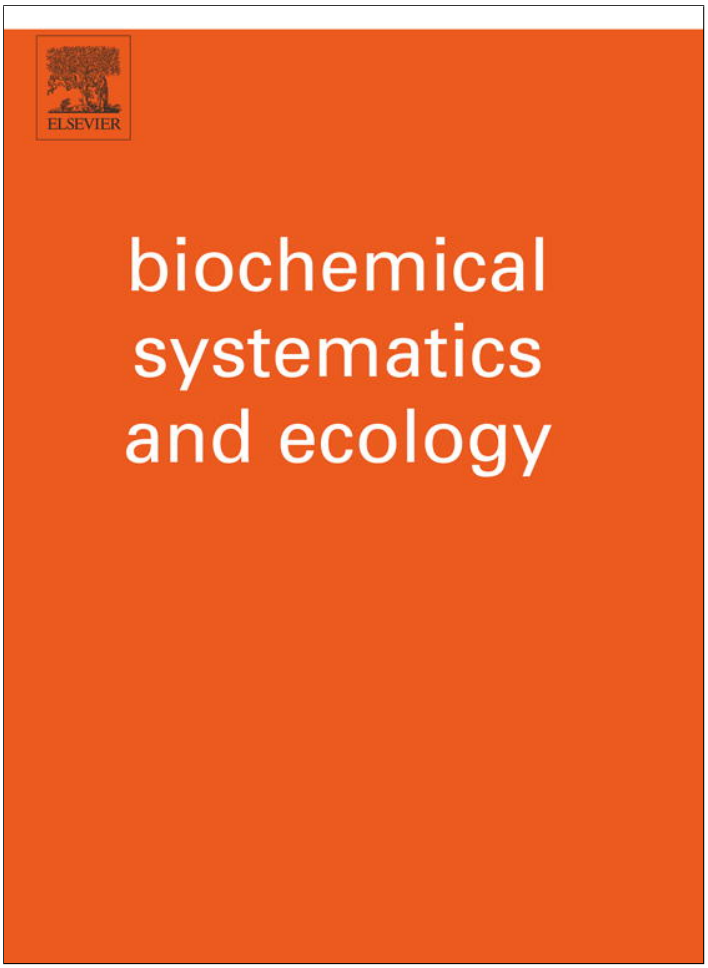

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Within-plant distribution of volatile compounds on the leaf surface of *Flourensia cernua*

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ABSTRACT

We are using *Flourensia cernua* as a shrub model to study how terpenes affect livestock herbivory. Two experiments were conducted to examine distribution of volatile chemicals within a plant in an effort to minimize sample variability. In Experiment 1, leaves (current year's growth) were collected from 20 tarbush plants. Two leaders were sampled from each of three positions (outer canopy, subcanopy, and basal) in all four quadrants (based on ordinal direction). In Experiment 2, 10 leaders of current year's growth were removed from another 20 plants. Leaders were collected from the outer canopy of each quadrant and separated into thirds before removing leaves, thereby creating three leaf age categories. Volatile compounds were extracted with ethanol and analyzed with gas chromatography/mass spectrometry. Ninety-four chemicals (including 15 unknowns) were present on the leaf surface of *F. cernua*. Although 14 and 21 compounds differed ($P < 0.05$) among quadrants in Experiments 1 and 2, respectively, no consistent quadrant effect was detected in either study. Leaf position differed ($P < 0.05$) for 52 chemicals in Experiment 1 but outer canopy and subcanopy leaves differed for only 10 compounds. In Experiment 2, 63 compounds differed among leaf age categories. Immature leaves contained greater concentrations of 46 chemicals ($P < 0.05$) than intermediate or mature age categories, but intermediate and mature leaves differed for only seven compounds. Estimated total concentration (i.e., cumulative concentration of all compounds) was not affected by leaf position but varied among leaf age categories ($P < 0.05$; immature > intermediate > mature). Differences in leaf position were attributed about equally to mono- and sesquiterpenes, whereas leaf age differences were primarily due to sesquiterpenes. Leaf position and age both affect terpene concentration and sampling variability for tarbush. However, little difference was detected between subcanopy and canopy positions. Thus, by avoiding basal sprouts and sampling from the mid-point of current year's growth, sampling variation should be minimal.

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1. Introduction

The role of mono- and sesquiterpenes in livestock herbivory is being examined at the Jornada Experimental Range using tarbush *(Flourensia cernua* DC) as a shrub model. Concentrations of epicuticular wax and several individual terpenes present on the leaf surface are related to tarbush consumption by sheep and goats (Estell et al., 1994a, 1998). However, concentrations of many volatile compounds on the leaf surface of tarbush are highly variable from plant to plant (Estell et al., 1994b).

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Concentrations and profiles of carbon-based plant secondary metabolites in woody plant species typically vary within and among plants (Barnola et al., 1997; Byrd et al., 1999). Biotic and abiotic factors and stressors (e.g., light intensity/wavelength, soil moisture/nutrients, herbivory/mechanical damage, etc.) can alter chemical profiles and concentrations of secondary compounds (Gershenzon and Croteau, 1991; Tingey et al., 1991; Kainulainen et al., 1992; Litvak and Monson, 1998; Thines et al., 2007; Burney and Jacobs, 2011). Plant age, growth stage, and phenology of a given species (Cedarleaf et al., 1983; Sinclair et al., 1988; Bryant et al., 1991; Vourc'h et al., 2002; Fredrickson et al., 2007) are also important drivers of plant chemistry. Two important factors that can affect chemical profiles in woody plants are leaf age and location within the plant (Meyer and Karasov, 1991; Barnola et al., 1997; Powell and Raffa, 1999; Laitinen et al., 2002). For many woody species, immature leaves contain higher concentrations of defense compounds such as terpenes (Meyer and Karasov, 1991), gallotannins/ellagitannins (Ossipov et al., 1997; Laitinen et al., 2002), and total phenolics (Massei et al., 2000).

A better understanding of how sampling methodology affects terpene concentration measurements will improve our ability to design studies, minimize sample size, reduce sample variation, and interpret results. Our specific objectives were to examine the effect of leaf age and within-plant sampling location on leaf surface terpene concentrations.

2. Materials and methods

2.1. Site description

The study was conducted on the Jornada Experimental Range in south-central New Mexico. Plants were in a 7.5 ha exclosure containing a dense stand of tarbush from which livestock had been excluded for five years prior to sampling. Soils on the study site are deep, well-drained Doña Ana–Reagan association, varying from sandy loam to loam (SCS, 1980). The site ranges from 1 to 5% slope. Long-term mean annual and growing season (July to September) precipitation is 247 and 131 mm, respectively. Mean monthly temperatures for the coldest (January) and warmest (July) months are 6 and 26 °C, respectively. Dominant vegetation on the site is tarbush and *Scleropogon brevifolius* Phil (burrograss). Tarbush is a deciduous, root-sprouting shrub that is dormant until after summer rainfall (Fredrickson et al., 2007).

2.2. Sample collection

Forty dormant tarbush plants were randomly selected during the spring of 1993 and labeled with an aluminum tag. Plants were divided into four quadrants (northeast, southeast, northwest, and southwest) with a compass and metal frame in early August and branches to be sampled were identified with colored wire. Sampling was conducted in late August at the approximate midpoint of active summer growth. Two experiments were conducted ($n = 20$ plants each). In Experiment 1, two leaders (current year's growth) were sampled from each of three positions (outer canopy, subcanopy [interior branches], and basal [base of plant near soil surface]) in each quadrant (two leaders per quadrant/position combination). Leaves (including petiole) were removed with forceps, composited, placed on dry ice, transported to the laboratory, and stored at −20 °C. In Experiment 2, 10 outer canopy leaders per quadrant were removed from each plant (severed at the base of branch with plant clippers) and handled as described above. Leaders were subsequently separated into thirds (proximal, medial, and terminal segments). Leaves were then removed with forceps and composited by quadrant for each plant to form three leaf age categories (immature, intermediate, and mature).

2.3. Laboratory analysis

In each experiment, samples were thawed and five whole leaves of uniform size and appearance from each sample were weighed and extracted for 5 min at room temperature in 5 ml of 100% ethanol containing 5 ηg/μl of 2-carene (internal standard) with occasional shaking, filtered through a fiberglass (Fisherbrand G8) filter and stored at 4 °C. Ten leaves were also removed for dry matter analysis in Experiment 2 (five leaves in duplicate at 100 °C for 24 h). Because of limited sample size, Experiment 1 data were adjusted to a DM basis using the mean DM value from Fredrickson et al. (2007). Plants in that companion study were located in the same exclosure and tarbush leaves were collected on the same day and year. Though absolute values may differ slightly from actual, differences among variables would not be affected.

Leaf surface terpenes were analyzed with gas chromatography-mass spectrometry using a Finnigan ion trap mass spectrometer (EI, 70 eV) in conjunction with a Varian model 3400 gas chromatograph and a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness 0.25 μm). Conditions were as follows: He carrier gas (1 ml/min), 1 μl injection, and a programmed (injector temp. 220 °C, transfer line temp. 240 °C, initial column temp. 60 °C, final column temp. 240 °C, 3 °C/min) temperature run (Adams, 1995; Tellez et al., 1997). Volatile compounds were identified by comparing mass spectra with authentic compounds when available or with mass spectral libraries (Adams, 1995) and by comparing relative retention times with those of authentic compounds or comparing retention indices to those in the literature (Adams, 1995). Individual compound concentrations were estimated with the internal standard and total volatile concentration was estimated from cumulative concentrations of all compounds in each sample. Ninety-four volatile compounds (including 15 unknowns) were present on the surface of tarbush leaves (Tables 1 and 2). Many of the unknowns eluted late and may have been diterpenes.

Table 1
Effect of leaf position on leaf terpenes in tarbush.[a]

Chemical[b,c]	RT[d]	Medians and interquartile ranges (μg/g DM)[e]			*P* value
		Canopy	Subcanopy	Basal	
Total volatiles[f,g]		8994.3 (5351.8, 12,622.3)	8948.0 (5477.0, 11,401.6)	8089.1 (5167.8, 11,147.9)	0.096
Santolina triene[c]	274	1.2 (0.7, 1.9)[j]	1.4 (0.6, 1.8)[j]	2.1 (1.0, 3.9)[i]	0.003
Tricyclene	299	3.4 (1.4, 8.0)	3.5 (1.4, 6.0)	2.2 (1.2, 5.7)	0.237
α-Thujene[c]	304	2.1 (1.0, 3.7)	1.5 (0.9, 2.8)	1.8 (1.1, 2.4)	0.147
α-Pinene[g,h]	314	18.5 (12.5, 28.5)[i]	18.3 (12.7, 27.5)[i]	10.3 (5.7, 13.7)[j]	< 0.001
Camphene	337	101.1 (80.5, 131.9)[i]	102.6 (58.5, 132.9)[i]	46.7 (31.1, 63.5)[j]	< 0.001
Sabinene	379	2.7 (1.5, 4.4)	2.0 (1.3, 3.1)	2.3 (1.4, 5.0)	0.068
β-Pinene	384	5.7 (2.4, 12.2)[i,j]	7.0 (3.7, 12.0)[i]	3.8 (2.1, 7.8)[j]	0.026
Myrcene[h]	405	18.4 (8.7, 28.0)[i,j]	9.3 (3.5, 27.5)[j]	19.8 (5.3, 38.0)[i]	0.025
Mesitylene[c,h]	412	0.7 (0.5, 1.3)[j]	0.8 (0.5, 1.2)[j]	1.0 (0.5, 1.5)[i]	0.033
Yomogi alcohol[c,g]	419	50.8 (30.9, 85.7)[i]	33.0 (18.6, 58.8)[i,j]	23.4 (6.2, 41.8)[j]	0.005
3-Carene[g,h]	443	14.1 (11.5, 20.8)[j]	17.4 (12.3, 21.5)[i]	18.0 (13.3, 24.3)[i]	0.011
α-Terpinene	455	1.2 (0.8, 1.7)[j]	1.2 (0.6, 1.9)[j]	1.3 (0.9, 3.0)[i]	0.003
p-Cymene	469	3.6 (1.8, 7.9)[i]	2.7 (1.5, 5.4)[j]	1.6 (0.8, 2.5)[k]	< 0.001
Limonene[h]	480	16.8 (10.2, 24.4)[i]	16.2 (10.4, 23.4)[i]	12.4 (9.0, 16.5)[j]	0.003
1,8-Cineole	484	9.9 (1.0, 57.1)[i]	9.1 (1.2, 35.1)[i]	6.3 (1.2, 13.2)[j]	0.018
(Z)-β-Ocimene	493	1.0 (0.6, 1.6)[j]	1.3 (0.7, 2.0)[i,j]	1.7 (0.8, 2.3)[i]	0.039
(E)-β-Ocimene[c]	519	1.0 (0.5, 1.8)[j]	1.0 (0.7, 1.7)[j]	2.2 (0.8, 3.9)[i]	0.006
trans-Decahydronaphthalene[c]	533	0.8 (0.4, 1.6)[j]	1.0 (0.5, 1.8)[j]	1.9 (0.9, 4.6)[i]	< 0.001
γ-Terpinene + Artemisia ketone[c]	543	1.1 (0.6, 1.9)	1.0 (0.6, 1.8)	1.2 (0.6, 1.8)	0.454
cis-Sabinene hydrate	560	4.9 (1.3, 12.7)[i]	4.6 (1.7, 8.3)[i]	2.0 (1.2, 4.8)[j]	< 0.001
Artemisia alcohol[c]	597	168.6 (118.9, 290.2)[i]	147.9 (98.2, 294.7)[i]	65.6 (33.8, 126.6)[j]	< 0.001
Terpinolene	609	0.8 (0.5, 1.4)[j]	0.8 (0.6, 1.7)[j]	2.2 (0.8, 3.7)[i]	< 0.001
trans-Sabinene hydrate[h]	633	2.1 (0.8, 7.3)	1.9 (1.0, 5.0)	1.2 (0.7, 2.6)	0.152
cis-*p*-Menth-2-en-1-ol[c]	682	5.8 (1.4, 10.1)	4.6 (2.0, 7.1)	2.8 (1.6, 6.4)	0.664
α-Campholenal[c]	693	1.7 (1.0, 2.9)	1.8 (1.2, 2.7)	1.8 (1.1, 2.4)	0.881
trans-Pinocarveol[c]	727	5.4 (3.5, 7.7)	4.9 (2.7, 7.6)	3.6 (1.4, 7.1)	0.068
Camphor + *trans*-Verbenol[c]	738	13.3 (8.8, 21.6)[i]	9.2 (5.0, 15.3)[j]	7.8 (3.2, 12.0)[j]	< 0.001
Isoborneol[c]	771	0.8 (0.5, 1.7)[j]	1.0 (0.3, 1.9)[j]	1.5 (0.9, 2.3)[i]	0.003
cis-Chrysanthenol[c] + Pinocarvone[c,g]	780	6.5 (3.6, 32.0)	5.3 (2.9, 20.0)	8.3 (3.3, 43.4)	0.173
Borneol[g]	793	152.5 (106.8, 200.3)[i]	132.2 (99.2, 180.4)[i]	100.1 (66.8, 134.0)[j]	< 0.001
Terpin-4-ol	822	3.7 (1.0, 6.3)	2.3 (1.1, 4.4)	2.3 (1.0, 4.4)	0.644
m-Cymen-8-ol[c]	831	0.7 (0.3, 1.3)[j]	0.9 (0.5, 1.3)[j]	1.8 (1.0, 2.9)[i]	< 0.001
p-Cymen-8-ol[c]	839	0.9 (0.5, 1.5)	0.9 (0.5, 1.4)	1.7 (0.5, 3.2)	0.057
α-Terpineol	856	1.5 (0.7, 2.7)[i]	1.1 (0.7, 2.0)[j]	1.5 (0.8, 3.3)[i]	0.019
Myrtenal[c]	868	1.1 (0.7, 1.8)[i]	0.9 (0.5, 1.5)[j]	1.4 (0.9, 2.4)[i]	0.001
Myrtenol[c]	870	0.7 (0.5, 1.3)[j]	0.9 (0.6, 1.5)[j]	1.4 (0.9, 2.5)[i]	0.004
cis-Chrysanthenyl acetate[c,g]	1040	1.3 (0.6, 2.6)[j]	1.6 (0.8, 2.7)[j]	2.3 (1.3, 5.1)[i]	0.002
Bornyl acetate[c,h]	1103	2.2 (0.8, 4.9)	2.1 (1.4, 3.3)	2.6 (1.0, 4.0)	0.376
Carvacrol[c]	1141	1.4 (0.7, 2.7)	1.3 (0.7, 2.6)	1.8 (1.1, 3.0)	0.187
α-Cubebene[c]	1270	1.0 (0.7, 2.1)[j]	0.9 (0.6, 2.2)[j]	2.4 (1.3, 3.6)[i]	< 0.001
Eugenol	1283	0.8 (0.4, 1.8)	1.0 (0.6, 1.8)	1.7 (0.8, 2.6)	0.181
Cyclosativene[c]	1315	3.7 (2.0, 5.3)	3.6 (1.8, 5.6)	2.8 (2.0, 4.0)	0.947
α-Copaene	1337	3.0 (1.2, 5.1)	3.5 (1.4, 6.2)	3.5 (1.6, 4.9)	0.448
β-Bourbonene[c]	1360	9.2 (6.0, 12.8)[i]	9.7 (5.6, 12.9)[i]	5.3 (2.4, 8.4)[j]	< 0.001
β-Cubebene[c]	1374	4.5 (2.1, 6.9)[i]	3.1 (1.3, 4.9)[j]	2.3 (1.2, 3.9)[j]	0.001
(Z)-Jasmone	1388	18.5 (7.2, 36.1)[i]	3.1 (0.9, 21.8)[j]	1.8 (0.9, 7.0)[j]	< 0.001
(E)-Caryophyllene	1447	23.1 (13.9, 39.4)[i]	19.8 (11.7, 31.5)[i]	14.3 (8.3, 21.1)[j]	0.006
α-Humulene	1530	10.3 (7.7, 17.2)[i]	8.8 (5.4, 13.1)[j]	7.7 (5.2, 12.2)[j]	< 0.001
Allo-Aromadendrene[c]	1551	4.4 (2.0, 7.8)	5.6 (2.6, 8.6)	5.3 (2.2, 7.9)	0.468
Drima-7,9(11)-diene[c]	1573	5.3 (2.7, 8.2)[i]	5.5 (3.4, 8.3)[i]	3.7 (1.9, 6.4)[j]	0.008
γ-Muurolene[c]	1589	6.6 (4.9, 9.5)[j]	8.9 (5.8, 12.1)[j]	11.1 (8.0, 16.2)[i]	< 0.001
Germacrene D[c]	1599	43.2 (25.1, 66.5)[i]	29.8 (17.9, 44.4)[j]	16.5 (10.8, 24.2)[k]	< 0.001
β-Selinene[c]	1615	16.0 (9.8, 22.7)[i]	14.2 (8.5, 22.1)[i]	10.1 (3.3, 15.0)[j]	< 0.001
epi-Cubebol[c,h]	1637	6.3 (1.7, 11.8)	3.4 (1.4, 12.5)	3.1 (1.7, 8.7)	0.377
Bicyclogermacrene[c]	1641	2.5 (0.5, 9.3)	1.8 (0.5, 6.2)	3.0 (0.9, 9.8)	0.154
α-Muurolene[c]	1652	14.5 (10.0, 19.3)	14.8 (11.1, 18.1)	15.8 (11.5, 22.3)	0.972
γ-Cadinene[c,h]	1687	3.6 (1.3, 9.5)	2.9 (0.9, 11.3)	1.9 (0.8, 4.6)	0.724
cis-Calamenene[c]	1702	3.1 (1.4, 7.8)	3.6 (0.9, 9.1)	2.4 (1.0, 7.5)	0.397
Δ-Cadinene[c]	1707	1.8 (0.8, 7.8)[j]	1.2 (0.7, 4.0)[j]	4.3 (0.9, 13.1)[i]	0.002
Cadina-1,4-diene[c,g,h]	1729	6.6 (4.8, 9.7)[i]	6.6 (4.0, 8.7)[i]	5.9 (3.8, 9.1)[j]	0.015
Elemol[c,g]	1766	18.0 (13.4, 29.2)[i]	10.7 (6.8, 18.0)[j]	4.5 (2.7, 10.2)[k]	< 0.001
Ledol[c]	1811	56.7 (27.9, 82.0)	57.7 (26.8, 80.8)	47.6 (35.6, 65.0)	0.828
Germacrene D-4-ol[c]	1831	28.1 (16.8, 38.6)[i]	20.5 (9.8, 30.6)[j]	16.6 (8.2, 26.2)[j]	0.018
Spathulenol[c]	1833	4.9 (0.9, 9.7)[j]	7.3 (2.4, 14.8)[i]	6.7 (2.1, 22.8)[i]	0.016
Caryophyllene oxide	1846	28.6 (14.6, 49.8)	27.0 (15.8, 44.5)	21.6 (13.2, 35.7)	0.997
Unknown-01	1867	343.7 (53.6, 965.7)[i]	294.6 (52.0, 921.4)[i,j]	212.1 (59.2, 370.4)[j]	0.045
Unknown-02[g]	1893	44.6 (22.6, 64.2)	42.9 (22.6, 65.6)	36.0 (20.5, 48.1)	0.656

Table 1 (*continued*)

Chemical[b,c]	RT[d]	Medians and interquartile ranges (μg/g DM)[e]			*P* value
		Canopy	Subcanopy	Basal	
β-Oplopenone[c,h]	1906	1.8 (0.8, 14.4)	5.5 (1.0, 15.1)	3.6 (1.2, 15.8)	0.125
1-epi-Cubenol[c,h]	1958	17.5 (0.7, 45.9)	16.9 (0.7, 42.9)	4.8 (0.8, 42.2)	0.953
epi-α-Muurolol[c]	1984	1.2 (0.6, 2.0)[j]	1.2 (0.6, 2.1)[j]	2.3 (1.2, 5.6)[i]	< 0.001
(Z)-methyl jasmonate[c,g,h]	1996	194.9 (118.3, 333.3)[i]	157.4 (92.5, 257.0)[i]	117.6 (32.9, 209.1)[j]	0.001
β-Eudesmol[c,g,h]	2002	65.3 (44.1, 116.9)	63.3 (36.8, 106.3)	56.3 (36.0, 80.4)	0.451
Selin-11-en-4-α-ol[c]	2014	122.5 (81.4, 175.0)	110.4 (72.4, 155.4)	95.9 (50.0, 146.9)	0.431
Unknown-03	2022	3.9 (1.1, 61.2)	3.8 (1.0, 46.8)	3.6 (1.2, 57.1)	0.871
Bulnesol[c]	2045	11.9 (4.4, 17.5)[i]	6.5 (3.7, 10.6)[j]	3.1 (2.1, 5.2)[k]	< 0.001
(Z)-Methyl epi-jasmonate[c]	2067	8.4 (1.0, 14.8)	7.1 (2.5, 14.2)	6.0 (2.0, 10.1)	0.549
α-Bisabolol[c,h]	2079	68.0 (39.1, 93.5)[i]	57.1 (3.7, 74.7)[i,j]	27.0 (3.1, 77.9)[j]	0.018
Oplopanone[c]	2195	11.3 (6.9, 16.0)[i]	9.0 (6.2, 13.4)[j]	6.2 (3.2, 9.3)[k]	< 0.001
Unknown-04	2206	50.3 (3.2, 229.8)	55.0 (2.2, 235.9)	32.3 (6.1, 185.6)	0.194
β-Acoradienol[c]	2247	19.7 (13.2, 28.4)	16.4 (10.1, 22.8)	14.7 (5.6, 22.4)	0.143
Nootkatone[c,h]	2336	10.1 (0.9, 15.7)	6.2 (0.9, 15.0)	6.5 (1.0, 10.1)	0.851
Cryptomeridiol[c]	2362	70.3 (45.0, 118.6)[i]	64.3 (30.0, 102.5)[j]	47.3 (22.3, 117.5)[k]	0.001
Flourensiadiol	2476	4255.8 (1481.1, 5713.6)	3884.9 (665.9, 5479.2)	3163.7 (1117.0, 5287.8)	0.169
Unknown-05	2592	32.5 (19.5, 43.7)	32.2 (16.8, 37.6)	25.5 (18.9, 34.4)	0.268
Unknown-06	2626	21.2 (5.9, 74.7)[i]	24.1 (4.3, 52.9)[i,j]	5.5 (2.2, 21.2)[j]	0.041
Unknown-07[g,h]	2754	125.2 (79.3, 181.3)	125.0 (84.9, 184.8)	164.2 (103.0, 211.7)	0.432
Unknown-08[h]	2798	61.3 (42.7, 86.9)[i]	55.7 (40.6, 77.5)[j]	74.1 (51.1, 98.0)[i,j]	0.049
Unknown-09	2876	405.6 (41.2, 626.4)	411.7 (39.8, 600.5)	390.6 (58.7, 680.9)	0.446
Unknown-10[g,h]	3113	1.2 (0.7, 2.1)	1.1 (0.6, 2.6)	2.4 (1.2, 4.2)	0.565
Unknown-11[g,h]	3234	8.7 (1.9, 56.4)	10.9 (2.1, 50.7)	14.9 (1.8, 187.9)	0.150
Unknown-12	3288	1016.3 (44.7, 2230.3)[j]	953.6 (61.3, 1980.2)[j]	1377.6 (167.8, 2397.3)[i]	< 0.001
Unknown-13	3332	51.3 (1.6, 132.3)[j]	47.1 (1.7, 156.3)[j]	87.8 (5.6, 128.0)[i]	0.018
Unknown-14	3420	90.3 (1.5, 234.2)	40.8 (1.2, 213.9)	87.4 (2.6, 256.3)	0.296
Unknown-15	3458	10.4 (3.7, 183.7)	14.5 (3.9, 179.8)	78.1 (7.5, 395.2)	0.144

Groups with different superscripts i,j,k differ ($P < 0.05$).

[a] Concentrations are untransformed medians (μg/g DM) ± interquartile ranges (25th percentile, 75th percentile) for each chemical and leaf position; statistical analyses were conducted on natural logarithms of concentration medians.

[b] Compounds were identified with kovats indices and mass spectral libraries; estimated concentrations were based on relative proportions of internal standard (2-carene).

[c] Tentatively identified based on Adams (1995); identity of other compounds verified with authentic standards.

[d] Retention time.

[e] $n = 20$ for each leaf position/quadrant combination except northeast/basal ($n = 5$), southeast/basal ($n = 9$), northwest/basal ($n = 10$), southwest/basal ($n = 10$), southwest subcanopy ($n = 19$), and southwest canopy ($n = 19$).

[f] Total volatiles = cumulative estimated concentrations of all compounds within a position category.

[g] A quadrant effect ($P < 0.05$) was observed for 14 compounds.

[h] A quadrant × leaf position interaction ($P < 0.05$) was observed for 21 compounds.

2.4. Statistical analysis

A linear mixed effects model was used to examine effects of quadrant and leaf position (Exp. 1) or quadrant and leaf age (Exp. 2) on concentration of each compound (SAS V9.3, SAS Institute, Cary, NC). Concentrations (μg/g DM) were transformed to natural logarithms before analyses. Quadrant, leaf position, and quadrant × leaf position interaction (Exp. 1) and quadrant, leaf age, and quadrant × leaf age interaction (Exp. 2) were modeled as fixed effects. Plants were modeled as random effects in both experiments. Levels of each effect were compared using Fisher's least significant difference test (LSD) in the event of a significant F test ($P < 0.05$). Because log-transformed data were reported on the original scale, medians and interquartile ranges were reported rather than means and standard errors (Ramsey and Schafer, 2002). Some outliers remained after log transformation; therefore, data were re-analyzed with suspected outliers removed to confirm results (Ramsey and Schafer, 2002). Because no changes in compound significance occurred during re-analysis, outliers were included in the final analysis. In Experiment 1, several plants had no basal leaves for one or more quadrants (Table 1). Thus, for the basal leaf category, $n = 5$, 9, 10, and 10 for northeast, southeast, northwest, and southwest quadrants, respectively.

3. Results

3.1. Experiment 1

Fifty-two compounds differed among leaf position categories ($P < 0.05$; Table 1). In over half of these cases, canopy and subcanopy leaves did not differ while basal leaves were either higher ($n = 16$) or lower ($n = 13$) than the other two categories. Canopy and subcanopy leaves differed for only 10 compounds ($P < 0.05$; Table 1). Estimated total volatile concentration did not differ among the three leaf types ($P > 0.05$; Table 1). Differences among leaf positions were about equally attributed to

Table 2
Effect of leaf age on leaf terpenes in tarbush.[a]

Chemical[b,c]	RT[d]	Medians and interquartile ranges (μg/g DM)[e]			*P* value
		Immature	Intermediate	Mature	
Total volatiles[f,g]		12,778.5 (9985.0, 19,316.2)[i]	9295.8 (7528.9, 11,192.7)[j]	8212.2 (6590.4, 10,871.4)[k]	< 0.001
Santolina triene[c]	274	1.4 (0.6, 3.4)[i]	0.7 (0.5, 1.2)[j]	0.7 (0.3, 1.3)[j]	< 0.001
Tricyclene	299	6.4 (3.1, 9.9)	5.4 (2.7, 8.7)	6.2 (2.8, 8.7)	0.554
α-Thujene[c,h]	304	5.5 (2.7, 9.8)[i]	3.5 (1.7, 7.0)[j]	3.2 (1.6, 6.8)[j]	< 0.001
α-Pinene	314	40.5 (21.4, 59.2)[i]	23.9 (12.9, 48.2)[j]	20.3 (12.7, 38.4)[j]	0.002
Camphene	337	115.4 (65.8, 183.5)[i]	109.1 (53.4, 178.3)[i,j]	96.7 (46.8, 140.7)[j]	0.013
Sabinene	379	6.7 (4.0, 12.9)[i]	5.1 (2.7, 8.7)[j]	4.9 (2.5, 9.3)[j]	0.002
β-Pinene	384	7.4 (1.4, 17.6)[i]	2.9 (0.9, 11.0)[j]	3.0 (0.9, 10.6)[j]	0.029
Myrcene	405	4.9 (3.3, 7.2)	4.1 (2.7, 5.4)	4.2 (3.2, 6.2)	0.128
Mesitylene[c]	412	0.6 (0.3, 1.8)[i]	0.6 (0.3, 1.5)[i]	0.4 (0.3, 0.9)[j]	0.004
Yomogi alcohol[c]	419	44.4 (17.7, 76.9)	45.3 (24.7, 65.7)	51.5 (16.8, 87.7)	0.531
3-Carene	443	8.4 (5.3, 12.2)	9.1 (6.7, 11.8)	8.5 (6.1, 11.6)	0.723
α-Terpinene	455	1.8 (0.8, 3.2)[i]	1.0 (0.5, 2.4)[j]	0.9 (0.5, 1.8)[j]	0.002
p-Cymene	469	6.5 (4.6, 14.1)	8.0 (4.5, 16.7)	7.9 (4.0, 15.3)	0.675
Limonene[g]	480	15.0 (8.4, 25.3)[i]	7.9 (5.1, 10.5)[j]	7.5 (5.2, 11.4)[j]	< 0.001
1,8-Cineole[h]	484	70.4 (28.6, 101.9)	53.6 (27.0, 94.0)	55.5 (21.4, 85.9)	0.581
(Z)-β-Ocimene	493	1.2 (0.6, 2.7)[i]	0.5 (0.3, 1.2)[j]	0.6 (0.3, 1.0)[j]	< 0.001
(E)-β-Ocimene[c]	519	0.5 (0.2, 0.7)	0.5 (0.3, 0.7)	0.3 (0.2, 0.6)	0.097
trans-Decahydronaphthalene[c]	533	0.5 (0.3, 1.0)	0.5 (0.2, 0.9)	0.5 (0.3, 0.8)	0.910
γ-Terpinene + Artemisia ketone[c]	543	4.0 (2.3, 7.1)[i]	2.7 (1.4, 6.0)[j]	2.4 (1.3, 5.6)[j]	< 0.001
cis-Sabinene hydrate	560	29.8 (10.5, 52.3)[i]	15.7 (7.5, 37.6)[j]	13.3 (6.6, 30.8)[j]	< 0.001
Artemisia alcohol[c]	597	386.0 (189.3, 648.6)[i]	244.2 (148.5, 369.0)[j]	215.7 (110.0, 337.3)[j]	< 0.001
Terpinolene	609	2.9 (1.4, 5.7)[i]	2.2 (1.3, 4.4)[i,j]	1.8 (0.8, 3.6)[j]	0.009
trans-Sabinene hydrate[h]	633	17.0 (7.8, 24.5)[i]	9.8 (6.2, 22.2)[j]	9.2 (5.6, 19.0)[j]	0.001
cis-*p*-Menth-2-en-1-ol[c]	682	6.1 (3.3, 12.8)[i]	4.1 (1.6, 7.5)[j]	4.6 (2.0, 8.6)[j]	0.005
α-Campholenal[c]	693	1.0 (0.5, 1.6)	1.2 (0.6, 2.1)	1.1 (0.8, 2.1)	0.534
trans-Pinocarveol[c]	727	5.7 (3.7, 8.5)	6.9 (3.7, 10.5)	7.0 (4.3, 10.0)	0.726
Camphor + *trans*-Verbenol[c,g]	738	8.4 (2.6, 14.3)	5.7 (2.7, 10.4)	5.8 (2.7, 9.8)	0.270
Isoborneol[c]	771	0.4 (0.2, 0.9)	0.4 (0.2, 0.8)	0.3 (0.2, 0.6)	0.171
cis-Chrysanthenol[c] + Pinocarvone[c]	780	1.6 (0.8, 73.7)	2.4 (1.0, 77.5)	2.6 (1.1, 5.3)	0.626
Borneol[g]	793	329.2 (178.8, 468.6)[i]	279.3 (125.9, 424.1)[j]	227.9 (122.5, 330.2)[j]	0.001
Terpin-4-ol	822	7.3 (3.4, 11.3)	5.5 (2.0, 10.7)	4.9 (1.7, 10.4)	0.066
m-Cymen-8-ol[c]	831	0.4 (0.2, 0.7)	0.3 (0.2, 0.5)	0.3 (0.2, 0.5)	0.171
p-Cymen-8-ol[c]	839	0.5 (0.3, 1.1)	0.5 (0.2, 0.7)	0.6 (0.2, 1.0)	0.127
α-Terpineol	856	0.8 (0.4, 1.8)	0.7 (0.3, 1.4)	0.7 (0.4, 1.4)	0.786
Myrtenal[c,g]	868	0.5 (0.3, 0.8)	0.7 (0.4, 1.2)	0.6 (0.3, 1.0)	0.075
Myrtenol[c]	870	0.9 (0.5, 1.8)	0.8 (0.4, 1.5)	0.7 (0.4, 1.6)	0.593
cis-Chrysanthenyl acetate[c]	1040	0.5 (0.2, 1.0)	0.4 (0.2, 0.8)	0.4 (0.2, 0.8)	0.427
Bornyl acetate[c,g]	1103	1.2 (0.7, 2.2)	1.2 (0.7, 1.9)	1.1 (0.7, 1.9)	0.474
Carvacrol[c]	1141	0.7 (0.5, 1.1)	0.8 (0.5, 1.3)	0.8 (0.5, 1.3)	0.623
α-Cubebene[c]	1270	8.3 (2.3, 14.6)[i]	2.4 (1.3, 3.8)[j]	1.8 (0.9, 3.4)[j]	< 0.001
Eugenol	1283	0.5 (0.3, 0.9)	0.5 (0.2, 0.7)	0.4 (0.2, 0.7)	0.414
Cyclosativene[c]	1315	1.1 (0.5, 6.1)[i]	0.8 (0.4, 1.7)[j]	0.5 (0.3, 1.2)[j]	< 0.001
α-Copaene	1337	12.4 (6.3, 19.5)[i]	4.3 (2.0, 7.0)[j]	3.8 (2.4, 5.7)[j]	< 0.001
β-Bourbonene[c]	1360	12.0 (7.6, 22.2)[i]	9.4 (3.8, 12.8)[j]	7.7 (4.0, 11.0)[j]	< 0.001
β-Cubebene[c]	1374	15.0 (6.7, 22.7)[i]	8.1 (5.7, 11.1)[j]	7.8 (4.6, 11.4)[j]	< 0.001
(Z)-Jasmone	1388	32.8 (13.3, 59.8)[i]	20.9 (5.1, 35.6)[j]	14.7 (0.8, 29.9)[k]	< 0.001
(E)-Caryophyllene[g]	1447	35.0 (24.7, 47.9)[i]	18.0 (13.5, 26.8)[j]	16.7 (10.9, 26.5)[j]	< 0.001
α-Humulene	1530	11.4 (8.2, 14.6)[i]	6.9 (4.3, 9.5)[j]	6.5 (4.2, 9.5)[j]	< 0.001
Allo-Aromadendrene[c]	1551	1.2 (0.5, 9.5)[i]	1.2 (0.3, 4.8)[j]	0.8 (0.3, 3.6)[j]	0.004
Drima-7,9(11)-diene[c,g]	1573	15.0 (10.2, 21.4)[i]	8.5 (5.2, 11.2)[j]	8.2 (5.5, 9.9)[j]	< 0.001
γ-Muurolene[c]	1589	26.8 (12.8, 44.2)[i]	7.7 (4.7, 11.7)[j]	7.8 (4.3, 10.4)[j]	< 0.001
Germacrene D[c,g]	1599	33.8 (22.5, 46.2)[i]	21.8 (13.5, 31.0)[j]	19.9 (12.0, 35.5)[j]	< 0.001
β-Selinene[c]	1615	32.9 (19.0, 48.2)[i]	13.3 (7.2, 18.2)[j]	10.1 (6.4, 15.6)[j]	< 0.001
epi-Cubebol[c]	1637	18.4 (8.4, 32.5)[i]	5.1 (2.1, 10.7)[j]	4.2 (2.2, 9.4)[j]	< 0.001
Bicyclogermacrene[c]	1641	0.4 (0.2, 0.9)	0.5 (0.3, 1.0)	0.5 (0.2, 0.9)	0.564
α-Muurolene[c]	1652	14.0 (1.8, 26.8)[i]	1.4 (0.5, 10.7)[j]	1.2 (0.3, 11.6)[j]	< 0.001
γ-Cadinene[c]	1687	33.4 (10.0, 56.8)[i]	6.7 (4.0, 17.0)[j]	7.9 (4.1, 16.7)[j]	< 0.001
cis-Calamenene[c]	1702	2.8 (0.7, 9.4)	3.0 (0.6, 5.8)	1.6 (0.5, 5.2)	0.068
Δ-Cadinene[c]	1707	13.8 (3.4, 26.3)[i]	2.4 (1.1, 4.6)[j]	2.4 (1.0, 4.2)[j]	< 0.001
Cadina-1,4-diene[c]	1729	3.5 (0.9, 7.3)[i]	1.8 (0.8, 3.6)[i,j]	2.0 (0.5, 4.7)[j]	0.006
Elemol[c]	1766	8.3 (3.7, 11.2)[i]	5.0 (2.1, 9.0)[j]	4.6 (2.0, 8.5)[j]	0.005
Ledol[c]	1811	5.8 (1.3, 101.8)[i]	3.5 (0.6, 72.3)[i,j]	1.4 (0.5, 61.3)[j]	0.013
Germacrene D-4-ol[c]	1831	5.1 (1.7, 8.8)[j]	6.3 (2.8, 9.1)[i,j]	6.0 (3.6, 9.1)[i]	0.050
Spathulenol[c]	1833	22.0 (14.3, 32.3)[i]	11.0 (8.0, 15.1)[j]	10.5 (6.8, 14.2)[j]	< 0.001
Caryophyllene oxide	1846	78.9 (57.5, 135.9)[i]	44.3 (28.2, 66.1)[j]	40.3 (23.4, 61.9)[j]	< 0.001
Unknown-01[g]	1867	170.8 (36.6, 412.5)[i]	101.5 (16.0, 264.1)[j]	95.4 (11.6, 208.6)[j]	< 0.001
Unknown-02	1893	71.6 (25.8, 108.9)[i]	47.5 (15.1, 70.0)[j]	45.4 (12.5, 63.8)[j]	0.006

Table 2 (*continued*)

Chemical[b,c]	RT[d]	Medians and interquartile ranges (μg/g DM)[e]			*P* value
		Immature	Intermediate	Mature	
β-Oplopenone[c]	1906	25.7 (15.6, 31.5)[i]	14.7 (8.7, 18.5)[j]	12.8 (8.0, 15.5)[k]	< 0.001
1-epi-Cubenol[c,g]	1958	64.8 (45.7, 86.1)[i]	45.4 (33.7, 59.1)[j]	38.7 (32.6, 52.1)[j]	0.001
epi-α-Muurolol[c]	1984	5.5 (2.0, 19.9)[i]	1.6 (0.8, 3.1)[j]	1.7 (1.0, 2.7)[j]	< 0.001
(Z)-methyl jasmonate[c]	1996	7.5 (2.4, 25.2)[i]	1.4 (0.6, 3.2)[j]	1.6 (0.5, 3.4)[j]	< 0.001
β-Eudesmol[c,g]	2002	239.5 (140.0, 293.8)	193.3 (129.0, 253.6)	167.0 (129.6, 233.8)	0.112
Selin-11-en-4-α-ol[c,g]	2014	211.7 (136.1, 346.3)[i]	91.3 (66.5, 135.3)[j]	89.5 (56.5, 119.0)[j]	< 0.001
Unknown-03	2022	50.5 (6.3, 170.9)[i]	21.6 (1.9, 81.1)[j]	16.5 (1.4, 60.8)[j]	< 0.001
Bulnesol[c]	2045	8.5 (4.3, 14.3)[i]	4.7 (2.2, 7.8)[j]	4.5 (2.5, 8.0)[j]	< 0.001
(Z)-Methyl epi-jasmonate[c]	2067	16.4 (7.2, 25.6)[i]	9.0 (3.8, 17.3)[j]	4.9 (2.5, 10.9)[k]	< 0.001
α-Bisabolol[c]	2079	133.6 (67.0, 196.6)[i]	56.9 (37.6, 96.8)[j]	47.0 (31.8, 85.5)[k]	< 0.001
Oplopanone[c]	2195	22.3 (15.4, 31.7)[i]	14.3 (8.7, 17.9)[j]	11.9 (7.4, 17.1)[k]	< 0.001
Unknown-04	2206	60.6 (5.8, 524.7)[i]	20.0 (3.0, 267.4)[j]	19.3 (2.6, 336.9)[j]	< 0.001
β-Acoradienol[c,g]	2247	74.9 (49.5, 106.2)[i]	51.3 (30.5, 61.4)[j]	45.5 (36.3, 56.9)[j]	< 0.001
Nootkatone[c]	2336	18.2 (9.1, 24.1)[i]	9.6 (3.3, 19.3)[j]	9.7 (4.8, 16.0)[j]	0.004
Cryptomeridiol[c]	2362	295.1 (76.5, 448.1)[i]	182.3 (61.0, 282.7)[j]	171.1 (58.4, 263.3)[j]	< 0.001
Flourensiadiol[g]	2476	2606.5 (24.3, 7102.3)	2978.0 (11.3, 4922.6)	2059.5 (7.4, 4040.1)	0.649
Unknown-05	2592	42.4 (7.4, 63.1)	36.3 (5.2, 53.2)	30.4 (3.8, 51.1)	0.152
Unknown-06	2626	359.4 (177.9, 707.2)[i]	171.9 (73.2, 279.1)[j]	164.0 (54.6, 253.1)[j]	< 0.001
Unknown-07[g]	2754	269.8 (168.6, 385.7)[i]	156.1 (102.6, 249.6)[j]	156.8 (89.6, 227.5)[j]	< 0.001
Unknown-08	2798	201.8 (116.7, 261.3)[i]	95.9 (66.5, 144.7)[j]	86.4 (57.5, 126.4)[j]	< 0.001
Unknown-09	2876	603.4 (92.3, 1359.6)	431.0 (72.1, 984.4)	400.0 (59.7, 914.5)	0.237
Unknown-10	3113	159.6 (118.3, 224.6)[i]	91.3 (68.8, 115.8)[j]	84.3 (63.1, 104.1)[j]	< 0.001
Unknown-11[g]	3234	710.7 (75.0, 1571.3)[i]	249.2 (45.9, 691.0)[j]	214.4 (36.6, 585.6)[j]	< 0.001
Unknown-12[g]	3288	2479.1 (88.6, 4110.5)	2132.8 (60.6, 3248.4)	2100.9 (90.6, 3137.5)	0.953
Unknown-13[g]	3332	146.3 (11.9, 292.2)[i]	113.6 (7.3, 218.2)[j]	110.6 (10.2, 215.6)[j]	0.027
Unknown-14[g]	3420	263.6 (6.9, 442.3)	216.3 (10.0, 338.8)	183.2 (6.4, 315.1)	0.537
Unknown-15[g]	3458	70.0 (2.6, 364.5)[i]	71.7 (2.7, 296.2)[i]	85.1 (1.6, 253.8)[j]	0.036

Groups with different superscripts i,j,k differ ($P < 0.05$).

[a] Concentrations are untransformed medians (μg/g DM) ± interquartile ranges (25th percentile, 75th percentile) for each chemical and leaf age; statistical analyses were conducted on natural logarithms of concentration medians.

[b] Compounds were identified with kovats indices and mass spectral libraries; estimated concentrations were based on relative proportions of internal standard (2-carene).

[c] Tentatively identified based on Adams (1995); identity of other compounds verified with authentic standards.

[d] Retention time.

[e] $n = 20$ for each leaf age/quadrant combination.

[f] Total volatiles = cumulative estimated concentrations of all compounds within an age category.

[g] A quadrant effect ($P < 0.05$) was observed for 21 compounds.

[h] A quadrant × leaf age interaction ($P < 0.05$) was observed for 3 compounds.

10-carbon and 15-carbon compounds (26 vs 21, in addition to five late eluting unknowns). A quadrant effect ($P < 0.05$; Table 1) was detected for 14 individual compounds (including four unknowns) and the estimated total concentration. A quadrant × leaf position interaction ($P < 0.05$) was detected for 21 compounds (Table 1).

3.2. Experiment 2

Sixty-three chemicals differed among leaf age categories ($P < 0.05$; Table 2). Concentrations of 46 of these compounds were greater for immature leaves than the other two age categories, while only seven compounds differed between intermediate and mature age categories ($P < 0.05$; Table 2). Differences among leaf age were represented to a greater extent by 15-carbon than 10-carbon compounds (35 vs 17, plus 11 late eluting unknowns). Total estimated concentration of volatiles differed among all three age categories, with immature > intermediate > mature leaves ($P < 0.05$; Table 2). A quadrant effect ($P < 0.05$; Table 2) was detected for 21 individual compounds (including six unknowns) as well as total volatile concentration. A quadrant × leaf age interaction ($P < 0.05$; Table 2) was detected for three compounds (α-thujene, 1,8-cineole, and *trans*-sabinene hydrate).

4. Discussion

Few differences were detected between canopy and subcanopy leaves, while basal leaves frequently differed from canopy and subcanopy leaf positions. Though location within plant has been reported to affect terpene distribution in some woody species (Barnola et al., 1997; Powell and Raffa, 1999), Byrd et al. (1999) reported no effect of leaf location on terpene distribution in sagebrush. Differences among quadrants were detected for 14 and 21 compounds in Experiments 1 and 2, respectively. However, no consistent pattern was observed in either study (data not shown). Only four compounds in each study contained a compound for which one quadrant differed from the other three, but the other three were not different (Exp. 1: yomogi alcohol lower in NW quadrant, borneol greater in NE quadrant, unknown 07 lower in SE quadrant, unknown

10 greater in NW quadrant; Exp. 2: camphor + *trans* verbenol greater in NW quadrant, and borneol, selin-11-en-4-α-ol, and β-acoradienol lower in NE quadrant). Light intensity has been shown to affect concentrations of some plant secondary metabolites (Tingey et al., 1991; Thines et al., 2007). Although we speculated light intensity may differ among quadrants and alter chemical patterns, no consistent patterns were detected. Several position × quadrant interactions were observed; however, they did not preclude interpretation of main effects. No interactions were detected for estimated total concentrations in either study. Numerous differences were detected for leaf age in Experiment 2, with almost all of these differences due to immature leaves. Furthermore, total estimated volatile concentrations were greater for immature than intermediate and mature leaves. These findings corroborate other studies showing greater concentrations of defense compounds in immature leaves of woody plant species (Meyer and Karasov, 1991; Laitinen et al., 2002). Though plant age was not examined, the plants in this study were in close proximity and of uniform size and shape, and were assumed to be of reasonably similar ages.

In summary, both leaf position and age affect terpene concentrations and sampling variability in *F. cernua*, although very few differences were detected between canopy and subcanopy leaves. Compounds that differed in Experiment 1 were represented about equally by mono- and sesquiterpenes, whereas compounds affected by leaf age were predominantly sesquiterpenes. Ordinal direction did not appear to influence sampling variability in a consistent manner. Sampling variation for tarbush leaf surface chemistry can be minimized by avoiding basal sprouts and by sampling from the mid-point of current year's growth.

Disclaimer

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