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Research Note

The Effect of *Raffaelea quercus-mongolicae* Inoculations on the Formation of Non-conductive Sapwood of *Quercus mongolica*

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Abstract In Korea, mass mortality of *Quercus mongolica* trees has become obvious since 2004. *Raffaelea quercus-mongolicae* is believed to be a causal fungus contributing the mortality. To evaluate the pathogenicity of the fungus to the trees, the fungus was multiple- and single-inoculated to the seedlings and twigs of the mature trees, respectively. In both the inoculations, the fungus was reisolated from more than 50% of inoculated twigs and seedlings. In the single inoculations, proportions of the transverse area of non-conductive sapwood at inoculation points and vertical lengths of discoloration expanded from the points were significantly different between the inoculation treatment and the control. In the multiple inoculations, no mortality was confirmed among the seedlings examined. These results showed that *R. quercus-mongolicae* can colonize sapwood, contribute to sapwood discoloration and disrupt sap flows around inoculation sites of *Q. mongolica*, although the pathogenicity of the fungus was not proven.

Keywords Discoloured sapwood, Non-conductive sapwood, Pathogenicity, *Quercus mongolica*, *Raffaelea quercus-mongolicae*

In Korea, mass mortality of *Quercus mongolica* trees has become obvious since 2004 and the intermediate to heavy infestation by the ambrosia beetle *Platypus koryoensis* is always observed on dead *Q. mongolica* trees [1, 2]. The trees infested by the beetles initially show wilting of foliage throughout the entire crown and eventually die [2]. Fungi were found to be associated with the beetle [3]. Among fungi associated with the beetle, one fungus was detected frequently on the dead trees and beetles. The fungus was soon identified and described as a new species, *Raffaelea quercus-mongolicae* [4]. Thus, both the beetle and the fungus are considered to contribute to the tree mortality. Although relationships between spatial attack patterns of the beetle on affected trees and degrees of damage of the trees have been reported [2, 5], no studies have yet conducted on either the actual association of the fungus with the tree mortality or the pathogenicity of the fungus to host trees.

Tree mortality associated with a member of the genus *Raffaelea* has occurred in Japan, Portugal and the United States [6-8]. In Japan, mass mortality of Fagaceae trees is caused by *Raffaelea quercivora* transmitted by the ambrosia beetle *Platypus quercivorus* via mycangia [9, 10]. In the United States, Lauraceae trees are affected by laurel wilt caused by *Raffaelea lauricola* carried by the ambrosia beetle *Xyleborus glabratus* [8, 11]. The pathogenicity of the *Raffaelea* fungus involved in each of the above diseases was confirmed mainly by observations of external symptoms (i.e., foliage wilting, mortality) resulting from artificial inoculations [8, 10, 12]. Moreover, the cause of the wilting was suggested to be xylem dysfunction and impaired water transport [12, 13]. However, the patterns of pathogen spread within the infected trees differ between the diseases.

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The hyphal distribution of *R. quercivora* and formation of discoloured and non-conductive sapwood in both artificially inoculated seedlings and naturally infected trees were limited vertically and horizontally to the area around inoculation points and tunnels bored by *P. quercivorus* [14, 15]. Thus, multiple inoculations with the fungal pathogen or mass attacks by the insect vector are needed to induce lethal disease development under artificial and natural conditions, respectively [10, 15]. On the other hand, *R. lauricola* can colonize systemically within susceptible host trees [8, 13]. Thus, a single inoculation with the pathogen or only a few vector beetles may be sufficient to induce tree mortality [8, 13, 16].

If *R. quercus-mongolicae* has the pathogenicity to *Q. mongolica*, the fungus should cause foliage wilt of the infected trees and the fungus may induce xylem dysfunction and impair water transport inside the trees. In the case of the mortality in Korea, mass attacks by *P. koryoensis* were found on dead trees [2]. Thus, the spread patterns of *R. quercus-mongolicae* within the host trees may be similar to those of the Japanese pathogen. If this is the case, non-conductive sapwood resulting from the fungal infection might form in a limited area, and multiple inoculations may be needed to evaluate the pathogenicity of the fungus. This study was undertaken to evaluate the pathogenicity of *R. quercus-mongolicae* to *Q. mongolica*.

Seventeen mature trees and 32 seedlings of *Q. mongolica* growing naturally within 15 ha from the Korea Forest Research Institute (37°36′ N, 127°03′ E) located in Seoul were used for this study (Supplementary Fig. 1). For the seedlings, 16 pairs of neighboring seedlings growing within 10 m were selected. The average diameters at breast height in mature trees (mean ± SD, n = 17) and root collar in seedlings (mean ± SD, n = 32) were 14.5 ± 7.0 cm and 13.1 ± 2.4 mm, respectively. As a fungal inoculum, *R. quercus-mongolicae* strain RQ 10. 210, which was isolated from a dead, mature *Q. mongolica* tree in Seoul in 2010 and preserved in the Division of Forest Insect Pests and Disease at the Korea Forest Research Institute, was subcultured at 25°C in the dark on half-strength potato dextrose agar until use. Wooden sticks, 3.5 mm long and 1.4 mm in diameter, were autoclave-sterilized. The sticks were then placed around fungal colonies and incubated at 25°C in the dark for a week so as to be colonized by the fungus.

To evaluate the extent of non-conductive sapwood induced by the *R. quercus-mongolicae* inoculation, single inoculation was conducted on two twigs of each mature tree on 3 July 2012. To minimize variations of tree responses due to genetic and micro-environmental conditions, we chose a pair of twigs at about the same height on individual trees; one twig for the fungal inoculation and the other one for control. A hole, 3.5 mm deep and 1.4 mm in diameter, was bored on a twig at a point where it was 8 mm in diameter, with a surface-sterilized hand drill. A fungus-colonized wooden stick was inserted into the hole and a sterilized wooden stick as a control. All holes were immediately sealed with plastic wrap and commercial packing tape. To investigate pathogenicity of *R. quercus-mongolicae* to *Q. mongolica*, multiple inoculations were conducted on the seedlings on 3 and 4 July 2012 in the same manner as described for the twigs of mature trees. On this occasion, twelve holes, 4 holes × 3 lines with 5 mm intervals between each line, were bored on a stem at the center line of inoculation sites where it was 8 mm in diameter [12]. Wooden sticks with the fungal inoculum were inserted into the holes in 16 seedlings, and sterilized wooden sticks were inserted into the holes on the neighboring seedlings as the controls. We conducted the fungal and control treatments on a pairs of neighboring seedlings to minimize variations of symptom expressions of the seedlings caused by micro-environmental conditions. After the inoculations, development of external symptoms was observed twice a week. We determined death as wilting and discoloration from greenish to brownish foliage of seedlings [2, 12]. At 55 days after the single inoculations and at 36 or 37 days after the multiple inoculations, all the twigs and the seedlings were harvested by cutting at locations more than 20 cm below inoculation points and by cutting at the base of the stem, respectively. To evaluate water conductance, we immersed the cut base of twigs and seedlings in a 1% (w/v) acid fuchsin solution for more than 12 hr [14].

In the single inoculations, we defined undyed areas as non-conductive sapwood, and the area was estimated as a proportion of a transverse section [14] at inoculation points

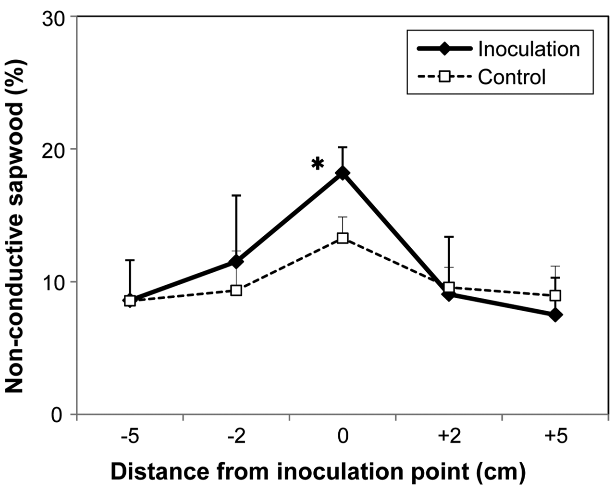

Fig. 1. Proportions of non-conductive sapwood in transverse sections of *Quercus mongolica* twigs after single inoculations with *Raffaelea quercus-mongolicae*. Zero on X-axis indicates inoculation points, and + and − indicate above and below the inoculation points, respectively. Values are mean + SE (n = 7 at 0 cm and n = 4 at the other height levels). Asterisks indicate significant differences in proportions of non-conductive sapwood between the inoculation treatment and the control at the same height level (one tailed paired t-test; $p < 0.05$).

(0 cm), 2, and 5 cm above and below inoculation points. Moreover, twigs were cut into half longitudinally along inoculation points and vertical lengths of discoloration were measured with a digital caliper. In transverse sections, the spatial distribution of *R. quercivora* hyphae was shown to coincide with non-conductive sapwood [15, 17, 18]. However, in longitudinal sections, the distribution coincided with the discoloured area [15, 19]. Therefore, we attempted to evaluate the vertical fungal distribution by the discoloration lengths. The lengths were determined as the distance from the edge of inoculation holes to the uppermost and lowermost of the discoloured area and both lengths were averaged for each inoculation [20]. In both inoculation and control treatments, the lengths were examined in twigs of all trees, but the proportions of non-conductive sapwood at inoculation points and at the other locations were examined in twigs of seven trees and four trees, respectively. In the multiple inoculations, we checked water conductance in transverse sections at three different height levels: at the marginal position of upper lines of inoculation sites, at 5 cm upper positions from the upper lines, and at the base of stems. For the fungal reisolation, several pieces (2 mm × 2 mm × 2 mm) were obtained from around inoculation sites of each twig and seedling. The small wood pieces were surface-sterilized sequentially with 70% ethanol and a sodium hypochlorite (1% of available chloride) solution [14]. They were then placed on half-strength potato dextrose agar and incubated for 7 days in the dark at 25°C. We checked for the presence of *R. quercus-mongolicae* based on the morphology of fungal colonies that emerged from the wood pieces. Differences in transverse proportions of non-conductive sapwood between the inoculation treatment and the control at each position and in vertical discoloration lengths between the treatments were analyzed by one tailed paired t-test ($p < 0.05$). Arcsine transformation was used to normalize data expressed as percentages. Statistical analyses were performed with PASW statistics ver. 18.0 software (SPSS Japan Inc., Tokyo, Japan).

Because *R. quercus-mongolicae* was not reisolated from six twigs and eight seedlings in the inoculation treatment, data from these samples as well as their pairs were omitted from further analyses. The fungus was reisolated from the rest of the inoculated twigs and seedlings but not from any of the controls (data not shown). Filamentous fungi, e.g., *Phomopsis* spp. and *Trichoderma* spp.-like fungi except for *R. quercus-mongolicae*, were occasionally isolated from both twigs and seedlings. In the single inoculations, no external symptoms were observed. In transverse sections, non-conductive areas coincided with discoloured areas. However, the non-conductive areas expanded beyond the discoloured areas in a longitudinal direction. The average transverse proportion of the non-conductive sapwood at the inoculation points in the inoculation treatment was significantly larger than that in the control (Fig. 1). However, no significant differences between the treatments were found in the proportions of the non-conductive sapwood area at 2 and 5 cm above and below inoculation points. The average vertical length of discoloration in the inoculation treatment was significantly longer than that in the control (Fig. 2). In the multiple inoculations, only one seedling inoculated with *R. quercus-mongolicae* wilted and died, but the fungus was not recovered. Thus, the seedling was discarded from the analysis. No wilting and mortality were found on the rest of the study seedlings until the end of the experiment period. Although wide discoloured and non-conductive areas were found in transverse sections at fungus-inoculation sites, the areas were not considerable in sections at the other height levels (Fig. 3).

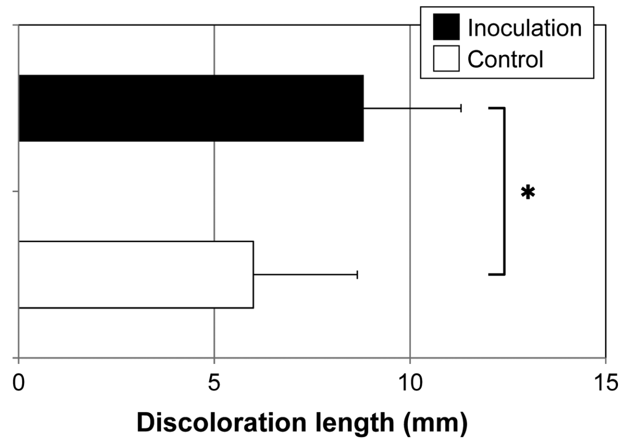

Fig. 2. Vertical discoloration lengths on twigs of *Quercus mongolica* after single inoculations with *Raffaelea quercus-mongolicae*. Values are mean + SD (n = 11). Asterisks indicate significant differences in discoloration lengths between the inoculation treatment and the control (one tailed paired t-test; $p < 0.05$).

In the present study, inoculated *R. quercus-mongolicae* was successfully reisolated from more than 50% of twigs and seedlings of *Q. mongolica*. The result indicates that the fungus can colonize both mature trees and seedlings of the species. Moreover, the results in evaluations of non-conductive and discoloured sapwood suggest that *R. quercus-mongolicae* is more likely to colonize and spread from where it is first established, and, thus, might have an ability to prevent sap flows in the xylem around inoculation sites. However, pathogenicity of the fungus was not proven in the multiple inoculations because neither wilting nor dead trees were found. The susceptibility of the Japanese oak species to *R. quercivora* was suggested to vary by when to inoculate such as summer versus autumn [18] and different months within summer (June or July) [21]. The variations might be due to the difference in the fungal growth affected by various temperatures [18]. Therefore, to clarify the mechanism of tree wilting and death of *Q. mongolica* infected by *R. quercus-mongolicae*, further multiple inoculation tests are needed to be conducted in different seasons with various temperature ranges.

In this study, the average vertical length of discoloured

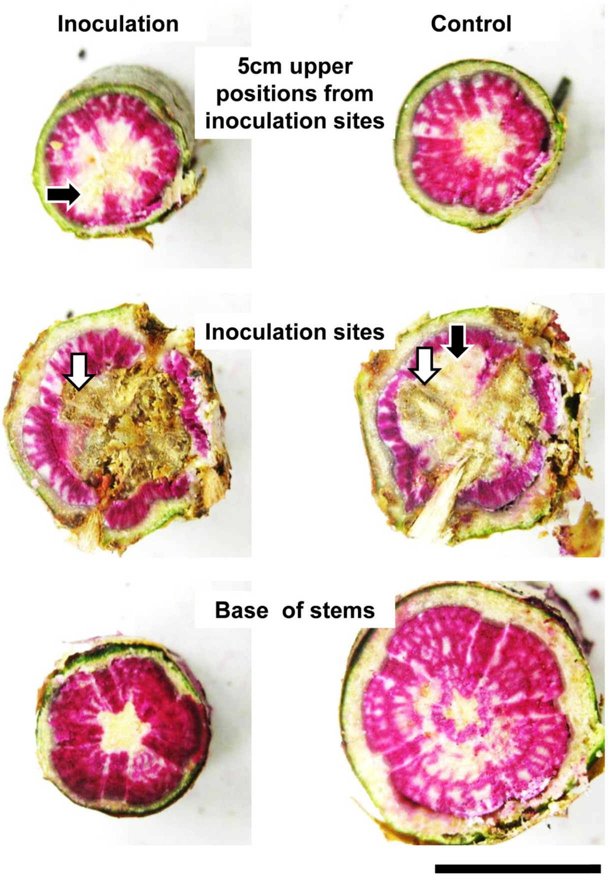

Fig. 3. Representative transverse sections of neighboring *Quercus mongolica* seedlings after multiple inoculations with *Raffaelea quercus-mongolicae* (scale bar = 10 mm). White and black arrows indicate non-conductive areas (undyed pink areas) with and without discoloration (dark brown areas), respectively. The areas were easily observed in transverse sections at inoculation sites but were slightly observed in sections at the other height levels.

sapwood from the inoculation points was 1 cm, and the transverse proportion of non-conductive sapwood at the inoculation points and at 5 cm above and below the points were 18% and 8%, respectively. In the case of the Japanese oak disease caused by *R. quercivora*, the same features in different Fagaceae species at the inoculation points were about 2~5 cm and 15~40%, respectively [14]. For laurel wilt caused by *R. lauricola*, the proportions of non-conductive sapwood at 5 cm above and below the inoculation points were about 60~70% [13]. In addition, the length of the discoloration on the sapwood sometimes exceeded 100 cm [16]. To our knowledge, no information is available for the Portuguese oak disease associated with *Raffaelea* spp., except for the presence of the fungi in dead trees [7]. The vertical discoloured lengths as well as the transverse proportions of non-conductive sapwood could have been affected by duration from inoculation to evaluation. In this respect, the duration in this study was consistent to for *R. quercivora* [14], but was even longer than that for *R. lauricola* [13, 16]. Therefore, the limited colonization of *R. quercus-mongolicae* in host trees may be very similar to the behavior of *R. quercivora* rather than with *R. lauricola*.

Overall, our study proved the stem colonization of *R. quercus-mongolicae* onto *Q. mongolica* by artificial inoculations for both mature trees and seedlings. Moreover, we showed the fungus induces the formation of non-conductive sapwood. However, the pathogenicity of the fungus remains to be confirmed. To clarify the underlying mechanisms of mass mortality of the oak trees in Korea, further inoculation tests of the fungus are needed.

ELECTRONIC SUPPLEMENTARY MATERIAL

Supplementary data including one figure can be found with this article online at http://www.mycobiology.or.kr/src/sm/mb-42-pp-s210.pdf.

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