Differences in Volatile Profiles Between Populations of *Ceratoides lanata* var. *subspinosa* (Rydb.) J.T. Howell

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Abstract: *Ceratoides lanata* (Rydb.) J.T. Howell, common winterfat, is valued for its nutrient content and palatability to livestock; however, the subspecies *Ceratoides lanata* var. *subspinosa* (Rydb.) J.T. Howell, ('subspinosa') is considered unpalatable. Curiously, observations of 'subspinosa' revealed several populations in central New Mexico that were heavily grazed. Volatile terpene profiles are associated with differences in palatability in many plant species. To determine whether differences in volatile profiles between 'subspinosa' populations would correlate with apparent differences in palatability, plants from 13 populations of 'subspinosa' were collected. Volatile analysis of shoot samples identified 33 compounds. Limonene, myrcene, and 3-carene were the most abundant volatiles in all populations, comprising 81 percent and 85 percent of the volatiles detected in heavily grazed and ungrazed plants, respectively.

Compounds present in at least half of either the heavily grazed or ungrazed plants were subjected to a stepwise discriminate analysis of relative quantities. The analysis identified a subset of 14 chromatographic peaks (myrcene, 3-hexenol, 3-carene, AR-curcumene, limonene, n-hexanol, p-cymene, a mixture thought to contain alpha-pinene, alpha-thujene, and tricyclene, a mixture thought to contain n-decanal, and five unknowns), which may distinguish between palatable and unpalatable phenotypes. When a discriminate rule based on these chemicals was applied to chemical data from individual plants at all sites, none of the plants were misclassified. The possibility that these varied oil profiles may be used to distinguish palatable from unpalatable phenotypes is discussed.

Introduction

Ceratoides lanata var. *lanata* (winterfat) is a widespread chenopod on western rangelands. The plant is valued for its nutrient content, grazing and drought tolerance, palatability to large herbivores, and ability to rapidly establish on disturbed soils (Stevens and others 1977). The subspecies *C. lanata* var. *subspinosa* ('subspinosa'), native to the southwestern U.S. and northern Mexico, is reputedly unpalatable to livestock (Gibbens 1999; Ueckert 2000). Although no controlled studies directly evaluating the palatability of 'subspinosa' have been found, its reputation for unpalatability has been indirectly supported in a high density stocking study at the Jornada Experimental Range in Southern New Mexico (Anderson 1990). In the study, cattle, sheep, and goats in mixed paddocks were more likely to consume tarbush (*Flourensia cernua* DC than 'subspinosa' (table 1). Tarbush is generally described as unpalatable, and has been used as a model "unpalatable" plant for studies evaluating diet selection by livestock (Estell and others 1994, 1996, 1998).

Curiously, observations of heavily grazed 'subspinosa' north of the Chihuahuan Desert suggest that some phenotypes of 'subspinosa' are palatable. Availability of palatable and unpalatable phenotypes could make *C. lanata* var. *subspinosa* a useful seed source for plant breeders seeking drought tolerant varieties and an interesting model plant for identification of secondary compounds influencing diet selection. Here we report the identification of 33 volatile compounds extracted from 'subspinosa' using solid-phase microextraction. Relative amounts of these compounds were examined in 106 plants from 13 populations. These compounds were subjected to a stepwise discriminate analysis in order to test the hypotheses that differences in volatile composition could be used to distinguish between heavily grazed and ungrazed populations of 'subspinosa.'

Materials and Methods

'Subspinosa' was collected from 12 sites in New Mexico. The GPS coordinates for these sites, as determined using a Garmin GPS 12 personal navigator, are listed in table 2. Additional samples were provided by Darrell Ueckert from the Texas Agricultural Experiment Station in San Angelo. Each site was characterized as either "heavily grazed" or "ungrazed" (table 2). The populations from Carrizozo, Magdalena, Willard, Dusty, and Winston were all collected from populations that extended into pastures stocked with cattle. Plants inside the stocked pastures had been grazed to less than 20 cm.

Populations from two sites on the NMSU-Corona ranch were in pastures from which livestock grazing had been limited. One site was an official grazing exclosure. However,

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Table 1. Summary of bite-count data resulting from high density feeding trials with cattle, sheep, and goats (Anderson, 1990). Initial forage composition was determined, then fixed numbers of livestock were placed in paddocks under continuous observation. The number of bites taken from each species of plant was recorded. Data reflecting "subspinosa" (*Ceratoides lanata var. subspinosa* [Rydb.] J.T. Howell) and tarbush (*Flourensia cernua* DC [comp.]) selection are shown here.

Paddock	1		2	
Plant species	**% composition**	**% of bites**	**% composition**	**% of bites**
'subspinosa'	4.5	1.6	1.1	0.2
tarbush	30.8	42.4	37.3	50

Table 2. Site locations, usage classification (heavily grazed or ungrazed, with "ungrazed" defined as populations that exhibited no clear evidence of grazing by livestock) of *Ceratoides lanata* var. *subspinosa* (Rydb.) J.T. Howell, ('subspinosa').

Site	Latitude	Longitude	Elevation (m)	Sampling date	Number of plants	Usage classification
Corona Range and Livestock Research Center	34°16.710'	105°23.612'	1,905	8/3/2000	10	grazed
Corona Range and Livestock Research Center	34°16.481'	105°19.862'	1,864	8/3/2000	10	grazed
Magdalena	33°51.515'	107°19.511'	1,912	8/10/2000	10	grazed
Willard	34° 22.992'	105°42.985'	1,972	8/3/2000	10	grazed
Dusty	33°57.297'	107°39.869'	2,273	9/29/2000	10	grazed
Winston	33°21.107'	107°33.382'	1,838	9/29/2000	10	grazed
Chihuahuan Desert Rangeland Research Reserve	32°35.236'	106° 55.703'	1,341	7/27/2000	8	ungrazed
Chihuahuan Desert Rangeland Research Reserve	32°33.823'	106° 54.269'	1,341	7/27/2000	2	ungrazed
Jornada Experimental Range	32° 43.295'	106°46.187'	1,352	7/25/2000	8	ungrazed
Jornada Experimental Range	32°31.142'	106°44.630'	1,325	7/7/2000	1	ungrazed
Jornada Experimental Range	32°31.933'	106°41.773'	1,344	7/30/2000 8/9/2000	7	ungrazed

plants on opposite sides of the fences to both pastures were accessible to sheep, and had been heavily grazed. Researchers working at the ranch stated that the winterfat populations were typically utilized by sheep, especially in the winter and early spring, when other forage was limited.

The samples from San Angelo were classified as unpalatable to cattle, sheep, and goats (Ueckert 2000). 'Subspinosa' populations from the Jornada Experimental Range, historically identified as common winterfat, have all been classified as unpalatable. One of these sites was previously utilized for the high density feeding study illustrated in table 1.

Two more populations were collected from the Chihuahuan Desert Rangeland Research Reserve near Las Cruces, NM. These populations were unusual in that they were small, both in number of plants (less than twenty plants each) and in size (less than 30 cm/plant). Observations of plants in the field revealed some evidence of light grazing. Approximately 5 percent of the shoots had been removed. The sharp edges remaining on removed shoots suggested grazing was by rabbits rather than livestock. We classified both these populations as "ungrazed."

All samples were collected in the fall of 2000 after flowering. Samples consisted of 10 ten cm leaders from each plant (table 2). When possible, 10 plants were sampled at each site. The year 2000 had little summer rainfall, and at some sites fewer than 10 plants had flowered. Samples were placed under dry ice upon harvesting. After transport, samples were stored at -20°C prior to volatile extraction. Taxonomic classification was verified at the Range Science Herbarium at New Mexico State University and voucher specimens were deposited there.

Preparation for Solid Phase Microextraction (SPME) analysis consisted of grinding all leaders from a single plant to a coarse powder under liquid nitrogen. SPME was performed by placing 0.2 g (fresh weight) of ground plant tissue into 4 mL glass screw-cap vials. Vials were sealed with pre-baked aluminum foil and ethanol-washed Viton septa, then equilibrated for 3.5 h at 50°C. SPME fibers (100 uM PDMS, Supelco, Bellefonte, PA) were exposed to vial headspace at a depth of 1 cm for 20 min using a manual fiber holder (also from Supelco). The fiber was injected into a Varian model 3400 GC with a DB-5 column (30 m x 0.25 mm fused silica capillary column, film thickness 0.25 μm) coupled to a Finnigan ion trap mass spectrometer (EI, 70 eV) and desorbed for 3 min. Blank injections followed each sample to verify the absence of residual compounds on the SPME fiber.

GC/MS analysis was performed using Helium at approximately 1 mL/min as a carrier gas. Injector and transfer line temperatures were set at 220°C and 260°C, respectively. The initial column temperature was 60°C and a linear temperature increase of 3°C/min was programmed into each run. Duplicate extractions of each sample were examined. Compounds were identified by comparing mass spectra and retention indices with literature data (Adams 1995, 2001) or with authentic standards.

Relative amounts of each compound detected were defined based on relative peak heights. [Total Ion Chromatogram (TIC) peak height]/[TIC peak height of 4 ng 3-carene] X 100. Compounds with relative heights greater than 0.01 detected in at least half of either the heavily grazed or ungrazed plants were subjected to a stepwise discriminate analysis of relative heights against palatability. The resulting discriminate rule was then applied individually to each plant sampled.

Results

A total of 62 unique volatiles were detected in SPME samples from winterfat. Those that comprised greater than 0.01 percent of the chromatographic peak area in at least 50 percent of the plants from either the grazed or the ungrazed populations are presented in table 3. Amounts shown represent either the relative peak height (RH), which was calculated as the peak height relative to the height of 4 ng of the monoterpene standard 3-carene, or the percent height, which represents the percent of the chromatogram comprised of that particular peak. Derived, rather than actual values were necessary because analytical grade standards were not available for all of the compounds found in winterfat.

Of the 33 volatiles listed in table 3, fourteen were positively identified by matching spectra and retention indices to library values. Several compounds consistently gave non-gaussian peaks and spectra that varied at points across the peaks. These are identified in the table with question marks indicating that the peaks detected were probably co eluting with unknown compounds.

Limonene was the dominant peak in all samples, comprising nearly 50 percent of the SPME-extractable fraction. Curiously, seven plants from the site at Carrizozo also

Table 3. Compounds found in at least 50 percent of either the heavily grazed or ungrazed 'subspinosa' populations. Values represent the mean peak height relative to the height of 4 ng 3-carene (Mean RH), the standard deviation of the Mean RH, the mean peak height expressed as a percent of the total chromatogram (Mean pH), and the standard deviation of the Mean pH. Compounds whose spectra did not match anything in available reference libraries were designated as CELA (*Ceratoides lanata*), followed by the retention time at which they eluted from the DB-5 column. Retention index values indicated with an asterisk (*) were determined by extrapolation, since they eluted prior to the retention time of the earliest hydrocarbon standard. Compounds with identification followed by "_mix?" were not spectrally pure and probably contained two or more chemicals.

Retention time	Compound ID	Heavily grazed shrubs				Ungrazed shrubs			
		Mean RH	Std RH	Mean PH	Std PH	Mean RH	Std RH	Mean RH	Std PH
597*	CELA1.482	0.01	0.01	0.39	0.35	0.01	0.01	0.32	0.22
639*	CELA1.62	0.10	0.13	1.85	1.32	0.08	0.11	1.42	1.57
669*	CELA1.756	0.00	0.01	0.28	0.22	0.01	0.01	0.26	0.22
693*	CELA1.884	0.05	0.03	1.05	0.63	0.05	0.04	1.30	1.14
716	CELA1.977	0.01	0.01	0.28	0.17	0.01	0.01	0.28	0.22
782	CELA2.508_mix?	0.00	0.01	0.19	0.08	0.00	0.00	0.16	0.11
806	CELA2.806	0.02	0.02	0.44	0.33	0.02	0.03	0.42	0.32
861	(Z)-3-hexenol	0.09	0.10	1.66	1.14	0.03	0.06	0.83	1.29
872	n-hexanol	0.02	0.03	0.48	0.36	0.01	0.02	0.22	0.15
931	pinene-thujene mix?	0.03	0.04	0.99	0.60	0.02	0.04	0.74	0.48
940	CELA5.25	0.03	0.03	1.06	0.41	0.03	0.03	0.91	0.33
977	sabinene_mix?	0.06	0.05	1.10	0.54	0.06	0.04	0.96	0.23
981	6-methyl-5-hepten-2-one	0.02	0.02	0.46	0.41	0.01	0.01	0.24	0.07
988	CELA6.6	0.01	0.01	0.22	0.09	0.01	0.03	0.26	0.18
992	myrcene	0.87	0.66	16.12	2.37	0.89	0.54	16.13	1.47
1012	CELA7.38	0.01	0.01	0.20	0.11	0.01	0.01	0.20	0.09
1013	3-carene	0.89	0.69	16.56	2.15	1.05	0.56	19.33	2.03
1027	p-cymene	0.03	0.02	0.55	0.47	0.02	0.02	0.31	0.08
1032	limonene	2.47	1.65	48.78	5.29	2.59	1.26	49.60	5.24
1043	1,8-cineole	0.01	0.01	0.31	0.34	0.00	0.00	0.16	0.08
1074	CELA9.38	0.03	0.02	0.67	0.49	0.01	0.01	0.19	0.09
1085	artemisia alcohol	0.01	0.02	0.26	0.15	0.02	0.02	0.45	0.33
1106	n-nonanal_mix?	0.01	0.03	0.70	1.17	0.02	0.04	1.40	1.35
1107	CELA10.71	0.05	0.04	1.29	1.05	0.08	0.07	1.68	1.13
1170	CELA13.2	0.01	0.01	0.34	0.63	0.00	0.00	0.15	0.09
1206	n-decanal_mix?	0.01	0.01	0.22	0.13	0.01	0.01	0.18	0.09
1220	CELA15.47	0.01	0.01	0.19	0.09	0.00	0.01	0.12	0.03
1419	beta-caryophyllene	0.02	0.04	0.78	1.38	0.02	0.03	0.66	0.57
1438	CELA24.6	0.00	0.01	0.18	0.08	0.02	0.03	0.78	0.66
1458	alpha-humulene	0.01	0.01	0.23	0.13	0.01	0.02	0.33	0.17
1485	germacreneD	0.01	0.02	0.38	0.33	0.02	0.03	0.39	0.32
1486	AR-curcumene	0.01	0.02	0.34	0.26	0.03	0.03	0.55	0.50
1512	(E)-beta-ionone	0.01	0.02	0.38	0.37	0.01	0.01	0.31	0.22

contained relatively large peaks of a compound thought to be sylvestrene. The retention times of limonene and sylvestrene vary only by 2s, and the only significant difference in their mass spectra is that limonene has a strong 93 peak with a base peak of 67, whereas sylvestrene has a strong 67 peak with a base peak of 93. Because of the difficulties in separating limonene, a major component, from this putative sylvestrene peak (PSP), the decision was made to report calculated peak heights rather than areas. This would reduce the potential for inaccurate calculation of limonene quantities when PSP was present. Unfortunately, the point for the maximum height of PSP always fell underneath the shoulder of the limonene peak, therefore, relative amounts of PSP are not reported.

Two other major peaks, myrcene and 3-carene, were present in every sample analyzed. The combination of myrcene, 3-carene, and limonene peaks resulted in a distinct chromatographic fingerprint, also seen in common winterfat (not shown), which may be unique to *Ceratoides*.

Stepwise multivariate analysis of the compounds identified in table 3 identified a subset of 14 chromatographic peaks that best distinguished between grazed and ungrazed populations. The subset included myrcene, 3-hexenol, 3-carene, AR-curcumene, limonene, n-hexanol, p-cymene, a mixture thought to contain alpha-pinene, alpha-thujene, and tricyclene, a mixture thought to contain n-decanal, as well as five unknowns (table 3). When a discriminate rule based on these chemicals was applied to chemical data from individual plants at all sites, none of the plants were misclassified.

Discussion

The three major compounds present in all volatile profiles observed were limonene, myrcene, and 3-carene. These three compounds are ubiquitous monoterpenes found in many of the plants we have previously analyzed and often serve as precursors to biosynthesis of other terpenoids. Although the large percentages of limonene, an industrially important compound, seem attractive, the overall yield of oil in 'subspinosa' was low. Attempts to harvest and quantify oil by steam distillation resulted in less than 5 uL oil per gram dry weight of leaf tissue, approximately half of which was limonene. Therefore, even though limonene is abundant relative to other volatiles, it is not likely that purification for industrial use would be cost effective due to the low yield per gram of plant tissue.

Fifteen of the 33 peaks listed in table 3 could not be identified, even though 14 of these were spectrally pure. This abundance of previously undescribed compounds is not surprising in natural products analysis due to the remarkable diversity of plant volatiles.

The identification of 14 compounds that distinguished grazed from ungrazed populations in this preliminary study may be seen as a starting point for additional research. Although some of the observed differences are undoubtedly due to differences in environment and grazing intensity at the various sites, it is encouraging to note that some of the compounds identified, namely limonene, myrcene, p-cymene, and alpha-pinene have also been associated with differences in livestock herbivory in tarbush [*Flourensia cernua* DC] (Estell and others 1996). Curiously, subsequent studies in which individual terpenes associated with low herbivory in tarbush were applied to alfalfa pellets in sheep feeding trials demonstrated only minimal reductions in herbivory (Estell and others 2002). It is possible that the mixture, rather than the individual compounds, is necessary for antiherbivory effects. If this is the case, a benefit of multivariate techniques is the ability to narrow down the possible combinations of compounds, permitting identification of groups of compounds, rather than individuals, which may act in conjunction to create an effect. Tests in which the compounds, combined in proportions defined in discriminate rules following multivariate analysis, need to be carried out.

Perhaps the most significant findings in this preliminary data include the abundance of *Ceratoides lanata* var. *subspinosa* (Rydb.) J.T. Howell in New Mexico, where it had not been previously described, and the observation that in some populations, 'subspinosa' showed evidence of heavy grazing. *C. lanata* has long been considered a desirable species on grazed lands and considerable effort has been devoted to development of germplasm suitable for revegetation in various environments. The availability of large and phenotypically diverse populations of 'subspinosa' may be useful in the development of germplasm for use in arid environments.

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