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Allopathic Effect of Black Locust (*Robinia pseudoacacia* L.) and Analysis for Its Allelochemicals[1]

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ABSTRACT

Allelopathic effects of black locust(*Robinia pseudoacacia* L.) was evaluated through germination tests using 13 different species including *R. pseudoacacia* itself. The water extract from leaf or root of *R. pseudoacacia* was separately tested. Seed germination of several species was inhibited in the presence of leaf or root extract, although the level of germination was in a wide range depending on the species. Seed germination of *R. pseudoacacia* was suppressed by 30 % in its leaf extract. Among tested tree species, *Hibiscus syriacus* and *Alnus firma* showed significantly inhibited germination in the root extracts whereas *Thuja orientalis* exhibited germination inhibition in the leaf extract. HPLC was carried out to identify allelochemicals in extracts of leaf and root using eight authentic phenolic compounds that are known to be responsible for allelopathy. The major phenolic compounds occupying about 40 % of total phenolic acids were gentisic acid in leaves and vanillic acid and syringic acid in roots. The leaves contained 7 times more phenolic acids than the roots. Among 8 tested phenolic compounds vanillin was detected only in roots and coumaric acid in leaves. Consequently, 3 out of 13 species showed significantly inhibited germination due to leaf or root extract. This germination test indicates that the inhibitory allelopathic effect by *R. pseudoacacia* is caused by chemical interaction not by nutritional competition and that the allelochemicals of black locust act species-specifically. No specific influence of the total amount of phenolic compounds on the allelopathic inhibitory germination and the synergic effect by each phenolic compound may play a role for the allelopathic effect by *R. pseudoacacia.*

KeyWords : allelopathy, chemical interaction, germination inhibition, phenolic compounds

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요 약

아까시나무에 있어서 알레로파시 효과를 분석하기 위해 아까시나무를 포함하여 13개 수종에 대한 종자발아 시험을 실시하였다. 아까시나무의 뿌리와 잎의 침출액을 실험에 사용하였다. 침출액에 대한 종자 발아실험 결과 수종에 따라 실험결과가 크게 다르게 나타났다. 아까시나무 잎 침출액에서 아까시나무 종자 발아는 30%가 억제되었다. 실험한 수종 가운데 무궁화와 오리나무는 뿌리 침출액에서 억제되었으나 누운측백나무는 잎의 침출액에서 발아가 억제되었다. 아까시나무의 잎과 뿌리 침출액을 HPLC분석 한 결과 알레로파시 효과를 가지고 있는 8종류의 authentic phenolic compounds를 찾아냈다. 잎에서는 전체 페놀물질 중 40%를 차지하고 있는 gentisic acid를 뿌리에서는 syringic acid와 vanillic acid를 발견하였다. 전체적으로 볼 때 뿌리에서보다 잎에서 7배가 많은 phenolic acids가 추출되었다. 조사된 8개 phenolic acid 중에서 뿌리에서는 vanillin만이 잎에서는 coumaric acid 만이 발견 되었다. 결과적으로 13수종 중에 3개 수종에서 잎과 뿌리의 침출액에서 종자 발아가 억제되었다. 종자발아 시험결과 아까시나무의 화학물질의 상호작용은 수종에 따라 차이가 많이 있음을 밝혀냈다. 아까시나무의 phenolic compound 총량은 알레로파시 역할에 큰 영향을 미치지 않았으나 이들 물질의 상승효과가 주요한 역할을 하는 것으로 추정하였다.

INTRODUCTION

Allelopathy is a term referring to biochemical interactions between plants including microorganisms, covering both stimulatory and inhibitory reciprocal interference (Molisch, 1937; Rice, 1984). Biochemicals originating from living plants or decomposing plant parts such as litter are known to drive such interference in allelopathy. The system using new classes of chemicals based on allelopathy, one of naturally occurring protective phenomena, have an advantage over synthetic chemicals in control of insects, weeds, and disease.

Evidence for allelopathy has been reported over years and many allelopathic chemicals have been identified and characterized in various plants especially crop and weed species (Bell, 1981; Duke, 1986; Putnam, 1988; Gross & Parthier, 1994; Seigler, 1996; Ito et al, 1998; Yamamoto et al, 1999; Kato- Noguchi, 2000; Ridenour and Callaway, 2001). There are thousands of such compounds but a limited number of them have been identified as toxins involved in allelopathy. Chemical compounds such as terpenes, alkaloids, flavonoids and cyanogenic glycoside are generally known as allelopathic substances (Rice, 1984; Swain, 1977).

In contrast to weeds and crop plants, there were a few allelopathic researches of forest species. Lodhi (1975) reported the production of phenolic phytotoxins such as ferulic, caffeic, gentisic and p -coumaric acids, scopolin and scopoletin by *Celtis laevigata*. Lee and Monsi (1963) identified tannin and p-coumaric acids as allelopathic agents in *Pinus densiflora*.

Growth inhibition was reported suggesting the possibility of an allolepathic effect of *Rhododendron maximum* (Nilsen et al, 1999) and *Larix leptolepis* (Park, 1994). Allelopathic effects of *Phellodendron camurense* on the growth of microorganisms and the responsible allelochemical was also reported (Park, 1996a; Park and Choi, 1999).

Black locust is known to be very useful from the aspect of soil conservation and its economical value as timber and honey production (Park, 1992, 1996b; Park et al, 1993). For this reason, it was introduced to Korea in 1897 and has quickly spread over the Korean peninsula. The artificial plantation occupies over 324,000 hectares in Korea (Lee, 1998). The presence of black locust may enhance the development of other vegetation through amelioration of the micro-climate and through nitrogen fixation. Scheidemann and Wetzel (1997) reported on the role of flavonoids in root extract of *R. pseudoacacia* in the development and maintenance of symbiosis with certain *Rhizobium* strains. In addition, several papers have mentioned the possible allelopathic effect of black locust (Perry, 1932; Scheidemann and Wetzel, 1997). However, there is no research that identified the allelopathic chemicals of black locust for the growth inhibition to other plantation. In this study, we tried to identify the allelopathic chemicals released from *R. pseudoacacia* and to determine their role in the growth of other plants.

MATERIALS AND METHODS

Preparation of water extracts

The leaves and roots were collected from the plantation of black locust at Daegu, Korea. Roots were cleaned of soil, cut the roots and leaves into pieces and submerged in distilled water in flasks to obtain water-soluble extracts. Two hundred grams (fresh weight) of root or leaf was added in 1000 mL of distilled water. The materials were then incubated in a shaker for 24 hours (26°C~28°C, 160rpm) after which the extracts from leaf and root were filtered using a filterholder (Nalgene) with Whatman filter paper and stored at 4°C .

Germination test

The germination test was conducted using 13 species; *Alnus firma, Alnus maximowiczii, Arundinella hirta., Hibiscus syriacus, Lactuca sativa* var. *capitata, Lespedeza bicolor, Pennisetum alopecuroides., Pinus densiflora, Pinus thumbergii, Pinus rigida,* x*Pinus rigitaeda, Robinia pseudoacacia* and *Thuja orientalis*. Seeds used in this study were collected at Youngyang, Bonghwa and Kyungpook National University, Kyongsangbuk-Do, Korea and carefully selected for fidelity. Seeds were washed with detergents three times and rinsed with tap water for 5 minutes and then with abundant distilled water. Seeds were then surface-sterilized in 3% (v/v) solution of commercial Chlorax for 20 min, rinsed in distilled water three times and finally soaked in 70% ethanol. After 10 min, floating seeds were discarded and sinking healthy seeds were collected for

use. After washing with distilled water three times, seeds were used in germination tests.

Fifteen seeds of each species were plated on a petridish with filter paper (Whatman, Ø90mm) and soaked in 10 ml of water extracts (200g/L) from leaves or roots. For the control, 10 ml of distilled water was added. Seeds were allowed to germinate in a culture room maintained at 26-28 °C with a 16 hr light period. The test was repeated 5 times. The number of germinated seeds was counted after 4 weeks of incubation and the percentage calculated.

Extraction of phenolic compounds for HPLC

Fresh leaves and roots were cut into pieces, dried at room temperature for 3 weeks, then homogenized. Drying was carried out in the dark as some polyphenols are sensitive to light. Two grams of homogenized plant powder were transferred to a flask with 100 ml of 2N HCl and extracted by boiling in a water bath for 45 min. The beaker was shaken every 3 minutes during extraction. Samples were then cooled down, filtered with filter paper (Whatman, No. 1) 2 times, and processed for the separation. Ether was added to each sample in a separating funnel, and the vessels were shaken carefully under a fume hood. Extraction with ether was repeated 3 times (60ml/60ml/40ml) and yellow-green upper phase of extracts was collected from each extraction. The extracts were then moved into a rotary evaporator flask and evaporated to remove ether under the ventilated hood overnight. The dry extracts were dissolved in 2 ml of absolute ethanol and stored in a sealed glass tube. They were then filtered through micropore (0.2 μm, Gelman), and used in HPLC analysis.

HPLC analysis

HPLC was conducted with Waters Breeze System using a Microbondapak C18 column (300×3.9mm, Waters). Ten μl aliquots of the extracts were injected and separated using an acetonitrile-water gradient elution method; Solvent A:0.5% acetic acid in distilled H_2O, solvent B: 0.5% acetic acid in acetonitrile, flow rate 1.5 ml/min, 0 to 20 of solvent B, in 45 min. The absorption spectra of the eluting compounds were analyzed with a multi-array diode detector (Waters) at 260, 280, and 320nm. Use of several detection ranges allows detection of all phenolic compounds present in extracts. Phenolic acids and their derivatives are identified by comparison with the retention time of pure standards (Protocatechuic Acid, Gentisic Acid, p-Hydroxybenzoic Acid, p-Hydroxybenzaldehyde, Vanillic Acid, Syringic Acid, Vanillin, p-Coumaric Acid; Sigma Co.). Extracts were then co-injected with the suspected standards, based on previous performance, for the confirmation of identity. The quantitative measurement of identified phenolic acids was conducted after the classical establishment of standard curves obtained using the reference compounds. The amount of phenolic compounds in leaf/root extract was calculated by the length and width of the curves corresponding to peaks in the UV spectra.

Table 1. Inhibitory germination on several species by allelopathy effects by black locust leaf or root extracts. Values are the mean of three replications.

Species	Germination (%)		
	Distilled water	Leaf extract	Root extract
Pinus densiflora	100 ± 0.00*	100 ± 0.00	100 ± 0.00
Pinus thunbergii	100 ± 0.00	100 ± 0.00	95 ± 1.48
Pinus rigida	100 ± 0.00	100 ± 0.00	100 ± 0.00
x *Pinus rigitaeda*	100 ± 0.00	84 ± 3.16	54 ± 4.64
Robinia pseudoacacia	100 ± 0.00	70 ± 2.74	100 ± 0.00
Alnus maximowiczii	100 ± 0.00	100 ± 0.00	97 ± 2.45
Alnus firma	100 ± 0.00	99 ± 1.00	45 ± 3.54
Lespedeza bicolor	97 ± 1.58	100 ± 0.00	91 ± 2.00
Arundinella hirta	100 ± 0.00	81 ± 2.00	100 ± 0.00
Pennisetum alopecuroides	100 ± 0.00	100 ± 0.00	100 ± 0.00
Thuja orientalis	90 ± 1.58	47 ± 0.25	57 ± 5.70
Hibiscus syriacus	97 ± 1.58	67 ± 3.52	10 ± 3.24
Lactuca sativa var. *capitata*	100 ± 0.00	0 ± 0.00	3 ± 1.58

* mean ± standard deviation

RESULTS AND DISCUSSION

Inhibitory effect on germination

Commercial lettuce seeds were used as a control for their known germination behavior. Germination was commenced in the control treatment lacking BL (black locust) extracts after 3 days and 100% germination had occurred after 4 weeks when germinated seeds were counted. Other species started germinating after 5-7 days and showed germination delays of 2-3 days in the presence of BL extracts.

Table 1 shows a summary of inhibitory effect on germination for 13 herbaceous or tree species due to the allelopathic effect of BL extracts. The germination rate for all but 3 tested species in the control treatment with distilled water was 100 %. Lettuce seeds were not germinated in BL leaf or root extract but there was no notable germination inhibition on other herbaceous species, *A. hirta* and *P. alopecuroides,* which are considered as weeds. Among the tested forest species, *H. syriacus,* and *A. firma* showed more than 50 % germination inhibition ($>I_{50}$) in BL root extract. *T. orientalis* and x*P. rigidateada* had inhibited germination ($=I_{50}$) in BL root extract. *T. orientalis* also had germination inhibition ($>I_{50}$) in BL leaf extract. The result of germination trials indicates that the inhibitory effects of BL extracts are species-specific depending on the source of extract.

Figure 1 and 2 showed the inhibitory effect of BL extracts on *T. orientalis, H. syriacus* and *L. sativa* according to the concentrations of leaf and root extracts of BL, respectively. The 50 % dose of extracts contain 100 g (fresh weight) of leaf or root in 1L of distilled water and 100 % dose contained 200 g (FW). In the case of *L. satira,* the 50 % dose of

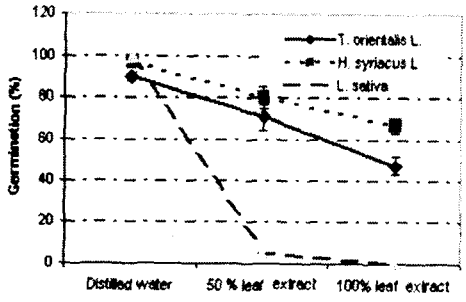

Figure 1. Inhibitory germination according to the concentrations of leaf extract of BL. 50 % extract: 100g/1L (FW/H_2O), 100 % extract: 200g/1L (FW/H_2O)

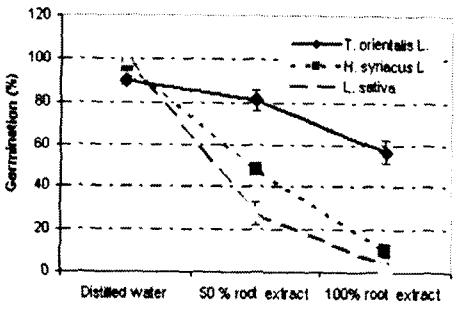

Figure 2. Inhibitory germination according to the concentrations of root extracts of BL. 50 % extract: 100g/1L (FW/H_2O), 100 % extract: 200g/1L (FW/H_2O)

leaf and root extract was sufficient for I_{50}. For *H. syriacus*, the germination inhibition (I_{50}) was observed at the 50 % dose of root extract. However, for *T. orientalis*, the 50 % dose of leaf extract caused only 29 % of germination inhibition and I_{50} was observed with the 100 % dose. According to this result, germination inhibition increases as the concentration of extract increases. Similar results have also been reported for other weed and tree species (Park 1996a, 1999; Kato-Noguchi, 2000). The concentration-dependent growth inhibition suggest that BL leaf and root extracts may contain allelochemicals and that, in turn, the inhibition of germination may be caused by allelopathic reaction rather than by nutritional competition. In addition, the experimental scheme excluded the interspecific competition for external resources by separating each species in a different dish.

Autotoxicity was observed by the leaf extract (100% dose) showing 30 % of germination inhibition. Autotoxicity was reported for pearl millet (Saxena et al., 1996), in which it was concentration-dependent. At lower concentrations, it tended to stimulate the growth, whereas at higher concentrations it inhibited the growth. BL could also exhibit similar autotoxicity on its seed germination, whereby seed germination occurs under a sparse canopy but growth is inhibited when the canopy is full.

Phenol compounds in BL leaf and root

BL leaf and root extracts were analyzed for phenolic acids and their derivatives by HPLC. Table 2 shows the characteristics of the phenolic compounds in BL leaf and root. Identification of each compound was conducted by comparing with retention times of the standards. Quantitative analysis for each detected compound was based on length and width of curves in UV spectra. Table 3 shows the amounts of 8 phenolic compounds per 1 g (dry weight) of BL leaf and root. Gentisic acid (40.1 %) was the

Table 2. Characteristics of phenolic compounds detected in black locust leaf and root.

Phenolic compound	Retention time (min)	Peaks in UV-spectra (nm)
Protocatechuic Acid (PTA)	19.000	220.3 259.0 293.4
Gentisic Acid (GEN)	23.430	216.0 325.4
p-Hydroxybenzoic Acid (PHA)	25.580	254.4
p-Hydroxybenzaldehyde (PHB)	29.210	221.5 283.9
Vanillic Acid (VAA)	30.760	220.3 260.3 292.2
Syringic Acid (SYR)	33.960	220.3 274.5
Vanillin (VAN)	35.690	203.9 229.7 279.2 308.8
p-Coumaricc Acid (PCA)	41.140	226.2 310.0

major component in leaf extract whereas vanillic acid (40.4 %) and syringic acid (36.1 %) were the major components in root extract. However, the total amount of the phenolic compounds in leaf was 7 times higher than that of root; gentisic acids was 4-fold, vanillic acid 3-fold, syringic acid 2-fold, protocatechuic acid 33-fold, p-hydroxybenzoic acid 17-fold, p-hydroxybenzaldehyde 4-fold. Interestingly, vanillin was detected only in root and p-coumaric acid was found only in leaf.

The phenolic compounds were reported causing inhibitory growth effect by allelopathy on various different species as described in the introduction. For black locust, Waks in 1936 reported that the extract of black locust had an allelopathic inhibitory growth effect to barley (Rice, 1984). In addition, Woo and Park (1996 unpublished) mentioned the germination inhibition of several species by the root extract of black locust in the Korean conference "Evaluation of fast-growing trees in Korea". Moreover, the allelopathic effect by black locust was confirmed in this study. According to our results, phenolic compounds in leaf and root appear to be responsible for such allelopathic effect by the synergic effect of each phenolic compound in leaf and root of black locust. Further research is needed to determine which compound is the active allelochemical for inhibitory allelopathy in black locust. The germination test using the pure phenolic compounds would be useful to confirm the allelopathy agent from black locust. In addition, studies of the role of other

Table 3. The amounts of phenolic compounds in black locust leaf and root.

Phenolic compounds	Amount (mg) 1 g dry weight			
	Root	%	Leaf	%
Protocateuic Acid (PTA)	0.061	1.3	2.021	6.3
Gentisic Acid (GEN)	0.532	11.4	12.936	40.1
p-Hydroxybenzoic Acid (PHA)	0.347	7.4	5.440	16.9
p-Hydroxybenzaldehyde (PHB)	0.035	0.7	0.131	0.4
Vanillic Acid (VAA)	1.884	40.4	4.214	13.1
Syringic Acid (SYR)	1.683	46.1	3.329	10.3
Vanillin (VAN)	0.125	2.7	0.000	0.0
p-Coumaric Acid (PCA)	0.000	0.0	4.210	13.0
Total	4.667	100.0	32.282	100.0

tissue such as humus, soil and litter on seedlings may help identify the specific origin of allelopathy of black locust.

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