Lessons from fungal inoculation experiments.
How oak trees wilt and die by the infection of Japanese oak wilt pathogen?

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1. Abstract

Japanese oak wilt (JOW) has been spreading throughout Japan since late 1980's, and is reported from 31 of 47 prefectures. We now know that a fungus *Raffaelea quercivora*, vectored by an ambrosia beetle *Platypus quercivorus*, has pathogenicity to varied species of Fagaceae trees based on artificial fungal inoculation tests. However, the wilting and later death of trees need a mass attack of the beetle and such a biological interaction makes difficult to manage with this disease in forests. In this talk, we demonstrate some evidences that we unveiled on the JOW based on a series of inoculation tests and shed light on our unsolved questions on this disease.

2. Introduction

JOW is a one of the most serious forest disease in Japan (Forestry agency 2012). If we look back history, ancient documents stated that JOW-like diseases occurred as old as in 1750 (Ida and Takahashi 2010). Thus, JOW seems to recur occasionally since several hundred years before in local areas. First unambiguous incidence of JOW appeared in an official report goes back to 1934 (Kumamoto Regional Forest Office 1941), and then many evidences were accumulated but the incidence of JOWs was temporal and localized before early 1980's (Ito and Yamada 1998). Moreover, epidemics of JOWs were occurred in areas along the Japan Sea and in several limited areas in the Pacific Ocean (Ito 2000). Incidences were mostly restricted in old naturally regenerated stands where used as woods and charcoal materials for local people and ceased within a few years in spite of a high mortality rate (Kobayashi and Ueda 2005).

However, a new outbreak of JOWs since late 1980's had a bit different from the previous ones because it has never come to end. Moreover, the disease had still been spreading over areas in Japan where no oak wilt has ever occurred before (Forestry agency 2012). Although the distribution of damaged areas was restricted to coastal areas along the Japan Sea during the 20th century, the disease is now reported not only coastal areas but also inland areas, in one case was from an island distanced from ca 200km from the main island (Forestry agency 2012). Since JOW is now distributed over 31 of 47 prefectures in Japan in 2012, we are suffering from this epidemic disease. Although the JOW is steadily spread over Japan, we are still not able to manage this disease appropriately. This might partly be caused by our lack of knowledge on the wilting and/or death mechanism of JOW. In this talk, we introduce our study progresses on JOW, especially focusing on biological interactions between host trees and associating fungi based on fungal inoculations.

3.1. Establishment of effective inoculation methods of *Raffaelea quercivora* isolates

A pathogen of JOW was firstly described as anamorphic ophiostomatoid ascomycete; *Raffaelea quercivora* (Kubono and Ito 2002) and later refined taxonomically its morphological and molecular characteristics (Matsuda et al. 2010, Seo et al. 2012). The fungus was supposed to be the pahogen because it was always isolated from not only damaged oak trees but also a dominantly emerging insect, *P. quercivorus*, from the trees in the field (Ito et al. 1998, Kinuura 2002, Endoh et al. 2011). In addition, the higher rate of wilting and/or death symptoms were appeared by the inoculation of fungal isolates into healthy trees compared with that of controls (Ito et al. 1998, Yamato et al. 2001). In the first step to understand the pathogenicity of *R. quercivora* against its colonizing host trees, it was needed to establish certain reproducible methods being less variable among studies as well as human errors. The aim of this inoculation experiment was to develop an inoculation protocol of *R. quercivora* isolates. For this purpose, different inoculation approaches and inoculum densities were applied to branches

and stems of one of susceptible tree species of *Quercus serrata*.

The study was conducted at the experimental forest of Mie Prefectural Forest Institute in Japan. To consider the effectiveness of fungal inoculum forms, both spore suspended solutions and mycelia grown on wood sticks were inoculated into *Q. serrata* branches. Two inoculation points were made at 2 cm in diameter of branches and both the forms were pairwisely applied at opposite directions. Fifteen and 3 individuals were used for *R. quercivora* inoculations and controls, respectively. For the fungal inoculated treatment, 3 branches were retrieved at 3, 5, 7, 14 and 21 days and the branches of the controls were harvested at only 21 days. Then the maximal vertical discoloration length from inoculation points was measured and fungal isolations were conducted with surface sterilized several mm^3 wood pieces at the border between discolored and non-discolored areas. The pieces were incubated at 20°C and the occurrence of *R. quercivora* colonized were analyzed following formula; (number of *R. quercivora* colonies/ number of wood pieces used)*100 (%).

Inoculum densities and its forms were examined inoculating into stems of *Q. serrata* trees. The inoculum forms were the same as the above (i.e. spores vs mycelia). Inoculated trees were ca 15 years old (11.8 cm in DBH, 8.1 m in height). Twelve, 3, 3 and 3 individual trees were used as fungal inoculation, control for mycelia, control for spore and no treatments, respectively. Configurations of inoculation holes were shown in Fig 1. Eight mm holes with a 2.5 cm depth were made either 3, 4 and 5 lines with 3 cm distances between the adjacent lines each other. The mycelial forms pregrown with rice-wheat bran media were inoculated into all the holes, but the spore forms were only applied for 5 line densities. As the wound control treatment, substrata without fungi or sterilized water were inoculated into the 5 line density. After the treatment, inoculated areas of all the trees were packed around with Parafilm and packing tape. Symptom developments (wilting/death) were also observed. The symptoms were divided into two categories; one was wilting, partial fading of the foliage color, or partial drying and curling of the foliage, and the other was death, discoloration of entire foliage from green to red brown. When inoculated trees were dead before the end of examination, they were cut down at the inoculation point and cross cuttings every 5 cm were obtained. Then wood disc was examined for the area of discoloration and of non-discoloration, and was applied for fungal isolation followed by the same protocol as for branches.

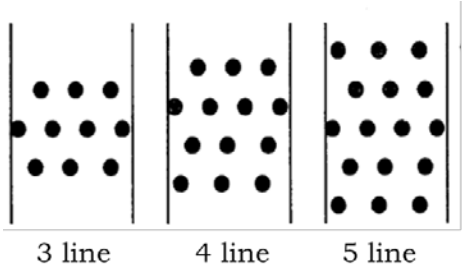

Fig 1. Configurations of inoculation holes made onto stems
● indicates inoculation holes that were distanced 3cm each other.

For the branch inoculation, discolored areas were confirmed at 5 days after the inoculation and no discolorations were detected from the controls (Fig 2). No significant differences in the discoloration length were detected between the inoculum forms (t-test, $p>0.05$). Colonies of *R. quercivora* was re-isolated from all the collection dates of both the inoculum forms but were not from the controls. These results indicated that inoculum forms of *R. quercivora* did not affected its colonization onto host trees. For the stem inoculations, 2 trees were dead in the 5 line density with mycelial forms, and one tree was wilted in 3 and 4 line densities with mycelial forms (Table1). The rest of trees in any treatments appeared to be healthy. One of 2 dead trees was used for fungal isolations. Although around the inoculation points were detected non-*Raffaelea* spp., *R. quercivora* was re-isolated within a

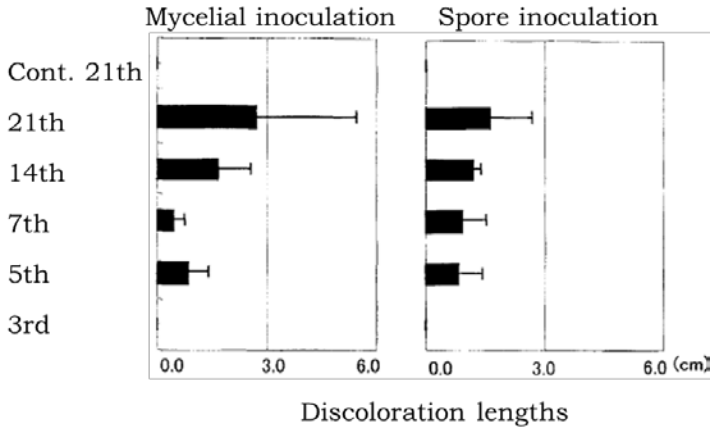

Fig 2. Temporal changes in discoloration lengths found in *Quercus serrata* branches

range of up to 95 cm. These results suggested that mycelial forms are effective to examine the pathogenicity of *R. quercivora* against its host trees and that relatively higher inoculum densities are needed to unequivocally recur the symptoms of JOWs.

Table 1. Numbers of wilt and dead trees after inoculation of *Raffaelea quercivora*

Inoculum forms, density	No of dead trees	No of wilt trees
Mycelial, 3 lines	0/3	1/3
Mycelial, 4 lines	0/3	1/3
Mycelial, 5 lines	2/3	0/3
Spore, 5 lines	0/3	0/3
No fungus, 5 lines	0/3	0/3
Water	0/3	0/3
No treatment	0/3	0/3

3.2. Variations of susceptibility of host trees against *R. quercivora*

In damaged areas, *R. quercivora* was detected as the one of dominant fungi derived from wilting and dead trees (Ito et al. 1998). Moreover, when *R. quercivora* isolates were inoculated artificially into mature *Q. crispula* trees, some of them were wilt and dead (Ito et al. 1998, Saito et al. 2001). These evidences thus indicated an intimate association of *R. quercivora* with JOW (Ito et al. 1998). However, since most of damaged trees were confined exclusively within one tree species, *Q. crispula*, at the initial era of mass mortalities of oaks, we did not known the difference of the susceptibility among host trees in spite of the rather larger number of potential hosts (ca 56 species) of *P. quercivorus* being recorded (Kobayashi and Ueda 2005). This let us move forward to understand how different to wilt and/or death by the colonization of *R. quercivora* among tree species. The aim of the present inoculation studies was to assess the susceptibility of species in the family Fagaceae to the fungus. For this purpose, we inoculated *R. quercivora* into seedlings and measured xylem pressure potential (XPP), discoloration or non-conducting sapwood areas.

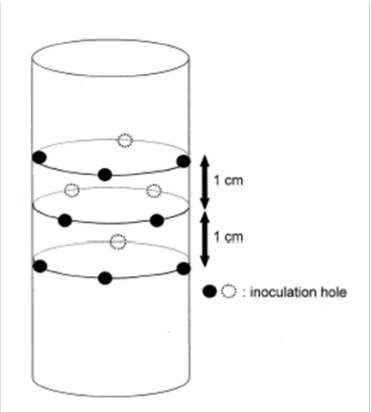

Fig 3. Locations of inoculation holes in seedling stems.

Five-year-old seedlings of 3 deciduous species (*Q. crispula*, *Q. serrata*, *Q. acutissima*) and evergreen species (*Q. phillyraeoides*, *Q. glauca*, *Castanopsis cuspidata* var. *sieboldii*) in the Fagaceae were applied for fungal inoculations. The isolate of *R. quercivora* was obtained from a dead *Q. crispula* tree in 2000, in Maizuru city, Kyoto Prefecture. Twelve holes (1mm in diameter) were bored around the stem of each seedling at three different heights (Fig 3). The wood sticks with *R. quercivora* were inserted into each hole as inocula. At each height, four holes were drilled at regular intervals. Inoculation sites and the depths of holes were determined based on the diameter of root collars. The symptom development of wilting and/or death was monitored daily for 4 months. The XPP of each seedling was measured weekly for 2 months and measured on one branch using a pressure chamber. The measurements of XPP were conducted during 10 to 12 pm.

Table 2. Number of days before wilting or seedling mortality was observed after inoculation with *Raffaelea quercivora*

	Q. crispula					*Q. serrata*	
	No. 1	No. 2	No. 3	No. 4	No. 7	No. 5	No. 7
Wilting	14	6	12	14	6	53	32
Death	17	11	15	17	11	56	–[a]

Neither wilting nor seedling mortality was found for two and five seedlings of *Q. crispula* and *Q. serrata*, respectively, and none of the other four species showed these symptoms

[a]Seedling mortality was not confirmed

No symptom development was observed in control and no treatment trees, and changes in XPP in the control were not significantly different from the "no treatment" seedlings (Kruskal -Wallis test, $p>0.05$). These

results indicate that the bored holes did not cause the symptom development. Five *Q. crispula* and one *Q. serrata* died, whereas no evergreen seedlings died within 2 months after inoculation. In the inoculation treatment, the first mortality was observed in *Q. crispula* seedlings on day 11 (Nos. 2 and 7), and on day 56 in a *Q. serrata* seedling (No. 5; Table 2). All dead *Q. crispula* seedlings died within 5 days after wilting began (Table 2). Although one inoculated *Q. serrata* seedling (No. 7) began to wilt on day 32, it was not clearly dead by the end of the experiment. No control seedlings showed any wilting symptoms. The number of dead seedlings differed significantly among the six species(x^2_{5df}=23.33, p=0.0003). In *Q. crispula*, the XPP of two inoculated seedlings had decreased by the 6th day after inoculation; a similar decrease in XPP occurred by the 13th day for three other seedlings (Fig 4). Two inoculated *Q. serrata* seedlings also showed lower XPP than the control seedlings by the 32nd and 52nd days after inoculation. In both species, some seedlings showed a sharp decrease in XPP before they died. In contrast, the XPP of two seedlings of *C. cuspidata* var. *sieboldii* showed a slight decrease by the 7th day after inoculation, but these values had recovered to normal levels within 1 week after the initial decrease. At the end of the experiment, no significant between-treatment differences in XPP were found in the surviving seedlings of each species, with the exception of the inoculated seedlings of *Q. crispula* (Kruskal-Wallis test, p>0.05; Fig 4). *R. quercivora* was re-isolated from the discolored sapwood of all inoculated seedlings, but was not re-isolated from any control seedlings. These results indicate that *R. quercivora* is pathogenic to *Q. crispula* and *Q. serrata* and that the susceptibility differed among the six Fagaceae species.

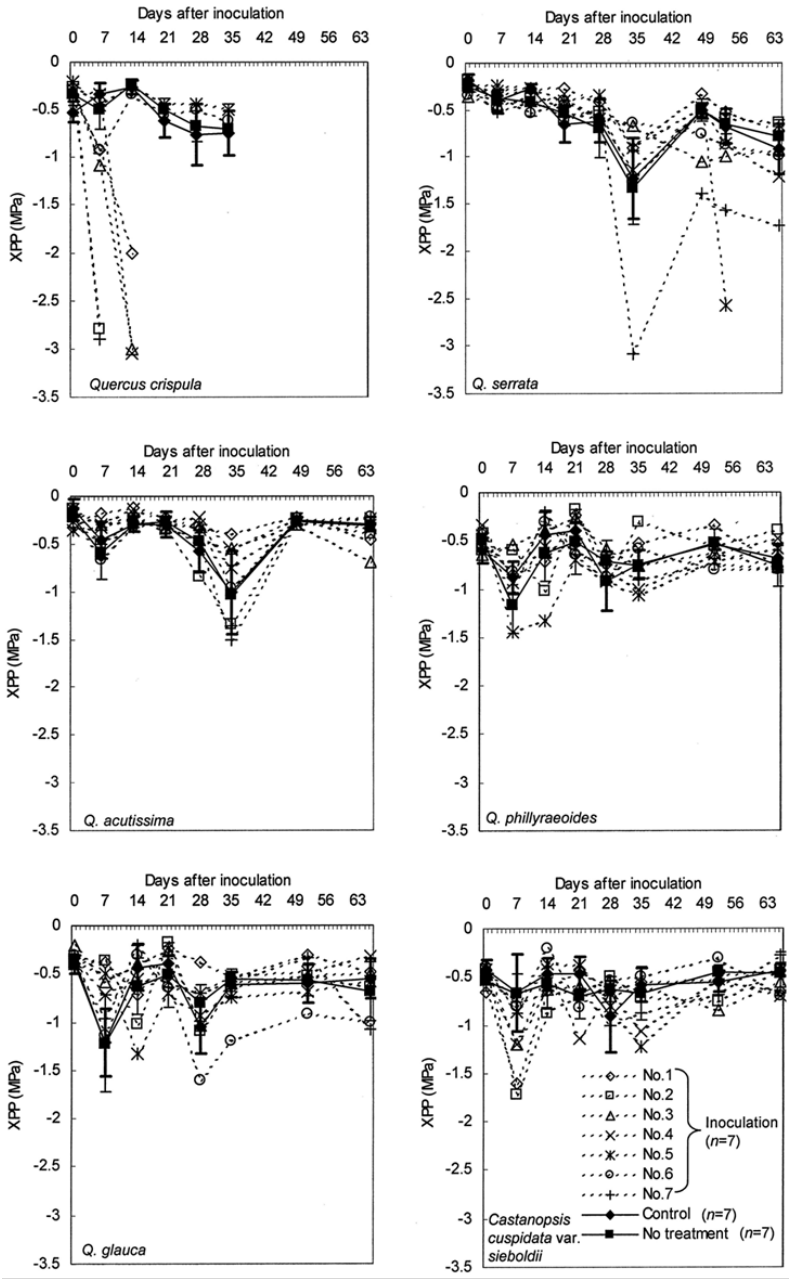

Fig 4. Changes in xylem pressure potential (XPP) of five *Quercus* species and one *Castanopsis* species after inoculation with *Rafelea quercivora*.

3.3. Discolored and non-conductive sapwood formations by *R. quercivora* inoculations against 6 Fagaceae species

We demonstrated the susceptibility of *R. quercivora* varied among oak species experimentally. However, fundamental wilting and death mechanisms were totally unknown. Enlarged

discoloration areas which hampered water conductance in xylem were always formed within stems of dead oaks, and thus these symptoms supposed to be related with wilting and further death processes (Kuroda and Yamada 1996). A next inoculation experiment was conducted to understand the effect of inoculation with *R. quercivora* on xylem discoloration and water conductivity.

Here, we used 6 different species of *Q. crispula*, *Q. serrata*, *Q. glauca*, *C. cuspidata* var. *sieboldii*, *Fagus crenata*, and *Pasania edulis*. Fourteen 5-year-old seedlings of each species were grown at the nursery of Mie University. An isolate of *R. quercivora* (RA1052) obtained in 2003 from the discolored sapwood of a dead *Q. glauca* in Wakayama Prefecture was used as the fungal inoculum. Two holes, each 1.2 mm in diameter and 4 mm deep, were bored on opposite sides of the stem of each seedling, at a point of 1 cm in diameter. A wood stick was inserted into each hole. Ten of the 14 seedlings of each species received *R. quercivora* colonized sticks, while four received sterilized wooden sticks as a control. The two-hole inoculation was applied in this case, rather than the mass inoculations since the principal aim of the experiment was to demonstrate the tissue response of each tree species to the fungus.

Table 3. Mean vertical discoloration lengths on six Fagaceae species inoculated with *Raffaelea quercivora*

	Inoculation treatments[2]		Control treatments		
Tree species	n	Mean discoloration length (cm)	n	Mean discoloration length (cm)	p-value[3]
Fagus crenata[1]	8	2.1 ± 0.9 ab	4	0.8 ± 0.1	0.017
Quercus crispula	10	5.2 ± 2.1 de	4	0.5 ± 0.2	0.001
Q. serrata	10	5.0 ± 2.6 cde	4	0.6 ± 0.2	<0.001
Q. glauca	10	5.0 ± 2.6 be	4	0.6 ± 0.2	0.002
Castanopsis cuspidata var. *sieboldii*	10	2.5 ± 1.5 abc	4	0.4 ± 0.5	0.022
Pasania edulis	10	1.7 ± 1.2 a	4	0.5 ± 0.1	0.065

n = number of seedlings.
[1]Two of 10 inoculated *F. crenata* seedlings were not measured because they died before the 56th day as a result of root rot.
[2]Inoculation treatments were compared in discoloration lengths among six species with the Kruskal–Wallis test ($\chi^2 = 28.49$, $p < 0.001$).
[3]Differences of vertical discoloration lengths between the inoculation and control treatments within tree species were analysed using Student's t-test.
Mean values (±SD) followed by different letters are significantly different (Mann–Whitney *U*-test adjusted for Bonferroni's inequality; $p < 0.05$).

All seedlings were observed for external symptoms. On the 56th day after inoculation, the seedlings were checked their water conductance by soaking in a 0.1% (w/v) acid fuchsin solution. After this, each seedling was cut across at the inoculation points and upward at 2-cm intervals until any brown or blackish discoloration disappeared. After that, a half of each 2-cm section was used for the measurement of both the sapwood area showing discoloration typical of that caused by *R. quercivora*, and the non-water-conducting area as determined by the absence of fuchsin dye. It was also used for the re-isolation test. All cross-sections were photographed and printed on paper. The non-conducting areas were measured with a hand-held digital planimeter. The non-conductive and discolored areas were normalized to the cross-sectional area as follows:

Table 4. Mean transverse discoloured areas on six Fagaceae species inoculated with *Raffaelea quercivora*

	Inoculation treatments[2]		Control treatments		
Tree species	n	Mean discoloured area (%)	n	Mean discoloured area (%)	p-value[1]
Fagus crenata[1]	8	13.7 ± 6.0 a	4	11.1 ± 5.9	0.479
Quercus crispula	10	40.2 ± 8.6 d	4	13.1 ± 2.5	<0.001
Q. serrata	10	35.1 ± 8.5 d	4	7.8 ± 2.5	<0.001
Q. glauca	10	23.2 ± 4.6 bc	4	9.0 ± 2.9	<0.001
Castanopsis cuspidata var. *sieboldii*	10	24.6 ± 8.4 c	4	9.0 ± 6.3	0.005
Pasania edulis	10	15.3 ± 4.6 ab	4	8.8 ± 2.5	0.015

n = number of seedlings.
[1]Please see Table 1.
[2]Inoculation treatments were compared among the six species with one-way analysis of variance (ANOVA) ($F = 20.87$, $p < 0.001$).
Mean values (±SD) followed by different letters are significantly different (Bonferroni's test, $p < 0.05$).

Non-conductive sapwood area (%)
= (Non-conductive sapwood area - bored area)/(Cross-section area - bored area) x 100

Discolored area (%) = (Discolored area - bored area) / (Cross-section area - bored area) x 100

In the five species except *P. edulis*, vertical discoloration lengths in the inoculation treatment were significantly larger than those in the control treatment (Table 3). In each species, the transverse discolored area in the inoculation treatment tended to be larger than that in the control treatment (Table 4). Significant differences between the inoculation and control treatments were found except for *F. crenata* (p=0.479). Following inoculation, the largest transverse discolored area was 40.2% in *Q. crispula*, and the smallest was 13.7% in *F. crenata* (Table 4). The relative area was significantly larger in *Q. crispula* and *Q. serrata* than in the other four species (p<0.05). Following inoculation, transverse non-conductive sapwood areas also tended to be larger than those in the control treatment (Table 5). These differences were significant except with *F. crenata* and *P. edulis*. The relative area in *Q. crispula* was significantly larger than that in *F. crenata*, *Q. glauca* and *P. edulis* (p<0.05, Table 5). In all inoculated seedlings in which the non-conductive sapwood area was measured, the transverse discolored area was positively correlated with the non-conductive sapwood area (n=40; r=0.93). These results suggested that the relative areas of transverse discoloration and of non-conductive sapwood might be associated with the susceptibility of Fagaceae species to *R. quercivora*.

Table 5. Mean non-conducting water areas on six Fagaceae species inoculated with *Raffaelea quercivora*

	Inoculation treatments[2]		Control treatments		
Tree species	n	Mean non-conductive sapwood area (%)	n	Mean non-conductive sapwood area (%)	p-value[3]
Fagus crenata[1]	5	21.1 ± 9.8 ab	3	12.4 ± 6.4	0.231
Quercus crispula	7	39.1 ± 8.3 d	3	13.5 ± 2.9	<0.001
Q. serrata	7	35.3 ± 6.4 cd	3	8.8 ± 2.0	<0.001
Q. glauca	7	24.1 ± 5.1 abc	3	8.2 ± 3.1	0.001
Castanopsis cuspidata var. *sieboldii*	7	27.8 ± 8.0 cd	3	12.1 ± 2.4	0.008
Pasania edulis	7	15.6 ± 9.7 ab	3	9.7 ± 2.1	0.086

n = number of seedlings.
[1]Two of seven inoculated *F. crenata* seedlings were not measured because they died before the 56th day as a result of root rot.
[2]Inoculation treatments were compared among the six species with one-way ANOVA ($F = 9.59$, $p < 0.001$).
[3]Mean values (±SD) followed by different letters are significantly different (Bonferroni's test, $p < 0.05$).

3.4. Temporal changes in transverse discolored and non-conductive sapwood among four Fagaceae species inoculated with *R. quercivora*

Discolorations in stems have been considered as a key feature caused by JOW. Although discolored areas do not transmit water, the discoloration does not always lead to deaths of host trees. In addition, the discolored areas showed a positive correlation with the level of water dysfunction (i.e. non-conductive sapwood areas) at a certain time. However, these relationships were not exactly traced during the course after fungal inoculation. Thus, the aim of this inoculation experiment was to clarify the temporal relationship of transverse discolored and non-conductive sapwoods.

We used 5 mature trees in 4 Fagaceae species (rather susceptible; *Q. crispula* and *Q. serrata*, and less susceptible; *Q. glauca* and *C. cuspidata* var. *sieboldii*) for the inoculation of *R. quercivora*. In this case, we used branches of each tree as replicates instead of individual saplings to avoid genetic variations among samplings. *R. quercivora* (RA1052) was used as an inoculum. To prepare the inoculum, sterilized 1-cm-long, 2-mm diameter wooden sticks were soaked previously in liquid potato dextrose medium for 24 h. These sticks were placed on PDA medium with the subcultured isolate and incubated at 25°C in the dark for 10 days. The sticks were inserted into a hole bored at branches in 1 cm diameter. One inoculated branch of each tree was cut off on days 3, 7, 10, 14, 21, 28 and 57, and external symptoms were assessed. Control branches were cut off only on day 57. To check their water conductance, they were soaked in a 0.1% (w/v) acid fuchsin solution for more than 12 h. After this, branches were cut across at the inoculation point. We defined brown to black discolored sapwood as transverse discolored sapwood, and not dyed areas by the solution as non-conductive sapwood. Those regions were estimated followed by Murata et al. (2007). Fungal isolations were conducted on a three wood pieces per branch, taken from the distal end of the discolored sapwood.

No external symptoms such as wilting or necrosis occurred after the inoculation. *R.*

quercivora was re-isolated from every inoculated branch in all species until day 14 after the inoculation. The rate of re-isolation then
decreased gradually, but was still at least 60% on day 57 in all species. The fungus was not isolated from control branches on day 57. Thus, *R. quercivora* was well colonized in almost all inoculated branches. Transverse regions of both discolored and non-conductive sapwood in inoculation treatment of all species were significantly larger than those in the control ($p<0.001$). In all species, non-conductive and discolored sapwood were recognized on days 3 and 7, respectively (Fig 5). Comparing the patterns in temporal changes between the regions of discolored and non-conductive sapwood through the all experiment period, the differences in *Q. crispula*, *Q. serrata* and *C. cuspidata* var. *sieboldii* were significant ($p<0.001$). The region of discolored sapwood was identical with that of non-conductive sapwood on day 21 in *Q. crispula*, on day 14 in *Q. serrata*, on day 7 in *Q. glauca*, and on day 10 in *C. cuspidata* var. *sieboldii*. On day 57, the both regions were largest in *Q. crispula* (43.3%) and smallest in *Q. glauca* (12.0%), and the differences among four species were significant ($p=0.002$, Table 6, Fig 5). On day 57, the index of susceptibility which was defined from the data on the death rate of *Q. crispula*, *Q. serrata*, *Q. glauca* and *C. cuspidata* var. *sieboldii* (Murata et al. 2005) was positively correlated with regions of the non-conductive sapwood ($n=17$; $r=0.76$; $p<0.001$). These results suggested that the expansion of the non-conductive sapwood occurred soon after the infection of *R. quercivora*, and the difference of the susceptibility among Fagaceae species to this fungus is determined in early times after inoculation.

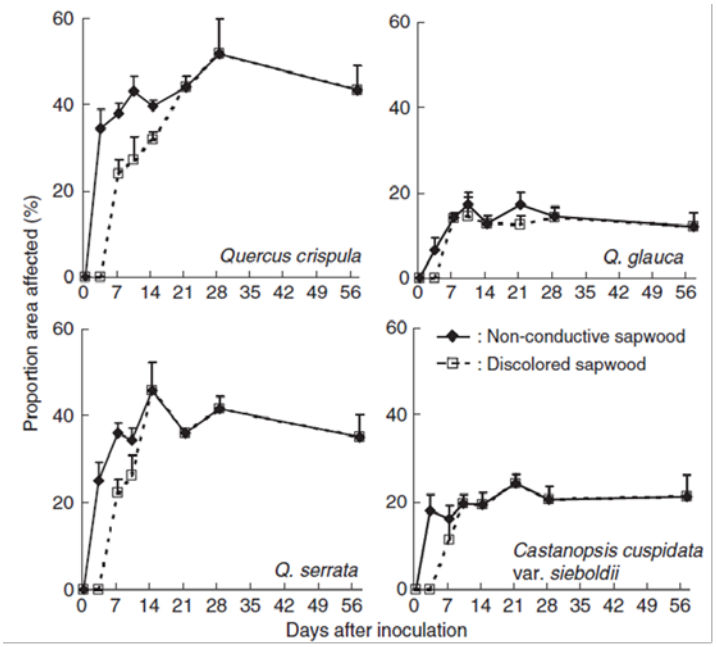

Fig 5. Changes in the proportions of discoloured and non-conductive sapwood in transverse sections of three *Quercus* species and one *Castanopsis* species after inoculation with *Raffaelea quercivora*. Values indicate mean (±SE) of five replicates.

Table 6. Mean proportions in transverse region of discoloured and non-conductive sapwood among four tree species on day 57 after inoculation with *Raffaelea quercivora*

Tree species	n	Discoloured sapwood (%)	n	Non-conductive sapwood (%)
Quercus crispula	4	43.3 ± 5.5 a	4	43.3 ± 5.5 a
Q. serrata	3	35.2 ± 4.7 a	3	35.2 ± 4.7 a
Q. glauca	5	12.0 ± 3.2 b	5	12.0 ± 3.2 b
Castanopsis cuspidata var. *sieboldii*	5	21.3 ± 4.6 ab	5	21.3 ± 4.6 ab
		$F = 8.97$, $p = 0.002$		$F = 8.97$, $p = 0.002$

Mean values (±SE) followed by different letters are significantly different by Scheffé's test at $p < 0.05$.
n = number of branches.

3.5. Distribution patterns of *R. quercivora* hyphae in host trees

The previous experiment (see 3.4) showed that the formation of non-conductive sapwoods preceded discoloration in the earliest stage, but both these regions coincided at several weeks after inoculation. Thus, the susceptibility to *R. quercivora* might be closely related to the transverse proportion of non-conductive sapwood at the inoculation point. Although previous studies have focused on the interaction between host trees and causal fungus at individual levels, few studies examined the actual fungal distribution within host trees or the relationship between hyphal growth and the formation of non-conductive sapwood at tissue levels. Therefore, this inoculation experiment was conducted to compare the distributions of *R. quercivora* hyphae within seedlings of a susceptible species, *Q. crispula* and a less susceptible species, *Q. glauca* (Murata et al. 2005, 2009).

Seedlings of two oak species, *Q. crispula* and *Q. glauca*, were grown in the nursery of Mie University. As an inoculum, *R. quercivora* (RA1309) isolated from the discolored sapwood of a dead *Q. serrata* collected in Aichi Prefecture in 2007 was used. Sterilized wooden sticks, 2 mm long and 1 mm thick, were placed on 1/2 PDA fungal culture and incubated at 25°C in the dark for a week. Ten seedlings of each species were inoculated on 28 July 2008 when most *P. quercivorus* were emerging from dead trees and infestation was declining and 10 more on 29 October when most beetles had ceased to fly (Saito et al. 2001, Esaki et al. 2002). We chose two inoculation times because the trees' response to hyphae may differ if their susceptibility to the fungus varies seasonally (Saito et al. 2001, Yamato et al. 2001). The two holes, each 1 mm in diameter and 2 mm deep, were bored on the opposite sides of each seedling stem. A wooden stick with fungal inoculum was inserted into the holes. All the holes were sealed with cling wrap and commercial packing tape. Five seedlings of each species were harvested at each of 1 and 2 weeks after inoculation. Their water conductance was examined by soaking the seedlings in a 1% (w/v) acid fuchsin solution for more than 12 h (Murata et al. 2007, 2009) and they were cut across at the inoculation point. Undyed areas were defined as non-conductive sapwood and estimated the proportion of the non-conductive sapwood in transverse section (Murata et al. 2007). Fungal re-isolation was carried out in all seedlings and incubated on 1/2 PDA media for 7 days in the dark at 25°C. On each harvest date, wood blocks cut from three of the five seedlings of each species were stored at -20°C and later examined histologically. From each sample, 10 transverse sections 30 µm thick were made at the inoculation point. All the sections were visualized staining with Calcofluor white stain and fixed in 10% (w / v) KOH solution (Harrington and Hageage 2003). Hyphae in the xylem were examined under both a light microscope and a fluorescence microscope with UV illumination.

Table 7. Results of three-way ANOVA for proportions of non-conductive sapwood in transverse sections

Source	Degrees of freedom	Mean squares	*F* values	p
Species	1	555.378	23.334	0.000
Inoculation time	1	142.478	5.986	0.021
Harvest date	1	0.921	0.039	0.845
Species × inoculation time	1	50.365	2.116	0.157
Species × harvest date	1	1.349	0.057	0.814
Inoculation time × harvest date	1	36.607	1.538	0.225
Species × inoculation time × harvested date	1	8.555	0.359	0.554
Error	28	23.801		

Since *R. quercivora* was not re-isolated from two *Q. crispula* seedlings harvested at 2 weeks after July inoculation or from one seedling harvested at 1 week after October inoculation, these seedlings were omitted from further analyses. For other seedlings, isolation frequency of the fungus was 10–90%, and the fungus was dominant in most of the seedlings. Statistical analyses showed that interactions among the factors (any possible combinations among tree species, inoculation time, and harvested date) were not significant, but proportions of non-conductive sapwood differed significantly between tree species and between inoculation times (Table 7). Proportions of non-conductive sapwood in *Q. crispula* were significantly larger than those in *Q. glauca* and proportions in July inoculation were significantly larger than those in October inoculation (Fig. 6). In July inoculation, hyphae were observed in the non-conductive sapwood in *Q. crispula*

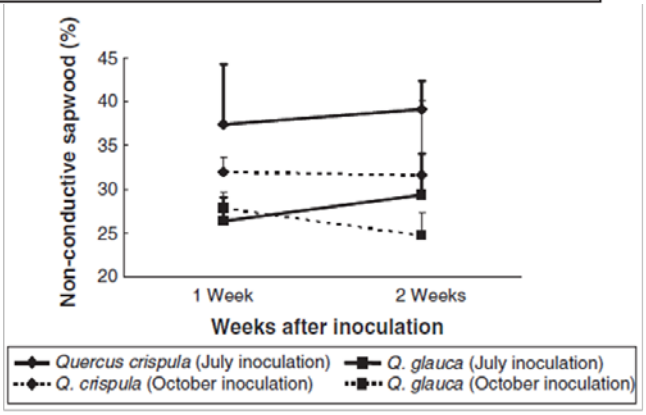

Fig 6. Mean proportions of non-conductive sapwood in transverse sections of *Quercus crispula* and *Q. glauca* after inoculation with *Raffaelea quercivora*. Values indicate mean±SD; n=5, except for *Q. crispula* at 2 weeks after July inoculation (n=3) and at 1 week after October inoculation (n=4), because seedlings where the fungus was not re-isolated were excluded. Proportions of non-conductive sapwood were significantly different between species and between inoculation times (three-way ANOVA; $p<0.05$, Table 7).

appeared to lie further from the inoculation points than those in *Q. glauca*, which clustered mainly around the inoculation points. In October inoculation, hyphae clustered mostly around the inoculation points in both species. These results suggested that non-conductive sapwood may spread with the hyphal growth of *R. quercivora* in both species and the hyphae can possible be grow beyond the non-conductive sapwood.

4. Future prospect

For clarifying the wilting and death mechanism of JOW, we have kept conducting fungal inoculation tests for a decade focusing on common susceptible trees, mainly species in the family Fagaceae. Our studies were well documented the pathogenicity of *R. quercivora*. Recently, various degrees of virulence among *R. quercivora* strains were suggested to susceptible host trees (Kusumoto et al. 2012). However, in a biological sense, we do know little about fundamental defense mechanisms of host trees against the pathogenic fungus behind the difference of the pathogenicity and virulence. Since JOW is a kind of epidemic disease that is now reported at most parts of Japan, and thus current research progresses might not allowed to prepare for developing stable strategies and for establishing applied practices recovering from the disease. However, we believe that the better understandings of fundamental mechanisms on tripartite relationship; host trees - insects – fungi, would be essential for the management of JOW. Now a similar disease becomes recognized as a serious problem in Korean forests where *Q. mongolica* is damaged by *R. quercus-mongolicae* carried by *P. koryoensis* (Kim et al. 2009). In a global scale, raffaelean disease was also reported from the USA (Fraedrich et al. 2008, Inch and Ploetz 2012). Considering the nature of causal fungi transmitted by their associating insects, they are quite possible to carry into far distant areas via logs' export and import procedures. This may be a potential hazard that JOW like diseases can suddenly occur in other countries. In fact, a foreign oak species was found to be dead by our preliminary inoculation of *R. quercivora*, experimentally (Fig 7; Torii et al. 2012).

Fig 7. A dead seedling of *Quercus rubra* inoculated with *Raffaelea quercivora*

Rapid shifts of vegetational compositions or unexpected tree deaths could lead to serious consequences to various organisms living in forest ecosystems that are organized by subtle biological interactions. For the management of JOW, there are a big gap between what we got and what we should understand. Although there are so many things remained to be done, lessons from fungal inoculation can provide insightful information for not only un-entangling complex biological interactions of this disease but also a basal step for JOW management.

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