Producing Spalted Alder Wood in Yunnan, China

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Abstract

Given the demand for environmentally friendly wood stains, dyeing by fungi has come to light as a suitable process for staining wood, textiles, and other materials. The identification of fungi capable of producing spalted wood merits considerable effort by researchers, and some spalted color or styles found on wood in the wild cannot be cultivated in the laboratory. To find additional fungal candidates and styles for spalting in China, we here collected and identified wood fungi in Yunnan and Guangxi in China. Fungi were purchased or isolated and then inoculated to alder wood blocks (*Alnus nepalensis* D. Don). Out of seven purchased strains, three formed zone lines, but it was unclear whether *Chlorociboria aeruginascens* cfcc 87397 could do so. Out of 20 strains, 15 species were isolated from wood forming zone lines with surface black pigments, and only *Diaporthe* sp. ZXH63-4 formed additional yellow pigments accompanied by zone lines throughout the wood, which is a new means of forming yellow pigments and black zone lines at the same time. Some fungi collected from stained wood samples showed reddish-brown zone lines, but they showed black zone lines when isolated and inoculated on alder.

Given the growing demand for natural, organic products and growing global market for natural colorants, fungi have come to be investigated as readily available sources of diverse chemical pigments and colorants (Robinson 2012, Caro et al. 2017, Rao et al. 2017). Robinson summarized color modifications induced by wood-staining fungi as ''spalting,'' as it is called in North America, and traced surviving spalted wood samples back to 700 years ago in the history of European decorative arts. Robinson concluded that spalted wood was quite common in decorative wooden objects throughout history (Robinson et al. 2016), and spalting was developed to improve pigment effects on both wood and textiles (Robinson et al. 2014a, 2014b; Hinsch and Robinson 2016; Robinson et al. 2017).

Studies have shown that some ascomycetes and basidiomycetes can spalt wood; *Xylaria polymorpha*, *Trametes versicolor*, *Bjerkandera adusta*, *Polyporus brumalis*, *Inonotus hispidus*, and *Phellinus weirii* produce black zone lines, and *X. polymorpha* is the most used one (Li 1983; Robinson et al. 2007, 2009; Robinson and Laks 2010a, 2010b, 2010c; Tudor et al. 2012). *Scytalidium cuboideum* (*Arthrographis cuboidea*) and *Eurotium* sp. produce penetrating red stain, two *Fusarium* spp. produce surface red stain (Robinson et al. 2011, Tudor et al. 2013, Galleguillos et al. 2015), *Chlorociboria* spp. forms a greenish stain on sugar maple (Robinson and Laks 2010a), and *Ceratocystis virescens* causes blue staining (Robinson et al. 2009). *Ceratocystis* spp. is a pioneer species on larch and spruce. *Ophiostoma* sp. and *Phialocephala* sp. are associated with the occurrence of black pigment and allow black pigment to penetrate *Pinus radiata* but not *Nothofagus obliqua* (Galleguillos et al. 2015). *Nigrospora sphaerica* showed significant internal black pigmentation on marupa (*Simarouba amara*), and *Cladosporium herbarum* showed a less pronounced but similar dark pattern on *Brosimum alicastrum* and *Matisia cordata* (Gutierrez and Robinson 2015).

In China, spalting was first researched in 2011, where *Xylaria* spp. (four species), *Daldinia childiae*, *Nemania diffusa*, *Phomopsis* sp., and *Stilbum* sp. formed black zone lines; *Nectria rigidiuscula* formed surface red pigment; and *Populus tomentosa* were easily spalted compared with *Betula alnoides*, *Alnus nepalensis*, and *Ochroma pyramidale* (He et al. 2014). Among these tree species, *A. nepalensis* is a good choice for spalting because of its low price, fast

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growth, short cutting cycle, limited edaphic requirements, wide range in southwestern China, resistance to fire, status as a pioneer species in recovery of *Eupatorium adenophorum* grassland, and dispersal corridors (Fan et al. 2009).

Various fungi create colorful and diversiform pigments. To find additional fungal candidates for spalting, this study aimed to collect spalting wood samples and isolate and inoculate the wood to produce spalted wood.

Methods

Fungi collection

Fungi were collected in two ways: purchased from the China Forestry Culture Collection Center and isolated from the naturally spalted wood. Methods of isolation and purification of fungi were performed as described by Fang (1999) and Wu et al. (2007); methods of traditional morphological observation were performed as described by Fang (1999); and molecular methods of identification of fungi were performed as described by Cao et al. (2010). The methods of inoculation and cultivation were performed as described by Robinson et al. (2011) and Liers et al. (2006). Methods are briefly described below.

To isolate fungi, wood splitters cut from spalted wood samples collected from the wild were immersed in 75 percent ethyl alcohol for a few seconds and then in 2.5 percent benzalkonii bromidum for 3 to 6 minutes, washed with sterile water three times, placed on potato dextrose agar (PDA) plates, and incubated at 24°C ± 2°C in darkness until the mycelium emerged (about 1 wk). Pure cultures were achieved through continual reculturing until only one species remained. All of the fungi were kept on PDA tubes and stored at 6°C ± 2°C before being inoculated onto woods.

Inoculation and incubation on wood

Alder (*A. nepalensis*) was harvested from Kunming Jindian Forest Farm in 2000 from trees that were about 15 years old; then alder panels were stored in an attic, which resulted in high temperature in daytime. The average air-dried moisture content was 10.45 percent, and average air-dried specific gravity was 0.503 kg m^{-3} (Qiu 2000). Wood samples 2 to 3 by 2 to 3 by 2 to 3 cm in size were placed in 4 by 4 by 12 cm culture flasks. Wood blocks were weighed and put in culture bottles. Then one block and 10 g perlite, and 20 mL water were placed in each bottle, autoclaved by Yamato SN510C (121°C, 0.1 Mpa, 30 min). After inoculating with fungal inoculums (all of the fungi isolations from the wild and purchased were about 2 by 2 cm PDA plate), wood samples were incubated at 24°C ± 2°C for 8 weeks (Fig. 1). Then they were cleaned, air-dried, polished, and photographed for examining spalted grains. Each strain was inoculated into at least three culture bottles.

Identification of fungi.—Selected fungi were identified based on morphology and internal transcribed spacer (ITS) sequences. Fungal DNA were extracted using EZNA Fungal DNA Kit, D3390-01 (Omega Bio-Tek). Primers ITS1 and ITS4 were used for polymerase chain reaction (PCR) amplification, PCR reaction system 50 μL:2 μL DNA template, 1.5 μL primers ITS1, 1.5 μL primers ITS4, 25 μL Taq Mix, deionized water 20 μL. PCR reaction procedure was as follows: 35 cycles of (1) 94°C for 4 minutes; (2) 94°C for 1 minute, 50°C for 45 seconds, 72°C for 1 minute; (3) followed by 72°C for 10 minutes. PCR amplification products were sent to Majorbio Technology Co. Ltd. for sequencing within 1 week. Sequences were rechecked using Bio Edit and BLAST in the National Center of Biotechnology Information (NCBI) database.

Figure 1.—Incubated wood in culture bottle.

Results

Fungi collection and cultivation

Seven strains were purchased and three of them spalted alder as shown in Table 1. *Chlorociboria aeruginascens* cfcc 87397, *Xylaria polymorpha* cfcc 84510, and *D. childiae* cfcc 88581were found to form black pigments and zone lines, but it is highly unlikely that *C. aeruginascens* caused zone lines.

Table 1.—List of purchased strains.

Storage no.	Latin name	Spalted form
cfcc 87397	*Chlorociboria aeruginascens*	Black pigments and zone lines
cfcc 84510	*Xylaria polymorpha*	Black pigments and zone lines
cfcc 87467	*Daldinia concentrica*	No pigments
cfcc 88581	*Daldinia childiae*	Black pigments and thick zone lines
cfcc 86102	*Phomopsis phyllanthicola*	No pigments
cfcc 88779	*Diaporthe vaccinii*	No pigments
cfcc 84577	*Gibberella avenacea*	No pigments

It has been reported that some strains of *C. aeruginascens* form blue-green stains on agar plates to which sugar maple (*Acer saccharum*), aspen (*Populus deltoids*), and tree of heaven (*Ailanthus altissima*) powder had been added (Robinson et al. 2012), and it has been reported that *Chlorociboria* sp. forms green stains on *Populus tremuloides*, *Acer saccharum*, and *Betula alleghaniensis*. However, *C. aeruginascens* could not be reisolated from samples due to overdrying, and microscopic photos of samples show melanin in wood cells causing the color but no xylindein (Fig. 2), so it appears likely to be a result of contamination or mutation of fungi.

Xylaria polymorpha and *D. childiae* were reported to form zone lines on wood (Campbell 1933, He et al. 2014); *Daldinia concentrica* and *D. childiae* belong to genus *Daldinia. Phomopsis phyllanthicola* and *Diaporthe vaccinii* belong to genus *Diaporthe*; but neither *D. concentrica* nor *P. phyllanthicola* stains alder. *Gibberella avenacea* and *N. rigidiuscula* in anamorph belong to genus *Fusarium. Nectria rigidiuscula* is a surface red pigment fungi effective on several kinds of wood (He et al. 2014), but *G. avenacea* did not stain alder.

Identification of fungi

There were more than 50 strains isolated from spalted wood samples. Examination showed that 20 strains among isolated fungi formed black pigments and zone lines.

Twenty fungal strains selected among isolated strains were identified as 15 species, including nine species of *Diaporthe* (the imperfect stage is *Phomopsis*), five species of *Xylaria*, and one species of *Beltrania*. Based on alignment of ITS, a phylogenetic tree was constructed using the neighbor joining (NJ) method (Fig. 3). Some plate cultures of fungi and spalted patterns were attached.

Table 2 shows the selected strains and the corresponding sample location, identification results, access number of each strain, and spalted character. All species formed black zone lines (thicknesses are slightly different between strains) with black pigmentations on alder, while *Diaporthe* sp. ZXH63-4 formed additional yellow pigmentations throughout the wood.

Discussion

The changes in the colors of zone lines merit study. Some collected spalted wood samples showed reddish-brown zone lines but showed black zone lines when isolated and inoculated to *A. nepalensis* for unknown reasons, and color zone lines in the wild are not found often. That may be due to pH or chemicals of woods or environment, as indicated by the fact that some pigment groups show different colors in different pH or connected to different groups. Pigments purified from *Fusarium chlamydosporum* showed various colors with variations in pH, which was revealed to be ''long train hydrocarbons with poly unsaturated groups'' (Soumya et al. 2018). Staining fungi *S. cuboideum*, *Monascus ruber*, and *C. aeruginascens* displayed a change in color with the pH variation on agar media, but no color change in the zone lines was recorded on beech or sugar maple inoculated with *X. polymorpha* or *T. versicolor* across various pH levels (Tudor et al. 2013). However, external pink zone lines were observed on *X. polymorpha*/*A. cuboidea*–inoculated block treated with 6.39×10^{-2} kg/m^3 copper sulfate treatment (Robinson and Laks 2010c). Zone lines formed by *X. polymorpha* did not change color when the pH of the substrate was changed. Some melanin was found in zone lines by oxidative polymerization of phenolic monomers or polyphenols from the wood substrate, so the color change of zone lines may be due to copper sulphate and *A. cuboidea* (Tudor et al. 2013, Soumya et al. 2018). *Chlorociboria aeruginascens* produced green stain (xylindein) on 2 percent malt agar media and media to which aspen and tree of heaven powder had been added, but it produced a yellow pigment when inoculated onto media to which sugar maple had been added (Robinson et al. 2012).

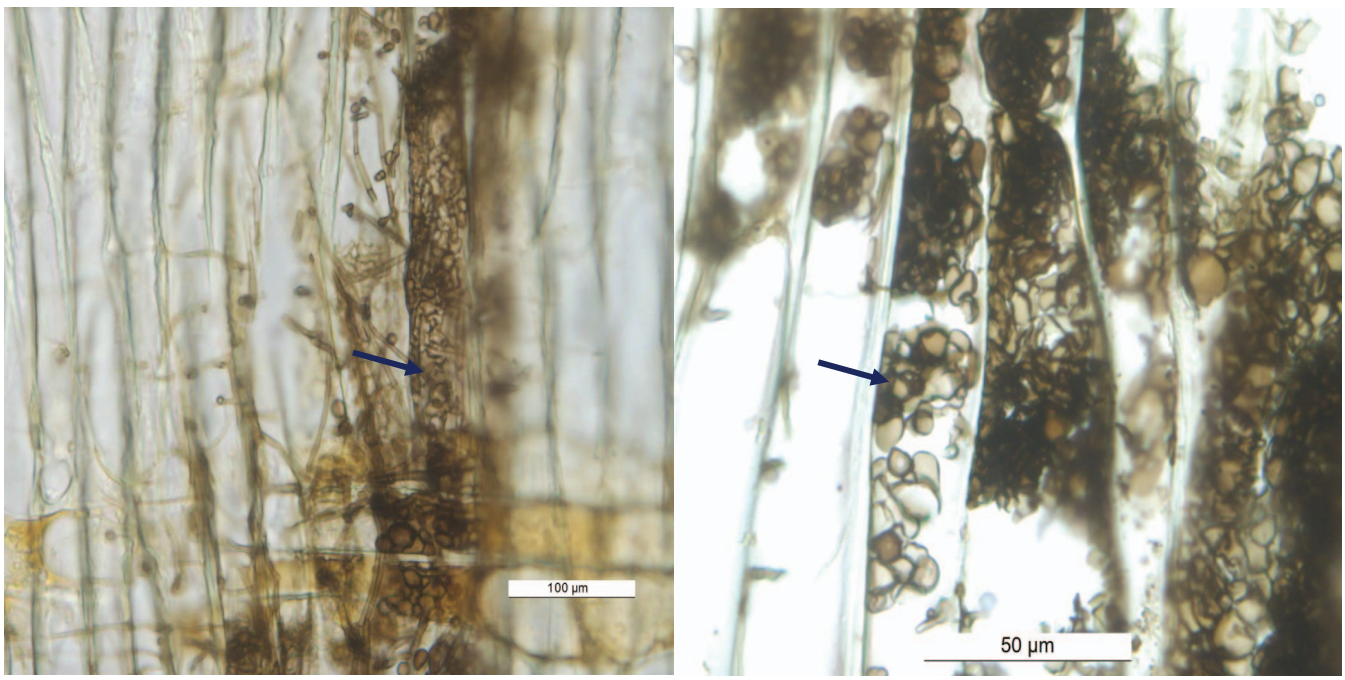

Figure 2.—The melanized hyphae in wood cells.

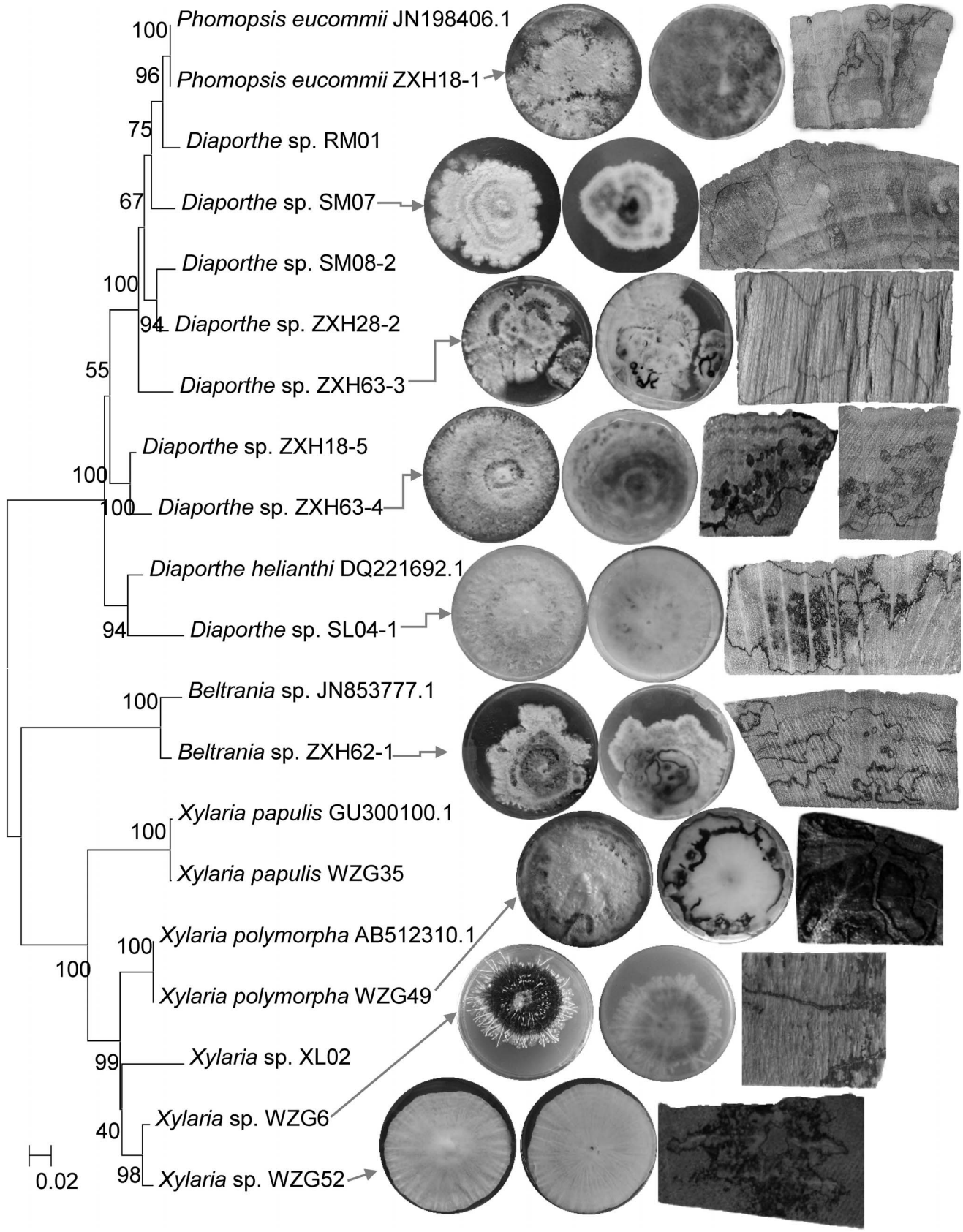

Figure 3.—The phylogenetic tree constructed based on fungi sequence and some fungi cultures and spalted patterns.

Table 2.—Strain location and spalting character description.[a]

Strain no.	Latin name	Access number of strains	Location	Surface spalted character	Internal spalted character
WZG35	*Xylaria papulis*	MK229146	XTBG	BZ, BP	BZ
WZG49	*Xylaria polymorpha*	MK229147	XTBG	BZ, BP	BZ
WZG52	*Xylaria* sp.	MK229148	XTBG	BZ, BP	BZ
WZG6	*Xylaria* sp.	MK229149	XTBG	BZ, BP	BZ
XL02	*Xylaria* sp.	MK229150	SWFU	BZ, BP	BZ
SM07	*Diaporthe* sp.	MK229151	NSFP	BZ, BP	BZ
RM01	*Diaporthe* sp.	MK229152	NPP	BZ, BP	BZ
SL04-1	*Diaporthe* sp.	MK229153	YAF	BZ, BP	BZ
SM08-2	*Diaporthe* sp.	MK229154	NSFP	BZ, BP	BZ
ZXH7-2, ZXH18-4, ZXH18-5, ZXH18-6, ZXH28	*Diaporthe* sp.	MK229155, MK229157, MK229158, MK229159, MK229160	XTBG	BZ, BP along wood rays	BZ
ZXH18-1	*Phomopsis eucommii*	MK229156	XTBG	BZ, BP	BZ
ZXH28-2	*Diaporthe* sp.	MK229161	XTBG	thin BZ, light BP	thin BZ
ZXH62-1, ZXH62-2	*Beltrania* sp.	MK229162, MK229163	XTBG	BZ, BP	BZ
ZXH63-3	*Diaporthe* sp.	MK229164	XTBG	BZ, BP	BZ
ZXH63-4	*Diaporthe* sp.	MK229165	XTBG	BZ, BP, yellow pigments	BZ, yellow pigments

[a] XTBG = Xishuangbanna Tropical Botanic Garden; SWFU = Southwest Forestry University; YAF = Yunnan Academy of Forestry; NSFP = Nanning Shimen Forest Park; NPP = Nanning People's Park; BZ = black zone lines; BP = black pigments.

Zone lines frequently appeared along edges of perlite and wood or the border between wood and the bottom of a flask, and finally enclosed a tridimensional area. The barrier of perlite or flask glass may stop hyphae from spreading or form an area of different moisture on wood. Fungi possibly self-isolate from an area with an unsuitable moisture content. This conjecture is similar to previously published hypotheses regarding spalting formation mechanism by other researchers. It has been reported that *X. polymorpha* and *T. versicolor* formed maximum amount of pigments on sugar maple and beech at low moisture content (about 30%) of wood (Tudor et al. 2012). It has been suggested that fungi secrete melanin in stressful environments (Henson et al. 1999, Gessler et al. 2014). Fungi cause zone lines, pigmentation, and white rot on wood substrates, and they can also stain fabric (Hinsch and Robinson 2016), suggesting that they are a suitable alternative to environmentally unfriendly dyes and that they can create desirable patterns.

Fungi have been seen to produce at least seven colors on wood, namely, black, red, purple, blue, green, yellow, orange, and brown, but in the laboratory they mainly produced black, red, blue, and yellow colors on wood. Finding a way to use fungi to create desirable stains and stable color in an environmentally friendly manner is worth exploring.

Conclusion

In this study, purchased and isolated fungi were inoculated to wood and screened out in China. Most strains of *Diaporthe* and *Xylaria* are established plant endophytes and phytopathogens and cause extremely slight decay after 8 weeks of cultivation. *Diaporthe* sp. ZXH63-4, especially, formed additional yellow pigments with black zone lines and pigments throughout wood, which can help to enrich spalted resources and patterns.

Spalting wood is a friendly way to paint wood, and different fungal stains can produce spalted wood in different types and colors.

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