

# A Common Fungal Associate of the Spruce Bark Beetle Metabolizes the Stilbene Defenses of Norway Spruce<sup>1</sup>[C][W][OA]

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Norway spruce (*Picea abies*) forests suffer periodic fatal attacks by the bark beetle *Ips typographus* and its fungal associate, *Ceratocystis polonica*. Norway spruce protects itself against fungal and bark beetle invasion by the production of terpenoid resins, but it is unclear whether resins or other defenses are effective against the fungus. We investigated stilbenes, a group of phenolic compounds found in Norway spruce bark with a diaryl-ethene skeleton with known antifungal properties. During *C. polonica* infection, stilbene biosynthesis was up-regulated, as evidenced by elevated transcript levels of stilbene synthase genes. However, stilbene concentrations actually declined during infection, and this was due to fungal metabolism. *C. polonica* converted stilbenes to ring-opened, deglycosylated, and dimeric products. Chromatographic separation of *C. polonica* protein extracts confirmed that these metabolites arose from specific fungal enzyme activities. Comparison of *C. polonica* strains showed that rapid conversion of host phenolics is associated with higher virulence. *C. polonica* is so well adapted to its host's chemical defenses that it is even able to use host phenolic compounds as its sole carbon source.

Norway spruce (*Picea abies*), a dominant tree species in European boreal, montane, and subalpine forests, is frequently subject to fatal attacks by the bark beetle *Ips typographus* (Wermelinger, 2004). During attacks, these scolytine beetles introduce fungal pathogens into their hosts. One of the most virulent spruce pathogens associated with bark beetle attacks is the blue-staining ascomycete *Ceratocystis polonica* (Krokene and Solheim, 1998). Tree death is thought to result either from bark beetle attack alone (Six and Wingfield, 2011) or from the combined action of the bark beetle and the fungus (Franceschi et al., 2005), where the beetles damage the

cambium by feeding and the fungus interrupts water flow in the xylem. Although the association between the fungus and the beetle might only be facultative (Six and Wingfield, 2011), bark beetles could potentially benefit from this necrotrophic fungus, which may help kill the host tree, unlock nutrients, and weaken host defense capacity (Paine et al., 1997).

Norway spruce trees are known to have several effective structural and chemical defense strategies against bark beetles that can ward off low-density attacks (Franceschi et al., 2005). The best known example of a chemical defense in this species is oleoresin (Keeling and Bohlmann, 2006; Schmidt et al., 2010). This viscous mixture of terpenoids stored in specialized ducts flows to the site of damage when ducts are severed and has been shown to increase in quantity after initial beetle attack. A less well-studied defense mechanism in spruce is the production of phenolic compounds in specialized cells in the bark. These substances are stored in phloem parenchyma cells (Franceschi et al., 2000; Li et al., 2012) that expand during wounding or fungal attack and show major cytological changes.

Stilbenes are a widespread group of phenolic compounds in spruce and other species of the family Pinaceae (Underwood and Pearce, 1992). They are reported to have antifungal properties and have been shown to contribute to plant disease resistance (Chong et al., 2009; Jeandet et al., 2010). In the genus *Picea*, the stilbene glucosides astringin and isorhapontin (Fig. 1A) occur in high concentrations in bark, roots, and foliage (Hammerbacher et al., 2011). Two stilbene synthase

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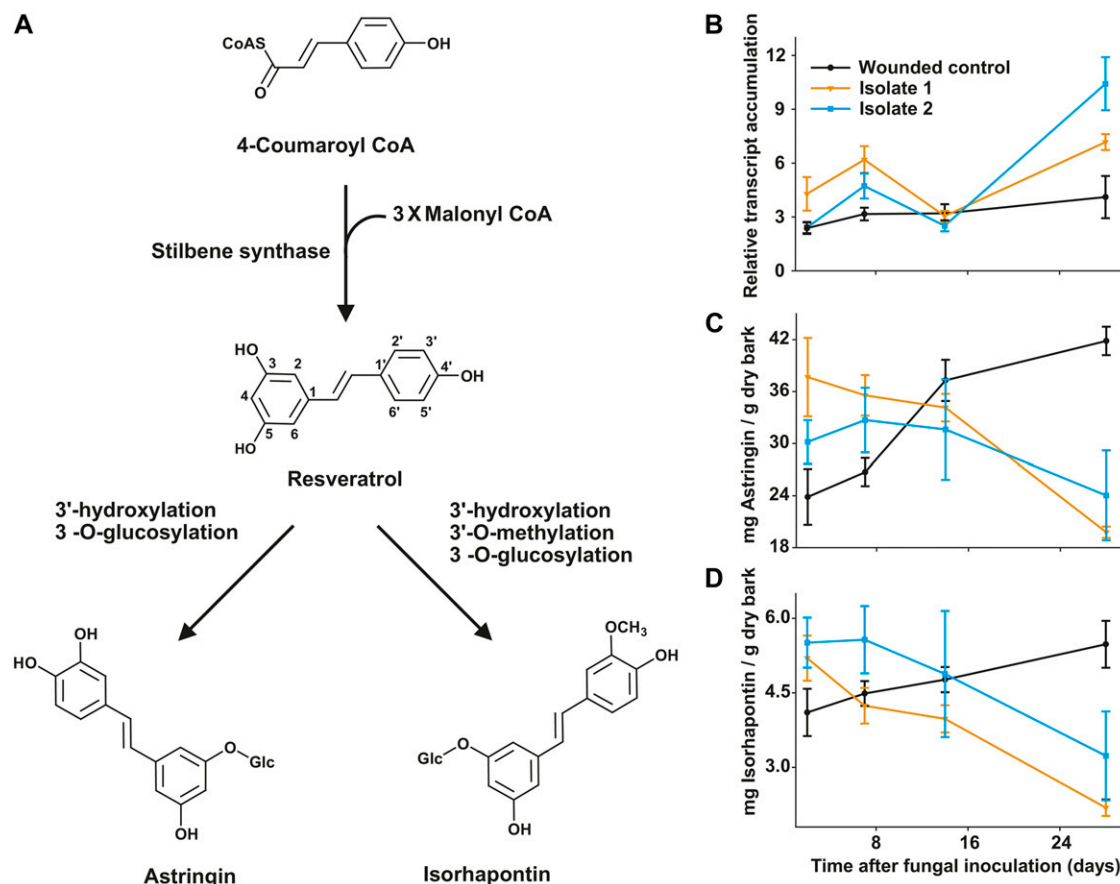
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**Figure 1.** Stilbene biosynthesis and stilbene concentrations in Norway spruce bark in response to fungal infection. A, Route of stilbene biosynthesis. STS transcription (B) and concentrations of the stilbene glucosides astringin (C) and isorhapontin (D) were measured in Norway spruce saplings after inoculation and infection by two *C. polonica* isolates and after control wounding treatment (inoculation with sterile agar). Error bars represent SE ( $n = 5$ ). Significant differences were detected between 14 and 28 d after treatment for both fungus-infected and wounded control saplings ( $P < 0.005$ ). [See online article for color version of this figure.]

(STS) enzymes that contribute to the biosynthesis of these compounds have been described in Norway spruce (Hammerbacher et al., 2011). These enzymes seem to play a role in tree defense, since fungal infection induced elevated amounts of STS transcript (Hammerbacher et al., 2011) and increased enzyme levels (Brignolas et al., 1995). However, there are contradictory reports on stilbene glucoside accumulation in spruce bark during fungal infection. Significant increases in astringin were observed after inoculation of *Picea glauca* saplings with avirulent *C. polonica* (Hammerbacher et al., 2011). However, stilbene glucoside concentrations in mature Norway spruce were stable or even declined during the course of *C. polonica* infection (Brignolas et al., 1995; Viiri et al., 2001; Li et al., 2012).

After host infection, fungi follow different strategies to gain access to host nutrients. Biotrophic fungi acquire nutrients directly from living cells by penetrating them and causing little visible damage (Voegelé and Mendgen, 2003). Necrotrophic fungi such as *C. polonica*

lyse cells in order to release nutrients that are then assimilated by the fungi (Oliver and Solomon, 2010). During plant cell lysis, potential antifungal defense compounds may come into contact with necrotrophic fungi. However, it is not yet known how effective Norway spruce defense compounds are against *C. polonica* and whether this specialized, bark beetle-vectored pathogen has developed any resistance to them. This missing information may be critical in understanding how *C. polonica* contributes to the success of bark beetle attacks and why so many mutualistic relationships between bark beetles and blue-staining fungi have evolved.

In this work, we demonstrate that *C. polonica* can circumvent the antifungal activity of Norway spruce stilbenes during infection. Although high levels of STS transcripts accumulate in fungus-infected bark, a net loss of stilbene glucosides was detected at the site of infection. This reduction in stilbenes was explained by fungal biotransformation processes, including the formation of stilbene dimers, aglycones, and ring-opened

lactones, which may represent the first step of the  $\beta$ -ketoacid pathway for microbial utilization of aromatic compounds as a carbon source. We could also show that different *C. polonica* isolates follow different stilbene biotransformation strategies and that rapid biotransformation and formation of ring-opened lactones is associated with greater levels of fungal virulence in Norway spruce bark.

## RESULTS

### Fungal Inoculation of Norway Spruce Bark Increases Stilbene Biosynthetic Gene Transcripts But Reduces Stilbene Accumulation

When Norway spruce saplings were inoculated with the blue-stain fungus *C. polonica*, the transcript levels of STS genes *PaSTS1* and *PaSTS2* (Hammerbacher et al., 2011) increased significantly compared with the sterile agar-inoculated control ( $P = 0.014$ ). The same pattern was observed for two different *C. polonica* isolates (Fig. 1B). However, the levels of the stilbene glucosides, astringin and isorhapontin, declined over the 28-d time course after inoculation of the two fungal isolates ( $P = 0.037$ ), while they increased in the sterile agar-inoculated control (Fig. 1, C and D).

To determine if *C. polonica* was the agent responsible for the reduced stilbene levels seen in infected bark, isolates 1 and 2 were grown in nutrient broth amended with 2 mg mL<sup>-1</sup> astringin. A significant decrease in astringin was observed in the fungal culture medium compared with a sterile control medium amended with astringin ( $P < 0.0001$ ) over a time course of 28 h (Table I). The rate of astringin decrease in the medium was significantly higher for isolate 2 compared with isolate 1 ( $P < 0.0001$ ).

### The Stilbene Astringin Is Metabolized by the Fungus to Ring-Opened, Deglucosylated, and Dimeric Products

When astringin was added to *C. polonica* cultures, several products of fungal biotransformation were detected in culture filtrates (Fig. 2), including the ring-opened

lactones **1** and **4**, the deglucosylated piceatannol **2**, as well as several dimeric products (**3**, **5**, and **6**). These compounds were identified after solid-phase extraction on RP-18 material followed by HPLC coupled to solid-phase extraction (SPE)-NMR spectroscopy.

To our knowledge, compounds **1** and **4** are reported here for the first time, and their structural elucidation is described in Supplemental Materials and Methods S1. The structures of **2a** and **2b** were identified as the deglucosylated derivatives of astringin, *E*- and *Z*-piceatannol in a ratio of 2:3, based on comparison of the mass spectrometry and <sup>1</sup>H-NMR data with reported data (Li et al., 2007). Two inseparable pairs of astringin dimers, **3a** and **3b**, were also isolated. Each pair comprises two diastereomers in a 1:1 ratio with *R,R* or *S,S* configurations at the indicated centers. Mass spectrometric and NMR spectroscopic analysis identified them as piceasides A and B and piceasides G and H (Li et al., 2008; Supplemental Figs. S2 and S3). Further pairs of dimers lacking one (**6a** and **6b**) or both (**5a** and **5b**) Glc moieties were also isolated and identified by mass spectrometry and <sup>1</sup>H-NMR spectroscopy. Again, each pair represents a mixture of diastereomers as described above.

### Separate Strains of *C. polonica* Metabolize Astringin at Different Rates Favoring Different Pathways

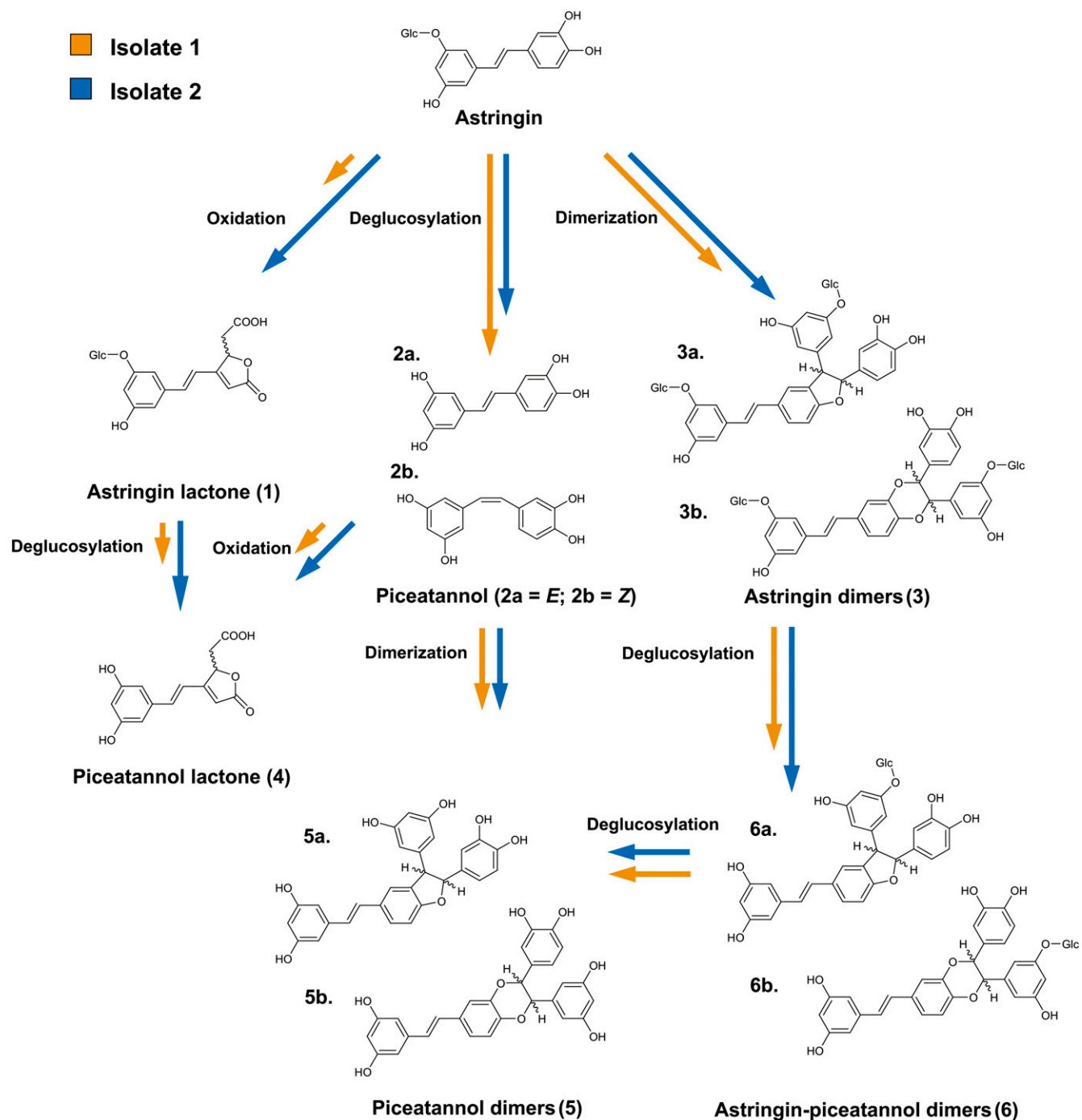
Although all 10 biotransformation products, some of which occurred as stereoisomers, could be detected in the cultures of both fungal isolates, the major pathways of stilbene breakdown differed between the two isolates. For example, the ring-opened lactone **1** (Fig. 2; Table I) was produced at significantly higher levels by isolate 2 than isolate 1 ( $P < 0.0001$ ). Lactone **4**, formed at lower concentrations than **1**, was also detected at higher levels in cultures from isolate 2 than isolate 1 ( $P < 0.0001$ ).

In contrast, *E*- and *Z*-piceatannol (**2a** and **2b**) were produced at higher levels by isolate 1 than by isolate 2 ( $P < 0.0001$ ). Moreover, a linear increase in piceatannol concentration could be observed in isolate 1 between 4 and 28 h of incubation, whereas a decrease in piceatannol content was noted in cultures colonized by isolate 2. Changes in piceatannol content in culture medium of

**Table I.** Biotransformation of the Norway spruce stilbene astringin to various metabolites by *C. polonica*

Listed are the percentages  $\pm$  SD of substrate recovered in individual biotransformation products 4 and 28 h after adding astringin to *C. polonica* isolate 1 and isolate 2 ( $n = 4$  replicates per isolate per time point). Control indicates medium without any inoculated fungus. Cultures were amended with 2 mg mL<sup>-1</sup> astringin. Metabolite numbers and structures are listed in Figure 2.

Astringin Metabolite	Isolate 1		Isolate 2		Control	
	4 h	28 h	4 h	28 h	4 h	28 h
Astringin lactone ( <b>1</b> )	0.05 $\pm$ 0.02	0.13 $\pm$ 0.04	6.54 $\pm$ 1.47	9.01 $\pm$ 1.28	0	0
Piceatannol ( <b>2a</b> and <b>2b</b> )	10.3 $\pm$ 1.14	20.7 $\pm$ 4.94	3.93 $\pm$ 0.74	0.32 $\pm$ 0.27	0	0
Astringin dimers ( <b>3a</b> and <b>3b</b> )	3.92 $\pm$ 0.50	13.9 $\pm$ 2.92	22.3 $\pm$ 3.21	10.1 $\pm$ 4.36	0.69 $\pm$ 0.11	2.14 $\pm$ 0.42
Piceatannol lactone ( <b>4</b> )	0.05 $\pm$ 0.01	0.08 $\pm$ 0.01	0.15 $\pm$ 0.04	0.54 $\pm$ 0.20	0	0
Piceatannol dimers ( <b>5a</b> and <b>5b</b> )	0.14 $\pm$ 0.02	1.12 $\pm$ 0.31	0.16 $\pm$ 0.01	0.15 $\pm$ 0.43	0	0
Astringin-piceatannol dimers ( <b>6a</b> and <b>6b</b> )	1.34 $\pm$ 0.87	15.0 $\pm$ 3.95	4.56 $\pm$ 0.87	3.83 $\pm$ 3.77	0	0
Total identified stilbene metabolites	15.8 $\pm$ 2.53	50.9 $\pm$ 12.2	37.6 $\pm$ 6.63	24.0 $\pm$ 10.3	0.69 $\pm$ 0.11	2.14 $\pm$ 0.42
Remaining astringin	77.5 $\pm$ 4.46	46.6 $\pm$ 11.6	53.7 $\pm$ 8.67	0.94 $\pm$ 0.27	96.86 $\pm$ 0.44	95.64 $\pm$ 0.27



**Figure 2.** Biotransformation of the Norway spruce stilbene astringin by *C. polonica* isolate 1 and isolate 2. The lengths of the arrows denote the relative velocity of each reaction when comparing the two isolates in the first 4 h after astringin was added to the growth medium.

both fungi were statistically significant between 4 and 28 h ( $P < 0.0001$ ).

Two pairs of diastereomeric astringin dimers (**3a** and **3b**) were observed in the culture medium of both fungal isolates as well as in noninoculated control medium, but the patterns of change differed. In cultures containing isolate 1 as well as in the sterile control,

a gradual increase in astringin dimer concentrations was observed, with levels significantly higher in the fungal cultures than in the control ( $P = 0.0006$ ). However, in cultures containing isolate 2, the highest concentration of astringin dimers was observed 4 h after the onset of the experiment, followed by a gradual decrease. Further biotransformation of astringin dimers

(diglycosides, **3a** and **3b**) to astringin-piceatannol dimers (monoglucosides, **6a** and **6b**) and piceatannol dimers (aglycones, **5a** and **5b**) followed the same relative kinetics observed for conversion to the astringin dimers, with statistically significant differences between fungal isolates 1 and 2 ( $P < 0.0001$ ).

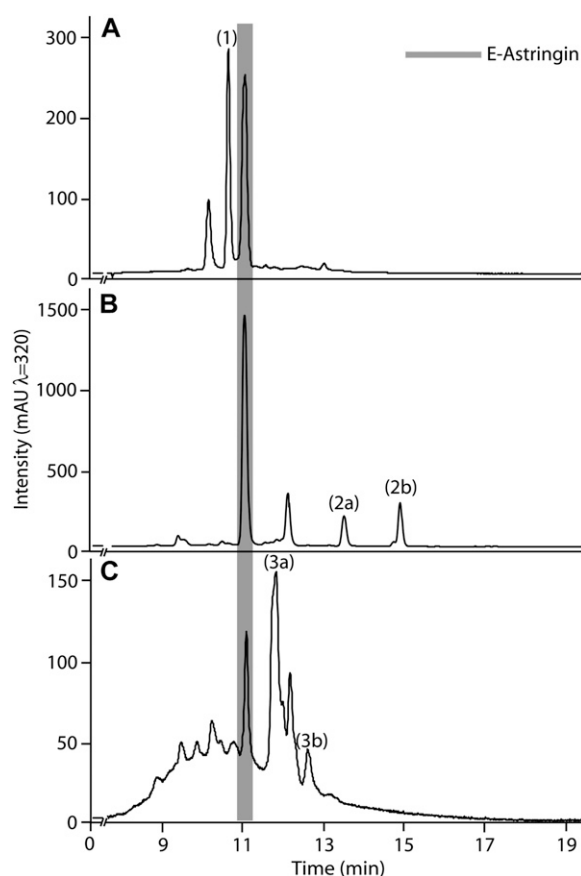
Consistent with the greater production of astringin metabolites **1**, **3a**, **3b**, and **4** in isolate 2 versus isolate 1, the overall rate of astringin degradation was faster in isolate 2 than isolate 1. After 28 h, less than 1% of the added astringin remained in isolate 2 cultures, while nearly 50% remained in isolate 1 cultures, and more than 95% of the astringin remained in the noninoculated medium (Table I).

### *C. polonica* Protein Extracts Metabolize Astringin in *Vitro* Assays

To verify that *C. polonica* had the capacity to biotransform stilbenes, the relevant astringin-metabolizing activities were sought in fungal enzyme extracts. After a two-step separation of a soluble protein extract from *C. polonica*, individual enzyme activities could be recovered for all three different types of biotransformation reactions observed in the *in vivo* study. After separation on the glycoprotein-binding matrix, concanavalin A-Sepharose, an unbound protein fraction hydrolyzed astringin to *E*- and *Z*-piceatannol **2** (Fig. 3B). Fractions that bound to concanavalin A-Sepharose were further purified by anion-exchange chromatography. One fraction from this second chromatographic step formed the ring-opened lactone **1** from astringin (Fig. 3A) via a putative catechol dioxygenase-like activity, while another fraction converted astringin to its dimers (**3a** and **3b**) via a putative laccase-like activity (Fig. 3C).

### Astringin Metabolism in *C. polonica* Is Induced by Contact with Stilbene-Rich Extracts of Norway Spruce

To test if *C. polonica* metabolism of stilbenes could be induced by contact with stilbenes, isolates 1 and 2 were grown for 4 d in medium amended with a stilbene-rich aqueous extract of Norway spruce. Contact with the spruce extract generally increased the rate of astringin degradation, as measured *in vitro* for all three reactions (Table II). For example, there was a 4-fold increase in the rate of formation of the ring-opened lactone by the protein fraction from isolate 2 relative to the control ( $P = 0.003$ ), although no significant change in the rate of lactone formation was observed for the same fraction from isolate 1. For the deglycosylation of astringin to piceatannol, a 2-fold increase in activity was noted for the protein fraction from isolate 1 after contact with spruce extract ( $P < 0.0001$ ). For isolate 2, astringin deglycosylation activity was 3-fold higher than for isolate 1 and showed a 1.5-fold increase after contact with spruce extract. The dimerization of astringin in isolate 1 was increased 2-fold by spruce extract compared with the untreated culture ( $P = 0.02$ ). No changes in the rate of dimerization



**Figure 3.** Stilbene biotransformation activity demonstrated in partially purified protein fractions from *C. polonica*. A two-step chromatography scheme (affinity separation on concanavalin A-Sepharose followed by anion exchange) separated the three astringin conversion activities indicated by the *in vivo* experiments: oxidation of astringin to the ring-opened lactone **1** (A); deglycosylation of astringin to form (*E*- and (*Z*)-piceatannol (**2a** and **2b**; B); and oxidation of astringin to form a mixture of dimeric products **3a** and **3b** (C). Assays for the activities in A and C were performed in 50 mM MOPS, pH 6.8, and 10% (v/v) glycerol with 1.2 mM astringin and analyzed by LC-ESI-MS and UV-diode array detector. Assays for the activity in B were performed in 50 mM Tris, pH 7.5, and 10% (v/v) glycerol. mAU, Milliabsorbance unit.

were observed for isolate 2 after treatment with spruce extract, but activity in both control and treated media was at a level similar to that of isolate 1 after treatment. In general, isolate 2 transformed astringin more efficiently than isolate 1 regardless of treatment with spruce extract (Table II). Conversion to the lactone **1** ( $P = 0.001$ ), piceatannol **2** ( $P < 0.0001$ ), as well as stilbene dimers **3** ( $P = 0.09$ ) in both control and treated medium was higher in enzyme assays from isolate 2 than from isolate 1.

### Astringin Metabolism Leads to Increased Growth on Astringin-Containing Medium

As previously shown, isolate 2 degraded astringin faster than isolate 1 both *in vivo* (Table I) and *in vitro*

**Table II.** Rates of astringin biotransformation by *C. polonica* after treatment with a phenolic extract from Norway spruce

The conversion of astringin to ring-opened lactone, aglycone, and dimeric products was measured in vitro as  $\mu\text{g product g}^{-1}$  fresh mycelium  $\text{h}^{-1} \pm \text{sd}$  for isolates 1 and 2. Treatment consisted of incubation with 40  $\mu\text{g}$  of Norway spruce methanol extract  $\text{mL}^{-1}$  culture for 4 d. Controls were grown in medium without spruce extract ( $n = 4$  replicates per isolate per time point).

Astringin Products	Rate of Conversion			
	Isolate 1		Isolate 2	
	Control	Treated	Control	Treated
Ring-opened lactone ( <b>1</b> )	0.021 $\pm$ 0.003	0.025 $\pm$ 0.004	0.043 $\pm$ 0.022	0.187 $\pm$ 0.043 <sup>a</sup>
Aglycones ( <b>2a</b> and <b>2b</b> )	0.112 $\pm$ 0.024	0.266 $\pm$ 0.027 <sup>a</sup>	0.411 $\pm$ 0.068	0.688 $\pm$ 0.035 <sup>a</sup>
Dimers ( <b>3a</b> and <b>3b</b> )	0.112 $\pm$ 0.093	0.263 $\pm$ 0.014 <sup>a</sup>	0.223 $\pm$ 0.056	0.275 $\pm$ 0.033

<sup>a</sup>Significant differences between control and treated rates ( $P < 0.05$ ). Activity for isolate 2 was always greater than for isolate 1 for each reaction (reactions 1 and 2,  $P < 0.05$ ; reaction 3,  $P = 0.09$ ).

(Table II). To determine if a greater rate of stilbene transformation was associated with increased fungal performance in the presence of astringin, the growth rate of the two *C. polonica* isolates was compared on artificial medium. On solid minimal medium, the growth rate of isolate 2 was higher than the growth rate of isolate 1 ( $P < 0.0001$ ), but the growth rates of both isolates declined similarly when the medium was amended with 100  $\mu\text{g}$  astringin  $\text{mL}^{-1}$  medium ( $P < 0.0001$ ). Thus, the relative growth rate of isolate 2 was higher than that of isolate 1 on artificial medium with astringin ( $P < 0.0001$ ; Fig. 4B).

### *C. polonica* Can Use Caffeic Acid as Its Sole Carbon Source

The ring-opened lactone **1** has structural similarities to muconolactone, an intermediate in the  $\beta$ -ketoacid pathway employed by microbes to utilize aromatic compounds as carbon sources (Harwood and Parales, 1996). To investigate if *C. polonica* could utilize stilbenes as a carbon source, an experiment was conducted where both isolates 1 and 2 were grown in sealed containers on medium containing different concentrations of [ $^{13}\text{C}$ ] caffeic acid, a phenolic compound containing the same *o*-dihydroxyphenyl moiety, and conjugated C=C bond as astringin. The head space containing  $\text{CO}_2$  arising from fungal respiration was sampled at intervals and measured by isotope ratio mass spectrometry. Increases in  $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$  ratios were observed in the head space of cultures containing isolate 2 for concentrations of  $^{13}\text{C}$ -labeled caffeic acid ranging from 10 to 0.08  $\mu\text{M}$ , while little or no change in  $^{13}\text{CO}_2$ - $^{12}\text{CO}_2$  ratios were detected in cultures containing isolate 1 (Fig. 4, C–F). The appearance of  $^{13}\text{CO}_2$  upon feeding [ $^{13}\text{C}$ ]caffeic acid was taken as evidence of caffeic acid metabolism.

### Utilization of Phenolics Is Associated with Fungal Virulence

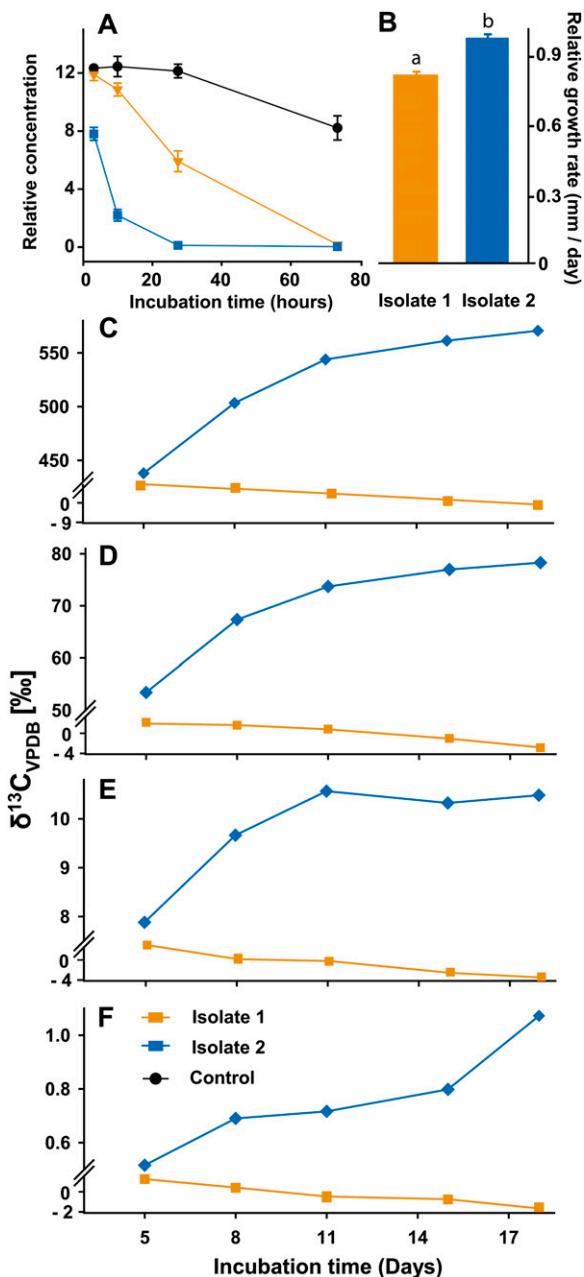
To determine if the ability of *C. polonica* to metabolize phenylpropanoids was associated with virulence in Norway spruce, the performance of four isolates

(including the isolates 1 and 2 used previously) was assessed after inoculation in the bark of Norway spruce saplings. Virulence was assessed by measurement of lesion lengths, represented by the area of damage after vertical growth upward and downward through the phloem. More virulent fungi should produce longer lesions. The lesion lengths created by isolate 2 were more than twice as long as those created by isolates 1 and 4 ( $P < 0.0001$ ), whereas isolate 3 created a lesion over 2.5 times that of isolate 2 ( $P < 0.0001$ ; Fig. 5A).

When these four isolates were grown on minimal medium containing caffeic acid as the sole carbon source, isolate 3 exhibited significantly greater mycelial growth ( $P < 0.0001$ ), followed by isolate 2. The least virulent isolates, 1 and 4, exhibited the poorest growth ( $P < 0.0001$ ; Fig. 5B). There is thus a correlation between fungal virulence and the utilization of aromatic compounds such as caffeic acid or astringin as a carbon source. Similar observations were made when an independent collection of *C. polonica* isolates, where the relative virulence is known (Krokene and Solheim, 2002), were compared for their growth rates on caffeic acid (Supplemental Fig. S5).

## DISCUSSION

The attack of bark beetles on their conifer hosts is frequently associated with infection by specialized fungi that are inoculated by the beetle. In this study, we investigated the biochemical adaptations that allow one such fungus, the ascomycete *C. polonica*, to colonize its host tree, Norway spruce. After inoculation into Norway spruce by the Eurasian spruce bark beetle (*Ips typographus*), *C. polonica* metabolizes the major antifungal phenolic compound produced in the bark. The stilbene astringin, a diaryl-ethene derivative, is metabolized to ring-opened lactones, aglycones, and dimers by fungal enzymes whose activities are induced when fungi are in a stilbene-rich environment. The ability of *C. polonica* to metabolize stilbenes and structurally similar phenolic compounds is directly correlated with its virulence in spruce bark and



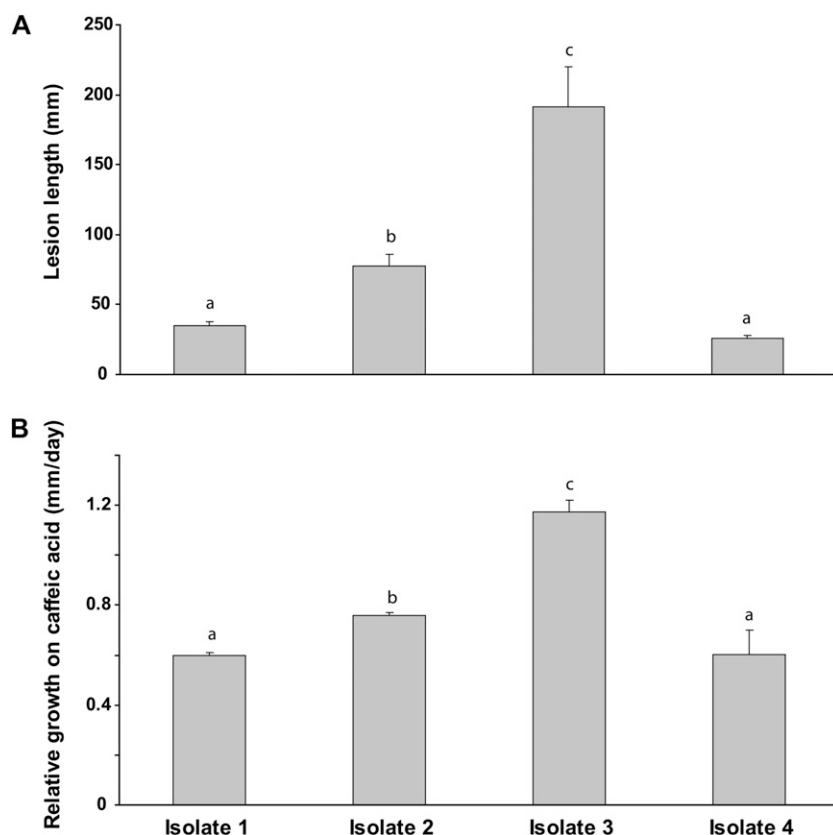
**Figure 4.** Relative growth of *C. polonica* isolates 1 and 2 in artificial medium amended with astringin in relation to the rates of phenolic biotransformation and the ability to utilize phenolics as sole carbon sources. A, Biotransformation. Depletion of astringin added to artificial medium over a period of 72 h was compared with changes in astringin concentration in sterile control medium ( $n = 4$ ). B, Growth. In vitro growth on solid minimal medium was determined in the presence of astringin relative to growth without astringin ( $n = 10$ ). Error bars in A and B represent SE. C to F. Utilization. Changes in the ratio of  $^{13}\text{CO}_2$  to  $^{12}\text{CO}_2$  were determined in fungal head space arising from respiration of  $[\text{U}-^{13}\text{C}]$ caffeic acid. Samples were collected over 18 d of fungal growth on agar containing 1 mM caffeic acid amended with 10  $\mu\text{M}$  (C), 2  $\mu\text{M}$  (D), 0.4  $\mu\text{M}$  (E), and 0.08  $\mu\text{M}$  (F)  $[\text{U}-^{13}\text{C}]$ caffeic acid ( $n = 1$ ). VPDB, Vienna Pee Dee Belemnite. [See online article for color version of this figure.]

appears to allow growth on stilbenes as a sole carbon source.

A number of previous studies had reported that the stilbene content of spruce bark declined during fungal attack (Brignolas et al., 1995; Viiri et al., 2001; Li et al., 2012). These declines were considered very puzzling because they occurred despite increases in the enzyme activity of STS (Brignolas et al., 1995), which catalyze the formation of the stilbene skeleton. Furthermore, increases in piceatannol concentrations (Viiri et al., 2001) and stilbene dimers (Li et al., 2012) were observed in spruce bark after *C. polonica* infection. Our results now clearly show that these metabolites originate from the fungal metabolism of host defense compounds and that *C. polonica* metabolism of stilbenes can override even increases in host stilbene biosynthesis.

Stilbenes have long been known as antifungal defenses in plants that inhibit fungal growth by interfering with microtubule assembly (Woods et al., 1995; Adrian et al., 1997), disrupting plasma membranes and uncoupling electron transport in fungal spores and germ tubes (Pont and Pezet, 1990; Adrian and Jeandet, 2012). It is not surprising that fungi specialized to live in stilbene-rich plant material, such as *C. polonica*, have developed mechanisms to circumvent the deleterious effects of these compounds. Here, we demonstrated that *C. polonica* not only metabolizes stilbenes but employs several different degradation routes. Using the tetrahydroxystilbene glucoside, astringin, which is produced in high amounts by Norway spruce, as a model, we identified ring-opened lactone, aglycone, and dimeric metabolites in *C. polonica* cultures. Some of these reaction types have been reported for other stilbenes (Breuil et al., 1998, 1999; Rodriguez-Bonilla et al., 2011). For example, stilbene dimer formation has been previously reported in the grape (*Vitis vinifera*) pathogen *Botrytis cinerea*. The dimerization of resveratrol and pterostilbenes has been shown to be an oxidative process involving the 4'-hydroxyl group of the stilbene skeleton and catalyzed by laccases and peroxidases (Breuil et al., 1998; Rodriguez-Bonilla et al., 2011).

Ring opening and lactonization has not previously been reported for stilbenes but is a logical early step of catabolism, since it results in a more polar product. Stilbenes exert their toxicity on fungi by diffusing through membranes into vegetative and reproductive structures (Adrian et al., 1998). A ring-opened and lactonized product with increased polarity should diffuse more slowly into fungal cells and, therefore, show decreased toxicity. Converting plant defense compounds to more polar products has been shown to be a successful detoxification strategy for other plant pathogens (Weltring and Barz, 1980; Esaki et al., 1998). A more recent study (Sobolev et al., 2006) reported the appearance of a novel stilbene phytoalexin in peanut (*Arachis hypogaea*) kernels infected by *Aspergillus* species. This compound has a prenylated but-2-enolide skeleton that bears a striking resemblance to the astringin lactone produced by *C. polonica* and so might also represent a fungal biotransformation product derived from a peanut phytoalexin.



**Figure 5.** Relative virulence of *C. polonica* isolates in Norway spruce bark in relation to their ability to utilize phenolics as sole carbon sources. A, Virulence. Lesion lengths were measured in the bark of spruce saplings 28 d after inoculation ( $n = 5$ ). B, Utilization. Performance was determined on solid minimal medium with caffeic acid as the sole carbon source. Growth on caffeic acid was measured relative to growth on medium amended with Glc ( $n = 5$ ). Lowercase letters denote statistically significant differences in growth.

In contrast to ring opening, deglycosylation is a seemingly disadvantageous detoxification process for the fungus, as this releases a less polar product. In fact, activation of glycoside defense compounds by deglycosylation when cell compartments are disrupted by herbivore or pathogen attack is a common feature of many plant defenses (Morant et al., 2008), and glycosylation is a known detoxifying mechanism of certain plant pathogens (Pedras et al., 2004). However, in a relatively nutrient-poor environment like Norway spruce phloem and sapwood, it might be advantageous for fungal pathogens with high tolerance to stilbenes to deglycosylate them and utilize the free Glc moieties as an energy source. Nevertheless, it is interesting that, in our study, the more virulent *C. polonica* strain relied less on deglycosylation and more on the other catabolic routes, ring-opened lactone formation and dimerization.

In this study, we demonstrated that metabolism of stilbenes was inducible by a crude stilbene-rich extract prepared from Norway spruce. The induction of detoxification pathways upon contact with plant antifungal defenses has been reported for other phytopathogenic fungi. Among phenolic compounds, caffeic acid induces the transcription of a gene cluster involved in the  $\beta$ -ketoacid pathway in *Cochliobolus heterostrophus*, the causal agent of southern corn leaf blight (Shanmugam et al., 2010), while phenolic compounds and pectin induce laccases responsible for stilbene dimerization in *B. cinerea* (Gigi et al., 1980). However, not all detoxification

pathways are inducible. In *Grosmannia clavigera*, a bark beetle-vectored fungal pathogen of pine (*Pinus* spp.), genes putatively involved in polyphenol detoxification were not observed to be induced by a pine phloem extract, which presumably would contain phenolic substances (Hesse-Orce et al., 2010).

The ability of plant pathogens to metabolize antifungal defenses has been shown to be an important virulence factor. For example, the virulence of pathogens on pea (*Pisum sativum*) plants depends on their ability to detoxify the pea phytoalexin pisatin (Van Etten et al., 2001), and the virulence of *B. cinerea* isolates on grape could be at least partially correlated with their ability to metabolize stilbenes (Sbaghi et al., 1996). Moreover, in *G. clavigera*, it was shown that the export of antifungal components of pine resin by an ATP-binding cassette transporter is essential for fungal growth in its host (Wang et al., 2013). In this study, we show that the virulence of *C. polonica* correlated with differential usage of the various pathways for stilbene biotransformation and the degradation of structurally similar compounds. Biotransformation pathways were generally induced in response to a spruce stilbene-containing extract. However, the more virulent isolate had higher constitutive enzyme activity for all three pathways, ring-opened lactone formation, deglycosylation, and dimerization, than the less virulent isolate. Moreover, application of spruce extract led to a significant increase in the rate of ring opening and deglycosylation.



The metabolism of Norway spruce stilbenes by *C. polonica* may serve not only to detoxify them but also to provide the fungus with nutrition. Other aromatic compounds, such as lignin breakdown products, have been shown to be metabolized by the  $\beta$ -keto adipate pathway in soil bacteria and fungi, yielding energy, reducing equivalents, and releasing CO<sub>2</sub> (Harwood and Parales, 1996). The structural similarities between the ring-opened lactone formed from astringin and an intermediate in the  $\beta$ -keto adipate pathway hinted that stilbenes might also be further catabolized by *C. polonica* via this process. Using caffeic acid, whose structure closely matches that of astringin in the region of the molecule giving rise to the ring-opened lactone, we established that virulent *C. polonica* isolates did employ this aromatic compound as an energy source based on release of <sup>13</sup>CO<sub>2</sub> from [U-<sup>13</sup>C]caffeic acid and growth on caffeic acid as a sole carbon source. Thus, *C. polonica* could conceivably use the stilbenes of its host tree as nutrients, resulting in an increased growth rate and more successful colonization of the tree. The use of host defense chemicals as a carbon source to support the growth of a bark beetle-associated fungus has also previously been reported for the mountain pine beetle associate *G. clavigera*, which can grow on monoterpene compounds of pine resin as a sole carbon source (DiGuistini et al., 2011).

The fungus *C. polonica* and the bark beetle are frequent associates that are usually viewed as killing their Norway spruce host by their combined efforts (Franceschi et al., 2005), but this opinion is not universally accepted (Six and Wingfield, 2011). In any case, the exact basis for their mutualism is not fully understood (Paine et al., 1997). It has been proposed that the fungus benefits by being dispersed to new hosts and gaining entry into the tree. The beetle, on the other hand, may benefit if the fungus helps weaken or kill the tree, since tree death is essential for beetle reproduction (Franceschi et al., 2005). Our study demonstrates that fungal growth in the stilbene-rich bark of Norway spruce depends on successful metabolism of these host defense compounds, which should also benefit the bark beetle by neutralizing tree defenses.

An alternative explanation for the cooccurrence of bark beetles with *C. polonica* might be the nutritional benefits associated with polyphenol biotransformation by the fungus. Degradation of stilbenes may benefit bark beetles by improving the quality of substrate available for feeding their larvae, since the stilbenes remaining in the bark could be toxic. For example, the catechol groups present in the astringin core structure may be spontaneously or enzymatically rearranged to form reactive quinones (Haruta et al., 2001; Lin et al., 2010) under the semialkaline conditions prevailing in beetle guts (Balogun, 1969). These quinones can then alkylate reactive nucleophiles such as sulfhydryl and amino groups in proteins or amino acids (Felton et al., 1992; Son et al., 2010). Protein alkylation may reduce the nutritional quality of ingested food or destabilize the peritrophic membrane in the bark beetle gut

(Barbehenn et al., 2008). Lactonization or dimerization of the catechol groups, as we showed for *C. polonica*, should prohibit quinone formation and thus protect bark beetles from the harmful effects of stilbene defense compounds.

We have shown in this study that *C. polonica* can transform phenolic defense compounds from Norway spruce and that it is even able to use these compounds as its sole carbon source. Further research should reveal more about how the biochemical capabilities of this fungus can contribute to its relationship with bark beetles and how its metabolism of spruce defenses modifies host tree resistance to both the fungus and the beetle.

## MATERIALS AND METHODS

### Inoculation of Norway Spruce Saplings with *Ceratocystis polonica*

Eight-year-old Norway spruce (*Picea abies*) saplings originating from the 3369-Schongau clone (Samenklinge and Pflanzengarten) were grown in an outdoor plot for 4 years prior to the experiment. Two *C. polonica* isolates (CMW 7749 = isolate 1 and CMW 7135 = isolate 2) were provided by the culture collection of the Forestry and Agricultural Biotechnology Institute (University of Pretoria). These were characterized as the least and most virulent, respectively, out of 12 isolates by measurement of lesion lengths created after inoculation of mature spruce trees (Marin, 2003). Both were grown on 2% (w/v) malt extract agar for 14 d at 25°C. Inoculations of saplings with *C. polonica* isolates 1 and 2 were performed 3 weeks after buds had broken during the flush of spring growth (June 10, 2008). A bark plug, 8 mm in diameter, was removed midway between the second and third branch whorl from the upper part of the sapling (2-year-old segment) with a cork borer. An 8-mm plug from one of the two *C. polonica* cultures was placed into the wound with the mycelium oriented toward the wood surface and sealed with Parafilm. For the control treatment, plugs of sterile malt extract agar were inserted into the wound. All treatments were applied during the same growth phase.

Bark tissue samples from five inoculated and five wounded saplings were harvested 2, 7, 14, and 28 d after the onset of the experiment. Five nonwounded control saplings were harvested at 2 d after the onset of the experiment. Fungal lesions were measured with a caliper. Bark material was flash frozen immediately after harvest in liquid nitrogen and stored at -80°C.

Inoculations of isolates 2, 3, and 4 were performed as described above using a 5-mm cork borer to wound 5-year-old clonal *Picea glauca* saplings. Lesion length data from this experiment were related to data from the first experiment by comparison of lesions of isolate 2 under the different conditions.

### Quantitative Real-Time PCR of STS from Norway Spruce

Total RNA from inoculated treatment and wounded control bark was isolated with the Invitrap Spin Plant RNA Mini Kit (Invitex) following the protocols of the manufacturer except that an additional DNA digestion step was included (RNase Free DNase set; Qiagen). RNA was quantified by spectrophotometry. Reverse transcription of 1  $\mu$ g of RNA into complementary DNA (cDNA) was achieved by using SuperScript II reverse transcriptase (Invitrogen) and 50 pmol of PolyT<sub>(12-18)</sub> primer (Invitrogen) in a reaction volume of 20  $\mu$ L. cDNA was diluted to 10% (v/v) with deionized water. One microliter of diluted cDNA was used as template for quantitative real-time PCR in a reaction mixture containing Brilliant SYBR Green QPCR Master Mix (Stratagene), 10 pmol of forward primer, and 10 pmol of reverse primer. *PaSTS* transcripts were amplified using the forward primer 5'-GTGGCGAGCAGAACACAGACTTC-3' and the reverse primer 5'-CAGCGATGGTACTCCATGAACG-3'. This primer pair was designed to amplify 140 bp of both *STS1* (GenBank accession no. JN400048) and *STS2* (GenBank accession no. JN400047) simultaneously. PCR was performed using a Stratagene MX3000P thermocycler using the following cycling parameters: 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a melting curve analysis from 55°C to 95°C. Reaction controls included non-template controls as well as non-reverse-transcribed RNA. *STS* gene abundance was normalized to the abundance of the Norway spruce ubiquitin gene

(Schmidt et al., 2010; GenBank accession no. EF681766) amplified with the forward primer 5'-GTTGATTTTCTGGCAAGC-3' and the reverse primer 5'-CACCTCTCAGACGAAGTAC-3'. Relative transcript abundance was calculated from three technical replicates of five biological replicates and calibrated against the transcript abundance of five nonwounded control saplings (relative transcript abundance = 1).

### Phenolic Extraction from Spruce to Investigate Changes after *C. polonica* Infection

For the extraction of phenolic compounds, spruce tissue was ground to a fine powder under liquid nitrogen and lyophilized. Approximately 40 mg of dried tissue was extracted with 2 mL of HPLC-grade methanol for 4 h at 4°C. Insoluble material was pelleted by centrifugation, and the supernatant was recovered. Insoluble material was reextracted with 1.5 mL of methanol for 16 h. Extracts were combined and evaporated to dryness under a stream of nitrogen. Dried samples were redissolved in 1 mL of methanol containing 100 µg mL<sup>-1</sup> of the internal standard apigenin glucoside. For liquid chromatography (LC)-electrospray ionization (ESI)-mass spectrometry (MS), samples were diluted to 20% (v/v) with water.

### HPLC-ESI-MS

Phenolic metabolites from spruce bark and astringin biotransformation products were separated on a Nucleodur Sphinx RP18ec column with dimensions of 250 × 4.6 mm and a particle size of 5 µm (Macherey-Nagel) using an 1100 series HPLC apparatus (Agilent Technologies). The total mobile phase flow rate for chromatographic separation was 1.0 mL min<sup>-1</sup>. The column temperature was maintained at 25°C. Phenolic compounds from spruce and fungal biotransformation were separated using 0.2% (v/v) formic acid and acetonitrile as mobile phases A and B, respectively, with the following elution profile: 0 to 1 min, 100% A; 1 to 25 min, 0% to 65% B in A; 25 to 28 min, 100% B; and 28.1 to 32 min, 100% A. Products from enzyme assays were separated with the following elution profile: 0 to 1 min, 100% A; 1 to 18 min, 0% to 100% B in A; 18 to 19 min, 100% B; and 19.1 to 22 min, 100% A.

Compound detection and quantification were accomplished with an Esquire 6000 ESI ion-trap mass spectrometer (Bruker Daltonics). Flow coming from the column was diverted in a ratio of 4:1 before entering the mass spectrometer electrospray chamber. The MS device was operated in negative mode scanning mass-to-charge ratio between 50 and 1,600 with an optimal target mass of mass-to-charge ratio 405. The mass spectrometer was operated using the following specifications: skimmer voltage, 60 V; capillary voltage, 4,200 V; nebulizer pressure, 35 p.s.i.; drying gas, 11 L min<sup>-1</sup>; gas temperature, 330°C. Capillary exit potential was kept at -121 V.

Compounds in chromatograms were identified based on retention time, their apparent molecular masses, and fragmentation spectra (Supplemental Table S3; Supplemental Fig. S4) and ultimately by NMR (see below). Bruker Daltonics Quant Analysis version 3.4 software was used for data processing and compound quantification using a standard smoothing width of 3 and Peak Detection Algorithm version 2. Linearity in ionization efficiencies was verified by analyzing serial dilutions of randomly selected samples. Ion suppression was controlled for by calculating analyte-to-internal standard ratios in serially diluted samples in which the internal standard was maintained at the same concentration. An external calibration curve for astringin was created by linear regression. Variability in the processing of individual samples was corrected by adjustment relative to the internal standard, the flavonoid apigenin glucoside.

### In Vivo Biotransformation of Astringin by *C. polonica* in Culture

*C. polonica* isolate 1 and isolate 2 were grown on 2% (w/v) malt extract agar for 12 d at 25°C in darkness. Agar plugs (diameter = 4 mm) from stationary cultures were placed in 15-mL test tubes for biotransformation assays. Then, 2 mL of sterile 2% (v/v) malt extract amended with astringin to a final concentration of 2 mg mL<sup>-1</sup> was added to the test tubes and incubated at 28°C with shaking at 220 rpm. Negative control treatments contained astringin-amended medium without fungus and fungus grown in medium without astringin. Cultures were harvested 4, 8, 24, and 72 h after the onset of the experiment. Biotransformation processes were stopped by adding 50 µL of HCl (2 N) to the test tubes. The internal standard apigenin-3-O-glucoside was

added to a final concentration of 0.1 mg mL<sup>-1</sup> prior to analysis by LC-ESI-MS. The concentrations of biotransformation products were calculated relative to the internal standard.

### Preparation of Astringin Biotransformation Products for NMR Analysis

Culture medium samples from biotransformation experiments were first subjected to SPE with RP-18 as the stationary phase. After loading, the columns were washed with water, dried with nitrogen gas, and finally eluted with methanol. The methanol extracts were separated by means of HPLC, and peaks of interest were collected online by postcolumn SPE. The HPLC-SPE system consisted of an Agilent 1100 chromatography system (Agilent Technologies) and a J&M photodiode array detector (J&M Analytik) connected to a Spark Prospekt 2 SPE device (Spark Holland) equipped with HySphere resin general phase cartridges (10 × 2 mm, 10 µm). Separations were performed with linear gradient elution, with water (A) and methanol (B) as solvents, both containing 0.1% formic acid: 0 to 1 min, 100% A; 1 to 20 min, 0% to 100% B in A; 20 to 25 min, 100% B; 25 to 27 min, 100% to 0% B in A; 27 to 34 min, 100% A. The column was a Macherey-Nagel Isis RP-18e column (250 × 4 mm). The make-up flow for postcolumn SPE trapping was set to 2.5 mL min<sup>-1</sup> using HPLC-grade water. The SPE cartridges were subsequently dried using pressurized nitrogen. HPLC-grade acetonitrile was used to extract the trapped analytes from the general phase cartridges into HPLC glass vials. After evaporation to dryness using nitrogen gas, the samples were reconstituted with 80 µL of MeOH-*d*<sub>4</sub> and DMSO-*d*<sub>6</sub>, transferred into 2-mm (i.d.) capillary NMR tubes, and subjected to NMR measurements. Hystar 3.2 software was used to coordinate the LC-SPE experiments, and Topspin 2.1 software was used to control the NMR spectrometer and to perform data processing.

### NMR and High-Resolution MS Analysis of Astringin Biotransformation Products

<sup>1</sup>H-NMR, <sup>13</sup>C-NMR, <sup>1</sup>H-<sup>1</sup>H-correlation spectroscopy, total correlated spectroscopy, heteronuclear multiple bond correlation, and heteronuclear single quantum coherence spectra were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin) operating at 500.13 MHz for <sup>1</sup>H and 125.75 MHz for <sup>13</sup>C. An inverse triple channel cryoprobe (5 mm) was used to measure spectra at a probe temperature of 300 K. Spectra are referenced to the residual solvent signals: for methanol-*d*<sub>4</sub> at δ 3.31/49.05 ppm and for DMSO-*d*<sub>6</sub> at δ 2.49/39.51 ppm. Capillary tubes (2 mm i.d.) were used for all NMR measurements.

Attempts to determine the structure of compound 1 in methanol-*d*<sub>4</sub> resulted in degradation during time-consuming two-dimensional heteronuclear experiments. Thus, DMSO-*d*<sub>6</sub> was used.

High-resolution MS was recorded on an ultra-performance liquid chromatography-MS/MS system consisting of an Ultimate 3000 series Rapid Separation LC (Dionex) system and an Orbitrap mass spectrometer (Thermo Fisher Scientific). Ultra-performance liquid chromatography was performed using a Dionex Acclaim C18 column (150 × 2.1 mm × 2.2 µm) at a constant flow rate of 300 µL min<sup>-1</sup>. A binary solvent system of water (A) and acetonitrile (B), both containing 0.1% formic acid, was used as follows: 0 min, 20% B in A; 0 to 6 min, 20% to 95% B in A; 6 to 10 min, 95% B; 10 to 14 min, 20% B in A. Full-scan mass spectra were generated using 30,000 resolving power: the mass accuracy was better than 3 ppm.

### Partial Purification of Fungal Protein Fractions Showing Stilbene Biotransformation Activity

*C. polonica* isolates 1 and 2 were cultured in 10% (v/v) carrot (*Daucus carota*) juice liquid culture for 6 d, harvested by centrifugation, and lyophilized. Dried material was finely ground using a vibrating ball mill. Powdered mycelium (0.5 g) was extracted with 10 mL of extraction buffer (50 mM Tris, pH 7.5, 5 mM ascorbic acid, 5 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 0.5 M NaCl, 10% glycerol, 1% polyvinylpyrrolidone [molecular weight of 360,000], 4% polyvinylpolypyrrolidone, and 0.1% Tween 20) at 4°C for 30 min with shaking. Crude fungal protein extract in extraction buffer was loaded onto an open column of concanavalin A-Sepharose (GE Healthcare) with a bed volume of 5 mL. The protein extract was incubated with the concanavalin A matrix for 30 min at 4°C before washing unbound proteins off with 5 bed volumes of washing buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, and

10% glycerol). Elution of bound proteins was achieved using 2 column volumes of washing buffer amended with 500 mM  $\alpha$ -D-methylglucopyranoside. Proteins from the column eluate were desalted at 4°C into 50 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO; pH 6.8) containing 10% glycerol using a HiPrep 26/10 (GE Healthcare) desalting column on an ÄKTA 900 chromatography system (GE Healthcare). The desalted protein fraction was loaded onto a 5-mL DEAE-Sepharose column and washed with 2 column volumes of DEAE washing buffer (50 mM MOPSO, pH 6.8, and 10% glycerol) at a flow rate of 5 mL min<sup>-1</sup>. Proteins were eluted from the column with DEAE wash buffer adjusted with NaCl using a step-wise gradient (100 mM, 200 mM, 300 mM, 500 mM, and 1 M NaCl). Elution steps and fraction volumes were 10 mL. Stilbene biotransformation activity was determined for crude extracts as well as for proteins that were eluted from the concanavalin A- and DEAE-Sepharose columns and for samples from the flow through of both columns. Enzyme activities were assayed in 300- $\mu$ L reaction volumes containing 200  $\mu$ L of enzyme from the purification steps described above and 100  $\mu$ g of astringin in DEAE washing buffer. Reaction mixtures were incubated at 30°C for 4 h before stopping the reaction with 10  $\mu$ L of 0.1 N HCl. After removing the protein by centrifugation, 20  $\mu$ L of the reaction mixture was analyzed by LC-ESI-MS.

### In Vitro Assay of Astringin Biotransformation Activity

Mycelium was cultured as above for 3 d. Each culture (10 mL) was subcultured in 50 mL of 10% (v/v) carrot medium. To determine if the biosynthesis of enzymes for stilbene biotransformation could be induced, the medium of four biological replicates was amended with 4 mg of crude spruce extract (methanol-soluble bark extract with solvent evaporated) in 2 mL of sterile water. As controls, four other replicates were subcultured after adding sterile water instead of spruce extract. Subcultured mycelium was harvested after 4 d by centrifugation and ground to a fine powder using a mortar and pestle. Then, 100 mg of ground mycelium was extracted with 5 mL of extraction buffer as above. Insoluble material was removed from extracts by centrifugation, and the resulting supernatant was filtered with a syringe filter with an exclusion size of 0.2  $\mu$ m.

Astringin biotransformation activity was assayed at 30°C in 2-mL reaction volumes containing fungal protein in extraction buffer and 100  $\mu$ g mL<sup>-1</sup> astringin. A 200- $\mu$ L subsample was taken from each assay reaction 45 min and 2, 4, and 8 h after the assays were initiated. The biotransformation reaction was stopped and analyzed as above.

### Assessment of Fungal Growth in the Presence of Astringin

Petri dishes with synthetic nutrient agar (Nirenberg and O'Donnell, 1998) amended with astringin were prepared by cooling the autoclaved medium to 55°C and adding astringin in ethanol to a final concentration of 100  $\mu$ g mL<sup>-1</sup> medium. Medium for negative control treatments was amended with ethanol only. Medium (25 mL) was dispensed in petri dishes (diameter = 10 cm). After the medium set, agar plugs (diameter = 4 mm) from 14-d-old *C. polonica* stationary cultures (isolate 1 or 2) were placed in the middle of each petri dish. Cultures were sealed with Parafilm and incubated at 26°C. Diameters of the expanding fungal cultures were measured every 24 h for 5 d. Growth rates were calculated using the slope of linear growth curves. Relative growth on astringin was calibrated against the mean growth rate of each isolate on control medium (100%) by calculating the fraction of each isolate's growth rate on astringin.

### Phenolic Extraction from Spruce to Induce Fungal Biotransformation Pathways

Finely ground lyophilized spruce bark (100 g) was extracted overnight with 400 mL of methanol. The mixture was filtered and the solvent was removed under reduced pressure in a rotary evaporator (Buechi Rotavapor R-114). The dried extract was weighed and redissolved in sterile water to a concentration of 2 mg mL<sup>-1</sup>.

### Measurement of Fungal Utilization of Caffeic Acid as a Carbon Source

Synthetic nutrient agar (Nirenberg and O'Donnell, 1998) was prepared. Modified synthetic nutrient agar was prepared by replacing Glc and Suc with

the equivalent amount of caffeic acid. Agar plugs (5-mm diameter) from fungal cultures (isolates 1–4 and 1994-169/113, 1980-53/7/A, 1993-208/115, 1980-53/7/B, 1980-53/7/C, and 1980-53/7/A'; Krokene and Solheim, 2002) were plated on both synthetic nutrient agar and modified synthetic nutrient agar and grown at 25°C in the dark for 14 d. Radial mycelial growth was measured every 2 to 5 d, and growth rates were calculated as above. Relative growth rates were determined by normalization with growth on synthetic nutrient agar.

Water agar (2.5%, w/v) amended with 1 mM caffeic acid was prepared. Filter-sterilized caffeic acid with a natural <sup>13</sup>C-<sup>12</sup>C carbon isotope ratio (Sigma) was mixed with [U-<sup>13</sup>C]caffeic acid (Campro Scientific) at ratios of 100:1, 100:0.2, 100:0.04, and 100:0.008 in different batches of medium. The growth medium (2 mL) was dispensed into heat-sterilized 10-mL vials (Exetainer; Labco). Mycelial plugs (length approximately 2 mm) from isolate 1 or isolate 2 stationary cultures, grown on modified synthetic nutrient agar containing 2 mM caffeic acid with a natural <sup>13</sup>C-<sup>12</sup>C carbon isotope ratio, were placed onto the medium inside the vials and sealed with a septum under open-air conditions. Using an autosampler (CTC Combi-PAL; CTC Zwingen), 200- $\mu$ L air aliquots were taken from each vial 5, 8, 11, 15, and 18 d after initiating the experiment. The aliquots were injected into a homemade, room temperature, constant pressure gas chromatograph modified from a commercial GC/general phase system (Thermo Fisher Scientific). Between the injector and a 30-m Poraplot Q capillary column, a Nafion (a sulfonated tetrafluoroethylene-based fluoropolymer; Perma Pure Products) online water-removal unit was placed. A second such unit was mounted postcolumn, followed by a third water-removal step realized by immersing the transfer line into a dry ice bath. The transfer line led to an active open splitter that was connected to an isotope ratio mass spectrometer (Delta<sup>+</sup> XL; Thermo Fisher Scientific). The open splitter reduces the effluent such that only the eluting CO<sub>2</sub> peak enters the mass spectrometer; other air constituents (mainly nitrogen, oxygen, and argon) are diverted to the vent and do not interfere with the isotopic analysis. Before each sample was injected, air from a continuously bleeding reference air tank, whose mixing ratio and isotopic composition were independently calibrated at the Max Planck Institute for Biogeochemistry, was analyzed. In addition, control samples were included for each <sup>13</sup>C-<sup>12</sup>C caffeic acid ratio from medium incubated without fungus.

The actual <sup>13</sup>CO<sub>2</sub>-<sup>12</sup>CO<sub>2</sub> ratio values in air samples from control and sample vials were calculated from the measured  $\delta^{13}\text{C}$  values using the International Union of Pure and Applied Chemistry reference value of 0.0111802 for <sup>13</sup>CO<sub>2</sub>-<sup>12</sup>CO<sub>2</sub> ratio in the international Vienna Pee Dee Belemnite reference (Zhang et al., 1990; Werner and Brand, 2001). Then, isotopic enrichment of fungal samples was obtained by subtracting the measured  $\delta^{13}\text{C}$  values of the blank samples without fungus.

### Statistical Analysis

The results are presented graphically as means  $\pm$  SE. Tabulated results are presented as means  $\pm$  SD. The normality of data was tested for with the Shapiro-Wilk test. The statistical significance of differences in *PaSTS* transcript accumulation and stilbene concentrations in living trees was determined using linear models with tree responses as the dependent variable and time as the explanatory variable on log-transformed data. Differences between fungal biotransformations of astringin as well as differences of fungal growth on astringin-amended medium were analyzed with the nonparametric Kruskal-Wallis test, as data could not be normalized. Differences in fungal growth in spruce bark and on caffeic acid were analyzed using one-way ANOVA. Differences in reaction rates of astringin biotransformation activity by the fungus measured in vitro were analyzed using two-way ANOVA of untransformed or reciprocally (1/ $\times$ ) transformed data. Following ANOVA, differences in means were calculated using Tukey's post-hoc pairwise comparisons test at a 95% confidence level. Analyses were conducted using the open-source software R (version 2.81) and the LAERCIO package for Tukey's pairwise comparisons.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Ring-opened lactone products derived from *C. polonica* transformation of astringin.

**Supplemental Figure S2.** NMR spectrum of one set of dimeric products (3a) derived from *C. polonica* biotransformation of astringin in this study.

**Supplemental Figure S3.** NMR spectrum of another set of dimeric products (3b) derived from *C. polonica* biotransformation of astringin in this study.

**Supplemental Figure S4.** Diagnostic mass spectral fragments of astringin and its biotransformation products.

**Supplemental Figure S5.** Relative virulence of six *C. polonica* isolates first described in Krokene and Solheim (2002) is correlated with their ability to grow on caffeic acid as the sole carbon source.

**Supplemental Table S1.** <sup>1</sup>H NMR (500 MHz) and <sup>13</sup>C NMR data (125 MHz) of compounds 1 in DMSO-*d*<sub>6</sub> and 4 in MeOH-*d*<sub>4</sub>.

**Supplemental Table S2.** Mass spectrometry data for astringin and identified astringin metabolites.

**Supplemental Materials and Methods S1.** Structural elucidation of compounds 1 and 4.

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