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

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

Benzimidazole fungicides have been used worldwide since the 1970s to control various fungal diseases, including those caused by *Botrytis* spp. In Hokkaido, benzimidazole fungicides, such as benomyl and thiophanate-methyl wettable powders, are sprayed on onions to control gray-mold neck rot and leaf blight caused by *Botrytis* spp. Benzimidazole inhibits fungal germ-tube elongation and mycelial growth by binding to the \( \beta \)-tubulin (Leroux et al. 1999). Benzimidazole-resistant strains show reduced binding affinity between fungicides and the \( \beta \)-tubulin (Davidse 1986), resulting in low control effectiveness. Resistance to benzimidazole is associated with mutations in the \( \beta \)-tubulin gene (Koenraadt and Jones 1993; Koenraadt et al. 1992). Highly resistant strains of *Botrytis cinerea* Persoon, a gray-mold pathogen of various crops, are reported to have point mutations at codon 198 in the \( \beta \)-tubulin gene resulting in changes from glutamic acid (GAG) to alanine (GCG), lysine (AAG), or valine (GTG); a moderately resistant strain had a point mutation at codon 200 resulting in a change from phenylalanine (TTC) to threonine (TAC) (Koenraadt and Jones 1993; Yarden and Katan 1993; Zhang et al. 2010).
Reports have described benzimidazole-resistant strains of the onion neck rot pathogen, *B. allii*, in Hyogo Prefecture, Japan (Nishiguchi et al. 2000) and New Zealand (Viljalinnen-Rollinson et al. 2007). Benzimidazole-resistant strains of onion neck rot pathogen have also been reported in Saga Prefecture, Japan, although the causal agent has not been identified at the species level (Yamaguchi et al. 2002). Taxonomic confusion between *B. aclada* and *B. allii* existed before Yohalem et al. (2003) clarified the phylogenetic placement of *B. allii*. Thus, the frequency and distribution of benzimidazole-resistant strains should be elucidated using populations of *Botrytis* spp. that conform to the current classification of Yohalem et al. (2003).

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

Culture medium assays were performed on 29 strains of *B. aclada* and 66 strains of *B. allii* isolated from onion bulbs with gray-mold neck rot symptoms in Hokkaido from 2009 to 2017 (Table 1). Previously, we identified 99 strains by PCR-RFLP (Notsu et al. (2020)), and 95 strains of them except for poorly growing 2 strains of *B. aclada* and 2 strains of *B. allii* were used for the assay. All strains were preserved in potato dextrose agar (PDA) slants at 5°C until use.
Fungicide was added to PDA medium at each of 13 different concentrations (0.39, 0.78, 1.56, 3.12, 6.24, 12.5, 25, 50, 100, 200, 400, 800, and 1,600 µg/mL) of commercial formulations of thiophanate-methyl (contents, 70% (w/w); Topsin M wettable powder, Nippon Soda, Tokyo, Japan) and media were autoclaved. Mycelial disks (6-mm diameter) were cut from the growing region and transferred to PDA plates (9 cm) amended with each concentration and incubated at 20°C in the dark. After 7 days, the hyphal elongation of each isolate was determined. The test was performed twice. From these results, the minimum inhibitory concentration (MIC) was determined for each strain.

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

PCR was performed using primers Bcb-F (5′-CACTGAGGGTGCTGAGCTTGT-3′) and Bcb-R (5′-GAAGCGGCCATCATGTTCTTA-3′), 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.
Fig. 1 shows partial sequences of the β-tubulin genes of six *B. aclada* strains, two *B. allii* strains, and four *B. cinerea* as reference strains. Five sensitive strains (three *B. aclada* and two *B. allii* strains) had GAG at codon 198 for glutamic acid, like the sensitive *B. cinerea* strain (Duan et al. 2018). Three highly resistant strains of *B. aclada* had a codon for lysin (AAG) at codon 198, as did the highly resistant *B. cinerea* strain. Thus, a point mutation of GAG to AAG occurred at codon 198 in highly resistant strains.

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

This study identified benzimidazole-resistant strains of onion gray-mold neck rot pathogen in Hokkaido. All 66 *B. allii* strains were sensitive, whereas 59% (17 of 29) of *B. aclada* strains were highly resistant. Thus, highly benzimidazole-resistant *B. aclada* is distributed widely in Hokkaido, and the development of benzimidazole resistance clearly differs between *B. allii* and *B. aclada*. Furthermore, we confirmed that thiophanate-methyl and benomyl had no effect in field tests involving the inoculation of plants with resistant strains (data not shown). Highly benzimidazole-resistant strains of *B. allii* have been reported in Hyogo Prefecture and Saga Prefecture, Japan (Nishiguchi et al. 2000; Yamaguchi et al. 2002) and in New Zealand (Viljalinnen-Rollinson et al. 2007). However, the species was not identified
clearly in either study. Thus, this report is the first to describe benzimidazole-resistant *B. aclada* strains that were accurately differentiated from *B. allii* using a molecular method. To our knowledge, no report has described the difference in fungicide resistance between these two species.

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

*B. allii* is allopolyploid hybrid of *B. aclada* and *B. byssioidea* (Nielsen and Yohalem 2001; Staats et al. 2005). The sequences of genomic DNA containing multiple alleles may be mixed when a recombinant is generated (Staats et al. 2005). In this study, the individual alleles were not sequenced; 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 $B.\text{allii}$ to $B.\text{aclada}$ 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 $B.\text{allii}$ and $B.\text{aclada}$. 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 $B.\text{aclada}$ increases in some years (Notsu et al. (2020)), monitoring of the resistant strain rate of each species is necessary.

References


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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–

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<sup>a</sup> |  |  |  |  |  |  |  |  |  |
|------------------------------|-------------------------------|---|---|---|---|---|---|---|---|
|                              | Botrytis allii |  |  |  |  |  |  |  |  |  |
|                              | Total | $S$
|                               | S | R | 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 |

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

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

<sup>c</sup> 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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Year of isolation</th>
<th>Geographic origin in Hokkaido</th>
<th>Species&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Culture medium assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>KF-Ba225</td>
<td>2009</td>
<td>Iwamizawa</td>
<td>B. allii</td>
<td>S&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BaI12-7</td>
<td>2012</td>
<td>Iwamizawa</td>
<td>B. allii</td>
<td>S</td>
</tr>
<tr>
<td>KF-Ba130</td>
<td>2009</td>
<td>Iwamizawa</td>
<td>B. aclada</td>
<td>R</td>
</tr>
<tr>
<td>BaNW11-a</td>
<td>2011</td>
<td>Naka-furano</td>
<td>B. aclada</td>
<td>R&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>BaTH11-A</td>
<td>2011</td>
<td>Takikawa</td>
<td>B. aclada</td>
<td>R</td>
</tr>
<tr>
<td>BFU1310</td>
<td>2013</td>
<td>Furano</td>
<td>B. aclada</td>
<td>S</td>
</tr>
<tr>
<td>BFU1316</td>
<td>2013</td>
<td>Furano</td>
<td>B. aclada</td>
<td>S</td>
</tr>
<tr>
<td>BaU2-8</td>
<td>2013</td>
<td>Yubetsu</td>
<td>B. aclada</td>
<td>S</td>
</tr>
</tbody>
</table>

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

<sup>b</sup> S: sensitive to benzimidazole (Minimum inhibitory concentration against thiophanate-methyl is ≤0.39 μg/mL)

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