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Defense Responses of Oak Trees against the Fungus *Raffaelea quercivora* Vectored by the Ambrosia Beetle *Platypus quercivorus*

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Abstract – Japanese oak wilt caused by *Raffaelea quercivora* has continually appeared in Japan since the 1980's. We investigated defensive responses of oak sapwood, i.e. the formation of reaction zone barriers. We observed tyloses suberization, but parenchyma was not suberized conspicuously. Insoluble deposits occluded fibre tracheids and small vessels. Staining tests showed that they consisted of lignin-like compounds, pectin, phenolics, quinones, and lipids. Wood fibre occlusion with deposits preceded tyloses and accumulation of parenchyma phenolics. Accumulation of condensed lignin suggested that the deposits consisted mainly of condensed lignin.

I. Introduction

Mass mortality of oak trees (mainly *Quercus serrata* and *Q. crispula*) has continually appeared in Japan since the 1980's [1]. The newly identified causal fungus *Raffaelea quercivora* is vectored by an ambrosia beetle *Platypus quercivorus* [2-5]. Blockage of xylem sap ascent induced by *R. quercivora* infection has been hypothesized to cause this mortality [6, 7]. Clarifying defense responses against the pathogen is necessary to understand interactions between the host trees and the pathogenic fungus, including wilt mechanisms. We are investigating the defense responses of oak sapwood against the pathogen, because it is the most important factor in the spread of both wood discoloration and the pathogen in sapwood.

II. Materials and Methods

A. *Materials*

We investigated the sapwood-discolored wood boundary, i.e., the reaction zone barrier (RZB), by measuring primarily histochemical responses on the following materials. Chemical analyses and bioassays with the fungus were also conducted on several materials.

1. Mature *Q. crispula* trees surviving natural attack by *P. quercivorus* / *R. quercivora* of various damage classes were harvested in Yamagata Prefecture in September. A mature *Q. serrata* tree surviving natural attack but without symptoms was also harvested in Kyoto Prefecture in October.
2. Sapwood of wounded *Q. serrata* branches planted at the Forestry and Forest Products Research Institute (FFPRI), Ibaraki Prefecture were wounded with a drill in July to elucidate the time-course of defense events.
3. Sapwood of fungal-inoculated *Q. crispula* trees

Young *Q. crispula* trees were wound inoculated with *R. quercivora* at Tohoku Res. Ctr., FFPRI in July [2], and were harvested 3 months after the inoculation.

B. *Histochemical tests*

Wood blocks were sectioned with a sliding microtome 20-30 μm thick and examined histochemically [8, 9].

Lignin was stained with phloroglucinol – hydrochloric acid (PG-HCl), Schiff's reagent or observed under UV illumination (U-mode). After quenching with PG-HCl, suberin was observed under UV illumination. Phenolic substances were stained using nitroso-phenol methods. Quinone was stained with *o*-tolidine. Lipid was stained with Nile blue or Sudan black B. Ruthenium red was used for pectin staining. Safranin O – Fast Green FCF double staining was also used. Then, sections were observed under the microscope. Staining for NAD diaphorase activity was conducted to assess the parenchyma cells activity.

Microtome sections were immersed in ethanol-benzene (1:2) and incubated at 25°C for 24 hr to check the solubility of the deposits.

Branches were investigated macroscopically under UV (360 nm) illumination 3 months after the wounding .

C. *Chemical analysis of lignin*

Wood slices for lignin analysis were obtained from fungus inoculated young trees, and occlusion of fibre-tracheids in RZB was confirmed. Lignin content was determined by the acetyl bromide method. Chemical structure of lignin was determined by alkaline nitrobenzene oxidation.

D. Assay for soluble inhibitory substances

Naturally attacked or fungal inoculated *Q. crispula* trees that survived were used for assays of antifungal substances. Wood chips were immersed in methanol and shaken at 30°C for 3 hr. Extractives loaded on TLC plate were developed with hexane – ethyl acetate (7:3). Spore suspensions of *R. quercivora* in medium were sprayed on developed TLC plates and incubated at 25°C in darkness for 3 days [10].

We sprayed 0.05% calcofluor white M2R in 67mM phosphate buffer, pH 8.0, on the samples and incubated them for 2 hours at 25°C. Inhibitory spots were examined under UV illumination (360 nm) [11].

III. Results

A. Naturally infected trees

Vessels in the RZB were occluded with tyloses, and cell walls of the tyloses were well suberized (Fig. 1). Suberization of parenchyma cell walls, however, was induced but not conspicuous in the RZB. Deposits were found to occlude fibre tracheids and small vessels in the RZB (Figs. 2, 3). Most of the deposits in microtome sections were insoluble to methanol or ethanol-benzene (1:2). Deposits in the wood fibre region fluoresced under UV illumination (Figs. 2a, 3c) and stained red with PG-HCl (Figs. 3a, b), suggesting the presence of lignin-like compounds. Additional staining tests showed that they consisted of pectin, phenolics, quinones, and lipids (Figs. 2b, c, d). Major components of the deposits appeared to differ between the fibre and the tracheid / small vessel regions. NAD diaphorase activity of the parenchyma cells increased in the RZB and was not detected in the discolored wood.

We observed a relationship between damage class and intensity of the defense responses. Responses in the RZB were conspicuous in the tree that appeared to be healthy in spite of the beetle attack.

Deposits that were PG-HCl positive fluoresced under UV illumination without staining. The fluorescence of the deposits was quenched by PG-HCl . Vessels which were not occluded with tyloses were abundant in the RZB and discolored wood. Although suberization of the tyloses cell walls progressed during tyloses formation, some tyloses remained unsuberized.

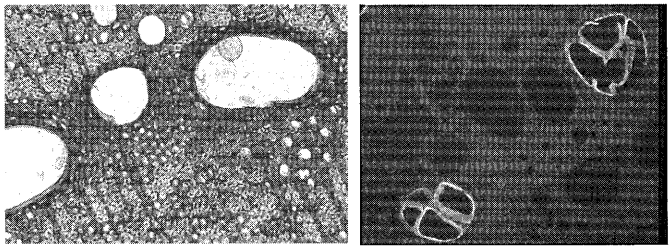

Fig. 1. Formation of tyloses (left) and their suberization (right-fluorescence) in the wood of naturally attacked *Q. crispula* trees.

B. Time course of defensive events in wounded branches

Results obtained from the sapwood of wounded *Q. serrata* branches are summarized in Table. 1. Wood fibres were partially occluded with deposits 3 days after wounding. Then, occlusion of fibre tracheids became remarkable to form a continuous barrier (Figs. 3a, b, c). Tyloses and their suberization were in progress 3 to 7 days after wounding (Fig. 1 left), and were completed after 2 weeks (Fig. 1 right). Accumulation of phenolic compounds was detected histochemically one week after wounding, and became remarkable after 2 weeks.

Fluorescence was not observed in discolored wood under UV illumination. Sound sapwood slightly fluoresced probably due to lignin in the cell walls. Fluorescence was prominent in the RZB which looked like normal sapwood (Fig. 4).

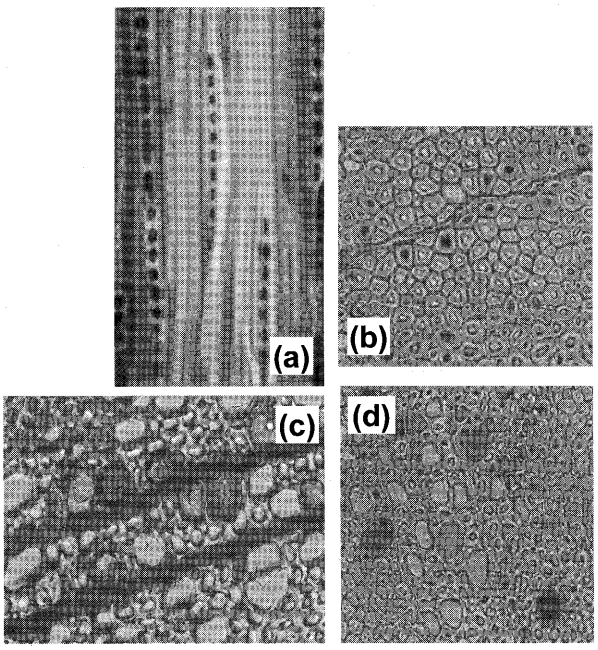

Fig. 2. Deposits in naturally attacked *Q. crispula* trees fluoresced under UV illumination (a), stained with Nile blue (b), stained for pectin (c), and stained for phenolics (d).

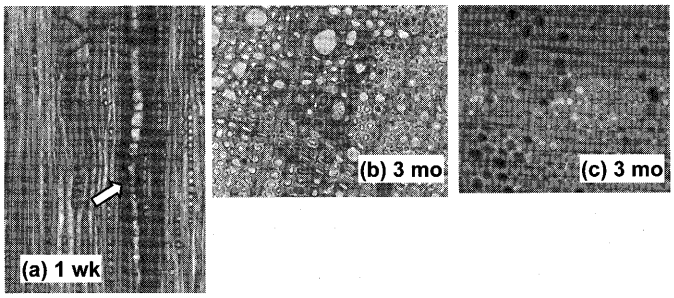

Fig. 3. Insoluble deposits in the wood of wounded *Q. serrata* trees stained red with phloroglucinol- HCl (a, b), and fluoresced blue-white under UV illumination (c).

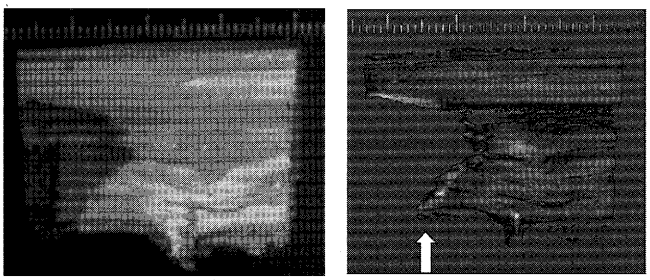

Fig. 4. Wood discoloration (left) and RZB fluorescence (arrow) under UV illumination (right) 3 months after wounding of *Q. serrata* trees.

TABLE 1
Time course of defensive events after wounding of *Q. serrata* trees.

	3 days	1 week	2 weeks, 1-3 months after wounding
Deposits in fibers and tracheids	±	++	++
Tyloses	±	±	+
Suberization of tyloses	–	±	+
Suberization of parenchyma	–	–	±
Phenolics accumulation	–	+	++

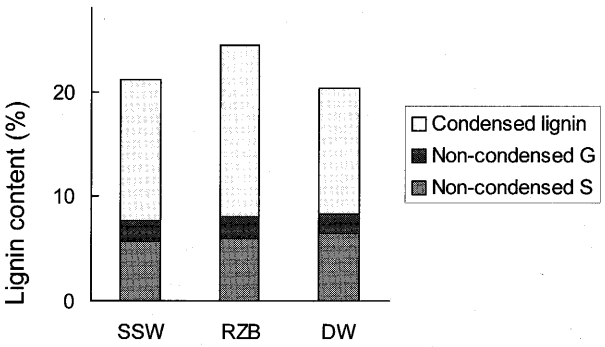

Fig. 5. Lignin accumulation in the RZB of fungal inoculated *Q. crispula* trees.
SSW, sound sapwood; RZB, reaction zone barrier; DW, discolored wood; G, guaiacyl-type lignin; S, syringyl-type lignin

C. Chemical analysis of lignin in the reaction zone barrier

Total lignin content was higher in the RZB than in sound sapwood (SSW) and discolored wood (DW) (Fig. 5). Most of the increased lignin in the RZB was attributed to condensed lignin (Fig. 5), which probably originated from guaiacyl-rich lignin.

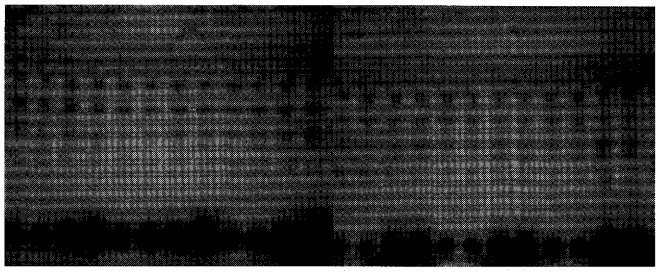

Fig. 6 TLC bioassay for soluble inhibitory substances from wounded *Q. serrata* trees with spore suspension of the pathogen. Inhibitory spots appear as dark areas.
C, control; W, wounded

TABLE 2
Changes in moisture and extractives content after wounding of *Q. serrata* trees

Period after the inoculation	Moisture content (%)		MeOH extractives content (%)	
	SSW	RZB	SSW	RZB
3 days	75.9	72.0	1.80	2.48
1 week	76.5	74.6	2.48	2.88
2 weeks	86.1	82.7	1.95	1.83
1 month	86.2	95.6	1.77	2.21
2 months	86.3	92.6	1.89	2.17
3 months	83.3	76.9	1.73	1.89

Two replicates.

D. Soluble inhibitory substances

Moisture content of the RZB did not differ from that of sound sapwood. Extractives content increased slightly in the RZB. We found several substances weakly inhibitory to the fungus even in sound sapwood extractives. Accumulation of soluble antifungal substances, however, was not detected in the RZB of naturally attacked mature trees nor branches of wounded trees (Table 2, Fig. 6).

IV. Discussion

Occlusion of xylem elements with insoluble compounds was the fastest and most conspicuous response in the RZB. This result suggests the importance of their deposition as a mechanism that inhibits the invasion of pathogens. Suberization of parenchyma cell walls was not remarkable, and tyloses formation alone and their suberization in large vessels was not sufficient as a continuous barrier against fungal invasion. Although the importance of suberization in the barrier zone is widely accepted [12], suberization seemed less important in the RZB, at least as an initial response of oak trees.

The absence of accumulated soluble inhibitory substances in the RZB suggests the possibility that sapwood defense of mature oak trees against *R. quercivora* does not depend on phytoalexins and that histological changes as a mechanical barrier are important.

Insoluble deposits in the fibre tracheid lumina had the same stainability as lignin. We think the PG-HCl positive materials are lignin-like compounds based on several other histochemical tests. Occlusion of xylem elements with insoluble compounds including lignin-like compounds has been widely observed [13, 14]. Similar materials composed of deposits or gel were also observed in *Prunus pensylvanica* [15], *Fagus sylvatica* [16] and oak trees [17]. Such deposition was apparently different from lignification of parenchyma cell walls and normal xylem elements. It is necessary to elucidate the mechanism of synthesis of occluding deposits and of its secretion into fibre and tracheid lumina. Various deposits observed in small vessels, tracheids and fibre lumina appeared to form by mixing of pectin, phenolics, quinones and lipids in addition to lignin-like compounds based on their stainability.

The zone where deposition and occlusion was most conspicuous, corresponding to the inner transition zone [18-20], looked healthy and parenchyma cells were living despite of starch disappearance. This suggests that the most important zone in the RZB is the transition zone, as in the gymnosperm Japanese cedar. The strong fluorescence we observed macroscopically in the RZB was certainly due primarily to PG-HCl positive materials.

Increased total lignin content suggested that lignin constituted the insoluble deposits in the RZB. Condensed lignin was likely the major constituent of the insoluble deposits in the RZB. Because condensed lignin is apparently hard to decompose, accumulation of condensed lignin must provide a strong barrier against fungal penetration. Assessment of the role of insoluble deposits as defensive barriers is necessary.

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