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Evaluation of Alfalfa Autotoxicity on Germination and Early Seedling Growth of 3 Cultivars

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ABSTRACT

Autotoxicity restricts reseeding of new alfalfa(*Medicago sativa* L.) after alfalfa until autotoxic chemical(s) breaks down or is dispersed into external environments, often requiring up to a year or more. One solution for reducing autotoxicity would be to select germplasms or cultivars with tolerance to the autotoxic chemical(s) and use genetically breeding program. Bioassay of seed germination and early seedling growth was conducted to evaluate autotoxic responses of 3 varieties of alfalfa to the water-soluble extracts(at 4 and 8g/L) from alfalfa 'Cody' leaf by using agar and filter paper medium in a petri-dish assay. Root length at 5 days after seeding was more sensitive to the extract than was hypocotyl length or seed germination, and was a better parameter of autotoxic effects of alfalfa leaf extracts. Use of an agar medium gave better sensitivity of root length than did use of filter paper. Evaluating tolerance with percent of control was more important indicator than was mean of root length, because of significant variation among varieties in root length of control treatment. Bioassay ranked varieties in the following order of tolerance on the basis of relative root length;" Cody" >" Pioneer 5373" >" Alfagraze". Seedling growth from old "Cody" seed was more sensitive to the autotoxic chemical(s) than was that from newly produced seed.

Key words : Alfalfa Autotoxicity, Growth Medium, Root Length, Relative Root Length.

INTRODUCTION

Allelopathy is defined as any direct or indirect harmful or beneficial effect of one plant on another through the production of chemical compounds that escape into the environment(Rice, 1984). Allelopathy plays a significant role in both natural and agro-ecosystems. Several weeds, crops, and trees have been shown to exert allelopathic influence on the crops, thus affecting their germination and growth adversely.

Autotoxicity is a specialized intraspecific type of allelopathy in which the donor and receptor plants are the same species and have a detrimental effect(Putnam, 1985). Reseeding of alfalfa is often not successful due to autotoxicity(Jensen et al., 1981). Alafalfa, as a perennial legume forage crop, has known to contain water-soluble substance(s) that are autotoxic to the same species(Chung and Miller, 1995a and 1995b; Hall and Henderlong, 1989; Hedge and Miller, 1992; Jensen

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et al., 1981; Klein and Miller, 1980; Miller, 1983) as well as allelopathic(Chung and Miller, 1995; Ells and McSay, 1991; Hedge and Miller, 1990; Miller, 1983) to other species.

Miller(1983) reported that the best preceding crop for alfalfa establishment is corn(*Zea mays* L.), followed by various grains, soybean(*Glycine max* L.) and the worst preceding crop is alfalfa. Klein and Miller(1980) indicated that alfalfa was difficult to establish without rotating to another crop first. When alfalfa was plowed in the fall and reseeded with alfalfa the following spring, yield and stand counts of alfalfa in the single cropping situation were markedly lower than when rotated with either corn or corn and soybeans.

Recently, some researchers have attempted new ideas for reducing autotoxicity, or as an additional weed management strategy. One approach has been to utilize rotational crops or companion plants in annual or perennial cropping system, especially, utilizing tolerant or resistant rotational varieties to the auto-allelochemicals(Klein and Miller, 1980; Miller, 1983). Another approach has been to screen for tolerant or resistant types in germplasm collections of crops, the idea being to transfer this character into varieties by either conventional breeding or genetic transfer technique(Chung and Miller, 1995b; Miller, 1992).

Genetic tolerance to the chemical(s) has been evaluated in the field without very much success. Chung and Miller(1995b) have recently classified some alfalfa varieties in order of decreasing inhibition to allelochemicals extracted from alfalfa herbage. Miller(1992) suggested that the autotoxicity problem in alfalfa might be solved by selecting new varieties that do not produce various allelochemicals or those that are resistant to these compounds. Substantial qualitative and quantitative variation in allelochemical content exists both within and between plant species. Selection for allelopathic genotypes among accession of crops has been attempted as a biological weed management strategy. Limitation information is available on breeding efforts in alfalfa for reducing autotoxicity or allelopathy. In terms of alfalfa tolerant to autotoxicity, it is thought that there were no cultivar differences for these characteristics(Goplen and Webster, 1969; Miller, 1983; Wyman-Simpson et al., 1991).

An appropriate bioassay that distinguishes autotoxic factors from competitive or inherent growth properties of alfalfa is needed to screen germplams, and to assess adoption of management practices. The bioassay will also allow more in-depth localizations of the mechanisms involved and will help with analytical procedures to determine the chemical(s) responsible. Autotoxic and allelopathic effects have been evaluated in petri plates with filter paper(Chung and Miller, 1995a; Cope, 1982; Hall and Henderlong, 1989; Hedge and Miller, 1990), but results can be inconsistent due to nonuniform moisture conditions or swelling of the paper in localized areas(Peters, 1968; Pederson, 1986). Bioassay techniques(Carlson et al., 1983; Dornbos and Spencer, 1990; Pederson, 1986) in which the extract is mixed in agar provide an alternatives and perhaps more sensitive evaluation of allelopathic effects(Dornbos and Spencer, 1990; Peterson, 1985).

The objective of this study is to develop best parameter of bioassay for general screening of the tolerant and susceptible genotypes or varieties to auto-allelochemicals through evaluating on autotoxic effect of alfalfa leaf extracts on 3 alfalfa varieties by using two different media.

MATERIALS AND METHODS

Sampling and Preparation of Extract

Three-year-old alfalfa(cv. Cody) plants was harvested at a vegetative stage from a field near West Plains, Missouri in November 1995 and oven dried at 40℃ for 5 days. Dried alfalfa plants were separated into leaves(blades and petioles) and stems. Leaf samples

were ground with a Wiley mill to pass a 1-mm screen and then stored until used. Twenty grams(dry weight) of tissue were extracted by soaking in 1 L distilled water for 24 h at 24℃ in a lighted room. The extract was filtered through four layers of cheesecloth to remove the fiber debris, then centrifuged at low speed(3,000 revolutions min^{-1}) for 4 hours.

Comparison of Growth Media

Our goal was to maximize root growth, ease of measurement, and precision of the assay. We evaluated root and hypocotyl growth on filter paper and agar when roots grew laterally. Two layers of Whatman No. 1 filter paper were placed in each 9-cm-diameter plastic petri-dish. Five milliliters of diluted extract were pipetted to the filter paper. The stock extract(20 g/L) was diluted appropriately with water to give the desired final concentration. Distilled water was used as a control. Difco Bacto agar(1.6%, w/v) was autoclaved for 25min at 125℃ and then equilibrated in a 50 ℃ water bath along with a flask of stock extract and one of sterile distilled water. The stock extract(20g/L) was diluted appropriately with water and then mixed in a 1:1 ratio with the agar solution to give the desired final concentration. About 10ml of extract-agar or water-agar(control) were poured into 9 cm-diameter plastic petri dishes, covered, and allowed to solidify for 4 hours at room temperature.

Bioassay

Seeds of Alfagraze, Cody(Cody II), and Pioneer 5373 collected in 1996 were used for the experiment. To compare sensitivities to the autotoxin with newly produced-Cody II seeds of Cody collected in 1994(Cody I) also were used. The seeds were surface sterilized for 15 min with sodium hypochlorite 5.25 %: water(10:90 v/v), rinsed, then imbibed for 12 h in deionized water at 25 ℃ and blotted by folding into a paper towel for 30 min. Twenty swelled seed were evenly placed on the agar or filter paper in each petri dish. The petri dishes were covered, sealed by wrapping in parafilm , and placed flat in a growth chamber programmed at 24 ℃ during the 14-h light period and 22 ℃ during the dark period. Plates were illuminated at 400 μmol photon $m^{-2}s^{-1}$ PAR provided by incandescent and fluorescent lamps. The number of germinated seed(radicles 1-mm long) was determined at 12 hour-intervals over a defined period. Hypocotyl and root length were measured on all seedlings in each petri dish 5 days after placing seeds on the medium. Data were transformed to percent of control for analysis. Also, we calculated root growth rate(mm/hour) through measuring root and hypocotyl length in agar and filter paper medium at 24-hour intervals over 120 hours. When the F-test was significant($p<0.05$) means were separated on the basis of least significant difference(LSD) at the 0.05 probability level. Autotoxic effect on root length of alfalfa expressed as percent of control was evaluated on three varieties of alfalfa using conditions of agar or filter paper, in light condition, with extract concentrations of 4 and 8 g kg-1.

RESULTS AND DISCUSSION

Seed Germination

Germination was counted as number of germinated seed per 20 seed and transformed to percent germination. Controls germinated by 90 % in 24 hour, but 8 g/L extracts apparently delayed seed germination of all varieties. Cody I had the lowest germination at no-extract control. Bioassay showed no significant differences in germination among varieties.

Hypocotyl Growth

Most hypocotyl growth ceased after 4 days and was only slightly decreased by extracts regardless of medium. Hypocotyl length of control was near 5.8 mm at 120 hours but it was considerably reduced(4.3 mm)

Table 1. Hypocotyl length of 3 alfalfa varieties as affected by different concentrations 5 days after seeding on agar or filter paper containing leaf extract from 'Cody' alfalfa.

Variety	Agar				Filter paper			
	Control	4g/L	8g/L	LSD 0.05	Control	4g/L	8g/L	LSD 0.05
Alfagraze	5.4(mm)	5.0(mm)	4.5(mm)	ns1)	5.8(mm)	5.1(mm)	4.9(mm)	ns
Cody I	5.4	4.4	4.1	0.5	5.9	4.6	4.5	0.7
Cody II	5.7	5.0	4.4	0.6	5.5	5.0	5.1	0.4
Pioneer5373	5.5	4.6	4.1	0.4	5.4	4.8	4.5	ns
LSD0.05	0.3	0.5	ns		ns	ns	ns	

[1)]No significance among treatments.

when exposed to extracts(Table 1).

Hypocotyl growth was not very sensitive to the autotoxic chemical(s) whether grown in agar or filter paper. Hypocotyls generally grow by cell division and especially cell elongation, and the final cell number and cell length are important regulations(Cavalieri and Boyer, 1982). During germination and early seedling growth, light alters the allocation of carbon, nitrogen, and mineral resources between the hypocotyl and primary roots. Even though the cotyledone turned green they likely contributed little photosynthesis. In a simplistic sense, the reduced growth of the hypocotyl in light would allow more resource, including photosynthate, to be allocated to root growth.

We regularly observed the hypocotyl arching upward shortly after its growth initiated, thus breaking direct contact with the extract. This corroborates data from earlier researches(Chung and Miller, 1995a; Hedge and Miller, 1990) indicating the hypocotyl growth was rather insensitive, probably because the tissue escaped contact. In the controls, slower hypocotyl growth with both agar and the paper flat method were due to exposure to the light. Our earlier study regarding the rolled paper-vertical method reported that hypocotyl growth actually was very sensitive to extract, but since most bioassay were conducted under light, hypocotyl growth was inhibited by light and made it less responsive to extracts(Chon, et al, 2000).

Root Growth and Root Growth Rate

Root lengths in agar medium were significantly inhibited at the extract concentrations of 4 and 8 g/L. Root length(32 mm) of control of cody II had the highest values at and at 4 g/L extract had the longest root length at 7.4 mm, but at 8 g/L extract treatment there was no significant differences among varieties. On

Table 2. Root length of 3 alfalfa varieties as affected by different concentrations 5 days after seeding on agar or filter paper containing leaf extract from 'Cody' alfalfa.

Cultivar	Agar				Filter paper			
	Control	4g/L	8g/L	LSD 0.05	Control	4g/L	8g/L	LSD 0.05
Alfagraze	31.5	5.0	3.8	3.4	24.6	19.3	17.9	4.4
Cody I	25.4	4.4	3.0	2.8	21.7	16.7	14.4	3.0
Cody II	31.8	7.4	3.8	4.2	23.1	21.3	21.2	ns[1)]
Pioneer5373	28.3	4.4	3.0	3.9	19.3	16.9	16.1	3.5
LSD 0.05	5.6	2.6	0.7		5.1	2.8	3.5	

[1)]No significance among treatments.

the other hand, root lengths in filter paper are insensitive to extracts compare to agar medium. Pioneer 5373 and Cody I had shorter root lengths at control(21.7 and 19.3 mm, respectively) and 4 g/L extracts(about 17 mm), and were susceptible varieties whereas Cody II had the longest root length at control(23.1 mm) and both concentrations of extracts(about 21 mm), and was the most tolerant variety(Table 2).

Root length increased steadily through 120 hours and was decreased as the extract concentrations increased. However, the data showed that it is necessary to use a relative value because root length at control was not consistent with those at extract treatment, derivating from significant variation among varieties. Root growth rates of Cody II and Alfagraze were higher than those of Cody I and Pioneer 5373 at control and extracts. Root growth rate of control treatment had highest values at Cody II(0.27 mm/h). When exposed to extracts responses against cultivars showed a similar tendency to controls. The results also very similar to those of root length(Table 3).

Relative Root length

We expressed our data as percent of control because of inherent genetic differences in root growth, and effect of small changes in environments or time which would alter absolute length. Calculating as percent of control, however, adds to experimental error due to the ratio of two numbers, each with an error, but allows data to be compared more reliably. This is most important when comparing germplasms(Chon et al, 1996). These results are similar to those of previous studies(Pederson, 1986; Carlson et al., 1983) in which greater root lengths were obtained on agar medium than on germination paper. Since maximization of root growth is important, the extract agar technique used in this study provides a more precise evaluation of allelopathic effects. Cultivars were ranked in the following order of tolerance on the basis of relative root length: "Cody II" > "Pioneer 5373" > "Alfagraze" > "Cody I". Root growth was more inhibited by autotoxin in agar than in filter paper indicating agar medium haas more sensitivity to extract. Use of an agar medium gave better root growth of controls and lower standard errors than did use of filter paper. Cody II was most tolerant cultivar to autotoxin of extracts and Cody I and Alfagraze were susceptible cultivars. Extract at 8 g/L inhibited root growth of Cody II by about 9 % while it inhibited root growth of Cody I and Alfagraze by 34 and 27 %, respectively(Fig. 1). These data suggested evaluation alfalfa autotoxicity was more influenced by variations in relative root length than by variations in final root length. In a routine screening program one may be able to use root length as a bioassay considering

Table 3. Root growth rate of 3 varieties as affected by different concentrations 6 days after seeding on agar or filter paper containing leaf extract from 'Cody' alfalfa.

Variety	On agar			On filter paper		
	0 g/L	4 g/L	8 g/L	0 g/L	4 g/L	8 g/L
	mm/hour					
Alfagraze	0.26	0.05	0.03	0.21	0.16	0.15
Cody I	0.21	0.04	0.03	0.18	0.14	0.12
Cody II	0.27	0.06	0.03	0.19	0.18	0.18
Pioneer	0.24	0.04	0.03	0.16	0.14	0.13
LSD(0.05)	0.05	0.02	ns*	0.04	0.02	0.03

* No significance among means within a column.

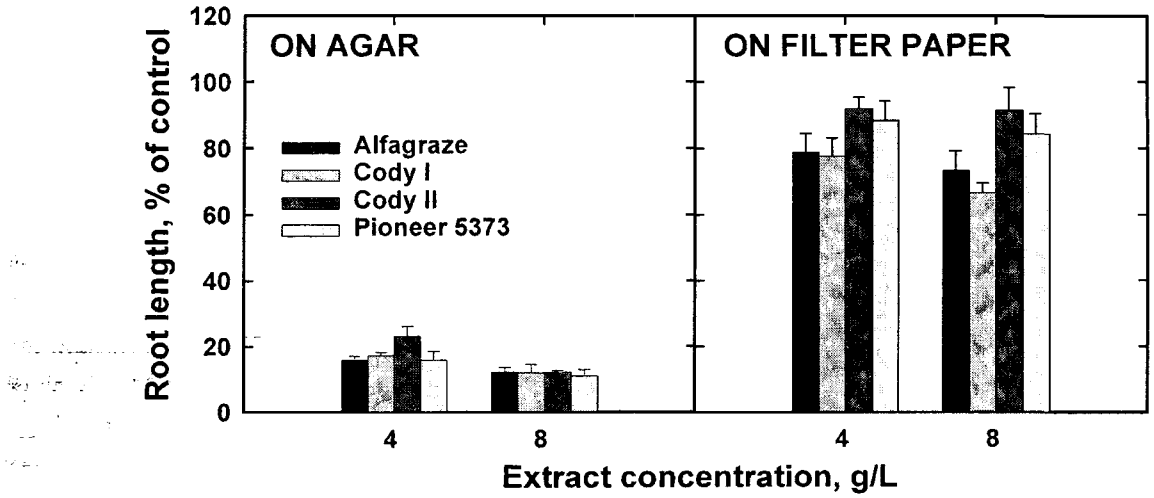

Fig. 1. Effect of extract concentrations on relative root length of 3 cultivars of alfalfa 120 hours after placing seeds on agar and filter paper. Each bar represents standard error of the mean.

that all seeds need to germinate on the same day to be equal in age at measurement. The seedling with the longest roots should be most tolerant. A control treatment would be needed to determine the relative toxicity level of the soil or bioassay environment.

Comparison of Growth Media

An aspect need to be considered, the effect of the media on the controls, and on response to the extract. The growing medium affected the control treatments, especially root length(Table 1, 2). Seedlings and roots were greater in the agar than in the filter paper. Controls grew least in the paper flat treatment, perhaps because the roots grew horizontally on the paper surface instead of more vertically as was the case in agar flat, and even more so in the agar vertical. Root growth on paper was primarily lateral and may have reduced root-media contact making it harder for the seedling to absorb water for growth. Hypocotyl length in both methods was much less affected by the extracts, remaining near 4 to 5 mm at both concentrations comparing with control(5 to 6 mm). Our research and research review of previous showed that most bioassays which use seeds placed on the medium did not show a clear response of hypocotyl growth to the extract due to avoiding contacting the surface of the extract-agar medium. Even though hypocotyl on the paper flat contacts easily to the surface of extract-wetted paper, it rapidly orients vertically and escapes from the surface. However, hypocotyl growth and root growth in the paper rolled-vertical method were more sensitive to extracts than in other methods due to continuous contact with the extracts.

Autotoxic effects of Cody alfalfa leaf extracts on 3 cultivars were evaluated on agar-medium containing extract. Genetic tolerance to the autotoxic chemical(s) would expand the use of a cultivar. Genetic tolerance of alfalfa seedlings to the chemical(s) has been evaluated in the field without very much success. For example, there was little difference among cultivars that differed in disease resistance(Hurt et al., 1996) or phytophthora resistance(Cosgrove, 1996). These more recent studies confirm earlier reports(Goplen and Webster, 1989; Miller, 1983). There have been few laboratory evaluations. Chung and Miller(1995b) evaluate d extracts from seven cultivars of alfalfa on seed germination and early seedling growth of the same cultivars. The data suggested genetic variation, but due

to the nature of the experiment, one can not determine if the difference is due to genetic variation in the production of the chemical, tolerance to the chemical, or a combination of both. The bioassay to select tolerant or susceptible germplasms to the autotoxin indicated that root length was more responsive than hypocotyl length or germination, and was sensitive to autotoxin among gerplasms.

These results are supported by that of Cope(1982), who reported that germination imbibition in legumes was not as good a measure of phytotoxicity as root growth. Peterson(1986) also concluded that root length score of white clover gave a better indication of the allelopathic effect of tall fescue than germination percentage.

In conclusion, root length was a more sensitive parameter to autotoxin than germination or hypocotyl length. Use of an agar medium gave better root growth of controls and lower standard errors than did use of filter paper. We evaluated 3 cultivars of alfalfa for root growth response to the autotoxin and found that bioassay ranked cultivars in the following order of tolerance on the basis of root growth; "Cody" > "Pioneer 5373" > "Alfagraze". Based on root length, plants were more sensitive to the alfalfa extract in the agar flat method than in the filter paper. Our observations indicated seedlings partially avoided the extracts due to uneven contact of root with extract and paper. Root length on filter paper method was reduced as the extract concentration increased, but hypocotyl length was less sensitive to the extract concentrations.

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