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Control of leafy spurge with natural chemical products

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Abstract:

Bioassays were used to evaluate the phytotoxicity of natural chemicals contained in extracts of plants and fungi for possible use as control agents of leafy spurge (*Euphorbia esula* L.). Sunnhemp (*Crotalaria juncea* L.) seeds contained a water-soluble substance that was highly phytotoxic in a Lemna bioassay, and that inhibited the growth of leafy spurge by 65%. Planting sunnhemp in pots with growing leafy spurge also inhibited this weed by 75 to 85%. *Alternaria angustiovoidea* (Simmons) growing in liquid culture produced substances that were phytotoxic to Lemna, but showed limited effects on leafy spurge.

Introduction

Among several strategies to control the proliferation of leafy spurge is the use of natural chemical products. This concept is not new and suggested exploitation has taken several forms including the use of these products as natural herbicides/plant growth regulators, as the structural basis for herbicides, or enhancement of the production of these chemicals in the producing organism (Einhellig and Leather, 1988). Research at this laboratory has demonstrated that selected crop plants can be used to reduce weeds (Leather, 1983) and, in certain situations, can control weeds to the same extent as applied synthetic herbicides (Leather, 1987).

Biological control (in the classical sense) of leafy spurge has had limited success, but with additional research has good potential (Carlson and Littlefield, 1983). The use of pathogenic fungi for leafy spurge control is currently under investigation (Littlefield, 1985), and a prospective *Alternaria* sp. has been identified (Krupinsky and Lorenz, 1983; Simmons, 1986). However, the requirements for infection with the fungus and the possi-

ble diversity of biotypes of leafy spurge (Yang *et al*., 1988), suggest that alternative approaches are necessary.

The objectives of this research were to determine the potential for using the palatable legume plant *Crotalaria juncea* (sunnhemp) and/or its chemical products for leafy spurge control and to identify any toxins produced by *Alternaria* sp. that may be used in a similar manner. There have been many toxins identified from *Alternaria* sp. and a few are host-specific (Nishimura and Kohmoto, 1983). With the development and use of highly sensitive bioassays (Leather and Einhellig, 1988), we hope to identify naturally produced chemicals that may be useful for the control of leafy spurge.

Materials and methods

Fungal culture

Alternaria angustiovoidea was grown on potato-dextrose agar (PDA). A block of agar with mycelium was cut from the margins of three- to five-day old cultures on PDA with a No. 3 cork borer and transferred to a 250 ml flask containing 50 ml of Fries medium (liquid). A total of 20 flasks were used on two occasions. The flasks were incubated in darkness at 25ºC for two weeks.

Extraction

After the culture period, the fungal mycelium was separated from the culture medium by filtration through a 0.25 um membrane filter, both freeze-dried and stored at 20ºC until extracted. For sunnhemp tissues were ground in a Wiley Mill at 20 mesh and stored at -20ºC until extracted.

The extraction procedure was the same for the *Alternaria* and sunnhemp. The freeze-dried material was extracted at 6ºC for 24 hours with 80% methanol/water. The slurry was centrifuged and the pellet discarded. The supernatant was partitioned with an equal volume of hexane three times. The hexane fraction was discarded after it was determined by bioassay to be void of active materials. The methanol/water fraction was evaporated at 35ºC under vacuum until only the water remained. The water extract was freeze-dried and an aliquot was bioassayed for phytotoxicity.

Three grams of the freeze-dried extract were reconstituted in water and layered on a pre-activated Amberlilte XAD-4 column. The extract was eluted with 200 ml water and collected in 50 ml fractions followed by 200 ml MeOH also collected in 50 ml fractions. The water fractions were freeze-dried, and the MeOH fractions were reduced to 2 ml. All fractions were stored at -20ºC until used for bioassay.

Bioassays

The primary bioassay for phytotoxicity was the *Lemna* (duckweed) bioassay described by Einhellig *et al*., (1985), using *Lemna obscura* (Leather and Einhellig, 1985). The bioassay employed a 24 well tissue culture plate with 1.5 ml of growth medium and

a beginning 4-frond *Lemna* plant. A 3 mg sample of each freeze-dried fraction was reconstituted in 100 ml of water and 5 ul of the solution was placed in a well giving a final concentration of 100 ppm. A 5 ul aliquot of the concentrated MeOH fractions was used directly for assay. After 7 days incubation, *Lemna* growth was determined by counting the fronds and measuring dry weight. Anthocyanin production was also determined when phytotoxicity was not evident. Each treatment was replicated four times.

The *Alternaria* fractions were also bioassayed by placing a 10 ul droplet of the solutions upon a puncture made by a needle through a detached leafy spurge leaf. The detached leaves were maintained on moist filter paper in a closed 100 by 15 mm petri plate and incubated at 28ºC under constant light. Each treatment was replicated four times.

Greenhouse assays of the fractions were accomplished by determining the regrowth of leafy spurge. Leafy spurge plants, growing in sand/Hoagland and Arnon's (1950) solution were severed 1 cm above the crown. After 3 days, when the regrowth of leafy spurge was evident, 10 ml of the reconstituted MeOH/HOH extract (1000 ppm) was used as a crown drench for each plant. The drench was repeated after a five-day interval. Each treatment was replicated six times.

A second greenhouse test was used to evaluate the influence of germinating and growing sunnhemp on the growth of leafy spurge. Two, four or eight sunnhemp seeds were distributed in pots around a single 6-week-old leafy spurge plant growing in sand culture. The sunnhemp seeds were covered with vermiculite and watered. Growth of the leafy spurge was determined after 7, 13, 24, and 35 days by the change in height measured from the sand surface. After 35 days the leafy spurge was severed 1 cm above the crown and dry weight determined for each plant. On day 35 the sunnhemp was also severed 0.5 cm above the cotyledonary node. The pots were maintained an additional 21 days and the dry weight of the leafy spurge regrowth was again determined. Each treatment was replicated six times.

Chromatographic analysis

Active fractions of the sunnhemp and *Alternaria* were further separated by High Performance Liquid Chromatography (HPLC) with a reverse-phase column and using ultraviolet absorption detector set at 254 nm. The mobile phase was water isocratic for 5 minutes; a linear gradient to 90% MeOH in 25 minutes; isocratic at 90% MeOH for 5 minutes; and a linear gradient to water in 5 minutes.

Results and discussion

Results of the primary bioassays using duckweed indicated that all tissue extracts of sunnhemp contained phytotoxic compounds. Extracts of the seed, however, contained a compound(s) that was highly toxic to the duckweed. Further separation indicated the toxic material was water-soluble and was eluted from the XAD-4 column in the first water fractions. HPLC analysis indicated that the fraction contained one major compound with a few minor peaks. The major peak eluted during the first 5 minutes while the mobile phase was at 100% water.

Extracts of the *Alternaria* mycelia and culture medium were also active in the duckweed bioassay; the mycelium had the greater phytoxicity. Further separation on the XAD-4 column indicated activity was not confined to a single fraction but eluted with both water and methanol. HPLC analysis confirmed a number of ill-defined peaks that were present in most fractions. Further research is needed to separate the active component(s) into cleaner fractions.

In the leafy spurge bioassay, the mycelium extracts did not produce a phytotoxic reaction. The extracts of medium, however, caused discoloration and/or necrosis 1 to 5 mm around the puncture in the leafy spurge leaf.

In greenhouse experiments, only the sunnhemp extract was phytotoxic to leafy spurge (Table 1). The dry weight of the leafy spurge regrowth was less than 50% of that of control plants. In addition to a stunted appearance, the sunnhemp extract caused a loss of chlorophyll (yellowing) and twisting and curling of the leaves and stem.

Table 1. Effect of natural compounds on the regrowth of leafy spurge in greenhouse culture.

Treatment	3 wk regrowth[a]
	(mg dry wt.)
Control	282.3 b
Alternaria angustiovoidea extract	255.3 b
Crotalaria juncea extract	132.0 a

[a]Means followed by the same letter do not differ significantly at the p=0.05 level.

Sunnhemp seeds and seedlings in pots with leafy spurge reduced the growth of the spurge plants, and this was significant after 13 days (Table 2). It was interesting that only two sunnhemp seeds were required to produce maximum growth inhibition of the leafy spurge. Overall inhibition after 35 days was 50 to 60% as determined by height. The dry weight of leafy spurge (Table 3) also reflects the overall inhibition after 35 days.

Table 2. Influence of sunnhemp on the growth of leafy spurge in sand culture.

Treatment	Change in height[a] Days			
Seeds/pot 1-7	7-13	13-24	24-35	0-35
	(cm)			
0	4.2 a	3.9 b	5.9 b	7.8 b 21.8 b
2	3.2 a	1.4 a	4.2 a	4.1 a 12.9 a
4	3.8 a	1.5 a	3.9 a	3.0 a 12.1 a
8	3.2 a	1.5 a	4.2 a	2.8 a 10.8 a

[a]Means in a column followed by the same letter do not differ significantly at the p=0.05 level.

Table 3. Dry weight of leafy spurge five and eight weeks after seeding with sunnhemp.

Treatment Seeds/pot	Leafy Spurge[a]	
	5 weeks	8 weeks[b]
	-------- (mg dry wt.) --------	
0	1042.5 b	738.7 b
2	368.2 a	188.5 a
4	510.0 a	126.7 a
8	337.0 a	86.0 a

[a]Means in a column followed by the same letter do not differ significantly at the p=0.05 level.
[b]Leafy spurge was severed 1 cm above crown and allowed to grow for 3 weeks after the initial 35-day growing period.

The sunnhemp plants were severed after 35 days to eliminate the possible influence of competition for light. Leafy spurge begins regrowth within 3 days of cutting while the sunnhemp requires 2 weeks before regrowth is visible. The dry weight of the leafy spurge growing with eight sunnhemp was 12% of that of control plants, while that growing with two sunnhemp was about 25% of controls. We suggest that chemicals leached from the seeds of sunnhemp, which from other bioassay data indicates high phytotoxicity, inhibit the growth of leafy spurge and that inhibition continues with the presence of the sunnhemp plant.

Initial results from this ongoing research suggest that sowing of sunnhemp on leafy spurge infestations could be an important factor in the biocontrol and management of this weed. Results also indicate that once identified, the phytotoxic component of the sunnhemp seed could be an important natural herbicide. Although there are phytotoxic compounds in the growth medium and the mycelium of *A. angustiovoidea*, further research is needed to determine the nature of those compounds and whether they are important in disease expression of this fungus on leafy spurge.

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