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Phytotoxic Effect of *Xanthium occidentale* Leaf Extract on Seed Germination and Early Seedling Growth of Alfalfa and Barnyard Grass

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ABSTRACT: Compositae plants are known to contain biologically active substances that are allelopathic to agricultural crops as well as weed species. Aqueous extracts from leaves of *Xanthium occidentale* were assayed against alfalfa (*Medicago sativa*) to determine their allelopathic effects, and the result showed that the extracts applied onto filter paper significantly inhibited seed germination as well as root growth of alfalfa. Untreated seeds germinated in 60 h, but extract concentrations greater than 30 g L^{-1} delayed seed germination. The extracts significantly inhibited seed germination of alfalfa, and β-amylase activity of alfalfa and barley seeds during 24-36 hours after treatment. Aqueous extracts of 40 g L^{-1} from *X. occidentale* were completely inhibited the hypocotyl and root growth of alfalfa. Aqueous leaf extracts showed the highest inhibitory effect and followed by root and stem extracts. Early seedling growth of both alfalfa and barnyard grass (*Echinochloa crus-galli*) was significantly reduced by methanol extracts. By means of high-performance liquid chromatography, chlorogenic acid and *trans*-cinnamic acid were quantified as the highest amounts from water and EtOAc fractions, respectively. BuOH and EtOAc fractions of *X. occidentale* reduced alfalfa root growth more than did hexane and water fractions. The findings of the bioassays for aqueous or methanol extracts reflected that the inhibitory effect of extract was closely related to the level of responsible allelochemicals found in plant extracts.

Keywords: *Xanthium occidentale*, plant extracts, bioassay, allelochemicals, herbicidal activity, HPLC.

Allelopathy was defined as any direct or indirect harmful or beneficial effect of one plant (donor plant) on another (recipient plant) through the production of chemical compounds that release into the environment (Rice, 1984). Most assessments of allelopathy involve bioassays of plant or soil extracts based on seed germination and seedling growth. Generally germination is less sensitive to the extracts than is seedling growth, especially root growth (Miller, 1996).

X. occidentale as an annual Compositae plant is one of the most competitive weeds in crop fields as well as wastelands and has been used as a medicinal plant in Korea (Research Institute of Natural Products, 1996). Allelopathic effects of several Compositae plant extracts or residues on crops and weeds have been reported. Inam *et al.* (1987) found that aqueous extracts of *X. strumarium* from different plant parts reduce germination, early growth and dry weight of *Brassica compestris*, *Lactuca sativa*, and *Pennisetum americanum*. Especially, *Parthenium hysterophours* is known to be very allelopathic to wheat (Kanchan & Jayachandra, 1979), soybean, and corn (Mersie & Singh, 1987). Their researches showed that extracts and residues of these plants significantly reduced germination and shoot and root dry weight of the test plants. Bendall (1975) studied on the ecological implication of allelopathy with 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 the areas infested with *Cirsium arvense* (L.) Scop. *Cirsium arvense* litters reduced the growth of *Amaranthus retroflexus* L. and *Setaria viridis* L. more than that of cucumber (*Cucumis sativus* L.) or barley (*Hordeum vulgare* L.) in a greenhouse experiment (Stachon & Zimdal, 1980). In their field experiment, high densities of *C. arvense* reduced the incidence of annual weeds in the vicinity of *C. arvense*.

The major biosynthetic pathways leading to the production of allelochemicals are probably shikimic acid or acetate pathways (Rice, 1984). 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 & 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 been known to reduce leaf water potential and stomatal conductance in grain sorghum (*Sorghum bicolor* (L.) Moench) and soybean (*Glycine max* L.) (Einhellig & Stille, 1979). Numerous studies have also shown that many phenolics are inhibitory (allelopathic) to germinating seeds or growing plants (National Academy of Sciences, 1971).

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The objectives of this research were (a) to determine allelopathic effects of aqueous and methanol extracts from *X. occidentale* on seed germination and seedling growth of alfalfa and barnyard grass, and (b) to quantify the causative allelopathic compounds by HPLC analysis. This research will promote a better understanding of allelopathy mechanisms in the natural ecosystem through bioassay and quantification of the causative allelochemicals in plant extracts.

MATERIALS AND METHODS

Sampling and preparation of extracts

X. occidentale plants grown in pastures of the Suncheon area, Korea were harvested at vegetative stage in May 2001. The plants were separately sampled into leaves, stems, and roots. The samples were immediately oven-dried at 60°C for 5 days (Chon & Nelson, 2001), ground with a Wiley mill to pass through a 1-mm screen, and stored in a refrigerator at 2 °C until required. To collect aqueous extracts forty grams of dried leaves were extracted with 1 L distilled water at 24°C 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 floating debris, and centrifuged at 5,000 rpm (× 4,530 g) for 2 h. The supernatant was vacuum filtered through Whatman No. 42 paper. Methanol extracts at 100 g L^{-1} from ground plant samples were used for the following bioassay and quantification of the causative allelochemicals.

Effect of aqueous plant extracts on seed germination and β-amylase activity

Alfalfa (cv. "Vernal") seeds were surface sterilized with 0.0525% sodium hypochlorite for 15 min. The seeds were rinsed four times with deionized water, imbibed in deionized water at 22°C for 12 h, and carefully removed surface-water using a folded paper towel. Fifty imbibed seeds of alfalfa were separately placed on a filter paper in a Petri-dish wetted with extracts of concentrations of 0, 10, 20, 30, and 40 g L^{-1}. The petri dishes were covered, sealed by wrapping with parafilm, and placed in a growth chamber at the controlled temperatures (24°/22°C, light/dark) with 14 hr photoperiod. Plates were illuminated with 400 μmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR), provided by a mixture of incandescent and fluorescent lamps. Cumulative germination was determined by counting the number of germinated seeds at 12-hour intervals over a 132-hour period, and transformed into percent germination.

To know responses in amylase activity of two different plants to the extracts, alfalfa as a protein-reserved seed and barley as a carbohydrate-reserved seed were used. Seeds of alfalfa and barley, when germination was initiated at 24 and 36 hours after seeding, respectively, were homogenized with some sea sand and 5 mL of 0.1 M phosphate buffer (pH 7.0) in a chilled mortar, and centrifuged at 5,000 rpm (× 4,530 g) for 30 min at 4°C. The supernatant was collected and immediately subjected to assays of amylase activity (Bernfeld, 1955). After reaction of 1 mL of 0.2% soluble starch with 1 mL of enzyme extractant from each sample for 30 min at 37°C, β-amylase activity was determined as the amount of reducing sugar by using a UV spectrophotometer. D-glucose was used as a standard.

Effect of aqueous plant extracts on alfalfa seedling growth

Two layers of Whatman No. 1 filter paper were placed in each petri-dish (dia. 9cm). Imbibed alfalfa seeds were evenly placed on the filter paper added with 4 mL of a series of aqueous plant extract concentrations of 0, 10, 20, 30, and 40 g L^{-1}. Bioassay procedures and conditions were same to the above mentioned. Root and hypocotyl lengths of all seedlings were measured at 144 hours after transfer of seeds on filter paper. There were two experiments, each with four replications.

Comparison of aqueous plant extracts from different plant parts for allelopathic effect

The plant samples separated into leaves, stems, and roots were separately extracted by soaking in 1L-distilled water at 24°C for 24 hours in a shaker to give a concentration of 40 g L^{-1}. Other experimental procedures were same to the previous work. Each stock extract was diluted with sterile distilled water to give the final concentrations of 10, 20, 30, and 40 g L^{-1}. Distilled water was the control. Bioassay procedures and conditions were same to the previous work. Root length was measured on all seedlings 5 days after placing seeds on the filter paper.

Effect of methanol plant extracts on alfalfa and barnyard grass root growth

Ground leaf samples of *X. occidentale* were extracted with 95% methanol at room temperature. The extract was then filtered through a Whatman No. 1 filter paper. The collected filtrates were evaporated to dryness under vacuum at 40°C using a rotary evaporator (N-1000V-W, Eyela, Japan). The yield of dried extract from the plant leaves was about 12.5%.

The dried samples were mixed with methanol to give final concentrations into 25, 50 and 100 g L^{-1}. Four milliliters of the methanol extracts and only methanol solution (95%)

without extracts as a control were pipetted onto Whatman No. 1 filter paper in a petri dish (dia. 9cm) and evaporated to dryness for 24 h at 24°C. After evaporation, four milliliters of distilled water was added again to the filter paper and then each of 15 imbibed seeds of alfalfa and barnyard grass were separately placed on the paper and grown for 6 days. Distilled water without methanol treatment was another control. Bioassay procedures and conditions were same to the previous work. Root length was measured for all seedlings in each petri dish.

Fractionation, identification and quantification of causative allelochemicals

For fractionation, crude methanol extracts were mixed with the same volume of distilled water and hexane to collect hexane extracts. After collecting the hexane, the distilled water fractions were mixed with ethylacetate (EtOAc) to obtain EtOAc fraction in the same way. The same procedure was used in preparing butanol (BuOH) and water fractions. The fractions were taken to dryness on a rotary evaporator at 40-50°C depending on the kind of solvent. The dried samples from hexane, EtOAc, BuOH, and water fractions were separately 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 and chlorogenic acid (Aldrich Co., USA). All of chemicals were purchased as high purity grade and the used solvents were HPLC spectral grade.

Allelopathic compounds were identified by a high performance liquid chromatography (HPLC) equipped with SPP 10AVP (Shimadzu, Tokyo, Japan) with a flow rate of 1 mL min^{-1}, the column was CAPCELL PAK C_{18} 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 wavelength of UV detector was set to 275 nm. Standard compounds were chromatographed alone and as mixtures. Retention times for the standard compounds and the major peaks from the extract were recorded and compared to calculate the concentration. Phenolic compounds such as coumarin, trans-cinnamic acid, and chlorogenic acid were identified and quantified by retention times and peak area compared to standards (Banwart *et al.*, 1985).

RESULTS AND DISCUSSION

Effect of aqueous plant extracts on seed germination and β-amylase activity

Untreated seeds germinated within 60 h, but extract concentrations greater than 30 g L^{-1} from plant extracts of *X. occidentale* significantly inhibited seed germination. Thus, the extract treatment with higher concentrations apparently delayed germination after imbibition, i.e., between onset of the germination process and emergence of the radicle from the seed coat. Germination was delayed at lower concentrations of 10 and 20 g L^{-1}, and inhibited over 132 h with the treatment of 30 and 40 g L^{-1} extract concentrations (Fig. 1). The result supports that difference in germination depends on the degree of uptake by seed in water or alfalfa leaf extract, and that differential germination rate of alfalfa seed is decreased by higher concentrations due to inhibition of seed germination (Chon *et al.*, 2004).

X. occidentale extracts at all concentrations inhibited amylase activity of alfalfa seeds during germination, showing 38-42% inhibition based on the amount of reducing sugars, while the extracts strongly inhibited enzyme activity of bar-

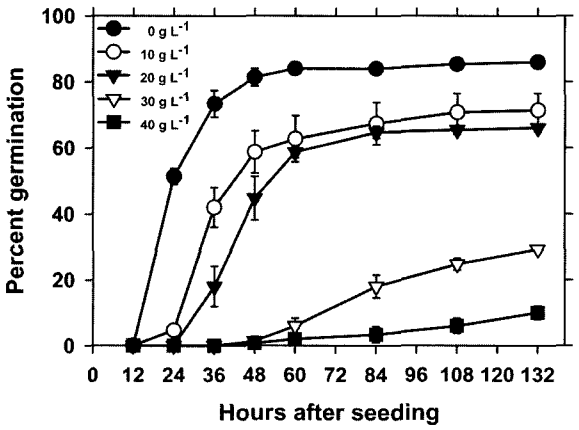

Fig. 1. Effect of aqueous *X. occidentale* extracts on cumulative percent seed germination of alfalfa over time.

Table 1. Effect of *X. occidentale* extracts on β-amylase activities of alfalfa and barley seeds during germination. β-amylase activity in alfalfa and barley seeds during germination was measured based on the released amounts of reducing sugars using DNS method.

Extract concentration	Amount of reducing sugars (mg g^{-1})	
	Alfalfa	Barley
0 g L^{-1}	55.0±5.0 (100.0)*	69.8±2.8 (100.0)
10 g L^{-1}	34.3±3.4 (62.4)	29.6±6.9 (42.4)
20 g L^{-1}	31.1±3.9 (56.5)	10.3±1.9 (14.7)
30 g L^{-1}	27.7±8.8 (50.4)	8.5±2.6 (12.2)
40 g L^{-1}	31.7±1.3 (57.7)	3.1±1.5 (4.4)

*Values in parentheses represent percent of control.

ley seeds during germination, showing 58-96% inhibition (Table 1). The result reflects that the β-amylase activity of barley seed was more vulnerable to the allelochemicals from *X. occidentale* than that of alfalfa. This result shows that inhibition of seed germination by the extracts is associated with the inhibition of β-amylase activity that is dependent on the level of reserved starch. The degree of inhibition in enzyme activity was increased with increasing concentration of the extract. It was thought that such differences between two species might be related to specific amounts of reserved carbohydrates in seeds (Bernfeld, 1955). Because barley seed contains more starch than alfalfa, the inhibition effect on amylase activity was stronger in barley than alfalfa.

Effect of aqueous plant extract on alfalfa seedling growth

At an extract concentration of 20 g L^{-1}, hypocotyl and root lengths of alfalfa were markedly reduced into 83 and 92%, respectively (Fig. 2), and extract concentrations greater than 30 g L^{-1} completely inhibited seedling growth. Root length of alfalfa was more highly reduced by the extracts treatments than was hypocotyls or seed germination. These results coincide with previous reports (Chon & Nelson, 2001; Chung & Miller, 1995) that alfalfa leaf extracts in agar are more inhibitory on root growth than on hypocotyl growth of alfalfa. Rare studies on allelopathic effects of extracts or residues from Compositae plants on some crops as well as on weeds have been reported. Inam *et al.* (1987) reported that aqueous extracts from different plant parts of *Xanthium strumarium* reduced germination, 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 & Jayachandra, 1979), soybean, and corn (Mersie & Singh, 1987).

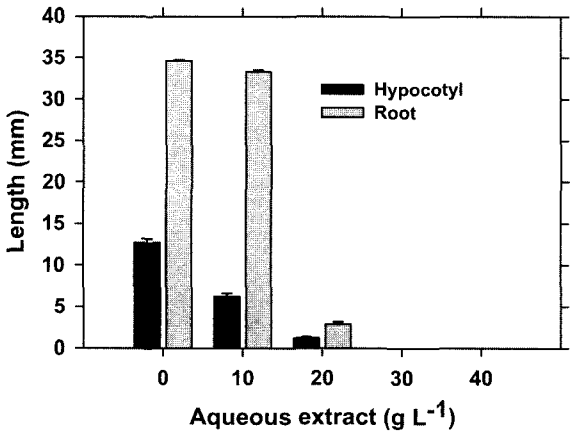

Fig. 2. Effect of aqueous *X. occidentale* extract on hypocotyl and root lengths of alfalfa at 6 d after placing on filter paper wetted with the extracts.

Comparison of aqueous plant extracts from different plant parts for allelopathic effect

Aqueous extracts from different plant parts inhibited seedling length of alfalfa. Leaf extracts above 30 g L^{-1} had the greatest inhibitory effect on root growth of test plant while stem extracts had the least effect. The degree of inhibition was enhanced with increment of the extract concentration. At the highest extract concentration of 40 g L^{-1}, leaf extract reduced root length by 90%, while stem extract stimulated root length of alfalfa by up to 40% over the control (Fig. 3). Such differences might be due to tissue-specific difference in the level of allelochemicals that endow the intensity of allelopathy. Chou & Leu (1992) reported that flowers among plant parts of *Delonix regia* had the highest inhibition effect against test plants. They also concluded that the inhibitory effect of extracts on seed germination and seedling growth in

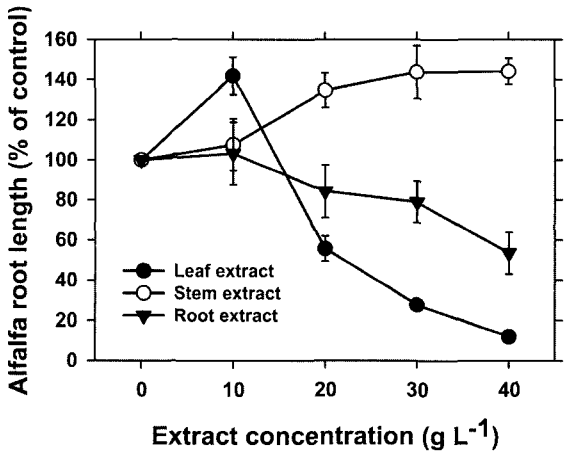

Fig. 3. Effects of *X. occidentale* leaf, stem, and root extracts on root length of alfalfa as affected by different extract concentrations.

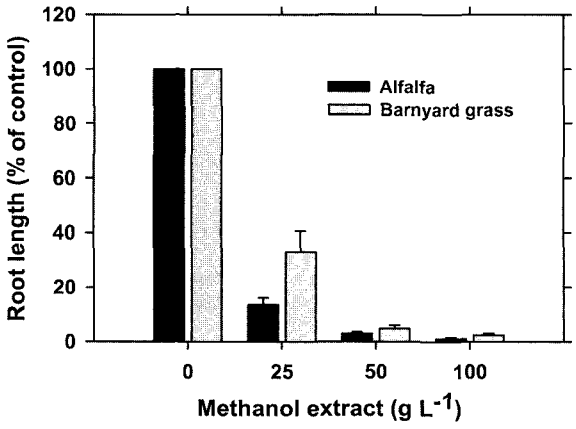

Fig. 4. Effect of methanol leaf extract from *X. occidentale* plant on root lengths of alfalfa and barnyard grass.

bioassay and the quantification of responsible allelopathic compounds found in *Delonix regia* were well correlated.

Effect of methanol plant extracts on alfalfa and barnyard grass root growth

Phytotoxic effect of methanol extracts from *X. occidentale* was assayed with the concentration range from 25 to 100 g L^{-1} against alfalfa and barnyard grass. Methanol extract significantly reduced seedling lengths of alfalfa and *E. crus-galli*. Alfalfa was more sensitive to the extract than was barnyard grass. At 25 g L^{-1}, the extract reduced root lengths of alfalfa and barnyard grass by 85 and 70%, respectively. The extracts at the concentration higher than 25 g L^{-1} completely (above 95% reduction) reduced root growth of two tested plant species (Fig. 4).

Fractionation, identification and quantification of causative allelochemicals

The major allelopathic substances present in the plant were analyzed by HPLC using standard compounds. The total content of phenol compounds from all fractions of *X. occidentale* was 76.8 mg 100 g^{-1}. In *X. occidentale*, three compounds, coumarin, *trans*-cinnamic acid, and chlorogenic acid were mainly detected in all the fractions. Of these, two compounds, chlorogenic acid (33.3 mg 100 g^{-1}) and *trans*-cinnamic acid (20.2 mg 100 g^{-1}) were detected with the greatest level in water and EtOAc fractions, respectively (Table 2). The type and amount of responsible allelochemicals found in the extracts were highly correlated with the inhibitory effects of extracts of the bioassay. Therefore, different allelopathic effects of each fraction from methanol extract would be due to both quantitative and qualitative differences of causative chemicals.

In conclusion, bioassays on allelopathic effects using aqueous or methanol extracts demonstrated that the *X. occidentale* had potent phytotoxic activity and various responsible allelochemicals, showing phytotoxic effects on early seedling growths of alfalfa or barnyard grass. A compound that causes allelopathy might be dependent on the type of plant tissue. HPLC analysis suggests that differential allelopathic effect of fractions might be related to the level of specific allelopathic compounds in certain fractions.

Table 2. Quantitative determination of some phenolic compounds present in leaves of *X. occidentale* by HPLC analysis.

Compound	Fraction			
	Hexane	EtOAc	BuOH	Water
	mg 100 g^{-1}			
Coumarin	0.1431	2.0653	2.0160	0.1845
trans-cinnamic acid	8.3932	20.1550	4.2026	0.2001
Chlorogenic acid	2.2418	0.2406	3.5899	33.3314
Total	10.7781	22.4609	9.8085	33.7160

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