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Allelopathic and Autotoxic Effects of Alfalfa Plant and Soil Extracts

Sang Uk Chon†

Biotechnology Industrialization Center, Dongshin University, Naju-Si, Jeonnam 520-811, South Korea

ABSTRACT: Alfalfa (*Medicago sativa* L.) plants have been reported to be autotoxic as well as allelopathic. Laboratory and greenhouse experiments through petri-dish and pot test were conducted to determine autotoxic effects of alfalfa leaf and soil extracts on the germination or early seedling growth of alfalfa, and to evaluate allelopathic effects of alfalfa leaf residues on alfalfa, barnyard grass, corn, eclipta and soybean. Alfalfa seed germination was delayed depending on aqueous extract concentration, with no difference in final germination after 48 hours. Alfalfa root length was more sensitive to the autotoxic chemicals from leaf extracts than was germination or shoot length. Root growth of alfalfa was significantly inhibited at extract concentration of more than 1 g dry tissue/L (g L^{-1}). Hypocotyl growth, however, was not affected by all the concentrations of leaf extracts. Soil extracts from 4-yr-old alfalfa stand significantly reduced alfalfa root length by 66%, while soil extracts from 0, 1, and 3 yr-old stand stimulated root length up to 14-32% over the control. Residue incorporation with dry matters of alfalfa leaf at 100 g kg^{-1} reduced seedling length of several crop and weed species, ranging from 53 to 87% inhibition. Addition of nutrient solution into alfalfa leaf extracts alleviated alfalfa autotoxic effect. This result indicates alfalfa leaf and soil extracts or residues could exert autotoxic as well as allelopathic substances into soil environments during and after establishment.

Keywords: Alfalfa, allelopathy, autotoxicity, herbicidal activity, plant extracts, residue incorporation, soil extracts

Allelopathy is a chemical interaction between plants or sometimes between microbes and higher plants that includes stimulatory as well as inhibitory influences (Molisch, 1937). Later it was defined as any direct or indirect, harmful or beneficial effect of one plant as a donor plant on another as a recipient plant through the production of chemical compounds that escape into the environment (Rice, 1984; Inderjit et al., 1999). Allelopathy principle plays a significant role under both natural and manipulated ecosystems (Rice, 1984). Autotoxicity is an intraspecific form of allelopathy in which the donor and receptor plants are the same species and have a negative effect (Putnam, 1985).

Alfalfa (*Medicago sativa* L.) has been known to contain water-soluble substance(s) that are autotoxic to the same species (Chung and Miller, 1995a; Jensen *et al.*, 1981; Miller, 1983) as well as allelopathic to bladygrass (*Imperata cylindrica*) (Abdul-Rahman and Habib, 1989), weed species (Chung and Miller, 1995b), cucumber (*Cucumis sativa*) (Ells and McSay, 1991), sorghum (*Sorghum bicolor*) (Hedge and Miller, 1990), corn (*Zea mays*), and soybean (*Glycin max*) (Miller, 1983). Most assessments of autotoxicity or allelopathy have involved bioassays with plant or soil extracts, and residue incorporation.

Autotoxicity of alfalfa during and after alfalfa establishment was first described by Jensen *et al.* (1981). They concluded autotoxicity exists where alfalfa has lower germination, poorer establishment, and lower productivity when grown after alfalfa compared with those after another species or after fallow. Jensen (1984) reported better germination achieved when alfalfa was germinated in 7 days rather than 2 or 4 days after seed treatment with an alfalfa-foliage extract in the laboratory. The common field recommendation is to delay seeding of alfalfa after alfalfa for at least 2 weeks and in some cases up to 2 years (Jennings, 1996).

So far some researchers have attempted to develop new ideas for reducing autotoxicity. Increasing fertilization has been suggested as a method to negate the action of allelochemicals. The interation between the phytotoxicity of certain phenolic compounds and deprivation of nutrients has investigated and demonstrated that the phenolic acids such as *p*-coumaric acid and vanillic acid were uniformly and significantly inhibitory only at low nutrient concentration of nitrogen and phophorous, indicating that allelopathy with phenolics seems most likely to occur in nutrient-deficient soils (Stowe and Osborn, 1980). Whitehead (1964) found in soil beneath a stand of bracken, was inhibitory in water culture, but only slightly inhibitory if nutrients were present in the solution. However, attempts to use added fertility to overcome allelopathy have had mixed results. Adding supplements of N and P to various allelopathic weed residues did not allelviate the deleterious effects of the residues on crop growth (Bhowmik and Doll, 1984).

Although many of allelochemicals are secondary products of plant metabolism, several are degradation products that occur in the presence of microbial enzymes. Probable major biosynthetic pathways leading to production of autotoxic

†Corresponding author: (Phone) +82-61-336-3109 (E-mail) chonsu@lycos.co.kr
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chemicals could be shikimic acid or acetate pathway (Rice, 1984; Macias *et al.,* 2003). Old alfalfa plants as donor plants release cinnamic acid and derivatives that are derived from aromatic amino acids through shikimic acid or acetate pathway (Rice, 1984). Autotoxic chemicals reported for alfalfa are mainly cinnamic acid and its derivatives such as ferulic acid, vanillic, hydroxybenzoic, *p*-coumaric, *trans*-cinnamic acid, caffeic acid (Miller, 1996), saponin (Miller, 1996), and medicarpin (Dornbos and Spencer, 1990). However, the causative chemicals have not been clearly identified.

The objective of this research was a) to evaluate autotoxic and allelopathic effects of alfalfa leaf and soil extracts and residues, in particular, variation in autotoxic effects of soil extracts as affected by different alfalfa growing years, and b) to know if addition of nutrient solution into aqueous extracts affects alfalfa autotoxicity. Short-term autotoxicity as well as allelopathy from alfalfa plant and soil extracts were investigated through laboratory and greenhouse experiments. This research will be useful for better understanding the mechanism of alfalfa autotoxicity or allelopathy in forage cropping system and fate of the chemical (s) in soil.

MATERIALS AND METHODS

Sampling and preparation of extracts

Entire 3-year-old alfalfa plants (cv. Vernal) grown at a field of West Plains, Missouri was harvested at a vegetative stage in November 1995. Fresh alfalfa plants were separated into leaves and stems. The plant samples were directly oven-dried at 40°C for 5 days. The samples were ground with a Wiley mill to pass a 1-mm screen. Twenty grams of dried leaves and stems were separately extracted by soaking in 1 L deionized water at 22°C for 24 hours in a lighted room to give a concentration of 20 g dry tissue L^{-1}. The extract was filtered through four layers of cheesecloth to remove the fiber debris, and centrifuged at 3,000 rpm (× 2063 g) for 4 hours. The supernatant was vacuum filtered through Whatman No. 42 paper. Stock extracts were made fresh for each experiment.

General bioassay

For all experiments seeds were surface sterilized with 0.525 g L^{-1} sodium hypochlorite for 15 min. Seeds were rinsed four times, imbibed in deionized water at 22°C for 12 hours, and carefully blotted using a folded paper towel. Seeds were evenly placed on agar containing extract in each petri dish. The petri dishes were covered, sealed by wrapping in Parafilm, and placed flat in a growth chamber held at 24°C during the14-hour light period and 22°C during the 10-hour dark period. Plates were illuminated at 400 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR) provided by a mixture of incandescent and fluorescent lamps.

Autotoxic effects of leaf extracts on seed germination and seedling growth

In agar bioassay, Difco Bacto agar at a concentration of 16 g L^{-1} was autoclaved at 125°C for 30 min and then equilibrated in a water bath at 50°C along with a flask of stock extract and another of sterile distilled water. The stock extracts were diluted appropriately with sterile warm water to twice each of the desired test concentration, then mixed with the agar solution in a 1 : 1 ratio to give the final concentrations at 1 and 4 g L^{-1}. About 10 mL of extract-agar or water-agar (control) were poured into a 9-cm-diameter plastic petri dish, and allowed to solidify at room temperature.

Fifty seeds of alfalfa Vernal were imbibed in distilled water for 12 hours and placed on the agar-extract surface. Agar containing distilled water was used for the control. Four replications were used in a randomized complete block design. Cumulative germination was determined by counting the number of germinated seeds at 12-hour intervals over a 144-h period and transformed into percent germination. To determine the phytotoxic effect of leaf stem extracts on seedling growth of alfalfa Vernal, twenty five seeds were imbibed in distilled water for 12 hours and placed on the agar surface with leaf extract concentration of 0, 1 and 4 g L^{-1}. Root and hypocotyl lengths of all seedlings in a petri dish were measured at 144 hours after seeding on agar-extract. Root and hypocotyl lengths or plant height of all seedlings were measured in 144 hours after seeding.

Autotoxic effect of soil extracts from different growing years

Bulk samples from the plow Carlow silty clay loam (fine, smetitic, mesic Vertic Endoaquolls) soils were collected to a depth of 15 cm from 0-, 1-, 3- and 4-yr-old alfalfa stand at fields of West Plains, Missouri in November of 1998. Each soil sample was air-dried for 3 weeks in the greenhouse and ground to pass through a 2.0-mesh screen. Chemical properties of alfalfa growing soils investigated as affected by growing year in 1998 were shown in Table 1. Forty grams of dried samples were separately extracted by soaking in 1 L deionized water. The extract was filtered through Whatman No. 1 to remove the fiber debris, and centrifuged at 3,000 rpm for 2 hours. Four milliliters of the extracts were pippetted to Whatman No. 2 filter paper in petri dish. Distilled water was the control. Alfalfa Vernal seeds were evenly placed on filter paper wetted with extract in each petri dish. Root and hypocotyls lengths were measured on all seedlings in each petri dish

Table 1. Chemical properties of alfalfa growing soils investigated as affected by growing year in 1998.

Growing year	pH		N.A. Meq	O.M.	Bray I	Bray II	Ca	Mg	K
	water	soil	100 g^{-1}	(%)	P mg kg^{-1}	P mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
0 (1998)	7.8	7.5	0.3	1.9	57	239	2632	166	199
1 (1997)	7.2	6.6	0.5	2.1	54	220	1680	128	266
3 (1995)	7.1	6.6	0.5	2.8	74	259	1735	149	350
4 (1994)	6.2	5.7	1.5	2.5	77	259	1290	165	336

6 days after placing seeds on the filter paper. The experiments were duplicated, each with four replications.

Effects of Residue Incorporation on Several Crops and Weeds

Residues of each plant part of black soybean were incorporated with a high organic matter-potting medium that contained 30% sphagnum peat moss, 50% vermiculite, 18% zeolite and 2% sand (v/v) per 200 cm^3 pot by vigorously shaking the components in plastic bags. The amount of plant residues in a soil medium used were; 0, 25, 50, 75 and 100 g kg^{-1}. After mixing, pots were filled with the medium mixture and five seeds per pot of alfalfa, barnyard grass, corn, and soybean were planted. The pots were saturated with water by subsurface irrigation. During plant growth, the growing medium was maintained near field capacity by subirrigation without nutrition solution. The experiments were conducted in glasshouse for 15 days at 28/22°C day/night temperatures. All plants were harvested to determine plant height and root length 15 days after seeding. Data were transformed to percent of control for analysis.

Effect of nutrient solutions on alfalfa autotoxicity

Laboratory experiment was designed to know if nutrient solution may reduce autotoxic detrimental effects of leaf extracts on alfalfa. The leaf extracts at 2 and 4 g L^{-1} mixed with half strength of Hoaglands solution were pippetted on Whatman No. 2 filter paper in Petri dish. Distilled water was the control. Alfalfa Vernal seeds were evenly placed on filter paper wetted with extract in each petri dish. Root and hypocotyl lengths were measured on all seedlings in each petri dish 6 days after placing seeds on the filter paper. The experiments were duplicated, each with four replications.

RESULTS AND DISCUSSION

Autotoxic effects of leaf extracts on seed germination and seedling growth

Seeds of alfalfa in control were all germinated within 36 hours. However, seed germination of alfalfa Vernal was

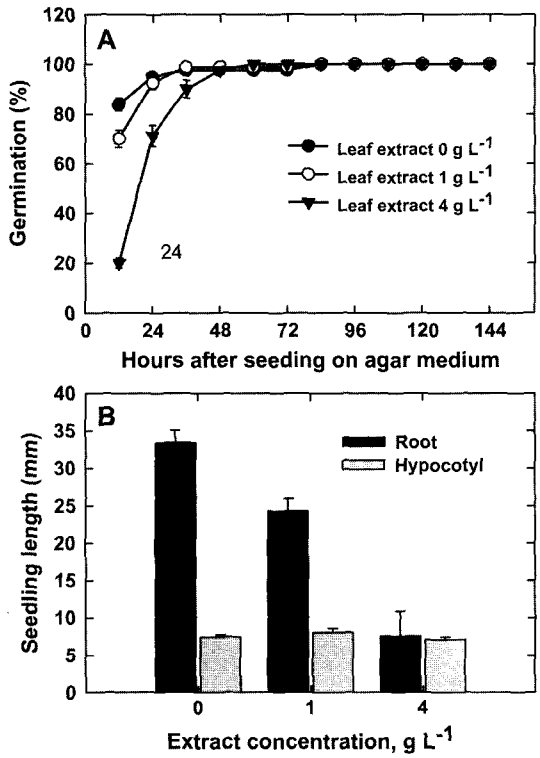

Fig. 1. Effects of alfalfa leaf extracts on accumulative percent germination (A) and root length of alfalfa (B) at 144 hours after placing on extract-agar medium.

delayed when treated with 1 and 4 g L^{-1} leaf extract-agar mixture, not stopped by extracts. Depending on extract concentration there was no differences in final germination at 72 hours (Fig. 1-A). This indicates that final germination was little affected but was delayed by the extract depending on the concentration. Agar-containing leaf extracts showed inhibitory effects on root growth with increasing of extract concentration. Root growth was more inhibited by leaf extracts than was hypocotyl growth (Fig. 1-B). Previous laboratory studies (Hedge and Miller, 1990; Chung and Miller, 1995a; Chon *et al.*, 2000) demonstrated that root elongation was more sensitive to the autotoxin than germination or shoot length.

Autotoxic effect of soil extracts from different growing years

No significant root growth reduction was observed, even

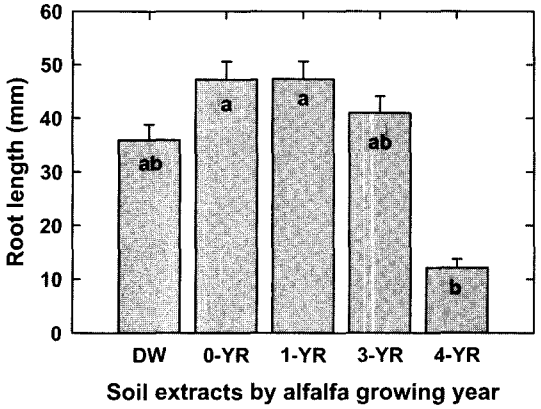

Fig. 2. Effects of alfalfa-growing soil extracts on root length of alfalfa by different growing years at 144 hours after seeding on filter paper. When the F-test for the growing years was significant ($p<0.05$), means were separated on the basis of least significant difference (LSD) at the 0.05 probability level. Means of alfalfa root length are not significantly different ($p<0.05$) from those with the same letter above the bar.

though root growth of alfalfa Vernal was enhanced in soil extracts from 0-, 1- and 3-yr old stands with 14-32% stimulation compared with distilled water as a control (Fig. 2). However, soil extracts from 4-yr-old alfalfa stand significantly reduced root length by 66%. It was thought that the soil extracts from 4-yr-old alfalfa stand could exert more autotoxic substances into soil environment. This confirms the earlier finding of Chung and Miller (1995a) that soil in which alfalfa had previously grown was the most inhibitory on alfalfa growth after 25 d of growth compared with soil where winter rye or hairy vetch had previously grown. Long-term autotoxicity and allelopathy of alfalfa were verified at Urbana, IL, by comparing the germination and growth of alfalfa and sorghum (*Sorghum bicolor* (L.) Moench) on silt loam soil previously cropped to alfalfa (alfalfa- soil) and sorghum (sorghum-soil) (Hedge and Miller, 1990). Plant height and fresh weight per plant of alfalfa and fresh weight per plant of sorghum were lower on alfalfa-soil than on sorghum-soil. Recently, the common field recommendation to avoid autotoxicity is to delay seeding of alfalfa after alfalfa for at least 2 weeks (Tesar, 1993), and in some cases up to 2 years or more (Jennings, 1996).

On the other hand, our experience showed that sterilization through 0.2 μ membrane filter after extraction does not affect autotoxic effects during 6-d assay (Data not shown). Chung and Miller (1995a) reported, in their greenhouse study, that no difference was observed in alfalfa autotoxicity between steam-sterilized and nonsterilized soil at the vegetative alfalfa stage.

Effects of Residue Incorporation on Several Crops and Weeds

Residue incorporation with ground alfalfa leaf materials

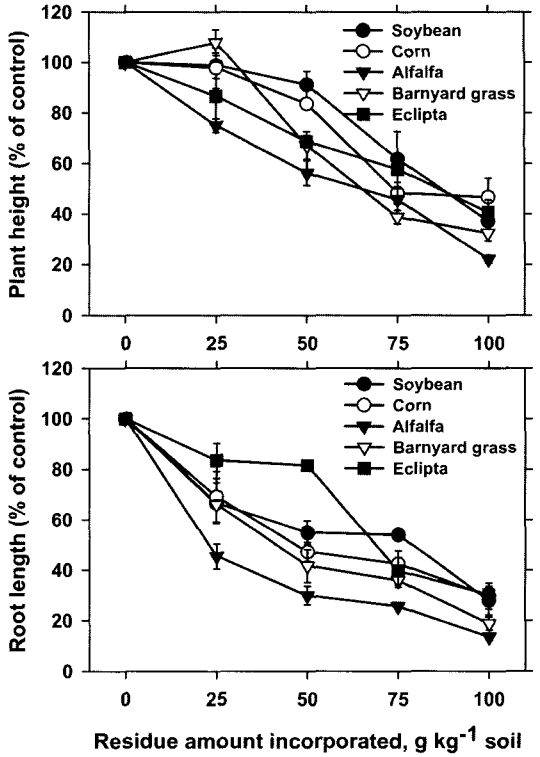

Fig. 3. Effects of residue incorporation with ground leaves on early seedling growth of several crops and weeds at 15 days after planting in pot.

significantly affected both shoot and root lengths of 5 test plants. The degree of inhibition increased with increasing of the incorporated amount of residue. Especially plant residues at 100 g kg^{-1} had the most inhibitory against alfalfa (Fig. 3). Residues at the highest amount of 100 g kg^{-1} reduced shoot growth of alfalfa and barnyard grass by 78 and 68%, respectively, while the residues reduced shoot growth of soybean, corn, and eclipta by 63, 59 and 53%, respectively (Fig. 3). The results showed that alfalfa leaf residues inhibited more root growth of test plants than the shoot growth, and that alfalfa autotoxicity seems to be more severe than allelopathy. Rose *et al.* (1984) reported, in their greenhouse study, that incorporation of 1% ground soybean dry matter into soil inhibited germination and dry weight of greenhouse grown velvetleaf an average of 46% each. The results also indicate that any inhibition of weed growth should be due primarily to the presence of toxic compounds or excessive solutes within the ground black soybean top growth.

Effect of nutrient solutions on alfalfa autotoxicity

To know whether certain allelopathic substances are rendered less inhibitory by the presence of certain nutrients, the interaction between autotoxic leaf extracts and nutrient deprivation was investigated through petri-dish bioassay. Root

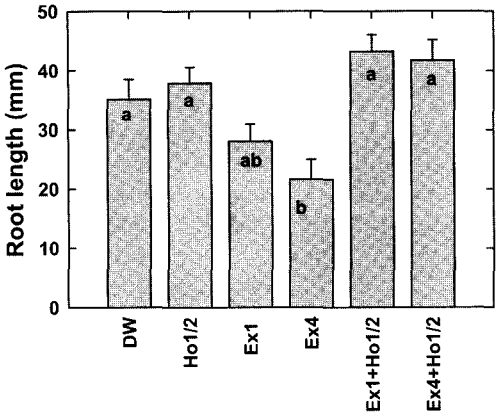

Fig. 4. Effects of nutrient solutions on autotoxicity of leaf extracts at 144 hours after placing on filter paper wetted with extract plus nutrient solution. When the F-test for treatments was significant ($p<0.05$), means were separated on the basis of least significant difference (LSD) at the 0.05 probability level. Means of alfalfa root length seedling length are not significantly different ($p<0.05$) from those with the same letter above the bar. DW : Distilled water, Ho1/2 : Half strength of Hoagland's nutrient solution, and Ex1 or 4 : Plant extracts at 1 or 4 g L^{-1}.

length was increased up to 23 and 19% over the control, respectively, when extracts at 2 and 4 g L^{-1} were mixed with half strength of nutrient solution, showing stimulation by interaction between alfalfa extracts and Hoagland's solution. The result shows that autotoxic effects could be reduced by fertilization. In the earlier study, Whitehead (1964) demonstrates that in soil beneath a stand of bracken was inhibitory in water culture, but only slightly inhibitory if nutrients were present in the solution. Alfalfa autotoxicity with certain chemical inhibitors seems most likely to occur in nutrient-poor soils.

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