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Allelopathic mechanisms in community resistance to exotic plants invasions: Allelopathic effects of *Pinus densiflora* Siebold and Zucc. and *P. thunbergii* Parlat. on the exotic invasive plant *Rhus typhina* L.

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ABSTRACT

In laboratory bioassay and pot culture, we tested the allelopathic effects of fresh leaf and stem aqueous extracts from the native species *Pinus densiflora* Siebold and Zucc. and the introduced species *Pinus thunbergii* Parlat. (origin East Asia) on the seed germination and seedling growth of exotic plant *Rhus typhina* L. In laboratory bioassay, the allelopathic inhibitory effects of *P. densiflora* were stronger than *P. thunbergii*, on the seed germination of *R. typhina* (except germination speed index (GSI) at high (0.1 g/mL) concentrations). Consistent with the laboratory bioassays, in pot culture experiments also the *P. densiflora* showed stronger inhibitory effects than *P. thunbergii* on seedling biomass accumulation of *R. typhina*. Further, we selected 9-common phenolic acids (*p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, salicylic acid, cinnamic acid) to determine their concentrations in *P. densiflora* and *P. thunbergii* soils. We found *P. densiflora* secreted more allelochemicals (except vanillic acid) into its surrounding soil compared to *P. thunbergii*. The results suggested that the allelopathy of some native dominant trees could affect the invasion potential of exotic plants in different forests types, implying that plant-plant interactions may play important role in determining the habitat invasion resistance.

Key words: Allelopathy, exotic plant, invasion resistance, lab bioassay, native dominant species, phenolic acids, *Pinus densiflora*., *Pinus thunbergii*, pot culture, *Rhus typhina*

In invasion ecology, the understanding the mechanisms underlying the community resistance to invasion has become a concern in current research (4,16,26). The biotic resistance of native communities against invasive species is enhanced by the competitive traits at early stage of establishment (35). Allelopathy is of great ecological interest and is very important competitive trait for plant-plant interactions (5,25). It has been widely studied as one of the mechanisms underlying the invasion success of exotic plant species (1,2,3,20). However, the allelopathic effects of native plant species on exotic plants have been largely neglected (6,15,21,23). Recently, some researchers indicated that the allelopathy of native species is important in the selection of native species to control invasive plants (5).

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Pinus densiflora Siebold and Zucc. (*P. densiflora*) (family), an evergreen Pinaceae conifer grows around the Yellow Sea [in Japan, Korea, northeastern China and Southeast Russia (42)]. It is common dominant tree species in early-succession stages of forest communities in hilly areas of Shandong Peninsula, China. *Pinus thunbergii* Parlat. (*P. thunbergii*) is native to Japan and Korea (origin East Asia) and has been introduced to China (8). Since *P. thunbergii* has capability to tolerate high salinity and drought, it is often used for afforestation in eastern coastal Chinese regions viz., Shandong and Jiangsu provinces (9,41). Field investigations in Shandong Peninsula had revealed that communities dominated by *P. thunbergii* were more vulnerable to exotic plant invasions than those dominated by *P. densiflora* (13). Both *P. densiflora* and *P. thunbergii* secretes allelopathic chemicals (18,19,32,37) by root exudation, leaching, decomposition of residues, volatilization, etc. However, it is unclear how the invaders are affected by the allelochemicals released by these two plant species? Do higher concentrations of allelochemicals in the rhizosphere soil surrounding *P. densiflora* explain the different invasion resistance than *P. thunbergii*?

Rhus typhina L., (family Anacardiaceae) is deciduous, large shrub or small tree native to eastern North America (38). It was introduced to China in 1959 to rehabilitate the degraded mountain areas in northern China (29) due to its high reproduction and spread rate. It is widely distributed and very harmful to other plants (30,32), hence has been identified as an invasive species in China (31).

This study aimed to provide evidence that allelopathy can play role in the resistance of native plant communities against invasion by exotic plants. In this study, we tested the allelopathic effects of fresh leaf and stem aqueous extracts from *P. densiflora* and *P. thunbergii* in laboratory bioassay and pot culture on the seed germination and seedling biomass accumulation of exotic plant *R. typhina*. We also determined the concentration of phenolic acids in the *P. densiflora* and *P. thunbergii* soils (top 10 cm soil layer underneath tree canopy).

METHODS AND MATERIALS

I. Study Site

Zhenshan Mountain (Yantai, Shandong) is located along the coast of Yellow Sea, on the Northern Shandong Peninsula, China. It has continental monsoon climate (mean annual rainfall: 740.3 mm) and daily mean annual temperature: 12°C. The mean height of Zhenshan Mountain (N37°30'-37°32', E121°19'-121°21') is 230-250 m above sea level. Due to frequent human activity, the original vegetation has been destroyed and the existing vegetation is mainly comprised of secondary types. The common dominant tree species are: *Robinia pseudoacacia* L., *P. thunbergii*, *P. densiflora* and *Quercus acutissima* Carruth. (39). In 2005, *R. typhina* was planted on Zhenshan Mountain along the roadside for ornamental purposes. Now it has rapidly spread and invaded the surrounding habitat, even becoming the single dominant specie in some habitats (13).

II. Laboratory Bioassays

In June 2016, fresh leaves and stems of *P. densiflora* and *P. thunbergii* were collected from Zhenshan Mountain. The leaves and stems were air-dried in shade for 2 weeks and 400 g plant sample was weighed and immersed in 4.0 L distilled water for 24 h. The extract was filtered to prepare the stock solution (0.1 $g \cdot mL^{-1}$), from which 3 concentrations (0.1 $g \cdot mL^{-1}$, 0.05 $g \cdot mL^{-1}$ and 0.025 $g \cdot mL^{-1}$) of aqueous extracts were prepared with distilled water. All solutions were stored in refrigerator until use.

Seeds of *R. typhina* were collected from Zhenshan Mountain and their dormancy was broken as under before use. The seeds were heated to high temperature (70-90°C) in hot water for 30 min, then temperature was lowered to 40°C and the seeds were naturally cooled and immersed in water for 24 h (34). After dormancy was broken, the seeds were disinfected with 0.3% potassium permanganate for 2 h and then washed with distilled water.

A laboratory bioassay was conducted to determine the allelopathic effects of leaf and stem aqueous extracts from *P. densiflora* and *P. thunbergii* on the seed germination and seedling growth of *R. typhina* in the laboratory. After breaking the dormancy of the *R. typhina* seeds, 20-seeds were placed in 9-cm-dia petri dishes lined with 2-layers of filter paper. The experiment was replicated six times. Initially, 5 mL of leaf and stem aqueous extracts were added and distilled water (Control) was added per petri plate as per treatments. The petri plates were kept in incubator [light 12 h (25°C)/dark 12 h (25°C) and relative humidity (65%)] for 10-days. The petri plates were observed daily and equal amount of extract was added to each petri plate as needed to prevent drying out of seeds or seedlings.

Seed germination was defined as 1 mm long radicle protrusion through the seed coat. The number of germinated seeds was recorded at 24 h intervals for 10-days. The seed germination viability of *R. typhina* was evaluated by SGR and GSI. These were calculated as under:

(i). Seed germination rate (SGR):

SGR = (Number of germinated seeds)/20×100%

(ii). Germination speed index (GSI):

$$GSI = 2(5X_1 + 4X_2 + 3X_3 + 2X_4 + X_5),$$

Where, X_i: Number of seeds germinated at 24-h intervals, X_1: Number of seeds germinated on day 1, X_2: Number of seeds germinated on day 2, etc. (36).

Allelopathic effects: The response index (RI) was calculated as under as per the method of Williamson and Richardson (33):

$$RI = 1 - C/T \ (T > C)$$

$$RI = T/C - 1 \ (T < C),$$

Where, T: Treatment data and C: Control data.

III. Pot culture

To further elucidate the effects of leaf and stem aqueous extracts from *P. densiflora* and *P. thunbergii* on *R. typhina* seedling growth, pot experiments were done in greenhouse [26°C/15°C (day/night)] with ambient light from July to September, 2016. The pots (8 cms height, 5 cms dia) were filled with the same, dry soil (180 g per pot) collected from the non-forested land on Zhenshan Mountain. The soil's chemical properties were pH 7.77, total C: 17.37 g/kg, total N: 0.98 g/kg, total P: 2.69 g/kg, total K: 15.67 g/kg, ammonium N: 14.298 mg/kg, nitrate N: 6.864 mg/kg, available P: 47.29 mg/kg and available K: 77.57 mg/kg. To obtain similar size seedlings, *R. typhina* dormancy broken seeds were sown in seedling bed. When the seedlings were 3 cms tall (10 days after sowing), uniform seedlings were selected and one seedling was transplanted per pot. Ten ml extracts of *P. densiflora* and *P. thunbergii* were added weekly to pots as per treatments and distilled water was used as control. Treatments were replicated 6 times. All the pots were watered with 5 mL distilled water once a week to keep the soil moist.

R. typhina was harvested after 50 days. Shoots were immediately clipped at the ground level. Roots were rinsed gently with water to remove any adhering soil particles. The shoots and roots of *R. typhina* were dried for 72 h at 60℃ and then weighed.

IV. Detection of phenolic acids in soil of *P. densiflora* and *P. thunbergii*

Soil samples were collected during April 2018 from a *P. densiflora* forest and a *P. thunbergii* forest. The distance between the two communities was > 100 m. The samples were collected from the top 10 cm soil layer. Every 6 sampling points were mixed and homogenised into one composite soil sample and there were a total of 3 soil samples for each forest type. The sampling site characteristics are shown in Table 1.

Table 1. Sampling sites characteristics

Sample plot	latitude	Longitude	Elevation (m)	Slope (°)	Slope aspect (°)
Pinus densiflora	37°31′18″-37°31′50″N	121°20′34″-121°21′0″E	167-171	8-15	180-196
Pinus thunbergii	37°30′32″-37°31′40″N	121°20′33″-121°21′26″E	135-140	10-18	200-210

The method used for soil extraction was modification of Hartley and Buchan (10). Briefly, 25 g fresh soil was added to 25 mL 1 mol/L NaOH solution. It was stored overnight and agitated for 30 min on reciprocal shaker next day. The soil suspension was centrifuged and the supernatant was filtered through filter paper. The suspension was then acidified to pH 2.5 with 12 mol/L HCL. After 2 h, the humic acid was removed by centrifugation and the supernatant was then filtered through 0.22-μm filter. The final extracts were analysed using high-performance liquid chromatography (HPLC) and the results were converted to dry soil weight.

Liquid chromatography was done in Waters HPLC system (Waters, e2695, USA) with a diode array detector, using a Uranus C18 column (250 × 4.6 mm, 5 μm) and guard column (20 ×4.6 mm, 5 μm). Phenolic compound standards were purchased from Sigma (St. Louis, MO). Detection was performed at 280 nm. The chromatographic data were recorded and processed with Waters empower workstation. Different phenolic acids were identified by their retention time with authentic standards. Standards of nine phenolic acids (*p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, salicylic acid, cinnamic acid) were prepared in different concentrations. The standard curve equations for the nine phenolic acids were obtained by taking the peak area of liquid chromatogram as Y and the sample concentrations as X (Table 2). The concentrations of each compound in the soil samples were obtained based on peak areas.

Table 2. Standard curve equations of the nine phenolic acids

Standard substances	Standard curve equations	Regression degree	Linear ranges
1. *p*-Coumaric acid	Y=31530x-28246	0.9999	0.001-0.1
2. Protocatechuic acid	Y=27889x-25769	0.9999	0.001-0.1
3. *p*-Hydroxybenzoic acid	Y=29916x-27528	0.9999	0.001-0.1
4. Vanillic acid	Y=35119x-31949	0.9999	0.001-0.1
5. Caffeic acid	Y=72852x-65440	0.9999	0.001-0.1
6. Syringic acid	Y=57725x-52334	0.9999	0.001-0.1
7. Ferulic acid	Y=52004x-46029	0.9999	0.001-0.1
8. Salicylic acid	Y=10682x-10920	0.9999	0.001-0.1
9. Cinnamic acid	Y=170030x-146718	0.9999	0.001-0.1

HPLC separations were done using the following mobile phase solutions: mobile phase of aqueous formic acid solution (0.1%, v/v) and acetonitrile, column temperature of 30 °C, 20 μL of injection volume, flow rate of 1.0 $mL \cdot min^{-1}$ and gradient of 6-10% acetonitrile (0-16 min), 10-22% (16-36 min), 22-50% (36-46 min), 50-100% (46-48 min) and held for 5 min. Column was equilibrated for 10 min between injections.

V. Statistical analysis

A one-way ANOVA was used to test the effects of each treatment on the SGR, GSI and total biomass of *R. typhina* and to test the effects of different soil origins on each phenolic acid concentration. A Duncan test was used to identify statistically significant differences at $P < 0.05$ using the SPSS 19.0 statistical software package. Figures were processed using EXCEL.

RESULTS AND DISCUSSION

I. Laboratory bioassays

The leaf and stem aqueous extracts from *P. densiflora* and *P. thunbergii* had variable effects on the germination parameters (SGR and GSI) of *R. typhina* compared to distilled water control (Figure 1,2). *P. densiflora* showed stronger inhibitory effects on the SGR of *R. typhina* at high (0.1 g/mL) concentrations than distilled water control and *P. densiflora* showed stronger inhibitory effects on the GSI of *R. typhina* at the high (0.1 g/mL) concentrations than control (Figure 1). There were no significant differences between the aqueous extracts of *P. densiflora* and *P. thunbergii* on seed germination and other treatments (Figure 1).

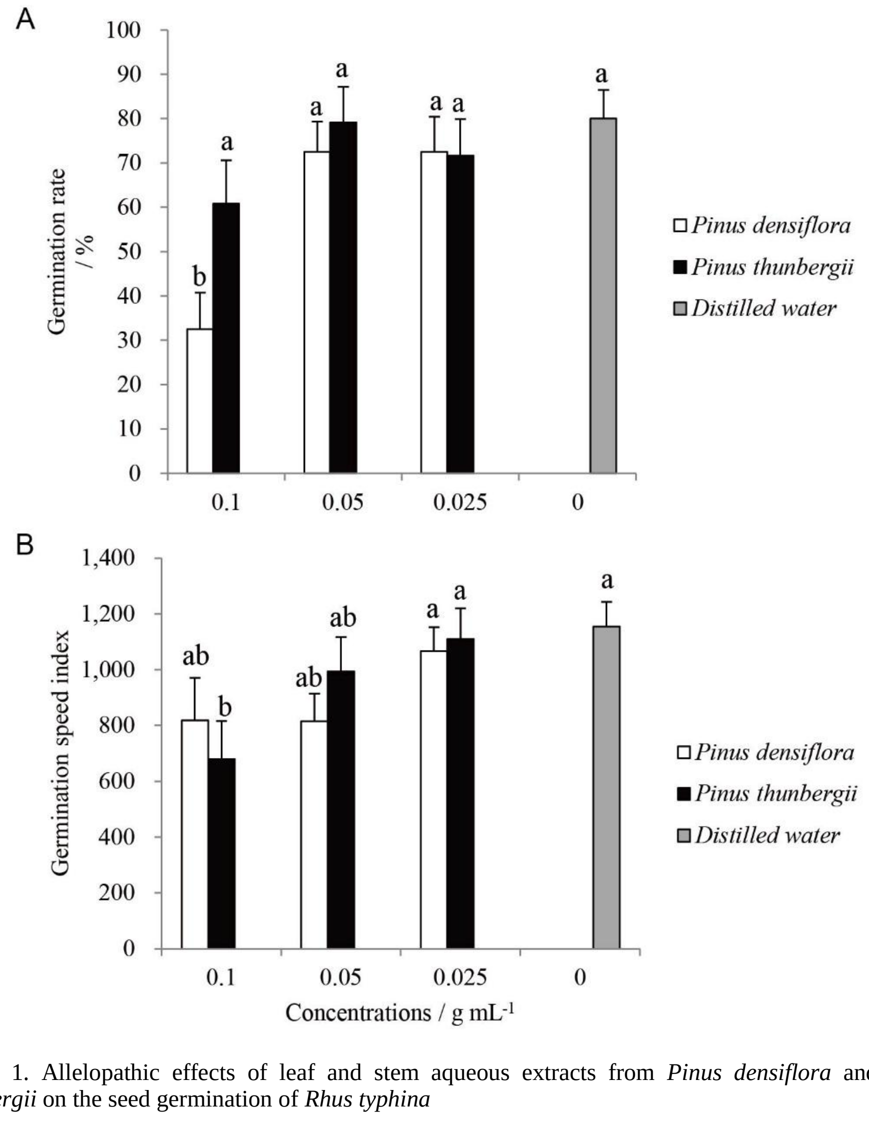

Figure 1. Allelopathic effects of leaf and stem aqueous extracts from *Pinus densiflora* and *P. thunbergii* on the seed germination of *Rhus typhina*

Seed germination is essential for plant species. The SGR and GSI greatly influence the future growth of species in plant community. A high SGR and GSI improve the early competitiveness for the aboveground and underground resources (28). In laboratory bioassay, the allelopathic inhibitory effects of *P. densiflora* were stronger on the seed germination of *R. typhina* than *P. thunbergii* (except for GSI under high concentration, (Figure 2); therefore, it will affect the survival and subsequent growth of exotic plant species.

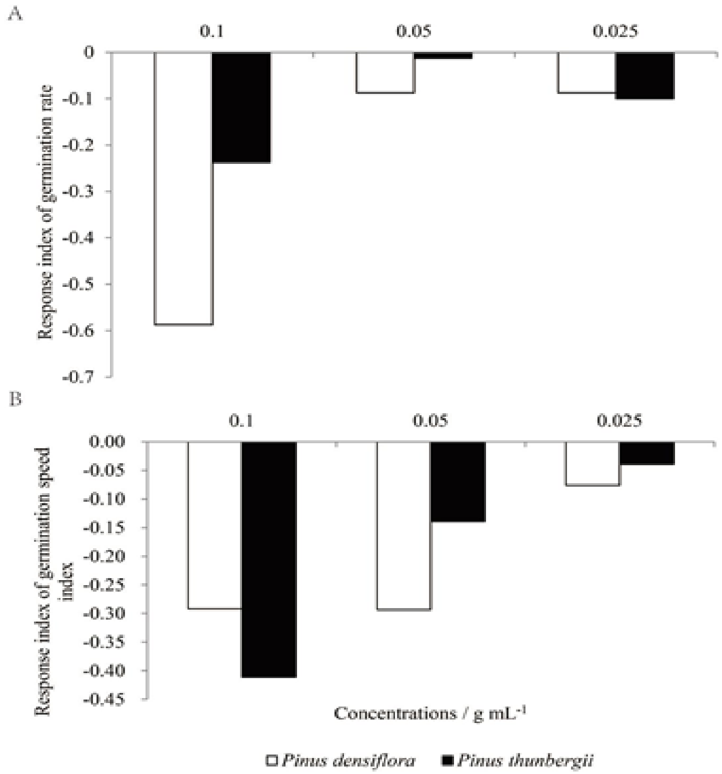

Figure 2. Response index of seed germination of *Rhus typhina* to the leaf and stem aqueous extracts from *Pinus densiflora* and *P. thunbergii*

II. Pot culture

Laboratory bioassay is useful method to study the plant allelopathic effects for shorter duration, however, it lacks a soil medium (24). Soil is most important mediums for plant-plant interactions, in which allelopathic chemicals function directly, or with help of soil biota (14,17). To further elucidate the effects of leaf and stem aqueous extracts from *Pinus densiflora* and *P. thunbergii* on *R. typhina* seedling growth, pot culture experiments were done in greenhouse. The results showed that there were no significant differences between the aqueous extracts from the two *Pinus* species and distilled water control on seedling biomass. However, compared to *P. thunbergii* the *P. densiflora* was more inhibitory to seedling biomass of *R. typhina* at low (0.025 g/mL) concentrations (Figure 3). There were no significant differences between the aqueous extracts from *P. densiflora* and *P. thunbergii* on seedling biomass at other concentrations (Figure 3). With regards to the RI of total biomass, *P. densiflora* was inhibitory at all three concentrations of extracts. Whereas, *P. thunbergii* was stimulatory to biomass accumulation of *R. typhina* at all extract concentrations (Figure 3). Overall, the results of pot culture indicated that the inhibitory effects of *P. densiflora* were stronger than *P. thunbergii* on *R. typhina* growth. In field investigations, less *R. typhina* invasions were found in *P. densiflora* forests than in *P. thunbergii* forests. One possible reason might be that the allelochemicals released by *P. densiflora* suppressed the seed germination and seedling growth of *R. typhina*.

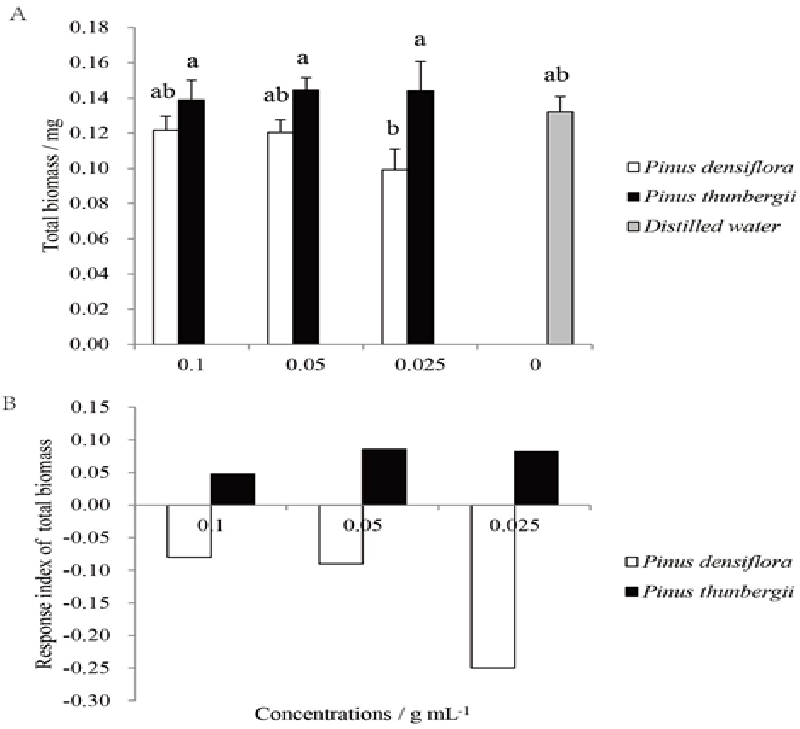

Figure 3. Allelopathic effects of leaf and stem aqueous extracts from *Pinus densiflora* and *P. thunbergii* on the seedling total biomass of *Rhus typhina*

III. Phenolic acids in soils

Phenolic acids are important allelopathic chemicals in soil and have been widely studied (11). Allelopathic chemicals are excreted directly into the soil, leached from plant tissues or released when dead plant material decays (12,22). Most of the allelochemicals eventually enter the soil and a series of interactions occur with soil abiotic factors (soil nutrients, temperature, moisture, etc.) and biotic factors (soil microbes, soil animals, etc.), creating lasting effects, indirectly affecting the performance of subsequent plants (10). Therefore, in this study, we selected nine common phenolic acids (*p*-coumaric acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, salicylic acid, cinnamic acid) to determine their concentrations in *P. densiflora* and *P. thunbergii* soils.

The results showed that the concentrations of *p*-coumaric acid and ferulic acid in *P. densiflora* soil were significantly higher than in *P. thunbergii* soil and the non-forested land control (Table 3). The concentration of *p*-coumaric acid in *P. densiflora* soil was 115 % and 276 % higher than in *P. thunbergii* soil and the control soil, respectively. The concentration of ferulic acid in *P. densiflora* soil was 170 % and 163 % higher than in *P. thunbergii* soil and control soil, respectively. The caffee acid and cinnamic acid followed the order : *P. densiflora* soil > *P. thunbergii* soil > blank control. *P. densiflora* soil had more caffee acid than control soil, but there was no difference between the *P. densiflora* and *P. thunbergii* soils (Table 3).

In contrast to above phenolic acids, the concentration of vanillic acid in *P. thunbergii* soil was 126 % higher than in *P. densiflora* soil and 67 % higher than control soil (Table 3). In addition, there was no significant difference in protocatechuic acid, *p*-hydroxybenzoic acid, syringic acid and salicylic acid among the three different soil origins viz., *P. densiflora, P. thunbergii* and control soils (Table 3).

Table 3. Detection of nine phenolic compounds in soil samples from the top 10 cm of soil in *P. densiflora* and *P. thunbergii* forests.

Phenolic compounds	Soil samples (µg g^{-1})		
	Blank control	*Pinus densiflora*	*Pinus thunbergii*
1. *p*-Coumaric acid	2.12±0.25b	7.98±1.78a	3.72±0.42b
2. Protocatechuic acid	1.21±0.04a	1.49±0.17a	1.35±0.11a
3. *p*-Hydroxybenzoic acid	1.19±0.03a	1.28±0.07a	2.62±0.72a
4. Vanillic acid	1.75±0.20b	1.29±0.14b	2.92±0.32a
5. Caffeic acid	2.38±0.25b	6.87±1.78a	3.55±0.40ab
6. Syringic acid	1.34±0.08a	1.24±0.08a	1.52±0.11a
7. Ferulic acid	1.96±0.50b	5.16±0.91a	1.91±0.37b
8. Salicylic acid	1.28±0.02a	51.31±37.15a	2.63±0.38a
9. Cinnamic acid	0.98±0.00b	1.82±0.11a	1.34±0.26ab

Data are means ± SE, n = 3, different lowercase letters in a row indicate statistically significant differences ($P < 0.05$).

The biotic resistance of native communities against invasive species at early stage of establishment is enhanced by certain competitive traits (35). Allelopathy is a very important competitive trait for plant-plant interactions (5,40). In the present study in laboratory bioassays, *P. densiflora* showed stronger inhibitory effects on the seed germination of *R. typhina*, especially at high concentrations (0.1 g/mL). In pot culture, there were no significant differences between the aqueous extracts from the two species and the distilled water control on seedling biomass. However, compared to *P. thunbergii* the *P. densiflora* still showed stronger inhibitory effects on the seedling biomass of *R. typhina* at some concentrations. Meanwhile, we found that *P. densiflora* could secrete more phenolic acids (except vanillic acid) into its surrounding soils compared to *P.*

thunbergii. Besides phenolic compounds, the mechanism of allelopathy involves the interaction of other classes of chemicals such as flavonoids, alkaloids, steroids, terpenoids and amino acids (22). Although only phenolic acids in the soil were measured in this study, the study of soil pot culture provides an evidence for the field phenomenon that there are far fewer invasive plants of *R. typhina* in *P. densiflora* forests than in *P. thunbergii* forests, perhaps due to some allelopathic interactions.

CONCLUSIONS

We found that the allelopathic effects of native species, *P. densiflora*, exerted more inhibitory effects on the growth of exotic species *R. typhina* than the introduced specie *P. thunbergii*. Thus the allelopathy of some native dominant trees could affect the invasion potential of exotic plants in different forests types. This study showed that plant-plant interactions played important role in determining the invasion resistance of habitat. Additionally, the phytotoxic potential of specific plant species can be further explored to develop new non-chemical herbicides to control exotic plants invasions.

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