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EPICUTICULAR WAX CONSTITUENTS OF NORTH AMERICAN AND EUROPEAN *EUPHORBIA ESULA* BIOTYPES

GARY D. MANNERS* and DAVID G. DAVIS†

*Western Regional Research Center, ARS, USDA, Berkeley, California, U.S.A.; †Metabolism and Radiation Research Laboratory, ARS, USDA, Fargo, North Dakota, U.S.A.

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Abstract—The epicuticular leaf wax of four North American and one Austrian *Euphorbia esula* biotypes was examined as a potential source of chemotaxonomic information relative to intraspecies classification. Analysis (GC and GC/MS) shows general similarity of wax constituent character among all biotypes but differences in specific component yields between the North American and Austrian biotypes. Distinctive variation in occurrence of five triterpenes (α- and β-amyrin, δ-amyrenone, 24-methylenecycloartenol and lupeyl acetate) was observed between the North American and Austrian biotypes.

INTRODUCTION

The perennial noxious weed *Euphorbia esula* (leafy spurge) infests 2.5 million acres of range and pasture land in the upper great plains of the United States. The plant is toxic to livestock [1], allelopathic to desirable forage plants [2] and poses a serious threat to livestock production on open range lands. Leafy spurge can be controlled by herbicides. However, the cost of control is high and continuous since the weed cannot be completely eradicated by chemical means [3]. Spurge is controlled naturally in Europe by indigenous insect predators, however, attempts to utilize these predators as biological control agents in North America have proved unsuccessful [4]. Several biotypes of *E. esula* have been identified [5, 6], suggesting the possible occurrence of separate North American and European leafy spurge species which cannot be differentiated morphologically, or the existence, within a single species, of intraspecies physiological or chemical differences between the biotypes. These differences may have significant effects on insects which are predators to this weed.

Intraspecies chemical and/or biochemical comparisons of the recognized *E. esula* biotypes may provide chemical taxonomic information relative to biological control methods. Individual *E. esula* biotypes have been previously examined chemically with the reported occurrence of *n*-alkanes (C_{25}–C_{32}) [7], long-chain alcohols (C_{26} and C_{28}) [7, 8], long-chain aldehydes (C_{26}, C_{28}, C_{30}) [9], sitosterol [7], triterpenes (24-methylenecycloartenol, cycloartenol, lupeol) [7, 10], flavanoid glycosides (kaempherol-3-glucuronide) [11] and phorbol esters (ingenol derivatives) [12–14]. This investigation is the first chemical comparison of North American and European *E. esula* biotypes to evaluate the feasibility of using epicuticular wax constituents as chemical taxonomic indicators.

RESULTS AND DISCUSSION

Yields of chloroform-soluble leaf wax from five spurge biotypes ranged from 0.8 to 1.4%. The major portion (~ 80%) of the wax consisted of hydrocarbons, long-chain alcohols and long-chain esters (Table 1).

The long-chain alcohols were the single most prominent (> 50%) group of compounds in the four North American leafy spurge waxes (biotypes 5, 13, 14 and 17). The amounts of alcohols, hydrocarbons, aldehydes, acids and triterpene esters occurring in these four biotypes were generally comparable while greater variations in yield were observed for the long-chain esters and triterpenes.

The long-chain alcohols (29%) were also the major components occurring in the Austrian spurge biotype (10), but the amount of alcohols was *ca* half that found in the North American plants. The wax of the European *E. esula* also contained higher amounts of hydrocarbons, aldehydes and terpenes than were present in the domestic leafy spurge waxes. The specific variation in the chemical constituents of these component classes provides evidence suggestive of chemical differences between the domestic and European biotypes.

The hydrocarbon constituents of the waxes occurred almost exclusively in the acetone-insoluble wax fraction. The domestic and European biotypes all contained the commonly occurring C_{29}, C_{31} and C_{33} alkanes [11] with a predominance of the C_{31}-constituent (Table 2). The domestic leafy spurge biotypes had generally similar amounts of the C_{29}- and C_{33}-alkanes, and the C_{31}-alkane occurred as more than 50% of the total hydrocarbon yield. The Austrian leafy spurge biotype contained the same hydrocarbons as the North American biotypes, with a similar yield of the C_{29}-alkane. This biotype, however, had the largest amount of C_{33}-alkane.

The long-chain alcohols and aldehydes were present primarily in the acetone-insoluble wax fraction; however, small amounts of these compounds also occurred in the acetone-soluble wax fraction. Quantitative data for these constituents (Table 3) reflect total yield from both fractions. All of the biotypes contained alcohols and aldehydes of C_{26}-, C_{28}- and C_{30}-chain lengths.

1-Hexacosanol (C_{26}) was the major alcohol in all of the

Table 1. Per cent composition and yield of epicuticular waxes of five biotypes of *Euphorbia esula*

	Biotype				
	North American				Austrian
Component	5	13	14	17	10
Hydrocarbons	12	18	16	14	25
Free alcohols	54	52	53	57	29
Aldehydes	1	1	2	1	4
Free acids	3	2	3	2	4
Esters	17	13	7	10	18
Triterpenes	3	5	10	7	11
Triterpene esters	2	2	4	3	2
Unidentified	8	7	5	6	7
Yield; % dry wt (mg)	0.9 (86)	1.1 (256)	1.2 (183)	1.2 (47)	0.1 (139)
Me_2CO-soluble (%)	5.8	7.8	13.7	10.6	11.5
Me_2CO-insoluble (%)	94.2	91.2	86.3	89.4	88.5

Table 2. Per cent composition of hydrocarbons of epicuticular wax of five biotypes of *Euphorbia esula*

	Biotype				
	North American				Austrian
Carbon No.	5	13	14	17	10
29	13	10	10	16	12
30	2	2	1	2	2
31	51	60	51	55	47
32	3	5	5	4	4
33	18	11	11	10	23
34	2	1	1	1	3
35	6	5	9	5	4
36			2		
37		1	4	1	1
38					
39			2		
Unidentified	5	5	4	6	4

biotypes. This alcohol represented *ca* 90% of the alcohols in the North American *E. esula* samples whereas the European spurge wax contained 71%. In addition, the Austrian sample also contained more 1-octacosanol (C_{28}, 14%) and 1-triacontanol (C_{30}, 15%) than the domestic spurge.

Octacosanol (C_{28}) was the major aldehyde (> 60%) in all of the spurge biotypes. Three of the domestic biotypes (5, 14 and 17) and the Austrian biotype had similar amounts of the C_{26}-, C_{28}- and C_{30}-aldehydes. The remaining domestic biotype (13) had a notably larger amount of the C_{30}-aldehyde and correspondingly less of the C_{26}-aldehyde than the other biotypes.

The methyl esters of the C_{26} to C_{34} even-carbon alkanoic acids were detected in the acetone-insoluble fractions (after methylation) of all of the spurge biotypes (Table 3). The C_{26}-, C_{28}- and C_{30}-acids represented more than 70% of the total acids present in the leaf wax. The yields of the long-chain acids were generally similar among biotypes 5, 10 and 14 with the three biotypes having the highest proportion of triacontanoic acid (C_{30}). Lower yields of the C_{30}-acid were found in biotypes 13 and 17, while these biotypes showed substantially higher yields of the C_{28}- and C_{26}-acids, respectively.

The unsaponified esters of the spurge waxes were classified after hydrolysis, methylation and GC/MS of sublimation fractions of the acetone-insoluble fraction. Although the sublimation did not yield pure fractions, comparative GC/MS analysis of the fractions indicated the chemical character of the long-chain esters. The ester composition of the domestic spurge waxes was generally similar while the Austrian spurge showed a higher yield of C_{26}, C_{28}-alcohol–C_{18}, C_{20}-acid esters and a correspondingly lower yield of the C_{26}, C_{28}-alcohol–C_{22}, C_{24}, C_{26}-acid esters (Table 4).

Table 3. Per cent composition of free alcohols, aldehydes and acids in five biotypes of *Euphorbia esula*

	Biotype														
	North American												Austrian		
	5			13			14			17			10		
Carbon No.	ALC	ALD	AC	ALC	ALD	AC	ALC	ALD	AC	ALC	ALD	AC	ALC	ALD	AC
26	91	24	24	91	14	24	91	34	22	88	34	37	71	27	18
28	8	69	30	7	62	40	8	61	22	11	65	26	14	64	31
30	1	7	30	2	24	22	1	5	37	1	10	16	15	9	35
32			12			8			12			12			11
34			4			6			7			9			5

Table 4. Per cent composition of esters of five biotypes of *Euphorbia esula*

	C_{26}, C_{28}-Alcohol Biotype					C_{26}, C_{28}, C_{30}-Alcohol Biotype				
	North American				Austrian	North American				Austrian
Acids	5	13	14	17	10	5	13	14	17	10
C_{16}, C_{18}	14	13	12	7	18					
C_{18}, C_{20}	16	16	17	14	29					
C_{22}, C_{24}	38	42	35	28	42	26	24	27	30	21
Unidentified	6	5	9	6	4					

The acetone-soluble fraction of the leaf waxes was composed primarily of terpenes and terpene esters. Only the free triterpenes in the fraction were quantified (Table 5). Five triterpenes represented *ca* 30–40% of the acetone-soluble wax fraction. These were α-amyrin, β-amyrin, δ-amyrenone, 24-methylenecycloartenol and lupeyl acetate. The comparative distribution of these compounds among the leafy spurge biotypes showed that all of the domestic plants contained high amounts of α-amyrin, low amounts of β-amyrin and moderate amounts of δ-amyrenone. In contrast, the European spurge plant contained large amounts of β-amyrin, moderate amounts of α-amyrin and no δ-amyrenone. Considerable amounts of 24-methylenecycloartenol occurred in all of the biotypes except 14 where much less of this tetracyclic triterpene was found. Smaller amounts of lupeyl acetate were also found in biotype 14 and the European biotype than were found in the three remaining domestic biotypes.

The analysis of epicuticular plant wax constituents has received continued attention since an early review [15] suggested their potential and advantages as chemical taxonomic indicators. Recent analytical data of plant waxes suggest that the panicoid and festucoid grasses may be differentiated on the basis of specific long-chain alcohol composition [16]. However, the use of these alcohols as chemotaxonomic indicators is considered to be limited to the characterization of groups of species or genera, but not to tribes [17]. Epicuticular triterpene methyl ethers have been employed in the chemotaxonomy of New Zealand grasses, with their applicability limited to intraspecies comparisons [10, 12].

The analysis of the leaf wax constituents of five separate *E. esula* biotypes showed, with minor variations, that all of the leafy spurge biotypes contained similar hydrocarbon compounds, had high yields of the same long-chain alcohols (particularly 1-hexacosanol), and were similar in both aldehyde and acid composition. These data may be chemotaxonomically characteristic of the genus *Euphorbia* and are comparable to the suggested chemotaxonomic criteria for separating the panicoid and festucoid grasses at the genus level [16].

Table 5. Per cent composition of free triterpenes of five biotypes of *Euphorbia esula*

	Biotype				
	North American				Austrian
Terpene	5	13	14	17	10
β-Amyrin	5	4	9	7	44
δ-Amyrenone	11	7	7	8	—
α-Amyrin	37	41	67	55	22
24-Methylenecycloartenol	28	31	5	19	23
Lupeyl acetate	4	5	1	4	2
Unidentified	15	12	11	7	9

The dramatic differences observed in the yields and occurrence of the triterpenes α-amyrin, β-amyrin and δ-amyrenone among the five *E. esula* biotypes provide evidence supporting the suggestion that North American leafy spurge may be an interspecies hybrid of *E. esula* and *E. virgata* [5], and further suggests the potential importance of the wax triterpenes as chemotaxonomic indicators in leafy spurge. A more detailed examination of the nature and distribution of the epicuticular wax triterpenes among *E. esula* could provide important information relative to the chemotaxonomic differentiation of leafy spurge.

EXPERIMENTAL

Plant source. Several leafy spurge plants (*E. esula* L.) displaying similar floral characteristics but variable leaf characteristics and growth habits were selected in the field. Numbers were assigned to the plants in the order of receipt, and the origin of the biotypes in this investigation were: Becker County, Minnesota (biotype 5); Kalkaska, Michigan (biotype 13); Baker, Oregon (biotype 14); Becker County, Minnesota (biotype 17). Biotype 10 originated from Krems, Austria and was obtained (along with biotypes 13 and 14) from a leafy spurge nursery maintained by Dr. Melvin McCarty, USDA, ARS, Lincoln, Nebraska. The selected biotypes displayed variable mature leaf size with average leaf areas of *ca* 480, 520, 660, 1070 and 650 mm^2 for biotypes 5, 10, 13, 14 and 17, respectively.

Root stock of the biotypes was transplanted to Fargo clay soil in a greenhouse in Fargo, North Dakota and grown under natural light with supplemental 40 W cool white fluorescent winter lighting to give 14 hr days. The plants were watered with tap water and fertilized occasionally with commercial fertilizers. Shoots were trimmed every 6–8 weeks and roots were repotted as necessary. All biotypes were grown under identical conditions and retained their distinctive growth habit and leaf characteristics over a 3 year period.

Wax collection. Preliminary scanning electron microscopic examination of leaf surfaces before and after dipping in $CHCl_3$, CH_2Cl_2, Et_2O and hexane established $CHCl_3$ as the most efficient leaf wax solvent. Shoots from 4- to 6- week-old greenhouse plants were excised for wax collection. A sample was taken for a dry wt determination and the remainder of the plant was weighed and dipped in $CHCl_3$ at room temp. for 30 sec. The

$CHCl_3$ soln was concd (*in vacuo*) to dryness, weighed, flushed with N_2 shipped to the analysis site (California) and stored at 0° prior to work-up.

Solvent partition of wax. The $CHCl_3$-soluble wax was dissolved in CH_2Cl_2 (800 ml), Me_2CO (200 ml) was added, and the soln concd (100°) to 25 ml. The conc. soln was removed and cooled to yield a waxy crystalline material. The crystalline material was collected by suction filtration (with difficulty) and washed with a small amount of Me_2CO. The filtrate was concd further and the procedure was repeated until minimal crystallization occurred. The final Me_2CO-soluble filtrate was concd to dryness under N_2 and weighed. The Me_2CO-insoluble materials were combined, dried (under N_2) and weighed. The percentage of the total wax occurring as Me_2CO-soluble and -insoluble material in the five biotypes is recorded in Table 1.

GC. Wax samples were analysed on a FID instrument with He carrier gas (19.1 cm/sec), utilizing a SCOT fused silica column (18 m × 0.25 i.d. mm, SE 30). The injection temp. was 275°, detector temp. 375°, and the oven programme was 170°–325° at 5°/min. Wax samples were dissolved in C_6H_6 and 0.5–1.0 μl samples were injected. Retention times (R_{TH}) are reported relative to the retention time of 1-hexacosanol [$R_{TH} = R_T$(obs.) − R_T(1-hexacosanol)]. Individual component yields were based upon integrated peak areas. Portions of the Me_2CO-soluble fraction were analysed immediately by GC or GC/MS. Me_2CO-insoluble material was methylated (CH_2N_2–Et_2O) prior to analysis.

GC/MS analysis of individual components from Me_2CO-soluble and -insoluble wax fractions was accomplished on a quadrupole mass spectrometer using a 15 m × 0.32 i.d. DB-1 fused silica capillary column with an ionizer temp. of 180°. Oven programme conditions were the same as for analytical GC runs. Additional GC/MS spectra were obtained using a magnetic sector instrument with a data system. A 15 m × 0.32 mm i.d. (Quadrex 077) non-polar fused silica capillary column was used with splitless injection and a 150°–300° (8°/min) programme.

Ester analysis from sublimation. Me_2CO-insoluble wax (30–50 mg) was subjected to a fractional sublimation at 0.025 mm/Hg according to the following schedule:

Fraction No.	Elapsed time (hr)	Oil bath temp. (°)
1	1	140
2	2	140
3	1	140
4	1	160
5	2	160
6	2	190
7	2	190
8	2	190

Material from each fraction was analysed by GC. Fractions containing long-chain esters (5, 6 and 7) were treated with MeOH–HCl according to ref. [20]. The hydrolysed–methylated ester mixtures were analysed by GC and GC/MS to determine acid and alcohol moiety chain lengths.

Acetone-insoluble wax analysis. Specific yields of hydrocarbons, alcohols, aldehydes, methylated acids and esters present in the Me_2CO-insoluble fractions were identified on the basis of comparison (GC and GC/MS) with authentic compounds. The long-chain esters had long retention times (R_{TH} = 24–51) while the remaining constituents occurred at R_{TH} = 0–12.

Acetone-soluble wax analysis. The fraction contained small amounts of C_{26}- and C_{28}-alcohols and -aldehydes. The amounts of these constituents were determined in terms of wt fractions; the total yields of these compounds were established on the basis of wt % of the total Me_2CO-soluble and -insoluble wax (Table 5). Five triterpenes were identified in the Me_2CO-soluble fraction on the basis of comparison (GC, GC/MS) with standard compounds or derivatives of standard compounds. β-Amyrin, R_{TH} = 10.43; MS *m/z* (rel. int.): 426 (6) $[M]^+$, 411 (2), 218 (100), 207 (2), 203 (26), 189 (10), 161 (4), 147 (5), 109 (13). δ-Amyrenone ($\Delta^{13(18)}$-oleanen-3-one), R_{TH} = 10.65; MS *m/z* (rel. int.): 424 (70) $[M]^+$, 409 (29, 308 (8), 245 (26), 232 (20), 217 (13), 205 (100), 191 (13), 189 (30), 177 (15), 175 (16), 163 (19), 161 (23), 109 (89). α-Amyrin, R_{TH} = 10.97; MS *m/z* (rel. int.): 426 (17) $[M]^+$, 411 (5), 218 (100), 207 (11), 203 (26), 189 (27), 161 (4), 147 (16), 109 (29), 175 (19), 161 (38), 147 (29), 135 (59). 24-Methylenecycloartenol, R_{TH} = 11.77; MS *m/z* (rel. int.): 440 (5) $[M]^+$, 425 (6), 422 (17), 407 (18), 379 (6), 300 (6), 288 (7), 203 (14), 189 (14) 187 (12), 175 (19), 173 (10), 161 (19), 134 (29), 55 (100). Lupeol acetate, R_{TH} = 12.27; MS *m/z* (rel. int.): 468 (55) $[M]^+$, 453 (11), 408 (27), 393 (12), 365 (20), 298 (12), 276 (13), 249 (16), 229 (17), 218 (15), 208 (16), 204 (45), 203 (36), 189 (100), 175 (20), 161 (29).

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