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1 **Distribution of benzimidazole-resistant strains of the onion gray-mold**
2 **neck rot pathogens, *Botrytis aclada* and *Botrytis allii*, in Hokkaido, Japan**

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19 The nucleotide sequence data reported are available in the DDBJ/EMBL
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24 *Botrytis aclada* and *Botrytis allii*, associated with onion gray-mold neck
25 rot and isolated in Hokkaido, were tested for sensitivity to benzimidazole. Of

26 the *B. aclada* strains, 59% were highly resistant and the remaining 41% were
27 sensitive; all strains of *B. allii* were sensitive. Resistant strains were
28 widespread in Hokkaido. We analyzed the sequences of the β -tubulin gene of
29 resistant strains and detected the replacement of glutamic acid (GAG) by
30 lysine (AAG) at codon 198. This is the first report of benzimidazole resistance
31 in *B. aclada*. This study revealed a difference in fungicide sensitivity between
32 the two *Botrytis* species.

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34 Keywords

35 benzimidazole resistance • *Botrytis aclada* • *Botrytis allii* • β -tubulin gene

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47 Hokkaido, the northernmost region of Japan, is a major area of onion
48 (*Allium cepa* L.) production. Two-month-old seedlings grown in greenhouses
49 are transplanted in open fields from late April to early May. The bulbs are
50 harvested from mid-August to late September, stored at low temperatures, and
51 shipped from October to April of the next year. Gray-mold neck rot caused by
52 *Botrytis* spp. during storage is an important disease in Hokkaido (Ishizaka
53 and Yanagita 1981). The pathogen infects onion leaves in the field during the
54 growing season, and then induces bulb rot during storage (Chilvers et al.
55 2004).

56 In Hokkaido, several *Botrytis* species are associated with onion
57 diseases, such as leaf blight pathogen *Botrytis squamosa* JC Walker and
58 mycelial neck rot pathogen *Botrytis byssoidea* JC Walker. *Botrytis allii* Munn
59 and *Botrytis aclada* Fresenius are the causal agents of gray-mold neck rot and
60 are distributed widely in Hokkaido (Notsu et al. (2020)).

61 *Botrytis* species, including *B. allii* and *B. aclada*, are difficult to
62 identify based on conidial morphology and the symptoms they cause in onion
63 (Beever and Weeds 2007). The identification of *Botrytis* species requires
64 molecular analysis (Nielsen et al. 2001; Staats et al. 2005). *B. allii* and *B.*
65 *aclada* can be distinguished using a polymerase chain reaction–restriction
66 fragment length polymorphism (PCR-RFLP) assay developed by Nielsen et al.
67 (2002). Previously, we identified 99 strains of onion gray-mold neck rot
68 pathogen using PCR-RFLP (Notsu et al. (2020)). Of the total 99 isolates, 63
69 were identified as *B. allii* and the remaining as *B. aclada*. *B. allii* was isolated

70 from all eight regions of Hokkaido, whereas *B. aclada* was isolated from six
71 regions. Both species were widely distributed in Hokkaido and coexisted in
72 each region. Very little is known about the differences in the ecological
73 characteristics of the two species.

74 Benzimidazole fungicides have been used worldwide since the 1970s
75 to control various fungal diseases, including those caused by *Botrytis* spp. In
76 Hokkaido, benzimidazole fungicides, such as benomyl and thiophanate-
77 methyl wettable powders, are sprayed on onions to control gray-mold neck
78 rot and leaf blight caused by *Botrytis* spp. Benzimidazole inhibits fungal
79 germ-tube elongation and mycelial growth by binding to the β -tubulin
80 (Leroux et al. 1999). Benzimidazole-resistant strains show reduced binding
81 affinity between fungicides and the β -tubulin (Davidse 1986), resulting in low
82 control effectiveness. Resistance to benzimidazole is associated with
83 mutations in the β -tubulin gene (Koenraadt and Jones 1993; Koenraadt et al.
84 1992). Highly resistant strains of *Botrytis cinerea* Persoon, a gray-mold
85 pathogen of various crops, are reported to have point mutations at codon 198
86 in the β -tubulin gene resulting in changes from glutamic acid (GAG) to
87 alanine (GCG), lysine (AAG), or valine (GTG); a moderately resistant strain
88 had a point mutation at codon 200 resulting in a change from phenylalanine
89 (TTC) to threonine (TAC) (Koenraadt and Jones 1993 ; Yarden and Katan
90 1993 ; Zhang et al. 2010).

91 Reports have described benzimidazole-resistant strains of the onion
92 neck rot pathogen, *B. allii*, in Hyogo Prefecture, Japan (Nishiguchi et al.
93 2000) and New Zealand (Viljalinnen-Rollinson et al. 2007). Benzimidazole-
94 resistant strains of onion neck rot pathogen have also been reported in Saga
95 Prefecture, Japan, although the causal agent has not been identified at the
96 species level (Yamaguchi et al. 2002). Taxonomic confusion between *B.*
97 *aclada* and *B. allii* existed before Yohalem et al. (2003) clarified the
98 phylogenetic placement of *B. allii*. Thus, the frequency and distribution of
99 benzimidazole-resistant strains should be elucidated using populations of
100 *Botrytis* spp. that conform to the current classification of Yohalem et al.
101 (2003).

102 In this study, we used a culture medium assay to clarify the distribution
103 of benzimidazole-resistant strains of the gray-mold neck rot pathogen in
104 Hokkaido, where *B. aclada* and *B. allii* coexist. We also analyzed the β -
105 tubulin gene sequences of the strains.

106 Culture medium assays were performed on 29 strains of *B. aclada* and
107 66 strains of *B. allii* isolated from onion bulbs with gray-mold neck rot
108 symptoms in Hokkaido from 2009 to 2017 (Table 1). Previously, we identified
109 99 strains by PCR-RFLP (Notsu et al. (2020)), and 95 strains of them except
110 for poorly growing 2 strains of *B. aclada* and 2 strains of *B. allii* were used
111 for the assay. All strains were preserved in potato dextrose agar (PDA) slants
112 at 5°C until use.

113 Fungicide was added to PDA medium at each of 13 different
114 concentrations (0.39, 0.78, 1.56, 3.12, 6.24, 12.5, 25, 50, 100, 200, 400, 800,
115 and 1,600 $\mu\text{g}/\text{mL}$) of commercial formulations of thiophanate-methyl
116 (contents, 70% (w/w); Topsin M wettable powder, Nippon Soda, Tokyo,
117 Japan) and media were autoclaved. Mycelial disks (6-mm diameter) were
118 cut from the growing region and transferred to PDA plates (9 cm) amended
119 with each concentration and incubated at 20°C in the dark. After 7 days, the
120 hyphal elongation of each isolate was determined. The test was performed
121 twice. From these results, the minimum inhibitory concentration (MIC) was
122 determined for each strain.

123 We analyzed the DNA sequences of the β -tubulin gene in six *B. aclada*
124 strains and two *B. allii* strains (Table 2) using the following method. The
125 strains were selected to include strains with different collection years,
126 geographical origins, and resistance. DNA was extracted from 7-day-old
127 mycelia grown at 25°C on PDA plates using a DNeasy Plant Mini Kit
128 (QIAGEN, Hilden, Germany). The extracted DNA was dissolved in 50 μL TE
129 buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at -30°C until use.
130 The DNA concentration was estimated based on the band density after agarose
131 gel electrophoresis and adjusted to a range of 10–100 ng/ μL .

132 PCR was performed using primers Bcb-F (5'-CACTGAGGGTGCT
133 GAGCTTGT-3') and Bcb-R (5'-GAAGCGGCCATCATGTTCTTA-3'),
134 designed to amplify a partial β -tubulin sequence that included codons 198 and

135 200 of *B. cinerea* (Zhang et al. 2010). The PCR products were purified using
136 the QIAquick PCR Purification Kit (QIAGEN), according to the
137 manufacturer's protocol. Sequencing was conducted by Hokkaido System
138 Science (Sapporo, Hokkaido, Japan), and the resulting sequences were
139 deposited in DDBJ/EMBL/GenBank as accessions LC576599–576606. Four
140 *B. cinerea* β -tubulin sequences (two highly resistant strains, one moderately
141 resistant strain, and one sensitive strain) were obtained from the
142 DDBJ/EMBL/GenBank databases and compared with our sequence data. The
143 sequences were aligned using MEGA X (<https://www.megasoftware.net/>).

144 In the culture medium assay, the MICs of 17 of 29 (59%) *B. aclada*
145 strains exceeded 1,600 $\mu\text{g}/\text{mL}$; those of the remaining 12 (41%) strains were
146 $\leq 0.39 \mu\text{g}/\text{mL}$. The MICs of all 66 *B. allii* strains were also $\leq 0.39 \mu\text{g}/\text{mL}$.
147 Koenraad et al. (1992) divided that highly resistant strains grow at ≥ 500
148 $\mu\text{g}/\text{mL}$, moderately resistant strains grow at 5 $\mu\text{g}/\text{mL}$ but not at 50 $\mu\text{g}/\text{mL}$, and
149 sensitive strains cannot grow at 0.5 $\mu\text{g}/\text{mL}$. Based on these criteria, the 17 *B.*
150 *aclada* strains with MICs $> 1,600 \mu\text{g}/\text{mL}$ were classified as highly resistant
151 and the 78 strains (12 *B. aclada* and 66 *B. allii* strains) with MICs ≤ 0.39
152 $\mu\text{g}/\text{mL}$ were classified as sensitive. The results of the resistance test using the
153 culture medium assay and the geographic origins of the strains are
154 summarized in Table 1. Previously, we found *B. aclada* in six areas of
155 Hokkaido (Notsu et al. (2020)); resistant strains were detected in all six areas
156 except Yubetsu, indicating that resistant strains are widespread in Hokkaido.

157 Fig. 1 shows partial sequences of the β -tubulin genes of six *B. aclada*
158 strains, two *B. allii* strains, and four *B. cinerea* as reference strains. Five
159 sensitive strains (three *B. aclada* and two *B. allii* strains) had GAG at codon
160 198 for glutamic acid, like the sensitive *B. cinerea* strain (Duan et al. 2018).
161 Three highly resistant strains of *B. aclada* had a codon for lysin (AAG) at
162 codon 198, as did the highly resistant *B. cinerea* strain. Thus, a point mutation
163 of GAG to AAG occurred at codon 198 in highly resistant strains.

164 All eight isolates had TTC at codon 200. The substitution of TAC for
165 TTC at codon 200, which corresponds to moderate resistance (Koenraad and
166 Jones 1993; Yarden and Katan 1993), was not found. In accordance, no
167 moderately resistant strain was found in the culture medium assays.

168 This study identified benzimidazole-resistant strains of onion gray-
169 mold neck rot pathogen in Hokkaido. All 66 *B. allii* strains were sensitive,
170 whereas 59% (17 of 29) of *B. aclada* strains were highly resistant. Thus,
171 highly benzimidazole-resistant *B. aclada* is distributed widely in Hokkaido,
172 and the development of benzimidazole resistance clearly differs between *B.*
173 *allii* and *B. aclada*. Furthermore, we confirmed that thiophanate-methyl and
174 benomyl had no effect in field tests involving the inoculation of plants with
175 resistant strains (data not shown). Highly benzimidazole-resistant strains of
176 *B. allii* have been reported in Hyogo Prefecture and Saga Prefecture, Japan
177 (Nishiguchi et al. 2000; Yamaguchi et al. 2002) and in New Zealand
178 (Viljalainen-Rollinson et al. 2007). However, the species was not identified

179 clearly in either study. Thus, this report is the first to describe benzimidazole-
180 resistant *B. aclada* strains that were accurately differentiated from *B. allii*
181 using a molecular method. To our knowledge, no report has described the
182 difference in fungicide resistance between these two species.

183 A mixture of thiophanate-methyl and diethofencarb (contents, 52.5%
184 (w/w) and 12.5% (w/w), respectively; Getter wettable powder, Nippon Soda)
185 is available in Japan for the control of onion neck rot. Diethofencarb is an N-
186 phenylcarbamate fungicide. In general, highly resistant isolates of
187 benzimidazole are sensitive to diethofencarb (Leroux et al. 2002). However,
188 benzimidazole-resistant field isolates of *B. cinerea* with cross-resistance to
189 diethofencarb have been detected (Malandrakis et al. 2011). Malandrakis et
190 al. (2011) revealed that a point mutation from GAG to AAG at codon 198 is
191 found in the strains resistant to zoxamide, carbendazim and diethofencarb.
192 Here, we found same mutation at codon 198 of the β -tubulin gene in a highly
193 resistant strain of *B. aclada*. Thus, although the diethofencarb sensitivity of
194 the highly benzimidazole-resistant strains identified in this study was not
195 tested, these strains might be resistant to diethofencarb.

196 *B. allii* is allopolyploid hybrid of *B. aclada* and *B. byssoidea* (Nielsen
197 and Yohalem 2001; Staats et al. 2005). The sequences of genomic DNA
198 containing multiple alleles may be mixed when a recombinant is generated
199 (Staats et al. 2005). In this study, the individual alleles were not sequenced;
200 only one sequence was obtained accurately at codons 198 and 200. We

201 hypothesized that (1) the primer matched only one of the alleles and was
202 selectively amplified or (2) the two alleles had identical sequences at these
203 codons. No β -tubulin gene sequence of *B. allii* or *B. aclada* was available in
204 the DDBJ/EMBL/GenBank databases, preventing comparison of our
205 sequences with those of other strains of *B. allii* and *B. aclada*. Accurate
206 determination of these sequences requires further analysis distinguishing
207 between alleles of each locus.

208 Ishizaka and Yanagita (1981) reported that the incidence of onion neck
209 rot bulbs in storage in major production areas in Hokkaido in 1980 was ca.
210 5%. In our 2009–2017 survey, no field with disease incidence > 5% was found
211 (data not shown). In the current field trial, the control effectiveness of
212 benzimidazole fungicide was remarkably low (0–31%: data not shown) on
213 inoculation with a highly resistant strain of *B. aclada*. No recent disease
214 outbreak has occurred, despite the wide distribution of highly resistant strains
215 in Hokkaido, which may be explained as follows. In Hokkaido, onions are
216 transplanted to the field in early May; fungicides with different modes of
217 action, including benzimidazole, are sprayed from July to September, before
218 the harvest (Iketani et al. 2016), to control leaf blight and neck rot caused by
219 *Botrytis* spp. Thus, fungicides other than benzimidazole may actually control
220 gray-mold neck rot in the regions with resistant strains. Otherwise, *B. allii*,
221 in which benzimidazole-resistant strain has not been found, is predominant in

222 Hokkaido (the ratio of *B. allii* to *B. aclada* is 68:31; Notsu et al. (2020)) may
223 explain the low incidence of this disease.

224 This study revealed a difference in the frequency of benzimidazole-
225 resistant strains of *B. allii* and *B. aclada*. At present, outbreaks of onion gray-
226 mold neck rot due to the development of resistant strains have not been
227 observed in Hokkaido. However, as the proportion of *B. aclada* increases in
228 some years (Notsu et al. (2020)), monitoring of the resistant strain rate of
229 each species is necessary.

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304

Table 1 Geographic origin of gray-mold neck rot pathogen used in this study and their sensitivity to benzimidazole

Geographic origin in Hokkaido	Number of strains ^a					
	<i>Botrytis allii</i>			<i>Botrytis aclada</i>		
	Total	S ^b	R ^c	Total	S	R
Takikawa	2	2	0	1	0	1
Naka-furano	1	1	0	2	0	2
Mikasa	16	16	0	0	0	0
Furano	5	5	0	10	3	7
Iwamizawa	19	19	0	4	0	4
Kitami	8	8	0	3	0	3
Kunneppu	11	11	0	0	0	0
Yubetsu	4	4	0	9	9	0
Total	66	66	0	29	12	17

a All strains were isolated and identified by Notsu et al. (2020) using PCR-RFLP

b S: sensitive to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is 0.39 µg/ml or below)

c R: highly resistant to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is higher than 1600 µg/ml)

Table 2 Strains used in this study for β -tublin sequence analysis

Strains	Year of isolation	Geographic origin in Hokkaido	Species ^a	Culture medium assay
KF-Ba225	2009	Iwamizawa	<i>B. allii</i>	S ^b
BaI12-7	2012	Iwamizawa	<i>B. allii</i>	S
KF-Ba130	2009	Iwamizawa	<i>B. aclada</i>	R
BaNW11-a	2011	Naka-furano	<i>B. aclada</i>	R ^c
BaTH11-A	2011	Takikawa	<i>B. aclada</i>	R
BFU1310	2013	Furano	<i>B. aclada</i>	S
BFU1316	2013	Furano	<i>B. aclada</i>	S
BaU2-8	2013	Yubetsu	<i>B. aclada</i>	S

a All strains were isolated and identified by Notsu et al. (2020) using PCR-RFLP

b S: sensitive to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is $\leq 0.39 \mu\text{g/mL}$)

c R: highly resistant to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is $> 1,600 \mu\text{g/mL}$)

a

Species/ strain Name	Sequence							
	codon		198		200			
1. <i>B.allii</i> KF-Ba225 (S)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
2. <i>B.allii</i> Ba12-7 (S)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
3. <i>B.aclada</i> KF-Ba130 (R)	C T C T G A C	A A G	A C C T	T	C T G T A T C G A T			
4. <i>B.aclada</i> BaNW11-a (R)	C T C T G A C	A A G	A C C T	T	C T G T A T C G A T			
5. <i>B.aclada</i> BaTH11-A (R)	C T C T G A C	A A G	A C C T	T	C T G T A T C G A T			
6. <i>B.aclada</i> BFU1310 (S)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
7. <i>B.aclada</i> BFU1316 (S)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
8. <i>B.aclada</i> BaU2-8 (S)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
9. <i>Botrytis cinerea</i> strain SD2(MG949128)	C T C T G A C	A A G	A C C T	T	C T G T A T C G A T			
10. <i>Botrytis cinerea</i> strain Bt4-1(MG949125)	C T C T G A C	G A G	A C C T	T	C T G T A T C G A T			
11. <i>Botrytis cinerea</i> strain GCY004 (MG949127)	C T C T G A C	G C G	A C C T	T	C T G T A T C G A T			
12. <i>Botrytis cinerea</i> strain B20 (MG949129)	C T C T G A C	G A G	A C C T	A	C T G T A T C G A T			

b

Species/ strain Name	amino acid position																						
	Q	L	V	E	N	S	D	198	E	T	F	C	I	D	N	E	A	L	Y	D	I	C	
1. <i>B.allii</i> KFBa225(S)
2. <i>B.allii</i> Ba12-7(S)
3. <i>B.aclada</i> KF-Ba130(R)	K
4. <i>B.aclada</i> BaNW11-a(R)	K
5. <i>B.aclada</i> BaTH11-A(R)	K
6. <i>B.aclada</i> BFU1310(S)
7. <i>B.aclada</i> BFU1316(S)
8. <i>B.aclada</i> BaU2-8(S)
9. <i>Botrytis cinerea</i> strain SD2(MG949128)	K
10. <i>Botrytis cinerea</i> strain Bt4-1(MG949125)
11. <i>Botrytis cinerea</i> strain GCY004(MG949127)	A
12. <i>Botrytis cinerea</i> strain B20(MG949129)	Y

Fig. 1. Partial sequences of the β -tubulin genes of strains of onion gray-mold neck rot pathogen collected in Hokkaido and *Botrytis cinerea*.

a DNA, b Amino acid

DNA sequences of at codon 198 and amino acid position 198 is boxed. The shaded sequence is the sequence at the position where mutation is confirmed due to benzimidazole-resistance. The letters in parentheses after the species / strain name of *B. allii* and *B. aclada* indicate susceptibility to benzimidazole (S, sensitive to benzimidazole; R, highly resistant to benzimidazole), and of *B. cinerea* indicate the accession numbers of DDBJ/EMBL/GenBank databases.

1-8 Onion gray-mold neck rot pathogens collected in Hokkaido , 9 *Botrytis cinerea* strain SD2 (highly resistant to benzimidazole), 10 *B. cinerea* strain Bt4-1 (sensitive to benzimidazole), 11 *B. cinerea* strain GCY004 (highly resistant to benzimidazole), 12 *B.*

cinerea strain B20 (moderately resistant to benzimidazole)