Title Characterization of host-fungus interactions among wood decay fungi associated with *Khaya senegalensis* (Desr.) A. Juss (Meliaceae) in Singapore

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Summary

Tree pruning creates wounds that are amenable for wood decay fungi colonization. To characterize the dynamic host-fungus interactions at this location in Senegal mahogany (*Khaya senegalensis*), *in vitro* and *in vivo* pathogenicity tests were conducted with wood

decay fungi associated with this tropical tree species. *Fomitiporella caryophylii*, *Hymenochaete murina*, and *Phellinus noxius* isolates were included in this experiment following their frequent isolation from Senegal mahogany pruning wounds. The evaluated isolates demonstrated unique host interactions in laboratory tests that suggest equally divergent prognoses for living Senegal mahoganies affected by these fungi. Although all evaluated fungal isolates successfully breached naturally induced reaction zones, *P. noxius* alone caused significant mass loss to incubated wood blocks. In addition, *P. noxius* caused extensive wood decay after inoculation in living hosts, successfully illustrating Koch's postulates for this host-fungus relationship. The wood decay ability, invasiveness, and facultative parasitism demonstrated by *P. noxius* suggest its dominant role in wood decay columns below pruning wounds on living Senegal mahoganies. These results highlight the importance of characterizing specific host-fungus interactions and their implications for wood decay severity below pruning wounds in living trees.

Keywords

Fomitiporella caryophylii, *Hymenochaete murina*, *Khaya senegalensis,* Pathogenicity, *Phellinus noxius*, Senegal mahogany

Introduction

Tree pruning is an important arboriculture maintenance activity that can improve the structure, extend the service life, and mitigate the risk of failure. While this activity serves a number of useful purposes, the resulting pruning wounds render trees vulnerable to infection by wood decay fungi. The dynamics of wood decay development and its restriction in trees have been studied extensively (Pearce 2000; Boddy 2001; Schwarze and Ferner 2003). The concept of compartmentalization, in particular, is widely used to describe the process by which trees restrict, through inherent and induced modifications of wood anatomy, the growth and development of wood decay columns in three dimensions (Pearce 1996; Smith 2006). The Compartmentalization of Decay in Trees ("CODIT") model promulgated by Shigo and

Marx (1977) is a widely recognized conceptual framework describing four distinct walls ("Walls 1-3" = reaction zones and "Wall 4" = barrier zone) that collectively prevent the expansion of wood decay columns in the axial, radial, and tangential directions. The accumulation of inhibitory compounds at the margins of wood decay columns, as well as the ability of certain fungi to circumvent or overcome these barriers, has been clearly illustrated (Pearce and Woodward 1986; Schwarze and Baum 2000; Baum and Schwarze 2002). The host-fungus interactions at these margins determine the ultimate infection severity and long-term prognosis of an affected tree. In addition to this model, complementary concepts have highlighted the importance of micro-environmental conditions (e.g., moisture content and aeration) and nutrient availability in delineating the favorability of tissue for colonization (Boddy and Rayner 1983). As a result, these barriers may also function as sealants against water loss and air infiltration that preserve hydraulic conductivity in the vascular cambium (Boddy and Rayner 1983).

In properly executed pruning cuts, the branch protection zone (BPZ) serves the same mutually compatible objectives of inhibiting the spread of infection and ingress of air (Green et al. 1981; Eisner et al. 2002b). This anatomically distinct region consists of shorter and narrower conduit elements (i.e., vessels and fibers) that comprise tissue undergoing an abrupt change in the axial direction at the point where the branch attachment turns downward to join the trunk (Ewers and Zimmerman 1984). This feature primarily regulates the flow of water into a living branch; the hydraulic conductivity measurements in branches that were smaller than the parent from which they arose were approximately one-half those measured in the trunk (Zimmerman 1978). The BPZ also provides a framework for reaction zone formation after branch senescence that, following induced cell wall alterations and chemical deposits, can further prevent discoloration and decay (Green et al. 1981; Pearce 2000). However, these natural defenses may be impaired by flush cuts or are altogether absent when branches that are relatively large compared to the trunk are removed (Eisner et al. 2002a). In these cases, the resulting extent of infection can increase significantly (Eisner et al. 2002a; Gilman and

Grabosky 2006). A similar outcome was reported after removing large-diameter branches, in absolute terms, as these wounds expose a large amount of non-living heartwood and create a greater volume of dysfunctional sapwood that is amenable to fungal colonization (Shigo 1986; Gilman and Grabosky 2006; Ow et al. 2013). A swollen collar can be observed around the base of tree branches when branch and trunk tissues experience temporally distinct secondary growth rates, and arborists can use this external visual cue, which is often associated with the BPZ, to successfully limit wood decay originating from pruning wounds (Eisner et al. 2002a). Collectively, these findings allow arborists to prune trees in a way that minimizes the biological consequences.

Still, the practical implications of wood decay on tree management issues often depend heavily on the unique interactions between the host and the fungus. Comparative studies of this process have provided useful information about the effect of specific decomposition patterns on tree hazard assessments (Schwarze 2001). Overall, there is a general deficit of related information about the unique host-pathogen relationships among tropical tree species that are used in urban plantings, and there have been relatively few studies focusing explicitly on wood decay columns associated with pruning wounds. There is a need to evaluate the extension and application of these plant defensive concepts specifically to this commonly encountered phenomenon, especially with underrepresented tree species. Detailed information about the host-fungus interactions near pruning wounds may provide useful information about the severity of wood material property changes within the affected tissue, facilitating conclusions about the impact on the attachment strength of nearby branches that are derived from dormant axillary or adventitious buds. In addition, this information will be fundamentally essential when evaluating the favorability of various control strategies for these infections, especially biological control.

The Senegal mahogany (*Khaya senegalensis*) is highly represented and intensively managed in Singapore's urban forest, and it is a useful candidate tree species for study. It is often

planted in Southeast Asian urban landscapes given its tolerance of diverse site characteristics, fast growth rates, and favorable aesthetic features. The tree comprises a large portion of Singapore's urban forest with more than 15,000 specimens representing approximately 4% of all street trees. As a result, a study was conceived and designed to characterize (1) the host-pathogen relationships for wood decay columns that are associated with pruning wounds in Senegal mahogany (*Khaya senegalensis* (Desr.) A. Juss) in Singapore and (2) the invasiveness of identified wood decay fungi using *in vitro* and *in vivo* pathogenicity tests. Specifically, a series of experiments was designed to progressively screen wood decay fungi associated with Senegal mahogany for their incidence rates, wood decay ability, invasiveness, and facultative host parasitism to ultimately identify one species exhibiting the most antagonistic form of all characteristics.

Materials and Methods

Isolation of wood decay fungi

Pure cultures of wood decay fungi were obtained from columns that are associated with branch pruning wounds in Senegal mahogany (*Khaya senegalensis*) growing at roadside locations throughout Singapore. Five branch sections descending one meter below existing pruning wounds were randomly selected from 15 trees during routine pruning and maintenance. Basic physical dimensions were recorded for each sample, including the wound diameter and wound wood coefficients according to Schubert et al. (2008). The branch sections were bisected longitudinally, and small subsurface wood shavings were removed with a sharp chisel from the advancing margin of the naturally occurring wood decay column (Fig. 1). The wood shavings were placed on a basidiomycete selective medium (BSM) modified from Sieber (1995), with each liter of media containing 50 g malt extract agar (MEA), 105.75 mg thiabendazole dissolved in 2 ml of concentrated lactic acid, 200 mg chloramphenicol, and 300 mg streptomycin sulfate. The fungal isolates recovered from the selective medium were initially grouped based on micro- and macro-morphological characteristics when grown at 30 °C in the dark on 2% MEA, and each of these isolates was

assigned a unique alphanumeric identification code. Subsequently, representative samples were drawn from the largest morphologically distinct groups using probability proportional to size.

Polymerase chain reaction

The selected fungal isolates were identified through polymerase chain reaction (PCR) amplification and sequencing of the rDNA ITS1-5.8S-ITS2 region. DNA was extracted from pure fungal cultures, grown at 30 °C in the dark in potato dextrose broth agitated with an orbital shaker at 150 rpm, using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was amplified using the fungus-specific primer ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and the eukaryotic-specific primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The 50 µl reaction volume consisted of 37.45 µl H_2O, 2.5 mM MgCl2, 5 µl 10X reaction buffer, 7.5 pM each primer, 0.2 mM dNTP mix, 0.3 µl Taq polymerase, and 0.3 ng·µl^{-1} template DNA. The PCR amplification reactions were performed in a thermal cycler (GeneAMP PCR System 2700, Thermo Fisher Scientific, Waltham, MA, USA) at 92 °C for 3 min; 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 10 min; and 72 °C for 15 min. The PCR products were subsequently run on a 2% agarose gel (HU10, Scie-Plas, Cambridge, UK) at 100 V for 1 hr, visualized by staining with ethidium bromide, and documented with the Syngene GBOX EF (Cambridge, UK). Forward and reverse sequences of the PCR products were obtained through Sanger sequencing using BigDye® Terminator (Thermo Fisher Scientific, Waltham, MA, USA) chemistry and capillary gel electrophoresis (3730*xl* DNA Analyzer, Thermo Fisher Scientific, Waltham, MA, USA) by AIT Biotech Pte. Ltd., Singapore. The sequence files were assembled, aligned, and edited using ChromasPro 1.7.5 (Technelysium, Brisbane, QLD, Australia), and the resulting consensus sequences were compared with available sequences produced during similar work. The obtained sequences were annotated broadly according to Keller et al. (2009) and deposited in GenBank (Table 1).

Inoculation of healthy sapwood blocks

The isolates were first inoculated in sapwood blocks under controlled conditions to characterize the wood decay rates. Test wood blocks (10 × 8 × 30 mm) were removed from healthy sapwood in Senegal mahogany. Individual wood blocks were oven-dried at 100 °C for 48 h, cooled in a vacuum desiccator, and assessed using a precision balance. The blocks were subsequently autoclaved twice at 121 °C for 30 min. Each wood block was placed on a 14-day-old pure culture of a selected fungal isolate growing on 2% MEA in a 90 mm Petri dish, covered, and sealed with plastic paraffin film (Parafilm®, Pechiney Plastic Packaging, Chicago, IL, USA). One additional set of wood blocks was placed onto un-inoculated media as a control. The test plates were incubated in the dark at 25 °C and 50-70 %RH for 12 wk. After the incubation period, random samples were extracted from wood blocks to confirm the presence of the causal organism. The wood blocks were subsequently cleaned by removing surface mycelia, oven-dried, cooled, and reassessed. The dry-mass loss was then determined for each wood block on a percentage basis.

Inoculation of reaction zone wood blocks

Subsequently, the isolates were artificially and broadly inoculated according to the procedures described by Schwarze and Fink (1998) and Baum and Schwarze (2002) into wood blocks containing naturally induced reaction zones to characterize their mode of action around the host defensive response. Test wood blocks (10 × 8 × 30 mm) containing the interface of a reaction zone and healthy tissue were removed from Senegal mahogany branches with naturally occurring wood decay columns that were inferior to existing pruning wounds (Fig. 2a-b). These reaction zone wood blocks were extracted from a single tree. The blocks were autoclaved twice at 121 °C for 30 min. After cooling, the blocks were warmed slightly and dipped into paraffin wax heated to 55 °C under aseptic conditions. The wood blocks were inserted to coat five surfaces with paraffin, leaving one remaining surface containing the reaction zone exposed. Each wood block was placed on a 14-day-old pure culture of a

selected fungal isolate growing on 2% MEA in a 90 mm Petri dish, covered, and sealed with paraffin film (Fig. 2c-d). One additional set of wood blocks was placed onto un-inoculated media as a control. The test plates were incubated in the dark at 25 °C and 50-70 %RH for 12 wk. After the incubation period, random samples were extracted from the wood block to confirm the presence of the causal organism.

The reaction zone wood blocks were subsequently cut into smaller sections (5 × 5 × 20 mm). These samples were fixed in a 2% glutaraldehyde solution buffered at 7.2-7.4 pH for 48 hr. While still immersed in the solution, a moderate vacuum (20- 30 kPa) was applied to remove the remaining air from the samples, after which the samples remained in the fixative solution for an additional 48 hr. Subsequently, the wood blocks were dehydrated with acetone and embedded in a methacrylate medium. The embedded samples were sectioned in the transverse and longitudinal planes into semi-thin sections (3- 5 µm) using a rotary microtome (Leica® 2040 Supercut, Leica, Baden-Wurttemberg, Germany) fitted with a diamond knife. The sections were consistently removed from the interface between the reaction zone and healthy sapwood tissue in each test block. The general observation of cell wall degradation and hyphal growth was accomplished using replicate sections that were stained for 12 hr in safranin, counter-stained for 3 min in methylene blue, and stained for 30 min in auramin. The early stages of selective delignification were assessed in replicate sections that were stained with safranin and Astra blue according to Srebotnik and Messner (1994). Histological examinations were conducted using a light microscope (Nikon Eclipse E200, Tokyo, Japan), and micrographs were taken using a digital camera system (Nikon Digital Sight DS-Fi1, Tokyo, Japan).

Inoculation of living sapwood

Finally, the most invasive isolate was selected based on the qualitative and quantitative outcomes of the preceding experiments for inoculation into living, undamaged *K. senegalensis* specimens to confirm the host-pathogen relationship. Twelve seed-propagated

Senegal mahoganies were planted in a 10 × 15 m rectangular grid with a 5 m on-center spacing in an open landscape near Yishun, Singapore. The average trunk diameter, at 1.37 m above the highest root, was 10.3 cm (SD 1), and the average tree height was 353.2 cm (SD 17). The trees were maintained under natural photoperiods without supplemental irrigation or fertilization for approximately one year after planting. The inoculum was prepared by incubating the selected fungal isolate on linden (*Tilia* spp.) sapwood dowels (19 mm diameter × 30 mm length) and coarse Senegal mahogany sawdust (9.5 cm^3). These two growth substrates were sterilized in an autoclave at 121 °C for 30 min and subsequently placed on a 14-day-old culture of the fungal isolate growing on MEA in a Petri dish. A separate set of wood dowels and sawdust were sterilized and placed onto un-inoculated media as a control treatment. The wood dowels and sawdust were then incubated in the dark at 25 °C and 50-70 %RH for five weeks.

The trees were inoculated at positions that were distributed equidistantly around the trunk circumference about two separate horizontal planes 1.0 and 1.5 m above ground. The inocula were introduced into the sapwood by drilling a 20 mm diameter hole radially towards the pith using an auger drill bit (up to a minimum depth of 32 mm) at each inoculation site. The prepared inocula were inserted into each of these holes and sealed with paraffin wax. Six of the trees received one of the inoculated substrates carrying the selected fungal isolate with three trees receiving sapwood dowels and another three receiving coarse sawdust. The remaining trees similarly received one of the un-inoculated control substrates.

The trees were felled and harvested after a twelve-month incubation period. The trunks were immediately cut into two 1.25 m long segments containing all of the inoculation points at one cross section. These trunk segments were immediately transported to a laboratory for further evaluation where they were split along the radial longitudinal plane bisecting each inoculation point. To confirm the presence of the intended fungal isolate, three small wood fragments were extracted from the original inoculum as well as the surrounding decayed and discolored

wood. These fragments were placed onto BSM, and the obtained isolates were sub-cultured onto MEA and identified using morphological characteristics.

The physical size of the fungal columns in all of the trees was determined by measuring the maximum longitudinal and radial extent of decay and discoloration. The assessed radial plane extended outward from the central pith through the inoculation point towards the bark. These lengths were recorded using a steel measuring tape in centimeters. In addition, small cylindrical wood samples (4 mm diameter × 10 mm length) were excised from the decayed wood using an increment borer (Haglöf, Two-Thread Increment Borer, Långsele, Sweden). Identical samples were removed from the healthy sapwood that was immediately adjacent to the position where the decayed sample was removed. All of the samples were immediately dried in an oven at 100°C for 48 hours, cooled in a vacuum desiccator, and assessed to determine the dry mass. The dry mass loss of the decayed wood was expressed as the difference between the healthy and decayed wood dry mass on a percentage basis.

Experimental design and data analysis

The *in vitro* pathogenicity tests were designed as one-way factorial ($fungus^4$) experiments with the completely random assignment of treatments to subjects with a balanced quintuplicate replication. The effect of the evaluated fungal isolates on the dry mass loss of the sapwood blocks was evaluated using a one-way analysis of variance (ANOVA) in SPSS 19.0 for Windows (IBM Corp. 2010). Post-hoc multiple comparisons were performed using Dunnett's test for treatment against the control values ($\alpha = 0.05$). The *in vivo* pathogenicity test was designed as a 2 × 2 factorial experiment with one random-effects factor ("host") nested within various combinations of the two fixed factors, "substrate" (sapwood dowels and coarse sawdust) and "fungus" (*Phellinus noxius* KSK6, control) with the completely random assignment of treatments to subjects with a balanced sextuplicate replication. These inoculations that were applied to each living tree were replicated among three separate hosts to evaluate the inherent heterozygosity as well as the correlated vitality and growth rates

among the seed-propagated trees. As a result, the response variables (i.e., dry mass loss and physical column extent) were analyzed using nested ANOVA where the random effects of the host were nested within the fixed effects of the evaluated inoculants. In all of the analyses, the assumptions of normality and homoscedasticity were checked using the Kolmogorov-Smirnov normality test and the Levene median equal variance test, respectively. Logarithmic (base 10) transformations, where necessary, were performed to stabilize the data for testing.

Results

Wood decay fungi on Senegal mahogany pruning wounds

The sampled pruning wounds had a mean diameter of 8.9 cm (SD 4), and the original cut surface that was concealed by the wound wood occlusion varied considerably between 0 and 100% with a mean of 62% (SD 36). In total, 189 fungal isolates were obtained from 37 branch pruning wound columns (Table 1). Representative samples of the largest morphologically distinct groups were identified as closely related members of the Hymenochaetaceae: *Fomitiporella caryophylii* (Racib.) T. Wagner & M. Fisch, *Hymenochaete murina* Bres., and *Phellinus noxius* (Corner) G. Cunn. (Tables 1 and 2).

Dry weight loss of healthy sapwood blocks

Among the Senegal mahogany sapwood blocks that were inoculated with the wood decay fungi *P. noxius* KSK6 caused the highest dry mass loss with a mean of 12.4% (SD 5) followed by *P. noxius* KSK8 with a mean of 11.5% (SD 6). In contrast, *H. murina* KSF3 and *F. caryophylii* KSF6 caused the lowest dry mass loss with a mean of 4.2% (SD 2) and 6.4% (SD 1), respectively. Among all five of the evaluated isolates, the average dry mass loss recorded was 7.9% (SD 6). The negligible dry mass losses were recorded among the control wood blocks with a mean of 1.8% (SD 1). An analysis of variance revealed that the *P. noxius* isolates KSK6 and KSK8 both caused significant dry mass losses to the sapwood blocks compared to the control treatment ($P < 0.05$) (Fig. 3). The sole presence of the intended inoculant was confirmed in all of the cases.

Anatomical features of Senegal mahogany wood

The diffuse porous wood of Senegal mahogany consists mainly of vessels and libriform wood fibers. Axial parenchyma cells formed a narrow, inconsistent sheath around the vessels (vasicentric paratracheal). The cells were also sparsely and irregularly distributed among the fibers (diffuse apotracheal) and arranged in marginal bands. Libriform wood fibers were mostly thick-walled and abundant. Xylem rays were between four and 10 rows wide (multiseriate) (Fig. 4a). Intercellular resin canals were also occasionally observed. In the reaction zone, vessel lumina were heavily occluded by polyphenolic deposits, which stained visibly reddish-brown with safranin, methylene blue, and auramin (Fig. 4b). Similar deposits nearly completely occluded the axial parenchyma, xylem ray parenchyma, and fibers. Overall, these deposits uniformly and completely obstructed every cell type within the reaction zone (Fig. 4b). These features were consistently absent from the healthy wood (Fig. 4a).

Fungal degradation patterns within the reaction zones

The paraffin wax barriers enveloping the reaction zone blocks remained intact throughout the entire incubation period, ensuring that each fungal isolate gained access to these blocks exclusively through the exposed surface containing a reaction zone. The exclusive presence of the intended fungal isolates within each test block, in contrast to those that induced the reaction zone's formation in the living tree, was verified by the absence of fungal hyphae from the controls. All of the introduced fungal isolates successfully colonized the test blocks and successfully breached the naturally induced reaction zones. However, two distinct strategies were employed to effectively overcome these barriers.

H. murina preferentially and strongly degraded the polyphenolic deposits that were initially within the xylem ray parenchyma where the partially degraded remnants of these deposits exhibited a distinct color change from reddish-brown to brown-black. After removing a

majority of these deposits, the fungus initiated cell wall decomposition near the hyphae growing within the lumina (Fig. 5a-d). At these locations, erosion troughs were formed by the simultaneous breakdown of the secondary cell wall constituents, evoking a general pattern of decomposition akin to simultaneous rot. Eventually, this entire process was repeated sequentially within the fibers, axial parenchyma, and vessels. The entire cellular structure ultimately showed substantial decomposition after the amalgamation of the erosion troughs that were formed by separate hyphae. The lateral movement of hyphae between adjacent cells occurred primarily through bore holes. The entire decomposition process occurred within a relatively small area of tissue approximately 200- 300 µm across (Fig. 5e-f).

In contrast, *F. caryophylii* and *P. noxius* preferentially altered and degraded the polyphenolic deposits that were initially in the lumina of libriform wood fibers. This breakdown was made similarly evident by the progressive darkening of partially degraded polyphenolic deposits in these locations. Simultaneously, the isolates initiated the decomposition of the secondary cell wall by forming helical erosion troughs that were aligned with the constituent cellulose microfibrils (Fig. 6a). Hyphae entered the cell walls by tunneling transversely from the lumina and then reorienting along the axial microfibrils within the S_1 and S_2 layers of the secondary cell wall by 'L-branching' and, occasionally, by 'T-branching', and these bore holes appeared to be the primary mechanism by which hyphae moved laterally. In the S_2layer, these tunnels appeared as helically-wound channels in the longitudinal sections and circular perforations in the transverse sections. The erosion tunnels in the S_1layer, conversely, appeared as peripherally oriented channels in the transverse sections and circular perforations in the longitudinal sections (Fig. 6b-c). During the initial stages of infection, the compound middle lamellae between the fibers, vessels, and parenchyma were largely unaltered. As the infection progressed, polyphenolic deposits were degraded similarly in the tracheary elements and parenchyma cells. Overall, the entire decomposition process occurred within a similarly narrow band of tissue approximately 200- 300 µm across (Fig 6d).

Fungal degradation patterns in living sapwood

After the one-year incubation period, the *K. senegalensis* that was inoculated with *P. noxius* KSK6 displayed distinctive external symptoms around the infection sites compared to the controls. Specifically, the inoculated trees exhibited greater wound wood callus tissue and cross-sectional bulging near the inoculation sites (Fig. 7). Most of these trees exhibited discolored or decayed columns adjacent to the inoculation points following harvest and dissection. In general, the infected wood appeared visibly dark brown to tan with scattered, irregular dark brown to black lines, and the columns were circumscribed by a barrier zone that appeared macroscopically as an approximately 1 cm wide red to pink margin. The separate columns originating from individual inoculation points often coalesced into one large infected area extending uniformly in all radial directions from the barrier zone to the pith (Fig. 8).

Among all of the trees, those that were inoculated with *P. noxius* KSK6 on the wood dowels and the sawdust media experienced higher dry mass losses and contained greater axial decay and discoloration compared to their respective control treatments. *P. noxius* KSK6 that was incubated in sawdust caused the highest dry mass losses with a mean of 42.2% (SD 13), and this was significantly more ($P = 0.0098$) than the respective control treatment with a mean of 20.3% (SD 12). Likewise, the trees that were inoculated with *P. noxius* KSK6 that was incubated in sawdust contained columns of comparatively greater axial extent with a mean of 50.5 cm (SD 15.6); this mean was significantly greater ($P = 0.0041$) than that of the associated control treatment with a mean of 12.3 cm (SD 4.3). However, the nested effect of the host, in this instance, exerted a significant influence ($P = 0.0021$) on the treatment means. In addition, those trees that were inoculated with *P. noxius* KSK6 that was incubated on wood dowels contained columns that expanded significantly more ($P = 0.0190$) in the axial direction than did the associated controls with mean values of 34.6 cm (SD 9.4) and 20.6 cm (SD 6.2), respectively (Table 3).

In contrast, there were no meaningful differences in the radial extent of discoloration and decay among all of the trees that were inoculated with the various treatments that were outlined. Although the columns that were associated with *P. noxius* KSK6 that was incubated in sawdust extended slightly farther, on average, in the radial direction, this difference was not significant ($P = 0.6102$). Furthermore, the nested effect of the host exerted a highly significant ($P< 0.0001$) influence on the treatment means for the radial column extent in both the sawdust and wood dowel treatments (Table 3).

Discussion

Although subject to ongoing taxonomic evaluation and revision (Wagner and Fischer 2002; Jeong et al. 2005; Larsson et al. 2006; Parmasto et al. 2013), the fungal isolates that were included in this study exhibited characteristics broadly representative of their family and order, including an absence of clamp connections, the production of setae (except for *F. caryophylii*), and wood colonization (Wagner and Fischer 2002). In general, this clade occurs primarily as saprotrophic wood decay, but a few species cause lethal infections for trees in disturbed landscapes (Larsson et al. 2006). In this study, *P. noxius* exhibited wood decay ability, invasiveness, and host parasitism in Senegal mahogany; *F. caryophylii* and *H. murina* demonstrated an ability to overcome the Senegal mahogany defensive response.

In general, the incidence and distribution of *P. noxius* diseases are well documented. This fungus, a pan-tropical pathogen that was originally described in Singapore by E.J.H. Corner (1932) as *Fomes noxius*, has been most frequently reported as the cause of a lethal root rot affecting numerous tree species (Bolland 1984; Chang 1995). The disease, usually called brown root rot, affects more than 300 species in 50 families (Ann et al. 2002; Mohd Farid et al. 2005; Farr and Rossman 2013). Several reports have notably detailed its etiology, pathogenicity, and induced host response on rubber (*Hevea brasiliensis*) cultivated in West Africa (Nicole et al. 1982, 1986a, 1986b, 1987; Geiger et al. 1986a; Geiger et al. 1986b; Nandris et al. 1987).

In contrast, there is a deficit of similar information about *F. caryophylii* and *H. murina*. Although there have been seven reported arboreal hosts for the former (Gilbert et al. 2008; Farr and Rossman 2013), there are no similar reports for the latter. Interestingly, *F. caryophylii* has been encountered in decayed wooden structural members of traditional Japanese houses (Toyoumi et al.2010) and Chilean churches (Ortiz et al. 2014).

The wood block inoculation tests broadly demonstrated the ability of *P. noxius* isolates to independently decompose Senegal mahogany sapwood. The measured wood decay rates were similar to the available reported values for these fungal species; Schwarze et al. (2012) reported that *P. noxius* caused a 6.3% average mass loss per month to sapwood blocks from four tree species, including bunya pine (*Araucaria bidwillii*), flame of the forest (*Delonix regia*), benjamin fig (*Ficus benjamina*), and jacaranda (*Jacaranda mimosifolia*); and Toyoumi et al. (2010) recorded a 2.0% average mass loss per month on Japanese beech (*Fagus crenata*) sapwood blocks that were inoculated with *F. caryophylii*. In our study, the wood blocks experienced, on average, a 3.9% and 2.1% mass loss per month after inoculation with *P. noxius* and *F. caryophylii*, respectively. Overall, the *P. noxius* isolates KSK6 and KSK8 caused a significantly higher mass loss than did the control wood blocks, indicating their greater wood decay ability in Senegal mahogany.

All of the isolates demonstrated some invasive potential by penetrating and overcoming the naturally induced reaction zones. However, two different colonization strategies were observed, including the preemptive degradation of chemical obstructions by *H. murina* to facilitate hyphal development in the cell lumen and the synchronous removal of polyphenolic deposits from the cell lumen alongside hyphal tunneling in the secondary cell walls by *F. caryophylii* and *P. noxius*. These patterns are obliquely similar to those that were observed for *Ganoderma adspersum* in European beech (*Fagus sylvatica*) where the predominant breakdown of polyphenolic deposits in the cell lumina was periodically supplemented by the

formation of helical cavities in the walls of fiber-tracheids during the advanced stages of infection (Schwarze and Baum 2000). However, hyphal tunneling through reaction zones has typically been reported as a means to achieve the physical isolation of developing hyphae from chemical obstructions; *Inonotus hispidus* and *Ustulina deusta*, for example, diverted around the occluded cell lumina in this manner without significantly degrading the polyphenolic deposits (Schwarze and Fink 1997; Schwarze and Baum 2000). These similarities outline the broadly analogous dynamics between the host and the fungi in the trunk and branches of trees.

The preferential, aggressive degradation of polyphenolic deposits by *H. murina* is similar to that of *Ganoderma adspersum* in London plane tree (*Platanus* × *acerifolia*) reported by Schwarze and Ferner (2003). The extensive damage observed in reaction zones colonized by this fungus contrasts starkly against the insignificant mass losses cause to healthy sapwood blocks. It is possible that *P. noxius*, similar to *G. adspersum*, may cause higher dry mass losses with a positive growth response elicited by the presence of polyphenols. It may have been possible to observe this distinction had dry mass measurements been recorded for the reaction zone wood blocks, and these comparisons should be a priority for future investigations.

The observation of hyphal tunneling by *F. caryophylii* and *P. noxius* in the secondary cell wall is interesting, and similar to reports of soft rot-like behavior occasionally exhibited by other basidiomycetes including *Fistulina hepatica*, *Ganoderma adspersum*, and *Inonotus hispidus* (Daniel et al. 1992; Schwarze et al. 1995; Schwarze et al. 2000; Schwarze 2007). This behavior was initially termed “facultative soft rot”, and its occurrence is often associated with cell lignification, polyphenolic deposits, or high moisture contents (Schwarze et al. 1995). Although simultaneous rot is the primary reported mode of action for *P. noxius* (Geiger 1986b), one possible explanation for this second mode is the extraction of cellulose

and hemicellulose to aid in the breakdown of polyphenolic deposits in the reaction zone (Schwarze et al. 2000).

These results contribute to existing knowledge about host-fungus interactions using excised, naturally induced reaction zones (Schwarze and Baum 2000; Baum and Schwarze 2002), but there are some important limitations that should be addressed in future work. In this study, neither hyphae nor cell wall decomposition were observed during histological examinations of uninoculated (control) reaction zones, but this technique is potentially vulnerable to confusion between natural wood decay and that caused by the artificially inoculated fungi. This vulnerability could be addressed by extracting histological sections from reaction zones in living trees artificially inoculated with the same wood decay fungus, and this complementary source of evidence should be included in future studies. In addition, the chemical properties of polyphenolic deposits in the reaction zones could be better preserved with gas (e.g. ethylene oxide) sterilization techniques instead of autoclaving.

There is a growing appreciation for the ability of certain fungi to change their mode of action in response to certain environmental or phenological stimuli, and most agree that it may no longer be useful to classify microbes discretely without a broader appreciation for dynamic host-pathogen relationships (Newton et al. 2010). For example, Olson et al. (2012) analyzed *Heterobasidion annosum* gene expression during periods of saprophytic and parasitic wood decay and illustrated the nutritive trade-offs that are confronted by this fungus to gain access to broader ecological niches. An overlapping physiological capacity for multiple trophic strategies could offer greater competitive, environmental, or ecological flexibility for the fungus in pursuit of reproductive success. As a result, neither the ability of *P. noxius* to employ a secondary mode of action nor to colonize branch sapwood should be viewed with alarm. Rather, these results build upon existing studies of *P. noxius* root system infections and provide broader insight into the ecological role of these wood decay fungi in disturbed urban landscapes.

The inoculation of living Senegal mahogany sapwood with *P. noxius* KSK6 supported observations of the invasive behavior of this wood decay fungus during *in vitro* tests. Axial decay and discoloration were significantly greater for the trees that were inoculated with *P. noxius* KSK6, and the sapwood dry mass losses were significantly greater for the trees that were inoculated with *P. noxius* KSK6 that was incubated on sawdust. Contrastingly, Deflorio et al. (2008) observed no differences between two inoculation substrates that were used to inoculate four tree species with six fungi, but it is possible that Senegal mahogany sawdust offered a superior host-specific incubation substrate compared to the linden sapwood dowels in this study. Overall, the extensive axial development of decay and discoloration in the sapwood that was inoculated with *P. noxius* KSK6 suggests that the reaction zones were invaded by similar means as were observed in the wood blocks. However, the barrier zone, which formed at the time of inoculation, remained largely intact and untouched by *P. noxius* KSK6, likely due to the physical changes that are associated with most barrier zones, mainly consisting of a circumferentially uniform barrier of suberized axial parenchyma cells, polyphenolic deposits, and smaller vessel diameters (Pearce and Rutherford 1981; Deflorio et al. 2009).

It is important to note that these conclusions may be slightly undermined by the use of small, seed-propagated trees. The small cross-sections limited the total volume of healthy wood that was available for colonization by *P. noxius*. Most of the columns coalesced from their respective inoculation points into one amalgamated column, and the radial development of the columns could have been more reliably assessed in larger trees with greater distances between the bark and the pith. In addition, the host defensive responses likely differed among the seed-propagated trees, whose vitality and wood anatomy may vary according to their genotype (Boddy 1992; Deflorio et al. 2009). However, these small trees more closely approximated the morphological and anatomical characteristics of the branches that were

investigated in this study, and these constraints are commonly encountered in investigations of slow processes in long-lived organisms.

The pathogenicity of *P. noxius* in Senegal mahogany was plainly established in these experiments. This is the first reported host association between Senegal mahogany and *P. noxius* supported by pathogenicity tests. In these experiments, *P. noxius* alone displayed attributes outlining a highly antagonistic relationship with Senegal mahogany, and the results suggest it is likely responsible for severe wood decay infections associated with pruning wounds on this tree. Still, the isolates in this study were obtained from less than one percent of all Senegal mahoganies in Singapore, and it is not possible to speculate about the prevalence of *P. noxius* infections in the entire population or the possibility of other wood decay fungi fostering similarly invasive relationship with this particular host. Although these infections are non-lethal, they have meaningful consequences for individual trees and the broader ecology of constructed urban landscapes. Practically, arborists can apply these results in the field when considering the long-term implications of pruning wounds on tree health and vitality. Pruning interventions that facilitate the introduction of wood decay in Senegal mahogany, such as large wounds or flush cuts, should be avoided to limit colonization opportunities for *P. noxius*. Collectively, these results demonstrate the host-pathogen relationship that is fostered by tree pruning in Senegal mahogany, and they add to the existing body of evidence outlining the biological consequences of this maintenance activity. Ultimately, the pruning interventions should be applied to meet clear biological, physical, or aesthetic objectives; arborists should avoid removing more than is required to meet these objectives to escape unnecessary costs.

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Table 1 Identity, origin, and accession numbers of the collected fungal strains that were used in the pathogenicity tests

Isolate	Identity	Host	Origin	Accession No. (ITS)	Reference	Identity (%)
KSF3	*Hymenochaete murina*	*Khaya senegalensis*	Clementi, Singapore	KJ769183	JQ716406 (He and Li 2013)	99
KSF6	*Fomitiporella caryophylii*	*Khaya senegalensis*	Clementi, Singapore	KJ769184	AY558611 (Jeong et al. 2005)	95
KSK6	*Phellinus noxius*	*Khaya senegalensis*	Bugis, Singapore	KF233592	FR821770 (Schwarze et al. 2012)	99
KSK8	*Phellinus noxius*	*Khaya senegalensis*	Bugis, Singapore	KF233591	FR821769 (Schwarze et al. 2012)	98

Table 2 Nested analysis of variance of dry mass loss and lesion size measurements observed among Senegal mahoganies inoculated with *Phellinus noxius* isolate KSK6, including means and standard deviations (SD) for all levels.

Response	Effect	df	*F*	*p*	Level	Mean (SD)
Dry Mass Loss (%)	(a) Wood Dowel Inocula					
	Fungus	1, 4	0.4629	0.5265	*Phellinus noxius* KSK6	14.3 (13.3)
					Control	8.2 (11.5)
	(Tree) Fungus	4, 24	0.6375	0.6732		
	(b) Sawdust Inocula					
	Fungus	1, 4	16.4160*	0.0098	*Phellinus noxius* KSK6	42.2 (13.2)
					Control	20.3 (11.9)
	(Tree) Fungus	4, 24	1.4809	0.2329		
Axial Decay and Discoloration (cm)	(a) Wood Dowel Inocula					
	Fungus	1, 4	11.6525*	0.0190	*Phellinus noxius* KSK6	34.6 (9.4)
					Control	20.6 (6.2)
	(Tree) Fungus	4, 24	2.3801	0.0689		
	(b) Sawdust Inocula					
	Fungus	1, 4	24.9978*	0.0041	*Phellinus noxius* KSK6	50.5 (15.6)
					Control	12.3 (4.3)
	(Tree) Fungus	4, 24	5.2648*	0.0021		
Radial Decay and Discoloration (cm)	(a) Wood Dowel Inocula					
	Fungus	1, 4	0.0097	0.9253	*Phellinus noxius* KSK6	3.5 (0.7)
					Control	3.5 (0.6)
	(Tree) Fungus	4, 24	10.3214*	<0.0001		
	(b) Sawdust Inocula					
	Fungus	1, 4	0.2952	0.6102	*Phellinus noxius* KSK6	3.7 (1.0)
					Control	3.3 (0.6)
	(Tree) Fungus	4, 24	18.3292*	<0.0001		

Figure 1 The fungal isolates that were used in this study were isolated from the advancing margins (pointers) of typically cone-shaped wood decay lesions that were inferior to the existing pruning wounds (dashed white lines) in Senegal mahogany growing as street trees in Singapore.

Figure 2 The test wood blocks containing the interface between a reaction zone and healthy tissue were extracted from Senegal mahogany, sterilized, and immersed in melted paraffin wax to seal all of the surfaces except for the one containing the reaction zone. These blocks were placed on actively growing wood decay cultures to assess the ability of various fungal isolates to penetrate and degrade the reaction zones (the figure was adapted from Schwarze and Baum, 2000).

Figure 3 Two *Phellinus noxius* strains caused significant dry weight losses to Senegal mahogany sapwood blocks after 12 weeks of incubation. Note: * denotes significant difference at the $\alpha = 0.05$ level.

Figure 4a A transverse section (T.S.) showing the typical anatomical features of Senegal mahogany sapwood, including vessels (V), axial parenchyma (AP), radial parenchyma (RP), and libriform wood fibers (F). Bar, 100 µm. **b A** T.S. of a Senegal mahogany reaction zone showing extensive, uniform polyphenolic deposits within all of the cellular constituents (arrows). Bar, 100 µm.

Figure 5a-d A tangential longitudinal section (T.L.S.) of Senegal mahogany reaction zones that were artificially inoculated with *Hymenochaete murina* KSF3 showing the preferential degradation of polyphenolic deposits in the xylem ray parenchyma (R) ahead of libriform wood fibers (F). Note the progressive removal of polyphenols from the xylem rays and the darkened color that was exhibited by their partially degraded remnants (arrows). After removing the polyphenols, the fungus rapidly degraded the xylem ray (R) and fiber (F) cell walls. Bars, 100 µm. **e** A transverse section (T.S.) showing an overview of reaction zone penetration by *H. murina* KSF3 in Senegal mahogany. The reddish-brown polyphenols in uninfected tissue (I) exhibited an obvious color change to brown-black following degradation by *H. murina* KSF3 (II) with their

removal soon followed by general cell wall decomposition (III). Bar, 100 μm. **f** A T.S. showing *H. murina* KSF3 growing in Senegal mahogany fiber lumina and localized erosion troughs formed near the hyphae. Bar, 10 μm.

Figure 6 a A T.S. of a Senegal mahogany reaction zone that was artificially inoculated with *Phellinus noxius* KSK6 showing hyphal tunneling and cell wall decomposition in the secondary cell wall of libriform wood fibers (arrows). Bar, 100 μm. **b-c** A T.S. and T.L.S. of Senegal mahogany reaction zones that were artificially inoculated with *P. noxius* KSK88 illustrating the distribution and alignment of hyphal tunnels with helically wound cellulose microfibrils in the S_1 (pointers) and S_2 (arrows) layers of the secondary cell wall. Bar, 10 μm. **d** A T.S. showing an overview of reaction zone penetration by *P. noxius* KSK6 in Senegal mahogany. The reddish-pink polyphenols that were deposited in the undamaged reaction zones (I) exhibited obvious darkening to brownish-black during enzymatic degradation by *P. noxius* KSK6 (II) and ultimately receded to reveal the original cellular structure (III). Bar, 100 μm.

Figure 7 Senegal mahoganies that were inoculated with *P. noxius* KSK6 (**a-c**) exhibited greater wound wood occlusion and local stem bulging around the infected areas compared to those of the controls (**d-f**).

Figure 8 Senegal mahoganies that were inoculated with *P. noxius* KSK6 (**a**) contained greater internal discolored tissue and suffered higher dry mass losses compared to those of the controls (**b**).

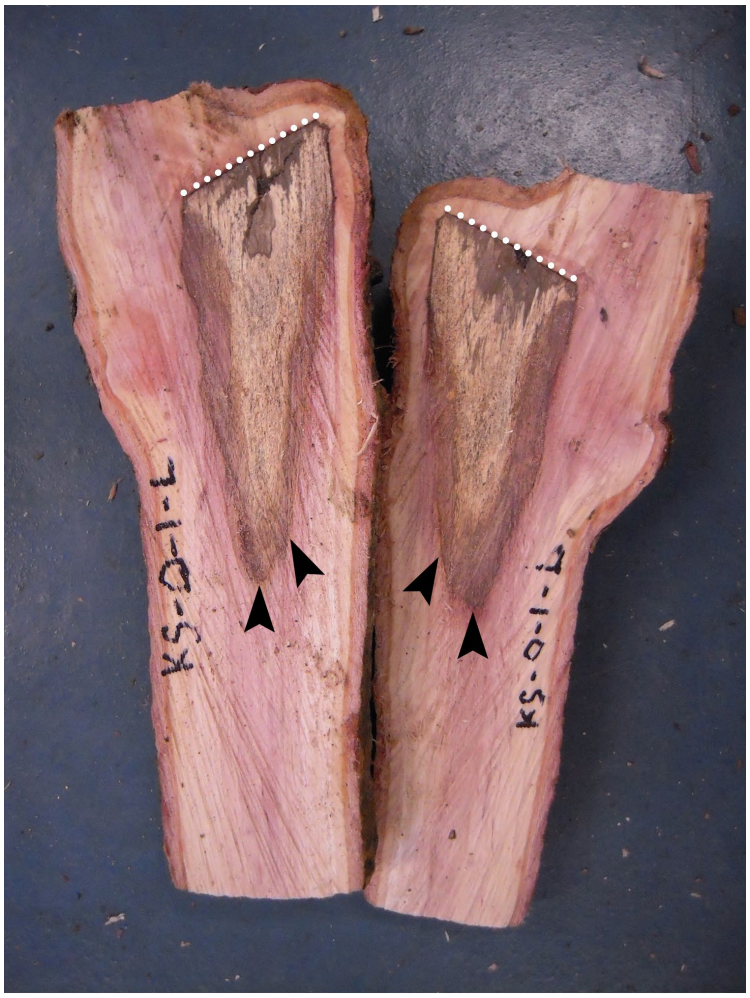

Figure 1

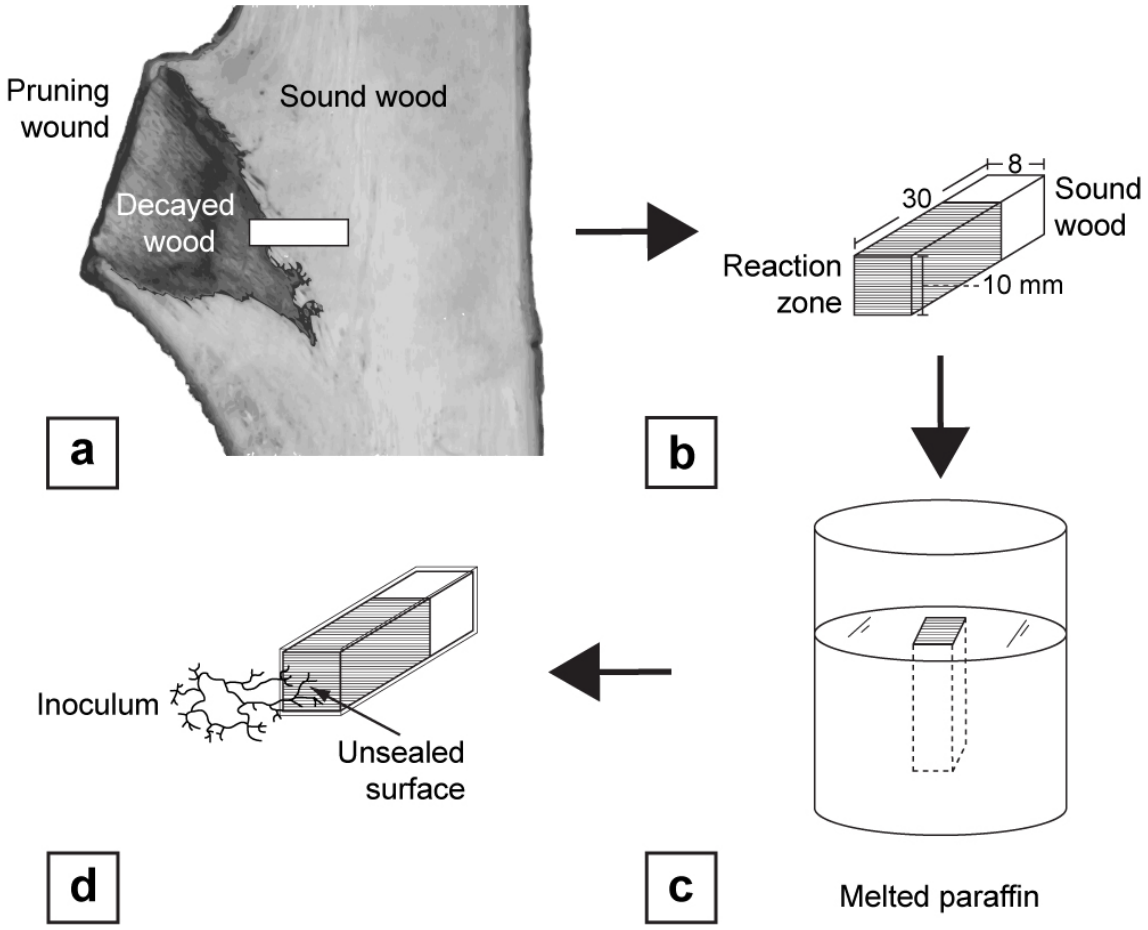
Pruning wound
Sound wood
Decayed wood
a
b
8
30
Sound wood
Reaction zone
10 mm
c
Melted paraffin
d
Inoculum
Unsealed surface

Figure 2

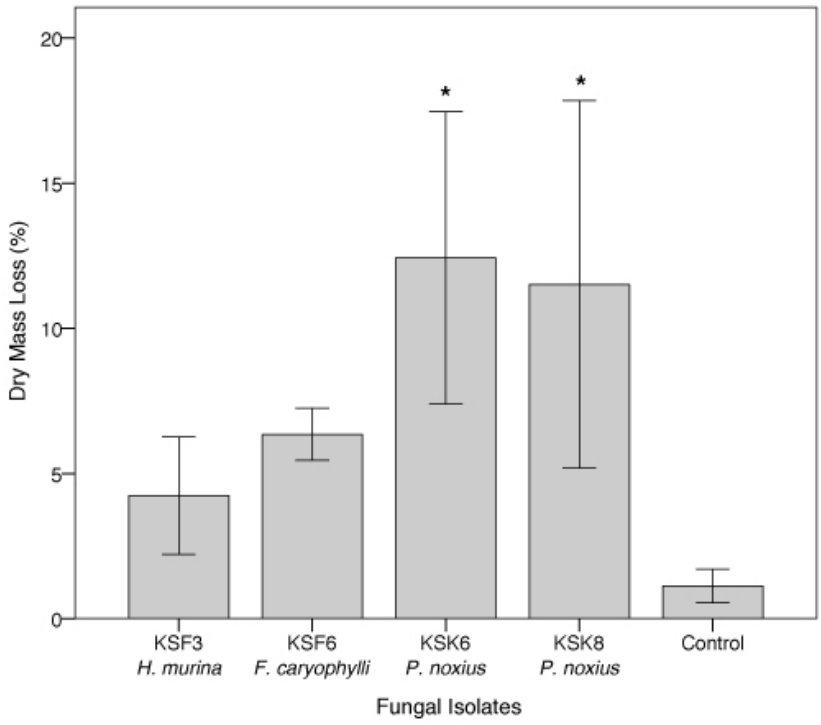
20
15
10
5
0
Dry Mass Loss (%)
*
*
KSF3
H. murina
KSF6
F. caryophylli
KSK6
P. noxius
KSK8
P. noxius
Control
Fungal Isolates

Figure 3

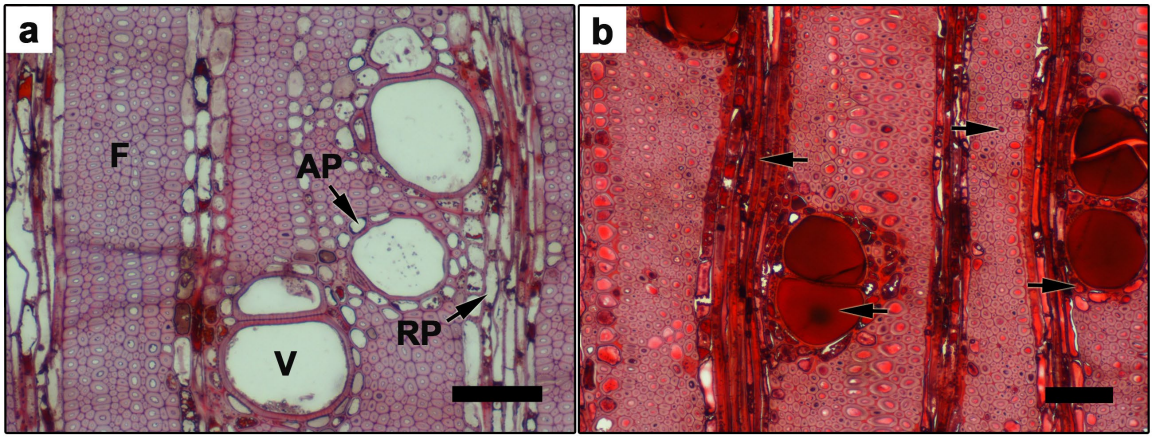

Figure 4

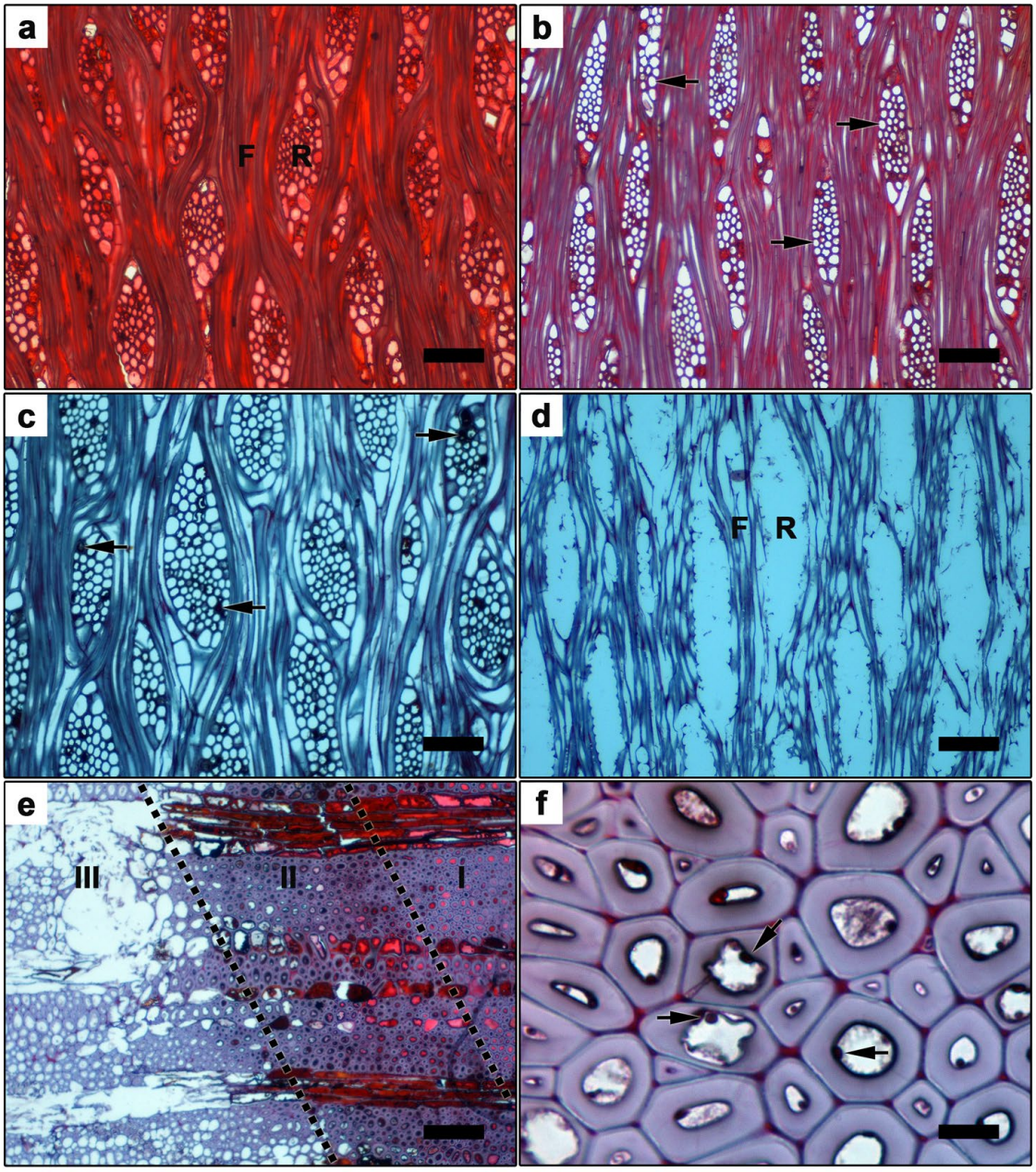

Figure 5

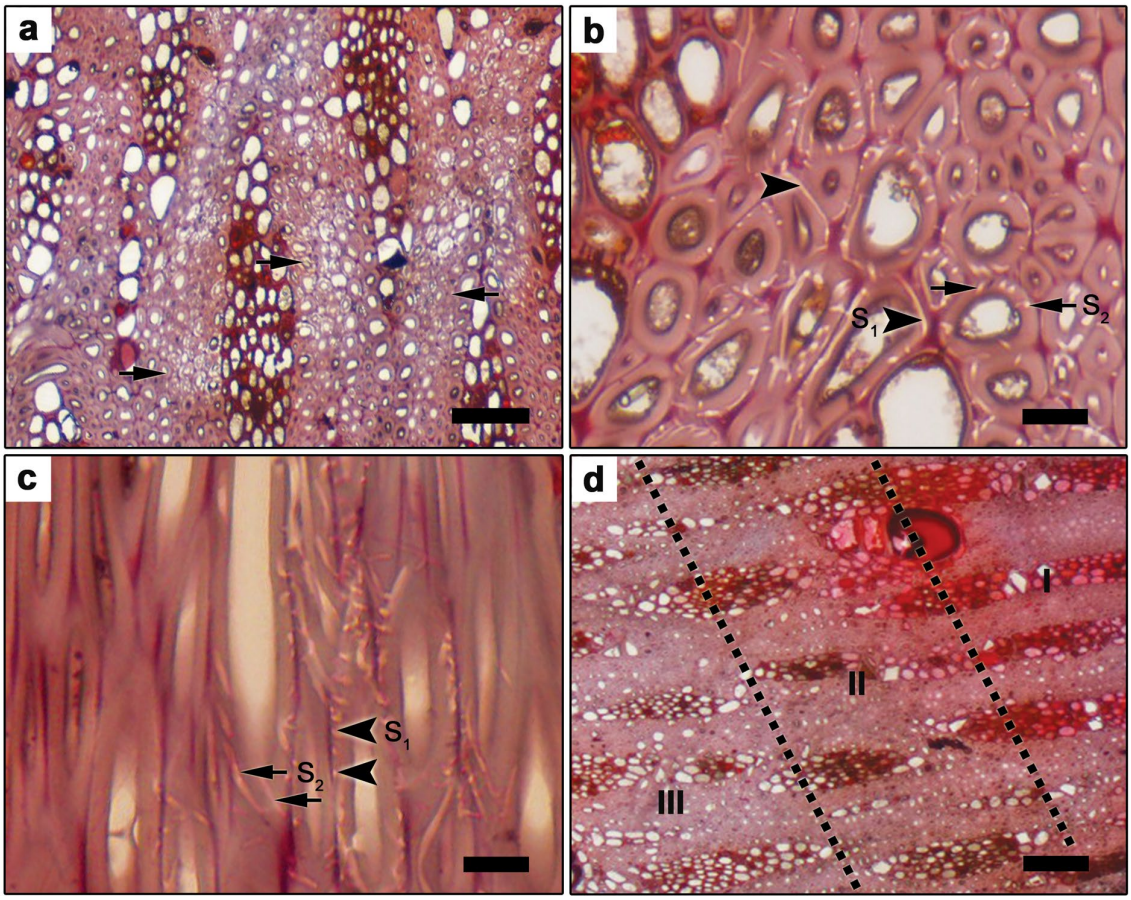

Figure 6

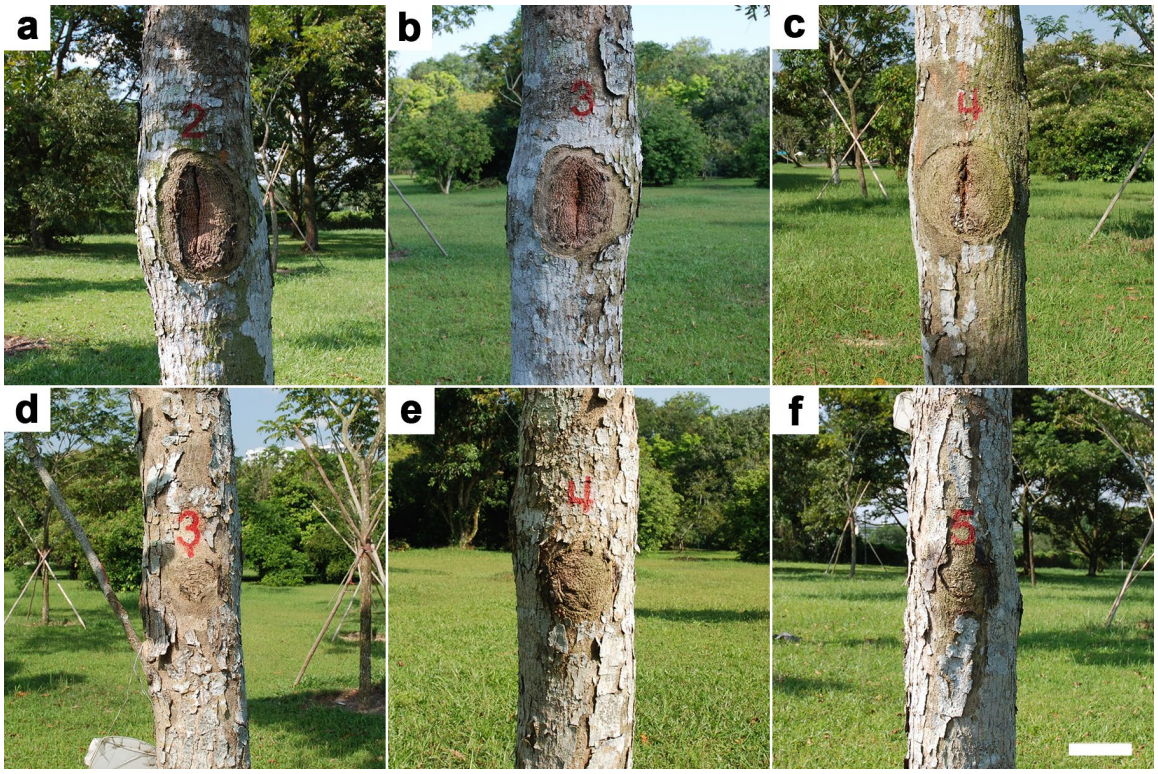

Figure 7

Figure 8