Allelochemicals in the Soil beneath Quercus mongolica
Fisch var. grosseserrata Rehd. Wils

Hai-Hang Li*, Labunmi Lajide*, Hiroyuki Nishimura**, Koji Hasegawa* and Junya Mizutani*

Abstract: The phenomenon that wild grasses did not grow beneath the tree of Quercus mongolica Fisch var. grosseserrata Rehd. Wils, while they grew vigorously beneath other species of trees around Q. mongolica was observed in many places in the Hokkaido district. The allelochemicals in the soil beneath Q. mongolica were studied. This soil inhibited the growth of lettuce (Lactuca scariola L. var. sativa Bisch), green amaranth (Amaranthus viridis L.), wheat (Triticum aestivum cv. Norin No. 59) and timothy (Phleum pratense L.) by 50-90% in a 30 day soil culture test in phytotron. Of four fractions of extracts, the ethyl acetate fraction of the phenolic extracts from the soil beneath Q. mongolica showed the highest inhibition on seed germination and growth of etiolated seedling of the four species. Nine allelochemicals, 3,4-dihydroxybenzoic, p-hydroxybenzoic, 3,4,5-trimethoxybenzoic, 3,4-dimethoxybenzoic, vanillic, p-coumaric, ferulic acids, p-hydroxybenzaldehyde, and kaempferol were identified in this fraction by HPLC, EI-MS and 'H-NMR. The contents of five main phenolic compounds, p-coumaric, ferulic, vanillic, p-hydroxybenzoic acids and p-hydroxybenzaldehyde and kaempferol in the soil were found to be 13,382, 3,542, 2,952, 2,164, 1,378 and 990 μg per 100 g soil, higher than those in the growing soils of Sasa cernua, Picea jezoensis, rice, corn, potato, carrot, soybean and beet, but lower than in the soil beneath red pine. These allelochemicals might have important roles in the distribution of forest species and understory grasses.

Key words: Allelochemicals, Quercus mongolica, phenolics, plant soil, growth inhibition

Introduction

The Quercus genus has over 300 species distributed throughout most of the temperate zone in the Northern Hemisphere. Most species of this genus make highly valuable forests and have a good quality of woods which has been used to make furniture, buildings, boats, etc.. Their fruits can be eaten or used as feed for animals. Investigation showed that two main species, Q. mongolica Fisch. var grosseserrata Rehd. et Wils. and Q. serrata Murr. of the Quercus genus grow widely in the Hokkaido area, forming pure stands or intermixed forests. Like some species of pine such as red pine (Pinus densiflora Sieb. et Zucc.), which have been demonstrated to have allelopathy15,16), in most areas beneath the pure forest of these two species there were very few or no weeds growing. In some intermixed forests where they have the same light, nutrient and humidity conditions, wild grasses grew vigorously under the trees other than Quercus, but did not grow under the Quercus species. This showed...
that the main reason for the lack of growing weeds beneath Quercus might not be the nutrient, light or humidity conditions, but allelopathy.

Allelochemicals have been shown to play important roles in agriculture, forestry and natural ecosystems. In this paper, the allelopathic effects in the soil beneath Q. mongolica were tested. The main allelochemicals were identified and their contents in the soil compared to the other species of trees and crops were analyzed.

Materials and Methods

Soil culture tests

Plant culture tests with soil were performed in a plastic culture box set as described before. Samples of soil (from the layer 5 to 15 cm from the surface) beneath Q. mongolica were collected in September, 1990 from forests where no other species of plants grew in the proximity. Two controls were used: Control I was vermiculite and Control II was normal soil taken from the upland of the Q. mongolica forest in which no Q. mongolica were growing. Three-day-old seedlings of lettuce (Lactuca scariola L. var. sativa Bisch), green amaranth (Amaranthus viridis L.), wheat (Triticum aestivum L.) and timothy (Phleum pratense L.) were planted in culture boxes and grown in phytotron for 30 days under conditions of 14h light (white fluorescence of 3.8×10^6 Ergs. cm⁻¹. sec⁻¹, 28°C) and 10h dark (20°C). The nutrient solution was distilled water during the first week and Hoagland's nutrient solution for the following days. Ten plants of each species were used. Each test was repeated two times.

Seed germination and seedling growth tests

All the seed germination and seedling growth tests were done on Toyo No. 1 filter paper in 33 mm diameter petri dishes with 1 ml test solution containing 100 ppm Tween 80. One hundred ppm Tween 80 aqueous solution was used in all the controls. For the seed germination test, thirty seeds for each test were added to dishes containing the test solution and germinated in the dark at 25°C. Germinated seeds were counted after a certain number of hours according to plant species. For the seedling growth test, 36 hr- (lettuce), 48 hr- (timothy and green amaranth) or 72 hr-old (barnyard grass) uniform etiolated seedlings with 3 mm hypocotyl or coleoptile and about 5 mm root were selected. For each test, six etiolated seedlings were planted in the petri dishes containing test solutions. The lengths of roots and hypocotyls or coleoptiles were measured after 48 hr culture in the dark at 25°C. Each test was repeated at least three times.

Direct bioassay on TLC plate

As described, one 10-mg sample of the neutral-ethyl acetate fraction was charged on a TLC plate (6.6×20 cm² with 0.25 mm thickness silica gel, Merck, Kiesel gel 60 F254) and developed to 15 cm from the original with toluene: ethyl acetate: methanol, 5:3:2, v/v/v. After evaporation of the solvents in a desiccator overnight, a 2 mm thickness of 0.8% agar layer was put on the TLC plate. The plate was then kept in a culture box saturated with water for one night to allow the compounds to diffuse into the agar. Plant seeds were then sowed on the agar plate in lines and the plate was put in an airtight container and cultured in the greenhouse at 25°C. Photos were taken on the fifth day.

Extraction, isolation and identification of the main allelochemicals in the soil beneath Q. mongolica

The soil taken from the forest of Q. mongolica was dried in a desiccator at room temperature. The extraction procedure is shown in Figure 1. The methanol soluble substances in the aqueous fractions of the two extracts, after being fractionated into ethyl acetate and n-butanol, did not show any plant growth activities in the preliminary experiments and were not used for further analysis. Four fractions, neutral-EtOAc (ethyl acetate), neutral-butanol, phenolic-EtOAc
and phenolic-butanol fractions were used for analysis of the plant growth activities.

The ethyl acetate fraction of the phenolic extracts, which showed the highest activity in bioassays, was analyzed and separated by HPLC column (YMC-packed ODS column 10 mm × 300 mm) with UV detection at 254 nm. The sample was eluted with A (H₂O : acetonitrile : acetic acid, 80 : 20 : 0.1, v/v/v) from 0-30 min., linear gradient to B (H₂O : acetonitrile, 1 : 9, v/v) from 30-60 min. and B after 60 min., at 2 ml/min. Purified compounds of the ordinary phenolic substances were identified by the retention times in HPLC and the spectra of ¹H-NMR compared to those of the standard compounds. Kampferol was identified by ¹H-NMR and EI-MS spectra analysis.
Determination of the contents of allelochemicals in the soils

Three soil samples for each species of plant were taken from different places of the pure stands or fields in October, 1992. Rice soil was taken from a paddy, and other crop soils were taken from dry lands after crops were harvested. The soils were air-dried at root temperature. Ten grams of each sample was extracted according to the method in Figure 1. The contents of the phenolic compounds in the ethyl acetate fraction of the phenolic extracts were detected by HPLC as described\(^\text{19}\). Control I was soil taken from a layer about 10 m below the surface of an area where no plants were growing at a mountain worksite. Other samples of soils were taken from layers between 5 and 10 cm from the soil surface. Control II was taken from the high land of a mountain where no plants were growing. And soil of a weed field was taken from an area where no crops had been planted for at least ten years and many species of weeds were growing vigorously.

Results

Effects of the soil beneath *Q. mongolica* on plant growth

Figure 2 shows the growth of 20-day-old plants of the four species tested in the soil from beneath *Q. mongolica* and in that of the two controls. Figure 3 shows that the soil beneath *Q. mongolica* strongly inhibited the growth of lettuce, green amaranth, wheat and timothy in soil tests cultured for 30 days. Two dicotyledonous plants, lettuce and green amaranth barely grew; their growth was inhibited by 90% or over in both fresh weight and dry weight. The growth of the two monocotyledonous plants, wheat and timothy, was inhibited by 70% or more in fresh and dry weight; these inhibitions were weaker than those of the two dicotyledonous plants tested. These assay results coincided with the natural phenomenon observed. However, in control tests with what we called the normal soil, some inhibitory

![Fig. 2. Effects of the soil beneath *Q. mongolica* on the growth of plants in soil assays after 20 day culture. A: lettuce, B: green amaranth, C: wheat, and D: timothy.](image)
Fig. 3. Effects of the soil beneath *Q. mongolica* on the fresh (a) and dry (b) weights of lettuce, green amaranth, wheat and timothy cultured in soil assays for 30 days. Control I: cultured in vermiculite, Control II: in normal soil, and Treatment: in the soil beneath *Q. mongolica*. Data are the percent over Control I.
Effect was also seen on the growth of lettuce, green amaranth and timothy, but not on wheat compared to the control in vermiculite.

**Effects of the extracts from the soil beneath *Quercus mongolica* on seed germination and etiolated seedling growth**

The effects of the four fractions of the extracts from the soil beneath *Q. mongolica* on seed germination and the growth of etiolated seedlings of lettuce, green amaranth, timothy and barnyard grass were tested. Two fractions, phenolic-EtOAc and phenolic-butanol fractions, showed strong growth inhibition on the four species of plants tested at the concentration of 500 ppm (Fig. 4), and this inhibition increased with the concentrations used (data not shown). The inhibitory activity of the phenolic-EtOAc fraction was stronger than that of the phenolic-butanol fraction on the growth of the four species. From Figure 4, it can be seen that the inhibitions of the phenolic-EtOAc and phenolic-butanol fractions on the elongation of root were stronger than that of hypocotyl or coleoptile in the four species of plants tested. Low concentrations (below 500 ppm) of the two fractions only inhibited root elongation. When the concentrations increased, they inhibited the elongation of both roots and hypocotyls or coleoptiles.

The phenolic-EtOAc fraction inhibited the seed germination of lettuce, green amaranth, timothy and barnyard grass at 500 and 1,000 ppm concentrations (Table 1), while other fractions did not.

![Fig. 4. Effects of the four fractions of the extracts from the soil beneath *Quercus mongolica* on the growth of etiolated seedlings of lettuce, green amaranth, timothy and barnyard grass at 500 ppm concentration. a: EtOAc-phenolic, b: butanol-phenolic, c: EtOAc-neutral, and d: butanol-neutral fractions. Data, indicated in percent over the controls, were the means of three independent repeats (each with 6 seedlings). Vertical bars indicate SE.](image-url)
show significant inhibition (data not shown).

The neutral-EtOAc and neutral-butanol fractions did not show significant inhibitions on the growth of the four plant species at the concentration of 500 ppm (Fig. 4) or 1,000 ppm (data not shown) in the bioassay in petri dishes. On the contrary, these two fractions had a growth promoting effect on timothy, but not on the other three species (Fig. 4). However, in the direct bioassay on TLC plate the neutral-EtOAc fraction, similar to the results with the soil of S. cernua, showed strong inhibition on seed germination and seedling growth of lettuce (Rf 0.25-0.55), but had little effect on the other three species of plants used (Fig. 5). The neutral-butanol fraction did not show this inhibition (data not shown).

Isolation and identification of the main allelochemicals from the soil beneath Q. mongolica

From the bioassay results of the four fractions of extracts from the soil beneath Q. mongolica, the phenolic-EtOAc fraction showed the strongest inhibitory activity on seed germination and seedling growth of the four species. The active substances in this fraction were analyzed and separated by HPLC column (Fig. 6). Following the bioassay of seed germination of lettuce, nine main inhibitory substances were purified. They were identified as 3,4-dihydroxybenzoic, p-hydroxybenzoic, 3,4,5-trimethoxybenzoic, 3,4-dimethoxybenzoic, vanillic, p-coumaric, ferulic acids,

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Treatment</th>
<th>Incubation time (hr)</th>
<th>Germination (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lettuce</td>
<td>Control</td>
<td>18</td>
<td>86±10</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>18</td>
<td>8±3</td>
</tr>
<tr>
<td></td>
<td>1,000 ppm</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Green amaranth</td>
<td>Control</td>
<td>48</td>
<td>80±9</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>48</td>
<td>10±3</td>
</tr>
<tr>
<td></td>
<td>1,000 ppm</td>
<td>48</td>
<td>3±3</td>
</tr>
<tr>
<td>Timothy</td>
<td>Control</td>
<td>48</td>
<td>67±10</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1,000 ppm</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>Barnyard grass</td>
<td>Control</td>
<td>72</td>
<td>65±15</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>72</td>
<td>30±10</td>
</tr>
<tr>
<td></td>
<td>1,000 ppm</td>
<td>72</td>
<td>10±9</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of the EtOAc-neutral fraction of the extracts from the soil beneath Q. mongolica on the growth of lettuce, green amaranth, timothy and barnyard grass on the TLC direct bioassay. Photograph was taken five days after seeds were sowed.
Fig. 6. HPLC analysis of the ethyl acetate fraction of the phenolic extracts from the soil beneath Q. mongolica.
Column: YMC-packed ODS column (10 mm × 300 mm), detector: UV detector at 254 nm, eluates: A (H₂O: acetonitrile : acetic acid, 80:20:0.1, v/v/v) from 0-30 min., A linear gradient to B (H₂O: acetonitrile, 10:90, v/v) from 30-60 min. and B after 60 min., flow rate: 2 ml/min.

Table 2. Contents of some phenolic compounds in the rhizosphere soils of some higher plants.

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>p-hydroxy-benzoic acid</th>
<th>p-hydroxy-benzaldehyde</th>
<th>vanillic acid</th>
<th>p-coumaric acid</th>
<th>ferulic acid</th>
<th>total amount of the five phenolics</th>
</tr>
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<tr>
<td>Control I</td>
<td>UD*</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
<td></td>
</tr>
<tr>
<td>Control II</td>
<td>144±20</td>
<td>76±18</td>
<td>72±25</td>
<td>468±50</td>
<td>108±22</td>
<td>864</td>
</tr>
<tr>
<td>Sasa cernua</td>
<td>810±30</td>
<td>630±90</td>
<td>860±80</td>
<td>5640±500</td>
<td>1060±120</td>
<td>9000</td>
</tr>
<tr>
<td>Quercus mongolica</td>
<td>2164±300</td>
<td>1378±140</td>
<td>2952±250</td>
<td>13382±1100</td>
<td>3542±320</td>
<td>23418</td>
</tr>
<tr>
<td>Pinus densiflora Sieb. et Zucc.</td>
<td>2917±260</td>
<td>3182±400</td>
<td>8221±720</td>
<td>15912±1280</td>
<td>2122±360</td>
<td>32354</td>
</tr>
<tr>
<td>Picea jezoensis Carr.</td>
<td>1135±120</td>
<td>487±85</td>
<td>1460±95</td>
<td>2109±170</td>
<td>811±60</td>
<td>6002</td>
</tr>
<tr>
<td>Solanum tuberosum L. (potato)</td>
<td>787±80</td>
<td>UD</td>
<td>1573±180</td>
<td>2622±280</td>
<td>6073±660</td>
<td>11055</td>
</tr>
<tr>
<td>Zea mays L. (corn)</td>
<td>1012±90</td>
<td>289±25</td>
<td>1157±75</td>
<td>3181±115</td>
<td>UD</td>
<td>5639</td>
</tr>
<tr>
<td>Oryza sativa L. (rice)</td>
<td>397±65</td>
<td>132±20</td>
<td>662±85</td>
<td>15358±2060</td>
<td>9665±1290</td>
<td>21214</td>
</tr>
<tr>
<td>Daucus carota L. var. sativa</td>
<td>789±35</td>
<td>UD</td>
<td>526±40</td>
<td>UD</td>
<td>UD</td>
<td>1315</td>
</tr>
<tr>
<td>Glycine max Merrill</td>
<td>408±25</td>
<td>272±30</td>
<td>544±55</td>
<td>680±75</td>
<td>816±120</td>
<td>2720</td>
</tr>
<tr>
<td>Beta vulgaris L. var. rapa</td>
<td>311±35</td>
<td>117±20</td>
<td>622±45</td>
<td>1867±140</td>
<td>1400±155</td>
<td>4317</td>
</tr>
<tr>
<td>Weed field</td>
<td>1611±85</td>
<td>691±35</td>
<td>2302±130</td>
<td>8748±670</td>
<td>7366±420</td>
<td>20718</td>
</tr>
</tbody>
</table>

* undetectable: the amount is too low to be detected.
p-hydroxybenzaldehyde and kaempferol. Compounds 1 to 8 were identified by their retention times in HPLC and 1H-NMR spectra compared with the standard compounds. Compound 9 was identified as kaempferol by MS and 1H-NMR spectra: yellow needles, mp 276-277°C, found M+ 286.0768, C15 H10O6, calcd 286.0788, EIMS 287 (M+), 286 (M+70), 258 (18), 229 (20), 215 (22), 153 (5), 105 (11), 93 (14), 43 (100). 1H-NMR (CD30D): 0.602 (1H, d, J=1.7 Hz C6-H), 6.4 (1H, d, J=1.7 Hz, C8-H), 6.85 (2H, d, J=9 Hz, C2'-H, C6'-H), 8.05 (2H, d, J=9 Hz, C3'-H, C5'-H).

The content of p-coumaric, ferulic, vanillic and p-hydroxybenzoic acids and p-hydroxybenzaldehyde in the soil was the highest among the nine compounds identified. The content of these five phenolics and kaempferol in the soil was: 13,382±1100, 3,542±320, 2,952±250, 2,164±300, 1,378±140 and 990±240 μg per 100 g soil. These phenolic compounds are very common inhibitory allelochemicals identified from many plant species.1,2,6,19,20,24,26,27) The total content of the five phenolics in the soil beneath Q. mongolica is higher than in the growing soils of S. cernua, Picea jezoensis, rice, corn, potato, carrot, soybean, beet and weed field, but lower than those of red pine (Table 2). These plants have been demonstrated to have allelopathy.1,3,5,7,19,25,27) Other growth inhibitors in the phenolic-butanol and neutral-EtOAc fractions are currently being identified.

The 80% methanol extracts from fresh leaves and dried fallen leaves showed strong inhibitions on seed germination and seedling growth of lettuce, green amaranth, timothy and barnyard grass (data not shown). They might be the main sources of the allelochemicals in the soil beneath Q. mongolica. The strong allelopathic effects produced by Q. mongolica might have important impact on the formation and distribution of the forestry communities and understory grasses. These allelochemicals might be washed away to the crop fields in the lower reaches of the forest and, in turn, inhibit the growth of crops.

The contents of the five phenolic compounds are very high in the soils of Q. mongolica, red pine and rice (Table 2). The allelopathic effect of red pine, which strongly inhibited the growth of other plants in its vicinity, was first reported by Banzan Kumazawa (ca. 1660's). Later, it was demonstrated that this effect was produced by phenolic compounds. Stevenson reported that soil of rice paddies in Japan and India contained high.
enough concentrations of aliphatic acid to inhibit the growth of rice and the growth of some other plants and nitrogen-fixing bacteria. p-Hydroxybenzoic, p-coumaric, vanillic, ferulic, o-hydroxyphenylacetic acids and some unidentified phenolic acids were isolated from the soil of rice fields and rice residues. This allelopathic inhibition, persistent for 4 months, was strongest in the first month of decomposition and declined thereafter. Increase of nitrogen fertilizer or rotating legume crops with rice or inoculating paddies with blue-green algae remarkably decreased the contents of the phenolic compounds, and increased rice yields by 30–100%. These results indicated that phenolic compounds are important in the allelopathy in red pine and rice. Our work showed that these five phenolic compounds may be the main, but not the only allelochemicals in the soil beneath *Q. mongolica*. The allelopathic effects and their allelochemicals of the other species of plants we used need to be studied further.

References


ミズナラ生育土壤中の他感作用物質
李 海航*••• • ラジデ ラブンミ* • 西村弘行** •
長谷川宏司*** • 水口純也*

摘 要
コナラ（Quercus）属の植物は約300種以上が北半球温帯に生育し、家具、建築、船舶、車両、酒樽等の製造に使われている重要な有用樹種である。その一種、ミズナラ（Quercus mongolica Fisch var. grosseserrata Rehd. Wils.）は北海道に多く見られる。この植物の純林と混交林とも、樹の下に雑草が成長しないことから、アレロパシーの存在が考えられたので、ミズナラの樹の下の土壌を用いて、レタス、アオヒユ、チモシーおよびコムギ植物の栽培実験によってアレロパシーを調べた。その結果、この土壌は植物の成長に対して強い抑制を示した。テストに用いた4種類の植物の成長はそれぞれ50%〜90%抑制された（Fig. 2, 3）。この土壌から中性和フェノール性物質を抽出し、それぞれ誘導体化とプレナールで分画した（Fig. 1）。レタス、アオヒユ、チモシーおよびビチの4種類の種子発芽と幼植物の生育テストにより、4つの分画の中で、フェノール性抽出物質を含む誘導体化成分は最も強い成長抑制を示した（Fig. 4, Table 1）。HPLC, EI-MS, および1H-NMR等によって、この分画から3,4-dihydroxybenzoic, p-hydroxybenzoic, 3,4,5-trime-
thoxybenzoic, 3,4-dimethoxybenzoic, vanillic, p-coumaric, ferulic acids, p-hydroxybenzaldehydeとkaempferolの9種の成長抑制物質が同定された。この内p-coumaric, ferulic, vanillic, p-hydroxybenzoic acids, p-hydroxybenzaldehydeとkaempferolは土壌中でそれぞれ13,382, 3,542, 2,952, 2,164, 1,378,と990 μg/100 g土壌と高い含量が示された。ミズナラの生育土壌中におけるこれらのフェノール性物質の総量（23,418 μg/100 g土壌）はSasa cernua（9,000）、Picea jezoensis（6,002）、イネ（21,214）、トウモロコシ（5,639）、ジャガイモ（11,055）、ニンジン（1,315）、大豆（2,720）、ビート（4,317）の生育土壌より高かったが、アカマツの生育土壌より低かった（Table 2）。以上の結果から、ミズナラの生育土壌中のフェノール性他感作用物質は森林種群および林下雑草の分布に重要な役割を果たしていると思われる。

キーワード：他感作用物質，ミズナラ，フェノール性物質，生育土壌，成長抑制