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Involvement of allelopathy in inhibition of understory growth in red pine forests

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ABSTRACT

Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) forests are characterized by sparse understory vegetation although sunlight intensity on the forest floor is sufficient for undergrowth. The possible involvement of pine allelopathy in the establishment of the sparse understory vegetation was investigated. The soil of the red pine forest floor had growth inhibitory activity on six test plant species including *Lolium multiflorum,* which was observed at the edge of the forest but not in the forest. Two growth inhibitory substances were isolated from the soil and characterized to be 15-hydroxy-7-oxodehydroabietate and 7-oxodehydroabietic acid. Those compounds are probably formed by degradation process of resin acids. Resin acids are produced by pine and delivered into the soil under the pine trees through balsam and defoliation. Threshold concentrations of 15-hydroxy-7-oxodehydroabietate and 7-oxodehydroabietic acid for the growth inhibition of *L. multiflorum* were 30 and 10 μM, respectively. The concentrations of 15-hydroxy-7-oxodehydroabietate and 7-oxodehydroabietic acid in the soil were 312 and 397 μM, respectively, which are sufficient concentrations to cause the growth inhibition because of the threshold. These results suggest that those compounds are able to work as allelopathic agents and may prevent from the invasion of herbaceous plants into the forests by inhibiting their growth. Therefore, allelopathy of red pine may be involved in the formation of the sparse understory vegetation.

1. Introduction

Pine species are the most widespread genus of Pinaceae family and form extensive forests in the northern hemisphere. Pine plants have needle-like leaves which help their adaptation to drought winter conditions. Fallen leaves accumulate and make litter layers on the forest floors. Pine forests have been known to form sparse understory vegetation since the Roman era, although sunlight intensity on the forest floor is sufficient for plant grow (Rice, 1984; Singh et al., 1999).

Plant litter has both positive and negative effects on the germination and growth of undergrowth plants (Bonanomi et al., 2006; Weidenhamer and Callaway, 2010). The litter layer on the soil of forest floors creates physical barriers which prevent plant seeds from reaching the soil (Wedin and Tilman, 1993; Bonanomi et al., 2006), and changes light intensity and the red- and far-red-light ratio of sunlight at the soil surface (Schimpf and Danz, 1999). The layer improves moisture content under water stressed conditions (Sohlberg and Bliss, 1984) and affects the microbial community, nutrient cycling and physical properties of the soil under the layer (Bais et al., 2006; Bonanomi et al., 2006). However, those effects of the litter are not unique to pine forests and cannot distinguish sparse understory vegetation of pine forests from other forests.

Plants produce a large number of secondary metabolites including unique compounds which are only produced by specific plant species. Some of those compounds have allelopathic potential such as germination and growth inhibitory activity against other plants (Gross and Parthier, 1994; Inderjit, 1996; Duke et al., 2000). After being liberated into the soil of forest floors by the decomposition processes of plant litter, some compounds with allelopathic potential affect the germination and growth of undergrowth plant species, and may interfere with the formation of forest plant communities (Rice, 1984; Caboun, 2006; Fernandez et al., 2006, 2009). Several pine species were reported to be allelopatic and allelopathy of those pine species was speculated to be one of the reasons for reduced understory growth (Rice, 1984; Harris et al., 2003; Nektarios et al., 2005; Caboun, 2006; Fernandez et al., 2013).

Red pine (*Pinus densiflora* Sieb. et Zucc.) is widespread in Eastern Asia, and forms sparse understory vegetation, and sunlight intensity on

Abbreviation: IC_{50}, the concentrations required for 50% growth inhibition

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the forest floor is relatively high (Rice, 1984). Lee and Monsi (1963) evaluated allelopathic potential of red pine against the seed germination of 15 test plant species and found that the pine leaf extracts significantly inhibited the germination of some of those plants. Several growth inhibitory substances including phenolic acids were isolated from the leaves and cones of red pine as putative allelopathic substances (Lodhi and Killingbeck, 1982; Kil et al., 1983; Node et al., 2003; Kato-Noguchi et al., 2009). However, it is unclear whether those compounds act as allelopathic agents of the red pine in the forest soil. Although plant tissues often contain potential allelopathic substances, only those liberated into the environment are able to act as allelopathic agents (Einhellig, 1999; Bais et al., 2006). The objective of this study was to investigate the allelopathic property of the red pine forest floor soil and identify potential allelopathic substances in the soil. The possible involvement of those substances in the formation of the red pine forest floor is discussed.

2. Materials and methods

2.1. Materials

Soil samples (red pine soil) were randomly collected from an around 20-year-old red pine (*Pinus densiflora* Sieb. et Zucc.) forest floor in Takamatsu, Japan in September, 2013. The area is temperate zone with an annual average temperature of 16.3 °C and average annual rainfall of 1082 mm (1981–2010; Japan Weather Association). About 100 g, 10 cm in depth of soil under litter layer of the red pine forest floors, were collected from each sampling point and about 5 kg of soil was accumulated in total. The soil moisture under the litter was 38 ± 7% (w/w; mean ± SE). After removal of organic matter and stones with a sieve (0.3 mm mesh), the soil was stored at −20 °C until extraction. Lettuce (*Lactuca sativa* L.), garden cress (*Lepidum sativum* L.) and alfalfa (*Medicago sativa* L.) were chosen for bioassay due to their stable germination rate. Weed species (*Lolium multiflorum* Lam., *Phleum pretense* L., and *Digitaria sanguinalis* L.) were also selected for bioassay. *L. multiflorum* was observed at the edge of several red pine forests.

2.2. Extraction and bioassay

After dried in the oven at 40 °C, a soil sample (100 g dry weight) was extracted with 2 L of 70% (v/v) aqueous methanol for 48 h with occasionally stirring by a spatula and filtered with filter paper (No. 2; Toyo Ltd., Tokyo). The extracts (0.2, 0.6, 2, 6 and 20 mL) were evaporated, dissolved in a 0.3 mL methanol and added onto a sheet of filter paper (No. 2) in a 3-cm Petri dish and the methanol was evaporated in a fume hood. Then, the filter paper in the Petri dishes was moistened with 0.8 mL of a 0.05% (v/v) Tween 20 solution. Ten seeds of garden cress, lettuce or alfalfa, or ten germinated seeds of *L. multiflorum*, *P. pretense* or *D. sanguinalis* were placed onto the Petri dishes as described by Kato-Noguchi et al. (2015). After incubation in the darkness at 25 °C for 48 h, the length of roots and shoots of those plants was measured and the percentage length of roots and shoots was determined by reference to those of control seedlings. For controls, 0.3 mL methanol was used instead of the extracts. The bioassay was repeated five times with 10 plants for each determination. The concentrations of the extracts required for 50% growth inhibition (defined as IC_{50}) of the test plants in the bioassay was determined by the regression equation of the concentration-response curves.

2.3. Isolation and identification of growth inhibitory substances

A soil sample (1600 g dry weight) was extracted and separated using columns of silica gel and Sephadex LH-20, reverse-phase C_{18} cartridge and HPLC as shown the process in Supplementary Fig. S1. The biological activity of all separated fractions after every purification steps was determined using a garden cress bioassay and the most active fraction was further purified. Two active fractions were found in the 60 and 80% fractions separated by reverse-phase C_{18} cartridge (Supplementary Fig. S1). The residue of the 60% fraction was finally purified by a reverse-phase HPLC (10 mm i.d. × 50 cm, ODS AQ-325; YMC Ltd.) with a flow rate of 1.5 mL min^{-1} with 65% aqueous methanol, detected at 220 nm. Inhibitory activity was found in a peak fraction eluted between 129 and 131 min (compound **1**). The residue of the 80% fraction was purified by a reverse-phase HPLC (ODS AQ-325) with a flow rate of 1.5 mL min^{-1} with 75% aqueous methanol, detected at 220 nm. Inhibitory activity was found in a peak fraction eluted between 122 and 124 min (compound **2**). Compound **1** and **2** were characterized by high-resolution mass, ^{1}H NMR and ^{13}C NMR spectra (TMS as internal standard) and optical rotation.

2.4. Bioassay of the isolated compounds in an aqueous solution

Compounds **1** and **2** (final assay concentrations: 1, 3, 10, 30, 100, 300 and 1000 μM) were dissolved in 0.2 mL of methanol, added to a sheet of filter paper (No. 2) in a 3-cm Petri dish. After evaporation of the methanol in a fume hood, the filter paper in the Petri dish was moistened with 0.8 mL of 0.05% (v/v) aqueous solution of Tween 20. Inhibitory activity and IC_{50} values of compound **1** and **2** were determined with garden cress and *L. multiflorum* as described above. The bioassay was repeated five times with 10 plants for each determination.

2.5. Concentration of compound 1 and 2 in forest soil

Red pine soil samples were collected under the litter layer of red pine forest floor and rocks and plant fragments were removed with a sieve as described above. Control soil samples were collected at the distance of 100 cm from the edge pine forest where no pine litter accumulated on the soil surface. The soil samples (100 g each) were centrifuged at 1000*g* for 20 min and the supernatant was separated. The supernatant was partitioned with ethyl acetate and the ethyl acetate fraction was then separated by a column of silica gel, a reverse-phase C_{18} cartridge and HPLC (Supplementary Fig. S2). HPLC conditions for compound 1 and 2 were used as described above. Quantification of compound **1** and **2** was performed by the interpolating the peak area on the HPLC chromatograms of samples to a standard curve constructed by the peak area of pure compound **1** and **2** isolated from pine soil. The experiments were replicated 9 times independently. The overall recovery of compound **1** and **2** (100 μM each) added to the soil sample was 67 ± 13 and 69 ± 11% (mean ± SE), respectively, as calculated from 9 replications.

2.6. Bioassay of the isolated compounds with forest soil

Soil samples were collected at the distance of 100 cm from the edge of the pine forest and remove rocks and plant fragments with a sieve. The soil was then dried at room temperature and 10 g of dried soil was placed in a 6-cm Petri dish. Compound **1** (7.7 μg: assay concentration; 3.7 μM, and 646 μg: assay concentration; 312 μM) and compound **2** (9.8 μg: assay concentration; 5.2 μM, and 750 μg: assay concentration; 397 μM) were dissolved in 1 mL of methanol, added to soil surface in the Petri dish, and the methanol was evaporated in a fume hood. The soil was mixed and moistened with 6 mL distilled water as described by Duke et al. (2009). Twenty seedlings of *L. multiflorum* were planted on the soil in the Petri dish and inhibitory activity of compounds **1** and **2** was determined as described earlier. For the control treatment, only methanol (1 mL) was used instead of the methanol solution of compounds. Control seedlings were then placed on the soil moistened with 6 mL distilled water. The bioassay was repeated three times with 20 plants for each determination.