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

Ayumi Notsu・Kayo Shirai・Norio Kondo

A. Notsu (✉)

Central Agricultural Experiment Station, Hokkaido Research Organization, Naganuma, Hokkaido 069-1395, Japan

notsu-ayumi@hro.or.jp

Tel. +81-123-89-2291; Fax+81-123-89-2060

K. Shirai

Tokachi Agricultural Experiment Station, Hokkaido Research Organization, Memuro, Hokkaido 082-0081, Japan

N. Kondo

Research Faculty of Agriculture, Hokkaido University, Sapporo, 060-8589, Japan

Total text pages: 14 pages

The number of tables and figures: 2 tables and 1 figure

The nucleotide sequence data reported are available in the DDBJ/EMBL /GenBank databases as accessions LC576599–576606.

Botrytis aclada and *Botrytis allii*, associated with onion gray-mold neck rot and isolated in Hokkaido, were tested for sensitivity to benzimidazole. Of

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

Keywords

benzimidazole resistance・ *Botrytis aclada*・*Botrytis allii*・β-tublin gene

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

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

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

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

In the culture medium assay, the MICs of 17 of 29 (59%) *B. aclada* strains exceeded 1,600 μg/mL; those of the remaining 12 (41%) strains were ≤0.39 μg/mL. The MICs of all 66 *B. allii* strains were also ≤0.39 μg/mL. Koenraadt et al. (1992) divided that highly resistant strains grow at ≥500 μg/mL, moderately resistant strains grow at 5 μg/mL but not at 50 μg/mL, and sensitive strains cannot grow at 0.5 μg/mL. Based on these criteria, the 17 *B. aclada* strains with MICs > 1,600 μg/mL were classified as highly resistant and the 78 strains (12 *B. aclada* and 66 *B. allii* strains) with MICs ≤ 0.39 μg/mL were classified as sensitive. The results of the resistance test using the culture medium assay and the geographic origins of the strains are summarized in Table 1. Previously, we found *B. aclada* in six areas of Hokkaido (Notsu et al. (2020)); resistant strains were detected in all six areas 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 (Koenraadt 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 (Viljalinnen-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

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

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

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

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

References

Beever RE, Weeds PL (2007) Taxonomy and genetic variation of *Botrytis* and *Botryotinia*. In: Elad Y et al. (eds) *Botrytis*: biology, pathology and control. Kluwer Academic Publishers, Dordrecht, Netherlands, pp 29–52

Chilvers MI, Hay FS, Wilson CR (2004) Survey for *Botrytis* species associated with onion bulb rot in northern Tasmania, Australia. Australas Plant Pathol. 33:419–422

Davidse LC (1986) Benzimidazole fungicides: mechanism of action and biological impact. Ann Rev Phytopathol 24: 43–65

Duan YB, Yang Y, Li MX, Li T, Fraaije BA, Zhou MG (2018) Development and application of a simple, rapid and sensitive method for detecting moderately carbendazim-resistant isolates in *Botrytis cinerea*. Ann Appl Biol 172: 355-365

Iketani-Saito M, Notsu A, Shirai K (2016) Susceptible growth stage of onion

246 to gray-mold neck rot judged by various inoculation period (in Japanese).
247 Ann Rpt Plant Prot North Jpn 67:108–111
248 Ishizaka N and Yanagita K (1981) Effect of foliar application of thiophanate-
249 methyl on control of neck rot in onions during the storage period. (in
250 Japanese with English summary). Ann Rept Plant Prot North Jpn 32:134-
251 135
252 Koenraadt H, Somerville SC, Jones AL (1992) Characterization of mutations
253 in the beta-tubulin gene of benomyl-resistant field strains of *Venturia*
254 *inaequalis* and other plant pathogenic fungi. Phytopathology 82:1348–1354
255 Koenraadt H, Jones AL (1993) Resistance to benomyl conferred by mutations
256 in codon 198 or 200 of the beta-tubulin gene of *Neurospora crassa* and
257 sensitivity to diethofencarb conferred by codon 198. Phytopathology 83:
258 850–854
259 Leroux P, Chapeland F, Desbrosses D, Gredt M (1999) Patterns of cross-
260 resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*)
261 isolates from French vineyards. Crop Prot 18:687–697.
262 Leroux P, Fritz R, Debieu D, Albertini C, Lanen C, Bach J, Gredt M,
263 Chapeland F (2002) Mechanisms of resistance to fungicides in field strains
264 of *Botrytis cinerea.* Pest Manag Sci 58:876–888
265 Malandrakis A, Markoglou A, Ziogas B (2011) Molecular characterization of
266 benzimidazole-resistant *B. cinerea* field isolates with reduced or enhanced
267 sensitivity to zoxamide and diethofencarb. Pestic Biochem Physiol 99:
268 118–124.
269 Nishiguchi S, Kanto T, Irie K, Osada Y, Kyuno T (2000) Detection of
270 benzimidazole resistant strain of *Botrytis allii* (onion gray-mold neck rot)

(abstract in Japanese) Jpn J Phytopathol 66: 305

Nielsen K, Justesen AF, Jensen DF, Yohalem DS (2001) Universally primed polymerase chain reaction alleles and internal transcribed spacer restriction fragment length polymorphisms distinguish two subgroups in *Botrytis aclada* distinct from *B. byssoidea*. Phytopathology 91:527–533

Nielsen K, Yohalem DS (2001) Origin of a polyploid *Botrytis* pathogen through interspecific hybridization between *Botrytis aclada* and *B. byssoidea*. Mycologia 93:1064–1071

Nielsen K, Yohalem DS, Jensen DF (2002) PCR detection and RFLP differentiation of *Botrytis* species associated with neck rot of onion. Plant Dis 86:682–686

Notsu A, Shirai K, Kodama F, Kondo N Distribution of *Botrytis* isolates associated with onion gray-mold neck rot in Hokkaido, northern Japan. J Gen Plant Pathol (2020)

Staats M, van Baarlen P, van Kan JAL (2005) Molecular phylogeny of the plant pathogenic genus *Botrytis* and the evolution of host specificity. Mol Biol Evol 22:333–346

Viljanen -Rollinson SLH, Marroni MV, Butler RC (2007) Reduced sensitivity to carbendazim in isolates of *Botrytis allii*. N Z Plant Prot 60:108–113

Yamaguchi J, Mikuriya H, Matsuzaki M (2002) Occurrence of thiophanate methyl resistant strains of onion gray-mold neck rot pathogen and its chemical control (abstract in Japanese). Kyushu Pl Prot Res 48:97

Yarden O, Katan T (1993) Mutations leading to substitutions at amino acids 198 and 200 of beta-tubulin that correlate with benomyl-resistance phenotypes of field strains of *Botrytis cinerea*. Phytopathology 83: 1478–

1483

Yohalem DS, Nielsen K, Nicolaisen M (2003) Taxonomic and nomenclatural clarification of the onion neck rotting *Botrytis* species. Mycotaxon 85:175–182

Zhang CQ, Liu YH, Zhu GN (2010) Detection and characterization of benzimidazole resistance of *Botrytis cinerea* in greenhouse vegetables. Eur J Plant Pathol 126:509-515

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]					
	Botrytis allii			*Botrytis aclada*		
	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)

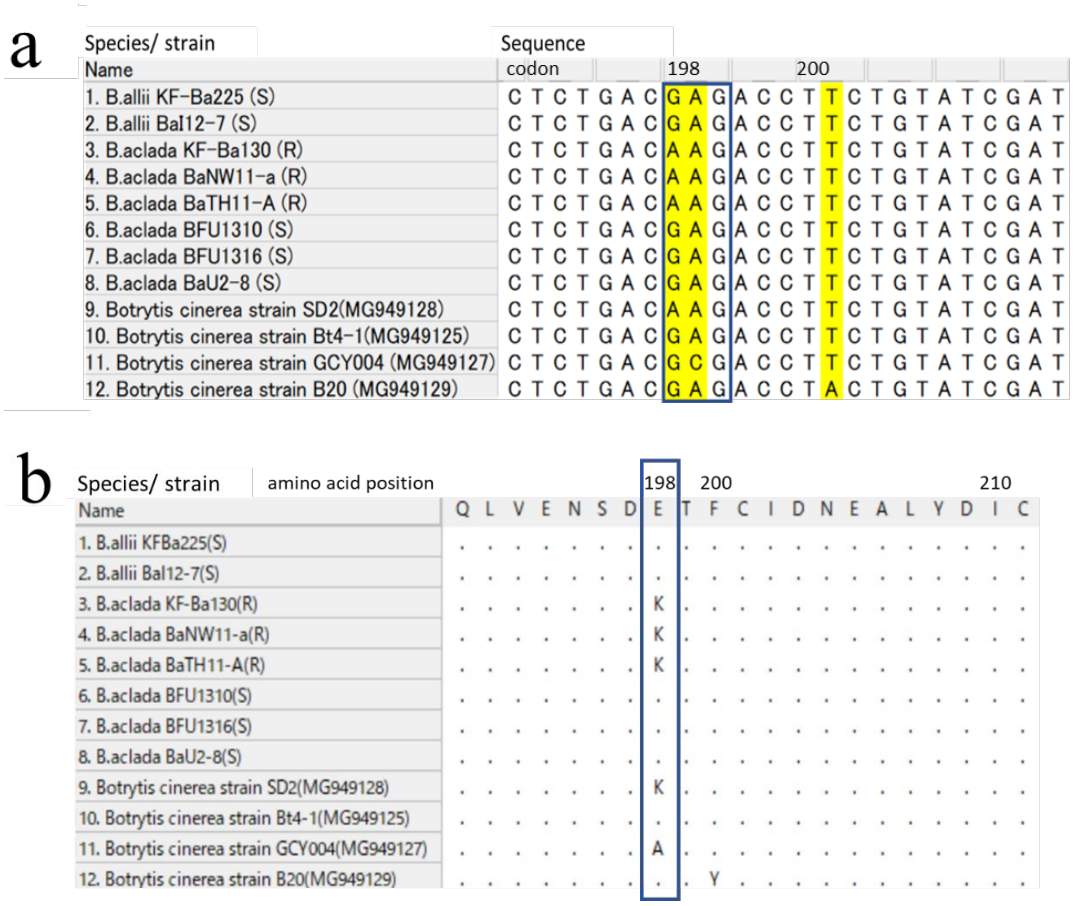
Table 2 Strains used in this study for β-tublin sequence analysis

Strains	Year of isolation	Geographic origin in Hokkaido	Species[a]	Culture medium assay
KF-Ba225	2009	Iwamizawa	*B. allii*	S[b]
BaI12-7	2012	Iwamizawa	*B. allii*	S
KF-Ba130	2009	Iwamizawa	*B. aclada*	R
BaNW11-a	2011	Naka-furano	*B. aclada*	R[c]
BaTH11-A	2011	Takikawa	*B. aclada*	R
BFU1310	2013	Furano	*B. aclada*	S
BFU1316	2013	Furano	*B. aclada*	S
BaU2-8	2013	Yubetsu	*B. aclada*	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 ≤0.39 μg/mL)

c R: highly resistant to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is > 1,600 μg/mL)



a

Species/ strain	Sequence																								
Name	codon							198						200											
1. B.allii 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. B.allii BaI12-7 (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. B.aclada 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. B.aclada 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. B.aclada 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. B.aclada 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. B.aclada 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. B.aclada 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. Botrytis cinerea 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. Botrytis cinerea 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. Botrytis cinerea 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. Botrytis cinerea 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	amino acid position							198		200									210		
Name	Q	L	V	E	N	S	D	E	T	F	C	I	D	N	E	A	L	Y	D	I	C
1. B.allii KFBa225(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. B.allii BaI12-7(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3. B.aclada KF-Ba130(R)	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.
4. B.aclada BaNW11-a(R)	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.
5. B.aclada BaTH11-A(R)	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.
6. B.aclada BFU1310(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7. B.aclada BFU1316(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8. B.aclada BaU2-8(S)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9. Botrytis cinerea strain SD2(MG949128)	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.
10. Botrytis cinerea strain Bt4-1(MG949125)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11. Botrytis cinerea strain GCY004(MG949127)	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
12. Botrytis cinerea 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)