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Seasonal Variation in Resource Allocation of Extractable Compounds in *Asclepias, Chrysothamnus* and *Grindelia*

ROBERT P. ADAMS and S. C. PRICE*

Biology Department, Box 204, Baylor University, Waco, TX 76798, U.S.A.;
*The Standard Oil Company, 4440 Warrensville Center Rd, Cleveland, OH 44128, U.S.A.

Key Word Index—*Asclepias*; *Chrysothamnus*; *Grindelia*; seasonal variation; resource allocation.

Abstract—The yields of whole plant hexane and methanol soluble extractables from *Asclepias speciosa, Chrysothamnus nauseosus* subsp. *consimilis* and *Grindelia squarrosa* were examined throughout the growing season. The yields of non-polar extractables remained relatively constant during the growing season with increases in late summer or early fall according to the species. Each of the three taxa showed a similar pattern with the methanol soluble fraction reaching a maximum in June. However, a second maximum was observed in *Asclepias* and *Grindelia* in August. Protein content was examined in *Asclepias* and *Grindelia* and was found to decline throughout the growing season. Individual components of the hexane extract of *Asclepias* were examined and several components were found to vary seasonally. Principal components analysis of the non-polar components revealed that the components with high intercorrelations were members of five chemical biosynthetic classes: alkanes, fatty acids, pentacyclic triterpenoids and tetracyclic sterols. Canonical variate analysis grouped the samples by date of sample from May to September–October.

Introduction

Resource allocation information is becoming increasingly important for our understanding of plant defences and adaptation [1, 2]. Annual and perennial species appear to show differences in allocation patterns [3] with annuals putting more resources into reproduction while perennials apparently allocate storage products to the roots [4–10]. Some of the differences between annuals and perennials may be related to their 'apparency' (=susceptibility to discovery [11]) particularly in regards to polyphenol production [3]. It is clear that there are many different lines of evolution and adaptation for which different taxa have evolved both life-time and seasonal allocation patterns to compete in their environment [12, 13].

Seasonal changes in resource allocation have shown considerable variation, particularly in storage products as exemplified by the classical study of Mooney and Billings [14]. Non-structural carbohydrates in *Carex lacustris* were found to shift from 16% in midsummer to 45% in late October [15]. Gallagher *et al.* [16] reported that 30% of the underground storage reserves in *Spartina alterniflora* were stored by mid-October and consumed by June of the following year. Dement and Mooney [17] found significant season changes in net photosynthesis but only small changes in total non-structural carbohydrates in the leaves of the perennial evergreen, mediterranean shrub, Toyon (*Heteromeles arbutifolia*).

Tannins have been found to vary seasonally in sugar maple and yellow birch [18], blackbrush [19], bracken fern [20], oaks [11, 21], *Heteromeles arbutifolia* [17] and birch [22] to name but a few of the many studies. Seasonal variation in protein content is well known [12, 23–27].

The literature is replete with studies showing seasonal variation in terpenoids, falvonoids, lipids, alkaloids, cyanogenic glycosides, saponins, etc. [1, 2, 12, 28–30] and will not be generally reviewed here. In one of the few studies involving total non-structural carbohydrate (TNC), crude protein (CP) and protection chemicals (terpenoids exterior to the leaves), Kelsey *et al.* [12] found a complex relationship among these three variables in the leaves of *Artemisia tridentata* subsp. *vaseyana*. Crude protein appeared to be negatively correlated with TNC and crude terpenoids (CT). This study

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[12] also showed that all the sesquiterpene lactones and some of the monoterpenoids appear to be localized in external glandular trichomes (see also ref. [31]). For example, washing the leaves for 5 min with chloroform removed 79–87% of the crude terpenoids, compared to the standard procedure of using a 16 h Soxhlet extraction with chloroform [12]. The same phenomenon has been observed in some sunflower species (Adams, R. P., unpublished).

A number of different classes of components have been analyzed for changes of allocation patterns under various stress conditions. Glucosinolates in mustards were found to decrease under low sulphur nutrition [32]. Alkaloids increased under moisture stress in *Phalaris aquatica* [33]. Phenolics have been examined in clover and isoflavones were found to double in concentration at low nitrogen supply levels [34]. Chlorogenic acid concentration was found to increase in *Helianthus annuus* with age and under mineral stress [35] and with nitrogen deficiency, drought and exposure to ultraviolet light [36]. Lehman and Rice [35] proposed that the increase in chlorogenic acid with age was important to increase the alleopathic effects. Kelley *et al.* [37] found an increase in both rubber and resin in drought stressed guayule plants. A more thorough examination of resource allocation of guayule [38], revealed that under moisture stress, levulins increased along with natural rubber in guayule; however, other carbohydrates remained fairly constant. Stress (drought) appeared to change the monoterpenes levels in Douglas fir and this resulted in differential susceptibility to budworm growth in Douglas fir [39]. The allocation of resources to monoterpenes in *Satureja douglasii* was little affected by moisture stress [40], nor was the leaf resin yield (mg/g leaf tissue) much affected by wet and dry treatments in *Hymenaea* [41]. This is in contrast to the resource allocation changes in the monoterpene composition of Douglas fir [39].

In an excellent study of seasonal variation of the cardenolides of *Asclepias eriocarpa*, cardenolide yields from the latices were found to reach a maximum in June, July and August, whereas they reached a maximum in the leaves in July, and declined from August through November [42]. Variations in phytosterols with water stress have been examined in *Digitalis lanata* [43]. Sitosterol, stigmasterol and cholesterol were found to increase at the onset of water stress, but decrease rapidly when the leaves reached the stage of irreversible injury [43]. Other less polar sterols were found to decrease until the plants lost their viability, and then increased in concentration.

The aforementioned changes in resource allocation not only impact the species defences but are important considerations in species that have been investigated for use in the production of industrial chemicals from biomass. Research on hydrocarbon crops has centered on latex-producing species such as *Asclepias* [44–46] and *Euphorbia* [47, 48]. However, the highest hydrocarbon yielding species are in the Asteraceae, with *Chrysothamnus* and *Grindelia* species consistently with the largest yields [49–51]. The purposes of this study were to investigate the seasonal variation in the hexane and methanol extractables from *Asclepias speciosa, Chrysothamnus nausesous* subsp. *consimilis* and *Grindelia squarrosa*, and examine the variations among individual components from the non-polar (hexane) extracts of *Asclepias speciosa* throughout a growing season.

Results and Discussion

The yields of hexane soluble components (referred as hexane extract hereafter) from the herbaceous perennial, *Asclepias speciosa*, showed no significant differences from the initial sample in June until the plants were becoming senescent in October (Fig. 1). The hexane extract is composed of alkanes, fatty acids, terpenoids and other non-polar components [45]. Part of the increase in the yield of the hexane extract in October may be due to the catabolism of the carbohydrates such that the percentage of dry matter is lowered and the hexane extract would then become a greater percent of the total dry weight.

In contrast, the yields of methanol soluble components (referred to as methanol extract hereafter) was greater than 24% initially, and declined through the season with an increase during seed setting in August (Fig. 1). The methanol extract contains simple sugars, free amino acids, and metabolic acids in addition to chlorophyll and other polar components [45].

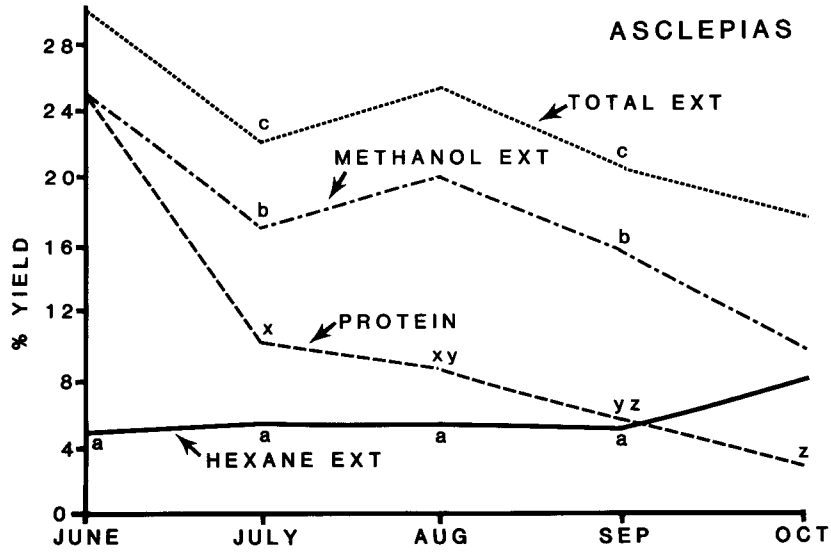

FIG. 1. SEASONAL VARIATION IN THE TOTAL EXTRACT, METHANOL AND HEXANE EXTRACTABLES, AND CRUDE PROTEIN FOR THE HERBACEOUS PERENNIAL, *ASCLEPIAS SPECIOSA*. For this and succeeding figures, any two or more data points with the same letter (e.g. c in July and September for total extract) are *not* significantly different by the Student–Newman–Keuls multiple range test at $P=0.05$. Percent yields in this and succeeding figures are on an oven dry (48 h, 100°) basis.

These metabolically active components show considerable turnover in these compounds during the growing season. The total yield reflects, chiefly, the methanol extract (Fig. 1). Crude protein yields followed the typical pattern observed in most species with a rapid decline as the young plants begin to grow (June to July) and then a more gradual decline throughout the season (Fig. 1).

The seasonal patterns (Fig. 2) for the herbaceous annual, *Grindelia squarrosa*, were very similar to *Asclepias* in the total and methanol extracts, and the crude protein (cf. Figs 1 and 2). The hexane extract showed a different pattern with a small, significant increase during the summer and a small decline in the fall (Fig. 2). The increase in the hexane extract during the summer is most likely due to the production of the external "gum" on the gumweed, *Grindelia*. This external gum is composed principally of diterpene acids of which grindelic acid is often the principal component in the genus [52–56]. As gumweed flowers (July in Salt Lake City), the flower heads become very sticky. The external diterpenes have been shown to be feeding deterrents for insects [54], so it seems reasonable that the species would allocate considerable carbon resources to the protection of the flowers since the next generation must come from seed. This carbon resource is apparently not retrievable for the plant. Interestingly, some of the largest yields (8–12% dry wt) of hexane extractives from terrestrial plants have been reported from *Grindelia* species [49, 51, 57].

In contrast with *Asclepias* and *Grindelia*, the woody perennial species, *Chrysothamnus nausesous* subsp. *consimilus*, exhibited a rather distinctive pattern with the total and methanol extracts showing a non-significant peak in June, but very stable patterns throughout the growing season (Fig. 3). The hexane extract showed a small, significant increase during the summer, with a return to springtime levels in October (Fig. 3). The hexane extract varied from about 4 to 6.5%.

Overall, the comparisons between a herbaceous perennial, a herbaceous annual and a woody perennial may be indicative of these modes of adaptation. Certainly the herbaceous species (*Asclepias* and *Grindelia*) showed a common pattern in their methanol soluble

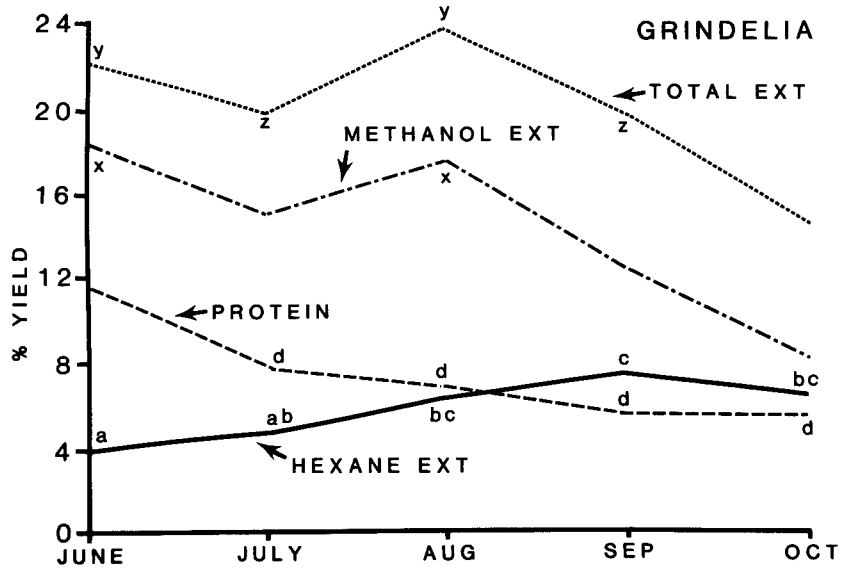

FIG. 2. SEASONAL VARIATION IN THE TOTAL EXTRACTIVES, METHANOL AND HEXANE EXTRACTIVES, AND CRUDE PROTEIN FOR THE HERBACEOUS ANNUAL, *GRINDELIA SQUARROSA*. The patterns for the extractives yields and protein are quite similar to that of *Asclepias* (Fig. 1). There is, however, a significant increase in the hexane extract throughout the summer, reaching a maximum in September.

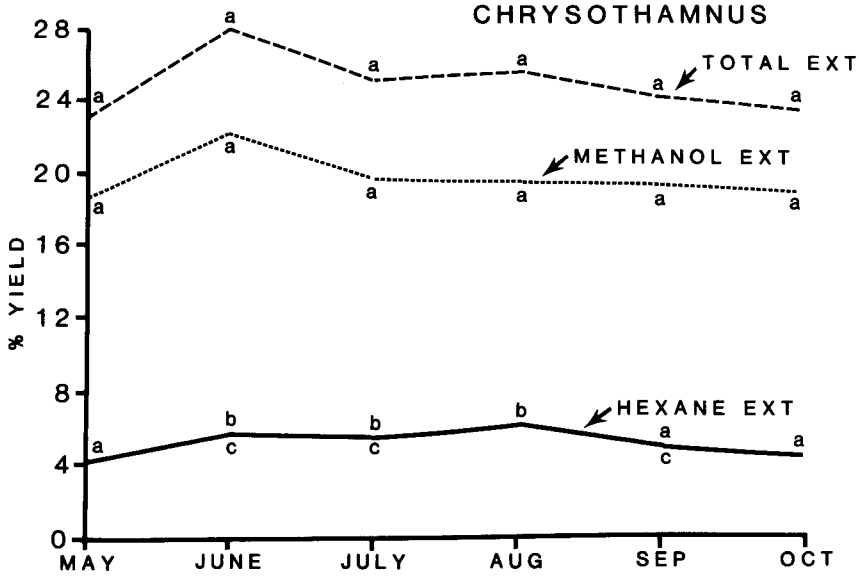

FIG. 3. SEASONAL VARIATION IN THE TOTAL EXTRACTIVES, METHANOL AND HEXANE EXTRACTIVES, AND CRUDE PROTEIN FOR THE WOODY PERENNIAL, *CHRYSOTHAMNUS NAUSESOUS* SUBSP. *CONSIMILIS*. Total and methanol extractives rose from May to June with the onset of leaf production and then declined as seen with *Asclepias* and *Grindelia*. The hexane extract showed a small, but significant, increase in June, remained relatively constant throughout the summer, and declined slightly in the fall.

carbohydrates that differed from the woody perennial (*Chrysothamnus*). The yields of non-polar extractables (hexane soluble) appear to be more stable during the growing season and their allocation appears to be expendable in the cases of *Asclepias* and *Grindelia* versus a life-time allocation pattern in *Chrysothamnus* (see refs [12, 13, 31] for other examples of life-time versus seasonal resource allocation patterns).

In the following year of this study, additional research was directed to monitor the growth and maturation of *Asclepias* and relate these changes to the variation within the composition of the hexane extract. Figure 4 shows the growth pattern for *Asclepias speciosa* grown under natural climatic and edaphic conditions in Layton, UT (see ref. [45] for test plot specifications). The maximum height was attained by July 22, at full flowering (i.e. all the plants were in flower at this time). Twenty plants were cut off to 6 cm height on July 2 and allowed to regrow under natural climatic and edaphic conditions. Ten of these were harvested to obtain the August 6, regrowth sample set (R1) and the other ten were harvested on August 26 to obtain the second regrowth sample set (R2). By August 26, the regrowth plants had reached a height of only 43 cm and approximately half of the leaves had defoliated due to water stress. The regrowth plants never produced flower buds even though they reached the height of the June 10 plants which did produce buds.

Sample sets from the aforementioned growth and regrowth harvest were used to investigate variation within the hexane extracts of *Asclepias*. Individual components previously identified [45] were quantified for statistical analyses. Analysis of variance for 34 compounds (greater than 0.5% concentration in at least one sample set) using eight sampling periods throughout the growing season resulted in six non-significant ($P=0.05$), five significant, and 23 highly significant compounds. Student–Newman–Keuls multiple range tests of significance for five compounds, typical of different chemical classes, is presented in Table 1. When viewed in conjunction with the graphs of the compounds (Fig. 5), the changes in linolenic acid are dramatic with a large decrease during the early bud stage and an increase during full flowering. Squalene and

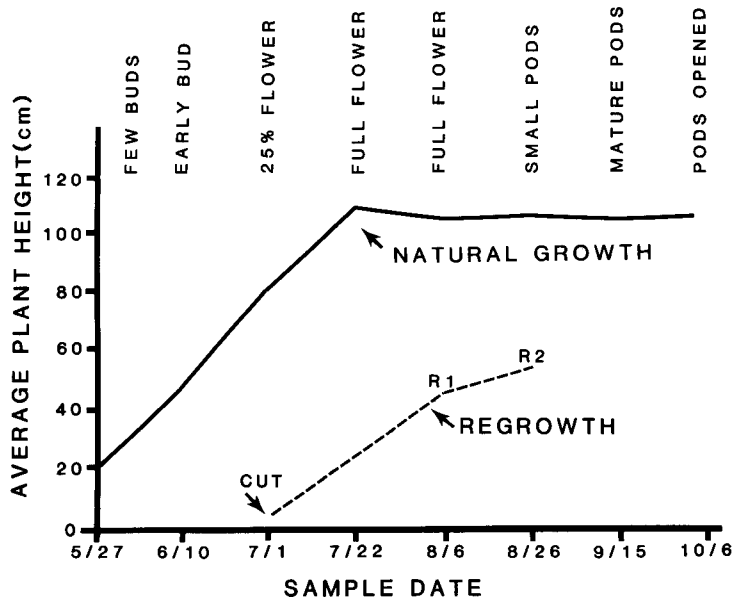

FIG. 4. GROWTH AND MATURATION OF *ASCLEPIAS SPECIOSA* UNDER NATURAL CLIMATIC AND EDAPHIC CONDITIONS IN A TEST PLOT IN LAYTON, UT. The solid line represents the average height of plants that were never cut back. The dotted line is the regrowth of plants which were cut to 6 cm height on July 2 and allowed to regrow under natural, dryland conditions. These plants were extremely stressed due to the very dry field conditions and had lost approximately one-half their leaves by Aug 26.

TABLE 1. STUDENT-NEWMAN-KEULS MULTIPLE RANGE TESTS OF SEASONAL VARIATION IN SELECTED COMPONENTS FROM THE HEXANE EXTRACT

Linolenic acid			F= 13.65, P= 0.0000002					
Date	5/27	8/6	8/26	7/22	7/1	6/10	10/6	9/15
Avg %	8.81	6.78	4.08	3.88	3.30	2.96	0.91	0.17
Squalene			F= 21.74, P= 0.00000001					
Date	5/27	6/10	7/22	8/6	7/1	8/26	10/6	9/15
Avg %	1.46	0.93	0.91	0.58	0.46	0.21	0.13	0.08
Sitosterol			F= 16.07, P= 0.00000008					
Date	5/27	6/10	7/22	8/6	7/1	8/26	9/15	10/6
Avg %	4.41	3.08	2.63	1.80	1.76	1.71	1.64	1.28
β-Amyrin			F= 2.24, P= 0.039					
Date	9/15	6/10	5/27	7/22	8/6	7/1	8/26	10/6
Avg %	2.87	2.77	2.67	2.52	2.32	2.11	1.87	1.86
β-Amyrin butyrate			F= 9.64, P= 0.000002					
Date	9/15	10/6	7/22	8/26	6/10	8/6	7/1	5/27
Avg %	1.53	1.28	0.62	0.60	0.57	0.55	0.53	0.51

Any means connected by a common line are not significantly different at P=0.05.

sitosterol show similar patterns with declines in the early growing season and a minor increase in full flowering and a steady decline into fruit maturation (Table 1 and Fig. 5). Beta-amyrin had no significant differences (from the SNK tests, Table 1), but had a barely significant F ratio from ANOVA (P=0.039). Beta-amyrin butyrate revealed a doubling in concentration in the fall as the pods matured which is similar to the pattern for beta-amyrin (Fig. 5). Biosynthesis, metabolism and translocation of beta-amyrin has been demonstrated in *Sorghum* [58], so some variation was not unexpected.

The covariation among the non-polar components was examined by principal components analysis of the pooled seasonal samples of *Asclepias* (eight sets of 10 individuals each). The components identified in the non-polar hexane extract [55] can be divided into four major chemical classes: alkanes, fatty acids, pentacyclic triterpenoids (including the esters of amyrin) and tetracyclic sterols. Principal component analysis revealed that the major chemical classes tended to cluster together (Fig. 6). The three tetracyclic sterols are sitosterol, stigmasterol, and campesterol, and they form a

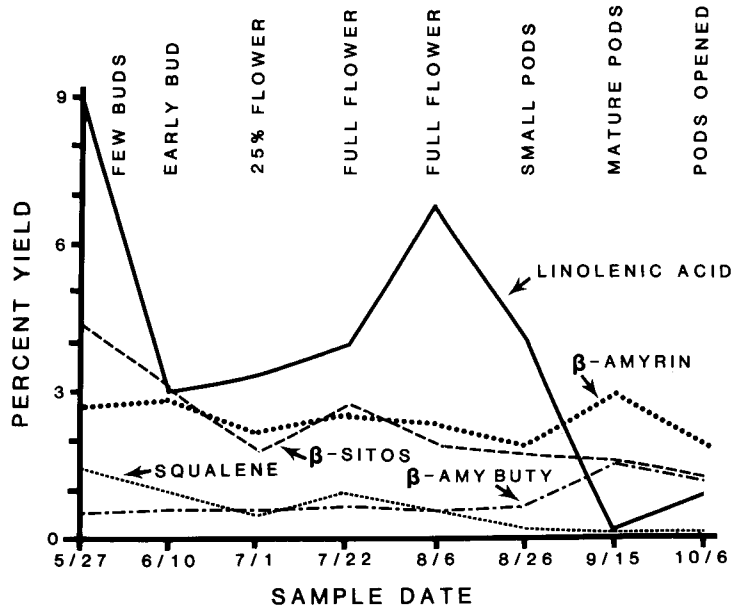

FIG. 5. SEASONAL VARIATION IN LINOLENIC ACID, BETA-AMYRIN, SITOSTEROL (β-SITOS), BETA-AMYRIN BUTYRATE (β-AMY BUTY) AND SQUALENE IN *ASCLEPIAS SPECIOSA*. Note the large decrease in linolenic acid as the plants begin to bud and a second peak associated with full flower. For statistical significance, see Table 1 and text for discussion.

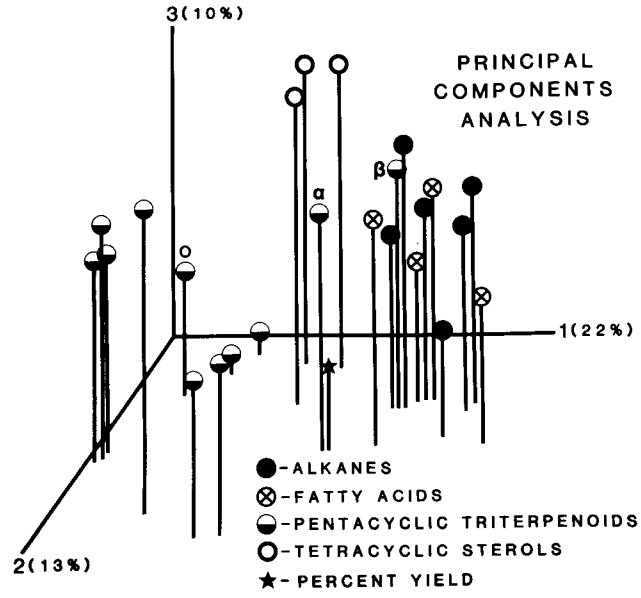

FIG. 6. PRINCIPAL COMPONENTS ANALYSIS (PCA) OF COMPONENTS FROM THE HEXANE EXTRACT OF *ASCLEPIAS SPECIOSA* FROM THE POOLED SAMPLE OF PLANTS COLLECTED AND ANALYZED THROUGHOUT THE GROWING SEASON. The two pentacyclic triterpenoids labeled alpha and beta are, respectively, α- and β-amyrin; the rest of the pentacyclic triterpenoids are esters of alpha- and beta-amyrin, except one component labeled 'o' (oleanoic acid). The covariation of components results in clusters of biosynthetically related compounds. The percentage value in parentheses on each axis is the amount of variance removed by that axis in the PCA.

distinct cluster (Fig. 6). The two pentacyclic triterpene alcohols (alpha- and beta-amyrin) appear to be more correlated with the alkanes and fatty acids than their corresponding amyrin esters (-acetate, -butyrate, -hexanoate, -palmitate). The alkanes and fatty acids cluster together (Fig. 6) as one would expect due to their common biosynthetic pathways [59, 60]. The percent yield of the total hexane extract was included in the analysis and it clusters rather intermediate. It is interesting to note that a few minor unidentified components were included in the PCA (not shown in Fig. 6) and some of these clustered very closely with the sterols and whereas others clustered very closely with the pentacyclic triterpene acid (oleanoic acid) which suggests that given the proper controls, experiments might be conducted to aid in the elucidation of biosynthetic pathways using principal component analysis.

The overall relationships among the seasonal samples and those cut and allowed to regrow were examined by canonical variate analysis (eight sampling dates of 10 plants each, two sampling dates of 10 plants which had regrown after having been cut off on July 2). The major trend is an ordination of the samples from spring to fall (Fig. 7). Forty-three percent of the variance among the sample sets (axis 1, Fig. 7) appears to be associated with this trend. The July 22 sample appears a little anomalous in that it shows affinity to the earlier samples (May). However, reexamination of Figure 4 shows that some of the components (sitosterol, squalene, and linolenic acid) have yields more like the May and June samples than the July 1 sample (of course this is exactly what the canonical variate analysis shows in Fig. 7). As the plants begin to develop the fruit pods (Aug 26 sample) and continuing into the fall, the sample sets cluster very tightly (see Aug 26, Sep 15, Oct 6 in Fig. 7).

The hexane soluble components from the regrowth plants (all were cut to 6 cm on July 2) were more similar (Fig. 7) to the samples harvested on the same date (R1=Aug 6; R2=Aug 26) than they were to plants with similar height and flowering characteristics (i.e.

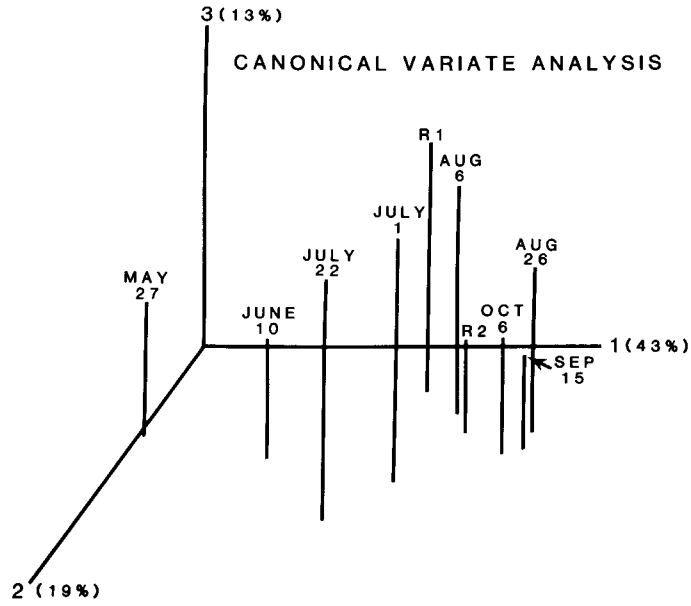

FIG. 7. CANONICAL VARIATE ANALYSIS (CVA) USING EACH OF THE SEASONAL SAMPLE SETS (10 PLANTS EACH) AND REGROWTH (R1, CUT JULY 2, HARVESTED AUG 6; R2, CUT JULY 2, HARVESTED AUG 26) SAMPLES. The CVA was based on 30 components from the hexane extract that had the largest F ratios in ANOVA of the seasonal samples. The major trend (43% of the variance) ordinates the seasonal samples with the growth from May to September–October. Regrowth clusters with the time of the season rather than the chronological age of the plants.

the May and June samples). Due to the severe drought conditions, the R2 samples (regrowth harvested Aug 26) had lost their lower leaves about half way up the stems. This also occurred with the never-cut plants between Aug 26 and Sep 15 and probably indicates that the regrowth samples (R1, R2) are physiologically comparable to the never-cut Aug 26 and Sep 15 samples. This is certainly borne out in the clustering pattern (Fig. 7).

In conclusion, we have found that plants with three different habits, herbaceous perennial, herbaceous annual and woody perennial, have different resource allocation patterns in their non-polar and polar extracts. The polar extracts contains considerable amounts of primary carbohydrates and were found to show a large degree of variation during the early growth stages. The non-polar extracts contain generally less metabolically active compounds and these extracts were generally more stable throughout the growing season. Components used for plant protection are, of course, found in both kinds of extracts. The waxes, presumedly used in conjunction with drought protection, would be expected to remain in place throughout the season. The major components of *Asclepias*, the amyrins and their esters, may be involved in a generalized plant defence and this might explain their relative constancy throughout the season. Both *Grindelia* and *Chrysothamnus* produce copious amounts of diterpenoids, some of which are deposited on the leaf surface. It appears that these components may not be metabolically retrievable for the plant and this may help explain the relative constancy of the non-polar extracts in these taxa.

Individual components of the hexane extract of *Asclepias speciosa* were found to vary seasonally with the fatty acids showing large changes that were correlated with growth and flowering. We have also shown that principal components analysis resolved the major classes of the components and may be useful in studying biosynthetic pathways. This area of research needs to be extended. Although there is variation within each of the seasonal sample sets, the samples tended to group according to

the date of harvest when the components from the hexane extract were analyzed by canonical variate analysis.

Experimental

The samples for the seasonal study in 1981 consisted of the entire above-ground portion from 10 *Asclepias speciosa* Torr. and 10 *Grindelia squarrosa* (Pursh) Dunal from a natural population in Salt Lake City, UT, with the samples from each taxon taken on June 4, July 6, August 4, September 4 and October 5, 1981. One plant of *Chrysothamnus nausesous* subsp. *consimilis* (Green) Hall & Clements was tagged and triplicate samples of leaves were collected on May 7, June 14, July 7, August 10, September 12 and October 11, 1982.

In 1982, a follow-up study was initiated to focus more closely on seasonal variation of the individual hexane soluble components of *A. speciosa* from a test plot in Layton, Utah where the plants were grown under natural climatic and edaphic conditions [46]. The entire above-ground portions of 10 plants were collected on May 27 (avg ht 20.3 cm, few plants had very small flower buds), June 10 (avg ht 45.7 cm, early bud stage), July 1 (avg ht 81.3 cm, 25% of the plants in full bloom), July 22 (avg ht 111.8 cm, plants in full bloom), August 6 (avg ht 104.1 cm, plants in full bloom), August 26 (avg ht 109.2 cm, early fruit pod stage), September 15 (avg ht 101.6 cm, fruit pods maturing, very few leaves on stems), and October 6, 1982 (avg ht 104.1 cm, fruit pods mature and seeds dispersing, stems yellow, very few leaves). In addition, 20 plants which had not been disturbed were cut off at 6 cm above the ground on July 2, 1982 and allowed to regrow under natural field conditions. 10 of these were harvested on August 6 (after 35 days of regrowth) and the other 10 were harvested on August 26 (after 48 days of regrowth).

In all cases, the harvested plants were air dried (60°) for 4 days and the material was ground in a Wiley Mill to pass a 2 mm seive. The material was Soxhlet extracted 20 h with hexane and then 20 h with MeOH. The material was dried (4 h at 70°) after the hexane extraction to remove the hexane before extraction with MeOH (see ref. [46] for detailed notes on the extraction protocol). The extracts were dried and percent yields were calculated on a dry weight basis (extracted residue dried for 48 h at 100°). Total dry weight was calculated as the sum of the extracts plus the dry extracted residue.

For the analyses of seasonal variation of individual components from the hexane extracts of *A. speciosa*, GLC analyses were performed on a Varian 1800 chromatograph with f.i.d. (350°) using a J&W fused quartz capillary column (DB1, 0.1 μ coating, 30 m × 0.32 mm with He at 30 cm/sec. All GLC analyses were performed in the split mode (25:1 split ratio) with the injector temperature at 275°. The oven temperature was programmed from 160° to 340° as follows: 8°/min for 12 min; 4°/min for 21 min; then isothermal at 340° for 9 min. Peak areas were quantified using a digital integrator. Peak identification follows the previous GC–MS analyses [44]. Several small components were found in this set of samples and coded as unknowns in this study.

The chemical data were coded and analyzed by one-way analysis of variance (ANOVA) with the appropriate degrees of freedom for each of the taxa analyzed for percent hexane extractables, percent methanol extractables, total yields, and percent protein (for *A. speciosa* and *C. n.* subsp. *consimilius*). Principal component analyses (PCA) follows the formulation of Veldman [61]. Canonical variate analyses follows Blackrith and Reyment (62).

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