Phytotoxic Effect of Lettuce (*Lactuca sativa* L.) Leaf Extract on Seedling Growth of Crops and Weeds

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ABSTRACT

Lettuce (*Lactuca sativa* L.) is known to contain water-soluble substances that are biologically active. Aqueous or methanol extracts and residues from leaves of lettuce plants were assayed to determine their allelopathic effects, and the causative allelochemicals from fractions were quantified by means of HPLC analysis and bioassayed. Extracts from oven-dried leaf samples were more phytotoxic than those from freeze-dried samples. Leaf extracts of 40 g L^{-1} were completely inhibitory on root growth of alfalfa (*Medicago sativa* L.), while root growths of barley (*Hordeum vulgare* L.) and soybean (*Glycine max* L.) were less sensitive. Early seedling growth of both alfalfa and barnyard grass (*Echinochloa crus-galli*) was significantly reduced by methanol leaf extracts. The major allelopathic substances analyzed by HPLC were coumarin, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid and chlorogenic acid. Of them *p*-coumaric acid was found as the greatest amount (8.9 mg 100 g^{-1}) in the EtOAc fraction; only coumarin was found in all the fractions. Hexane and EtOAc fractions of *L. sativa* reduced alfalfa root growth more than did BuOH and water fractions. These results suggest that lettuce had potent herbicidal activity, and that its activity differed depending on type and amount of causative compounds by fraction.

Keywords **:** Lettuce, plant extracts, bioassay, allelopathy, fractionation, HPLC.

INTRODUCTION

Allelopathy, defined by Molisch (1937), is the chemical interaction between plants, including stimulatory as well as inhibitory influences. Allelopathy plays an important role in both natural and agro-ecosystems. Suitable manipulation of the phenomenon towards improvement of crop productivity and environmental protection through eco-friendly control of weeds, pests, crop diseases, conservation of nitrogen in crop land, and synthesis of novel agrochemicals based on natural products have gained prominent attention of scientist engaged in allelopathic research.

Lettuce is an annual herbaceous plant of Compositae, one of the largest and most diverse families of flowering plants. Allelopathic effect of lettuce plant has not been documented well. Only few studies on allelopathic effects of other Compositae plant extracts

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or residues on some agronomic crops and weeds have been reported. Bendall (1975) studied water and ethanol extracts and residues in soil and concluded that an allelopathic mechanism might be involved in the exclusion of some annual thistle (*Carduus crispus* L.), pasture, and crop species in areas infested with *Cirsium arvense* (L.) Scop. *C. arvense* litter reduced the growth of *Amaranthus retroflexus* L. and *Setaria viridis* L. more than that of cucumber (*Cucumis sativus* L.) or barley in their greenhouse experiments (Stachon and Zimdal, 1980). In their field experiment, high densities of *C. arvense* reduced the incidence of annual weeds growing in the same vicinity of *C. arvense*.

To identify and quantify compounds contained in plant extracts or residues is an important part of the process of discovering agents of allelopathy. Plants contain thousands of natural products, but not all are implicated as being allelopathic (Bell and Charlwood, 1980; Rice, 1984). The major biosynthetic pathways leading to the production of allelochemicals are known to be shikimic acid or acetate pathways (Rice, 1984). Phenolic acids in the literature on allelopathy are often mentioned as putative allelochemicals and are perhaps the most commonly investigated compounds among potential allelochemicals. They are found in a wide range of soils or plants, and their phytotoxic potential against various test plants has been demonstrated under controlled conditions. Phenolic compounds are among the most abundant groups of secondary metabolites in plants (Harborne, 1980). Penolics bear hydroxlated aromatic rings including simple phenols, phenolic acids, phenylpropanoids, coumarins, quinones, flavonoids, tannins and other miscellaneous phenols (Harborne, 1980). They are known to be of significance in allelopathy (Inderjit, 1996).

Phenolic acids such as p-hydroxybenzoic, vanillic, *p*-coumaric, syringic and ferulic acids are a main category of allelochemicals. These phenolic acids have been identified as allelopathic agents in natural and agroecosystems (Guenzi and McCalla, 1966; Blum *et al.*, 1991; Ben-Hammouda *et al.*, 1995). Einhellig *et al.* (1970) reported that a coumarin derivative, scopoletin, inhibited dry matter production, leaf area expansion, and photosynthesis in tobacco (*Nicotiana tabacum* L.), sunflower (*Helianthus annuus* L.) and *A. retroflexus*. Ferulic acid and *p*-coumaric acid have known to reduce leaf water potential and stomatal diffusive conductance in grain sorghum (*Sorghum bicolor* (L.) Moench.) and soybean (*Glycine max* L.) (Einhellig and Stille, 1979). Other numerous studies have also shown that many phenolics are inhibitory (allelopathic) to germinating seeds or growing plants (National Academy of Sciences, 1971).

The objective of this research was a) to determine allelopathic effects of aqueous extracts or methanol extracts, and residues from lettuce plants through Petri dish and pot tests, and b) to quantify the causative allelopathic compounds by means of HPLC analysis from fractions of the lettuce. This research will promote a better understanding of allelopathy mechanisms in the agro-ecosystem through investigating the allelopathic effect and quantification of causative allelochemicals.

MATERIALS AND METHODS

Sampling and Preparation of Extracts

Top growth of lettuce plants grown in crop fields of the Suncheon area, Korea were harvested at a vegetative stage in May 2001. The plants were separately sampled into leaves, stems, and roots. The samples were immediately oven-dried at 60℃ for 5 days (Chon and Nelson, 2001), ground with a Wiley mill to pass through a 1-mm screen, and stored in a refrigerator at 2 ℃ until required. Forty grams of dried leaves were extracted by soaking in 1L-distilled water at 24℃ for 24 h in a shaker to give a concentration of 40 g dry tissue L^{-1} (hereafter referred to as '$g\ L^{-1}$'). The extract was filtered through two layers of cheesecloth to remove the

fibre debris, and centrifuged at 5000 rpm (x 4530 *g*) for 2 h. The supernatant was vacuum filtered again through Whatman No. 42 paper. Methanol extracts from ground plant samples were used for the following bioassay and fractionation.

Phytotoxic Effects of Aqueous Plant Extracts by Drying Method

To compare the sensitivities of test plant to the extracts as affected by drying method of plant sample, the plant samples were divided into two groups. One group was oven-dried at 40℃ for 5 days and the other was freeze-dried at -26℃ for 5 days. Phytotoxic effects of extracts from two groups on alfalfa were assayed in petri-dish.

Alfalfa (cv. "Vernal") seeds were surface sterilized with 0.525 g L^{-1} sodium hypochlorite for 15 min. The seeds were rinsed four times with deionized water, imbibed in deionized water at 22℃ for 12 h, and carefully blotted using a folded paper towel. Twenty imbibed seeds of alfalfa were separately placed on filter paper wetted with extract concentrations of 0, 10, 20, 30, and 40 g L^{-1}. The Petri dishes were covered, sealed by wrapping in parafilm, and placed flat in a growth chamber at 24℃ during the 14-h light period and 22℃ during the 10-h dark period. Plates were illuminated with 400 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR), provided by a mixture of incandescent and fluorescent lamps. Root length was measured for all seedlings in each Petri dish 6 days after seeding, and transformed to percent of control.

Effects of Aqueous Plant Extracts on Root Growth of Alfalfa, Barley and Soybean

To know the response of upland crops against alfalfa leaf extracts, leaf extracts of *L. sativa* L. were prepared. Two layers of Whatman No. 1 filter paper were placed in each 9-cm-diameter plastic petri-dish. Five milliliters of diluted extract were pippetted to the filter paper. Imbibed seeds of alfalfa (cv. Vernal), barley (cv. Saessalbori), and soybean (cv. Gwangan) were separately placed on filter paper wetted with aqueous plant extract concentrations of 0, 10, 20, 30, and 40 g L^{-1}. Bioassay procedures and conditions were same to the previous work. Root lengths on all seedlings were measured at 144 hours after transfer of seed on filter paper. Data were transformed to percent of control for analysis as used. There were two experiments, each with four replications.

Effects of Methanol Plant Extracts on Alfalfa and Barnyard Grass Root Growth

Ground leaf samples of lettuce were extracted with 95% methanol at room temperature. The extract was then filtered through a Whatman No. 1 filter paper. The collected filtrate was evaporated to dryness under vacuum at 40℃ using a rotary evaporator (N-1000V-W, Eyela, Japan). The yield of dried extracts from the original plant leaves was 10-15%.

To determine phytotoxic effects of methanol extracts from the lettuce plants, four milliliters of methanol extracts at 25, 50 and 100 g L^{-1} and only methanol solution as a control were poured on Whatman No. 1 filter paper in Petri dish and evaporated to dryness for 24 h at 24℃. After evaporation, four milliliters of distilled water were pipetted to the filter paper and then 15 imbibed seeds of alfalfa and barnyard grass were separately placed on the paper and grown for 6 days. Bioassay procedures and conditions were same to the previous work. Root length was measured for all seedlings in each Petri dish, and transformed to percent of control.

Fractionation, Identification and Quantification of Causative Allelochemicals

For fractionation, crude methanol extracts were mixed with distilled water and hexane to collect hexane extracts. After hexane collection, the distilled water

fractions were added with ethylacetate (EtOAc) to obtain EtOAc extracts in the same way. The same procedure was used in preparing butanol and water extraction. The fractions were dried on a rotary evaporator at 40-50℃, and transferred into vacuum freeze dryer to obtain dry matters. The four dried samples from hexane, EtOAc, BuOH, and water fractions were dissolved in HPLC grade MeOH to give 1,000 ppm for HPLC analysis. The standard phenol compounds used for HPLC analysis were coumarin, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, and chlorogenic acid (Aldrich Co., USA). All of chemicals were purchased as high purity standards and the used solvents were HPLC spectral grade. All solvents and distilled water were degassed before use. All solvent ratios were based on volume.

Allelopathic compounds were identified by a high-performance liquid chromatography (HPLC) using SPP 10AVP (Shimadzu, Tokyo, Japan) with a flow rate of 1 mL min^{-1}, the column was CAPCELL PAK C18 SG120 (4.6 × 250 mm) and an autoinjector with a 10 μl sample loop was employed. The mobile phase consisted of water, methanol and acetic acid in the ratio of 12:15:1 volume, respectively. The UV detector wavelength was set at 275 nm. Standard compounds were chromatographed alone and as mixtures. Retention times for the standard compounds and the major peaks in the extract were recorded.

Phenolic compounds such as coumarin, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, salicylic acid, and chlorogenic acid were identified by retention times or standard addition, and amounts were calculated by comparing peak area with those of standards (Benwart et al., 1985).

Phytotoxic Effects of 4 Fractions against Alfalfa Root Growth

The four dried samples, hexane, EtOAc, butanol, and water fractions, as mentioned before, were dissolved in MeOH for bioassay. To compare phytotoxic effects of the methanol extracts from four fractions of the plant samples, 4 mL of methanol extracts at 25, 50, 75 and 100 g L^{-1} and only methanol solution as a control were pipetted to Whatman No. 1 filter paper in Petri dish and evaporated to dryness for 24 h at 24℃. After evaporation, four milliliters of distilled water was pipetted to the filter paper and then 15 imbibed seeds of alfalfa were placed on the paper and grown for 6 days. Bioassay procedures and conditions were same to the previous work. Root length was measured for all seedlings in each Petri dish. The data were transformed into % of control and analyzed.

RESULTS AND DISCUSSION

Phytotoxic Effects of Aqueous Plant Extracts by Drying Method

Extracts from oven-dried samples significantly inhibited root growth more than did those from freeze-dried samples (Fig. 1). At 20 g L^{-1}, the difference in autotoxic effect was greatest between the two drying methods. Leaf extracts of 20 and 30 g L^{-1} from the oven-dried samples inhibited root growth by 72 and 90 %, respectively, while those from freeze-dried samples by

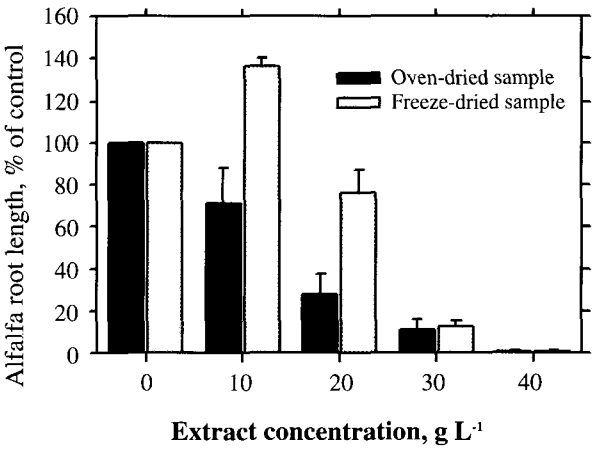

Fig. 1. Effects of lettuce extracts from oven- and freeze-dried leaf samples on alfalfa root length.

24 and 87 %, respectively.

Our previous data indicate that freeze-dried samples of alfalfa plant were less inhibitory to seed germination and hypocotyl and root growth than oven-dried samples (Chon and Nelson, 2001). These results might be because oven drying increases solubility or activity of the extracts while freeze-drying allows allelochemical (s) breakdown during drying or assay. Perkins (1961) and Nelson and Smith (1972) also support these results that chemical compositions such as amino acids change during storage of dried samples.

Effects of Aqueous Plant Extracts on Root Growth of Alfalfa, Barley and Soybean

Root length of alfalfa was more reduced by the extracts treatments than that of barley or soybean, especially root growth of soybean was stimulated by lettuce extracts. At highest extract concentration of 40 g L^{-1}, the root growth of alfalfa was markedly reduced, while root growth of barley and soybean was less reduced, showing each 60% reduction (Fig. 2). In other studies, allelopathic effects of several Compositae plant extracts or residues on some agronomic crops and weeds have been reported. Inam et al. (1987) reported that aqueous extracts of *Xanthium strumarium* from different plant parts reduce germination, the early growth and dry weight of *Brassica compestris, Lactuca sativa*, and *Pennisetum americanum. Parthenium hysterophours* is also known to be very allelopathic to wheat (Kanchan and Jayachandra, 1979), soybean, and corn (Mersie and Singh, 1987).

Effects of Methanol Extracts on Alfalfa and Barnyard Grass Root Growth

Methanol extracts of the lettuce plant samples were assayed against alfalfa and barnyard grass. No significant difference was observed between the two controls, only between methanol solution and the distilled water (Data not shown). Methanol extracts significantly reduced seedling lengths of both alfalfa and barnyard grass with increasing of extract concentration, even though they were less phytotoxic than the aqueous extracts. Early root growth of alfalfa and barnyard grass were significantly inhibited by extracts, compared to untreated controls. Alfalfa was more sensitive to all the extracts than was barnyard grass. At 100 g L^{-1}, the extracts reduced root lengths of alfalfa and barnyard grass by 47 and 43%, respectively (Fig. 3).

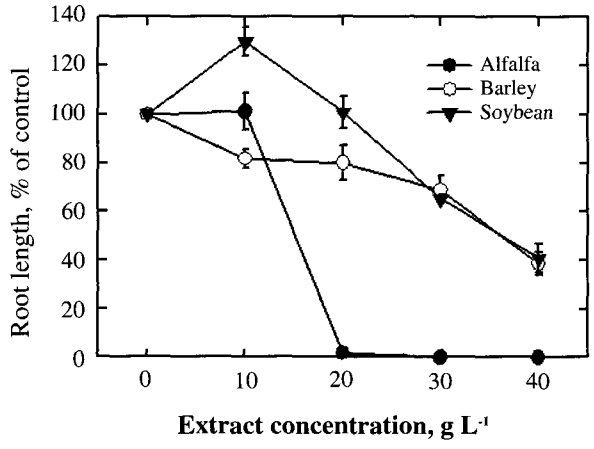

Fig. 2. Effects of lettuce extracts on root lengths of alfalfa, barley, and soybean at 6 d after placing on filter paper wetted with the extracts.

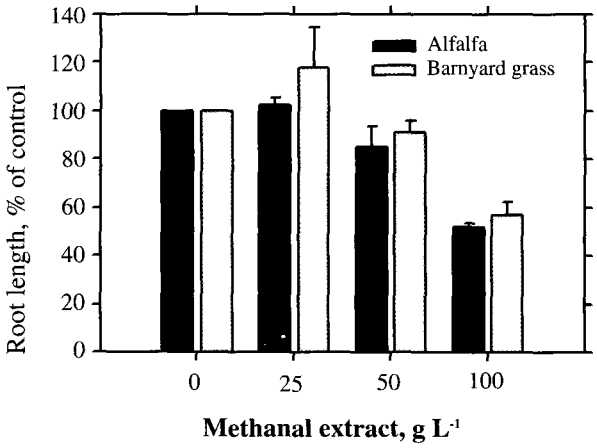

Fig. 3. Effects of methanol leaf extracts from lettuce on root lengths of alfalfa and barnyard grass.

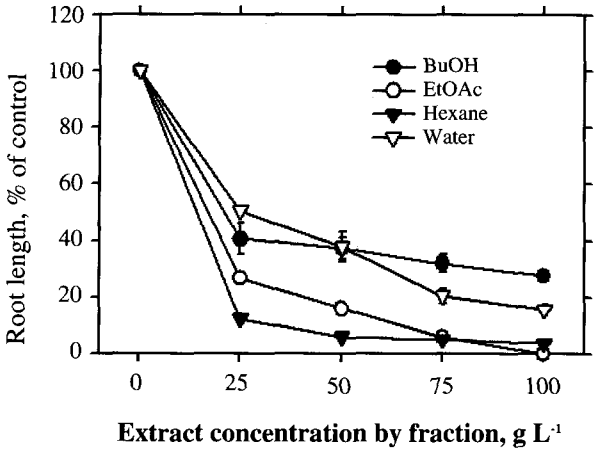

Fig. 4. Effects of methanol extracts from various fractions of lettuce leaves on alfalfa root length 6 days after seeding.

Fractionation, Identification and Quantification of Causative Allelochemicals

The major allelopathic substances present in the lettuce plant were analyzed by HPLC using standard compounds and were recorded as total phenol compounds of all fractions in *L. sativa* by 18.4 mg 100 g^{-1}. The individual compounds identified were coumarin, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, and chlorogenic acid. In aqueous leaf extracts of *L. sativa*, *p*-coumaric acid was found in the greatest amount (8.9 mg 100 g^{-1}) at EtOAc fraction; coumarin was detected in all the fractions (Table 1). Taking together, the findings of the bioassay were highly correlated with the amount of responsible allelochemicals found in plant extracts. Differential allelopathic effect of each species would be due to quantitative as well as qualitative matters of the causative chemicals, suggesting various types and amount of allelochemicals were detected from different plant species.

Phytotoxic Effects of 4 Fractions against Alfalfa Root Growth

Methanol extracts of lettuce samples by fraction were assayed against alfalfa. Methanol extracts through fractionation significantly reduced root lengths of alfalfa. Methanol extracts from hexane and EtOAc fractions of *L. sativa* reduced alfalfa root growth more than those of BuOH and water fractions. Especially, methanol extracts from hexane and EtOAc fractions at 50 g L^{-1} reduced root growth by 94 and 84 %, respectively, while a treatment at same concentration of BuOH and water fractions reduced root growth by each 63%, respectively. The result shows that the causative allelochemicals were more detected in the hexane and EtOAc fractions than in the BuOH and water fractions.

In conclusion, a bioassay on allelopathic effects of lettuce plant extracts demonstrated that lettuce had potent herbicidal activity on early seedling growths of alfalfa and barnyard grass and its activity depended on type and amount of the causative allelochemicals in

Table 1. Quantitative determination of HPLC analysis of some phenolic compounds present in leaves of *L. sativa*.

Compound	Fractions				
	Hexane	EtOAc	BuOH	Water	Total
			(mg 100 g-1)		
Coumarin	0.2345	0.4017	0.1364	0.0787	0.8513
trans-cinnamic acid		0.5964	0.2363	0.0762	0.9089
o-coumaric acid		1.6057	0.4253		2.0310
p-coumaric acid	0.0875	8.9405	1.5799		10.6079
Chlorogenic acid	0.1949		2.9120	0.9046	4.0115
Total	0.5169	11.5443	5.2899	1.0595	18.4106

plant. Such differences might be related to specific allelopathic compounds being produced in larger quantities in certain fraction, imparting a higher level of allelopathy. The allelopathic potential in extracts and residues of lettuce may be a valuable means of biological weed control based on natural plant extracts.

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