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Phytochemical variation within populations of *Echinacea angustifolia* (Asteraceae)

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Abstract

Quantitative evaluation of phytochemical diversity in *Echinacea angustifolia* DC. populations from different natural geographic areas supports the existence of distinct natural chemotypes within the species. Consumers, growers and manufacturers of phytomedicines are interested in chemotype identification for prediction of phytochemical content in cultivar development. Six month old *E. angustifolia* roots, grown from nine different wild seed sources in a controlled environment, were extracted into 70% ethanol and 28 reported phytochemicals were measured by HPLC separation. Two-way ANOVA between the nine populations revealed quantitative differences ($p<0.05$) in the caffeic acid derivatives 2,3-*O*-dicaffeoyl tartaric acid (cichoric acid), 2-*O*-caffeoyl tartaric acid (caftaric acid), 1,3-dicaffeoyl-quinic acid (cynarin), echinacoside and ten reported alkamides. Canonical discriminant analysis determined the phytochemical variables which contributed the most towards chemotype distinction for five of the nine populations: undeca-2E,4Z-diene-8,10-diynoic acid-2-methylbutylamide*, dodeca-2E,4E-dienoic acid isobutylamide*, dodeca-2E-ene-8,10-diynoic acid isobutylamide**, hexadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide*, cichoric acid**, caftaric acid*, and echinacoside** (*$p<0.0001$, **$p<0.05$). Five of those compounds were also significantly associated with latitudinal variation by regression analyses ($p<0.05$). © 2002 Published by Elsevier Science Ltd.

Keywords: Echinacea angustifolia; Secondary metabolism; Phytochemical variation; Chemoraces; Alkamides; Caffeic acid derivatives; Polyacetylenes

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1. Introduction

Plants of the genus *Echinacea* are native to North America, with wild populations ranging from the US Great Plains north to the Canadian prairies, east to the Appalachian uplands and the southeastern coastal plains (McGregor, 1968). As one of the most geographically-widespread species in the genus, *E. angustifolia* has adapted naturally to different habitats from Texas to Saskatchewan, but it remains one of the more difficult *Echinacea* species to cultivate successfully. Traditionally, the roots of *E. angustifolia* were the most frequently-used medicine among most First Nations groups of the Great Plains region (Kindscher, 1989; Shemluck, 1982). In the last decade, *Echinacea* species have regained this popularity as the top-selling medicinal market botanical (Brevoort, 1998). Wild-harvested *E. angustifolia* roots have the highest market value of all *Echinacea* material sold as phytomedicine.

Since worldwide economic demand for *E. angustifolia* in particular has far exceeded the capacity for sustainable wild root harvesting, effective cultivation of all *Echinacea* species is imperative. Growers of *E. angustifolia* must take an interest in genetic preservation to protect their native sources of germplasm diversity from the consequences of urban development, herbicide use and overgrazing (Price-Hurlburt, 2000). Morphological races from a range of wild *E. angustifolia* seed sources have been identified in a common garden based on significant variation in aerial yield, height, seed survival, lodging/disease, growth habit, flowering and seed maturity (Little, 1999). Genetic variation within and among natural populations of *E. angustifolia* is reportedly extremely high (Feghahati and Reese, 1994). Viles and Reese (1996) suggested that such genetic variability was responsible in part for reported phytochemical variability (Bauer and Wagner, 1991). As a measure of phytochemical diversity among wild seed sources, Viles and Reese (1996) studied allelochemical activity in a greenhouse study, with the conclusion that natural selection was acting to differentiate wild populations of *E. angustifolia* genetically, through shifting environmental pressures such as herbivory.

Cultivation of *E. purpurea* (L.) Moench has already enjoyed significant increases through traditional selective breeding practices worldwide. Morphologically superior *E. purpurea* lines from germplasm with demonstrated high genetic variability (Baum et al., 1999) resulted in doubled average phytochemical content in each of the major chemical classes when compared to non-selected lines (Letchamo et al., 1998). Similar selection of superior cultivars by phytochemical prediction would enhance *E. angustifolia* cultivation.

We demonstrated pathway induction for the *Echinacea* secondary phytochemicals, alkamides and ketoalken/ynes, using a naturally-occurring chemical mediator in *E. pallida* (Binns et al., 2001). This phenomenon may be a mechanism under environmental control, which effectively minimizes the energetic costs of defense. However, there are no reports of genetic adaptation leading to differences in plant secondary phytochemical metabolism, although the ability to direct resources to primary metabolism (growth and differentiation) may evolve through stable genetic polymorphisms

in plant species with a broad range of habitats and selection pressures (Herms and Mattson, 1992).

The objective of the current study was quantitative evaluation of the genetic component in phytochemical diversity of *E. angustifolia* populations from different geographic areas. We have evaluated the quantity and type of phytochemicals accumulated in young *E. angustifolia* roots from a range of wild populations, grown under uniform environmental conditions. We also explored the association between the observed phytochemical variability and latitude of germplasm sources.

2. Methods

2.1. Achene germination

Achenes from nine native populations of *E. angustifolia* were harvested from August to October 1997 and 1998, then stored in a freezer at −20 °C. Voucher specimens are deposited at the Department of Agriculture Ontario Herbarium, code DAO (Table 1). Botanical identification of source plants was performed in the field according to McGregor (1968) and confirmed in a concurrent morphometric taxonomic revision (Binns et al., in press), where only one population (no. 6) exhibited introgressant morphological characteristics (Table 1). Under sterile conditions, 50 achenes from each accession were placed in a sealed container with holes (smaller diameter than achenes, approx. 1 mm) and soaked in Plant Preservation Mixture (PPM)™ (10 ml/l in dH_2O) for 24 h on a rotary shaker. They were rinsed three times with dH_2O and placed in petri dishes on filter paper (Whatman no. 1) soaked with 1 ml/l ethylene (Ethrel™) in distilled H_2O. Petri dishes were sealed with parafilm and placed in a growth chamber under constant light (5 $\mu M/m^2/s$, incandescent)

Table 1
Location, latitude, accession labels (corresponding to voucher specimens, coll. S. Binns, DAO) and number of individuals for nine populations of *Echinacea angustifolia*

Population	No. plants (*n*)	Label (DAO)	Latitude	Location
1	16	EST0822B	34.368 N	OK: Carter Co, Fox
2	10	EST0823	34.419 N	OK: Carter Co, Alma
3	16	EA23909	34.717 N	OK: Comanche Co
4	8	EST0823	35.009 N	OK: Cleveland Co
5	12	EA421331	36.643 N	OK: Logan Co
6[a]	16	EA014	37.313 N	KS: Cowley Co
7	8	EA24058	39.179 N	KS: Pottawatomie Co
8	17	EA421332	40.065 N	NE: Richardson Co
9	12	EA23930	42.917 N	IA: Clay Co

[a] Introgressant characteristics from possible past hybridization between *E. pallida* X *E. angustifolia* according to morphometic taxonomic analyses (Binns et al., in press).

at 4 °C for 14 days. Gradually, over 5 days, the temperature was raised to 25 °C and germination occurred within 10 days following temperature increase.

Mature germlings were potted in a mix of 5:3:1 vermiculite: Promix: quartz sand (large grains) in cell packs for several weeks in the growth chamber (25 °C and 16 h days) and eventually transplanted into individual 3 in. pots in a greenhouse (25 °C and 16 h days). The final sample size per population (Table 1) varied according to survival, which was between 21–78%. We used a completely randomized design on a single greenhouse bench.

2.2. Extraction and isolation

Fresh roots were harvested at the age of 6 months, washed thoroughly and covered with 60% EtOH. They were coarsely chopped in a blender (Osterizer), followed immediately by 60 s on the medium–high setting of a Polytron (Brinkmann Instruments, Westbury, NY) to completely release cell contents into solution. The root–ethanol slurry was shaken at medium speed (70 rpm) for 20 h, and filtered by Buchner filter (Whatman no. 1). Extracted root material was dried in an oven (50 °C), then weighed and discarded. The filtrate was rotary evaporated to dryness and re-dissolved in fresh 60% EtOH with the appropriate volume to achieve standardized 0.5g/ml extracts. These were filtered for HPLC using 0.2 μm nylon membranes.

A validated method was used for HPLC phytochemical separations (Bergeron et al., 2000). Hydrophilic chromatography was achieved using a solvent system of acetonitrile: 50 mM NaH_2PO_4 pH 2.95, at a flow rate of 1.5 ml/min following a linear gradient of 5–25% acetonitrile over 7 min. Lipophilic chromatography was achieved using a solvent system of acetonitrile:water, at a flow rate of 1.0 ml/min following a linear gradient of 40–80% acetonitrile over 15 min. In both systems, 5 μl of sample was injected on a 7.5×4.6 mm C-18 column (3 μm particle size) (Lichrospher, Merck BDH Toronto, Canada). Lipophilic compounds were detected at 210 and 260 nm and hydrophilic compounds were detected at 326 nm.

2.3. Identification and quantitation

Reference standards of the first 11 compounds were obtained through isolation in our laboratory or purchase as follows: undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide **1**, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (tetraenes) **2**+**3**, pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide **5**, 2,3-*O*-dicaffeoyltartaric acid (cichoric acid) **6** and echinacoside **7** were isolated by column chromatography on silica gel and assessed for purity by ^{1}H and ^{13}C NMR spectral data (Bergeron et al., 2000). Two others compounds were isolated by the same method (Bergeron et al., unpublished): dodeca-2E,4E-dienoic acid isobutylamide **4** (by online UV spectra; Bauer and Remiger, 1989) and cynarin **10** (by ^{1}H and ^{13}C NMR, Cheminat et al., 1988). We purchased caftaric acid **9** (Dalton Chemical Laboratories Inc., Toronto, Canada), and both caffeic acid **8** and chlorogenic acid **11** (Sigma Aldrich, St. Louis, USA). We followed the method of other researchers (Perry, 1997), and identified compounds **12–28** by their relative retention time to the marker compounds dodeca-

2E,4E,8Z,10E/Z-tetraenoic acid isobutylamides **2**+**3,** and online photodiode array UV spectra (c.f. Bauer and Remiger, 1989), since they are not available for purchase and isolation of adequate quantities has proven costly.

We calculated a response factor from the standard curve of each of the compounds **1–11**, and used the response factor for tetraenes **2**+**3** to quantify compounds **12–28** in tetraene equivalents, which was acceptable for the purpose of mean quantitative comparisons within and between populations in the present study. For each eluted compound in experimental samples, we multiplied peak area by the appropriate response factor to give μg compound/ml extract, then divided by the original concentration of 0.5g extracted dried root/ml sample and multiplied by 10^3 to reach mg/g dry wt.

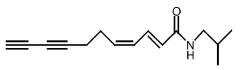
2.4. Statistical analyses

The null hypothesis that there was no significant difference in phytochemical quantity between populations (nine locations) was tested for the 26 phytochemicals singly and collectively in 115 individual plants, as follows. The effect of each individual phytochemical on variation between populations was assessed by one-way ANOVA using Systat software version 7.0 (SPSS Inc., Chicago, IL, 1999). Tukey's pairwise differences between the nine populations were also calculated for each individual phytochemical. Then, canonical discriminant analysis of the 26 phytochemicals using SAS CANDISC procedure (SAS Institute Inc., Cary, NC, 1999) assessed squared Mahalanobis distances between populations, measured the variation within and between populations, and ascertained which phytochemical(s) contributed the most towards that variation (Sneath and Sokal, 1973). The resulting matrix of Mahalanobis distances was subjected to principal coordinate analysis and single linkage clustering using NT-SYS-pc software (Rohlf (1999)) to provide several graphical representations of the relative distances between the centroids of each population.

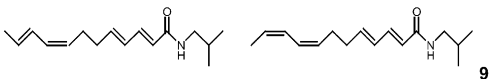
The relationship between geographical latitude and phytochemical content was determined using simple linear regression (Systat v.7) of the population means for each phytochemical (one at a time) on the latitude of the corresponding germplasm source (in Table 1). When assumptions of normality and homoscedasticity were violated, we used either the logarithmic transformation or weighting to correct for different variances and sample sizes (n) between populations. The justified weighting procedure, in this particular program, consisted of multiplication of the population means (log-transformed) by the inverse of sample variance so that means with smaller variances received more weight in the analysis.

3. Results

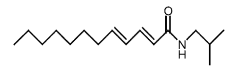
The mean levels of 28 constituents in representative samples from nine different *E. angustifolia* populations are presented in Figs. 1–5. There was significant quantitative variation (ANOVA $p<0.05$) between the mean levels of several lipophilic and hydrophilic compounds across all nine populations (Table 2).



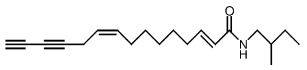
1 Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide



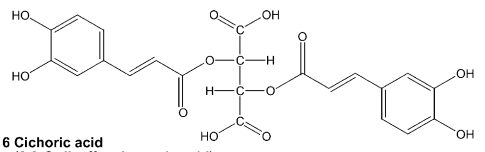
2+3 Dodeca-2E,4E, 8Z,10E/Z-tetraenoic acid isobutylamide (tetraene)



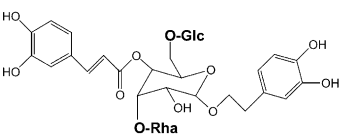
4 Dodeca-2E,4E-dienoic acid isobutylamide



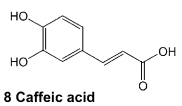
5 Pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide



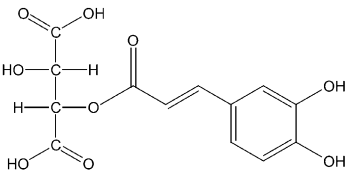
6 Cichoric acid
(2,3-O-dicaffeoyl tartaric acid)



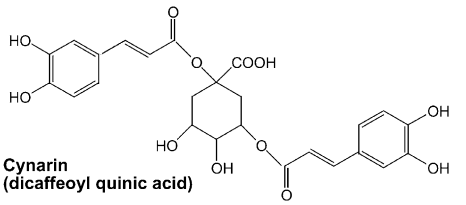
7 Echinacoside



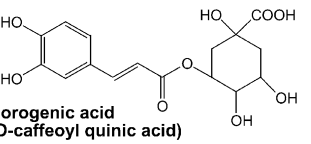
8 Caffeic acid



9 Caftaric acid
(2-O-caffeoyl tartaric acid)



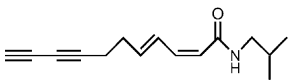
10 Cynarin
(dicaffeoyl quinic acid)



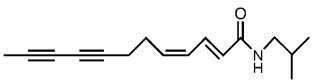
11 Chlorogenic acid
(3-O-caffeoyl quinic acid)

All mean accumulations of phenolics were significantly different by ANOVA between the nine populations, except for caffeic **8** and chlorogenic **11** acids. Among those compounds with significantly-different means (Table 2), the maximum mean accumulations of cichoric acid **6** were observed in no. 9, of echinacoside **7** in no. 3, of caftaric acid **9** in no. 6, and cynarin **10** in no. 8 (Fig. 1).

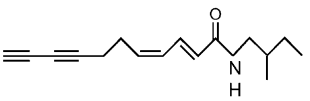
Populations nos. 8 and 9 contained the maximum mean concentration of the following alkamides: **4, 5, 13, 14, 15, 16, 17, 18, 19**, and each of those was significantly



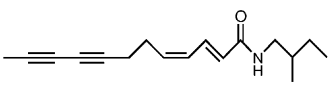
12 Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide



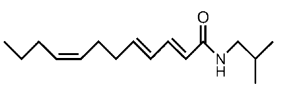
13 Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide



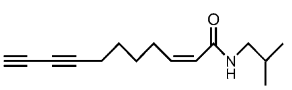
14 Undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide



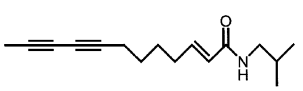
15 Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide



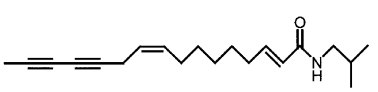
16 Dodeca-2E,4E,8Z-trienoic acid isobutylamide



17 Undeca-2Z-ene-8,10-diynoic acid isobutylamide



18 Dodeca-2E-ene-8,10-diynoic acid isobutylamide



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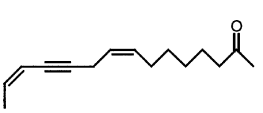
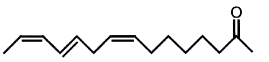


20 Pentadeca-8Z,13Z-diene-11-yn-2-one



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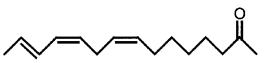
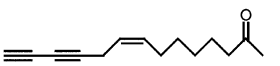
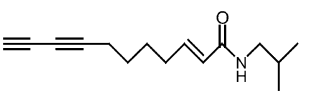


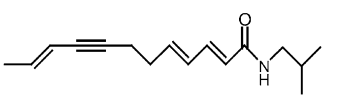
21B Pentadeca-8Z,11Z,13E-triene-2-one



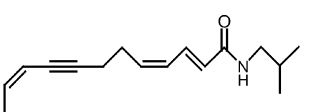
22 Tetradeca-8Z-ene-11,13-diyn-2-one



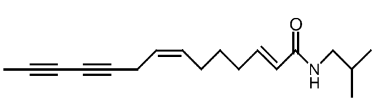
23 Undeca-2E-ene-8,10-diynoic acid isobutylamide



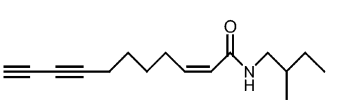
24 Dodeca-2E,4E,10E-triene-8-ynoic acid isobutylamide



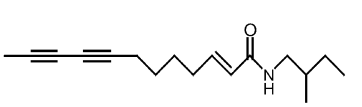
25 Dodeca-2E,4Z,10Z-triene-8-ynoic acid isobutylamide



26 Trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide



27 Undeca-2Z-ene-8,10-diynoic acid isobutylamide



28 Dodeca-2E-ene-8,10-diynoic acid isobutylamide

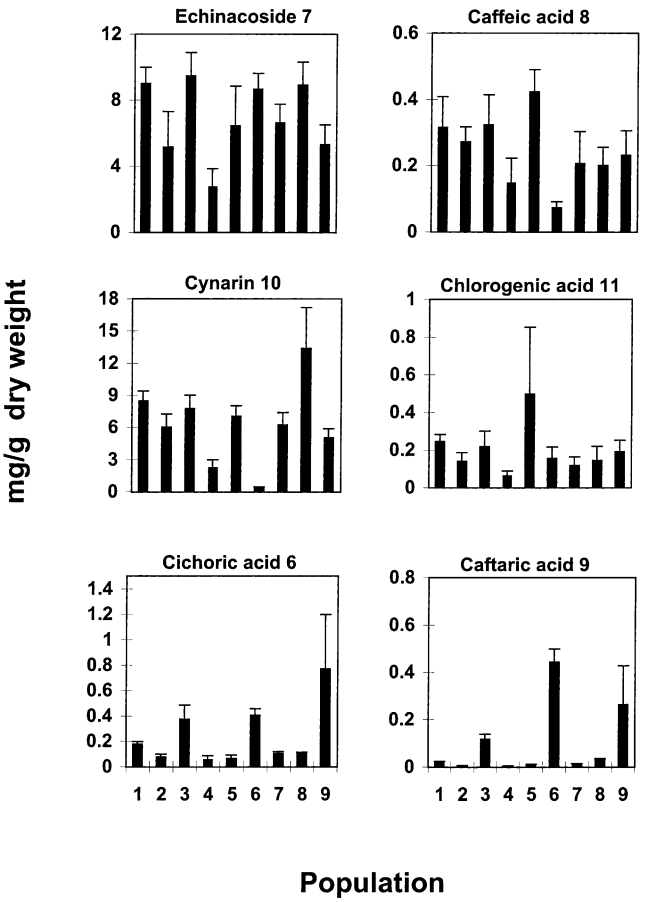


Fig. 1. Caffeic acid derivatives **6, 7, 8, 9, 10, 11** in *E. angustifolia* populations from Table 1 (mean mg/g dwt±SEM).

different across all populations according to ANOVA (Table 2). The major alkamides, **2**+**3** were found in similar quantity among all populations, except no. 7 (not significantly different). Also, ANOVA of the mean differences in ketoalkene/ynes across all populations was not statistically significant, although **20** and **21** were highest in population no. 7 and compound **22** was unique to nos. 3, 5 and 6.

3.1. Regression

A positive relationship between increasing phytochemical quantity and increasing latitude (of parental populations) was statistically significant for the phenolics **6** and **7,** and alkamide **14** ($p<0.05$, Table 3). However, there was a significant inverse

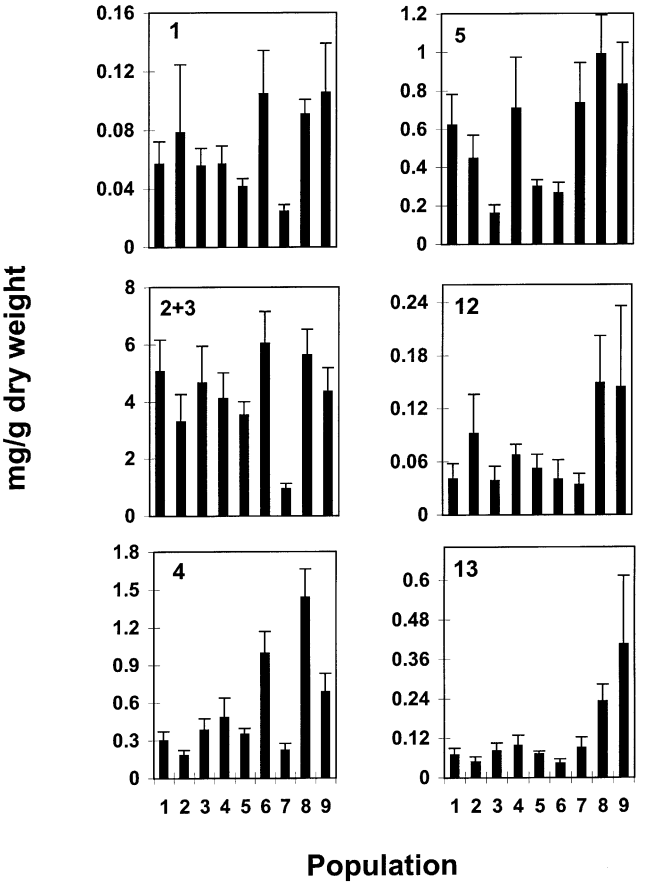


Fig. 2. Alkamides **1, 2+3, 4, 5, 12, 13** in *E. angustifolia* populations from Table 1 (mean mg/g dwt±SEM).

relationship between the quantity of phenolic **9** and alkamide **19** with increasing latitude (Table 3).

3.2. Canonical discriminant analysis

A canonical discriminant analysis is considered acceptable when at least 75% of the variation is explained by the three axes (Sneath and Sokal, 1973). Here, the first three canonical axes explain 79% of the phytochemical variation between populations. An *F*-statistic of the squared Mahalanobis distances between population centroids (Fig. 6) determined that all were significantly distant from one another at $p<0.001$ or $p<0.01$ except the following pairs of populations; nos. 1 and 2 ($p=0.7850$), nos. 1 and 5 ($p=0.1914$), nos. 2 and 4 ($p=0.4075$) and nos. 2 and 5 ($p=0.2553$). Population no. 6 was most easily distinguished from the others. The

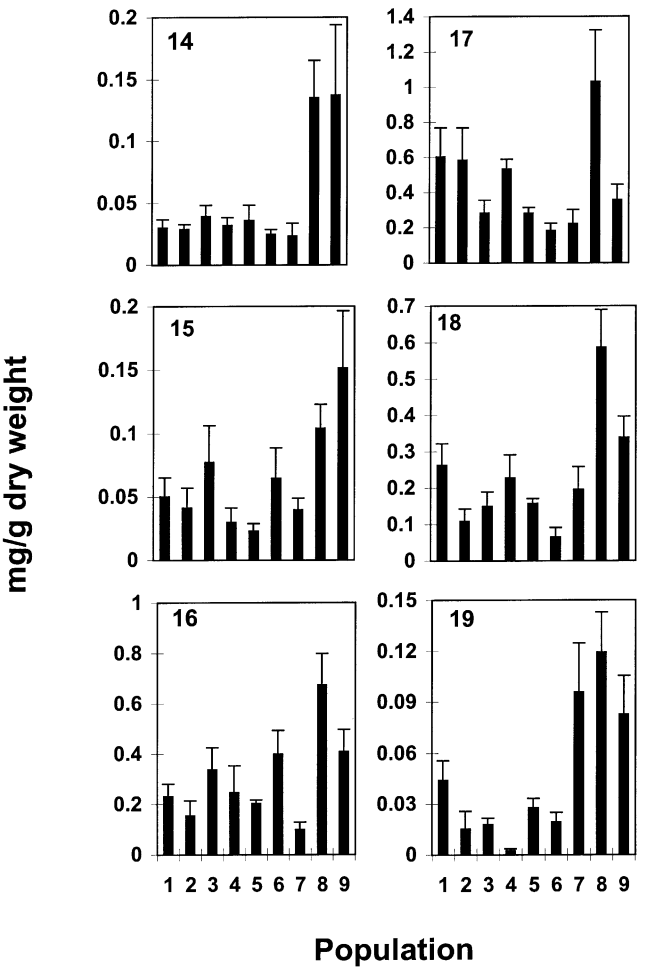


Fig. 3. Alkamides **14, 15, 16, 17, 18, 19** in *E. angustifolia* populations from Table 1 (mean mg/g dwt±SEM).

centroid of population no. 8 was also significantly segregated from other populations ($p<0.0001$), and even from its closest neighbour, no. 9 ($p=0.0026$). Fig. 7 is a three dimensional representation of the principle coordinates for the variation of each population along the first three canonical axes, which facilitates the visualization of quantitative differences between the multivariate population means.

4. Discussion

In the current study of wild *E. angustifolia* germplasm, quantitative phytochemical production varied as a function of geographical distance between nine populations.

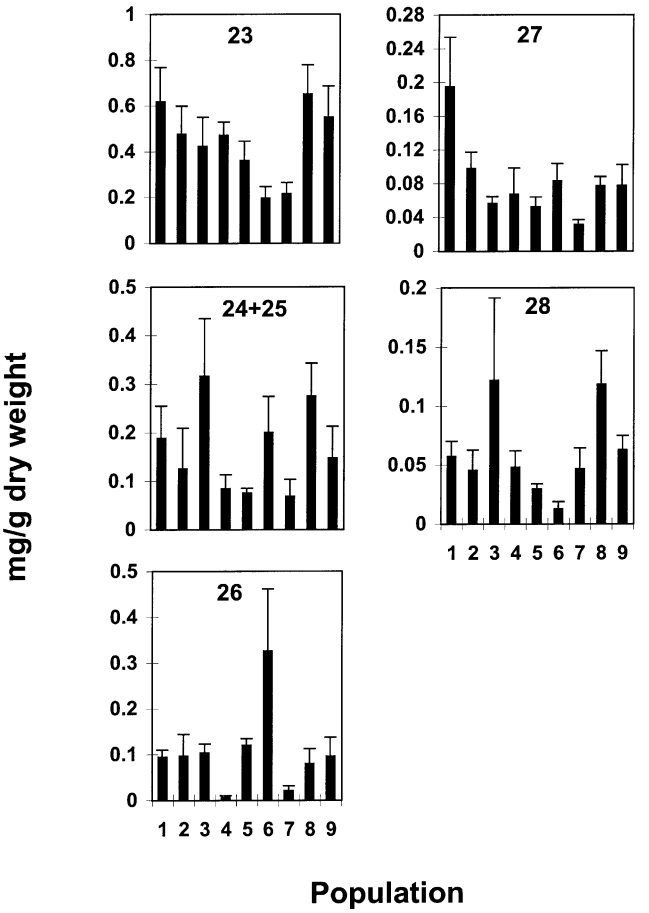


Fig. 4. Alkamides **23, 24+25, 26, 27, 28** in *E. angustifolia* populations from Table 1 (meanmg/g dwt±SEM).

Population nos. 3, 6, 7, 8 and 9 were the most significantly different according to several statistical analyses. Compared to the above, much smaller geographical distances separated populations nos. 1, 2, 4 and 5 (Table 1), and these same populations were the least distinguishable by ordination.

The most distinct chemotype was population no. 6 according to the multivariate statistical results (Figs. 6 and 7). Population no. 6 contained only trace amounts of *E. angustifolia* species markers, cynarin **10** and alkamides **17, 18, 23**, but a large amount of ketone **22** (a commercial *E. pallida* marker) and echinacoside **7** (which was reported from both species in Bauer and Wagner, 1991; Binns, 2001). Considering both its morphological characteristics (Binns et al., in press) and its phytochemical profile presented here, the chemotype of population no. 6 may be a result of hybridization/introgression between *E. pallida* (Nutt.) Nutt. and *E. angustifolia* DC.

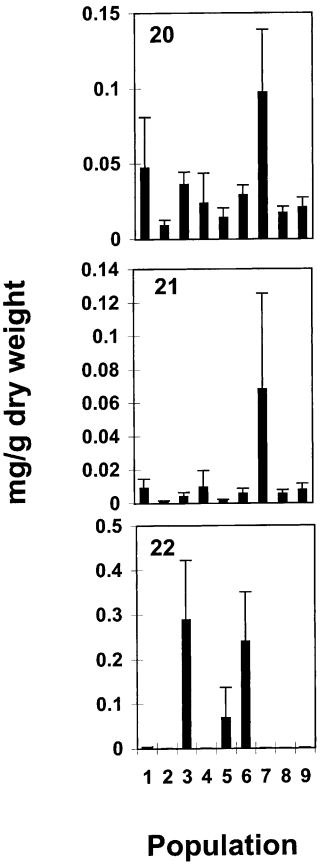


Fig. 5. Ketoalkene/ynes **20, 21, 22** in *E. angustifolia* populations from Table 1 (mean mg/g dwt±SEM).

Population no. 3 also slightly resembled *E. pallida* root profiles (Bauer et al., 1988) with an unusual level of **22**, as did no. 7 with the highest levels of **20** and **21**. However, there was no morphological or ecological indication that populations nos. 3 and 7 were possible hybrids or introgressants, and the phytochemical evidence may be due to the young age of the plants in the current study.

Plant developmental stage influences secondary metabolism; defense compounds are generally more concentrated and diverse when plants are young and more "apparent" to herbivores, but they are known to decrease with age as structural defenses are developed (Feeny, 1976). Therefore, as roots in the present study were still young, and they yielded a greater variety of chemical defenses than what is reported for older commercial *E. angustifolia* roots (typically >2 years) (Bauer and Wagner, 1991), this might explain why populations nos. 3 and 7 had intermediate profiles.

It is useful to determine which constituents may be responsible for most of the differences between potential chemotypes in a multivariate analysis. In the present study, phytochemical variables that contributed the most to the squared Mahalanobis

Table 2
Significantly different mean phytochemical contents [mg/g±SEM] in *Echinacea angustifolia* populations (two-tailed ANOVA $p<0.05$) from south to north by latitude (Table 1)[a],[b]

Phenolics					Alkamides								
Pop	**cich** [c]	**ech** [c]	**caft** [c*]	**cyn***	**4** [c*]	**5** [c*]	**13**	**14** [c*]	**15**	**16***	**17**	**18***	**19** [c*]
1	0.18[a]	9.00[abc]	0.02[a]	8.47[ac]	0.3[a]	0.62[abc]	0.07[ac]	0.03[a]	0.05[abc]	0.23[a]	0.60[ab]	0.26[ac]	0.04[ab]
	[0.02]	[0.98]	[0.01]	[0.92]	[0.07]	[0.16]	[0.02]	[0.01]	[0.01]	[0.05]	[0.16]	[0.06]	[0.01]
2	0.08[a]	5.14[abc]	0.003[a]	6.00[abc]	0.18[ae]	0.45[abc]	0.05[ac]	0.03[ac]	0.04[abc]	0.16[a]	0.58[ab]	0.11[ab]	0.02[ac]
	[0.02]	[2.15]	[0.002]	[1.23]	[0.04]	[0.12]	[0.02]	[0.00]	[0.02]	[0.06]	[0.18]	[0.03]	[0.01]
3	0.37[ab]	9.47[b]	0.12	7.78[ac]	0.38[ac]	0.16[a]	0.08[ab]	0.04[abc]	0.08[abc]	0.34[ab]	0.28[a]	0.15[ab]	0.02[ac]
	[0.11]	[1.42]	[0.02]	[1.20]	[0.09]	[0.05]	[0.02]	[0.01]	[0.03]	[0.09]	[0.07]	[0.04]	[0.00]
4	0.06[a]	2.79[c]	0.003[a]	2.33[ab]	0.48[ab]	0.71[ab]	0.1[abc]	0.03[ac]	0.03[abc]	0.25[ab]	0.53[ab]	0.23[abc]	0.002[a]
	[0.03]	[1.04]	[0.002]	[0.67]	[0.15]	[0.26]	[0.03]	[0.01]	[0.01]	[0.11]	[0.06]	[0.06]	[0.00]
5	0.06[ab]	6.43[c]	0.01[a]	7.03[ae]	0.35[a]	0.30[ab]	0.07[ab]	0.04[abc]	0.02[b]	0.20[c]	0.28[a]	0.16[ab]	0.03[ab]
	[0.03]	[2.43]	[0.00]	[0.98]	[0.05]	[0.03]	[0.01]	[0.01]	[0.005]	[0.01]	[0.03]	[0.01]	[0.01]
6	0.41[ab]	8.67[abc]	0.44	0.37[b]	1.00[bcd]	0.27[ac]	0.04[bc]	0.02[b]	0.06[abc]	0.40[ab]	0.18[ab]	0.06[b]	0.02[ad]
	[0.05]	[0.96]	[0.06]	[0.08]	[0.17]	[0.06]	[0.01]	[0.00]	[0.02]	[0.10]	[0.04]	[0.03]	[0.01]
7	0.10[ab]	6.61[abc]	0.01[a]	6.22[abc]	0.22[a]	0.74[ac]	0.09[abc]	0.02[abc]	0.04[abc]	0.10[a]	0.22[a]	0.20[abc]	0.10[b]
	[0.01]	[1.15]	[0.005]	[1.16]	[0.05]	[0.21]	[0.03]	[0.01]	[0.01]	[0.03]	[0.08]	[0.06]	[0.03]
8	0.10[ab]	8.9[abc]	0.03[a]	13.36[c]	1.44[c]	0.99[b]	0.23[abc]	0.14	0.10[abc]	0.67[b]	1.03[b]	0.59[c]	0.12[bd]
	[0.01]	[1.39]	[0.01]	3.81	[0.22]	[0.20]	[0.05]	[0.03]	[0.02]	[0.13]	[0.29]	[0.10]	[0.02]
9	0.77[b]	5.29[abc]	0.26[a]	5.03[a]	0.69[ade]	0.83[bc]	0.41[c]	0.14[c]	0.15[c]	0.41[ab]	0.36[ab]	0.34[ac]	0.08[bc]
	[0.43]	[1.21]	[0.16]	[0.83]	[0.14]	[0.22]	[0.21]	[0.06]	[0.04]	[0.09]	[0.09]	[0.06]	[0.02]

[a] Results followed by the same bold, superscript letter in the same column were not significantly different by Tukey's pairwise comparison.
[b] cich, cichoric acid **6**; ech, echinacoside **7**; caft, caftaric acid **9**; cyn, cynarin **10**; Pop, population.
[c] Compounds that contributed most significantly to segregation by canonical discriminant analysis.
* Significantly different by two-tailed ANOVA $p<0.001$.

Table 3
Simple or weighted regression of the mean phytochemical quantities per population versus latitude of germplasm source for those compounds found to be significantly different by ANOVA (Table 2)

Compound	r^2 (slope)	*p*-value	Weighting/transformation
2+3	0	0.882	none
4	0.276	0.146	LOGmean[a]
5	0.066	0.0504	LOGmean
6	0.038	0.036	None
7	0.528	0.027	None
8	0.017	0.742	None
9	0.509	0.031	LOGmean×LOGVAR^{-1}[b]
10	0.094	0.422	None
13	(-)[c]	(-)	(-)
14	0.477	0.039	None
15	0.375	0.08	None
16	(-)	(-)	(-)
17	0.141	0.319	LOGmean
18	0.005	0.858	LOGmean
19	0.695	0.005	LOGmean×LOGVAR^{-1}[b]
27	0.107	0.391	LOGmean

[a] LOGmean=mean transformed by the logarithm base 10.
[b] LOGVAR^{-1}=inverse variance of the LOGmean.
[c] (-)=no results where normality assumption was violated in all trials.

distances between populations in the canonical discriminant analysis were alkamides **4**∗, **5**∗∗, **14**∗, **19**∗, and cichoric acid **6**∗∗, echinacoside **7**∗∗, and caftaric acid **9**∗ (*F*-statistic probabilities $^{*}p<0.0001$, $^{**}p<0.05$). Both alkamide **18**, and echinacoside **7** are currently used for commercial standardization of *E. angustifolia*, so their significant variation as chemotype determinants is economically significant. Interestingly, the root concentrations of five of those same compounds, **6**, **7**, **9**, **14**, and **19** varied significantly in relation to latitudinal variation (Table 3). Populations nos. 8 and 9 are potentially superior chemotypes with significantly increased concentrations of compounds **6, 7** and **14** at higher latitudes and including the overall increasing latitudinal trends of most alkamides and phenolics (Figs. 1–5). As well, we caution that the significant inverse relationship of **9** and **19** with increasing latitude may be an artifact of the regression, because there were no matching trends in the mean population data (Figs. 1 and 4).

Latitudinal and quantitative variation of cichoric acid **6** and echinacoside **7** promises to be useful. Echinacoside **7** has been reported from all varieties and species of *Echinacea*, except *E. purpurea* (Bauer and Wagner, 1991; Binns, 2001). Here, we indicated that seed source within a single species (and the effects of latitude on that location) influences the overall levels of echinacoside **7** in plant materials used for herbal product manufacturing. It is therefore misleading to standardize *Echinacea* products by % echinacoside concentration, which is often listed on herbal product labels.

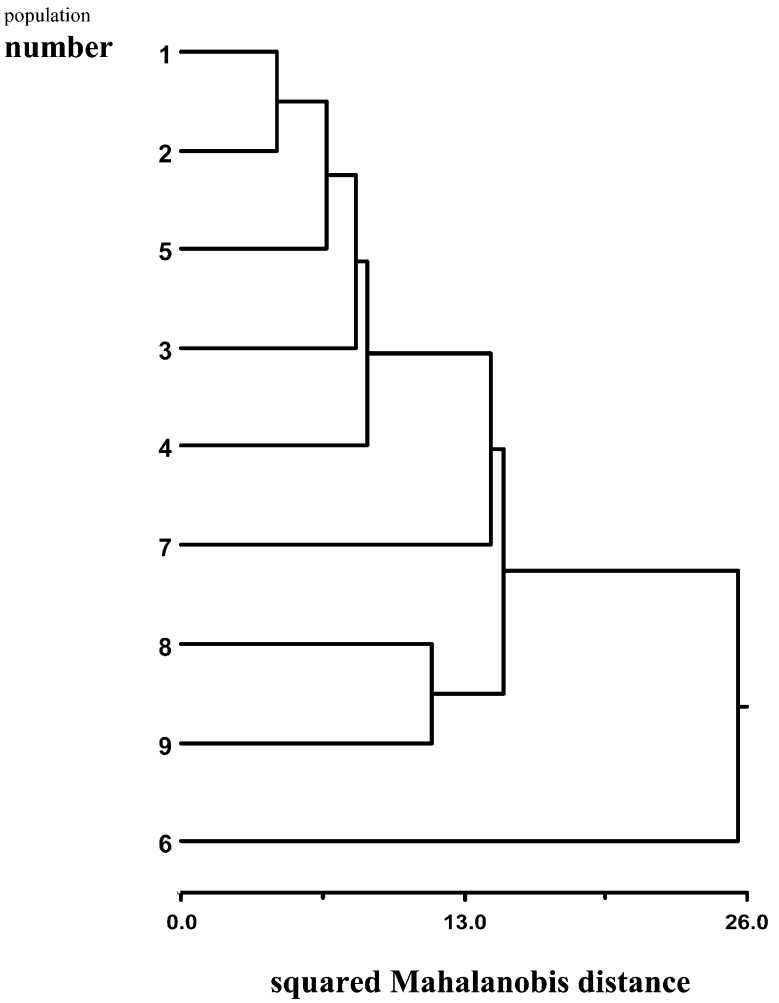


Fig. 6. Single linkage phenogram of the squared Mahalanobis distances between centroids of *E. angustifolia* populations numbered as in Table 1.

Immunostimulant activity was reported for cichoric acid **6**, although it was not measurable for caftaric acid **9** in tests of phagocytosis induction (Bauer, 1998). Cichoric acid **6** is the most abundant constituent in the flowers of all *Echinacea* species (Bauer, 1998; Binns, 2001) with the highest quantity in young *E. purprea* flower buds (Letchamo et al., 1998). In addition, *E. purpurea* roots contain **6** in large amounts (Bauer and Wagner, 1991; Bauer, 1998; Binns, 2001) compared to much smaller trace amounts in *E. pallida* and *E. angustifolia* roots where the major compounds were echinacoside **7** and cynarin **10** (see Results; Binns, 2001). Therefore, demonstrated latitudinal influence on cichoric acid accumulation in the roots of wild *E. angustifolia* accessions, while not as quantitatively significant for this particular

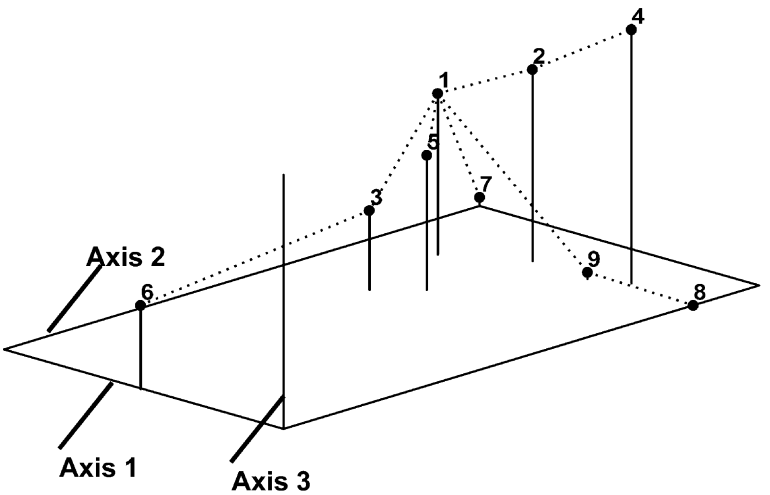


Fig. 7. Principal coordinates of nine *E. angustifolia* populations representing the variation on the first three canonical axes superimposed by a minimum spanning tree of the squared Mahalanobis distances (dashed lines).

species, has implications for potentially high-yielding *Echinacea* cultivar development, especially if the same latitudinal variation can be determined among cultivated seed sources for *E. purpurea*.

Finally, the significant quantitative variation in *E. angustifolia* populations (see Table 2) with respect to caftaric acid **9** and cynarin **10** may impact the *Echinacea* products derived from different seed sources, once the pharmacological activities of these pure compounds are investigated.

Increased phytochemical production in northern plant populations has been reported for the phenolic DIMBOA in corn (*Zea mays* L.; Levin and York (1978)). This research measured higher levels of DIMBOA from temperate germplasm sources compared to the tropical landraces. High levels of DIMBOA were associated with increased insect resistance in temperate varieties of corn (Xie (1991)). Similarly, in the present study, ecological gradients that exist across a latitudinal cline helped to create germplasm heterogeneity in wild *E. angustifolia* populations which affected the phytochemical accumulations of both phenolics and alkamides in a controlled environment. These phenomena are best explained by stable or impermanent genetic polymorphisms among populations of the widespread species *E. angustifolia* in response to selection pressures, such as habitat, competition and herbivory.

Those chemotypes which were most distinct in the current study may be identified by their US county names: no. 3=Comanche (OK); no. 6=Cowley (KS); no. 7=Pottawatomie (KS); no. 8=Richardson (NE); and no. 9=Clay (IA) (Table 1). This information is particularly useful when combined with traditional breeding studies for desirable morphology and disease resistance to improve cultivation of *E. angustifolia* and thereby help to insure germplasm conservation. To provide tools for the improvement of modern phytomedicines, future investigations should target the effect of

genetically-variant wild and cultivated sources of all *Echinacea* species and varieties on biologically-active phytochemical production.

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