations at the  $Q_o$  site, G143A and F129L. The  
55 G143A mutation produces a very high level of resistance and has caused a loss in field efficacy in at least  
56 42 pathogens (Sierotzki, 2015). Although less common than G143A and associated with lower resistance  
57 factors, the F129L mutation is the main cause of strobilurin resistance in *Phakopsora pachyrhizi* (soybean  
58 rust), *Pyrenophora teres* (net blotch in barley), *Alternaria solani* (potato/tomato early blight) and *Pythium*  
59 *aphanidermatum* (Pythium blight of turfgrass) (Sierotzki, 2015; Klosowski et al., 2016).

60 The picolinamide fungicides fenpicoxamid, florylpicoxamid and metarylpicoxamid represent a new  
61 chemical class and are the first QiIs to target ascomycete and basidiomycete pathogens. Fenpicoxamid is  
62 derived from the natural product UK-2A and was recently introduced to control Septoria wheat leaf  
63 blotch (*Zymoseptoria tritici*) and black Sigatoka (*Pseudocercospora fijiensis*) in banana (Owen et al.,  
64 2017). Inspired by UK-2A, florylpicoxamid is a fully synthetic molecule which shares the same key  
65 binding interactions at the  $Q_i$  site as the natural product (Meyer et al., 2021a, 2021b). Florylpicoxamid  
66 will extend the use of picolinamides to control additional pathogens, including *Botrytis*, *Colletotrichum*  
67 and *Alternaria* species, as well as powdery mildew diseases (Yao et al., 2021). The development of a  
68 third picolinamide, metarylpicoxamid, was recently announced for control of asian soybean rust (Meyer  
69 et al., 2022).

70 It can be assumed that target site-based resistance to picolinamides will eventually emerge in the field  
71 as it has for other complex III inhibitors, and the particular mutations will be determined by the level of  
72 resistance and any associated fitness penalty. Lab mutants with resistance to picolinamides have been  
73 identified in yeast and *Z. tritici*. In yeast, N31K, G37L/I/C/V/M and L198F conferred resistance (Young  
74 et al., 2018, 2022) while G37V was isolated in *Z. tritici* (Fouché et al., 2022). Most diseases which will be  
75 managed by application of picolinamide fungicides involve pathogens in which strobilurin resistance is  
76 already present in the population. In this context, for resistance risk assessment and management strategy  
77 it is important to consider the phenotype associated with Q<sub>i</sub> site mutations in both QoI sensitive and  
78 resistant genetic backgrounds. Since the Q<sub>o</sub> and Q<sub>i</sub> sites are spatially separated, a mutation at one site  
79 cannot directly impact binding of an inhibitor at the opposite site. However, the two sites are functionally  
80 linked and actively coupled such that ligand binding at one site might impact the opposite site (Cooley et  
81 al., 2009). Intriguingly, negatively-correlated cross-resistance between picolinamides and strobilurins has  
82 been reported in *Zymoseptoria tritici* (Fouché et al., 2022). Also, double mutants of *Z. tritici* containing  
83 both the G37V Q<sub>i</sub> site mutation and the G143A Q<sub>o</sub> site mutation showed a reduced level of resistance to  
84 both QiI and QoI fungicides as compared to the corresponding single mutants.

85 The commercial use of QoI and QiI fungicides to control the same diseases presents a unique situation  
86 among fungicides in which two inhibitors act at different sites on the same target protein. In this study,  
87 using the yeast *Saccharomyces cerevisiae* as a model organism, we explore the relationship between  
88 effects of mutations at the Q<sub>i</sub> and Q<sub>o</sub> sites on growth rate, as a measure of “fitness”, and altered sensitivity  
89 to fungicides. To assess the phenotype of Q<sub>i</sub> site mutants in strobilurin-resistant genetic backgrounds we  
90 also examine the levels of resistance and growth rate of strains containing combinations of Q<sub>o</sub> and Q<sub>i</sub> site  
91 mutations. Possible implications of our findings for resistance development and strategies for use of QiI  
92 and QoI fungicides in resistance management are discussed.

93

94

## 95 2. Materials and methods

### 96 2.1. Strains and media

97 The following media were used for the growth of *S. cerevisiae*: YPD (1% yeast extract, 2% peptone,  
98 3% glucose), YPG (1% yeast extract, 2% peptone, 3% glycerol), and transformation medium (0.7% yeast  
99 nitrogen base, 3% glucose, 2% agar, 1 M sorbitol and 0.8 g of a complete supplement mixture minus  
100 uracil per litre, supplied by Anachem).

101 Mutants containing the single *cyt b* changes G37C, L198F and N31K were isolated by selection on  
102 fenpicoxamid or antimycin A (Young et al., 2018). All other mutants were generated by site-directed  
103 mutagenesis and mitochondrial transformation. The following *S. cerevisiae* strains were used for  
104 transformation and screening: i) W303-1B MAT alpha *ura3 his3 leu2 ade2 trp1 rho*<sup>0</sup> (lacking  
105 mitochondrial DNA), used as recipient strain for biolistic transformation; ii) CKPF3 MAT a *leu1 kar1-1*  
106 with an intronless mitochondrial DNA and a short deletion ( $\Delta$ 158-161) in *CYTB*, the *cyt b* encoding gene,  
107 rendering the cells unable to growth on respiratory medium. The strain used for the direct selection of  
108 fungicide resistant mutants (Young et al., 2018) was a diploid issued from the crossing between W303-1B  
109 MAT alpha *ura3 his3 leu2 ade2 trp1 rho*<sup>0</sup> and CKWT MAT a *leu1 kar1-1* with an intronless  
110 mitochondrial DNA (and wild-type *CYTB*), thus isogenic to CKPF3 with the exception of the *CYTB*  
111 deletion.

### 112 2.2. Site-directed mutagenesis, biolistic transformation, and selection of mitochondrial mutants

113 Wild-type intronless *CYTB* with its 350 bp upstream sequence was cloned in the pCRscript vector  
114 (Stratagene). The chosen *CYTB* mutations were then introduced using the Quickchange site-directed  
115 mutagenesis method (Stratagene) according to the manufacturer's recommendations. After verification of  
116 the sequence, the plasmids carrying the mutated *CYTB* were used for biolistic transformation.

117 Mitochondrial transformation by microprojectile bombardment was adapted from Bonnefoy et al., 2007.  
118 Approximately 3  $\mu$ g of the plasmids carrying mutated *CYTB* and 0.5  $\mu$ g of plasmid YEP352 (which  
119 contains the *URA3* gene, allowing the selection of Ura<sup>+</sup> nuclear transformants) were mixed with 50  $\mu$ l of  
120 0.7- $\mu$ m tungsten particles (at a concentration of 60 mg ml<sup>-1</sup>). The particles (supplied by Bio-Rad) were

121 prepared and coated with DNA according to the manufacturer's protocol. Aliquots of the coated particles  
122 were used for transformation of the recipient strain W303-1B rho<sup>0</sup>. Around 10<sup>7</sup> cells of the recipient strain  
123 were evenly spread on transformation medium (selective for Ura<sup>+</sup> transformants). The particle  
124 bombardments were performed with a Biolistic PDS-1000/He particle delivery system (Bio-Rad)  
125 according to the manufacturer's recommendations. Colonies of Ura<sup>+</sup> transformants (which have received  
126 plasmid YEP352) appeared after 4 to 5 days of incubation. Ura<sup>+</sup> colonies (>1,000) were then crossed with  
127 the respiratory-deficient CKPF3 and the diploids replica-plated on respiratory medium (YPG).  
128 Respiration-competent diploids (usually 1/500 diploids tested) were selected and subcloned. Their  
129 respiratory competence resulted from homologous recombination between *CYTB* carrying Δ158-161 and  
130 plasmid-derived *CYTB* carrying the chosen mutations. The sequencing of *CYTB* from the respiratory  
131 competent diploids showed that the new mutations were incorporated while Δ158-161 was replaced by  
132 the wild-type sequence.

### 133 2.3. *Evaluation of kinetic growth rate*

134 The method used to measure the growth rate of yeast strains in 96-well microtiter plates was based on  
135 the technique described by Held in an application note from Agilent Technologies  
136 (<https://www.agilent.com/cs/library/applications/monitoring-growth-of-saccharomyces-cerevisiae-5994-3284EN-agilent.pdf>). Cells from overnight cultures grown on YPD medium at 30 °C were suspended in  
137 YPEth broth (10 g of yeast extract, 20 g of Bacto peptone and 2% (v/v) of ethanol per liter) and adjusted  
138 to A<sub>600</sub> = 0.3 in a 1 cm cuvette. Cell suspensions (100 μl) were added to wells of a 96-well flat-bottom  
139 tissue culture plate (Cat. No. 655180, Greiner Bio-One, Monroe, NC, USA) with 8 replicate wells per  
140 strain. The plate was sealed with Breathe-Easy<sup>TM</sup> sealing film (Cat. No. BEM-1, Diversified Biotech,  
141 Dedham, MA, USA). Growth curves were measured in a Cytation 3 instrument (BioTek Instruments,  
142 Winooski, VT, USA) at 30 °C using continuous double orbital shaking at 425 cpm with 3 mm amplitude  
143 and A<sub>600</sub> measurements taken at 1 h intervals. Growth rates were determined during exponential growth  
144 over the 6 - 11 h period after start of the experiment using Excel as described by Bio-Resource  
145 (<https://www.youtube.com/watch?v=adYWnTPPWIM>) and used to calculate doubling time (Dt), as a

147 more intuitive expression of growth, using the equation  $Dt = \ln(2)/\text{growth rate}$ . All experiments were  
148 repeated at least twice for each strain with 8 replicates per experiment and the mean growth rates  $\pm$  SD  
149 were determined across all replicates.

#### 150 2.4. *Fungicide sensitivity of cytochrome b mutants*

151 The experimental system used to measure kinetic growth rate was also employed in fungicide  
152 sensitivity tests so that relationships between the two parameters could be compared under the same  
153 growth conditions. CAS-649, the active metabolite of florylpicoxamid, and azoxystrobin were chosen to  
154 represent the QiI and QoI fungicide classes, respectively. CAS-649 (Chemical Abstracts Service Registry  
155 Number: 1961312-07-1) was preferred in this study over the natural product UK-2A, the active  
156 metabolite of fenpicoxamid, due to its greater stability in culture medium (unpublished results).  
157 Azoxystrobin was selected as a strobilurin with similar *in vitro* fungitoxicity to CAS-649 against yeast.  
158 CAS-649 was synthesized at Corteva Agriscience and azoxystrobin was obtained from Chem Service,  
159 Inc., West Chester, PA. CAS-649 or azoxystrobin were dissolved in DMSO and diluted 1000-fold into  
160 YPEth broth to the desired concentration. Two-fold dilutions were prepared in YPEth broth containing  
161 0.1% DMSO then 50  $\mu$ l aliquots were dispensed into a 96-well plate. Wells were inoculated with 50  $\mu$ l of  
162 cell suspension prepared at  $A_{600} = 0.6$  with 3 replicate wells at each concentration per strain. The plate  
163 was sealed and incubated for 19 h in the Cytation 3 instrument, as described above. 50% Effective  
164 concentration ( $EC_{50}$ ) values for growth inhibition were determined based on initial (1 h measurement) and  
165 final  $A_{600}$  readings using the CALCUSYN program (Biosoft, Cambridge, UK). Strains were tested in two  
166 or more independent experiments and average  $EC_{50}$  values  $\pm$  SD determined across all replicates.  
167 Sensitivity to cycloheximide (product number C7698, Sigma-Aldrich, Inc., St. Louis, MO) was  
168 determined similarly except that each strain was tested in one experiment with 3 replicate wells at each  
169 concentration.

#### 170 2.5. *Statistical Analysis*

171 The SigmaStat module of SigmaPlot v 14.5 (Inpixon) was used for all analyses. The Holm-Sidak multiple  
172 comparison procedure was used to compare the effects of mutations on doubling time and fungicide



173 sensitivity. A nonlinear regression model of exponential growth was used to examine the relationship  
174 between fungicide sensitivity and growth rate. Differences in mean doubling time and fungicide  
175 resistance factors between single Qi site mutants and double mutants containing Qo and Qi site mutations  
176 were analyzed using the Student's *t*-test.

177

### 178 **3. Results**

#### 179 *3.1. Effect of amino acid exchange at the Q<sub>o</sub> site on growth rate and sensitivity to CAS-649*

180 Doubling time (Dt) for the parent strain was 4.26 h and Dt values for the Q<sub>o</sub> site mutants ranged from  
181 4.04 to 15.53 h (Fig. 1A). Strains with the G143A and F129L mutations showed the smallest changes in  
182 growth rate as compared to the parent strain (Dt = 4.26 h); Dt of the F129L mutant increased to 5.24 h  
183 and did not change significantly for the G143A mutant (Dt = 4.04 h).

184 Amino acid changes at the Q<sub>o</sub> site increased sensitivity to CAS-649 by 1.5 to 7.5-fold (Fig. 1B, Table  
185 1), and the increase in sensitivity was correlated ( $R^2 = 0.793$ ) with a reduction in growth rate (Fig. 2). The  
186 F129L mutant showed a 2.9-fold increase in sensitivity compared to the parent strain and, interestingly,  
187 the G143A mutant was also slightly more sensitive (1.5-fold) than the parent although its growth rate was  
188 not reduced (Fig. 1B, Table 1).

189 For Q<sub>o</sub> site mutants, the correlation between growth penalty and increased sensitivity to CAS-649  
190 resulted in negatively correlated cross-resistance between azoxystrobin and CAS-649 in the case of  
191 mutations which cause strobilurin-resistance and also carry a growth penalty, e.g. F129L and G143S  
192 (Table 1). However, there was no overall correlation between sensitivity of the mutant set to azoxystrobin  
193 and to CAS-649 (Table 1). This is consistent with discrete binding sites for QoI and QiI fungicides which  
194 implies that a mutation at one site which causes resistance by interfering with fungicide binding should  
195 not affect ligand binding interactions at the opposite site.

#### 196 *3.2. Effect of amino acid exchange at the Qi site on growth rate and sensitivity to azoxystrobin*

197 Since the mutations known to affect resistance to the picolinamide fungicides involve N31, G37 and  
198 L198, a set of mutants with changes at these positions and different growth rates (Young et al., 2022) was  
199 chosen for study.

200 Dt values for the  $Q_i$  site mutants ranged from 4.46 to 7.14 h (Fig. 3A), and mutants displaying a  
201 growth penalty showed substantially increased sensitivity to azoxystrobin, ranging from 5.8 (N31G) to  
202 15.2-fold (G37V) (Fig. 3B, Table 2). Mutants which did not show a statistically significant change in  
203 growth rate exhibited only small changes in sensitivity to azoxystrobin, ranging from 0.8 (L198V) to 3.2-  
204 fold (L198G). As in the case of  $Q_o$  site mutants, a significant correlation ( $R^2 = 0.800$ ) was observed  
205 between the increase in sensitivity of  $Q_i$  site mutants to the inhibitor acting at the opposite site  
206 (azoxystrobin) and a reduction in growth rate (Fig. 2). Also, similar to the  $Q_o$  site mutation effects, while  
207 there was no overall correlation between sensitivity to CAS-649 and to azoxystrobin among the  $Q_i$  site  
208 mutants (Table 2), those mutations which conferred resistance to CAS-649 and also carried a growth  
209 penalty (e.g. G37V/C and N31K) caused negatively correlated cross-resistance to azoxystrobin.

210

### 211 3.3. *Growth rate and fungicide sensitivity of double mutants with amino acid exchange at both $Q_i$ and* 212 *$Q_o$ sites*

213 L198F, G37C, G37V and N31K changes at the  $Q_i$  site confer resistance to picolinamide fungicides  
214 (Young et al., 2018, 2022). Strains containing these single changes were compared with corresponding  
215 double mutant strains which also contained the G143A or F129L changes at the  $Q_o$  site.

216 All double mutants containing a  $Q_i$  site mutation in combination with F129L showed a significant  
217 increase in Dt as compared to corresponding single  $Q_i$  site mutants (Fig. 4). With the exception of the  
218 N31K/F129L double mutant, the level of resistance to CAS-649 was lower in all F129L double mutants  
219 (2.0 to 4.6-fold) than in corresponding single mutants (Table 3), and the levels of resistance to  
220 azoxystrobin were lower by 1.5 to 6.4-fold (Table 4).

221 G143A-containing double mutants with  $Q_i$  site changes G37V and N31K showed an increase in Dt  
222 relative to the respective single  $Q_i$  mutant strains, but the combination of G143A with G37C or L198F did

223 not significantly affect Dt (Fig. 4). As in the case of F129L double mutants, with the exception of the  
224 N31K/G143A double mutant, the level of resistance to CAS-649 was lower in all G143A double mutants  
225 (1.6 to 2.6-fold) than in corresponding single mutants (Table 3), and levels of resistance to azoxystrobin  
226 were lowered by 1.5 to 13.5-fold (Table 4).

227 Single and double mutants were also tested for sensitivity to cycloheximide to assess whether the  
228 slower-growing mutants might show a general increase in sensitivity to fungicidal compounds. No  
229 relationship was found between sensitivity to cycloheximide and Dt (Supplemental Fig. S1).

230

#### 231 **4. Discussion**

232 Analysis of Q<sub>o</sub> site mutations showed an increase in sensitivity to CAS-649 for all mutants and a clear  
233 correlation between growth penalty and sensitivity. These data raise the question of whether the  
234 introduction of picolinamide fungicides might alter the proportion of strobilurin-resistant and sensitive  
235 genotypes in pathogen populations. Sensitivity of the G143A mutant to CAS-649 increased only slightly,  
236 by 1.5-fold. If a similar increase in sensitivity occurred in a pathogen containing G143A, this would be  
237 unlikely to affect the size of the resistant population given the general fitness of G143A genotypes and  
238 very high resistance factors, which provide a very strong selection pressure in the presence of a  
239 strobilurin. Unlike G143A, the F129L mutation did cause a significant growth penalty in yeast and a  
240 greater increase (2.9-fold) in sensitivity to CAS-649. Consistent with the increased Dt of the yeast mutant,  
241 in pathogens it is generally assumed that F129L carries a greater fitness penalty than G143A (Sierotzki,  
242 2015). Doubling time of the yeast F129L mutant increased to 5.24 h as compared to 4.26 h for the parent  
243 strain. Over multiple generations in the absence of fungicide selection pressure, a similar growth penalty  
244 could translate to a considerable difference in population size; in yeast for example this difference in  
245 doubling time would translate to a 3.6-fold difference in population size between the parent and resistant  
246 strains over 10 generations of the parent, when growing exponentially. Given the much lower resistance  
247 factors associated with F129L-containing pathogen populations, which imply lower selection pressure  
248 than for G143A, and reduced fitness, even a small increase in sensitivity to picolinamide fungicides might

249 reduce the size of F129L strobilurin-resistant populations and perhaps help to maintain longer term utility  
250 of strobilurins. It would be interesting to test this hypothesis in field experiments against pathogens where  
251 F129L causes strobilurin resistance.

252 Similar to the effect of Q<sub>o</sub> site mutations on sensitivity to CAS-649, there was a clear correlation  
253 between reduced growth of Q<sub>i</sub> site mutants and increased sensitivity to azoxystrobin. Earlier studies  
254 (Young et al., 2018, 2022) showed that certain amino acid changes involving N31, G37 and L198 were  
255 associated with resistance to picolinamides in yeast and since changes to N31 were not tolerated without a  
256 large growth penalty, ones involving G37 and L198F seem to be of greatest potential concern for  
257 resistance development (Young et al., 2022). In the case of *Z. tritici*, lab studies suggest that the G37V  
258 mutation may be the most likely resistance mutation to develop in the field (Fouché et al., 2022). Unlike  
259 in yeast, the G37V mutation did not cause a growth penalty in *Z. tritici*, highlighting the fact that a given  
260 mutation will not necessarily produce the same fitness phenotype in different organisms, and that  
261 resistance mutations which emerge in the field might differ between pathogens.

262 The use of fungicide mixtures, or alternation, is a common strategy to delay resistance development  
263 and the negative cross-resistance between QiI and QoI fungicides observed for target site mutants which  
264 carry a growth penalty suggests that strobilurins could be particularly effective in delaying resistance to  
265 picolinamides. However, a QiI-QoI combination would be most advantageous in delaying resistance  
266 caused by Q<sub>i</sub> site mutations which confer a larger fitness penalty and might not apply in the case of a  
267 mutant with no fitness penalty. A recent development in QoI chemistry has been the discovery of  
268 molecules like metarylpicoxamid which are almost unaffected by the G143A and F129L mutations  
269 (Matsuzaki et al., 2019). Many recent patent applications also reveal ongoing interest from Ag companies  
270 in new QoIs, particularly in compounds which control resistant soybean rust strains containing the F129L  
271 mutation. As novel QoIs which overcome existing resistance are introduced, resistance is likely to  
272 develop via new Q<sub>o</sub> site mutations. The higher efficacy of picolinamides against Q<sub>o</sub> site mutants suggests  
273 their utility for resistance management in fungicide programs to slow the emergence of such mutants.

274 The introduction of picolinamides to control pathogens where a proportion of the population is already  
275 resistant to strobilurins could lead to  $Q_i$  site resistance mutations in QoI-sensitive, QoI-resistant or both  
276 genotypes. Analysis of yeast mutants containing both  $Q_o$  and  $Q_i$  site changes showed an increased growth  
277 penalty for most double mutants as compared to single mutants. This was true for all combinations of  $Q_i$   
278 site mutation with F129L and for the combination of N31K or G37V with G143A. L198F and G37C had  
279 little effect on Dt in single mutants, and were the only  $Q_i$  site changes which in combination with G143A  
280 did not produce a statistically significant growth penalty. Double mutants containing G37C/V or L198F  
281 were more sensitive to both CAS-649 and azoxystrobin than corresponding single mutants. The combined  
282 effects of increased growth penalty for most double mutants and lower resistance factors suggest that  
283 resistance to picolinamides may be more likely to emerge in strobilurin-sensitive than in resistant  
284 populations. Nevertheless, strains resistant to both picolinamides and strobilurins could develop if the  
285 individual  $Q_i$  and  $Q_o$  site mutations carried little or no fitness penalty. Assuming a greater fitness penalty  
286 for F129L than for G143A, double mutants resistant to both QoI and QiI fungicides seem less likely to  
287 appear in pathogens where F129L is the cause of strobilurin resistance than in those where it results from  
288 the G143A change.

289 The mechanism by which a mutation at the  $Q_i$  or  $Q_o$  site increases sensitivity to an inhibitor acting at  
290 the opposite site is unclear. However, the observed correlation between increased sensitivity and growth  
291 penalty, and lack of such correlation for cycloheximide, strongly suggests the effect is specific to  
292 cytochrome  $bc_1$  inhibitors. The simplest explanation is that the increased sensitivity of  $Q_o$  mutants to  
293 CAS-649, and of  $Q_i$  mutants to azoxystrobin, results from decreased activity of the cytochrome  $bc_1$   
294 complex making the cell more dependent on the residual activity and consequently more sensitive to  
295 inhibitors. An alternative explanation is based on interactions between the  $Q_o$  and  $Q_i$  sites (Cooley et al.,  
296 2009; Cooley, 2010). It was reported that in yeast, when stigmatellin was bound at the  $Q_o$  site, only one of  
297 the two  $Q_i$  sites was accessible to the  $Q_i$  site inhibitor antimycin and that a conformational change was  
298 needed to enable binding to the remaining  $Q_i$  site (Covian and Trumpower, 2006). Also, inhibitor binding  
299 or mutations at the  $Q_i$  site of *Rhodobacter capsulatus* have been shown to increase susceptibility of the

300 Rieske iron-sulfur protein to proteolysis (Valkova-Valchanova et al., 2000) and affect the interaction  
301 between the Rieske protein and the Q<sub>o</sub> site (Cooley et al., 2005). Thus, despite their locations on opposite  
302 sides of the membrane there is communication between the Q<sub>o</sub> and Q<sub>i</sub> sites involving conformational  
303 change which could be affected by mutations and result in altered inhibitor sensitivity. These two possible  
304 explanations are not mutually exclusive and detailed biochemical studies will be needed to explore the  
305 underlying mechanism(s) responsible for the observed mutation effects.

306 An important question is the extent to which results from a yeast model will translate to plant  
307 pathogens. Cytochrome *bc*<sub>1</sub> is highly conserved across eukaryotic organisms and although some  
308 mechanistic details remain unclear, it can be assumed to function similarly in yeast and in fungal  
309 pathogens. Consequently, in pathogens similar effects of mutations at the Q<sub>i</sub> or Q<sub>o</sub> site on sensitivity to  
310 inhibitors acting at the opposite site are expected. This is supported by analysis of *Z. tritici* lab mutants  
311 containing the G37V Q<sub>i</sub> site change, where negative cross-resistance was found between azoxystrobin  
312 and the picolinamide UK-2A for G37V and G143A mutants (Fouché et al., 2022). Also, as in the current  
313 yeast work, *Z. tritici* double mutants with G37V and G143A had lower resistance factors for both Q<sub>i</sub>Is  
314 and Q<sub>o</sub>Is. The fact that this could be observed in *Z. tritici* where G143A is not generally believed to carry  
315 a fitness penalty in the field is noteworthy. The slight increase in sensitivity to picolinamides in G143A  
316 mutants of both yeast (Fig. 1B) and *Z. tritici* (Fouché et al., 2022) may reflect a small effect of the G143A  
317 mutation on cytochrome *bc*<sub>1</sub> activity which did not result in a detectable effect on growth.

318 QoI and QiI fungicides are also used to control Oomycete diseases and resistance has emerged to both  
319 fungicide classes. In grape downy mildew, resistance to the QiI cyazofamid is associated with L201S and  
320 with two insertions, E203-DE-V204 and E203-VE-V204 (R4P, 2020). Another Q<sub>i</sub> site mutation, S34L,  
321 confers resistance to ametoctradin (Fontaine et al., 2019), which is believed to act at both the Q<sub>o</sub> and Q<sub>i</sub>  
322 sites (Dreinert et al., 2018). These mutations are found in some of the same populations of *Plasmopara*  
323 *viticola* where strobilurin-resistance exists due to the G143A mutation. In *P. viticola*, the G143A mutation  
324 does appear to cause some loss in fitness since QoI-resistant strains have been shown to revert to  
325 sensitivity in the absence of selection pressure from strobilurins (Genet et al., 2006). To our knowledge,

326 the combination of G143A with Q<sub>i</sub> site resistance mutations in the same strain has not yet been reported  
327 in *P. viticola*. Considering the lower growth rates observed for Q<sub>o</sub>/Q<sub>i</sub> double mutants in yeast, it will be  
328 interesting to see whether or not double mutants do eventually contribute to resistance problems in grape  
329 downy mildew in the field since a greater fitness penalty could be expected.

330 The picolinamide Q<sub>i</sub> fungicides provide a new mode of action against many ascomycete and  
331 basidiomycete pathogens and it is imperative to preserve their efficacy for as long as possible by  
332 implementing appropriate resistance management strategies. One element of resistance management is the  
333 use of mixtures or alternation with fungicides which have a different mode of action to reduce selection  
334 pressure. The present studies in yeast reveal specific interactions between Q<sub>o</sub> and Q<sub>i</sub> site mutations which  
335 could be exploited through the use of picolinamide-strobilurin mixtures or alternation for resistance  
336 management.

337

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345

### 346 **Supplementary material**

347 Supplementary data may be found in the online version of this article.

348

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- 422

423 **FIGURE LEGENDS**

424

425 **Fig. 1.** Effect of Q<sub>o</sub> site amino acid exchange on doubling time (A) and sensitivity to CAS-649 (B). Data  
426 are mean values ± SD. Different letters next to bars indicate a significant difference between strains ( $\alpha =$   
427 0.05).

428

429 **Fig. 2.** Correlation between growth rate of Q<sub>o</sub> and Q<sub>i</sub> site mutants and their sensitivity to fungicides  
430 acting at the opposite site. Circles represent growth rate of Q<sub>o</sub> site mutants and their sensitivity to CAS-  
431 649; crosses represent growth rate of Q<sub>i</sub> site mutants and their sensitivity to azoxystrobin. R<sup>2</sup> values were  
432 determined using an exponential growth nonlinear regression model.

433

434 **Fig. 3.** Effect of Q<sub>i</sub> site amino acid exchange on doubling time (A) and sensitivity to azoxystrobin (B).  
435 Data are mean values ± SD. Different letters next to bars indicate a significant difference between strains  
436 ( $\alpha = 0.05$ ).

437

438 **Fig. 4.** Doubling time of mutants containing Q<sub>i</sub> site mutations, with and without the Q<sub>o</sub> site mutations  
439 G143A and F129L. Data are mean values ± SD. Asterisks indicate statistically significant differences  
440 between double mutants with G143A or F129L and strains with no Q<sub>o</sub> site mutation and the  
441 corresponding Q<sub>i</sub> site allele (Student's t-test,  $P = < 0.001$ ).

442

443

444 **Table 1**445 Sensitivity of cytochrome *b* mutants with Q<sub>o</sub> site amino acid exchange to CAS-649 and azoxystrobin.

446

Amino Acid Change	CAS-649 EC50 ± SD (µg ml <sup>-1</sup> )	Azoxystrobin EC50 ± SD (µg ml <sup>-1</sup> )
None	0.0082 ± 0.0020	0.0076 ± 0.001
G143A	0.0054 ± 0.0008	16.30 ± 1.95
F129L	0.0028 ± 0.0002	0.38 ± 0.05
F129S	0.0022 ± 0.0001	0.052 ± 0.005
L275S	0.0017 ± 0.0005	0.071 ± 0.003
G143S	0.0013 ± 0.0006	>20
Y132C	0.0011 ± 0.0005	0.036 ± 0.01
Y279S	0.0011 ± 0.0003	0.042 ± 0.007

447

448

449 **Table 2**450 Sensitivity of cytochrome *b* mutants with Q<sub>i</sub> site amino acid exchange to azoxystrobin and CAS-649.

451

Amino Acid Change	Azoxystrobin EC50 ± SD (µg ml <sup>-1</sup> )	CAS-649 EC50 ± SD (µg ml <sup>-1</sup> )
None	0.0076 ± 0.0010	0.0082 ± 0.0020
L198V	0.0095 ± 0.0017	0.0089 ± 0.0006
L198F	0.0059 ± 0.0011	0.22 ± 0.05
G37A	0.0058 ± 0.0011	0.069 ± 0.006
G37C	0.0027 ± 0.0002	0.21 ± 0.02
L198G	0.0024 ± 0.0008	0.0063 ± 0.0006
N31G	0.0013 ± 0.0001	0.047 ± 0.015
N31K	0.0009 ± 0.0001	1.96 ± 0.22
N31S	0.0008 ± 0.0001	0.022 ± 0.003
G37V	0.0005 ± 0.0001	0.45 ± 0.12

452

453

454

455 **Table 3**

456 Resistance factors for CAS-649 in single Qi mutants and corresponding double (Qi + Qo) mutants

457 containing G143A or F129L.

458

Qi site change	Qo site change	Resistance Factor $\pm$ SD
none	none	1.00 $\pm$ 0.24
none	G143A	0.67 $\pm$ 0.10*
none	F129L	0.35 $\pm$ 0.03*
L198F	none	26.63 $\pm$ 6.00
L198F	G143A	13.30 $\pm$ 3.08*
L198F	F129L	5.81 $\pm$ 0.78*
G37C	none	26.32 $\pm$ 2.57
G37C	G143A	16.05 $\pm$ 2.38*
G37C	F129L	12.90 $\pm$ 4.43*
G37V	none	54.72 $\pm$ 14.48
G37V	G143A	21.04 $\pm$ 6.05*
G37V	F129L	21.60 $\pm$ 6.95*
N31K	none	240.3 $\pm$ 26.5
N31K	G143A	220.1 $\pm$ 41.3 NS
N31K	F129L	260.9 $\pm$ 56.2 NS

Asterisks indicate statistically significant differences in resistance factor between the double mutants with G143A or F129L and the corresponding strain with no Qo site mutation (Student's t-test,  $P = < 0.001$ ). NS = not significant.

459

**Table 4**

Resistance factors for azoxystrobin in single Qo mutants and corresponding double (Qo + Qi) mutants containing L198F, G37C/V and N31K.

Qo site change	Qi site change	Resistance Factor $\pm$ SD
none	none	1.00 $\pm$ 0.13
none	L198F	0.77 $\pm$ 0.14 NS
none	G37C	0.35 $\pm$ 0.03*
none	G37V	0.061 $\pm$ 0.01*
none	N31K	0.12 $\pm$ 0.02 *
G143A	none	2139.4 $\pm$ 255.9
G143A	L198F	1412.4 $\pm$ 250.9*
G143A	G37C	487.5 $\pm$ 101.3*
G143A	G37V	157.9 $\pm$ 35.4*
G143A	N31K	427.5 $\pm$ 174.3*
F129L	none	49.80 $\pm$ 6.76
F129L	L198F	33.61 $\pm$ 3.98*
F129L	G37C	28.61 $\pm$ 6.19*
F129L	G37V	7.80 $\pm$ 5.17*
F129L	N31K	17.95 $\pm$ 7.16*

460

461 Asterisks indicate statistically significant differences in resistance factor between the double mutants with

462 L198F, G37C/V and N31K and the corresponding strain with no Qi site mutation (Student's t-test, P = &lt;

463 0.001).

464