Comparison of Sapwood Discoloration in Fagaceae Trees After Inoculation with Isolates of *Raffaelea quercivora*, Cause of Mass Mortality of Japanese Oak Trees

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Abstract

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The mass mortality of oak trees has been prevalent in Japan since the late 1980s. The fungus *Raffaelea quercivora* is transmitted by an ambrosia beetle, *Platypus quercivorus*, which causes mortality. The beetle is able to bore galleries into the sapwood of most Fagaceae trees in Japan; however, the level of mortality caused by *R. quercivora* and *P. quercivorus* differs greatly among tree species. Previous studies by our research group have demonstrated that the virulence of *R. quercivora* differs among isolates when inoculated into *Quercus serrata* logs. However, interactions between the virulence of *R. quercivora* isolates and the susceptibility of other fagaceous species have yet to be elucidated. In this study, we inoculated the fresh logs of 11 fagaceous species with isolates of low and high virulence, and measured the tangen-

tial widths of discolored sapwoods 3 weeks after inoculation. Although the discoloration widths of *Q. crispula* sapwood were similar among all isolates, those of *Q. serrata* and *Q. acutissima* tended to increase with the more virulent isolates. Sapwood discoloration in *Q. glauca*, *Q. acuta*, *Q. salicina*, *Lethocarpus edulis*, and *Castanopsis sieboldii* was greatly increased by highly virulent isolates. Discoloration in *Fagus japonica* was not influenced by any of the isolates. The logs of *Q. crispula* and *Q. serrata* but not *Q. glauca* were significantly more discolored by a low-virulence isolate compared with standing trees. The various virulent isolates induced unique sapwood discoloration characteristics in each species, which may explain species-specific differences in mortality rates.

Over the last 20 years, fungi carried by ambrosia beetles have caused the mass mortality and dieback of woody plants worldwide, threatening their survival. Examples include laurel wilt in the United States (2) and Fusarium dieback in Israel and the United States (1,3), along with the mass mortality of oak trees in Korea (7) and Japan (5). Consequently, these pathogens have had major ecological and economic impacts on the forests and orchards of many of the regions of the world.

The mass mortality of oak trees has been prevalent in Japan since the late 1980s, with the range in mortality continuing to expand. Causal agents of this mortality are the ambrosia beetle *Platypus quercivorus* (Murayama) and the associated fungus *Raffaelea quercivora* Kubono et Shin. Ito (13). Mortality caused by *P. quercivorus* was first reported in 1934 (5). Subsequently, outbreaks

occurred intermittently in restricted areas, primarily in stands that were over 40 years old (5). Each outbreak lasted a few years, with intervals of several decades between outbreaks until the mid-1980s. Moreover, documents dating back to 1790 in Nagano Prefecture recorded similar damage of oak trees in a shrine forest (4). From the features of incidence before the mid-1980s, *P. quercivorus* has been considered to be a native species in Japan. Two reasonable hypotheses for the recent epidemic have been proposed. The first hypothesis is an increase in the size and age of oak trees in abandoned coppice forests, which are preferable for the reproduction of beetles (12). The second hypothesis is global warming, which potentially shifts the distribution of beetles to more northerly latitudes and higher altitudes (6).

In early summer, *P. quercivorus* attacks the trunks of living oak trees and bores galleries into the sapwood. The female beetle has mycangia in the pronotum (8) and, thus, introduces *R. quercivora* into the galleries during its boring activities (9). Introduced *R. quercivora* induces sapwood discoloration, along with the loss of water conductance around the galleries (14,15). Consequently, the oak trees wilt and die when the ascent of sap is completely blocked at any cross-section of the trunk. Because sapwood discoloration induced by *R. quercivora* is limited to a narrow area around the galleries (34), mass attacks by *P. quercivorus* are required to kill oak trees (9).

P. quercivorus and *R. quercivora* cause different levels of mortality among the Fagaceae species. *Quercus crispula* exhibits the

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highest mortality in the field, ranging from 50 to 100% mortality at the stand level, followed by *Q. serrata*, with mortality ranging from 10 to 60% (6,10,22,26,28). There are also many records of *P. quercivorus* outbreaks in stands of evergreen oak species such as *Quercus*, *Castanopsis*, and *Lithocarpus*. However, these species exhibit much lower levels of mortality than deciduous oaks such as *Q. crispula* and *Q. serrata* (19,30,32,33). In contrast, dead trees belonging to the genus *Fagus* have not been found yet, despite these trees being attacked by *P. quercivorus* (12).

The mortality of oak trees depends on complex interactions among the host tree, the ambrosia beetle, and the pathogenic fungus. However, our understanding about tree–fungus interactions remains limited compared with tree–beetle interactions. Murata et al. (20,21) artificially inoculated *R. quercivora* into the seedlings and branches of several Fagaceae species, and found that *Q. crispula* and *Q. serrata* had larger areas of discolored and nonconductive sapwood in transverse sections compared with *Q. glauca*, *Castanopsis sieboldii*, *Lithocarpus edulis*, and *Fagus crenata*. In addition, the authors suggested that the xylem vessel arrangement in fagaceous trees (i.e., ring-, radial-, and diffuse-porous wood) is associated with differences in the tangential spread of sapwood discoloration among host species. Torii et al. (35) found that the hyphae of *R. quercivora* spread farther from the inoculation point in the sapwood of *Q. crispula* than that of *Q. glauca*, and that the different widths of nonconductive sapwoods reflect different hyphal growth between the two host species. These articles indicate that the nonconductive area induced by a single inoculation is correlated with the mortality of each species. Furthermore, Kusumoto et al. (16,17) collected fungal isolates from Japan and other Asian countries and showed that the most virulent isolate caused twice as much discoloration in *Q. serrata* logs than the least virulent isolate.

Although our previous studies have indicated that there is a considerably large difference of virulence among *R. quercivora* isolates, their effect on the discoloration of the other fagaceous species has yet to be elucidated. In the present study, *R. quercivora* isolates with different levels of virulence were inoculated into the logs of 11 Fagaceae trees to evaluate the relationship between fungal virulence and host susceptibility. In addition, we compared differences in sapwood discoloration between logs and standing trees to confirm the validity of log inoculation. The results of this study are expected to help estimate the contribution of this pathogen to the mortality of oak trees.

Materials and Methods

Preparation of inocula. The *R. quercivora* isolates were isolated from the body surface of *P. quercivorus* collected in Japan, Taiwan, Vietnam, Thailand, and Indonesia (Table 1). All of the isolates were prepared as monospore isolates that originated from a conidium, and were identified by the nucleotide sequence of the internal transcribed spacer 2 and D1-D2 large subunit region of nuclear ribosomal DNA. Isolates with different virulence were selected based on the results of previous studies (Table 1) (16,17). Phytosanitary certificates and research permissions were obtained from the governmental authorities in each country (16).

Autoclaved birch toothpicks (65 mm in length and 2 mm in diameter) were incubated with the mycelia of each isolate on 2%

malt extract agar plates for 2 weeks at 25°C. After incubation, *R. quercivora*-proliferated toothpicks were used as inocula. Autoclaved toothpicks incubated without *R. quercivora* were used as negative controls.

Experiment 1: Inoculation of the logs of six *Quercus* spp. in 2012. Three individual trees of *Q. acuta*, *Q. acutissima*, *Q. glauca*, *Q. salicina*, and *Q. serrata* were harvested in natural forest stands in the University of Tokyo Chiba Forest (UTCBF) in Chiba Prefecture, Japan on 2 July 2012. Three individual trees of *Q. crispula* were harvested at the University of Tokyo Chichibu Forest (UTCF) in Saitama Prefecture, Japan on 4 July 2012. After harvesting, the trees were cut into five logs (50 cm in length and 10 to 15 cm in diameter), and both ends of the logs were immediately sealed with paraffin to prevent desiccation from the cross sections.

Log inoculation was carried out on 4 July 2012. Six holes (2 cm in depth and 2.7 mm in diameter) were drilled from six directions at regular intervals along the circumference of the middle part of the individual logs. Five *R. quercivora* isolates (CXP5-2, IP95-1, nR24, Ta28, and Vt42-1) proliferating in the birch toothpicks and a sterilized toothpick were inserted into each of the six holes on each log. The parts of the toothpicks that were sticking out from the log were cut off. The holes were sealed with Parafilm and adhesive tape to prevent the inocula from drying out. Finally, the inoculations were carried out on 15 logs per tree species. The logs were incubated at 25°C in darkness. The inoculation and incubation were carried out inside incubators that were set in a laboratory at Kagemori Nursery of the UTCF under a biosecurity guideline by the Plant Protection Law, Ministry of Agriculture, Forestry, and Fisheries, Japan.

The logs were cut across at the inoculation points after a 3-week incubation period. The cross-sections were photographed using a digital camera (PowerShot SX110 IS; Canon). Then, the areas and lengths of the photographed discolored sapwoods were measured using the graphics software package ImageJ (<http://rsbweb.nih.gov/ij/>). The tangential widths of discoloration from the edge of the inoculation holes were calculated as the average values of the discolored area of the sapwood on the right and left sides of the inoculation hole divided by the radial length of the discolored sapwood where the area was measured.

Experiment 2: Inoculation to the logs of eight Fagaceae species in 2013. Three individual trees of *Castanea crenata*, *Castanopsis sieboldii*, *L. edulis*, *Q. glauca*, and *Q. serrata* were harvested in natural forest stands in the UTCBF. Three individual trees of *F. japonica* and *Q. crispula* were harvested in the UTCF. Three trees of *Q. phillyraeoides* were harvested in the nursery of a commercial gardening company in Sayama City, Saitama Prefecture, Japan. All trees were harvested on 3 July 2013. Four logs (50 cm in length and 10 to 15 cm in diameter) were obtained from each harvested tree. Both ends of the logs were immediately sealed with paraffin to prevent desiccation from the cross sections.

Log inoculation was conducted on 9 July 2013, following the same procedure as experiment 1. Finally, 12 repeats of the inoculated logs were prepared per tree species. After a 3-week incubation period, the tangential widths of the discolored sapwoods were measured. Some logs showed unexpected discoloration as a result of contamination in experiment 2. In such cases, the data

Table 1. *Raffaella quercivora* isolates used for the inoculation experiment on logs and standing trees

Isolate number ^a	Other number ^b	Date	Origin	Relative virulence ^c
G37	FFPRI411062	March 2003	Ibusuki, Kagoshima, Japan	Low
nR24	FFPRI411059	September 2006	Kisakata, Akita, Japan	Moderate
Rem10	...	2 September 2009	Naruko, Miyagi, Japan	This is the first time using
IP95-1	...	23 April 2007	Cibodas, Indonesia	Low
Ta28	...	14 January 2009	Chiang Mai, Thailand	Low
Vt42-1	...	14 October 2010	Lao Cai, Vietnam	Low
CXP5-2	...	18 January 2006	Taitung, Taiwan	High

^a Reference number for cultures by H. Masuya.

^b FFPRI: culture collection of Forestry & Forest Products Research Institute, Japan.

^c Relative virulence is based on Kusumoto et al. (16).

affected by the contamination were excluded from all subsequent analyses.

Experiment 3: Comparison of discoloration between logs and standing trees. Three logs of *Q. crispula*, *Q. glauca*, and *Q. serrata* were prepared from the same trees used in experiment 2. Eight holes (2 cm in depth and 2.7 mm in diameter) were drilled midway up the individual logs on 9 July 2013 and were distributed at regular intervals around the circumference. Toothpicks of four inocula (G37, nR24, Rem10, and negative control) were inserted into two of eight holes on a single log, respectively. Inoculations were repeated on three logs of each tree species. The holes were sealed with Parafilm and adhesive tape and the logs were incubated at 25°C in darkness. After a 3-week incubation period, the tangential widths of the discolored sapwoods were measured.

A similar inoculation technique was used on standing trees of *Q. crispula* in a natural forest of UTCF on 9 July 2013 and those of *Q.*

glauca and *Q. serrata* in UTCBF on 10 July. Three fungal isolates and a sterilized inoculum were inoculated into the trunks of three standing trees from each species, following the same procedure that was used for the log inoculation. Three weeks later, the trees were harvested and cut across at the inoculation points to measure the extent of sapwood discoloration.

Statistical analysis. The tangential widths of discolored sapwoods were compared among the inocula in each tree species or among the tree species for each inoculum using nonparametric all-pair comparisons for joint ranks (Dunn's test). Differences between the logs and the standing trees in experiment 3 were analyzed using Welch's *t* test. All statistical analyses were conducted using JMP Pro 10 software (SAS Institute Inc.).

Results

Experiment 1. Photographs of cross-sections near the inoculation points and the boxplots of the tangential widths of the discolored sapwoods from the logs of six tree species belonging to the genus *Quercus* after the *R. quercivora* inoculations in 2012 are shown in Figures 1 and 2, respectively. The mean tangential widths of the discolored sapwoods caused by nonfungal inoculations (i.e., the negative control) ranged from 0.44 mm in *Q. salicina* to 1.43 mm in *Q. crispula*. All *R. quercivora* isolate inoculations significantly increased the discoloration widths of *Q. crispula* compared with the control but there was no significance among the fungal isolates. For *Q. serrata* and *Q. acutissima*, sapwood discoloration was affected by isolate virulence. The mean widths of the discoloration caused by the most virulent isolate, CXP5-2, were six times as large as those in the control for both species. Inoculation with Ta28, IP95-1, and Vt42-1 slightly increased the discoloration widths (to no more than 2 mm) of *Q. glauca*, *Q. acuta*, and *Q. salicina*, while inoculation with nR24 and CXP5-2 caused large discoloration. Greater variability in discoloration widths was obtained from inoculations with higher virulence isolates, particularly in *Q. acutissima*.

In comparison with the discoloration widths among host tree species by each inoculum, the discoloration widths in the three deciduous ring-porous species (*Q. crispula*, *Q. serrata*, and *Q.*

Fig. 1. Cross-sections of the logs of six *Quercus* spp. near the inoculation points with the *Raffaelea quercivora* isolates. Numbers beside the inoculation points indicate 1: sterilized toothpick (negative control), 2: nR24, 3: Ta28, 4: CXP5-2, 5: Vt42-1, 6: IP95-1. **a**, *Quercus crispula*; **b**, *Q. serrata*; **c**, *Q. acutissima*; **d**, *Q. glauca*; **e**, *Q. acuta*; **f**, *Q. salicina*.

Fig. 2. Boxplots of the tangential widths of the discolored sapwoods from the logs of six *Quercus* spp. inoculated with low- to high-virulence isolates of *Raffaelea quercivora* in 2012 (experiment 1). Open circles and plus signs indicate outliers and mean, respectively. Vessel arrangement is indicated as ring-porous or radial-porous. Different letters indicate significant differences among the inocula in each tree species by Dunn's nonparametric test ($P < 0.05$).

acutissima) were larger than the three evergreen radial-porous species (*Q. glauca*, *Q. acuta*, and *Q. salicina*) when Ta28, IP95-1, and Vt42-1 were inoculated (Supplementary Fig. S1). By inoculation with nR24 and CXP5-2, the discoloration width of *Q. crispula* was similar to the three evergreen radial-porous species.

Experiment 2. Photographs of the cross-sections near the inoculation points and the boxplots of the tangential widths of the discolored sapwoods from the logs of the eight fagaceous species after the *R. quercivora* inoculations in 2013 are shown in Figures 3 and 4, respectively. Compared with the control, all isolates greatly increased the discoloration widths of *Q. serrata*. In comparison, the discoloration widths of *Q. crispula* and *Q. phillyraeoides* gradually increased with the virulence of the fungal isolates. In *Q. glauca*, *L. edulis*, and *C. sieboldii*, all fungal inoculations (except CXP5-2) caused a slight increase in discoloration width. In contrast, CXP5-2 inoculations induced large discoloration, with great variability, compared with the other isolates. The fungal inocula-

Fig. 3. Cross-sections of the logs of eight Fagaceae species near the inoculation points with the *Raffaelea quercivora* isolates. A letter and the numbers beside the inoculation points indicate C: sterilized toothpick (negative control), 1: nR24, 2: Ta28, 3: CXP5-2, 4: IP95-1, 5: Vt42-1. a, *Quercus crispula*; b, *Q. serrata*; c, *Q. phillyraeoides*; d, *Q. glauca*; e, *Lithocarpus edulis*; f, *Castanopsis sieboldii*; g, *Castanea crenata*; h, *Fagus japonica*.

tions increased the discoloration widths of *Castanea crenata* but the extent of discoloration did not parallel the virulence of the isolates. Of all eight fagaceous species, *F. japonica* was the least discolored by the nonfungal inoculation (negative control) (Supplementary Fig. S2). In addition, the discoloration widths of *F. japonica* were not influenced by any of the isolates, including the isolate with the highest virulence (CXP5-2).

The tangential widths of the discoloration caused by each fungal inoculation in *Q. crispula* and *Q. serrata* were smaller in experiment 2 compared with the corresponding inoculations in experiment 1. However, the discoloration widths of *Q. glauca* were similar in both experiments.

Experiment 3. The tangential widths of the discolored sapwood in logs and standing trees of *Q. crispula*, *Q. serrata*, and *Q. glauca* induced by the *R. quercivora* inoculations are shown in Figure 5. The tangential widths of discoloration significantly differed in some pair comparisons between the logs and the standing trees inoculated with the same isolates. In the logs of *Q. crispula* and *Q. serrata*, the sterilized inoculum, G37, and Rem10 induced significantly larger discoloration than in the standing trees. In contrast, only the most virulent isolate, Rem10, induced significantly larger discoloration in the logs of *Q. glauca* than in the standing trees.

Discussion

Q. crispula shows the highest mortality by *P. quercivorus*-*R. quercivora* infestation based on field observations. Our results indicated that nonfungal inoculation (sterilized toothpicks) caused *Q. crispula* sapwood to form relatively large areas of discoloration, with fungal inoculations causing greater discoloration. *Q. serrata* and *Q. acutissima* were more sensitive to fungal inoculations than *Q. crispula*, showing the greatest variation in discoloration widths among all isolates. Field-based observations have reported that *Q. serrata* has lower mortality than *Q. crispula* (6,10,22,26,28), and that *Q. acutissima* has lower mortality than *Q. serrata* (27). In the present study, susceptibility based on the size of the discoloration in the three deciduous oak trees did not coincide with the levels of mortality in the field. The mortality of oak trees is related to the

Fig. 4. Boxplots of the tangential widths of the discolored sapwoods from the logs of eight Fagaceae species inoculated with low- to high-virulence isolates of *Raffaelea quercivora* in 2013 (experiment 2). Open circles and plus signs indicate outliers and mean, respectively. Different letters indicate significant differences among the inocula in each tree species by Dunn's nonparametric test ($P < 0.05$).

size of discoloration by *R. quercivora* infection, in addition to the density of beetle galleries in the sapwood. Compared with *Q. crispula*, the sap of *Q. serrata* is known to flow more frequently from the holes bored by *P. quercivorus*, which kills boring beetles and reduces beetle reproduction (11). Different levels of mortality among *Q. crispula*, *Q. serrata*, and *Q. acutissima* may be more affected by differing levels of susceptibility to beetle infestation rather than their susceptibility to *R. quercivora* infection.

Evergreen radial-porous trees, belonging to the subgenus *Cyclobalanopsis* (genus *Quercus*) and the genera *Lithocarpus* and *Castanopsis*, were minimally affected by the low-virulence isolates. The sapwood discoloration widths of these species were comparatively smaller than the deciduous ring-porous *Quercus* spp. These differences in discoloration may relate to the lower mortality levels of evergreen oaks in the field, because most *R. quercivora* isolates used in our previous studies were categorized as having low virulence (16,17). In comparison, *Q. phillyraeoides* has evergreen leaves and radial-porous wood, like *Q. glauca*, *L. edulis*, and *Castanopsis sieboldii*; yet, this species responded in a similar manner to *Q. crispula*.

The tangential width of sapwood discoloration induced by the low-virulence isolates (Ta28, IP95-1, and Vt42-1) in the *Castanea crenata* logs was relatively large compared with the other species.

Fig. 5. Mean ($\pm$ standard error) tangential widths of the discolored sapwoods from the logs and standing trees of three *Quercus* spp. inoculated with low- to high-virulence isolates of *Raffaella quercivora* (experiment 3); *, **, and *** indicate significant differences between the logs and standing trees at $P < 0.05$, 0.01, and 0.001, respectively, by Welch's *t* test; ns indicates no significant difference.

However, the shape of discoloration differed from that of the other species (i.e., the discoloration was very narrow near the cambium, and tended to widen toward the heartwood; Fig. 3). In ring-porous trees, including *C. crenata*, water ascent mainly occurs in a current annual ring (31). Therefore, the loss of water conductance induced by *R. quercivora* infection is probably limited in *C. crenata*.

F. japonica showed the least sapwood discoloration of all eight fagaceous species in response to all of the inoculations in experiment 2. In addition, the discoloration widths induced by *R. quercivora* inoculations were similar to those of the nonfungal inoculation. This result may explain why trees belonging to the *Fagus* genus are not killed by *P. quercivorus* infestations.

The physiological status of a host tree is assumed to be a very important factor in its susceptibility to pathogenic infection (36). In experiment 3, the sapwood discoloration widths of logs were significantly larger than those of standing trees in some comparisons. This result suggests that physiological activities related to defense responses are weakened in logs because a supply of photosynthetic carbohydrates is required to maintain quantitative disease resistance (23). In addition, the mortality of *Q. crispula* and *Q. serrata* by the artificial inoculation of *R. quercivora* is influenced by the phenology of host trees (25,35,37), which tends to depend on host physiology. In the present study, the sapwood discoloration widths of these trees differed between experiment 1 (conducted in 2012) and experiment 2 (conducted in 2013), even though the discoloration widths of *Q. glauca* remained similar. These results suggest that the susceptibility of *Q. crispula* and *Q. serrata* to *R. quercivora* infection is more influenced by the physiological status of host trees than that of *Q. glauca*.

In conclusion, the present study demonstrated that the sapwood of different Fagaceae species responds differently to the inoculation of low- and high-virulence isolates of *R. quercivora*. One of the host-pathogen interactions based on sapwood discoloration corresponded to the levels of mortality in the field, but there were exceptions. In addition, host-pathogen interactions are likely to reflect taxonomic classification rather than the vessel arrangement in the xylem (Supplementary Table S1). The mortality of oak trees caused by the *P. quercivorus*-*R. quercivora* complex is often called Japanese oak wilt. However, colonization by *R. quercivora* is limited to a narrow range of the xylem (34), tangentially within a few millimeters and longitudinally up to 20 cm or less (17,37), which differs from other wilt diseases associated with bark and ambrosia beetles, such as Dutch elm disease and laurel wilt. The pathogens of these diseases are invasive fungi, and can colonize the tree body systemically. The occurrence and severity of the disease mainly depends on the interaction between host resistance and pathogen virulence (24,29). In contrast, *R. quercivora* and *P. quercivorus* are regarded as species native to Japan. Mass attack by *P. quercivorus* and the mass introduction of *R. quercivora* are required for tree death (9). Thus, the mortality of oak trees in Japan is likely to depend on both the interactions of host resistance-beetle aggressiveness and host resistance-fungal virulence (18). To determine what causes variation in the levels of mortality among tree species, and to estimate the risk of mass mortality in oak forests, further studies are required, focusing on host-beetle interactions, comparisons of populations with isolates of differing virulence, and the physiological status of host trees.

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