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Relationship of tarbush leaf surface secondary chemistry to livestock herbivory

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Abstract

Tarbush (*Flourensia cernua* DC.) is an abundant but generally unpalatable shrub native to the Chihuahuan Desert. The objective of this study was to examine the leaf surface chemistry of tarbush in relation to degree of use by ruminants. Mature tarbush leaves were collected on 2 sites during 2 periods approximately 2 weeks apart from plants exhibiting either high (>45%) or low (<10%) use when browsed by cattle, sheep, and goats confined to a small area (5 plants per use category for each site/period combination). A greater ($P<0.05$) concentration of epicuticular wax was detected on the leaf surface of plants that were used to a lower degree (8.2 and 10.3% of the leaf dry matter for high- and low-use plants, respectively). Several leaf surface compounds were related to period, while site and degree of use were seldom related to individual mono- and sesquiterpenes measured in this study. Camphene and 10 unidentified compounds differed between periods ($P<0.10$). Two unidentified compounds were related to site ($P<0.10$) and 2 others were related to use ($P<0.10$). In summary, individual leaf surface compounds on tarbush do not appear to greatly affect degree of use of tarbush by livestock, but collectively (based on epicuticular wax data), these compounds may influence the diet selected by browsing ruminants.

Keywords: *Flourensia cernua*, leaf surface chemistry, diet selection, epicuticular wax, monoterpenes, sesquiterpenes

Many shrubs receive limited use by herbivores (Owen-Smith and Cooper 1987); consequently, they maintain a competitive advantage over frequently or intensively grazed plant species. Secondary metabolites often serve as defense against herbivory (Freeland 1991), and in desert ecosystems, this phenomenon may be applicable to woody species. Woody species appear to rely on a chemically diverse array of specific compounds, rather than classes of compounds, for defense (Bryant et al. 1991). Phytochemicals may combine with other abiotic and biotic factors to influence niche expansion of shrubs, and plant chemistry may be involved in shrub dominance and desertification.

Desert shrubs typically exist in a high carbon/low nitrogen environment, which should favor a carbon-based plant defense (Bryant et al. 1985) rather than biomass production. Antiherbivore compounds in desert woody perennials are primarily terpenoids and phenols (Meyer and Karasov 1991). Some terpenoids are involved in metabolic processes, membrane structure, surface properties, or sensory cues for insects, but others appear to function mainly in defense (Meyer and Karasov 1991). Monoterpenes, especially oxygenated monoterpenes, have been shown to deter mammalian herbivory (Schwartz et al. 1980, Elliott and Loudon 1987, Gershenzon and Croteau 1991).

We postulate that an understanding of the secondary chemistry of shrubs as it relates to herbivore selectivity and environmental factors will lead to improved methods of range management. Increased shrub use, reduced shrub competitiveness, disruption of desertification, and reduced herbicide use are potential benefits. We chose tarbush (*Flourensia cernua* DC.) as a model for evaluating these relationships because although use as a forage by livestock is generally low, tarbush has comprised 12% of cattle diets during early summer (Anderson and Holechek 1983). Our objective was to evaluate the phylloplane secondary metabolite profile of tarbush as related to plant phenology, site, and degree of use of individual plants by livestock. Our hypothesis was that preferential use of specific tarbush plants from the population was determined by combinations and concentrations of secondary phylloplane compounds.

Materials and Methods

Study Site

The study site was located in an area heavily infested with tarbush on the Jornada Experimental Range (JER) in New Mexico. The area was excluded from livestock grazing in 1988. Prior to that time, the area had been exposed to light to moderate stocking rates over the past 75 years. The long-term (1915-1990) mean annual and seasonal (July, August, and September) rainfall is 241 and 130 mm, respectively. The annual and seasonal rainfall for 1990 was 292 and 164 mm, respectively.

Sampling Protocol

Tarbush leaf samples were collected in conjunction with a 2-year study of tarbush utilization by cattle, sheep, and goats (Anderson et al. 1991). In this study, high-density stocking in small paddocks for short time periods (9 days) resulted in a high percentage of tarbush in ruminant diets, and differential use of individual tarbush plants. During the second year, tarbush samples were collected from 4 paddocks (0.6 ha) at 2 sites on 2 collection dates approximately 2 weeks apart (mature leaf stage). The 2 sites were approximately 1.6 km

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apart and the 2 paddocks on each site were within 604 m of each other. Leaf samples were collected from 10 tarbush plants in 1 paddock at each site on each of 2 sampling days (23 August and 7 September 1990). Sampling days coincided with the fourth day of exposure of paddocks to livestock. Ten plants (5 plants in each use class) exhibiting high (>45%) or low (<10%) use (ocular estimation to the nearest 5% use class by an experienced observer) were sampled in each site/collection date combination. Approximately 50 g of leaves were removed from each of the 40 plants and immediately placed on dry ice. Subsequently, samples were stored at -10°C. A voucher specimen of *Flourensia cernua* DC. was placed in the Experimental Range herbarium located in Las Cruces, N.M.

Sampling on day 4 was an attempt to circumvent the paradox of sampling leaf material from plants which have been highly defoliated at the end of the grazing period; thus the assumption was made that patterns of use on day 4 reflect use at the end of the 9-day period. Even by day 4, occasional plants were so defoliated that no sample could by obtained; thus, we may not have sampled the most preferred plants in every case. Also, inherent in this assumption is that no (or minimal) within plant selection occurred. Although distribution of secondary chemicals within a plant is probably not uniform, it is likely minimal compared to differences among plants, because many plants were almost completely used by day 9, while others were virtually intact.

Chemical Analyses

Mature leaves of uniform size and appearance from the midpoint of current years leader growth were subjected to chemical analyses. Dry matter was analyzed in duplicate on 10 whole leaves from each sample. Epicuticular wax (modification of the procedure of Mayeaux et al. 1981) was analyzed in duplicate on 10 whole leaves from each sample by extracting with 20 ml of chloroform for 20 sec. Surface compounds from 5 whole thawed leaves were extracted at room temperature with 5 ml of 95% ethanol for 5 min and refrigerated. A detailed description of plant sampling and extraction protocol is described elsewhere (Estell et al. 1994).

Surface mono- and sesquiterpenes were analyzed using gas chromatography/ion trap mass spectrometry (electron impact ionization source, DB-5 column, 5% phenyl, 95% methyl silicone, 30 m, 0.32 mm I.D., 0.25 µm film thickness, helium as carrier gas at 1 ml/min, 240 sec filament multiplier time, initial column temperature of 60°C, 3 min isothermal, 3°C/min ramp to final column temperature of 240°C, 5 min isothermal, 68 min total run time, 1µl injection volume). Tentative identification of leaf surface terpenes was based on comparison of unidentified peak retention times and spectra to the spectral library amassed by Adams (1989). Positive identification of terpenes was based on comparison of retention times and spectra to those of authentic standards. We were unable to locate purified compounds to verify the identity of several tentatively identified compounds. Concentrations of several unidentified peaks were estimated from peak area using the standard curve of β caryophyllene. Rationales and methodologies for tentative and positive identification, analysis, and quantitation (or estimation) of leaf surface terpenes are described by Estell et al. (1994).

Statistical Analyses

Leaf dry matter, ash, epicuticular wax, and individual terpene concentrations (known and estimated) were analyzed using GLM procedures of SAS (1985), with plant as the experimental unit. The experiment was conducted in a split-plot design with time period as the whole-plot treatment assigned to site, and degree of use as the subplot treatment assigned within time period. Treatment effects were evaluated by an F test with site x period as the error term for both site and period, and 3-way interaction used to test degree of use. Due to period x use interactions ($P<0.05$), degree of use was analyzed within period. Average content of leaf dry matter, ash, epicuticular wax, and individual terpene concentrations were estimated for each time period, by degree of use, and by degree of use within each time period (SAS 1985).

Discriminant analysis, using DISCRIM procedure of SAS (1985), determined the set of variables which best discriminated between the 2 use categories. Due to the large number of individual terpenes identified, a subset variables was subjected to the discriminant analysis. The subset included variables which affected degree of use ($P<0.05$) and significant correlates ($P<0.05$). Equal prior probabilities were specified, and the significance level for the likelihood ratio test of the homogeneity of within group covariance matrices was set at 0.1.

Results

Epicuticular wax concentration was greater ($P<0.05$) on the surface of low-use plants (Table 1). Approximately 20% more epicuticular wax was present on leaves of tarbush plants receiving low use than on plants browsed to a greater extent. No difference in epicuticular wax was attributed to site or period ($P>0.10$). Use was also higher for plants with lower dry matter ($P<0.05$) and ash ($P<0.10$) concentration (Table 1), although neither moisture nor ash content was related to site or period ($P>0.10$).

Previous efforts resulted in identification of 10 mono- and sesquiterpenes from the leaf surface of tarbush (Estell et al. 1994). Numerical differences associated with degree of use and period were often large for these 10 volatile compounds (Table 2), but generally values were quite variable and little of the variation in concentration of individual terpenes was explained by the model. Camphene exhibited a period effect ($P<0.10$). Several of the identified volatile compounds were present in numerically higher concentrations in Period 1 compared to Period 2, but standard errors were often quite large. None of the previously identified volatile compounds were related to

Table 1. Mean epicuticular wax, dry matter, and ash content of the leaves from differentially used tarbush plants during forced browsing in a high density, mixed grazing study.

	Period			Use			OSL[c]		
	1	2	SEm[b]	>45%	<10%	SEm[b]	Period	Site	Use
Epicuticular wax[a], %	9.7	8.8	1.11	8.2	10.3	0.61	0.680	0.417	0.022
Dry matter, %	43.6	45.5	0.35	42.9	46.2	1.13	0.155	0.439	0.044
Ash[a], %	14.4	13.9	0.56	13.6	14.7	0.40	0.644	0.687	0.068

[a] Dry matter basis.
[b] SEm=standard error of least square means, *n*=40.
[c] OSL=observed significance level.

Table 2. Mean mono- and sesquiterpene concentration and estimated unidentified compound concentration by period and use level on the leaf surface of differentially used tarbush plants during forced browsing in a high density, mixed grazing study.

			Period			Use			OSL[c]		
Compound	RT[a]	*n*	1	2	SEm[b]	>45%	<10%	SEm[b]	Site	Period	Use
	(sec)	----------------------------------(ug/g DM)------									
Camphene	467	40	374	293	7.7	323	344	39.1	0.109	0.086	0.696
β Myrcene	565	40	124	91	14.0	113	102	6.5	0.743	0.347	0.251
3-Carene	614	40	205	150	33.5	178	177	21.1	0.573	0.454	0.954
Limonene	671	40	128	24	13.3	73	78	17.5	0.239	0.114	0.843
1,8-Cineole	679	40	94	81	24.3	91	85	17.9	0.782	0.773	0.798
Borneol	1081	40	992	513	73.5	724	782	73.7	0.502	0.136	0.584
cis Jasmone	1466	40	24	5	7.8	5	24	8.5	0.303	0.329	0.122
β Caryophllene	1482	40	190	203	8.4	187	205	18.5	0.150	0.459	0.499
Caryophyllene oxide	1561	40	20	7	5.6	19	8	8.07	0.229	0.332	0.332
Globulol	1565	40	570	339	208.9	403	507	207.5	0.728	0.578	0.724
Unknown #1	585	40	91	40	22.3	60	71	13.8	0.373	0.352	0.570
Unknown #2[d,e]	660	40	17	9	1.3	13	13	2.3	0.912	0.160	0.898
Unknown #3	788	40	37	12	1.0	27	22	6.9	0.896	0.037	0.609
Unknown #4[d]	816	40	740	214	84.4	473	481	82.1	0.768	0.142	0.951
Unknown #5[d]	1006	40	8	4	3.1	7	6	1.2	0.549	0.521	0.499
Unknown #6[d]	1056	40	105	86	32.8	86	106	49.9	0.582	0.745	0.780
Unknown #7[d]	1430	40	13	6	0.4	8	10	1.5	0.285	0.056	0.433
Unknown #8	1452	40	22	12	1.1	14	19	3.2	0.382	0.098	0.279
Unknown #9[d]	1459	40	23	7	7.3	12	17	4.1	0.631	0.364	0.409
Unknown #10[d]	1461	39	21	10	0.1	14	17	2.4	0.230	0.007	0.432
Unknown #11[d]	1502	34	13	8	4.8	12	9	2.5	0.911	0.553	0.380
Unknown #12[d]	1512	40	29	18	2.6	19	28	2.8	0.253	0.209	0.040
Unknown #13[d]	1516	40	63	26	2.9	43	46	9.0	0.218	0.069	0.835
Unknown #14[d,e]	1519	39	43	17	0.1	32	28	4.1	0.050	0.002	0.432
Unknown #15	1523	39	51	15	0.5	27	39	9.6	0.209	0.012	0.366
Unknown #16	1527	40	35	24	0.1	26	33	5.0	0.030	0.007	0.329
Unknown #17[d]	1556	40	115	52	20.0	82	86	17.3	0.743	0.269	0.871
Unknown #18	1569	40	127	85	5.5	110	102	19.2	0.117	0.114	0.775
Unknown #19[d]	1589	40	328	149	23.6	195	283	33.3	0.241	0.117	0.070
Unknown #20	1620	39	869	346	246.5	427	788	289.1	0.714	0.370	0.378
Unknown #21	1647	40	260	198	58.4	258	200	44.8	0.802	0.587	0.362
Unknown #22	1668	40	6905	6611	661.1	6095	7421	887.8	0.980	0.806	0.402
Unknown #23	1734	40	780	524	63.2	682	622	199.0	0.466	0.214	0.852
Unknown #24	1746	40	1173	1065	193.1	997	1241	165.1	0.908	0.761	0.303
Unknown #25	1873	39	1618	0	171.2	693	925	275.7	0.500	0.093	0.551
Unknown #26	1888	38	193	1057	85.6	585	665	187.6	0.266	0.087	0.765
Unknown #27	1904	39	3319	2435	175.1	2504	3248	476.1	0.170	0.171	0.270
Unknown #28	1975	39	398	285	50.1	305	379	62.4	0.345	0.352	0.405
Unknown #29	1984	39	202	243	65.3	149	296	73.1	0.497	0.731	0.157

[a] RT=retention time.

[b] SEm=standard error of least square means. Column least square means based on chemical analysis of 20 plants. Estimates of concentration of unidentified compounds obtained using standard curve of β caryophyllene.

[c] OSL=observed significance level.

[d] Tentative identification of unknowns #2, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 17, and 19 are *p*-cymene, artemisia alcohol, camphor, trans verbenol, α copeane, β bourbonene, β cubebene α humulene, gamma cadinene, gamma cadinene, viridiflorene or valencene, ledol, and β eudesmol or viridiflorol, respectively.

[e] Period x use interaction was observed ($P<0.05$).

site or degree of use ($P>0.10$).

The relationship of estimated concentration of a limited number of unidentified peaks (Table 2) with season, site, and degree of use by grazing ruminants was examined. Again, variability among plants in estimated concentration was high. Unknown concentrations were consistently greater for Period 1 than Period 2, and differences ($P<0.10$) were detected in 10 of 29 unknown estimates. One exception was unknown No. 26, which was higher in Period 2. This compound appeared to be structurally related to unknown No. 25 because the mass spectra were nearly identical.

No obvious pattern was noted for site or degree of use, although site did affect estimated concentration of 2 compounds ($P<0.10$). Two volatile compounds (unknown Nos. 12 and 19) exhibited a relationship with use ($P<0.10$), with greater concentration in low-use plants. Numerically, mean concentrations of other unidentified compounds were generally higher in low-use plants, but relationships were usually very weak. Two compounds (unknown Nos. 2 and 14) exhibited a period x use interaction ($P<0.05$), with a lower concentration of both compounds in Period 2 for high-use plants, but no change in low-use plants. Main effects for unknown No. 2 were not significant, but site and period effects were observed for No. 14 ($P<0.05$).

Listed in the order entered into the model, discriminant analysis provided the following predictor variables of high- and low-use plants: leaf dry matter, concentration of unknown No. 19, ash content of leaf dry matter, concentration of unknown No. 24, weight of epicuticular wax (organic matter basis) and concentrations of unknown No. 5 and unknown No. 14 ($R^2 = 0.56$). Using these variables, 15 leaf samples from plants designated as high use were correctly classified as being from high-use plants ($n = 20$), while 14 samples from low-use plants were correctly classified ($n = 19$).

Discussion

Epicuticular wax is deposited on the leaf cuticle surface of semi-arid shrubs, and contains a variety of compounds, including terpenoids. The leaf surface has recently been recognized as a plant compartment, and is the site of deposition of numerous compounds (Zobel et al. 1991). Many terpenoids are compartmentalized in glandular trichomes, and are exposed to herbivore sense organs (Personius 1987). Levin (1976) described volatile substances as particularly effective herbivory defense mechanisms because herbivores are repelled before plant damage occurs. Thus, it appears 1 or more terpenoids could affect diet selection, since epicuticular wax was related to selectivity in this study, and leaf surface extracts of tarbush revealed an abundance of terpenoids. However, volatile compounds are only a fraction of the total surface constituents, and other non-volatile components could also be involved in diet selection.

In general, we observed a relationship between total surface compounds (based on epicuticular wax) and degree of use. Other workers have reported negative relationships for deer diet selection and crude terpenoid content (Longhurst et al. 1968), volatile odor concentration (Elliott and Loudon 1987), and concentrations of oxygenated monoterpenes and total volatile oil or monoterpene concentration (Schwartz et al. 1980, Goralka and Langenheim 1991). Zang and States (1991) noted avoidance of trees by Albert squirrels was related to total concentration and diversity of terpenes, as well as presence and concentration of specific terpenes. Monoterpenes have also been associated with diet selection for voles (Bucyanayandi et al. 1990) and hares (Reichardt et al. 1985). Little work with domestic ruminants is available. Yabann et al. (1985) reported that sheep selected individual sagebrush plants with a lower total monoterpenoid concentration, and Morrison et al. (1987) reported altering preference of cattle for 3 *Panicum* spp. with compounds present in the volatile fraction. Possibly, application of solutions to alter or remove epicuticuler wax could lead to enhanced use of tarbush by livestock.

Some trends for relationships of individual compounds with use appeared to exist, but were inconclusive due to variability. It is not clear why unknowns Nos. 5, 14, and 24 did not appear to influence degree of use but contributed to the models discriminating power between high- and low-use plants, or why unknown No. 12 was related to use but did not enter the model during discriminant analysis. Possibly, chemical differences in unused leaves and leaves selected by livestock might contribute to this discrepancy. It is also possible that these compounds may be correlated with other plant attributes influencing animal preference, or work synergistically with other compounds to affect chemosensory perception. Mixtures of chemosensory stimuli can influence chemical perception within (Colucci and Grovum 1993) and among (Livermore et al. 1992) sensory modalities differently than a specific stimulant alone. Given that herbivory can induce terpene synthesis in some plants (Gershenzon and Croteau 1991), it is conceivable that during the 4 days required to distinguish between high- and low-use plants, induction of terpene synthesis could potentially mask differences which may have been more pronounced on day 1.

Leaf moisture content appeared to influence plant palatability, with increasing water content having a positive influence on palatability. The relationship of plant water content and grazing preference was described earlier by Archibald et al. (1943). Components of ash such as sodium can have either a positive or negative influence on animal preferences depending on the post ingestive consequences of previous dietary choices (Grovum and Chapman 1988). In our study, ash content was negatively related to use. Although these observations may suggest a relationship between plant succulence and plant use, any compounds volatilized during oven drying would appear as moisture loss; consequently, the association of moisture content with plant use could be an artifact of a relationship of volatile compound content and use.

Chemical defense of woody plants generally varies with growth stage and plant part within growth stage (Bryant et al. 1991). Concentrations of several terpenoids are greater in immature compared to mature leaves for a variety of plant species (Gershenzon and Croteau 1991). Monoterpenoid concentration in sagebrush has been shown to vary seasonally (Cedarleaf et al. 1983, Yabann et al. 985). Also, epicuticular wax has been shown to vary seasonally in both amount and composition in other plant species (Rao and Reddy 1980, Mayeaux and Jordan 1984, Jacoby et al. 1990). However, in the 2 weeks between periods in the present study, no difference in epicuticular wax was observed, even though several individual compounds were related to period.

Environmental factors and nutrient availability affect terpenoid concentration and composition in plants as well (Gershenzon and Croteau 1991). Although the biosynthesis of phytochemicals is ultimately dependent on the plants genetic makeup, the plastic expression of genetic capabilities is driven by many, constantly changing factors, including soil moisture availability (Kainulainen et al. 1992), incoming radiation (Fitter and Hay 1987), and pathogenicity (Misaghi 1982). Environmental stress can increase carbon-based secondary chemistry concentration in plants; water stress often increases terpenoid content in shrubs (Gershenzon and Croteau 1991). Herbivory also affects secondary chemistry, and biotic stresses such as herbivory can cause a rapid induction of terpene synthesis (Carroll and Hoffman 1980, Gershenzon and Croteau 1991). Based on these general observations of phenological and environmental effects on plant phytochemistry, period and site differences in leaf surface secondary chemistry noted for several mono- and sesquiterpenoids in this study were not surprising. Period differences suggest timing of browsing could be an important consideration for enhancing tarbush utilization by livestock.

Conclusions

These results suggest a relationship of the surface chloroform soluble fraction of tarbush leaves and degree of use by livestock. Few differences in identified or unidentified compounds due to degree of herbivory were evident. Some individual compounds differed due to period, and only limited differences due to site were noted. We hope to capitalize on the plant-to-plant variability in chemical makeup of tarbush and differential use of tarbush by browsing livestock to gain insight into the relationship of phytochemistry and diet selection by herbivores. Possibly, 1 or more of the volatile compounds which were not examined or the non-volatile constituents of the wax fraction could be important to the selection process. A combination of 2 or more compounds or the cumulative total concentration may also be involved in diet selection. Because relationships do not necessarily indicate cause and effect, bioassay studies are needed to verify that epicuticular wax or a component of the wax truly affects diet selection. The relationship of leaf surface chemistry with other environmental factors should be explored (insect infestation, resource distribution, etc.). Other compounds present in the epicuticular wax which were not examined as well as internal compounds may respond to environmental cues.

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