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## Leafy spurge research at WRRC/ARS

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The following report summarizes continuing research efforts conducted at the Western Regional Research Center, Agricultural Research Service, Albany, California.

### Artificial diets for *Aphthona*

Research observations (1) have led to the projected use of the flea beetle (*Aphthona* sp.) as a biological control agent for leafy spurge. The projected introduction of this bio-control insect has defined a need to develop an artificial diet for the mass rearing of this insect to provide sufficient quantities for large releases in spurge infested areas. In this report we will summarize the initial attempts to formulate an effective artificial diet for these insects in the WRRC laboratory. It should be noted that the diapause of *Aphthona* represents a major obstacle in the continuous evaluation of potential diets for these insects since only one generation is available each year.

Eleven artificial diets have been reported (2) for members of the Chrysomelidae family of which *Aphthona* is a member. These diets were formulated in the WRRC laboratory and applied to *Aphthona* sp. None of these formulations were found to be effective. Chemical extractives of leafy spurge roots were considered as a possible source of feeding stimulants for the flea beetle and four extracts of the roots were evaluated as additives to a base diet for *Aphthona flava* (Table 1). The results of these feedings show no phagostimulatory effects for the total extracts.

**Table 1. Effect of extracts of leafy spurge on survival of larvae of *Aphthona flava* \*.**

Extract	Yield (% dry wt.)	Survival (mean no. of days)
Hexane	10.9	1.6 <sup>a</sup>
Acetone	1.4	1.4 <sup>a</sup>
Methanol	4.4	1.8 <sup>a</sup>
Water	0.6	0.8 <sup>a</sup>
Hexane + acetone	-	0.6 <sup>a</sup>
Methanol + water	-	0.6 <sup>a</sup>
Control diet	-	3.8 <sup>b</sup>

\*2nd Instar Larvae, 10 replicates/treatment of 2 larvae/container.

When freeze dried secondary leafy spurge roots were ground and added to a 1.7% agar media, larval feeding was observed to increase with increasing concentration of the

ground root material (Table 2). A mean survival of 4.2 days was observed at a concentration of 100 mg/ml of agar. Higher concentration of ground root material could not be suspended in the agar.

**Table 2. Effect of leafy spurge root powders in agar on survival of larvae *Aphthona flava*.**

Conc. (mg root powder/ml)	Survival (Mean No. of days)
100	4.2 <sup>a</sup>
50	3.8 <sup>a</sup>
10	3.0 <sup>a</sup>
5	2.2 <sup>b</sup>

\*Freeze-dried secondary roots in 1.7% agar.

Fresh root material proved to be most suitable material for rearing the *Aphthona flava* larva (Table 3). Both chopped and ground fresh root material exposed to larva on filter paper produced many pupae and 20 or more day survival times. Chopped and ground fresh root material added to 1.7% agar showed fewer pupae or no pupae and shorter survival times. It is apparent that the agar media does have some adverse effect on the feeding ability of larva.

**Table 3. Effect of leafy spurge fresh root preparations on survival of larvae of *Aphthona flava*.**

Root Preparation	Survival (Mean No. of Days)
Chopped Fresh .25-.50 cm. (on filter paper)	20+ (many pupae)
Ground by Polytron (on filter paper)	20+ (many pupae)
Chopped Fresh .25-.50 cm. (in 1.7% agar)	15+ (few pupae)
Ground by Polytron (in 1.7% agar)	10+ (no pupae)

The results of these preliminary investigations indicate that fresh root tissue contain the phago-stimulants and nutrients necessary for flea beetle development. A closer examination of this tissue for specific chemical or biochemical factors is planned.

### **Characterization of Leafy Spurge Volatiles**

The introduction of monophagous insect predators to North American leafy spurge has been complicated by the inability to correlate susceptible leafy spurge accessions to insect predation. The diverse morphological character of North American spurge accessions has thwarted definitive taxonomic differentiation of the plant by classical means. Chemical taxonomic investigations of leaf waxes (3), latex (4) and root (5,6) extractives

of leafy spurge have shown variations in diterpenes and triterpenes among spurge accessions which may be useful in their taxonomic separation. Information about the volatile constituents of leafy spurge accessions could offer additional chemo-taxonomic information and provide baseline data relative to insect attraction or feeding on leafy spurge plants.

Aerial plant tissue from field grown (mixed accessions) and greenhouse-grown (accessions 1979ND1, 1978A1 and 1978OP1) leafy spurge plants were blended in water, vacuum codistilled, liquid-liquid extracted (ether) and concentrated. The concentrated distillate was subjected to GC/MS analysis and separated components were compared to reference library data. Preliminary identification of compounds was verified by comparison to authentic reference compounds.

A total of 119 volatile compounds were found to be present in trace or greater levels for one or more of the four leafy spurge concentrates. Six-carbon alcohols were the predominate constituents in the concentrates and major differences (mostly quantitative) were found between the field-grown and greenhouse samples. The greenhouse samples contained higher concentrations of specific components while the field-grown samples had more trace-level components. The 17 components present in 1% or greater concentration in at least one of the four concentrates are included in Table 4. Greenhouse-grown plants produced 11-13 compounds that occurred in concentrations  $\geq$  1%. Field-grown plants produced 5-8 compounds that were  $\geq$  1%.

**Table 4. Major components ( $\geq$  1% of total FID area) of leafy spurge concentrates.**

Component	Greenhouse		Field	
	G-1 Area %	G-2 Area %	F-1 Area %	F-2 Area %
<b>Alcohols</b>				
ethanol	0.4	1.2	0.7	1.8
pentanol	1.2	0.7	tr	0.3
hexanol	5.1	3.8	3.7	5.5
heptanol	5.5	3.8	tr	tr
octanol	2.8	1.4	tr	0.1
nonanol	19.0	12.4	tr	0.1
methylbutanol,3-	1.5	1.4	1.1	3.1
cyclohexanol	11.0	18.9	tr	
hex-2-enol, (E)-	7.1	7.9	22.6	21.5
hex-3-enol, (Z)-	28.6	24.5	63.5	51.4
<b>Aldehydes</b>				
heptanal	1.0	0.2		tr
nonanal	7.4	1.1		tr
hex-2-enal,(E)-	0.8	0.4	1.7	1.2
<b>Esters</b>				
ethyl formate	tr	6.4		tr
ethyl acetate	0.3	7.4	0.5	2.4
hex-3-enyl acetate,(Z)-	0.9	1.9	0.8	1.7

tr=concentration  $<$ 0.12% from GC/FID area percent measurements.

Alcohols were the predominant volatiles detected in the spurge concentrates with (Z)-hex-3-enol occurring as a major component in all concentrates. (E)-Hex-2-enol and hexanol were also detected as major components in both the field-grown and greenhouse concentrates. Cyclohexanol and nonanol were abundant in the greenhouse samples but were essentially absent in the field samples. Cyclohexanol is not commonly found in plant tissue concentrates and its presence in spurge will be confirmed in later studies of the volatiles in this plant. The homologous series of normal primary alcohols and a number of branched acyclic primary and secondary alcohols occur in low concentrations. Several unsaturated alcohols were identified and with the exception of the abundant hexenols, their yield was less than 1%. Three aldehydes derived from prominent alcohols were detected and three esters, several ketones, pyridine, limonene and p-cymene, several non-terpene hydrocarbons and carboxylic acids were found in the concentrates.

The results of this investigation show that reproducible volatile profiles can be obtained from leafy spurge shoots. They further indicate differences in the volatile constituents of the greenhouse accessions and the field-grown samples which include the effect of the growing environment. The results provide the framework for an examination of individual accessions grown under identical conditions and also describe chemical components occurring in leafy spurge which should be evaluated in insect tests as attractants or feedants.

### **The biochemical basis of the allelopathic interaction of leafy spurge and small everlasting**

The early observation that the low-growing plant small everlasting produced allelochemicals which inhibited the spread of leafy spurge (7) prompted a cooperative investigation (ARS/WRRC and NDSU) (8) which characterized phenolic compounds (arbutin, hydroquinone and caffeic acid) occurring in the chemical extractives of small everlasting. Hydroquinone, which occurs in low yield in small everlasting, was shown to be a potent phytotoxin to leafy spurge and lettuce seedlings. The research findings suggested that hydroquinone, derived from a large arbutin pool by hydrolysis, may serve as a chronic allelochemical toward leafy spurge. The chemical investigation indicated that more conclusive evidence of the mode of action of allelochemicals toward leafy spurge might be obtained through an examination of the biochemical processes of phytotoxicity at the cellular level. A cooperative investigation (ARS/WRRC, MRRL and NDSU) has now been undertaken to investigate these processes.

Suspension culture cells, obtained from ARS/MRRL stock, have been treated with varying concentrations of hydroquinone and arbutin to confirm prior toxicity data and determine the appropriate concentration level to assess chronic exposure. Exposure of the cells to hydroquinone (Table 5) clearly confirm the toxicity of hydroquinone at the cellular level and establish  $5 \times 10^{-4}M$  as the concentration level for chronic exposure. Toxicity results for exposure of leafy spurge cells to arbutin at the same concentrations showed no difference from control at all levels.

**Table 5. Summary of initial concentration experiments with leafy spurge suspension culture cells.**

Treatment	Mean fresh weight (g)	
	Day 4	Day 8
1% Methanol Control	10.92	19.61
10 <sup>-5</sup> M Hydroquinone	11.16	15.90
10 <sup>-4</sup> M Hydroquinone	7.35	14.44
10 <sup>-3</sup> M Hydroquinone	1.35	1.46
10 <sup>-2</sup> M Hydroquinone	cells dead	

Notes:

Based on these experiments,  $5 \times 10^{-4}$  M hydroquinone was chosen as a reasonable concentration for further experiments.

Ether extractions of the media from the above samples showed no traces of hydroquinone, with the exception of the 10<sup>-2</sup> M treatment, in which the cells appeared brown and dead.

Based upon the exposure results, the ability of leafy spurge to detoxify hydroquinone through glucosylation at  $5 \times 10^{-4}$  M and 10<sup>-3</sup> M at the cellular level was evaluated (Table 6). The results show that hydroquinone is successfully glucosylated to form arbutin but that the efficiency of the detoxification is reduced at increased concentrations. Enzyme assays with extracts from the treated cells suggest the presence of a UDPG-dependent glucosyltransferase enzyme capable of catalyzing the hydroquinone to arbutin reaction. At higher concentrations, the glucosylation reaction efficiency is reduced; suggesting the presence of toxic levels of the phytotoxin. Based upon these preliminary results, cell culture experiments have been designed to evaluate the effect of chronic exposure of hydroquinone to leafy spurge. Further purification of the glucosyltransferase catalyzing the hydroquinone to arbutin transformation should yield specific characterization of the enzyme.

**Table 6. Conversion of hydroquinone to arbutin in leafy spurge suspension culture cells.**

Treatment	Fresh Weight	Arbutin (ug/10 ul)	Percent Conversion
Methanol Control	18.35	0	--
$5 \times 10^{-4}$ M Hydroquinone	12.67	11.53	85%
$1 \times 10^{-3}$ M Hydroquinone	10.46	19.31	71%

Notes:

Hydroquinone was added to the cells 24 hours after inoculation. Fresh weight was measured seven days after inoculation.

Arbutin was chromatographed on a C18 reverse phase HPLC column and detected by UV absorbance (280 nm).

Evaluation of the phytotoxicity of secondary metabolites produced by small everlasting toward leafy spurge at the cellular level would substantiate field and laboratory observations and would allow access to biochemical observations which could describe the mode of action of the phytotoxins. Callus derived from small everlasting leaf tissue has now been successfully cultured in our laboratory thereby providing the means for the evaluation of secondary metabolites produced at the cellular level as phytotoxins to leafy spurge. The evaluation of lettuce seed germination on media supporting small everlasting and leafy spurge (Table 7) indicates that small everlasting culture exudes phytotoxic material into the support media.

**Table 7. Lettuce seed germination bioassay on callus culture media.**

Treatment	Percent germination
Control	97%
Leafy spurge agar	85%
<i>Antennaria</i> agar	19%

Notes: Data were collected after sterile seeds had been placed on the agar for 48 hours and the emerging radicals were at least 2 mm long.

Attempts are now being made to isolate a bioactive fraction from this *Antennaria* agar.

Co-culturing experiments with small everlasting and leafy spurge callus (Table 8) reveal that leafy spurge callus growth is reduced by about 50% when co-cultured with small everlasting or grown on media (on which small everlasting was grown).

**Table 8. Co-culturing experiments with callus cultures of *Antennaria microphylla* and *Euphorbia esula*.**

Treatment	Percent increase in growth
I. leafy spurge control	27.21 <sup>c</sup>
II. leafy spurge, co-cultured with <i>Antennaria</i>	13.84 <sup>b</sup>
III. leafy spurge on agar from which <i>Antennaria</i> was removed after one week	14.25 <sup>b</sup>
IV. <i>Antennaria</i> , co-cultured with leafy spurge	31.45 <sup>c</sup>
V. <i>Antennaria</i> control	27.70 <sup>c</sup>

Notes:

<sup>a</sup>After one week of growth at 28° C in 24 hour darkness.

<sup>b</sup>Significantly different from the leafy spurge control at the 0.05 percent level.

<sup>c</sup>The controls are not significantly different from each other, or from treatment IV. The same experiment set up in divided petri dishes produced no significant differences between any of the treatments, i.e. there is not a volatile phytotoxin (data not shown).

Small everlasting, in contrast, is not affected by the presence of leafy spurge in the same culture media. These data are consistent with the earlier reports of similar experiments with whole plants and provide substantial evidence of small everlasting's ability to produce phytotoxins which are particularly effective against leafy spurge. Analysis of the media on which the small everlasting is grown is presently underway in an effort to characterize specific compounds which are present and compare them to the phenolics obtained from the whole plant. The successful isolation and characterization of specific allelochemicals from cultured plant cells would provide the first information relative to the definitive description of allelopathic mechanisms at the cellular level.

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