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6 Evaluation of antifungal activities of woody plant barks using an incubation system with *Trametes*
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Abstract

Tree barks protect inside of tree bodies against pathogens invasion, but their chemical constitutive defense mechanisms remain largely unexplored. Therefore, the antifungal activities of bark tissues of 25 tree species acquired from Hokkaido, Japan, were examined to investigate the constitutive chemical defense mechanisms of tree barks. Bark pieces were placed on potato dextrose agar with *Trametes versicolor* after sterilization with gamma rays at 15 kGy. Bark tissues of most species exhibited limited activities. However, the whole and inner barks of *Sorbus commixta* completely inhibited the mycelial growth of *T. versicolor*. Furthermore, the inner bark of *S. commixta* on media with grooves also inhibited the mycelial growth of *T. versicolor*, indicating that antifungal substances generated from the inner bark of *S. commixta* are volatile. Among other species, *Cerasus sargentii* var. *sargentii*, *Picea jezoensis* var. *jezoensis*, and *Magnolia obovate* showed lower antifungal activities than *S. commixta*. We also examined the validity of 15 kGy for sterilizing the bark pieces of 16 species among 25 species. A dose of 12 kGy was sufficient to sterilize the bark pieces of all species, except *Abies sachalinensis*, and 15 kGy was necessary for the bark pieces of *A. sachalinensis*. In conclusion, this incubation system can be applied to select tree species, such as *S. commixta*, with high antifungal activities, and 15 kGy was sufficient for this incubation system to sterilize the barks of tree species in Hokkaido. This incubation system is useful to find out tree species that form bark tissues with unique chemical defense mechanisms.

Keywords

Antifungal substances, Bark function, Constitutive chemical defense, Gamma sterilization, *Sorbus commixta*, White-rot fungus

Key Message

The novel incubation system where antifungal activities of bark tissues against *Trametes versicolor* were evaluated revealed that the inner bark of *Sorbus commixta* strongly inhibits mycelial growth of *T. versicolor*.

Introduction

Tree bark is defined as the tissues outside the vascular cambium (Angyalossy et al. 2016) and is considered to act as the primary constitutive defense against pest and pathogen invasions (Telford et al. 2015). Constitutive defense in the bark is extremely effective in preventing pathogen infection in a wide range of tree species because most fungi cannot penetrate wood through intact barks, and fungal growth can be suppressed within bark tissues without penetrating the vascular cambium (Biggs 1992; Woodward and Pearce 1988). Constitutive defense includes the inhibition of pathogen penetration into the tree by a structural barrier, such as suberized cork tissue (Biggs 1992), and the inhibition of pathogen growth by chemical substances, such as phenolic compounds (Franceschi et al. 1998, 2005), terpenoids (Seki et al. 2012; Soliman et al. 2015; Soliman and Raizada 2020), glycosides (Woodward and Pearce 1988), and resins (Rosner and Führer 2002). Most fungi cannot degrade suberin easily (Morris et al. 2020). Regarding structural barriers, information on the quantity (Leite and Pereira 2017), localization, and function (Alonso-Serra et al. 2019; Biggs 1984; Danti et al. 2018) of suberin in cork tissue is available. Contrastingly, studies investigating the role of constitutive antifungal substances in defense mechanisms are limited (Biggs 1992; Wittstock and Gershenzon 2002), probably because experimentally manipulating constitutive compounds in plants is difficult (Wittstock and Gershenzon 2002). Although previous studies have reported various constitutive antifungal substances from bark tissues, most were

1 aimed at identifying extractives for industrial and pharmaceutical applications (Biggs 1992). Gaining
2 knowledge of the constitutive chemical defense mechanisms of barks could not only provide an
3 understanding of complete defense strategies, including induced defense mechanisms, which function
4 after detecting the intrusion of foreign enemies (Franceschi et al. 2005), but also assist in identifying
5 measurable phenotypic markers that can be used to predict a tree's response to pests or pathogens attacks
6 (Telford et al. 2015) and contribute to enhanced tree breeding (Seki et al. 2012).

7 A common method for examining the antifungal activity of wood is to investigate the antifungal
8 activity of extracts obtained using various solvents (Laireiter et al. 2013; Munir et al. 2020a). Previous
9 studies examining the antifungal activity of barks have also used extraction-based methods (Alfredsen
10 et al. 2008; Lomeli-Ramírez et al. 2016; Mori et al. 1995, 1997). However, the extraction-based methods
11 do not consider the bark anatomy, and the extraction process may alter the active substances (Munir et
12 al. 2020a, b). The bark anatomy should be considered when evaluating the constitutive defense
13 mechanism, because the constitutive defense of barks is based on a combination of toxic substances,
14 anatomical structure, and the location of the substances (Seki et al. 2012). Additionally, as one of the
15 few examples of the constitutive chemical defense mechanisms of barks, a previous study showed that
16 when the bark of *Picea sitchensis* is inoculated with pathogenic or decay fungi, the fungal enzyme (β -
17 glucosidase) degrades the glycosides (astringin and rhaponticin) stored in the bark tissues (Woodward
18 and Pearce 1988). Consequently, antifungal aglycones (astringenin and isorhapontigenin) are released,
19 creating an unfavorable environment for fungal growth (Woodward and Pearce 1988). Another study
20 showed that *Taxus* tree ray parenchyma in the wood and phloem permanently accumulates taxol encased
21 in hydrophobic bodies (HBs) to avoid cytotoxicity (Soliman and Raizada 2020) and taxol is assumed to
22 be released from HBs to the outer bark in response to pathogenic invasion (Soliman and Raizada 2020;
23 Wagner and Flores 1994). Considering these examples, evaluating the antifungal activity of bark tissues
24 with relatively preserved chemical components in their native state is important to elucidate the
25 constitutive defense mechanisms. One method is to evaluate the antifungal activity of the bark by placing
26 sterilized bark tissues directly on an agar with a tester fungal strain (Munir et al. 2020a). In this method,
27 chemicals are diffused from the bark pieces into the agar, and their effect on the fungal growth is
28 observed (Munir et al. 2020a). This method requires the sterilization of bark samples to eliminate the
29 contribution of microorganisms (e.g., endophytes or other microbes attached to the bark surface) to the

antifungal activity. For example, an endophytic fungus from the inner tissues of *Taxus* tree branches, *Paraconiothyrium* SSM001, which produces taxol, can inhibit the growth of decay-causing fungi *in vitro* (Soliman et al. 2011, 2015). In this study, gamma sterilization was used because the loss of active chemicals by gamma irradiation is minimal compared to that by autoclave sterilization (Munir et al. 2020a).

The present study aimed to assess the antifungal activity of gamma-sterilized bark pieces from 25 tree species in Hokkaido against *Trametes versicolor*. The reason for using *T. versicolor* as a test fungus is based on the target of tree's constitutive defense. The constitutive defense mechanism is thought to be effective not against specific fungi, but against a wide range of fungi that are floating around an external environment, looking for opportunities to invade the inside trees (Franceschi et al. 2005). *T. versicolor* is a common white-rot fungus in Hokkaido (Takahashi 2012) that occurs on dead stumps, fallen trees, and hardwoods trunks (Moore et al. 2011; Takahashi 2012). It has been previously used for wood decay (Bazyar 2012; Sakai et al. 2020) and antifungal activity tests (Lomelí-Ramírez et al. 2016; Mori et al. 1995, 1997; Quiñones et al. 1995); therefore, it was considered as a suitable test fungus for conducting antifungal activity tests. Additionally, we examined the lowest possible gamma ray irradiation dose necessary to sterilize the bark pieces. The abundance of microorganisms depends on the gamma irradiation dose. However, high gamma irradiation may affect antifungal activities, for example, by degrading high molecular weight compounds to low molecular weight compounds that contribute to antimicrobial activities (Khattak and Rahman 2016). To eliminate as many complications as possible, minimizing the gamma irradiation dose for sterilization is desirable. Hence, we examined the validity of 15 kGy for sterilizing bark pieces by comparing it with other intensities of gamma irradiation.

Material and Methods

Sample preparation

The barks of 25 species were collected from Hokkaido, Japan, in summer when vascular cambium was active; hence peeling the barks from the stem was easy (Table 1). Rectangular bark pieces (axial direction × tangential direction = 30 cm × 3 cm) were cut at heights between 50 and 210 cm above the ground. The specimens were immediately wrapped in wet paper towels and placed in plastic bags to

prevent drying. They were then taken to the laboratory and cut into 3 cm × 3 cm pieces. Some bark pieces were divided into outer and inner barks, and three types of bark samples (whole, outer, and inner barks) were prepared. In the whole bark of *Phellodendron amurense* var. *amurense* and *Syringa reticulata*, the outer and inner barks were easily detached, and thus they were stitched together. All bark pieces were stored in a deep freezer at −80 °C. The frozen bark pieces were freeze-dried for 24 h at 3–10 Pa (FDU-2200, EYELA). This freeze-drying treatment was performed to eliminate the induced defense response by killing living cells, and to exclude the effect of the high moisture content of the bark tissues. Subsequently, the freeze-dried bark pieces were sent to Radia Industry Co., Ltd. (Takasaki, Japan) via refrigerated shipping (−15 °C) to sterilize them by gamma irradiation at a dose of 15 kGy. The sterilized samples were sent back to the laboratory from the company in the same manner as mentioned previously and were stored in a thermostatic chamber at −10 °C until further use.

Antifungal activity test

A potato dextrose agar (PDA, Difco™) medium was poured into Petri dishes (9 cm diameter, 2 cm height). The tester fungal strain *T. versicolor* (NBRC 30340) was used and maintained on PDA slants at 25 °C under dark conditions. The fungi were then transferred from the slants to Petri dishes containing PDA. After the fungi spread concentrically and covered most of the plate surface, small inoculum discs of the medium containing the fungi were punched out with a cork borer (7 mm in diameter) and placed on the agar medium. The sterilized bark pieces were then placed on the agar, with the outer surface directed upward (Fig. 1). Subsequently, the plates were incubated at 25 °C under dark conditions. The diameter of mycelial growth was measured every 2 d until the mycelia reached the edge of the bark piece (Fig. 2). Each treatment was performed using one replicate of n = 4–7, and the mean value of the mycelial growth diameter for each treatment was calculated and substituted as GC or GT into the equation given below. Antifungal activity (AFA) was determined using the following equation (Mori et al. 1995, 1997; Quiñones et al. 1995):

$$\text{AFA (\%)} = 100 \times \frac{(\text{GC} - \text{GT})}{\text{GC}}$$

1 where GC is the mycelial growth on the control medium (without bark) and GT is the mycelial growth
2 on the test medium (with bark).

3 Petri dishes containing PDA with a groove in the center of the medium were also prepared by
4 removing the medium (approximately 1 cm wide) using a flame-sterilized scalpel or sterilized
5 inoculation needle to check whether the antifungal substances were volatile (Fig. S1). The inner bark
6 pieces of *Sorbus commixta* and the whole bark of *P. jezoensis* were placed on one side of the divided
7 medium and the fungus was placed on the other side and incubated at 25°C under dark conditions. The
8 diameter of the mycelial growth was measured. Each treatment was performed using one replicate of n
9 = 3. AFA was determined as described above.

12 *Gamma sterilization test*

13 Bark pieces were collected from the stems of 16 species and 3 cm × 3 cm (axial direction ×
14 tangential direction) pieces were trimmed as described above (Tables 1 and 2). In the bark of *Larix*
15 *kaempferi*, *Cerasus sargentii* var. *sargentii*, *P. amurense* var. *amurense*, and *Betula platyphylla* var.
16 *japonica*, the outer and inner barks could be easily detached and were thus, stitched together. Frozen
17 bark pieces were freeze-dried for 24 h at approximately 4 Pa after freezing and stored in a deep freezer
18 at −80 °C as stated above. Some of the freeze-dried bark specimens were stored in a deep freezer at
19 −80 °C as a control test (0 kGy) without sterilization, until further use. The remaining bark specimens
20 were sterilized by gamma irradiation at 3, 6, 9, 12, or 15 kGy as described above. The sterilized samples
21 were stored in a thermostatic chamber at −10 °C until further use. After the non-sterilized or sterilized
22 bark pieces were placed on the center of the agar (Figure 1), the plates were incubated at 25°C under
23 dark condition for 10 d. The number of plates on which any microorganisms appeared were counted,
24 and the percentage of sterilized bark pieces in each gamma dose was calculated.

27 **Results**

28 The whole and inner barks of *S. commixta* strongly inhibited mycelial growth (Days 2–6: AFA =
29 100%; Table 3, S1), and the outer bark of *S. commixta* exhibited a lower AFA (Day 2: 57%, Day 4: 38%,

1 and Day 6: 25%; Table 3, S1). The inner bark of *S. commixta* also inhibited the mycelial growth in the
2 media containing grooves (Days 2–6: AFA = 100%; Table 4, Fig. S1).

3 In other species, the outer bark of *C. sargentii* var. *sargentii* showed a lower AFA (Day 2: 36%,
4 Day 4: 25%) than that of *S. commixta* (Table 3, S1). The inner barks of *C. sargentii* var. *sargentii* showed
5 almost half of the AFA in the outer bark (Table 3, S1). Contrastingly, a minimal AFA was observed in
6 the whole bark of *C. sargentii* var. *sargentii* (<10%; Table 3, S1). In the case of *P. jezoensis*, the whole,
7 outer, and inner barks contained approximately 10–20% AFAs (Table 3, S1). However, the whole bark
8 of *P. jezoensis* did not significantly inhibit mycelial growth in media containing grooves (AFA<10%;
9 Table 4). In the case of *Magnolia obovate*, the inner bark contained 13% AFA on Day 4 (Table 3, S1).
10 Contrastingly, the other 21 tree species showed no or minimal AFAs (<10%; Table 3, S1).

11 In gamma irradiation tests, no microorganisms appeared on the medium when the bark pieces of all
12 tree species, except *Abies sachalinensis*, were sterilized at a dose of 12 kGy and incubated for 10 d (Table
13 5). Moreover, microorganisms were not observed in the medium when the bark pieces of *A. sachalinensis*
14 were sterilized at 15 kGy and incubated for 10 d (Table 5). Additionally, many angiospermous species
15 could be sterilized at 6 kGy, whereas coniferous species required a dose of 9 kGy or more (Table 5).

18 Discussion

19 Mechanisms of AFAs in three species showing high AFAs

20 The inner and whole barks of *S. commixta* showed 100% AFAs, whereas the outer bark exhibited
21 a lower AFA (Table 3, S1). These results suggested that the inner bark of *S. commixta* contains antifungal
22 substances that can strongly suppress the mycelial growth of *T. versicolor*. The causal substance could
23 be volatile substances, rather than water-soluble substance, that can diffuse through the PDA medium,
24 because the fungal growth was also inhibited on the medium with a groove between the fungi and the
25 bark specimen (Table 4, Fig. S1).

26 It has been reported that various tissues of *S. commixta* (e.g., seeds, bark, buds, wood, and roots)
27 contain cyanogenic glycosides, i.e., amygdalin and prunasin, which are hydrolyzed by enzymes (β -
28 glucosidase) to produce hydrogen cyanide (HCN) and benzaldehyde (Takaishi and Kuwajima 1976;
29 Takaishi et al. 1977). HCN causes high toxicity by inhibiting the mitochondrial respiration pathway

(Siegień and Bogatek 2006); additionally, it has a defensive function (Davis 1991; Machingura et al. 2016). Therefore, it can be hypothesized that HCN was generated and released from the inner bark of *S. commixta* on the medium because of the degradation of cyanogenic glycosides by β -glucosidase and contributed to the high AFA of the bark of *S. commixta* against *T. versicolor*. Further, the HCN generated from amygdalin and prunasin likely contributed to the AFA of the outer bark. However, the AFA of the outer bark was much lower than that of inner bark (Table 3, S1). A possible reason for this difference was that the quantities of amygdalin and prunasin would be extremely low in the outer bark, which consists primarily of dead phellem and a few living cells of phellogen and phelloderm (Angyalossy et al. 2016).

The whole, outer, and inner barks of *P. jezoensis* showed low AFAs (11–21%), whereas the whole bark of *P. jezoensis* on media with grooves showed a minimal AFA (Table 3, 4, S1). These results implied that the antifungal substances in the whole bark of *P. jezoensis* are not volatile and affect fungal growth by diffusing into the PDA medium. Previous studies have shown that the bark of *P. jezoensis* is rich in terpenoids (Tanaka et al. 1995, 1996, 2003) and stilbenes (Suprun et al. 2021). These bioactive compounds may have contributed to the AFA in this study. Additionally, the outer bark of *C. sargentii* var. *sargentii* showed 36% AFA on Day 2 and 25% AFA on Day 4, whereas the inner bark showed a lower AFA than the outer bark (Table 3, S1). These results indicated that the outer bark of *C. sargentii* var. *sargentii* contains antifungal substances against *T. versicolor*. Flavonoids, such as neosakuranin and taxifolin, have been reported as antioxidative substances in the outer bark of *C. sargentii* var. *sargentii* (Park and Bae 2012). Another study showed that the ethanol extracts from the wood of *C. sargentii* var. *sargentii* inhibit mycelial growth of the white-rot fungus, *Pleurotus cornucopiae* (PERS) ROLLAND, and contains flavanones, including sakuranetin, isosakuranetin, naringenin, pinocembrin, and flavanonols, including dihydrokaempferide and aromadendrin (katuranin), and a flavone, genkwanin (Miura et al. 1985). If such flavonoids are present in the outer bark, they could contribute to the AFA of the outer bark of *C. sargentii* var. *sargentii* against *T. versicolor*. Although we observed AFAs in the outer and inner barks of *C. sargentii* var. *sargentii*, the AFA in the whole bark showed a minimal AFA (Table 3, S1). A possible explanation for this difference is that the antifungal compounds present in the inner bark of *C. sargentii* var. *sargentii* may have accumulated near the boundary between the outer and the inner barks and effectively released into the air when the inner bark surface was exposed after peeling

1 the outer bark from the inner bark. However, whether the antifungal compounds present in the inner bark
2 of *C. sargentii* var. *sargentii* are volatile needs further investigation.

3 4 *Comparison of AFAs between this study and previous studies*

5 Previous studies have examined AFAs of acetone extracts of barks of *S. commixta* and *C. sargentii*
6 var. *sargentii* against *T. versicolor* and showed 0% AFAs against *T. versicolor* (Mori et al. 1997), but in
7 the present study, high AFAs were observed in the inner bark of *S. commixta* and the outer bark of *C.*
8 *sargentii* var. *sargentii* (Table 3, S1). A possible explanation for this discrepancy is that antifungal
9 substances, which are not extractable in acetone or are lost during extraction, contributed to the high
10 AFA. Furthermore, the assay system used in this study was useful in identifying AFAs exhibited by
11 tissues, without the bias of extraction methods. Conversely, Mori et al. (1995, 1997) found that the
12 acetone extracts of the barks of *M. kobus* var. *borealis*, *M. obovate*, and *Taxus cuspidata*, which had the
13 minimal AFAs in this study (Table 3, S1), exhibited the high AFAs (25–100%). One hypothesis is that
14 the number of antifungal substances in the acetone extracts in the medium used in previous studies (Mori
15 et al. 1995, 1997) may have been more concentrated than the amounts exploited from the actual tissues.
16 Moreover, *Ulmus davidiana* var. *japonica*, *Fraxinus lanuginosa* f. *serrata*, *F. mandshurica*, and *S.*
17 *reticulata* showed minimal AFAs in this study (Table 3, S1) and in a previous study (Mori et al. 1997).
18 Minimal AFAs may indicate that these trees are susceptible to fungal attack or that the antifungal
19 substances in the bark involved in the tree's constitutive defense are limited, as discussed for the low
20 AFA of the bark extracts of *B. pubescens* in a previous study (Alfredsen et al. 2008). Alternatively, these
21 tree species could contain antifungal substances that are not extracted by acetone and cannot easily
22 permeate the PDA medium from the bark pieces before the fungi spread to the PDA medium, probably
23 because of their low water solubility (Scorzoni et al. 2007) or low diffusion rate (van Vuuren 2008).
24 Overall, combining the extraction-based method with this assay system is useful for a better
25 understanding of the chemical defense mechanisms in a wide range of tree species and for finding an
26 effective parameter for screening barks with superior chemical defense.

27 28 *Trade-off between bark and xylem defenses*

1 In the present study, the barks of *S. commixta* and *C. sargentii* var. *sargentii* showed high AFAs
2 against *T. versicolor* (Table 3, S1), indicating that their barks had superior constitutive chemical defense
3 mechanisms. Interestingly, *S. commixta* and *Cerasus* spp. are known host tree species of *T. versicolor*
4 (Kamei and Igarashi 1960; Yasuda 1908). Furthermore, *S. commixta* and *C. sargentii* var. *sargentii*, both
5 of which are widely planted in Hokkaido, Japan, are vulnerable to damage by cankers and dieback from
6 different pathogens, such as *Leucostoma massariana* and *L. personii* (Oguchi 1981). Previous studies
7 have shown that bark and xylem defenses are trade-offs, and it is highly unlikely that both tissues have
8 strong constitutive and induced defenses (Biggs 1986; Morris et al. 2020; Romero and Bolker 2008).
9 Overall, it can be hypothesized that the barks of *S. commixta* and *C. sargentii* var. *sargentii* may have
10 effective defenses against pathogens or fungi, including white-rot fungi, but the xylem may have a low
11 defense ability, and the lesions may spread easily once fungi or pathogens invade the xylem.

12 13 *Appropriate gamma dose for sterilization of bark pieces*

14 In this study, sterilization of bark pieces was necessary to evaluate the AFAs of natural substances
15 in the bark tissues to exclude the effects of microorganisms, such as endophytes. In our preliminary
16 study, we attempted autoclave sterilization at 121 °C for 15 min. However, no AFAs were detected in
17 the whole or inner bark of *S. commixta*, probably because the antifungal compounds were lost or altered
18 during autoclave sterilization (data not shown). Therefore, we adopted gamma sterilization at 15 kGy
19 for the antifungal activity test, according to Pointing et al. (1998). In this study, we examined the
20 necessary and sufficient doses for 16 tree species and found that 12 kGy was sufficient for 15 out of the
21 16 species, and 15 kGy was necessary for the remaining one species (Table 5). Furthermore, coniferous
22 species seem to tend to require more gamma doses than angiospermous species (Table 5). Thus, although
23 15 kGy is a sufficient dose for the sterilization of bark tissues of most tree species from temperate zones
24 especially angiospermous species, it is assumed that 18 kGy is more desirable for coniferous species to
25 completely sterilize them.

Conclusion

- The inner bark of *Sorbus commixta* showed extremely strong AFAs in an attempt to evaluate the AFAs of bark tissues from a total of 25 tree species using an incubation system in which mycelial growth of *Trametes versicolor* was monitored.
- The incubation system is useful to find out tree species that bear bark tissues with strong/unique chemical defense mechanisms, which may be undetectable by other methods.
- In the incubation system, gamma sterilization of bark sample is necessary as a pretreatment of bark specimens. The radiation dose of 15 kGy is sufficient to completely sterilize the bark specimens for most tree species growing in temperate regions.

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14 **Statements & Declarations**

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19 **Competing interests**

20 The authors have no relevant financial or non-financial interests to disclose.

23 **Author contributions**

24 All authors contributed to the study conception and design. Material preparation, data collection
25 and analysis were performed by Masato Koyama, Yutaka Tamai, Kengo Shigetomi, and Yuzou Sano.
26 The first draft of the manuscript was written by Masato Koyama and all authors commented on previous
27 versions of the manuscript. All authors read and approved the final manuscript.

1

2 **Data availability**

3 The datasets generated during and/or analyzed during the current study are available from the
4 corresponding authors on reasonable request.

5

6

7 **Figure caption:**

8 **Fig. 1** Schematic diagram of the culture system for gamma sterilization test and antifungal activity test.

9 The dotted line indicates a line drawn on the bottom of a Petri dish. The diameter of the Petri dish
10 is 9 cm

11

12 **Fig. 2** *Trametes versicolor* incubated with (A) and without (A') inner bark pieces of *Phellodendron*
13 *amurense*, with (B) and without (B') outer bark of *Cerasus sargentii* var. *sargentii*, and with (C)
14 and without (C') inner bark of *Sorbus commixta* 4 d after inoculation. GT: mycelial growth with
15 a bark tissue, GC: mycelial growth without a bark tissue. Scale bars = 3 cm

16

Tables and Figures

Table 1 Trees sampled for the antifungal activity (AFA) test against *T. versicolor*

Species	Family	DBH (cm) ^a	Date	Site ^b
Rosids				
<i>Betula platyphylla</i> Sukaczew var. <i>japonica</i> (Miq.) H.Hara	Betulaceae	18	3 July 2020	Tomakomai
<i>Maackia amurensis</i> Rupr. et Maxim.*	Fabaceae	28	29 July 2021	Tomakomai
<i>Fagus crenata</i> Blume*	Fagaceae	9.9	8 July 2021	Sapporo ₋₁
<i>Quercus crispula</i> Blume var. <i>crispula</i>	Fagaceae	31	3 July 2020	Tomakomai
<i>Juglans mandshurica</i> Maxim. var. <i>sachalinensis</i> (Komatsu) Kitam.*	Juglandaceae	35	8 July 2021	Sapporo ₋₁
<i>Aria alnifolia</i> (Siebold et Zucc.) Decne.*	Rosaceae	27.5	29 July 2021	Tomakomai
<i>Cerasus sargentii</i> (Rehder) H.Ohba var. <i>sargentii</i> *	Rosaceae	31	29 July 2021	Tomakomai
<i>Sorbus commixta</i> Hedl.	Rosaceae	36	24 June 2020	Sapporo ₋₁
<i>Phellodendron amurense</i> Rupr. var. <i>amurense</i>	Rutaceae	36	3 July 2020	Tomakomai
<i>Salix caprea</i> L.	Salicaceae	12	10 June 2022	Tomakomai
<i>Salix integra</i> Thumb.	Salicaceae	4.4	10 June 2022	Tomakomai
<i>Salix udensis</i> Trautv. et C.A.Mey.	Salicaceae	27	10 June 2022	Tomakomai
<i>Ailanthus altissima</i> (Mill.) Swingle	Simaroubaceae	26.9	9 July 2020	Sapporo ₋₁
<i>Ulmus davidiana</i> Planch. var. <i>japonica</i> (Rehder) Nakai	Ulmaceae	20.2	4 July 2020	Sapporo ₋₁
Magnolids				
<i>Magnolia kobus</i> DC. var. <i>borealis</i> Sarg.*	Magnoliaceae	16	29 July 2021	Tomakomai
<i>Magnolia obovata</i> Thunb.	Magnoliaceae	15	3 July 2020	Tomakomai
Asterids				
<i>Sambucus racemosa</i> L. subsp. <i>kamtschatica</i> (E.L.Wolf) Hultén*	Viburnaceae	11	29 July 2021	Tomakomai
<i>Fraxinus lanuginosa</i> Koidz. f. <i>serrata</i> (Nakai) Murata	Oleaceae	16	3 July 2020	Tomakomai
<i>Fraxinus mandshurica</i> Rupr.	Oleaceae	14.6	4 July 2020	Sapporo ₋₁
<i>Syringa reticulata</i> (Blume) H.Hara*	Oleaceae	39	4 June 2021	Sapporo ₋₂
Pinales				
<i>Abies sachalinensis</i> (F.Schmidt) Mast.	Pinaceae	21.5	4 July 2020	Sapporo ₋₁
<i>Larix kaempferi</i> (Lamb.) Carrière	Pinaceae	21	25 August 2020	Sapporo ₋₁
<i>Picea glehnii</i> (F.Schmidt) Mast.	Pinaceae	21.6	3 July 2020	Tomakomai
<i>Picea jezoensis</i> (Siebold et Zucc.) Carrière var. <i>jezoensis</i> *	Pinaceae	48	29 July 2021	Tomakomai
<i>Taxus cuspidata</i> Siebold et Zucc.*	Taxaceae	16	21 July 2021	Sapporo ₋₁

^aDBH, diameter at breast height.

^bSapporo₋₁: Sapporo Experimental Forest of Hokkaido University, ^bSapporo₋₂: Sapporo campus of Hokkaido University.

*Sample trees that were also used for gamma sterilization test

Table 2 Trees sampled for gamma sterilization test.

Species*	Family	DBH (cm) ^a	Date	Site ^b
Rosids				
<i>Betula platyphylla</i> var. <i>japonica</i>	Betulaceae	16	29 July 2021	Tomakomai
<i>Sorbus commixta</i>	Rosaceae	33	21 July 2021	Sapporo. ₁
<i>Phellodendron amurense</i> var. <i>amurense</i>	Rutaceae	24.5	29 July 2021	Tomakomai
Magnolids				
<i>Magnolia obovate</i>	Magnoliaceae	30	29 July 2021	Tomakomai
Pinales				
<i>Abies sachalinensis</i>	Pinaceae	28	8 July 2021	Sapporo. ₁
<i>Larix kaempferi</i>	Pinaceae	29.5	8 July 2021	Sapporo. ₁

^aDBH, diameter at breast height. ^bSapporo.₁: Sapporo Experimental Forest of Hokkaido University.

*Sample trees that were also used for AFA test are listed in Table 1

Table 3 AFAs of bark tissues against *T. versicolor* (%)

Species	Day 2 ^a			Day 4 ^a			Day 6 ^a		
	WB ^b	OB ^b	IB ^b	WB ^b	OB ^b	IB ^b	WB ^b	OB ^b	IB ^b
Rosids									
<i>Betula platyphylla</i> var. <i>japonica</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Maackia amurensis</i>	—	—	—	n.d.	—	n.d.	n.d.	n.d.	n.d.
<i>Fagus crenata</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Quercus crispula</i> var. <i>crispula</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Juglans mandshurica</i> var. <i>sachalinensis</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Aria alnifolia</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Cerasus sargentii</i> var. <i>sargentii</i>	—	36	17	—	25	12	n.d.	n.d.	n.d.
<i>Sorbus commixta</i>	100	57	100	100	38	100	100	25	100
<i>Phellodendron amurense</i> var. <i>amurense</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Salix caprea</i>	—	—	—	—	—	n.d.	n.d.	n.d.	n.d.
<i>Salix integra</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Salix udensis</i>	—	—	—	—	n.d.	—	n.d.	n.d.	n.d.
<i>Ailanthus altissima</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Ulmus davidiana</i> var. <i>japonica</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
Magnolids									
<i>Magnolia kobus</i> var. <i>borealis</i>	—	—	—	n.d.	n.d.	—	n.d.	n.d.	n.d.
<i>Magnolia obovate</i>	—	—	—	—	—	13	n.d.	n.d.	n.d.
Asterids									
<i>Sambucus racemosa</i>	—	—	—	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
<i>Fraxinus lanuginose</i> f. <i>serrata</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Fraxinus mandshurica</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Syringa reticulata</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
Pinales									
<i>Abies sachalinensis</i>	—	—	—	—	—	—	n.d.	—	n.d.
<i>Larix kaempferi</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Picea glehnii</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.
<i>Picea jezoensis</i> var. <i>jezoensis</i>	13	11	21	19	12	17	n.d.	n.d.	n.d.
<i>Taxus cuspidata</i>	—	—	—	—	—	—	n.d.	n.d.	n.d.

^aNumber of days elapsed after inoculation. ^bWB; whole bark, ^bOB; outer bark, ^bIB; inner bark. —; AFA<10%, n.d.; not-determined (It was difficult to measure the amount of mycelial growth because the mycelia reached the edge of the bark piece)

Table 4 AFAs of bark tissues against *T. versicolor* inoculated on media with grooves (%)

Species	Day 2 ^a	Day 4 ^a	Day 6 ^a
<i>Sorbus commixta</i>	100	100	100
<i>Picea jezoensis</i> var. <i>jezoensis</i>	—	n.d.	n.d.

^aNumber of days elapsed after inoculation. —; AFA<10%, n.d.; not determined (It was difficult to measure the amount of mycelial growth because the mycelia reached the edge of a groove)

Table 5 Gamma irradiation dose required for sterilization of freeze-dried bark pieces incubated for 10 d

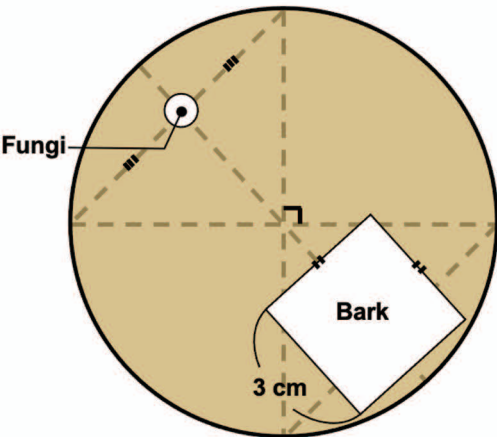
Species	Minimum dose (kGy) for 100% sterilization	Percentage of sterilized bark pieces (%) ^a					
		0 kGy	3 kGy	6 kGy	9 kGy	12 kGy	15 kGy
Rosids							
<i>Betula platyphylla</i> var. <i>japonica</i>	6	0	0	100	100	100	100
<i>Maackia amurensis</i>	6	0	0	100	100	100	100
<i>Fagus crenata</i>	6	0	0	100	100	100	100
<i>Juglans mandshurica</i> var. <i>sachalinensis</i>	6	0	0	100	100	100	100
<i>Aria alnifolia</i>	9	0	0	80	100	100	100
<i>Cerasus sargentii</i> var. <i>sargentii</i>	6	0	60	100	100	100	100
<i>Phellodendron amurense</i> var. <i>amurense</i>	6	0	20	100	100	100	100
<i>Sorbus commixta</i>	6	0	80	100	100	100	100
<i>Phellodendron amurense</i> var. <i>amurense</i>	6	0	20	100	100	100	100
Magnolids							
<i>Magnolia kobus</i> var. <i>borealis</i>	6	0	0	100	100	100	100
<i>Magnolia obovata</i>	9	0	0	60	100	100	100
Asterids							
<i>Sambucus racemosa</i>	12	0	0	0	60	100	100
<i>Syringa reticulata</i>	6	0	0	100	100	100	100
Pinales							
<i>Abies sachalinensis</i>	15	0	0	0	0	60	100
<i>Larix kaempferi</i>	9	0	0	60	100	100	100
<i>Picea jezoensis</i> var. <i>jezoensis</i>	12	0	0	0	80	100	100
<i>Taxus cuspidata</i>	9	0	0	0	100	100	100

^aThe percentage was calculated by counting the number of plates on which any organisms appeared. Five types of media were used for each treatment

Fig. 1 Schematic diagram of the culture system for gamma sterilization test and antifungal activity test. The dotted line indicates a line drawn on the bottom of a Petri dish. The diameter of the Petri dish is 9 cm

Fig. 2 *Trametes versicolor* incubated with (A) and without (A') inner bark pieces of *Phellodendron amurense*, with (B) and without (B') outer bark of *Cerasus sargentii* var. *sargentii*, and with (C) and without (C') inner bark of *Sorbus commixta* 4 d after inoculation. GT: mycelial growth with a bark tissue, GC: mycelial growth without a bark tissue.

Antifungal activity test



Gamma sterilization test

