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Changes in hydraulic conductivity of *Quercus crispula* seedlings surviving the inoculation of *Raffaelea quercivora*

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Introduction

Mass mortality of oak trees (mainly *Quercus serrata* and *Q. crispula*) induced by the boring of *Platypus quercivorus* (MURAYAMA) (Coleoptera: Platypodidae) is epidemic in Japan especially along the Japan Sea (ITO and YAMADA, 1998). This disease was first recognized in 1934 in the Kyushu district, southern Japan. Following this, sudden but temporary outbreaks occurred repeatedly in geographically isolated parts of southern Kyushu and along the Japan Sea. Transition into a continuous occurrence has been observed since late 1980's, and it has spread further into areas where this disease has not been observed before.

Tree death by this disease has been explained being caused by the pathogenic fungus *Raffaelea quercivora* KUBONO *et* Shin-ITO (ITO *et al.*, 1998, SAITO *et al.*, 2001, KUBONO and ITO, 2002), which is introduced into the wood by *P. quercivorus*. Wilting usually occurs about two weeks to one month following the mass attack of *P. quercivorus* on *Q. serrata* and *Q. crispula* mature trees. Artificial inoculation of *R. quercivora* to mature *Q. crispula* trees also induced wilting about one month after the inoculation (ITO *et al.*, 1998, SAITO *et al.*, 2001). *Q. serrata* and *Q. crispula* seedlings wilted one week to one month after the inoculation (YAMATO *et al.*, 2001). Such rapid death is characteristic of this disease. However, *Quercus* trees often survive the mass attack of the beetles and fungal infection in nature and by artificial inoculation.

The causal mechanism of wilt and death of infected trees has been suggested as blockage of sap ascent in the area infected by the fungus, because the blockage occurs in discolored wood of infected trees (KURODA and YAMADA, 1996). To confirm this hypothesis, we have conducted further study on the process of wilting and death. A parameter of chlorophyll fluorescence (Fv / Fm) decreased rapidly at the very early stage of wilting in inoculated oak seedlings, similarly to seedlings which were mechanically cut at the stem base (YAMATO *et al.*, 2001). The appearance of the wilting process of trees which were cut at the stem base was same as fungus-inoculated trees in both seedlings (YAMATO *et al.*, 2001) and young trees (ICHIHARA and KUBONO, personal communication) and was also similar to the naturally infected trees.

Further, at the beginning of slight wilt symptoms after the *R. quercivora* inoculation, hydraulic conductivity was maintained at the upper stem part distal from the inoculation site and wilt symptom development was suppressed when the basal end of stem cuttings from the inoculated seedlings were placed in water (YAMATO *et al.*, 2001). These results evidently showed blockage of sap ascent around the fungus-infected site as the wilting mechanism. In this previous study,

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hydraulic conductivity was evaluated qualitatively by the infusion of a dye (acid fuchsin) into seedlings. In the present study, we have quantitatively evaluated hydraulic conductivity in the inoculated seedlings using a high pressure flowmeter (HPFM) (TYREE *et al.*, 1993, 1995). To clarify the wilting mechanisms, hydraulic conductivity of seedlings which survived from *R. quercivora*-inoculation is reported in this paper, in comparison to previous studies in which severely infected plants died.

Materials and methods

1. Inoculation

Three-year-old *Quercus crispula* seedlings planted in a nursery of the Experimental Station at Tanashi, the University Forests, the University of Tokyo, were inoculated with *R. quercivora* (isolate MAFF 410918, deposited in the MAFF Gene Bank, Ministry of Agriculture, Forestry and Fisheries (Tsukuba, Japan)) on July 3. *R. quercivora* was subcultured on potato dextrose agar (PDA) at 25 °C in the dark. Mycelial plugs were transplanted onto a rice-wheat bran medium (rice bran - wheat bran - water, 1:1:2) and incubated to make inoculum at 25 °C in the dark for 2 weeks. At 5 cm above ground level, bark was peeled from half of the stem circumference in a 5 mm strip, and then bark was similarly peeled 5 mm above on the opposite side. Inoculum was placed onto the exposed stem wound and Parafilm was tied around the stem. Sterile rice-wheat bran medium was inoculated as a wound control.

2. Chlorophyll fluorescence, dissection and fungal isolation

A parameter of chlorophyll fluorescence, Fv / Fm (maximum quantum yield of photosystem II, which in turn is highly correlated with the quantum yield of net photosynthesis) was measured in predawn darkness (3:00 a.m.) with a Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany) for 18 days after the fungal inoculation. Modified Tukey's HSD test (Spjotvoll-Stoline test) was used to determine the differences in Fv / Fm. (STATISTICA2000, StatSoft, OK, USA).

Each 4 of wound control seedlings, inoculated seedlings without changes in Fv / Fm, and inoculated seedlings with decreased Fv /Fm were dissected 2 months after inoculation and the length of bark necrosis and wood discoloration was recorded. For fungal isolation, wood blocks (ca. 3 mm cubic) from each seedling were surface-sterilized with sodium hypochlorite solution (available chlorine 1%) for 3 minutes and incubated on PDA at 25 °C in the dark for 2 weeks. Acid fuchsin solution (0.2 %) was infused from the cut end at stem base of half of seedlings harvested. Differences in length of bark necrosis, area of wood discoloration and the blockage of water conduction were tested by Tukey's HSD test of log-transformed data (STATISTICA2000).

3. Xylem hydraulic conductance and microscopic observation

Four non-treated intact seedlings, 3 wound control seedlings, 3 inoculated seedlings without changes in Fv /Fm and 2 inoculated seedlings with decreased Fv / Fm were dissected 4 months after inoculation, and hydraulic conductivity was determined with a high pressure flowmeter

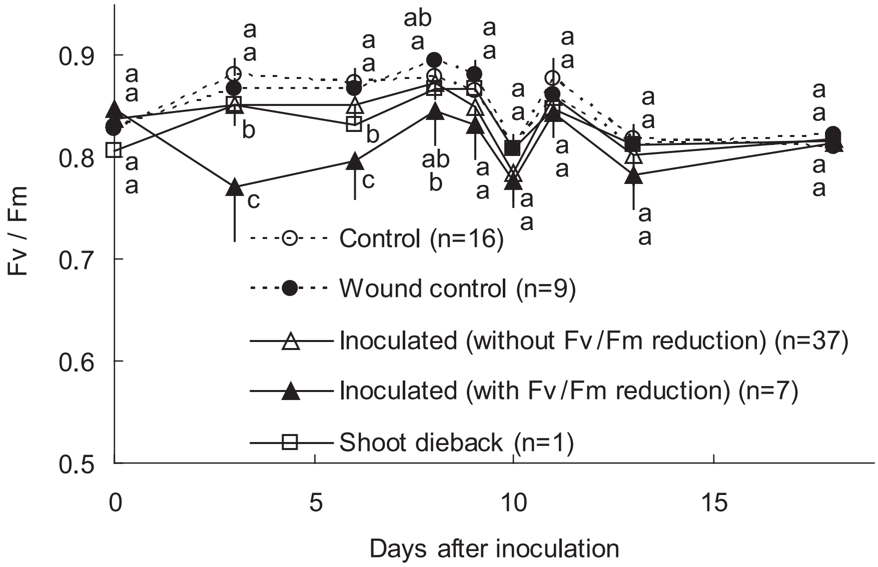

Fig. 1. Changes in Fv / Fm of *Raffaelea quercivora*-inoculated *Quercus crispula* seedlings. Different letters indicate significant differences by modified Tukey's HSD test (Spjotvoll-Stoline test) (p = 0.05).

(HPFM, Dynamax, Inc., Houston, TX, USA). Stem lengths (10 cm long) taken from 0-10 cm and 25-35 cm above the inoculation site were used for determination of hydraulic conductance. The stump (including roots) was also tested. Current annual ring was removed on the lower end connecting to HPFM from the stem length adjacent to inoculation site, and then hydraulic conductance was measured. For histological observation, samples were taken from disks at 0 cm and 25 cm above the inoculation site. Sections of 20-25 μm thick were made with a freezing microtome, and stained with safranin O - fast green FCF (JENSEN, 1962). Differences in hydraulic conductivity were tested by modified Tukey's HSD test (Spjotvoll-Stoline test) of log-transformed data (STATISTICA2000).

Results

1. Chlorophyll fluorescence

No seedlings wilted during the measurement period of chlorophyll fluorescence after *R. quercivora* inoculation. Fv / Fm did not decrease in non-treated intact seedlings (16 seedlings) and wound control (10 seedlings). Seven of the 44 seedlings which were inoculated with the fungus showed more than 5 % reduction of Fv / Fm within 6 days after inoculation. Changes in Fv / Fm were shown in Fig.1. Recovery of Fv / Fm to normal level were observed in these seedlings thereafter (Fig. 1). One seedling of them, however, wilted until the measurement of hydraulic conductance with HPFM was conducted.

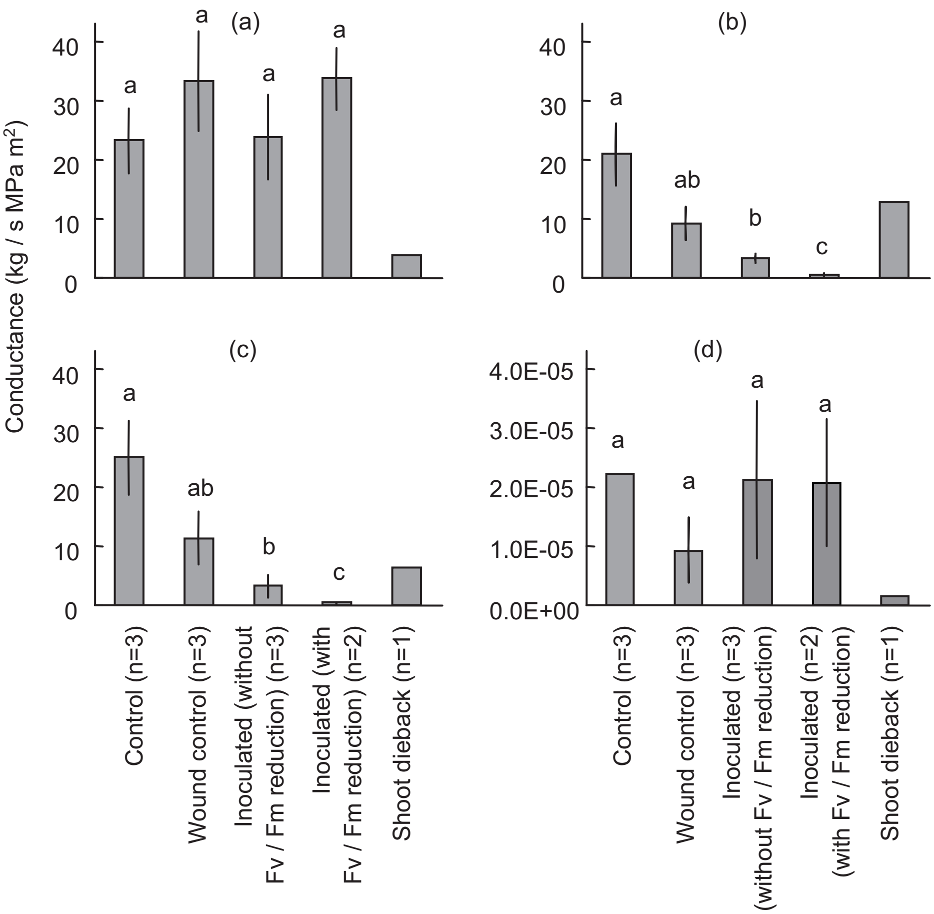

Fig. 2. Comparison of hydraulic conductivity in *Quercus crispula* seedlings 4 months after *Raffaelea quercivora*-inoculation. (a) Segment 25 cm distal from inoculation site. (b) Segment adjacent to the inoculation site. (c) Segment adjacent to the inoculation site without current annual ring. (d) Roots with stump. Vertical bars represent standard deviations. Different letters indicate significant differences by modified Tukey's HSD test (Spjotvoll-Stoline test) of log-transformed data (p = 0.05).

2. Xylem hydraulic conductivity and spread of lesion and inoculated fungus

Xylem hydraulic conductivity decreased conspicuously near the inoculation site of *R. quercivora*, especially in seedlings with reduced Fv / Fm (Fig. 2b). Macroscopic disorder, however, was not observed 25 cm distal from the inoculation site and roots, and hydraulic conductivity did not decrease (Fig. 2a). Reduction of hydraulic conductivity was observed similarly in wounded seedlings and fungally inoculated seedlings when the current annual ring was removed (Fig. 2c). For a control seedling showing dieback, hydraulic conductivity was greatly decreased in both the roots (Fig. 2d) and the upper stem (Fig. 2a). In this seedling, wood discoloration was observed in the center of the stem, but Fv / Fm was not different from other non-treated control and wound control seedlings.

The area of wood discoloration on the stem cross section was large in fungally inoculated seedlings, while it was small in wound control seedlings. Further, seedlings with temporarily decreased Fv / Fm showed no or little recovery by new wood formation, i.e. progress of wound closure, even 4 months after inoculation (Fig. 3). Wound closure was well extended in both wounded seedlings and inoculated ones without reduction of Fv / Fm.

No difference was observed in the extent of blockage of sap ascent in axial direction

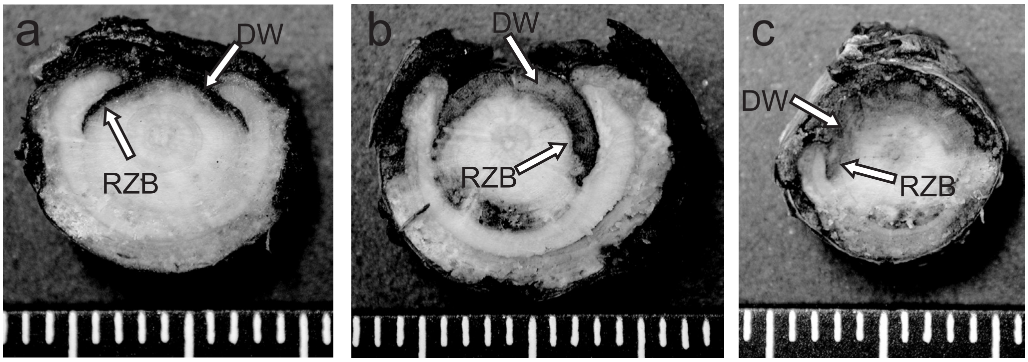

Fig. 3. Transverse sections of *Quercus crispula* seedlings adjacent to *Raffaelea quercivora*-inoculation site observed at 4 months of the inoculation. The ruler is graduated in mm. (a) Wound control. (b) Inoculated seedlings without effect on Fv / Fm. (c) Inoculated seedlings with decreased Fv / Fm. DW, discolored wood; RZB, reaction zone barrier.

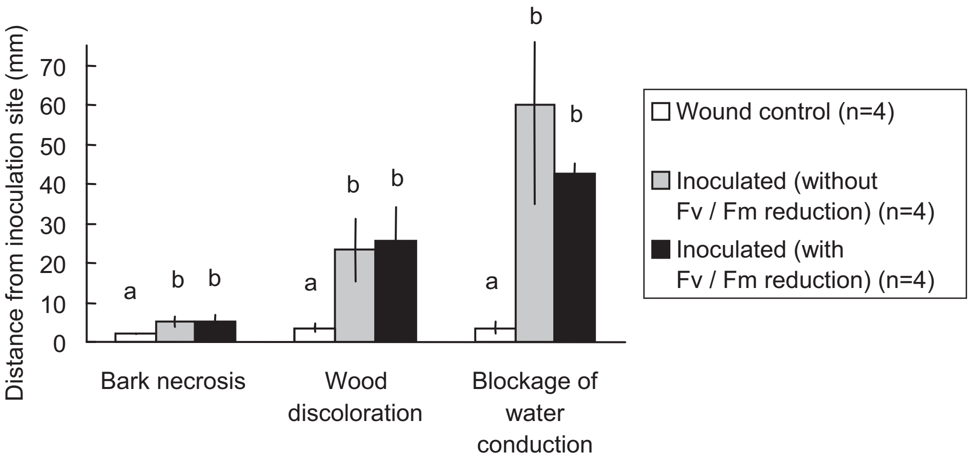

Fig. 4. Effect of inoculation on the development of bark lesion and wood discoloration in *Quercus crispula* seedlings 2 months after *Raffaelea quercivora*-inoculation. Vertical bars represent standard deviations. Different letters indicate significant differences by Tukey's HSD test of log-transformed data ($p = 0.05$).

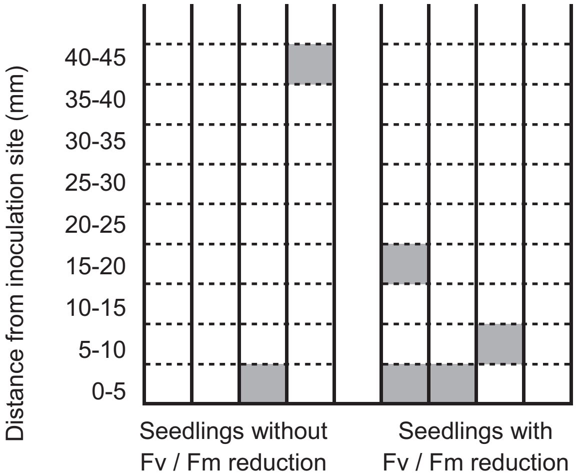

Fig. 5. Relationship between reduction of Fv / Fm and the pathogen distribution in *Quercus crispula* seedlings 2 months after *Raffaelea quercivora*-inoculation. Each vertical column represents each seedling. Gray zone indicates the xylem tissue where *R. quercivora* was re-isolated.

determined with acid fuchsin infusion in seedlings with decreased Fv / Fm compared with ones without Fv / Fm reduction. There was no relationship in inoculated seedlings between axial extent of bark lesion / wood discoloration and Fv / Fm reduction (Fig. 4).

No relationship was detected between reduction of Fv / Fm and whether the fungus was re-isolated or not, and also between reduction of Fv / Fm and the extent of re-isolation (Fig. 5). Wood discoloration extended even in the cases where the inoculated fungus was not re-isolated, which was the same as cases where re-isolation was successful.

3. Anatomical observation

Similar reaction zone (boundary of xylem lesion) features were observed histologically among seedlings of every treatment. Deposits occluding fibre tracheids at the reaction zone barrier were poorly stained with Sudan black B (for lipids), but sparsely stained by nitrosophenol reaction (for phenols and tannins). The parenchyma cell wall at the reaction zone barrier was sparsely stained with Sudan black B.

Effects of the wounding were not observed macroscopically 25 cm distal from the wounded site for the wound control (Fig. 6a). Superficial xylem parenchyma necrosis near the cambial zone and occlusion of xylem elements (tyloses of large vessels and plugging of small vessels and fibre tracheids) was observed adjacent to the wounds (Fig. 6b). Only a very small part of the cross section was necrotic and occluded 25 cm distal from *R. quercivora*-inoculation site, even in seedlings with Fv / Fm reduction (Fig. 6c). An example was slight tyloses at outer part of xylem.

At the vicinity of the inoculation site, only the surface of xylem was necrotic and extended wound closure was observed in seedlings without Fv / Fm reduction and in wound control (Fig.

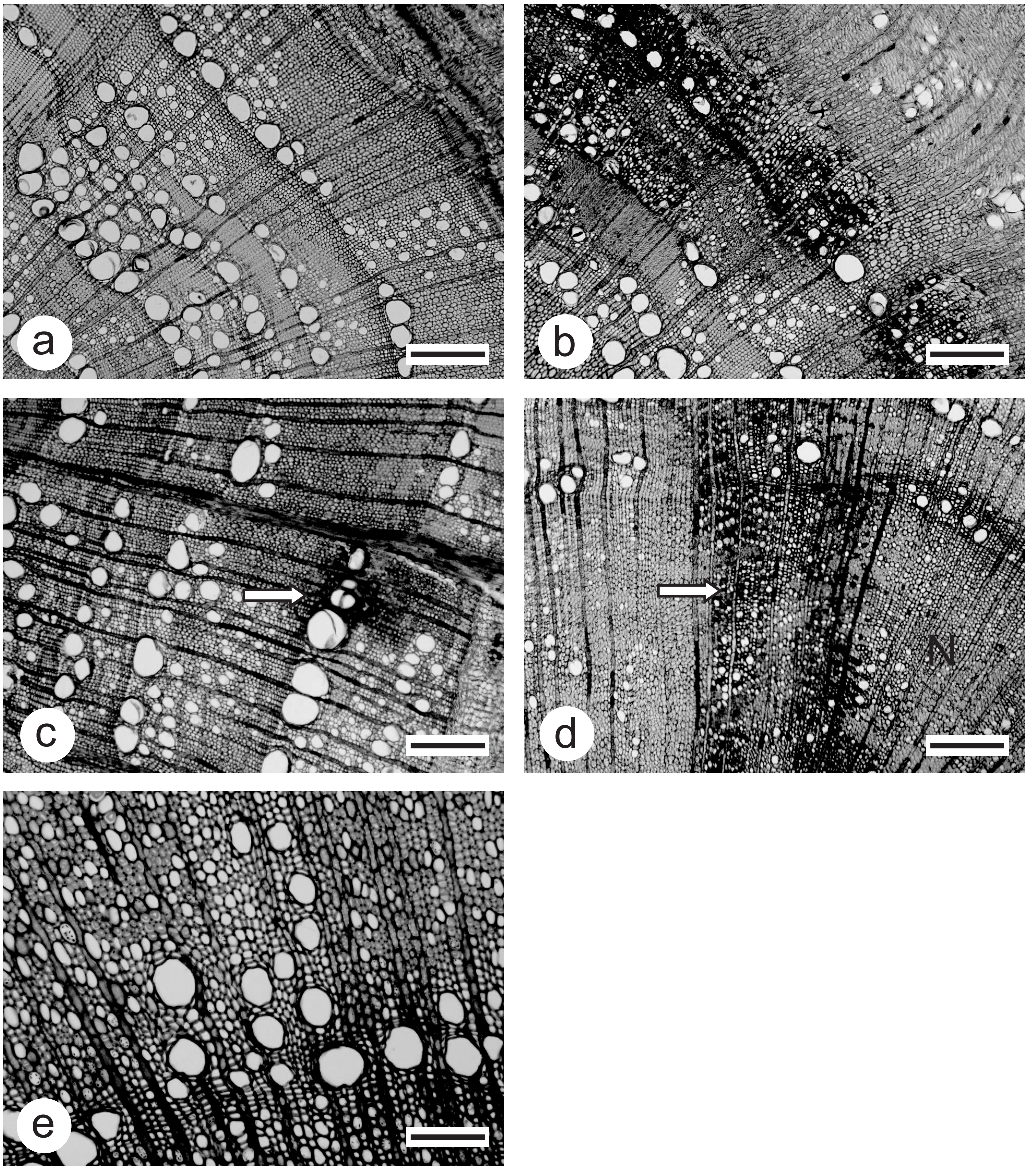

Fig. 6. Anatomical observation of *Quercus crispula* seedlings 4 months after *Raffaelea quercivora*-inoculation. (a) Sound sapwood without deposits, pluggings and tyloses distal from wound in wound control. (b) Superficial necrosis and xylem elements pluggings observed adjacent to wound in wound control. (c) Slight effect (arrow) of inoculation straight up on distal part of the seedling with Fv / Fm reduction. (d) Reaction zone barrier (arrow) and necrotic xylem tissue (N) in Fv / Fm-decreased seedlings. (e) Magnification of necrotic xylem tissue in Fv / Fm-decreased seedlings. Bars = 250 μm (a)-(d), 100 μm (e)

3a, b). Necrosis in the vicinity of pith was observed in seedlings with Fv / Fm reduction, and the extent of wound closure was slight or not observed at all (Fig. 3c). Occlusion of xylem elements was conspicuous in the reaction zone barrier (Fig. 6d), while it was hardly observed in the central part of discolored sapwood (necrotic xylem area) (Fig. 6e). No obvious difference was observed of anatomical features in reaction zone barrier among seedlings, i.e. wounded, inoculated, with and without Fv / Fm reduction.

Discussion

Fv / Fm is an index of efficiency of photo-utilization at photosystem II, which generally decreases in stressed plants (MORI, 1990, KRAUSE and WEIS, 1991, ROHÁČEK and BATÁK, 1999). Reduction of Fv / Fm in *Quercus suber* has been observed with the effect of pathogenesis and also water relation disruption (LUQUE *et al.*, 1999). Fv / Fm was also thought to be an index of the level of the disease development caused by *R. quercivora* in the previous study (YAMATO *et al.*, 2001) because severe water stress was induced by blockage of water conduction. Although extensive permanent blockage of water conduction was recognized and new xylem formation delayed around the inoculation site of seedlings where Fv / Fm decreased temporarily, hydraulic conductivity did not decrease in the upper part of stems. This result further supported a hypothesis by the previous report (YAMATO *et al.*, 2001), in which blockage of sap ascent occurred only around the fungally infected area. It was also suggested that only extreme reduction of stem hydraulic conductivity could affect the efficiency of photosystem. Further reduction of conductivity would be necessary to cause death of the seedlings. In other words, the possibility was shown that the seedlings survived and recovered from severe stresses which reduced photochemical efficiency.

In the previous study (YAMATO *et al.*, 2001), all *Q. crispula* seedlings wilted which were inoculated in the beginning of June, whereas no *Q. crispula* seedling wilted when inoculated in the mid of July. The season of inoculation and the size of inoculation wound might be the factors why almost all seedlings survived in the present study. Reduction of Fv / Fm was observed immediately before wilting symptom became visually noticeable. It indicated that inoculated seedlings with Fv / Fm reduction in the present study were around the threshold for wilting.

Blockage of water conduction had been investigated qualitatively by infusing acid fuchsin solution in the previous study (YAMATO *et al.*, 2001). Reduction of hydraulic conductivity was evaluated quantitatively for the first time in the present study. The present study also revealed several characteristics of water conduction in *R. quercivora*-infected seedlings, i.e. reduction of water conductivity observed even in conducting area near the infection site, normal conductivity in the distant part, and comparison with shoot dieback. These facts could become clear only by quantitative measurement. The possibility remained that standard measurement technique with HPFM did not detect recoverable cavitation if it existed, therefore blockage was underestimated. Even if the level of water blockage was underestimated in the present study, the relative level of blockage among treatments could be determined.

Reduction of hydraulic conductivity was expected to be severe in annual rings older than one year old, because of lateral increment growth, i.e. functional sapwood formation, after the inoculation. However, eliminating current annual ring revealed no obvious difference between two measurements, with and without current annual ring, indicating little recovery of water conduction per conducting area. Of course, water conducting area, therefore total capacity for conduction, increased by new wood formation in seedling which escaped severe damage. Increasing conducting area could ensure subsequent survival and growth.

The possibility that the pathogenic fungus or toxic substances produced by the fungus reached shoot and leaves and then cause systemic wilting could not be excluded completely in the previous and present studies. Wilting, however, can be well explained simply by the blockage at the infection site and surrounding discolored wood. External symptom development, i.e. browning and wilting of leaves, was the same between young (18-year-old) *Q. crispula* trees inoculated and severed at the stem base (ICHIHARA and KUBONO, personal communication). The process of wilting was delayed or suppressed in seedlings for which cut-ends were dipped into water when slight wilting occurred after the inoculation with *R. quercivora* (YAMATO *et al.*, 2001). These observations suggest that water deficit in distal part, but not stem base or roots, from infection site was the direct cause of death in *R. quercivora*-infected trees. Root hydraulic conductivity remarkably decreased in a control seedling with shoot dieback, suggesting that dysfunction in the water absorption into roots (for example, root-rot). Thus, this seedling wilted by a different mechanism from that in seedlings inoculated with *R. quercivora*.

Systemic runaway embolism is thought to be requisite for wilting in pine wilt disease (IKEDA and KIYOHARA, 1995, IKEDA, 1996a, b). In several cases of inoculation test on pine wilt, reduction of hydraulic conductivity and histological changes preceded in roots apart from inoculation site (HASHIMOTO, 1981, 1983, SAKUTA *et al.*, 1994a, b). However, it is concluded that *R. quercivora* induces localized blockage of sap ascent at the infection site, thus killing the whole tree.

No relationship was observed between reduction of Fv / Fm and axial distribution of fungi, blockage of water conduction and wood discoloration, but observed between reduction of Fv / Fm and extent of wood discoloration in transverse section. Further, few histological changes were observed at distal portions from the inoculation site. These results support the hypothesis that localized blockage of water conduction at *R. quercivora*-infection site causes wilting of oak trees. *R. quercivora* was not re-isolated from several seedlings, in which wood discoloration spread as far as that in fungus re-isolated ones. This suggested that the pathogen had spread, but then died or the density was reduced, for example, by infestation of other fungi.

The mechanism of blocking water conduction in *R. quercivora*-infected tree has not been clarified yet. Heavy occlusion of xylem elements by tyloses and deposits at reaction zone barrier in surviving tree might suggest that such occlusion caused blockage of water conduction. No remarkable occlusion, however, was observed in xylem necrotic area surrounded by reaction zone barriers. Further, reaction zone barrier formation could occur later than the onset of blockage of water conduction. Thus, the mechanism of blockage in surviving tree, as well as wilting tree, is still unclear. The extent of physical blockage with such substances should be determined in wilting trees, especially rapidly wilting trees, to clarify the wilting mechanism. Loss of conductivity seemed to be severe in visually intact sapwood area, suggesting the damage in living xylem tissue without visible anatomical feature. Such discrepancy was also recognized in Scots pine (GUÉRARD *et al.*, 2000). Comparing the sequential changes in water relations would also be required among different portions of the trees, especially hydraulic conductivity, water potential and transpiration, during the development of wilt, combined with detailed anatomical observation.

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Summary

Mass mortality of oaks, mainly *Quercus serrata* and *Q. crispula*, has been found along the Japan Sea since 1980’s. Mortality is associated with wood discoloration and xylem blockage, induced by the fungus *Raffaelea quercivora*, which is vectored by ambrosia beetle *Platypus quercivorus*. To reveal the wilting mechanisms of the disease, lower stems of *Q. crispula* seedlings were inoculated with *R. quercivora* in summer, and stem hydraulic conductivity of these seedlings which survived the inoculation was investigated with the high pressure flowmeter (HPFM). Hydraulic conductivity around the fungus-inoculation site greatly decreased, especially of seedlings of which Fv / Fm, maximum quantum yield of photosystem II, decreased temporarily. Hydraulic conductivity of distal part from inoculation site or roots, however, did not change. Dye treatment also revealed that blockage of sap-flow occurred only around the inoculation points. These results suggested that the distal part of seedlings was not directly affected by the fungal inoculation, and that wilt was induced by the blockage of sap ascent around the inoculation point. A control seedling with dieback had a much reduced hydraulic conductivity near the dead stem part and in the roots, while the conductivity was maintained in the lower stem. It was suggested that the dieback resulted from lower hydraulic conductivity in the roots, and the wilting mechanism differed from *R. quercivora*-inoculated seedlings.

Key words: Japanese oak wilt, *Quercus crispula*, *Raffaelea quercivora*, hydraulic conductivity, chlorophyll fluorescence

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Raffaelea quercivora を接種して生残した *Quercus crispula* 苗の水分通導性の変化

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要　　旨

コナラ，ミズナラを主な被害樹種とするブナ科樹木萎凋病は 1980 年代から日本海側で流行している。枯死は材変色や木部通導阻害と関連し，養菌性キクイムシ *Platypus quercivorus* が伝搬する病原菌 *Raffaelea quercivora* によって起こる。萎凋機構を明らかにするために，ミズナラ苗の主軸下部に *R. quercivora* を接種し，生残した苗木の主軸の水分通導性を HPFM を用いて測定した。水分通導性は菌接種部の近くで大幅に低下した。これは特に光合成系 II の最大量子収率 Fv / Fm が一時的に低下した苗木で顕著であった。しかし菌接種部から離れた部位や根の水分通導性は変わらなかった。酸性フクシン処理で観察した通水阻害は菌接種部周囲のみで発生した。これらの結果は，菌接種は離れた部位には直接影響を及ぼさないこと，萎凋は接種部付近での通水阻害によって生じることを示唆している。先枯れ症状を呈した対照苗では，水分通導性は壊死部位の近くと根で低下したが，主軸下部では維持されていた。先枯れ症状は根の水分通導性の低下により起こり，*R. quercivora* 感染による萎凋機構はこれとは異なることが示唆された。

キーワード： ブナ科樹木萎凋病・ミズナラ・*Raffaelea quercivora*・水分通導性・クロロフィル蛍光