0971-4693/94 US $ 5.00
Allelopathy Journal 15(1): 57-66 (2005)
Tables: 2, Figs : 3

Effects of Phenolics on seedling growth and ^{15}N nitrate absorption of *Cunninghamia lanceolata*

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(Received in revised form: November 26, 2004)

ABSTRACT

Chinese fir (*Cunninghamia lanceolata*) has replant problem under field conditions and has been attributed to phenolic allelochemicals, which cause its autotoxicity. To determine the mechanism of inhibitory effects of phenolics on Chinese fir, we studied their effects on growth and ^{15}N nitrate absorption by each organ of its seedlings, cultured in pots using ^{15}N-labelled isotope trace technique. The 1×10^{-3} $mol\cdot L^{-1}$ vanillic acid and phenolics mixture (0.5×10^{-3} $mol\cdot L^{-1}$ vanillic acid and 0.5×10^{-3} $mol\cdot L^{-1}$ ρ-hydroxybenzoic acid) significantly inhibited the seedling growth of Chinese fir. Phenolics solution of 1×10^{-2} $mol\cdot L^{-1}$ concentration decreased total N content of roots, stems and leaves. ^{15}N derived from the fertilizer (NDFF) in roots, stems and leaves treated with 1×10^{-2} $mol\cdot L^{-1}$ vanillic acid was 36.1, 42.7 and 38.5% less, respectively, and corresponding values for phenolics mixture at the same concentration were 14.1, 17.2, and 17.0% lower, respectively, compared to control. In contrast, vanillic acid and ρ-hydroxybenzoic acid at 1×10^{-5} $mol\cdot L^{-1}$ stimulated the seedling growth, enhanced total ^{15}N content and increased ^{15}N nitrate absorption. These treatments also increased the ^{15}N nitrate distribution in roots and reduced its distribution in stems and leaves. We suggest that accumulated phenolics in soil could inhibit seedling growth through reducing nutrient absorption by Chinese fir and consequently leading to low productivity of replanted Chinese fir.

Key Words: Allelochemicals, Chinese fir, ρ-hydroxybenzoic acid, ^{15}N, Phenolics, Total N content, Tracer, Vanillic acid.

INTRODUCTION

Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook.] is a fast-growing tree specie native to China and is planted commercially for timber (3). Due to its growing demand for timber, its pure plantations have been extensively replanted on the same site in successive rotations. This has resulted in serious problems of soil fertility degradation and productivity decline that draw the attention of foresters, microbiologists, soil scientists and ecologists (2,16,21). The amount of nitrates decreased during the growth and development period of pure Chinese fir plantation (2,3,23). Simultaneously, the population of soil microbes also declined year by year, biochemical activity and oxidative-metabolic capacity was also reduced (2,23) and the soil structure degraded seriously in second and third rotations (21). The resultant soil degradation, has led to poor establishment and decreased productivity of Chinese fir plantations.

Recent studies indicated that the inhibitory effect of allelochemicals in the Chinese fir plantations may be one of the causes of soil degradation (4,12,13), poor establishment and lower productivity (5,17). Growth of Chinese fir seedlings is inhibited by the extracts of soil and decomposing roots in the replanted area (25). Zhang (26) have isolated and identified five allelochemicals, including *ρ*-hydroxybenzoic acid, *ρ*-coumaric acid, gallic acid, α-coumaric acid and vanillic acid in the soil extracts from the second and third rotation woodlands of Chinese fir. Huang (12,13) showed that compounds (vanillic acid, *ρ*-hydroxybenzoic acid and ferulic acid) extracted from stump roots of Chinese fir inhibited the growth of its own seedlings. Most studies have determined the inhibitory effects on seedling growth and few studies have focussed on the growth-inhibiting mechanism of allelochemicals on Chinese fir.

Yu and Matsui (24) found that cinnamic acid, a main component of root exudates of cucumber, can inhibit ion uptake of NO_3^-, SO_4^{2-}, K^+, Ca^{2+} and Fe^{2+} in nutrient culture. Nutrient absorption, especially N, is very important for the growth, development, flowering and fruiting of Chinese fir and other plants. Hence, its absorption, transport, transfer and distribution have been studied using ^{15}N-labelled isotope trace techniques (1,7,9,10,18).

This study aims to further elucidate the mechanism of Chinese fir allelopathy, hence, we examined the effects of phenolics, vanillic acid and *ρ*-hydroxybenzoic acid on seedling growth and the effect of these phenolics on absorption and distribution of nitrogen.

MATERIALS AND METHODS

The experiment was conducted at Huitong Forest Ecology Experimental Station, Huitong County, Hunan Province (longitude 109°26´ to 110°08´ E and latitude 26°40´ to 27°09´ N, 400-600 m above sea level; Annual mean temperature: 16.5 ºC, relative humidity: 80%, and annual mean rainfall: 1200 to 1400 mm). Soil is red-yellow.

Pot Culture

The experiment consisted of two factors: three compounds (vanillic acid, *ρ*-hydroxybenzoic acid, and their mixture) and their five concentrations (0, 1×10^{-2}, 1×10^{-3}, 1×10^{-4} and 1×10^{-5} mol·L^{-1} phenolic solution).

To obtain 1 mol·L^{-1} mother solution, weighed 15.2 g vanillic acid and 13.8 g *ρ*-hydroxybenzoic acid (Analytical Grade, Sigma Co.), dissolved them in 100 ml ethanol, respectively, and then the solutions of 1 M concentration of vanillic acid and *ρ*-hydroxybenzoic acid were obtained. Keep them in Refrigerator at –4^{0}C. When used, the mother solutions of phenolics acid were diluted to provide 1×10^{-2} mol·L^{-1}, 1×10^{-3} mol·L^{-1}, 1×10^{-4} mol·L^{-1} and 1×10^{-5} mol· L^{-1} solutions having the same ethanol concentration (1×10^{-2} mol·L^{-1}).

Treatment concentrations of 1×10^{-2} mol·L^{-1} were prepared by transferring 0.5 ml of both mother solutions of vanillic acid and *ρ*-hydroxybenzoic acid into 100 ml of distilled water. Similar procedures were used to obtain the 1×10^{-3} mol·L^{-1}, 1×10^{-4} mol·L^{-1} and 1×10^{-5} mol·L^{-1} concentrations. Ethanol concentration in all solutions was 1×10^{-2} mol·L^{-1}, including the distilled water control.

Experimental Procedure

The soil used for seedling pots was obtained from *Schima superba* plantation. Stones and roots were removed and the soil was mixed with fine sand (soil: sand in ratio of 2: 1). Pots (30 cm dia, 25 cm depth) were filled with soil from evergreen-broad forest. Vigorous, uniform one year old Chinese fir seedlings bought from Guangping nursery in Huitong County, Hunan province, were planted in pots from February 18 to 28, 2000. The seedlings pot were kept in greenhouse and irrigated with 100 ml of single phenolic compound solution or mixed phenolic solution as per treatments every two weeks. Relative humidity was maintained at 80 % and soil moisture content at 65 %. The treatments were replicated five times. After the establishment of seedlings in April, every pot was fertilized with 200 mg $Na^{15}NO_3$ and the seedlings were harvested after 8 months. The ^{15}N nutrition was $Na^{15}NO_3$ at 21.97 atom %, made by Shanghai Academy of Chemical Technology. When seedlings growth ceased in December, measurements were made of seedling heights and stem diameter. Then, all seedlings were harvested and partitioned into roots, stems and leaves and oven-dried at 65 ºC for 48 h and their dry weights were determined.

Chemical Analysis of Plant Samples

Every parts of seedlings were milled into powder and the each parts powder was mixed evenly. Care was taken to avoid contamination from each other sample. All samples were analyzed for total N and ^{15}N using Isotope Ratio Mass Spectrometry (MAT-251, USA). Replicates of each treatment were mixed and one representative sample was analyzed, hence, calculation of analysis of variance of ^{15}N absorption and distribution data was not possible.

Data Analysis

The percentage of ^{15}N-labelled fertilizer in roots, stems, and leaves was calculated from ^{15}N abundance in the former samples using Eq. (1):

$$\%\ \text{NDFF} = \left(\frac{\text{atom \% }^{15}\text{N excess in each organs}}{\text{atom \% }^{15}\text{N excess in fertilizer}} \right) \times 100 \qquad (1)$$

Where, % NDFF is the percentage of ^{15}N derived from the fertilizer.

Nitrogen derived from fertilizer (mg/pot) in each seedling organ coming from the labeled NO_3^- of the fertilizer was calculated using Eq. (2):

$$\text{NDFF} = \left(\frac{\text{atom \% }^{15}\text{N excess in each organs}}{\text{atom \% }^{15}\text{N excess in fertilizer}} \right) \times (\text{yield of N in each organs}) \times 1000 \qquad (2)$$

Where, NDFF is the yield of ^{15}N derived from the fertilizer in each seedling organ. Atom % ^{15}N excess in fertilizer is 21.97%.

For seedlings treated with vanillic acid, *ρ*-hydroxybenzoic acid and phenolics mixture, the ratio of ^{15}N absorption in each seedling organ to ^{15}N absorption in all organs was calculated using Eq. (3):

$$\left(\begin{array}{l} \text{Ratio of }^{15}\text{N absorption in each organ} \\ \text{to }^{15}\text{N absorption in all organs} \end{array} \right) = \left(\frac{\text{NDFF in each seedling organ}}{\text{the sum of NDFF in roots, stems and leaves}} \right) \qquad (3)$$

RESULTS

The heights of seedlings treated with 1×10^{-3} mol·L^{-1} and 1×10^{-2} mol·L^{-1} vanillic acid were significantly reduced by 7.6 % and 4.7 % ($p < 0.1$), respectively, over the control (Fig. 1).

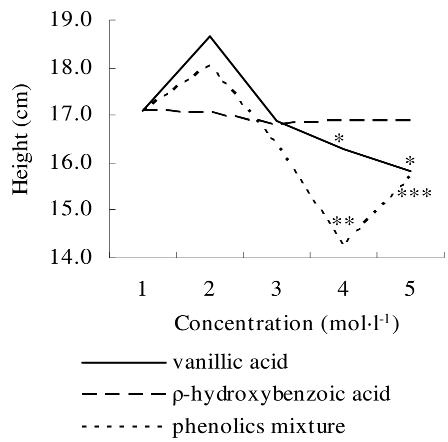

Fig. 1 Effects of vanillic acid and *ρ*-hydroxybenzoic acid and their mixture on height of Chinese fir seedlings. Statistical significance difference using the student t-test between adjacent treatments are indicated (*p<0.1, **p<0.05, ***p<0.01). 1= control; 2 = 1×10^{-5} mol·L^{-1}; 3 = 1×10^{-4} mol·L^{-1}; 4 = 1×10^{-3} mol·L^{-1}; 5 = 1×10^{-2} mol·L^{-1}.

The phenolics mixture at 1×10^{-2} mol·L^{-1} decreased (8.2 %) the height of fir seedlings than control ($p < 0.01$). Likewise, phenolics mixture at 1×10^{-3} mol·L^{-1} also significantly reduced the fir seedling height than control ($p < 0.05$). However different concentrations of *ρ*-hydroxybenzoic acid did not significantly reduced the seedlings height over the control.

Effects of phenolics on total N of each organ

Higher concentration of phenolics can affect the total N content of each seedling organ (Fig. 2). Phenolics solution concentration (1×10^{-2} mol·L^{-1}) reduced the total N content of roots, stems and leaves. Vanillic acid concentration of 1×10^{-2} mol·L^{-1} reduced the total N content of stems and leaves by 10.1 and 8.3 %, respectively, than control. While *ρ*-hydroxybenzoic acid solution at the same concentration decreased the total N content of roots, stems and leaves by 6.3, 2.2, and 10.4 % than control, respectively. Total N content of roots, stems and leaves treated with 1×10^{-2} mol·L^{-1} phenolics mixture solution were 8.4, 30.2, and 30.0 % lower than the control, respectively.

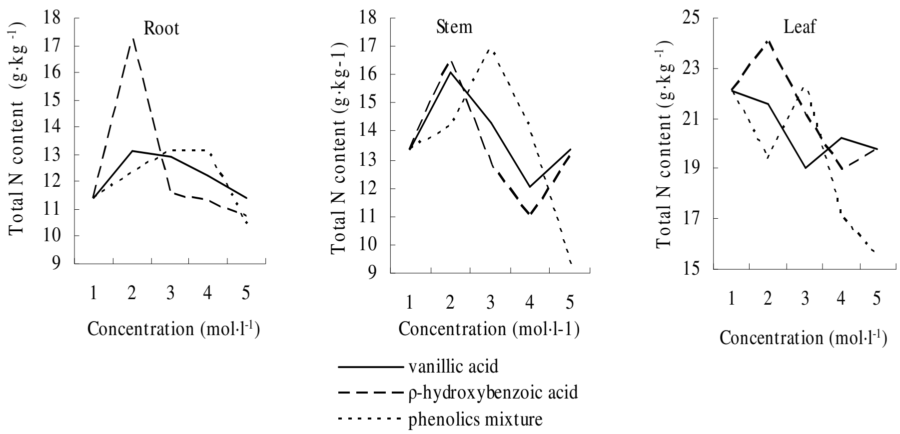

Fig. 2. Effects of vanillic acid and *ρ*-hydroxybenzoic acid and their mixture on total N content of each organ of Chinese fir seedlings. 1 = control; 2 = 1×10^{-5} mol·L^{-1}; 3 = 1×10^{-4} mol·L^{-1}; 4 = 1×10^{-3} mol·L^{-1}; 5 = 1×10^{-2} mol·L^{-1}

Compared with vanillic acid and *ρ*-hydroxybenzoic acid, the maximum total N content of seedlings with phenolics mixture treatment was at lower concentrations. Total N content of each seedling organ treated with 1×10^{-5} mol·L^{-1} vanillic acid and *ρ*-hydroxybenzoic acid was greater than that treated with phenolics mixture. However, total N content of each seedling organ with 1×10^{-4} mol·L^{-1} phenolics mixture was greater than at other concentrations.

^{15}N absorption by seedlings

Phenolics at lower concentrations, accelerated the ^{15}N absorption by Chinese fir seedlings, but phenolics at higher concentrations may inhibit ^{15}N absorption (Table 1). %NDFF in roots, stems and leaves treated with 1×10^{-2} mol L^{-1} vanillic acid was 36.1, 42.7, and 38.5 % lower, respectively, compared with control. %NDFF in roots, stems and leaves treated with 1×10^{-2} mol·L^{-1} *ρ*-hydroxybenzoic acid was 0.1, 3.3, and 2.2 % lower, respectively, as compared to the control, and %NDFF in roots, stems and leaves treated with 1×10^{-2} mol·L^{-1} phenolics mixture were 14.1, 17.2, and 17.0 % lower than the control , respectively. %NDFF in roots, stems and leaves treated with 1×10^{-5} mol·L^{-1} vanillic acid, *ρ*-hydroxybenzoic acid and phenolics mixture were all higher than the control.

NDFF in roots, stems and leaves treated with 1×10^{-2} mol·L^{-1} vanillic acid were 38.5, 48.1, and 46.5 % lower, respectively, as compared to the control. NDFF in roots, stems and leaves treated with 1×10^{-2} mol·L^{-1} phenolics mixture were decreased by 34.3, 58.4, and 49.3 % than the control, respectively. NDFF in roots, stems and leaves treated with 1×10^{-5} mol·L^{-1} vanillic acid, *ρ*-hydroxybenzoic acid and phenolics mixture were all higher than the control, especially. Whereas NDFF in roots, stems and leaves treated with 1×10^{-5} mol·L^{-1} *ρ*-hydroxybenzoic acid were 195.8, 132.5, and 104.2 percent higher than the control, respectively (Table 1).

Table 1. Effects of phenolics on absorption of ^{15}N by Chinese fir seedlings

Treatment	Concentration ($mol \cdot L^{-1}$)	Roots		Stems		Leaves	
		%NDFF	NDFF (mg)	%NDFF	NDFF (mg)	%NDFF	NDFF (mg)
Control	-	7.37	1.43	6.46	0.77	5.58	2.15
Vanillic acid	1×10^{-5}	9.12	2.11	7.34	1.02	6.16	2.34
	1×10^{-4}	9.30	2.20	7.51	1.08	6.20	2.20
	1×10^{-3}	8.24	1.28	7.34	0.68	4.62	1.54
	1×10^{-2}	4.71	0.88	3.70	0.40	3.43	1.15
Mean		7.84	1.62	6.47	0.80	5.10	1.81
ρ-hydroxybenzoic acid	1×10^{-5}	10.71	4.23	7.05	1.79	5.69	4.39
	1×10^{-4}	7.64	2.04	6.54	1.22	5.88	4.06
	1×10^{-3}	8.34	2.06	7.40	1.02	6.15	2.60
	1×10^{-2}	7.36	1.72	6.25	0.94	5.46	2.24
Mean		7.78	1.94	6.73	1.06	5.83	2.97
Phenolics mixture	1×10^{-5}	8.16	1.85	7.17	1.19	6.47	2.49
	1×10^{-4}	8.49	1.97	6.78	1.01	5.99	2.29
	1×10^{-3}	7.26	1.07	4.86	0.39	4.07	0.95
	1×10^{-2}	6.33	0.94	5.34	0.32	4.63	1.09
Mean		7.36	1.33	5.66	0.57	4.90	1.44

% NDFF is the percentage of ^{15}N derived from the fertilizer, and NDFF is the yield of ^{15}N in each seedling organ

Effects of phenolics on ^{15}N nitrate distribution in seedlings

The ratios of ^{15}N absorption in each seedling organ to ^{15}N absorption in all organs treated with vanillic acid, *ρ*-hydroxybenzoic acid and phenolics mixture are shown in Fig. 3. Three sets of simple equations were listed in Table 2.

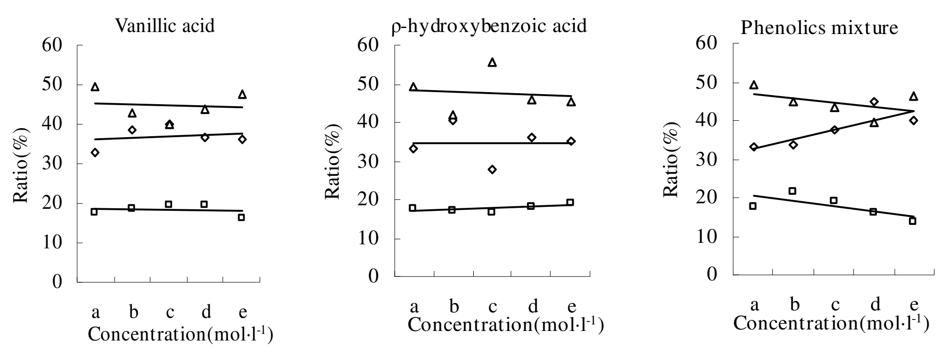

Fig. 3. Effects of vanillic acid and *ρ*-hydroxybenzoic acid and their mixture on ^{15}N nitrate distribution by Chinese fir seedlings. 1= control; 2 = 1×10^{-5} $mol \cdot L^{-1}$; 3 = 1×10^{-4} $mol \cdot L^{-1}$; 4 = 1×10^{-3} $mol \cdot L^{-1}$; 5 = 1×10^{-2} $mol \cdot L^{-1}$; = root; = stem; = leaf.

Table 2. The ratio of ^{15}N absorption in each organ to ^{15}N absorption in all organs treated with different phenolics

Treatment	Roots	Stems	Leaves
Vanillic acid	y = 0.4302 x + 35.595	y = -0.1797 x + 18.941	y = - 0.2505 x + 45.464
ρ-hydroxybenzoic acid	y = - 0.0083 x + 34.57	y = 0.37 x + 16.635	y = - 0.3616 x + 48.794
Phenolics mixture	y = 2.5024 x + 30.168	y = - 1.3525 x +21.675	y = - 1.1489 x + 48.157

The simple equations based on relationships in Fig. 3 showed that higher phenolics concentration, increased the proportion of ^{15}N absorbed in roots and decreased that absorbed in stems and leaves. The linear coefficients of equations of stems with vanillic acid and phenolics mixture were also negative and the linear coefficients of equations of roots with vanillic acid and phenolics mixture were positive. It showed that the ^{15}N distribution in stems gradually decreases and the ^{15}N distribution in roots of Chinese fir seedlings increased with concentration of vanillic acid and phenolics mixture.

DISCUSSION

Phenolics are often regarded as allelochemicals to accelerate the growth of plants at lower concentrations and inhibit at higher concentrations (8,11,12,14,22,27). Results showed that both vanillic acid and phenolics mixture (vanillic acid and *ρ*-hydroxybenzoic acid) at concentrations of 1×10^{-2} mol·L^{-1} and 1×10^{-3} mol·L^{-1} significantly inhibited the height of Chinese fir seedlings compared with control. Phenolics have inhibitory allelopathic effects on Chinese fir seedlings.

Nitrogen is most important for plants (19) and reduction in its absorption directly affect the seedlings growth of plants. Results showed that phenolics influence the total nitrogen contents of roots, stems, and leaves of Chinese fir seedlings. %NDFF and NDFF in each organ with 1×10^{-2} mol·L^{-1} phenolic treatment was lower than control, indicating that phenolics can inhibit nitrogen absorption by Chinese fir seedlings and differences between treatments of phenolics were evident. The inhibition caused by phenolic treatment reduced productivity of Chinese fir seedlings. It is known that phenolics, such as vanillic acid, decreases the activity of root system (6), which is an important index of the capacity for nitrate absorption and metabolism of organic compounds. The inhibitory effects of phenolics on seedlings growth of Chinese fir may be due to decline in activity of root system, which adversely decreases the nutrient absorption. Other studies suggested that vanillic and *ρ*-hydroxybenzoic acids at higher concentrations inhibit the availability of soil mineral nutrients including N, P and K (4). Zhang (26) reported that soil extracts from replanted woodlands significantly inhibited the net soil nitrogen mineralization rates, which decreased the available nitrogen content and consequently influenced nitrogen absorption by Chinese fir for their seedlings growth.

Phenolics also affected the ^{15}N nitrate distribution in each organ of Chinese fir. The linear coefficients of above equations of leaves were negative, which indicates that ratios of ^{15}N absorption in leaves to ^{15}N absorption in all organs were reduced gradually as concentration of phenolics increased, especially treated with phenolics mixture. That is, higher concentrations of phenolics can decrease the ^{15}N distribution in leaves of Chinese fir seedlings. Phenolics concentration of 1×10^{-2} mol·L^{-1} enhanced the ^{15}N nitrate distribution in roots but reduced its distribution in stems and leaves. With increase in the concentration of vanillic acid and phenolics mixture, the ^{15}N distribution in stems gradually decreased but increased in roots. The poorer the site, the greater is the proportional allocation of photosynthate to the underground parts (20). Phenolics significantly affects the availability of soil nutrients and made the soil poor in Chinese fir plantation (4,15). The influence of phenolics on nitrogen distribution increased the metabolized substance and nitrogen distribution in roots and as a result, phenolics inhibited the seedlings growth and thus decreased the productivity of aboveground parts of Chinese fir.

ACKNOWLEDGEMENTS

This research was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (KZCX3-SW-418), the National Natural Science Foundation of China (30270268) and the Knowledge Innovation Program of the Institute of Applied Ecology, Chinese Academy of Sciences (C12SISLYQY0405506). The authors gratefully acknowledge the assistance of Professor Emeritus John A. Helms, Department of Forest Science, University of California, Berkeley, for his valuable comments and suggestions.

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