Title: Ecoregion drives intraspecific variation of secondary chemistry in a big sagebrush common garden

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Open Research Statement: Code and data for the analysis and figures is available on Github in a public repository at https://github.com/elleteri/commongarden. For the final version, all data and code will be made available through a Zenodo link with a permanent DOI upon acceptance of the manuscript.

Key Words - *Artemisia tridentata*, climate, foundation shrub, volatiles, non-volatiles, phytochemistry.

Abstract - Phytochemistry plays an integral role in the health and survival of plants and can mediate community interactions, communication with neighboring plants, protection against disease and herbivores, and attraction of pollinators. We measured non-volatile compounds using liquid chromatography-mass spectrometry (LC-MS) and volatile compounds using gas chromatography (GC) at two different time points from leaves of three subspecies of *Artemisia tridentata* that were grown in a common garden from seeds sourced from 44 sites. Similar explanatory factors drove the variation in both classes of phytochemicals. While there was an effect of subspecies and cytotype designation, we found that the ecoregion from where the seeds were sourced explained the largest portion of the variation in non-volatile and volatile compounds. Within subspecies, both separation by distance and local climatic factors were correlated with non-volatile chemical profiles. Our results indicate that *A. tridentata* phytochemistry is genetically determined and differs among individuals of the same subspecies based on the location of origin. These results highlight strong intraspecific variation in phytochemistry for a widespread foundational plant species. Future work may identify and explore the functions of specific compounds, providing further insights into the ecological drivers of *A. tridentata* phytochemistry.

Introduction

Phytochemicals, the secondary compounds that include various classes of chemicals (*i.e.* terpenes, phenolics, alkaloids), play a large role in ecosystems. Plants partition resources to produce bioactive secondary compounds that protect them against abiotic and biotic stressors (Loreto and Schnitzler 2010) and mediate complex interactions within their ecosystems. Plants can emit volatile organic compounds (VOCs) to attract pollinators (Raguso 2008) and herbivores (Finnerty et al. 2017; Skopec et al. 2019) and also repel herbivores (De Moraes et al. 2001; Finnerty et al. 2024). VOCs from plants can signal an herbivore attack to their distant plant parts, and neighboring plants can detect this signal to prepare for an anticipatory attack (Heil and Bueno 2007; Karban et al. 2012). Phytochemicals also defend against bacterial or fungal pathogens (Huang et al. 2012; Bouhlali et al. 2021). Phytochemistry clearly has important, widespread implications across trophic levels. However, little is known to what extent phytochemistry differs across individual plants within the same non-model species, how phytochemistry changes with environment over time, and how this might impact essential ecosystem processes.

A few recent studies have begun to tackle the question of how intraspecific variation in phytochemistry has evolved and the selective pressures involved. While phytochemicals are under genetic control (Allevato et al. 2019; Rabelo et al. 2023), biotic and abiotic stimuli can also influence plastic intraspecific variation (Glassmire et al. 2016; Hartmann 1996). For example, phytochemical variation can be driven by resource availability over just tens of meters (Glassmire et al. 2023). Abiotic factors, such as precipitation and temperature, explained variation in the chemical composition of essential oils in laurels (*Laurus nobilis L.*) across locations (Stefanova et al. 2020). Biotic factors, such as herbivore presence, can also alter

phytochemical differences at relatively small scales. Invasive plant species in their introduced range may differ in their phytochemistry because their previous co-evolved natural herbivores are not present (Wang et al. 2012). In subpopulations of a single plant species, *Piper kelleyi*, differences in phytochemicals were correlated with differences in associated herbivore communities, showcasing how intraspecific chemical variation may be driven by this multitrophic interaction to provide different niches for host-associated herbivores (Glassmire et al. 2016). Here, we sought to extend the knowledge of how phytochemistry can differ across space and time within a single foundational plant species, *Artemisia tridentata* (Nutt., Asteraceae), and explore the mechanisms driving that intraspecific variation.

Whole genome duplication and hybridization have important roles in determining the environmental breadth, and potentially, the phytochemistry of *A. tridentata.* Three common subspecies *A. t.* subsp. *tridentata* (basin big sagebrush), *A. t.* subsp. *wyomingensis* (Wyoming big sagebrush), and *A. t. vaseyana* (mountain big sagebrush), are, in part, defined by cytotype: diploid (2n = 2x = 18) or tetraploid (2n = 4x = 36). For example, *A. t.* subsp. *wyomingensis* is exclusively tetraploid (E. Durant McArthur and Sanderson 1999). Moreover, subspecies have defined niches within the range of this species (Still and Richardson 2015; Mahalovich and McArthur 2004). Between these niches, known as ecotones, diploid hybridization or allopolyploids may contribute to the formation of mosaic hybrid zones (Massatti et al. 2025). Evidence of hybridization has also been found in structure of the genome (Melton et al. 2022), morphology (Freeman et al. 1991), phytochemistry (E. D. McArthur et al. 1988), and population genomic structure (Grossfurthner et al. 2023; Massatti et al. 2025). Cytotypes of *A. tridentata* have functional implications related to a variety of morphological and ecophysiological traits (Richardson et al. 2021; Lazarus et al. 2019; Roop et al. 2025). Similarly, research has found that

92 a small proportion of *A. tridentata* volatile organic compounds (VOCs) differentiate subspecies
93 with hybrid plants displaying an intermediate position among the taxa, suggesting that VOCs
94 have an important role in adaptation among these subspecies (Jaeger et al. 2016).

95 To advance our understanding of the mechanisms driving intraspecific variation of
96 phytochemicals, this study assessed how factors related to the plant's genotype or region of
97 origin explained spatial and temporal variation of non-volatile and volatile compounds in three
98 subspecies of *A. tridentata* within a common garden experiment. Based on the distinct
99 environmental associations of species and populations of *A. tridentata*, we hypothesized that
100 subspecies combined with cytotype designation, hereafter, subspecies:cytotype, would explain
101 the most variation in leaf phytochemistry with less variation attributed to seed source-ecoregion
102 and time. To address this hypothesis, we used a common garden experiment to limit the
103 influence of phenotypic plasticity among individuals, because all plants experience a similar
104 environment despite their different evolutionary backgrounds (Berend et al. 2019). To explore
105 phytochemical compounds, we used Liquid Chromatography - Mass Spectrometry (LC-MS) to
106 measure non-volatile compounds, including flavonoids and coumarins, and we used Gas
107 Chromatography (GC) to measure volatile compounds. Inclusion of both phytochemical classes
108 provides a more comprehensive analysis of *A. tridentata* phytochemistry. With our experimental
109 design, we were able to investigate how *A. tridentata* phytochemistry varied across
110 subspecies:cytotype, ecoregion, spatial distance, and climate, and, how phytochemistry may have
111 plastically changed within individuals over two time points spanning a period of nine years. This
112 study addresses a large knowledge gap of the extent to which individual plant species vary in
113 their phytochemistry across natural populations and the mechanisms driving intraspecific
114 differences.

Methods and Materials

Study Species

Artemisia tridentata (big sagebrush) is a foundational species in the sagebrush steppe ecosystem and has a broad biogeographic extent containing multiple subspecies that often have overlapping ranges. *Artemisia* has distinctive and complex phenolics, monoterpenes, and sesquiterpene lactones that can differentiate species and populations (Jaeger et al. 2016; Kelsey et al. 1976; Turi et al. 2014). Some of these chemicals influence habitat use and foraging of herbivores including those of conservation concern (Crowell et al. 2018; Frye et al. 2013; Ulappa et al. 2014) and of economic value (*i.e.* game species and domestic species) (Ngugi et al. 1995; Striby et al. 1983).

Common Garden

A common-garden experiment was established in 2010 in Orchard, Idaho, representing a climate typical of *A. t.* subsp. *wyomingensis* that was warm and arid with a mean annual precipitation of ~28 cm (Jaeger et al. 2016). Seeds were collected from various wild-source populations throughout the range of *A. tridentata* (Figure 1). The original populations were from Arizona, California, Colorado, Idaho, Montana, New Mexico, Nevada, Oregon, Utah, Washington and Wyoming (described in Richardson et al. 2015) and span across 20 ecologically distinct ecoregions. Ecoregions (defined for this study as Level III Ecoregions by the United States Geological Survey) are relatively small ecological areas that are differentiated from each other based on local ecological characteristics.

Subspecies identification was conducted using UV fluorescence, growth morphology, and DNA amplicon sequencing, and cytotype was determined with flow cytometry for a subset of the individuals in the common garden and on parental individuals from the seed locations (Richardson et al. 2012). During the period of this study, between 2012 and 2021, 82 plants in the common garden died, most of which were *A. t.* subsp. *vaseyana*, which is least compatible with the climate of Orchard, Idaho.

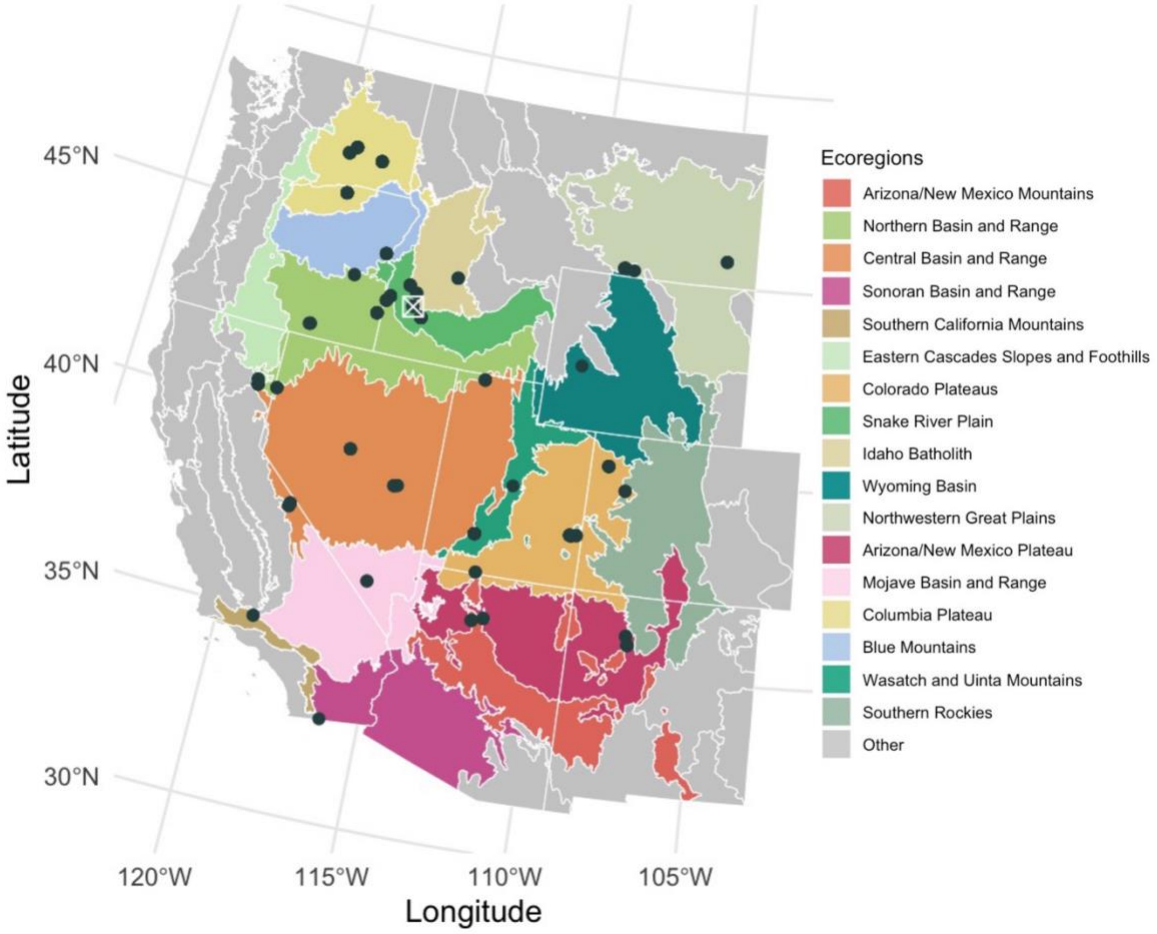


Figure 1: Map showing the locations of *A. tridentata* populations where seeds were collected. Dark grey points show original collection sites from distinct ecoregions and the white square with an X is the location of the common garden in Orchard, ID. Level III Ecoregions where

seeds were collected are colored according to the legend and ecoregions where there was no collection site are grey. State lines are included for reference.

Sample collection

We sampled from over 70 plants of the three subspecies of *A. tridentata* (subsp. *tridentata*, *vaseyana*, and *wyomingensis*) at the Orchard common garden. Individuals that survived through both collection years were sampled twice, nine years apart, in mid-June 2012 and mid-June 2021 (Dataset S1).

In 2012, three to four sprigs were collected using garden shears from different parts of each plant, avoiding inflorescences. Samples were placed into plastic bags with a seal (Ziploc), stored on ice, and then put into a -20 °C freezer on the day of collection, and stored there until later processing. In 2021, leaves from different parts of each plant were collected using tweezers sterilized with 70% ethanol, also avoiding inflorescences and placed in sterile bags (Whirlpak), kept on ice, and stored in a -80°C freezer on the day of collection until later processing. Samples had leaves of varying counts, intending to have enough leaf biomass to perform multiple chemical analyses.

Chemistry data collection

We performed Liquid Chromatography-Mass Spectrometry (LC-MS) and Gas Chromatography (GC) for the leaves from the 2012 and 2021 collections. We used LC-MS for the analysis of polar compounds extracted in methanol to quantify non-volatiles, where the majority of these are phenolics, given the solvent used for extraction. We used GC headspace analysis to quantify volatile organic compounds, where the majority of these were monoterpenes but may also

include sesquiterpenoids, green leaf volatiles, and heterocyclic compounds as found in previous work (Jaeger et al. 2016).

We performed LC-MS on 40 samples of *A. tridentata* leaves from the 2012 collection and 71 samples from 2021 collections in June 2023. LC-MS methods were adapted from Dadabay et al. (2019). To prepare samples, leaves were ground to a fine powder with mortar and pestle in liquid nitrogen. To ground leaves (100 mg), extraction solvent containing 90% methanol, 9.5% dH_2O, and 0.5% acetic acid (4mL) was added (adapted from Sakakibira et al. 2003, Dadabay et al. 2019). Samples were sonicated for 2-3 minutes, centrifuged to sediment solids, and the solvent was removed into a clean tube. The remaining plant solids were re-extracted in the same manner twice more with fresh solvent, then all extractions were combined. Solvent was evaporated under nitrogen gas, 1.0mL of fresh methanol added, then extract filtered through a 0.45 μm PTFE filter. Ground leaves were stored at -20°C when not in use. LC-MS analysis was carried out using a HPLC (Agilent 1260 Infinity), photodiode array detector, and single quadrupole mass spectrometer with electrospray ionization source (ESI, Agilent G6125C). For HPLC separation of plant extracts, a Phenomenex Luna Omega 3um Polar C18, 100Å, 50 x 3.0mm column was used at 40ºC. Extracts (3 μL) were applied to the column and eluted at a flow rate of 0.7mL/min as follows: 0-9 min with a linear gradient from initial 84.9% water, 15% methanol, 0.1% formic acid to 24.9% water, 75% methanol, 0.1% formic acid; then switched to 100% methanol from 9-9.5 minutes, and 100% methanol to 13 minutes. All elutions were monitored for absorbance at 270nm. For mass spectrometry, the ESI was operated under the following conditions: positive ion mode; nebulizer pressure 15psi; flow rate of drying gas (N 2) 7.0 L/min; drying gas temperature 300 °C; capillary voltage 4000V positive and negative setpoints. Acquisition mode was 75m/z min to 650 m/z max, scan dwell time of 500ms. Retention times and peak areas (area

under the curve, AUC) were calculated (Agilent CDS Openlab version 2.6; Dadabay et al. 2019; Sakakibara et al. 2003).

We performed GC on 147 samples from 2012 and 70 samples from 2021 re-collections. The weight of our samples ranged between 66.1 to 110.3 mg with the majority of our samples at around 100 mg. Samples from 2012 were separated from any stem material and leaves were ground in liquid nitrogen and were run in 2013. Samples from 2021 only contained leaf tissue from sampling and were ground in liquid nitrogen and were run in 2024. We used headspace analysis to quantify volatile organic compounds, where the majority of these were monoterpenes but may also include sesquiterpenoids, green leaf volatiles, and heterocyclic compounds as found in previous work (Jaegar et al.). Concentrations of VOCs were quantified using an Agilent 7694 headspace sampler and an Agilent 6890N gas chromatograph. A subsample of sagebrush leaf material (100 mg) was weighed into 20 mL glass headspace vials. For each sample, one ml of headspace gas was injected into a J&W DB-5 capillary column (30m x 250µm x 0.25µm). Temperature settings for the headspace auto-sampler were at 100°C for oven temperature, 110°C for loop temperature and 120°C for transfer line temperature. We used a vial equilibrium time of 20 min, a pressurization time of 0.20 min, a loop fill time of 0.50 min, a loop equilibrium time of 0.20 min and an injection time of 0.20 min. The settings for the gas chromatograph included the splitless injector at 250°C and the flame ionization detector at 300°C. The oven temperature was initially at 40°C for 2 min and then increased by 3°C/min to 60°C, then 5°C/min to 120°C, then 20°C/min to 300°C where the oven was held for 7 min. The inlet pressure was at 80 KP with a flow rate of 1.0 mL/min. The gas chromatograph used nitrogen for the make-up gas, and helium for the carrier gas. We identified compounds using a cocktail of monoterpene standards (alpha-pinene, camphene, beta-pinene, p-cymene, 1,8-cineole, and camphor) to generate reference

retention times (min). Not all compounds could be identified through co-elution and unknown compounds were labeled based on retention times. Retention times and peak areas (area under the curve, AUC) were calculated using Hewlett-Packard ChemStation version B.01.00 (Agilent Technologies).

Given the complexity of chemicals found in *Artemisia* species (Turi et al. 2014) and challenges identifying chemicals from varying taxa and environmental conditions, chemical identification is difficult without rigorous mass spectrometry and standards. Without standards, we are unlikely to identify chemicals. The small biomass of leaves from plants that are part of a larger and continuing study in the common garden and use of headspace analysis (which volatilizes the chemicals from the leaves and cannot be reran without variation in AUC) prevents us from being able to rerun the exact samples for identification of volatiles. Even in using GCMS, previous authors found that 35% of 72 VOCs required the use of commercial standards to identify chemicals which we do not have and 43% were only putatively identified using NIST software (Jaeger et al. 2016).

Statistical analysis

All subsequent data analyses and figures were produced using R Studio v4.2.3 (R Core Team 2023) with the *lme4* (Bates et al. 2015)*, vegan* (Oksanen et al. 2022), *pairwiseAdonis* (Arbizu 2024), *ggplot2* (Wickham et al., n.d.), *pheatmap* (Kolde 2019), *ANCOM-BC2* (Lin and Peddada 2020), and *sf* (Pebesma 2018) packages.

As described in the introduction, subspecies and cytotype were combined as one predictor variable (subspecies:cytotype) for our analyses to create five distinct levels of the three distinct subspecies, two of which differ in cytotype (diploid [2n] or tetraploid [4n]). To assess if there

were differences in the number of compounds (sum of detected peaks) between subspecies:cytotype and year, we used generalized linear mixed-effects models (GLMMs) with Gamma distributions and log link functions for each chemical class. Compound richness (i.e. the number of compounds) served as the coarsest metric of phytochemical diversity. We chose to focus on ecoregion rather than the individual populations, both to measure a broader ecological effect and to ensure a large enough sample size. Our models used compound richness as the response variable, year, subspecies:cytotype, and ecoregion as fixed-effects explanatory variables, and plant ID as a random effect (e.g., compounds ~ year + subspecies:cytotype + ecoregion + (1|Plant ID)), unless otherwise specified. We used a Tukey HSD test to assess pairwise differences in compound richness between subspecies:cytotype levels.

We used the *betadisper* function in the *vegan* package to evaluate dispersion between ecoregions since we know the data between ecoregions is likely unbalanced and could be in part due to differences we may observe between ecoregions. We followed our betadispersion tests with a Tukey HSD test to compare pairwise differences in dispersion between ecoregions. To identify differences in chemical composition among subspecies:cytotype, year, and ecoregions, we used Principal Component Analysis (PCA) coupled with Permutational Multivariate Analysis of Variance (PERMANOVAs) for each chemical class. PCAs are commonly used for multidimensional chemistry data to identify similarities and differences across samples (Wenderski et al. 2015). For PCAs, pre-processing included separating each subspecies:cytotype group and defining a threshold where compounds that were not found in twenty percent of each group were removed and the remaining data were scaled using the *scale* function in base R to one standard deviation (Yang et al. 2015). PCAs were run using *prcomp* function and the approximated eigenvalues were plotted for figures. PERMANOVAs were run using function

adonis2 in the *vegan* package to measure differences in chemical composition between subspecies:cytotype, year, and ecoregion. The normalized LC-MS and GC datasets were used as the dependent variable in each PERMANOVA, and the significance was assessed using 999 permutations. The models included source-population ecoregion, subspecies:cytotype, and year as explanatory variables and a euclidean distance method was used (method = "euclidean"). We controlled for the effects of individual source-population within ecoregions by including a nested variable for populations within ecoregion (e.g., chemistry ~ subspecies:cytotype + year + ecoregion + population %in% ecoregion). Variables were placed in order for what we hypothesized would best account for the chemical variation as our models measured sequentially (by = "term"). For post hoc analyses, we used a multilevel pairwise comparison from the package *pairwiseAdonis* based on the PERMANOVAs to identify which subspecies:cytotype group significantly differed from another in chemical composition.

To further understand how the effects of individual populations and their distance from each other vs. the climate at their seed source locations may be influencing phytochemistry within subspecies:cytotypes, we subset our data into separate subspecies:cytotype groups and ran PCAs on climate and spatial distance (latitude and longitude) data collected from the seed source-population sites (Figure 1). We ran Partial Mantel tests, with method "spearman", using both the spatial distance matrices and the climate-related distance matrices against the LC-MS and GC data. Partial Mantel tests were done for each subspecies:cytotype group separated by year to test for relationships with spatial distance while controlling for climate and vice versa. The separation of year and subspecies:cytotype was done to focus on specific groups and avoid confounding factors.

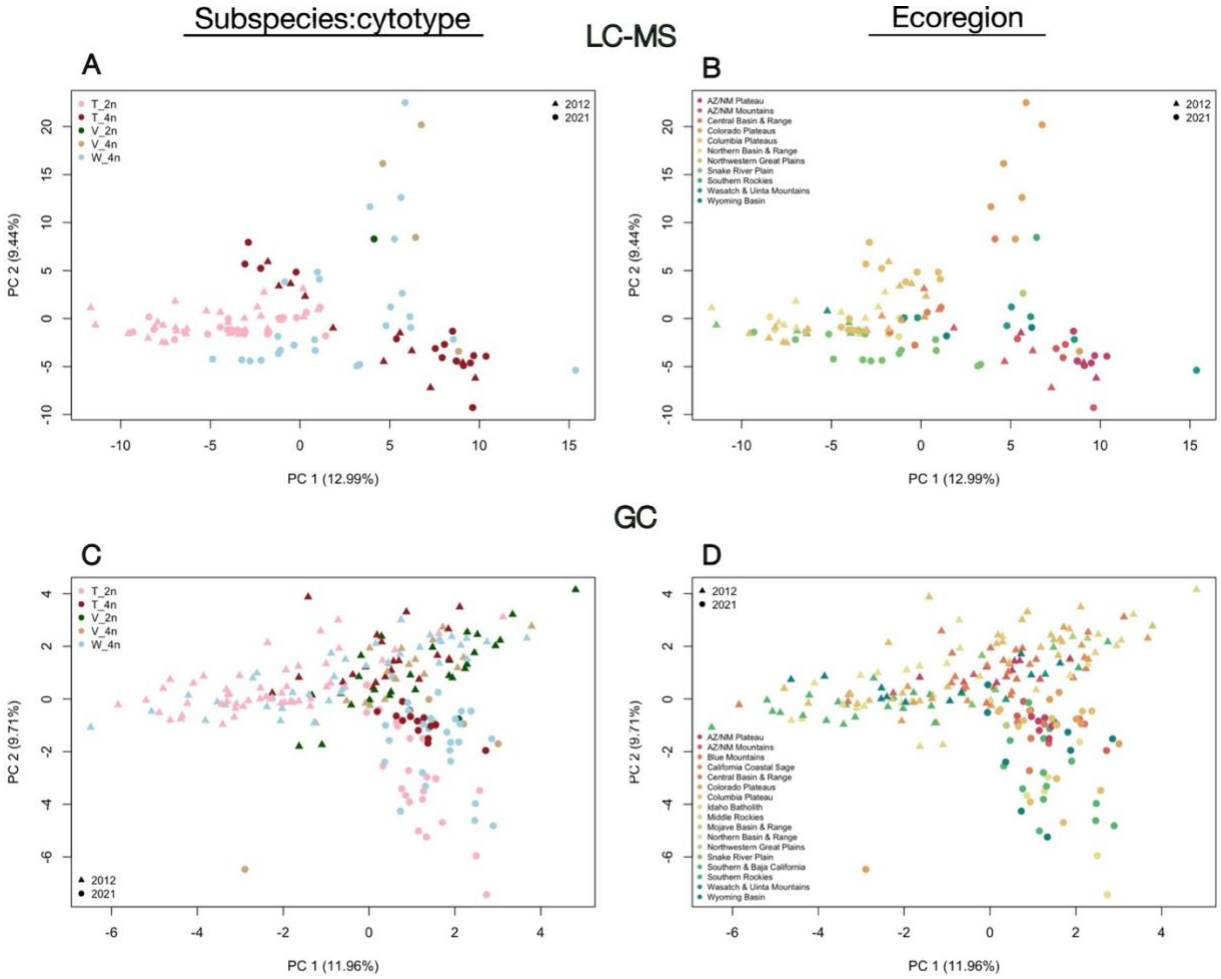
Based on the Partial Mantel test results, we ran further PERMANOVAs on the LC-MS *A. t.* subsp. *tridentata* 2n group. The PERMANOVA model used the three climate variables from our climate PCA that strongly separated populations (Table S2; Figure S2): growing season precipitation balance (PRATIO), minimum temperature in the coldest month (MTCM), and maximum temperature in the warmest month (MTWM). We also ran *ordisurf* analyses (a type of generalized additive model) from the *vegan* package to fit and plot smooth surfaces of the three environmental variables onto our PCA ordinations.

To look at compound-level differential abundance across samples for leaf chemistry explained by the different ecoregions and subspecies:cytotype, we used Analysis of Composition of Microbiomes with Bias Correction 2 (ANCOM-BC2). ANCOM-BC2 is a statistical method that normalizes the read counts (in this case, abundance derived from AUC of peaks), controls for the false discovery rate and returns differentially abundant species (or in this case, compounds) between variables of interest. The LC-MS ANCOM-BC2 model was run for the *A. t.* subsp. *tridentata* 2n group as it had the largest sample size spanning across the most ecoregions. This model was run using an alpha of 0.001 and a prevalence cut-off of 0.20. We selected a conservative alpha value to focus on the strongest patterns in our results and still identified many compounds as differentially abundant between five different ecoregions for *A. t.* subsp. *tridentata* 2n group. We did not separate the LC-MS into 2012 and 2021 models due to only a few ecoregions having sufficient sample size in both years and the minimal effect of year on composition. We used the package *pheatmap* to plot heatmaps displaying the natural log + 1 mean abundances of the differentially abundant compounds from our ANCOM-BC2 models across ecoregions using the LC-MS data for *A. t.* subsp. *tridentata* 2n subspecies:cytotype group.

Finally, to test for correlations between our LC-MS and GC matrices we performed a Procrustes analysis using the *protest* function in base R. We chose to use a Procrustes test as it more powerful for matrix association detecting than a Mantel test (Peres-Neto and Jackson 2001).

Results

Surprisingly, while we found significant variation in *A. tridentata* phytochemistry across subspecies:cytotype groups, the most variation was explained by seed source-ecoregion where the seeds for the common garden were collected based on our PERMANOVA results (Figure 2). We visualized these statistical differences with our PCAs. Our LC-MS PCAs showed clearer separation by ecoregion and subspecies:cytotype (Figure 2A and B) than GC, but our GC PCAs showed the clearest clustering by sample year along the PC2 axis (Figure 2C and D).



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312 Figure 2: Chemical composition differed among subspecies:cytotype levels and the ecoregions
313 where seeds were collected. All graphs show Principal Component Analyses (PCAs) of either
314 Liquid Chromatography – Mass Spectrometry (LC-MS; top) or Gas Chromatography (GC;
315 bottom) chemistry composition. A) Points representing LC-MS samples show statistically
316 significant clustering across subspecies:cytotype (colors; $R^2 = 0.172$, $p < 0.01$) and across years
317 (shapes; $R^2 = 0.037$, $p < 0.01$). B) Points representing LC-MS samples show significant
318 clustering across ecoregions (colors; $R^2 = 0.203$, $p < 0.01$). C) Points representing GC samples
319 explain less variance than LC-MS but have statistically significant clustering across
320 subspecies:cytotype (colors; $R^2 = 0.077$, $p < 0.01$) and year (shapes; $R^2 = 0.081$, $p < 0.01$). D)

Points representing GC samples show significant clustering across ecoregions (colors; R^2 = 0.128, $p < 0.01$).

Compound richness for LC-MS

A total of 302 compounds were detected from the 111 leaf samples from 87 individuals using LC-MS. There were 25 sample replicates between 2012 and 2021. After applying the twenty percent minimum occurrence threshold *A. t.* subsp. *tridentata* had 80 samples with 297 possible compounds, *A. t.* subsp. *wyomingensis* had 26 samples with 299 possible compounds, and *A. t.* subsp. *vaseyana* had 5 samples with 294 possible compounds.

A GLMM used to predict the relationship between compound richness detected from the LC-MS results based on subspecies:cytotype, ecoregion, and year predictor variables (deviance = 0.261, DF = 94, AIC = 698.7) revealed that year was not a significant factor predicting compound richness ($\beta = 0.005$, SE = 0.013, $t = 0.404$, $p = 0.69$; Figure S1A), however select ecoregions and subspecies:cytotypes were significant. Qualitatively, subspecies with a higher cytotype number had higher compound richness (Figure S1C). In support of this finding, a Tukey HSD post-hoc test revealed that *A. t.* subsp. *tridentata* 2n contained significantly fewer compounds than *A. t.* subsp. *vaseyana* 4n (diff = 17.6, $p < 0.01$), *A. t.* subsp. *tridentata* 4n (diff = 11.9, $p < 0.01$) and *A. t.* subsp. *wyomingensis* 4n (diff = 16.5, $p < 0.01$).

Compound richness for GC

Of the 224 leaf samples from 156 plant individuals, 74 compounds were detected in the GC data. There were 68 sample replicates between 2012 and 2021. A GLMM analogous to that used for LC-MS data was used to predict compound richness detected from the GC data. Results

(deviance = 5.67, DF = 199, AIC = 1066.9) revealed that compound richness remained consistent across all ecoregions. Individuals of *A. t.* subsp. *vaseyana* 2n had significantly fewer compounds than *A. t.* subsp. *tridentata* 2n (diff = -3.63, $p < 0.01$; Figure S1D). In addition, year 2021 was associated with fewer compounds detected than 2012 ($\beta = -0.031$, SE = 0.027, $z = -11.4$, $p < 0.01$).

Ecoregion, subspecies:cytotype, and year differences for LC-MS and GC analyses

For chemical composition of both LC-MS and GC we found that the largest portion of variation was explained by ecoregion, even after accounting for all the variation explained by subspecies:cytotype and year. The LC-MS and GC PERMANOVA analyses testing for compositional variation across ecoregion, subspecies:cytotype, and year revealed that LC-MS and GC profiles were significantly different across ecoregions, among subspecies:cytotype levels, and across years (Table 1). For both LC-MS and GC we also found the second largest portion of the variation in chemical composition was attributed to populations, when nested in ecoregions (LC-MS: $R^2 = 0.174$, $p < 0.01$; GC: $R^2 = 0.116$, $p < 0.01$).

We found significant differences in betadispersion across ecoregions in both the LC-MS ($F = 3.53$, $p < 0.01$) and GC data ($F = 3.53$, $p < 0.01$). Our Tukey HSD test looking at the LC-MS data revealed that the Southern Rockies and the Northwestern Great Plains differed from the Colorado Plateaus (vs. Southern Rockies: diff = -1.6e+01, $p < 0.05$; vs. Northwestern Great Plains: diff = -1.6e+01, $p < 0.05$) and the Arizona/New Mexico Mountains (vs. Southern Rockies: diff = -1.7e+01, $p < 0.05$; vs. Northwestern Great Plains diff = -1.7e+01, $p < 0.05$). Our Tukey HSD test for the GC data found no significant pairwise differences between ecoregions.

Due to the differences in betadispersion, we recognize that our PERMANOVA results may be due in part by the differences in betadispersion across ecoregions.

Table 1: PERMANOVA results (Type I, sequential term effects) for LC-MS and GC data testing how year, subspecies:cytotype, and ecoregion affect phytochemistry.

Data	Variables	R^2	F Model	p-value
All LC-MS	Subspecies:cytotype	0.172	7.994	0.001
	Year	0.037	6.886	0.001
	Ecoregion	0.203	3.781	0.001
	Population within Ecoregion	0.174	1.699	0.001
All GC	Subspecies:cytotype	0.077	5.598	0.001
	Year	0.081	23.47	0.001
	Ecoregion	0.128	2.199	0.001
	Population within Ecoregion	0.116	1.307	0.006

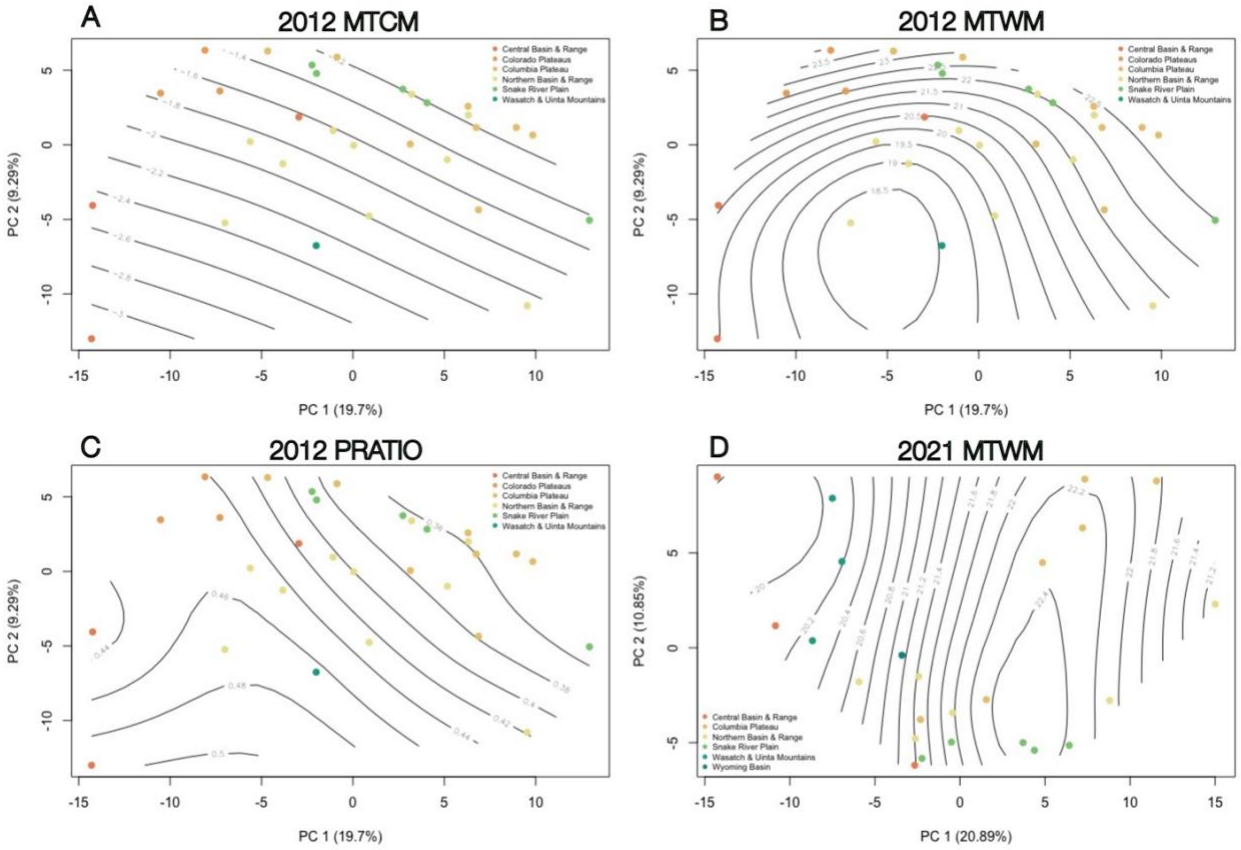
Further investigation of LC-MS and GC differences across ecoregions

Because we found that the strongest relationship for LC-MS and GC profiles was with seed source-ecoregion, we wanted to further explore more detailed location-related variables driving these results, while controlling for subspecies:cytotype and year differences. Thus, we subset our datasets to 2012 and 2021 and investigated how the effects of climate and distance between source-population sites affected the phytochemistry of our two subspecies:cytotype groups with the most samples: *A. t.* subsp. *tridentata* 2n and *A. t.* subsp. *wyomingensis* 4n. We conducted Partial Mantel tests on each subset to test for the effect of climate while controlling for spatial distance and, conversely, to test for the effect of spatial distance while controlling for climate.

Spatial distance (while controlling for climate), was significantly correlated with the 2012 GC chemical composition for *A. t.* subsp. *wyomingensis* 4n ($r = 0.226$, $p < 0.01$) and LC-MS

chemical composition for the 2012 *A. t.* subsp. *tridentata* 2n ($r = 0.37$, $p < 0.01$), 2021 *A. t.* subsp. *tridentata* 2n ($r = 0.393$, $p < 0.01$), and 2021 *A. t.* subsp. *wyomingensis* 4n ($r = 0.205$, $p = 0.02$). However, we also found that climatic differences (while controlling for spatial distance) were significantly correlated with LC-MS chemical composition for 2012 *A. t.* subsp. *tridentata* 2n ($r = 0.153$, $p = 0.02$) and 2021 *A. t.* subsp. *tridentata* 2n ($r = 0.123$, $p = 0.05$).

We further investigated which variables underly climate or environment-based differentiation of LC-MS phytochemistry in *A. t.* subsp. *tridentata* 2n. PERMANOVA results showed that the 2012 *A. t.* subsp. *tridentata* 2n LC-MS chemical composition was significantly explained by all three of our climate variables (Table 2; PRATIO: $R^2 = 0.188$, $p < 0.01$; MTWM: $R^2 = 0.152$, $p < 0.01$; MTCM: $R^2 = 0.091$, $p = 0.01$; Figure 3A, 3B, and 3C) while the smaller 2021 *A. t.* subsp. *tridentata* 2n LC-MS data was marginally significantly explained by only MTWM (Table 2; $R^2 = 0.082$, $p = 0.058$; Figure 3D). We used *ordisurf* on the LC-MS *A. t.* subsp. *tridentata* 2n PCAs for the results outlined above to illustrate how the environmental variables related to the phytochemistry results from our samples (Figure 3). In contrast with the PERMANOVA results, the *ordisurf* models showed higher R^2 values and significant relationships for all explanatory variables except MTCM in 2012. These slight differences in results are likely due to *ordisurf* using generalized additive models and a separate model for each variable, instead of controlling for all other variables and looking at marginal effects as was done in the PERMANOVAs.



397

398 Figure 3. *Artemisia tridentata* subsp. *tridentata* 2n plants differ in their chemical profiles across
399 climate-related variables. All plots show Principal Component Analyses (PCAs) of LC-MS
400 chemical composition with points colored by ecoregion. A) 2012 samples by the minimum
401 temperature of the coldest month (MTCM) of the seed source-ecoregion (*ordisurf*: $R^2 = 0.121$, p
402 = 0.075). B) 2012 samples by the maximum temperature in the warmest month (MTWM) of the
403 seed source-ecoregion (*ordisurf*: $R^2 = 0.527$, $p < 0.01$). C) 2012 samples with the growing season
404 precipitation balance (PRATIO) of the seed source-ecoregion overlaid (*ordisurf*: $R^2 = 0.501$, $p <$
405 0.01). D) PCA of the 2021 LC-MS chemical composition by MTWM of the ecoregion where the
406 seeds were sourced (*ordisurf*: $R^2 = 0.339$, $p = 0.015$). In all plots points are colored by ecoregion.

Table 2: Results of PERMANOVAs (Type III, marginal effects) and *ordisurf* models (a separate model for each variable) for 2012 and 2021 LC-MS *A. t.* subsp. *tridentata* 2n testing the effects of three important climate variables at source-population sites.

Data	Variables	PERMANOVA R^2	PERMANOVA p-value	*ordisurf* R^2 adjusted	*ordisurf* p-value
2012 LC-MS for *A. t.* subsp. *tridentata* 2n	MTCM	0.085	0.009	0.121	0.075
	MTWM	0.064	0.044	0.527	0.001
	PRATIO	0.246	0.001	0.501	0.001
2021 LC-MS for *A. t.* subsp. *tridentata* 2n	MTCM	0.043	0.276	0.313	0.034
	MTWM	0.082	0.058	0.339	0.015
	PRATIO	0.054	0.200	0.248	0.043

We ran an ANCOM-BC2 model to further explore which compounds were differentially abundant in the *A. t.* subsp. *tridentata* 2n data across different ecoregions. There were 16 compounds that were differentially abundant across five ecoregions having at least four samples within each ecoregion (Figure 4). Samples from more northern ecoregions of the United States (Columbia Plateau, Northern Basin and Range, and Snake River Plain) shared more of these compounds (Figure 4). This is consistent with results from the PCAs where the LC-MS chemical composition clustered closely when looking across ecoregions (Figure 2B). Samples from the ecoregions spanning the Central Basin and Range and the Wasatch and Uinta Mountains shared similarities in chemical profiles, but three compounds (C050, C196, C202) were more enriched in the Central Basin and Range ecoregion and thus more similar to the three more northern ecoregions. These three compounds had lower differential abundance in the Wasatch and Uinta Mountain samples compared with any other ecoregion, highlighting a difference in phytochemistry associated with this ecoregion (Figure 4). Of the 16 compounds found to be significant across ecoregions, compounds were grouped into their suspected compound subclass

of non-volatile compounds based on retention times. Any compounds with retention times between 0–3-minute were categorized as a coumarin glycosylated, 3 to 6 minutes as a coumarin, 6 to 8 minutes as a flavonoid, and above 8 minutes as a flavonoid glycosylated (Table S3). Our results did not find notable patterns related to the differentially abundant compounds and compound subclass.

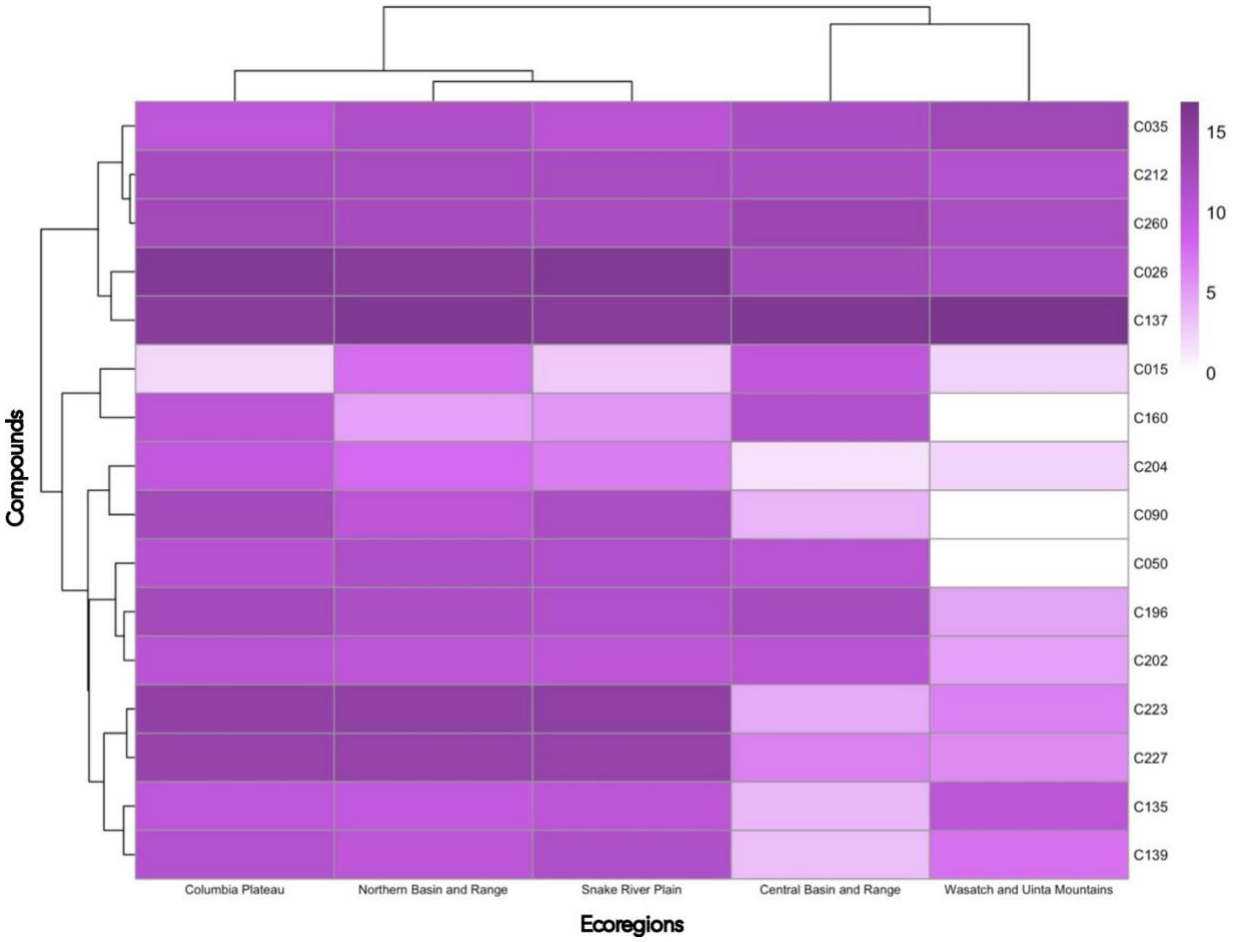


Figure 4: Differentially abundant LC-MS compounds across *A. t.* subsp. *tridentata* 2n ecoregions separate more northern from more southern populations. The heatmap shows the significantly differentially abundant compounds from LC-MS outputs across the different *A. t.* subsp. *tridentata* 2n ecoregions using the natural log +1 of normalized compound abundances. Darker

435 purple represents higher abundance while lighter purple represents a lower abundance. Both
436 ecoregions and compounds are clustered based on similarity.

437 *GC and LC-MS profiles are correlated*

438 In addition to our primary findings, we found a significant correlation between the LC-MS and
439 GC chemical profiles when using a Procrustes analysis to compare the compound matrices
440 across both methods (symmetric Procrustes rotation = 0.6514, p = 0.001; Figure S3). This
441 relatively high correlation suggests that both methods captured similar patterns in chemical
442 compounds across samples, while the deviations seen in the Procrustes plot may be attributed to
443 the differences we see in subspecies:cytotype or ecoregions across the two methods.

444 **Discussion**

445 Intraspecific differences in phytochemistry may shape species interactions and communities
446 across multiple trophic levels (Glassmire et al. 2016), but these impacts will remain hidden if
447 only species-level differences are investigated. While there is increasing evidence for climatic
448 variation influencing plant metabolomics, the extent to which individual plant species vary
449 within a natural population and the mechanisms driving these differences in the environment are
450 not well understood (Sedio et al. 2021). Our common garden study provides new evidence for
451 large differences in phytochemistry across ecoregions within a single species, that can be driven
452 in part by subspecies:cytotype and climate variation among seed source locations.

453 Overall, in contrast with our initial hypothesis, we found that seed-source ecoregion of *A.*
454 *tridentata* had more explanatory power for phytochemistry than the subspecies:cytotype levels
455 (Figure 1, Table 1). Even when controlling for subspecies:cytotype and for sampling across a

nine-year span, phytochemical compositions were best explained by the original ecoregion of the seeds. Ecoregion accounted for the largest amount of variation in both LC-MS and GC composition (Table 1), indicating that *A. tridentata* phytochemistry has strong genetic determination, that remained consistent over time, potentially due to maternal effects or related to adaptation to sites of origin. Evolved phytochemical differences related to ecoregion could either be due to isolation by physical distance or due to selection by the local environment. In our study, we found evidence for both isolation and selection. These results align with previous research into *A. tridentata* volatiles (Jaeger et al. 2016) but moves beyond investigating subspecies differences and volatile compounds to include non-volatile compounds and ecoregion. Our results show that both non-volatile and volatile profiles have diverged — even within individual subspecies:cytotype groups — across ecoregions likely via adaptation to the local environment, isolation by distance, or maternal effects.

Beyond ecoregion differences, we also found significant differences in LC-MS and GC chemical composition across subspecies:cytotype levels and across the 9-year sampling interval. The differences over time suggest that non-volatile and volatile compounds may also be affected by the current environment or by a changing environment. While sampling year was significant in explaining both the LC-MS and GC compositions, the amount of variation explained by year was the lowest contribution for LC-MS and explained under four percent. In contrast, for GC, year explained nearly eight percent of the variation, which was more than subspecies:cytotype (Table 1). The lower variation explained by subspecies:cytotype in the GC data compared to the LC-MS data may suggest that volatiles are more genetically conserved in *A. tridentata* which is supported by previous research into volatile organic compounds biosynthetic pathways (Picazo-Aragonés et al. 2020). One explanation for the year effect in the GC PERMANOVA and PCA

results is that volatile compounds can decrease over time, even in frozen leaves. For both years, GC samples were analyzed 1–2 years post-sampling, which is likely to yield more comparable results than analyzing one set 9–10 years post sampling, however the sample storage methods were poorer for the older samples compared to newer samples. However, because 2021 leaves were, stored differently, analyzed at a different time and with a slightly different acquisition method, and due to the possibility of differences in volatile loss over time, it is not clear if the differences between the two years are real aspects of the plant chemistry, or due to methodological differences. Previous search supports that VOCs shift in response to the current environment, as they often are triggered by species interactions such as herbivory or by abiotic conditions (Jaeger et al. 2016; Picazo-Aragonés et al. 2020).

In terms of compound richness, we found that subspecies:cytotype level significantly influenced LC-MS chemistry, while year did not. Qualitatively, 4n plants had larger compound numbers than *A. t.* subsp. *tridentata* 2n and *A. t.* subsp. *vaseyana* 2n plants and there were significantly fewer compounds detected in *A. t.* subsp. *tridentata* 2n than detected in *A. t.* subsp. *vaseyana* 4n or *A. t.* subsp. *wyomingensis* 4n in our post-hoc test (Figure S1C). Due to *A. t.* subsp. *vaseyana* 2n and *A. t.* subsp. *vaseyana* 4n having low sample size, the interpretation of compound diversity is limited. However, our results align with previous research that polyploidy enhances chemical diversity, potentially conferring adaptive advantages such as possible improved chemical defense mechanisms (Chen et al. 2024). The higher chemical variation found in 4n plants may reflect their ancestry. In *A. tridentata,* 4n plants typically form an intermediate group between *A. t.* subsp. *vaseyana* 2n and *A. t.* subsp. *tridentata* 2n*,* suggesting admixture of 4n plants (Grossfurthner et al. 2023) and thus higher chemical variation.

The 2012 *A. t.* subsp. *wyomingensis* 4n GC data were significantly correlated with spatial distance. Recent research provides evidence that individuals from *A. t.* subsp. *wyomingensis* 4n are genetically distinct in different parts of the range and could have originated from separate hybridization events (Grossfurthner et al. 2023). These genetic differences also likely influence the plants' secondary metabolite profiles.

For *A. t.* subsp. *tridentata* 2n, both the 2012 and 2021 LC-MS chemical compositions were correlated with spatial distance as well, but for this subspecies we found additional correlations with the climate of the seed source-ecoregion. When testing for effects of three important climate variables, we found that maximum temperature in the warmest month (MTWM) was a significant driver of non-volatile compound chemistry within this subspecies:cytotype group in both 2012 and 2021 (Table 2; Figure 3). Minimum temperature in the coldest month (MTCM) and growing-season precipitation balance (PRATIO) were found to be significant in the 2012 samples. Strong abiotic selective pressures such as hot and cold extremes can shift plant chemistry through adaptive responses. Previous research in *A. tridentata* has found increased survival in plants whose population of origin had colder winters and decreased survival in plants whose population of origin was warmer and drier (Chaney et al. 2017). Together, these findings highlight how genetic differences driven by the climate of the seed origin can affect plant survival, emphasizing that considering the climate of where the seeds were sourced, which could be indicated by the compositions of non-volatile compounds, can enhance restoration or conservation success over a landscape.

In a different plant system, a study investigating coumarin and alkaloid concentrations across the genus *Pilocarpus* found that variation was more strongly associated with adaptive environmental convergence than phylogenetic relationships, although both factors played a role in shaping the

chemical variation (Allevato et al. 2019). The best predictive model for *Pilocarpus* was between coumarin concentration and precipitation in the coldest quarter (Allevato et al. 2019). Similarly, a 2024 study in *Plantago lanceolata* found that the concentrations of chemical defense compounds across both native and introduced ranges were affected by geographic origin, with a correlation to place-of-origin climate conditions (Medina-van Berkum et al. 2024). These results align with results here, indicating that local climate conditions can affect plant chemistry.

Individual non-volatile compounds in *A. t.* subsp. *tridentata* 2n plants were particularly abundant or sparse depending on the ecoregion the seeds were collected from (Figure 4). The clustering patterns observed across both the ecoregions suggest that ecoregions with presumed similar environmental conditions tend to exhibit comparable chemical profiles (Figure 3). Although the phytochemicals targeted by our methods are not directly involved in plant growth or reproduction, these secondary metabolites could indirectly enhance plant tolerance to abiotic factors (e.g., drought, salinity, UV radiation, heat) (Salam et al. 2023). Further investigation into the identity and function of these secondary metabolites could provide insights into the mechanisms (e.g., herbivore-defense, pollination, or associated microorganisms) underlying local adaptation or plastic responses to stress.

The significant correlation between our GC and LC-MS datasets suggests similarity in the overall chemical profiles detected by each method. However, our results also highlight the importance of considering the limitations of each method for gaining a more comprehensive understanding of what may drive intraspecific chemistry variation. Despite their correlation, we found differences in the amount of variation explained across variables with climate and spatial distance explaining more variation in non-volatile compounds for 2012 *A. t.* subsp. *tridentata* 2n, 2021 *A. t.* subsp. *tridentata* 2n, and 2021 *A. t.* subsp. *wyomingensis* 4n, while only spatial

distance explained variation in the volatile compounds detected in 2021 *A. t.* subsp. *wyomingensis* 4n. The inclusion of both these chemical profiles offers the potential to identify which chemical phenotypes are indicators of climate adaptation, which will likely be important as climates continue to shift.

This study provides insights into the drivers of intraspecific phytochemical variation. The ecoregion where *A. tridentata* seeds were sourced was particularly important for determining secondary chemistry, with spatial separation being most important for one subspecies:cytotype group while another was significantly affected by climatic conditions in the place-of-origin. This confirms that there are persistent, genetically based phytochemical differences within *A. tridentata* subspecies, with both isolation by distance and adaptation to local climatic factors affecting intraspecific variation. Our results in this foundational plant system suggest that strong intraspecific differences may also exist in other plant species and should be considered when investigating the ecological causes and consequences of stable or dynamic chemical phenotypes. Furthermore, future research should work towards identifying secondary metabolite compounds and their functional roles to better understand the relationship between climate, geography, plant physiology, species interactions, and chemistry.

Acknowledgements

The authors thank Jacob Heil, Molly Garrett, Lukas Grossfurthner, and Lisa Lam for help with sample collection. We acknowledge the support from Boise State University, Idaho State University, and College of Western Idaho. We thank all the volunteers, Bureau of Land Management, and USDA Forest Service.

Funding

This research was funded by the National Science Foundation (NSF) Idaho EPSCoR program under award number OIA-1757324 and OIA-1826801. LSB and ETH were also supported by NSF CAREER award DEB-2236782. This research was funded in part by USDA Forest Service, Rocky Mountain Research Station.

Author Contributions

ET & LSB performed statistical analyses and wrote the manuscript. LSB, BAR, JSF, and KGT provided edits for the manuscript and contributed statistical knowledge. LSB, JSF, DC, BB, CD, and ET processed samples and ET, LSB, JSF, DC, BB, CD, and BAR contributed data.

Competing Interests

The authors declare no conflicts of interest.

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