Enhanced fungicide resistance in *Isaria fumosorosea* following ionizing radiation-induced mutagenesis

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**Title:**
Enhanced fungicide resistance in *Isaria fumosorosea* following ionizing radiation-induced mutagenesis

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**Keywords:**
benzimidazoles; resistance level; fungicide compatibility; irradiation dose

**Running title:**
Ionizing radiation-induced mutagenesis in *Isaria fumosorosea*

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Abstract

The application of entomopathogenic fungi such as *Isaria fumosorosea* to combat insect pests on plants is complicated by their sensitivity to commonly used fungicides. In this study, *I. fumosorosea* mutants with enhanced resistance to the fungicide benomyl were induced by irradiation using either ion beams, gamma rays, or a combination of the two. When grown on agar containing benomyl, mycelial growth was observed for five of the six mutant isolates at benomyl concentrations that were more than 2000-fold those observed for the wild-type isolate (EC$_{50}$: > 5000 mg L$^{-1}$ c.f. EC$_{50}$: 2.5 mg L$^{-1}$ for the wild-type isolate). The mutant isolates evaluated also showed enhanced resistance to other fungicides at recommended field application rates. No differences were observed at the β-tubulin locus between the wild-type and the mutant isolates, suggesting that the enhanced benomyl resistance was not attributable to mutations in that gene. Ion beams and gamma rays are thus potentially useful tools for inducing beneficial fungal mutations and thereby improving the potential for application of entomopathogenic fungi as microbial control agents.
Introduction

Entomopathogenic fungi, such as *Isaria fumosorosea*, *Beauveria bassiana*, *Metarhizium anisopliae* and *Lecanicillium* spp., are all commercially available as microbial pesticides (Copping, 2009). However, entomopathogenic fungi can be negatively affected by fungicides, with the result that their biological control potential can be reduced if both products are used together (Clark *et al*., 1982; Loria *et al*., 1983; Saito & Yabuta, 1996; Pell *et al*., 2010; D’Alessandro *et al*., 2011). While the harmful effects of fungicides on entomopathogenic fungi are typically avoided by employing extended intervals between the application of each agent (Gardner *et al*., 1984; Bruck, 2009), these intervals can complicate the effective and practical use of both products in the field. One way to overcome this problem has been to develop fungicide-resistant isolates of entomopathogenic fungi by selection on chemically amended media (Shapiro-Ilan *et al*., 2002, 2011; Butters *et al*., 2003), by transformation (Pfeifer & Khachatourians, 1992; Inglis *et al*., 1999), or by exposure to mutagenic agents such as NaNO₂ (Zou *et al*., 2006; Song *et al*., 2011). Irradiation with ion beams is also a useful method for mutagenesis in microorganisms (Matuo *et al*., 2006; Tanaka *et al*., 2010; Toyoshima *et al*., 2012). For example, exposure of the entomopathogenic fungus *Cordyceps militaris* to ion beams successfully generated a mutant isolate capable of enhanced production of cordycepin, a medicinal adenosine analogue (Das *et al*., 2008, 2010). However, there is relatively little information in the literature on the use of ion-beam irradiation to induce mutations in entomopathogenic fungi and none considering induction of mutations conferring resistance to fungicides. Gamma-ray irradiation has also been demonstrated as a successful mutagenic agent in entomopathogenic fungi (Paccola-Meirelles & Azevedo, 1991; Kava-Cordeiro *et al*., 1995), though there are no reports describing enhanced fungicide-resistance induced by gamma-ray irradiation. In this study, an isolate of *I. fumosorosea* was subjected to ion-beam and/or gamma-ray irradiation to encourage the production of mutants with the potential for increased resistance to benomyl as a model fungicide. Levels of benomyl resistance in the mutants and their responses to other fungicides at recommended field application rates were also examined. Moreover, the β-tubulin locus of the mutants was sequenced, as benomyl resistance in *B. bassiana* is known to be conferred by a mutation at this locus (Butters *et al*., 2003; Zou *et al*., 2006).

Material and methods

Fungal preparation
A wild-type isolate (PF-3110) of *I. fumosorosea* that had been isolated from the sweet potato whitefly *Bemisia tabaci* B biotype in 1990 was cultured in Petri dishes (90 mm diameter) containing Sabouraud’s dextrose agar (SDA) (Difco, BD Biosciences, NJ) at 23 ± 1 °C for three weeks in darkness. Conidial suspensions were prepared by scraping the conidia/mycelia into sterile 0.1% Tween 80 and then filtering the mixture through sterile cloth (0.2 mm mesh size) to provide a suspension of conidia. Before experimentation the germination rate of conidia was determined on SDA at 23 ± 1 °C (>95%).

### Ion-beam and gamma-ray irradiation

Conidia of the wild-type isolate were irradiated with carbon-ion beams ($^{12}$C$^{5+}$, 121.8 keV µm$^{-1}$) accelerated by an azimuthally varying field cyclotron at the Takasaki Ion Accelerators for Advanced Radiation Application site (Gunma, Japan) and/or gamma rays ($^{60}$Co, 0.2 keV µm$^{-1}$) at the Food Irradiation Facility, Japan Atomic Energy Agency (Gunma, Japan), respectively.

### Fungicides against which mutants were evaluated

Eight commercial fungicides were used: benomyl [50% wettable powder (WP), Sumitomo Chemical, Japan], thiophanate-methyl (70% WP, Nippon Soda, Japan), iprodione (50% WP, Nippon Soda, Japan), diethofencarb (25% WP, Sumitomo Chemical, Japan), chlorothalonil (40% WP, Kumiai Chemical Industry, Japan), polycarbamate (75% WP, Dow Chemical Japan, Japan), myclobutanil [25% emulsifiable concentrate (EC), Dow Chemical Japan, Japan] and triflumizole (15% EC, Ishihara Sangyo Kaisha, Japan). Each fungicide was added to autoclaved SDA once it had cooled to below 50 °C and mixed using a stirrer before pouring the agar and allowing it to set. The eight fungicides used in this experiment belong to different classes of chemical, except benomyl and thiophanate-methyl, which are both benzimidazoles.

### Relationship between irradiation dose and survival rates

The relationship between irradiation dose and conidial survival rate was determined as follows. For ion-beam irradiation, 50 µL of the conidial suspension ($2.5 \times 10^3$ conidia mL$^{-1}$) were spread on 20 mL SDA in each replicate plastic Petri dish (60 mm diameter). The dishes were covered with a polyimide film (Kapton 30EN, Du Pont-To-ray, Japan) and irradiated at a range of doses (0, 50, 100, 200, 300, 400, 500, 600 Gy). For
gamma-ray irradiation 100 µL of the conidial suspension (2.0 × 10³ conidia mL⁻¹) were used in each Petri dish (90 mm diameter), the dishes were covered with plastic lids and the range of doses tested were 0, 30, 100, 300, 1000 and 3000 Gy. All dishes were incubated at 23 ± 1 °C in darkness and five replicates were made for each dose. Survival rates were determined based on the number of colonies that grew on SDA after three days and used to select appropriate doses for the production of mutants.

Production of mutants with potential resistance to benomyl

Conidia from 3 ml of a suspension (1.0 × 10⁸ conidia mL⁻¹) of the wild-type isolate of *I. fumosorosea* were transferred to each of three replicate 47 mm diameter cellulose membrane filters with a pore size of 0.45 µm (Millipore, Merck Millipore, Germany) using filtration equipment (Swinnex, Merck Millipore, Germany). The filters were individually placed in plastic Petri dishes (60 mm diameter for ion-beam irradiation and 50 mm diameter for gamma-ray irradiation). Prior to ion-beam irradiation the Petri dish lids were replaced with polyimide film.

Conidia collected on the membrane filters were either irradiated with ion beams or gamma rays at a range of doses selected from the relationship between irradiation dose and survival rate determined in the previous experiment (n = 3 per dose). Each treated filter was then transferred to a vial containing 3 mL of sterile Sabouraud’s dextrose broth and agitated with a sterile glass rod to detach the conidia from the filters. The resulting conidial suspensions were incubated at 20 ± 1 °C in darkness overnight to remove unstable mutations through cell division (germination). From each suspension, 200 µL were spread on to SDA into which 1000 mg L⁻¹ benomyl had been incorporated, in each of five/six Petri dishes (90 mm diameter). The dishes were then incubated at 23 ± 1 °C in darkness for seven days. Well grown colonies were assumed to be benomyl-resistant mutants and isolated onto fresh SDA for further evaluation; colonies that differed in shape and colour from the wild-type isolate were excluded.

In addition, the effectiveness of dual irradiation with both gamma rays and ion beams for inducing mutations was examined in the wild-type isolate; irradiation doses of 0, 50 or 500 Gy of gamma rays were followed by 0, 100 or 200 Gy of ion beams, respectively. After the conidia on the membrane filters were irradiated with gamma rays they were placed in Petri dishes containing SDA at 23 ± 1 °C and incubated in darkness overnight to remove unstable mutations through cell division (germination). This was followed by ion-beam irradiation and subsequent treatment procedures were as described previously for single ion-beam irradiation.
Evaluation of levels of resistance to benomyl in mutants

The EC$_{50}$ (Effective concentration of benomyl to reduce mycelial growth by 50%) values were determined for wild-type and mutant isolates based on mycelial growth on SDA that contained different quantities of benomyl. Mycelial plugs (4 mm diameter) of each isolate were excised from the margins of colonies growing on SDA. The plugs were then placed individually into the centre of Petri dishes (90 mm diameter) containing 30 mL SDA to which benomyl had been added (0, 0.1, 0.3, 1, 3, 10 or 30 mg L$^{-1}$ for the wild-type isolate and 0, 300, 1000, 3000 or 5000 mg L$^{-1}$ for the mutant isolates; n = 3 per dose). Dishes were incubated at 25 ± 1 °C in darkness for seven days and mean colony diameter was determined from two perpendicular measurements of each colony, excluding 4 mm to account for the diameter of the original inoculation plug. Each colony diameter was then divided by the mean colony diameter obtained for the same isolate when grown on SDA containing no fungicide, to provide relative growth rates. The EC$_{50}$ value for each isolate was estimated using the growth rates obtained for each dose by linear regression using the software package SPSS Statistics (SPSS, 2009).

Resistance of mutants to other fungicides

Growth of benomyl-resistant mutants on SDA to which one of eight fungicides, including benomyl, had been incorporated at the recommended field application rate was evaluated. The methods were the same as described above, except that dishes were incubated for eight days. Each isolate was also cultured in the absence of fungicides (control) and there were five replicate dishes per fungicide. The resistance ratio (RR) was obtained using the following formula: (mean colony diameter for fungicide/mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild type. For each mutant, the mean colony diameter for each fungicide was statistically compared to the mean colony diameter for the control using a Mann-Whitney’s U-test (SPSS, 2009).

DNA sequencing of β-tubulin locus

After culturing the wild-type and mutant isolates on SDA at 20 °C for three days, genomic DNA was extracted from the conidia of each isolate using a FastDNA Spin Kit (MP Biomedicals, UK). To isolate the partial β-tubulin locus, PCR was conducted using a PrimeSTAR GXL DNA polymerase (Takara Bio, Japan), the purified genomic DNA as a template and two specific primers, Isaria_beta-tub-R and Metarhizium_beta-tub-F2.
under the following conditions: 1 cycle of 98 °C for 20 s, 30 cycles of 98 °C for
10 s, 68 °C for 2 min, 1 cycle of 68 °C for 5 min and then storage at 4 °C. The PCR
products were purified using a MinElute PCR Purification Kit (Qiagen, Netherlands). To
identify the mutation sites in the β-tubulin locus, DNA sequencing was performed using
a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, CA) and six
specific primers (Table 1) with an ABI Prism 377 DNA Sequencer (Life Technologies,
CA).

Results and Discussion

Production of benomyl-resistant mutants and fungal resistance levels

Ion-beam irradiation with a dose of 50 Gy was associated with high survival rates
(64.6%), 500 Gy with very low survival rates (0.3%) and 600 Gy killed all the conidia
(Table 2). Gamma-ray irradiation with doses below 300 Gy only slightly affected
survival rates (> 78.1%), 1000 Gy was associated with very low survival rates (0.5%),
and 3000 Gy killed all the conidia (Table 3). In previous studies, the highest mutation
frequency obtained by ion beam irradiation was associated with survival rates ranging
from 1-10% in Saccharomyces cerevisiae (Matuo et al., 2006) and Aspergillus oryzae
(Toyoshima et al., 2012). Consequently, we selected irradiation doses of 100, 200, 300,
400 and 500 Gy for ion-beam irradiation and 400, 600, 800 and 1000 Gy for gamma-ray
irradiation when attempting to produce mutants.

Six benomyl-resistant isolates were established from colonies that appeared normal
(Fig. 1); Ib-34 (200 Gy) and Ib-421 (300 Gy) from ion-beam irradiation, Gr-5 and Gr-22
(both 1000 Gy) from gamma-ray irradiation and GrIb-8 and GrIb-9, both from dual
irradiation by 500 Gy of gamma rays followed by 200 Gy of ion beams. The wild-type
isolate had an EC50 value of 2.5 mg L⁻¹, while five mutant isolates, Ib-34, Ib-421, Gr-5,
Gr-22 and GrIb-8, had EC50 values > 5000 mg L⁻¹ (> 2000-fold more resistant than the
wild-type) (Table 4). Another mutant isolate, GrIb-9, also had a high EC50 value of 1855
mg L⁻¹ (742-fold more resistant than the wild-type) (Table 4). This considerably
enhanced resistance in the mutant isolates was greater than that of I. fumosorosea
benzimidazole-resistant isolates produced using other methods. For example,
carbendazim-resistant mutants generated using the mutagen NaNO₂ exhibited a
maximum EC50 value of only > 1000 mg L⁻¹ which was still 830-fold more resistant
than the wild-type (Song et al., 2011). Furthermore, benomyl-resistant transformants
generated using a polyethylene glycol-mediated procedure had a minimum inhibitory
concentration of only 20 mg L⁻¹ compared with 7.5 mg L⁻¹ for the wild-type (Inglis et
al.], 1999). Enhanced resistance in our mutants may be sufficient to avoid the negative
effects resulting from benomyl application in the field (500 mg L⁻¹ is the recommended
application rate). This is the first study to demonstrate that ion-beam and gamma-ray
irradiation are potentially useful tools for inducing mutations that enhance fungicide
resistance in entomopathogenic fungi. Since the increase in resistance obtained
separately with either irradiation method alone was considerable, combined irradiation
may be unnecessary. Ion-beam irradiation facilities are now available worldwide. This
method creates different characteristics in entomopathogenic fungi, because it causes a
high mutation frequency, a broad mutation spectrum and creates point mutations in
genes (Matuo et al., 2006; Zengliang, 2006; Tanaka et al., 2010; Toyoshima et al.,
2012).

Resistance of mutants to other fungicides

The wild-type and mutant isolates produced significantly smaller colonies on most of
the fungicides at a field application rate compared with the control (P < 0.01 or P <
0.05) (Table 5). As expected, however, all the mutant isolates had large RR values (4.48
to 6.46) when grown on media containing benomyl. Similarly, the mutants also had
comparatively large RR values (1.30 to 1.69) when grown on media containing
thiophanate-methyl which, like benomyl, is a benzimidazole fungicide. For the other
fungicides, the mutant isolates generally had RR values around 1 which indicated they
had similar resistance levels to the wild-type isolate, except for iprodione (RR: 1.05 to
2.05) and myclobutanil (RR: 1.16 to 4.99). These findings suggest that the mutant
isolates may have multiple mechanisms conferring resistance to several fungicides
including benzimidazoles.

Benzimidazole fungicides, such as benomyl, are negatively cross resistant to
N-phenylcarbamate fungicides, such as diethofencarb (Fujimura et al., 1992a; Ziogas &
Girgis, 1993; Leroux et al., 1999). However, our benomyl-resistant isolates exhibited
similar or little more sensitivity to diethofencarb (RR: 0.84 to 1.09) than the wild-type
isolate. Regardless of this, all mutants should be screened for undesirable mutations that
may have occurred alongside the desired mutation conferring benomyl resistance. For
example, it will be important to compare the virulence of mutant and wild-type isolates
against target pests to ensure any benefits associated with benomyl resistance are not
counteracted by any loss in virulence; these studies are currently under way in our
laboratories.

Sequence of β-tubulin locus
The β-tubulin locus of the wild-type and six mutant isolates was amplified by PCR. Two of the mutant isolates, Ib-34 and Gr-5, produced very weak amplification signals and were excluded from further analysis. The β-tubulin sequence in *I. fumosorosea* is 1727 bp (Song et al., 2011), but only partial genomic DNA sequences of the β-tubulin gene (1424 bp at nucleotide position from 304 to 1727) were determined for the wild-type and four mutant isolates and the β-tubulin sequences in the four mutant isolates, Ib-421, Gr-22, GrIb-8 and GrIb-9, were identical to those of the wild-type.

Most of the molecular studies on benzimidazole-resistant phytopathogenic fungi have focused on the replacement of amino acids at codon 198 and/or 200 in the β-tubulin locus (Fujimura et al., 1992b; Koenraadt & Jones, 1993; Yarden & Katan, 1993; Davidson et al., 2006; Schmidt et al., 2006; Kongtragoul et al., 2011). A similar mutation at codon 198 has been reported in NaNO₂-induced benomyl-resistant mutants of *B. bassiana* (Butters et al., 2003; Zou et al., 2006). However, none of these mutations in the β-tubulin locus were detected in our mutant isolates, suggesting that other mechanisms may be responsible for the observed enhancement in benomyl-resistance.

In another study, Song et al. (2011) showed that, although benomyl-resistant *I. fumosorosea* mutants did not possess any mutations at the β-tubulin locus, mutations were observed in the promoter region of the ABC transporter gene (*ifT1*). It is thus possible that similar mutations may have occurred in our benomyl-resistant mutant isolates. These findings indicate that different mechanisms are responsible for conferring benomyl-resistance in *B. bassiana* and *I. fumosorosea*. Future studies will examine the possibility of mutations in other genes including *ifT1* in our mutants.

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selection for fungicide resistance on Beauveria bassiana and Metarhizium brunneum.

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carbendazim arises from the overexpression of an ATP-binding cassette transporter


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Ionizing radiation produced fungicide-resistant mutants (arrow) in the entomopathogenic fungus *Isaria fumosorosea*.
Figure legends

**Fig. 1.** Benomyl-resistant mutant colony (indicated by arrow) derived from *I. fumosorosea* wild-type conidia irradiated with ion beams (400 Gy) plated on SDA to which benomyl had been added (1000 mg L$^{-1}$).
<table>
<thead>
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<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isaria_beta-tub-R</td>
<td>TTACATGGGCTCCTCAGCCTCA</td>
<td>PCR and sequencing</td>
</tr>
<tr>
<td>Beauveria_beta-tub-R2</td>
<td>CTTCATGGCAACCTACCAC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>Beauveria_beta-tub-R3</td>
<td>GAACAACGTCGAGGACCTG</td>
<td>Sequencing</td>
</tr>
<tr>
<td>Metarhizium_beta-tub-F2</td>
<td>CCAATTGGTGCTGCTTTTCCTGG</td>
<td>PCR and sequencing</td>
</tr>
<tr>
<td>Beauveria_beta-tub-F2</td>
<td>CAGGTTTCCAGATCACCC</td>
<td>Sequencing</td>
</tr>
<tr>
<td>Beauveria_beta-tub-F3</td>
<td>GAGGACCAGATGCGTAATGTG</td>
<td>Sequencing</td>
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Table 2. Relationship between ion-beam irradiation doses and conidial survival rates in the wild-type of *I. fumosorosea*

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>No. colonies per Petri dish (mean ± SE)</th>
<th>Survival rate&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>88.7 ± 4.3</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>57.3 ± 10.5</td>
<td>64.6</td>
</tr>
<tr>
<td>100</td>
<td>33.3 ± 2.4</td>
<td>37.5</td>
</tr>
<tr>
<td>200</td>
<td>12.0 ± 1.0</td>
<td>13.5</td>
</tr>
<tr>
<td>300</td>
<td>3.7 ± 0.3</td>
<td>4.2</td>
</tr>
<tr>
<td>400</td>
<td>1.7 ± 0.3</td>
<td>1.9</td>
</tr>
<tr>
<td>500</td>
<td>0.3 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>600</td>
<td>0.3 ± 0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>700</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Survival rate = (mean number of irradiated colonies/mean number of control colonies) × 100.
Table 3. Relationship between gamma-ray irradiation doses and conidial survival rates in the wild-type of *I. fumosorosea*

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>No. colonies per Petri dish (mean ± SE)</th>
<th>Survival rate$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>140.0 ± 2.0</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>124.0 ± 9.0</td>
<td>88.6</td>
</tr>
<tr>
<td>100</td>
<td>115.7 ± 16.8</td>
<td>82.6</td>
</tr>
<tr>
<td>300</td>
<td>109.3 ± 5.9</td>
<td>78.1</td>
</tr>
<tr>
<td>1000</td>
<td>0.7 ± 0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>3000</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Survival rate = (mean number of irradiated colonies/mean number of control colonies) × 100.
**Table 4.** Benomyl-resistance in the wild-type and mutant isolates of *I. fumosorosea*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>EC$_{50}$ (mg L$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td>2.5</td>
</tr>
<tr>
<td>Ib-34</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>Ib-421</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>Gr-5</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>Gr-22</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>GrIb-8</td>
<td>&gt; 5000</td>
</tr>
<tr>
<td>GrIb-9</td>
<td>1855</td>
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</table>
Table 5. Colony diameters (mm, mean ± SE) of the wild-type and mutant isolates of *I. fumosorosea* on SDA containing each fungicide at the recommended application rate and the resistance ratios (RR)\(^a\) in parentheses

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Benomyl (500 mg L(^{-1}))</th>
<th>Thiophanatemethyl (700 mg L(^{-1}))</th>
<th>Iprodione (500 mg L(^{-1}))</th>
<th>Diethofencarb (250 mg L(^{-1}))</th>
<th>Chlorothalonil (400 mg L(^{-1}))</th>
<th>Polycarbamate (937 mg L(^{-1}))</th>
<th>Myclobutanil (625 mg L(^{-1}))</th>
<th>Triflumizole (150 mg L(^{-1}))</th>
<th>Control (No fungicide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.4 ± 0.1** (1)</td>
<td>14.0 ± 0.2** (1)</td>
<td>6.2 ± 0.3** (1)</td>
<td>21.6 ± 1.2* (1)</td>
<td>16.3 ± 0.5** (1)</td>
<td>9.6 ± 0.5** (1)</td>
<td>2.0 ± 1.0** (1)</td>
<td>0.6 ± 0.1** (1)</td>
<td>25.4 ± 0.4</td>
</tr>
<tr>
<td>Ib-34</td>
<td>7.7 ± 0.3** (5.70)</td>
<td>9.4 ± 0.4 (1.69)</td>
<td>4.3 ± 0.2** (1.74)</td>
<td>7.2 ± 0.2** (0.84)</td>
<td>9.4 ± 0.2 (1.45)</td>
<td>5.6 ± 0.4** (1.47)</td>
<td>3.3 ± 0.2** (4.15)</td>
<td>0** (0)</td>
<td>10.1 ± 0.4</td>
</tr>
<tr>
<td>Ib-421</td>
<td>12.7 ± 0.9** (4.70)</td>
<td>14.5 ± 0.6** (1.30)</td>
<td>5.2 ± 0.2** (1.05)</td>
<td>18.7 ± 0.6 (1.09)</td>
<td>13.2 ± 1.0** (1.02)</td>
<td>9.1 ± 0.2** (1.19)</td>
<td>4.9 ± 1.2** (3.08)</td>
<td>0.1 ± 0.1** (0.21)</td>
<td>20.2 ± 0.1</td>
</tr>
<tr>
<td>Gr-5</td>
<td>17.9 ± 0.2** (5.31)</td>
<td>22.4 ± 0.2** (1.61)</td>
<td>9.2 ± 0.7** (1.50)</td>
<td>19.1 ± 0.2** (0.89)</td>
<td>16.7 ± 0.4** (1.03)</td>
<td>7.9 ± 0.4** (0.83)</td>
<td>2.3 ± 0.2** (1.16)</td>
<td>0.7 ± 0.2** (1.18)</td>
<td>25.2 ± 0.1</td>
</tr>
<tr>
<td>Gr-22</td>
<td>11.4 ± 0.4** (4.48)</td>
<td>16.1 ± 0.2** (1.54)</td>
<td>6.0 ± 0.3** (1.29)</td>
<td>15.9 ± 0.2** (0.98)</td>
<td>11.9 ± 0.2** (0.98)</td>
<td>6.2 ± 0.1** (0.86)</td>
<td>6.2 ± 0.7** (4.14)</td>
<td>0.2 ± 0.2** (0.45)</td>
<td>19.0 ± 0.1</td>
</tr>
<tr>
<td>GrIb-8</td>
<td>7.7 ± 0.4* (6.46)</td>
<td>7.9 ± 0.2* (1.61)</td>
<td>3.7 ± 0.3** (1.70)</td>
<td>7.3 ± 0.2* (0.96)</td>
<td>7.7 ± 0.3* (1.35)</td>
<td>4.7 ± 0.2** (1.40)</td>
<td>3.5 ± 0.2** (4.99)</td>
<td>0** (0)</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>GrIb-9</td>
<td>6.2 ± 0.2** (4.73)</td>
<td>8.5 ± 0.4* (1.57)</td>
<td>4.9 ± 0.3** (2.05)</td>
<td>7.4 ± 0.1** (0.89)</td>
<td>8.6 ± 0.3* (1.37)</td>
<td>4.6 ± 0.2** (1.24)</td>
<td>3.2 ± 0.3** (4.15)</td>
<td>0** (0)</td>
<td>9.8 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\)RR = (mean colony diameter for fungicide/mean colony diameter for control) mutant/(mean colony diameter for fungicide/mean colony diameter for control) wild-type.

* and ** in each isolate indicate significant differences from control at \(P = 0.05\) and \(P = 0.01\), respectively.