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ORIGINAL ARTICLE

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Responses of *Quercus* sapwood to infection with the pathogenic fungus of a new wilt disease vectored by the ambrosia beetle *Platypus quercivorus*

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Abstract *Quercus serrata* and *Q. crispula* wilt during the summer in wide areas along the Sea of Japan. Mass attacks of trees by an ambrosia beetle (*Platypus quercivorus*) are characteristic before appearance of the wilting symptoms. This study investigated the pathogenic effects of a fungus detected specifically in the wilting trees. This hyphomycete fungus, *Raffaelea* sp., has a distribution that correlates with the discolored xylem area called wound heartwood in which vessels are dysfunctional. Tylosis formation around the hyphae indicates vessel dysfunction. In areas with discoloration, the fungal hyphae were invading living ray parenchyma cells from the vessel lumen. As a protective reaction the ray cells exuded yellow substances into the vessels, but these substances seemed ineffective against the fungal activity, probably because the fungus disperses along the beetle's gallery before enough substance can accumulate. It should allow wide discoloration in sapwood. Cambium was not necrotic around the fungus. The cytological process in the host was as follows: (1) synthesis of secondary metabolites by the stimuli of oak fungus; (2) exudation of yellow substances into vessels; and (3) dysfunction of vessels and wound heartwood formation. In regard to wilting of trees, the pathogenicity of the fungus should be assessed by its ability to stop sap flow.

Key words Xylem discoloration · Wound heartwood · Vessel dysfunction · Hyphomycetes · *Raffaelea*

Introduction

Mass outbreaks of oak wilting during the summer have been reported in a wide area along the Sea of Japan every year since the late 1980s.[1,2] An ambrosia beetle, *Platypus quercivorus* (Murayama),[3] attacked in swarms before the wilting incidence of the deciduous oak trees *Quercus serrata* Thunb. and *Q. crispula* Blume,[4] which are native to Japan. The *Platypus* beetle does not seem to kill trees by itself.[5,6] Ito et al.[7,8] found that a specific hyphomycete fungus was always present in oak trees that were wilting after the beetle attack and concluded that the fungus must be closely related to the mass mortality of oak trees, especially in view of the fact that healthy oak trees died after being inoculated with that fungus. The fungus is a new species that belongs to the genus *Raffaelea*, and is called *Raffaelea* sp. here.[9] This fungus must be vectored by the beetle because the fungus was also detected in mycangia (the organ that keeps the spores of symbiotic fungi) on the back of the female beetle.[8]

To discuss the pathogenicity of this fungus in regard to its ability to kill oak trees, information on fungal activity in the infected trunks is critical. Kuroda and Yamada[10] have reported that the wilting oak trees contain a dark-colored xylem that almost covers the cross section of the lower trunks where the beetle's galleries are mostly found, and sap ascent had completely stopped above those areas. Such a discolored area is called wound heartwood or wound wood[11,12] and is as dysfunctional as heartwood. This result suggested the key to resolving the wilting mechanism is to find the causal factor for wound heartwood formation in a wide area. The contribution of fungal hyphae to the discoloration was suggested in that report. Distribution of *Raffaelea* sp. and its effects on host tissue should be clarified. This study focuses on the cytological aspects of sapwood infected with the oak fungus and the correlation of the oak fungus distribution with xylem dysfunction.

Materials and methods

Specimens

Thirty-six oak trees (*Quercus crispula* and *Q. serrata*) were cut from deciduous forests in Fukui and Shiga Prefectures[10]

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Table 1. Condition of sample tree A, *Quercus crispula*

Site	Fukui Prefecture
Harvest date	July 22, 1992
Age and DBH	ca. 50 years, 14.5 cm
Beetle attack	From previous year; extensive attack in the current year
Beetle galleries	Mostly <1.6 m
Wilting symptom	None at harvest
Range of xylem discoloration	0–4 m (height)

DBH, Diameter of breast height

during wilting season and were classified into three types: wilting, not wilting after beetle (*P. quercivorus*) attack, and healthy with no beetle attack. Anatomical observations were made. Tree A (Table 1), *Q. crispula*, was selected for detailed investigation. This tree had been attacked by the beetle in swarms but had shown no wilting symptoms at the time of harvest in July. To check its ability to conduct water, a dye-injection test with 1% fuchsin acid solution was conducted on tree A (Table 1) and a healthy tree before cutting it down.[10,13] On the harvest day of tree A, two disks 3 cm in thickness were cut from the tree 50 cm above the ground. One was used for isolation of fungi, and the other was used for anatomical study (Fig. 1).

Isolation of microorganisms

A xylem block (2 × 2 × 2 cm) was cut from each of six sites on the disk from tree A: normal sapwood without discoloration (Fig. 1A), the boundary of the dark brown and slightly colored areas (Fig. 1C,E), and a slightly colored area (Fig. 1B,D,F). Cube-shaped xylem pieces of about 3 mm made from the blocks were washed in 70% ethanol, surface-sterilized in 0.5% sodium hypochlorite for 3 min, rinsed once with sterile distilled water, and transferred to PDA (potato dextrose agar). They were then incubated at 20°C and checked for *Raffaelea* sp.

Anatomical observation

Six sample blocks were taken from the disk symmetrically opposite to the site of the above-mentioned fungus isolation (Fig. 1A–E). Blocks were fixed in FAA (formalin/acetic acid/50% ethanol, 5:5:90 v/v) and washed under tap water. Xylem blocks were also prepared from other sample trees including wilting and healthy trees. Following the macroscopic observation of blocks under a binocular microscope, transverse, radial, and tangential sections 25–30 μm in thickness were cut with a sliding microtome. Some were stained with safranin-fast green for observation of xylem cells, and others were stained with toluidine blue O for observation of fungal hyphae. Some sections were left unstained and mounted on slides with gum syrup for observation of colored substances. Stained sections were dehydrated through an ethanol series and xylene. Sections were observed by light microscopy.

Results

Raffaelea sp. distribution, xylem discoloration, dysfunction

In the trees that were wilting after a *P. quercivorus* attack, the hyphae, which were well observed in their discoloring xylem, were assumed to be those of the *Raffaelea* sp. based on the characteristics of the mycelia.[8] To study the physiological effects of the *Raffaelea* sp. on oak tissue, data obtained for tree A (Table 1) were used, and the distribution of the *Raffaelea* sp. was confirmed by isolation.

Figure 1 shows the disk surface of tree A immediately after harvest. The color of the xylem at sites B–E became darker after harvest probably because of oxidization. No fungus was isolated from the sapwood without discoloration (Fig. 1A). Moreover, no fungal hyphae were found on tissue sections by light microscopy. The *Raffaelea* sp. was dominantly isolated from the dark-colored areas at sites C and E. Those areas, which were discolored brown to black (Fig. 2), were adjacent to older galleries formed the previous year by the initial attack of the beetles. Hyphae were found in vessels by microscopy. Fungi other than the *Raffaelea* sp. were rarely isolated from xylem pieces. Areas slightly colored a grayish tan hue (sites B, D, and F) included galleries formed during the current year, probably in July. A light-brown area extended about 5 mm above and below the new horizontal galleries. The *Raffaelea* sp. was dominantly isolated from site B, and hyphae were observed in the galleries and in some of the large and small vessels by microscopy (Fig. 3). No fungus was isolated on PDA from sites D and F, and hyphae were sparsely found by microscopy. Necrosis of cambium has not occurred in these areas from sites B–F (Fig. 2).

The amount of dye absorption at a slightly discolored area (Fig. 1E) was half that of a healthy tree in the same stand. Sap flow depended on small vessels (Fig. 2) when the large vessels were plugged with tylosis; therefore the ability of sap to ascend had significantly decreased.

Cytological aspects in and around discolored areas

In areas that had completely discolored to dark brown in the sapwood (Figs. 1C,E, 2), characteristically tan to brown substances had accumulated in the vessels, and lignified tyloses had plugged large vessels. The hyphae in the vessels were thick and brown. Ray parenchyma cells had lost nuclei, were necrotic, and contained yellow to tan substances. The longitudinal area of discoloration around horizontal galleries was more than several centimeters and it was wider than the radial or tangential area. Phloem and cambial cells adjacent to those areas were not necrotic.

Young hyphae were observed in vessels of the slightly discolored area, such as sites B and D (Fig. 3), and were invading the living ray parenchyma cells (Fig. 3) from the vessel lumen, penetrating the pits on the cell walls (Fig. 4). Tyloses were budding from ray cells into vessels around those young hyphae (Fig. 3). Some parenchyma cells

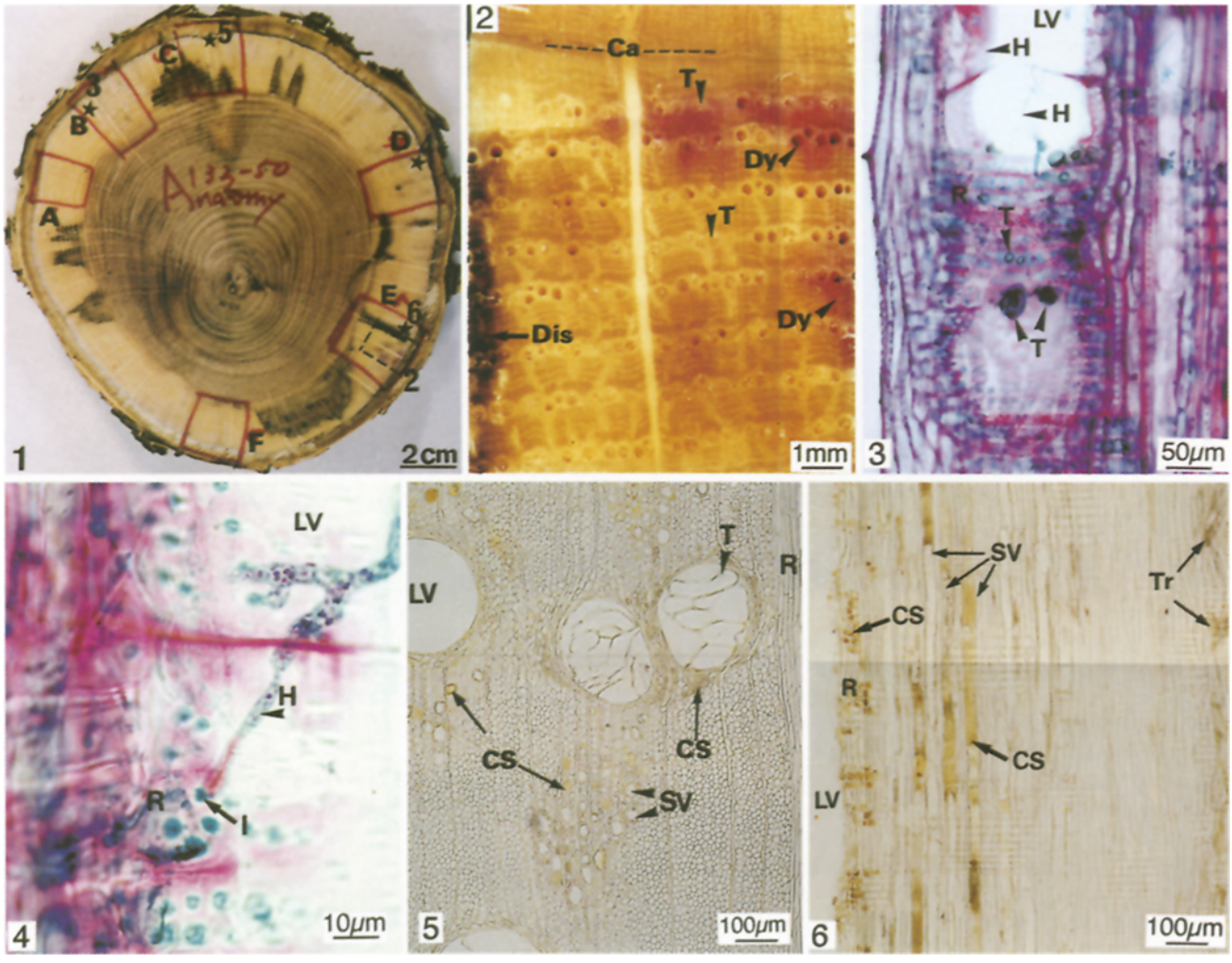

Fig. 1. Surface of a disk cut from tree A, *Quercus crispula*. Blocks to study anatomy were taken from sites without discoloration (*A*), with slight discoloration (*B*, *D*, *F*), and with borders of dark and slight discoloration (*C*, *E*). Fungus isolation was made from the exact sites adjacent to *A–F* of the other disk. Enclosed areas indicated by *broken lines* or *stars with numbers* indicate the sites of the photographs in Figs. 2 to 6

Fig. 2. Transverse view of block E in Fig. 1 magnified with a binocular microscope. Note the dark discoloration on the left side (*Dis*) and slight discoloration on the rest of the xylem. Dye absorption demonstrates that sap flow depends on small vessels when large vessels are nonconductive and plugged with tyloses. *Ca*, cambium; *T*, tylosis; *Dy*, dye absorption

Fig. 3. Hyphae distribution and tylosis budding in the slightly discolored area (site B in Fig. 1, ★3). Radial section stained with safranin-fast green. *LV*, large vessel; *H*, hyphae; *R*, ray parenchyma cells

Fig. 4. Young hyphae invading ray cells (site D, Fig. 1, ★4). Radial section stained with safranin-fast green. *I*, hyphae invasion

Fig. 5. Occlusion of small vessels with colored substances and large-vessel plugging with tylosis at site C (Fig. 1, ★5) where the sapwood is grayish tan. Third and fourth rings from the current annual ring. Cross section without staining. *CS*, colored substance; *SV*, small vessel

Fig. 6. Yellow to tan substance has accumulated in ray cells and is exuded into vessels near the boundary of the discolored area in site E (Fig. 1, ★6). Radial section without staining. *Tr*, tracheids

around the fungal invasion still looked alive, judging from the large, round nuclei and the cytoplasm stained blue with fast green. Hyphal elongation was active inside the sapwood and was not observed in the cambial zone.

Occlusion of small vessels or tracheids with yellow substances (Fig. 5) was found along ray tissues, where parenchyma cells had started having physiological changes, such as tyloses budding (Fig. 3) and production of pale-yellow droplets. Those physiological reactions were conspicuous in the slightly colored area around new galleries. Yellow substances are insoluble in water and partially soluble in xylene. Occlusion was abundant and darker-colored near the boundary of dark discoloration at sites E (Fig. 6) and C.

In healthy trees, tyloses were observed only in heartwood. In contrast, in tree A, large vessels were partially plugged with newly formed tyloses with unlignified walls in the current annual ring that had not apparently discolored (Fig. 2) or even at site A without hyphae distribution. Small vessels look like they are conducting in such areas (Fig. 2). Because large vessels are extending to some length,[14] some vessels that were dysfunctional in the infected area often penetrate an intact area. Dissection of trunk xylem in the longitudinal direction revealed this fact.

In trees with *P. quercivorus* galleries other than tree A, the above-mentioned phenomena in sapwood were observed with hyphae assumed to be from the "oak fungus."

Discussion

In the present investigation, abundant hyphae observed by microscopy were judged to be those of *Raffaelea* sp. in specimens from tree A in which *Raffaelea* sp. was dominantly or uniquely isolated on PDA. Ito et al.[8] emphasized the contribution of *Raffaelea* sp. to the wilting phenomenon because the inoculated fungus successfully killed trees.

Distribution of the *Raffaelea* sp. coincided with the area of discolored sapwood. Mechanical wounding causes xylem discoloration but in the limited area around the wounds.[11] Wide discoloration in the wilting oaks is not attributable to physical wounds by the beetle alone.[10] The dark-colored xylem around galleries of previous years is the complete wound heartwood.[11,12] All parenchyma cells were necrotic, and all vessels were dysfunctional in those areas. In the slightly discolored area surrounding a new gallery, ray parenchyma cells may be in the midst of physiological changes, judging from the production of pale-yellow droplets and tylosis budding. Invasion of hyphae into living cells indicated that this fungus is not saprophytic. This fungus, however, does not invade the cambial zone, so cambium necrosis did not occur before wilting.

The yellow substances found in ray parenchyma cells and vessels are secondary metabolites assumed to be oily and to contain phenolic substances and terpenoids.[11] Exudates from ray cells turned brown owing to oxidization and polymerization in vessel lumens, contributing to coloring the xylem. The close relation between *Raffaelea* sp. distribution and the progressive discoloration of the xylem was confirmed by microscopic observations. The increase in secondary metabolites is usually thought to be a protective reaction against fungal infection or insect feeding. However, the substances did not prevent the distribution of oak fungus in this case. Pearce et al.[15] reported that it takes trees 1–2 weeks to increase their protective substances. The density of beetle invasion is high in the lower trunks, and galleries extend long distances in the tangential, radial, and vertical directions for breeding.[10,16] The so-called barrier zone proposed by Shigo[17] does not work if present because the fungus uses these densely formed galleries as distribution routes and grows rapidly[8] before enough secondary metabolite accumulates. Instead, such a protective reaction occurring synchronously around many galleries should have induced discoloration and dysfunction throughout the sapwood within a few months after infection.[10] Where the discolored area was narrow for the sparse gallery formation, trees survived[10] or were killed after repeated infection the next year. The density of galleries seems important for oak fungus distribution in a trunk.

Tylosis is ballooning of the cell wall, with cytoplasm from ray parenchyma cells through the pits.[18] Tyloses are formed when water is absent from the lumen of vessels.[19] Vessel plugging with tylosis is not the cause but the result of a dysfunction.[14] Sperry and Tyree[20] demonstrated that substances with low surface tension (e.g., butanol) added to the xylem sap promotes cavitation (embolism) in water conduits. Kuroda[21] had proposed that pathologically induced volatile exudates from ray cells may be a cause of xylem dysfunction. With oak wilting a secondary metabolite exuded from parenchyma cells may be involved in the dysfunction. Some plugged vessels were observed even in noncolored areas. This means that dysfunctional vessels extend vertically beyond the boundary of hyphae distribution. Dysfunction of large vessels in the current annual ring should result in a significant decrease in sap flow in ring-porous species.

The present observations suggest that the cytological process in host tissue after *Raffaelea* sp. infection is as follows: (1) synthesis of secondary metabolites by the stimulation of hyphae; (2) exudation of yellow substances into vessels; and (3) dysfunction of vessels and wound heartwood formation. Complete blockage of sap ascent in the trunk[10] must be related to the rapid and wide spreading of this fungus through the beetle's galleries. Similar wilting phenomena associated with barkbeetles have been reported for conifers.[22,25] The same type of discoloration associated with fungi and the activities of ambrosia beetles has been reported in the case of *Nothofagus* and *Weinmannia*.[5,6,24] In those reports, however, the cytological aspects in host trees against infection were not noted. This report demonstrated that distribution of the pathogenic fungus *Raffaelea* sp. is closely related to xylem dysfunction and discoloration. The strategy of this fungus for distribution in the host should be noted, although the fungus did not kill cambium. Regarding the wilting incidence in trees, pathogenicity should be assessed by the organism's ability to stop sap flow; the ability to kill cambium does not seem essential. Inoculation experiments with this fungus will provide more detailed information about how the sapwood dysfunction occurs.

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References

1. Nunokawa K (1993) Distribution of oak trees attacked by *Platypus quercivorus* in Niigata Prefecture (in Japanese). For Pests 42:210–213
2. Ito S, Yamada T, Kuroda K (1992) Investigation of oak trees mortality in the Kansai district (in Japanese). Ann Phytopathol Soc Jpn 58:545
3. Wood SL, Bright Jr DE (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic Index. Great Basin Naturalist Memoirs 13:1–1553
4. Hijii N, Kajimura H, Urano T, Kinuura H, Itami H (1991) The mass mortality of oak trees induced by *Platypus quercivorus* (Murayama) and *Platypus calamus* Blandford (Coleoptera: Platypodidae): the density and spatial distribution of attack by the beetles. J Jpn For Soc 73:471–476
5. Kile GA, Eliott HJ, Candy SG, Hall MF (1992) Treatments influencing susceptibility of *Nothofagus cunninghamii* to the am-

brosia beetle *Platypus subgranosus* in Australia. Can J For Bot 22:769–775
6. Kile GA, Hall MF (1988) Assessment of *Platypus subgranosus* as a vector of *Chalara australis* causal agent of a vascular disease of *Nothofagus cunninghamii*. NZ J For Sci 18:166–186
7. Ito S, Kuroda K, Yamada T, Miura Y, Inoue S (1993) Investigation of fungi associated with the mass mortality of oak trees (in Japanese). Ann Phytopathol Soc Jpn 59:290–291
8. Ito S, Kubono T, Sahashi N, Yamada T (1998) Associated fungi with the mass mortality of oak trees (in Japanese with English summary). J Jpn For Soc 80:170–175
9. Ito S, Kubono T, Yamada T (2000) Mass mortality of oak trees in Japan. Proceedings of the 3rd regional workshop of IUFRO 7.03.08, Forest protection in Northeast Asia, July 31–August 4, 2000, Chiayi, Taiwan, pp. 182–185
10. Kuroda K, Yamada T (1996) Discoloration of sapwood and blockage of xylem sap ascent in the trunks of wilting *Quercus* spp. following attack by *Platypus quercivorus* (in Japanese with English summary) J Jpn For Soc 78:84–88
11. Hillis WE (1987) Heartwood and tree exudates. Springer-Verlag, Berlin, p 268
12. Shigo AL, Hillis WE (1973) Heartwood, discolored wood, and microorganisms in living trees. Annu Rev Phytopathol 11:197–222
13. Kozlowski TT, Winget CH (1963) Patterns of water movement in forest trees. Bot Gasette 124:301–311
14. Zimmermann MH (1983) Xylem structure and the ascent of sap. Springer-Verlag, Berlin, p 143
15. Pearce RB (1996) Tansley review no. 87: antimicrobial defenses in the wood of living trees. New Phytol 132:202–233
16. Sone K, Mori T, Ide M (1998) Life history of the oak borer, *Platypus quercivorus* (Murayama) (Coleoptera: Platypodidae). Appl Entomol Zool 33:67–75
17. Shigo AL (1984) Compartmentalization: a conceptual framework for understanding how trees grow and defend themselves. Annu Rev Phytopathol 22:189–214
18. Shibata N, Harada H, Saiki H (1980) Formation and wall structure of tyloses in *Castanea crenata*. Bull Kyoto Univ For 52:206–215
19. Sinclair W, Lyon HH, Johnson WT (1987) Diseases of trees and shrubs. Cornell University Press, Ithaca, NY, pp 364–365
20. Sperry JS, Tyree MT (1988) Mechanism of water stress-induced xylem embolism. Plant Physiol 88:581–587
21. Kuroda K (1991) Mechanism of cavitation development in the pine wilt disease. Eur J For Pathol 21:82–89
22. Christiansen E, Solheim H (1990) The barkbeetle-associated blue-stain fungus *Ophiostoma polonicum* can kill various spruces and Douglas fir. Eur J For Pathol 20:436–446
23. Elliot HJ, Kile GA, Candy SG, Patkowsky KA (1987) The incidence and spatial pattern of *Nothofagus cunninghamii* (Hook) Oerst. attacked by *Platypus subgranosus* Schedl in Tasmania's cool temperate rainforest. Aust J Ecol 12:125–138
24. Payton IJ (1989) Fungal (*Sporothrix*) induced mortality of kamahi (*Weinmannia racemos*a) after attack by pinhole borer (*Platypus* spp.) NZ J Bot 27:359–368
25. Solheim H (1992) The early stages of fungal invasion in Norway spruce infested by the bark beetle *Ips typographus*. Can J Bot 70:1–5