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Phenylheptatriyne variation in bidens alba var. radiata leaves

Emily Gayle Cantonwine
Florida International University

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

PHENYLHEPTATRIYNE VARIATION IN BIDENS ALBA VAR. RADIATA LEAVES

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by

Emily Gayle Cantonwine

1999

To: Dean Arthur W. Herriott
College of Arts and Sciences

This thesis, written by Emily Gayle Cantonwine, and entitled Phenylheptatriyne variation in Bidens alba var. radiata leaves, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Bradley C. Bennett

David W. Lee

Kelsey R. Downum, Major Professor

Date of Defense: June 25, 1999

The thesis of Emily Gayle Cantonwine is approved.

Dean Arthur W. Herriott
College of Arts and Sciences

Dean Richard L. Campbell
Division of Graduate Studies

Florida International University, 1999

DEDICATION

I dedicate this thesis to Scott Gregor. His love and support helped me survive hot days, hurricanes and statistical blues. My research and this thesis are better because of his presence in my life.

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I would like to thank my major professor, Dr. Kelsey R. Downum, for his support and guidance with my research, and his expertise and willingness to help me through administrative hurdles. I would also like to thank to my committee members, Dr. David Lee and Dr. Bradley Bennett, for their confidence and easy going nature. Additional thanks goes to Scott Gregor for field support, Victor Apanius and Paulette Johnson for statistical guidance and University of Florida's Tropical Research and Education Center for land to conduct experiments. This work was funded by a grant from the Tropical Biology Program from the Department of Biological Sciences at Florida International University.

ABSTRACT OF THE THESIS

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Professor Kelsey R. Downum, Major Professor

Variation of phenylheptatriyne (PHT) concentrations in leaves of *Bidens alba* (Linn.) var. *radiata* (Schultz-Bip.) was investigated across it's Florida range, throughout the year and in response to photoenvironment. Natural surveys of PHT concentrations in *B. alba* leaves were done at 13 sites in Florida and three sites throughout the year. PHT concentrations were significantly different between populations ($p<0.001$) but showed little relationship with latitude ($R^2=0.024$) and none with longitude. Concentrations in leaves fluctuated throughout the year ($p<0.001$). They were highest in October, followed by June and lowest in January and April. Photoenvironmental influences were experimentally tested. PHT concentrations decreased under low R/FR treatments and increased under filtered UV treatments. Low light quantity did not significantly influence PHT concentrations but decreased PHT levels and leaf biomass. The results suggests that PHT concentrations in *B. alba* leaves vary in nature and that light quality, R/FR and UV, may effect PHT biosynthesis.

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INTRODUCTION

Secondary compounds vary quantitatively and qualitatively within species. Some of the variation between plants may be genetic, however, Bryant et al. (1983) proposed that much of the quantitative variation in phytochemical concentrations within a plant species may be due to the availability of resources for their production. For example, when available carbon is limited by low light, Bryant's model suggests that the allocation of carbon would go into growth over carbon based secondary compounds - resulting in lower levels of defensive allelochemicals.

Light quantity is not the only factor that can influence the synthesis of secondary compounds. Light quality, red far-red ratios (R/FR) and ultra-violet (UV) radiation, can also influence the levels of secondary compounds by activating enzymes (Tietjen and Matern, 1983) or genes coding for compound biosynthesis (Chappell and Halbrock, 1984; Lois et al., 1989; Schulze-Lefert et al., 1989). This is true for coumarins and flavonoids, both products of the shikimate pathway with known photomediated mechanisms (Beier and Oertli, 1983; Hahlbrock and Scheel, 1989; Harborne, 1993).

Polyacetylenes (PA) are secondary metabolites derived from oleic acid, a common fatty acid found in all plants. There are about 1000 known PA from 18 plant families and the basidiomycetes (Bohlmann et al., 1973). They are most commonly found in Asteraceae, Arialaceae, Campanulaceae, Santalaceae and Apiaceae. PAs in Asteraceae are characterized by conjugated systems of double and triple bonds, often with cyclic or heterocyclic structures. Some PAs are biologically active against detrimental organisms and many are phototoxic compounds that require UV radiation for activity.

Little is known regarding how PAs vary in nature or how their biosynthesis or storage is influenced by environmental factors, including light (Ichiara and Noda, 1977; Norton and Towers, 1985; Towers, 1984), though unlike the shikimate pathway, there is no evidence that the enzymes in the fatty acid pathway are regulated by light.

Phenylheptatriyne (1-phenylhepta-1, 3, 5-triyne, PHT) (Figure 1) is a phototoxic PA activated by UV-A radiation (Wat et al., 1979; Wat & Towers, 1980; Weir et al., 1985). UV excited PHT degrades membranes resulting in broad spectrum bioactivity against competing organisms, including herbivorous insect larvae (Wat et al., 1981; Arnason et al., 1981), fungi (Bourque et al., 1985; Arnason et al., 1980), bacteria (Geissberger & Sequin, 1991; Towers et al., 1979; Wat et al., 1980; Rabe and Van Studen, 1997; Towers and Hudson, 1987), membrane bound viruses (Hudson et al., 1986; Towers and Hudson, 1987) and other plants (Campbell et al., 1982). Phototoxicity is a function of compound concentration and intensity of UV-radiation (Downum, 1992) suggesting that variations in PHT concentrations and light could impact plant fitness.

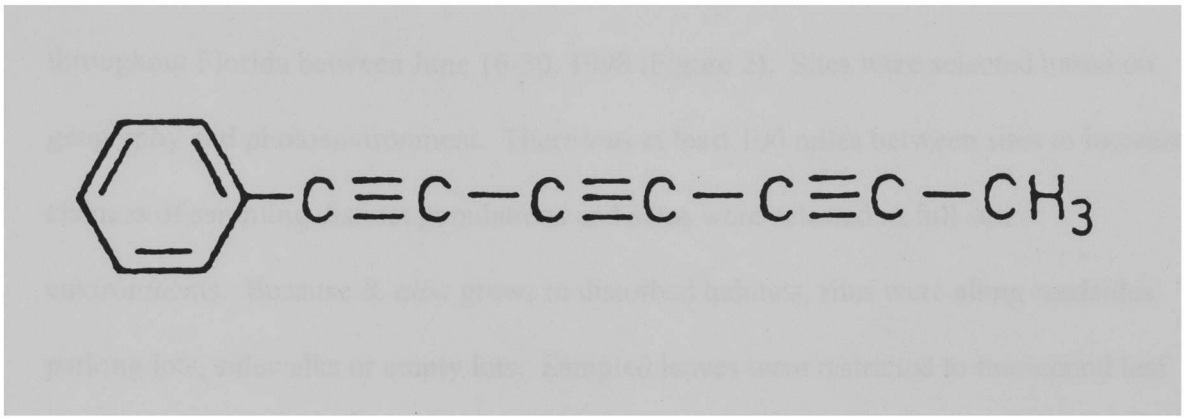
PHT is found in all parts of *Bidens alba* var. *radiata* (Asteraceae) though the highest concentrations occur in the leaves (Bourque et al., 1985). *B. alba* ranges throughout Florida, the Caribbean Islands and the East Coast of Mexico (Ballard, 1986). Part of the *Bidens pilosa* (L.) complex (Ballard, 1986), all of which are invasive weeds in over 40 countries (Holm et al., 1977), *B. alba* is used as food and medicine throughout the world (e.g., Wat et al., 1980; Ugarte, 1997). Much of the research done on *B. alba* and *B. pilosa* as a weed or medicine have acknowledged PHT as a potentially significant

ecological and medicinal factor (Campbell et al., 1982; Meissner and Beyers, 1986; Geissberger and Sequin, 1991; Wat et al., 1980).

It is not clear where PHT or other PAs are synthesized but there have been reports of the involvement of chloroplasts (Ichihara and Noda, 1977), endodermal cell walls (Van Fleet, 1970) and roots (Norton and Towers, 1985). PHT has been detected in cuticular extractions of *B. alba* (unpublished data) and *B. pilosa* leaves (Wat et al., 1979) and appears to be in all cells of *B. pilosa* leaves including the epidermis, trichomes, resin canals and underlying cells (Wat et al., 1979). The location of PHT in *B. alba* leaves is not known.

This paper investigates the natural variation and the influence of photoenvironment on PHT concentrations in *B. alba* leaves. Plants were sampled from sites throughout Florida and throughout the year to monitor natural variation in PHT levels from a consistent leaf position and developmental stage. Photoenvironmental experiments manipulated light quantity, photosynthetic photon flux density (PPFD), and two spectral regions, UV-radiation and R/FR, to test the effect of light on PHT accumulation in leaves. These light factors vary in nature and have been shown to mediate phenotypic responses in plants, including the synthesis of some secondary compounds. We predicted that levels of PHT would follow the model proposed by Bryant (1983) for carbon based compounds, and decrease as PPFD decreased. Investigation of light quality effects may provide additional information regarding PA biosynthesis since the enzymes responsible for the acetylenic bonds have not been isolated and since PHT bioactivity is dependent on UV intensity.

Figure 1: Chemical structure of PHT.



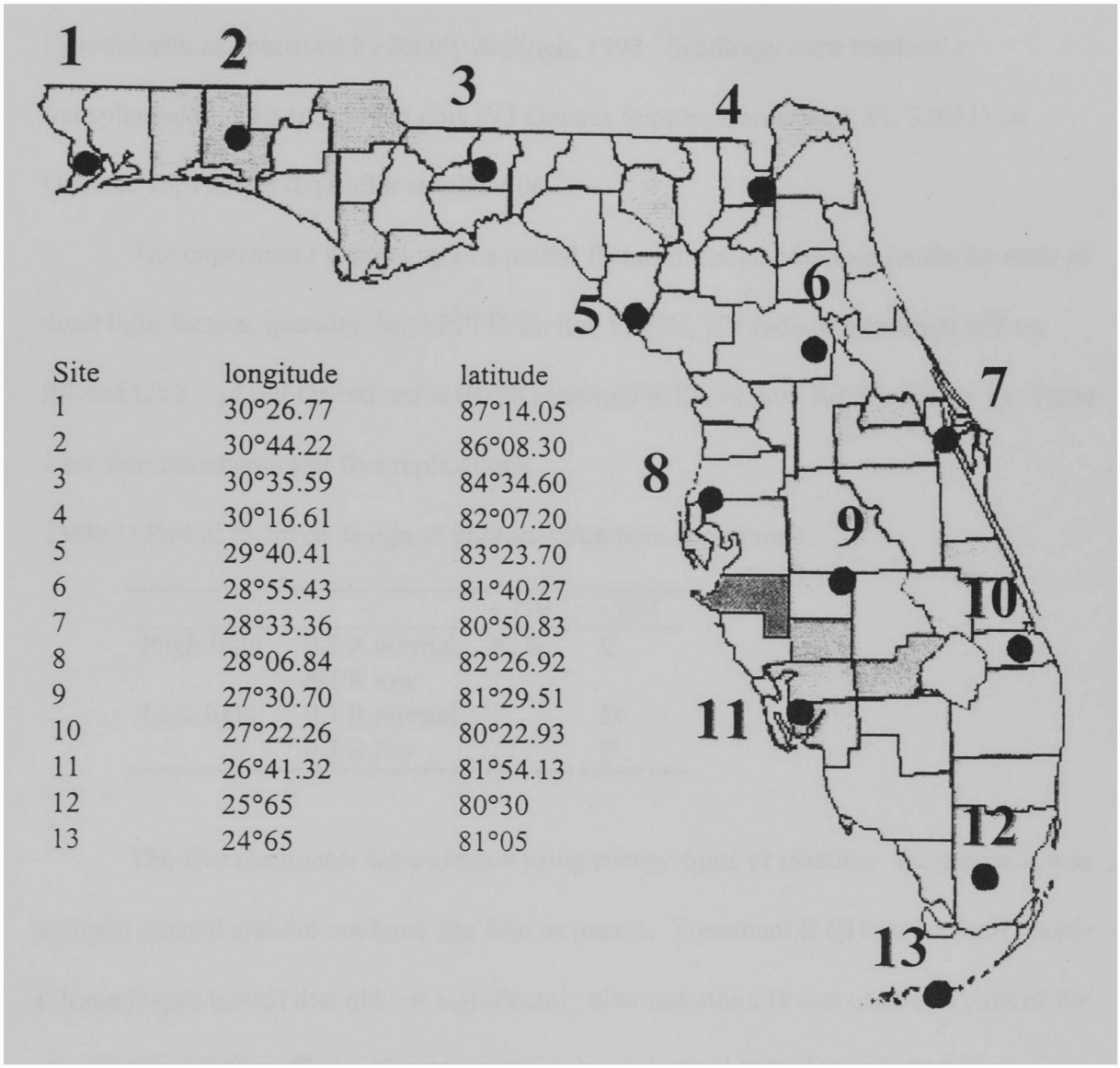
MATERIALS AND METHODS

Protocol for Geographic and Seasonal Studies

Thirty plants were randomly sampled along a 50m transect at thirteen sites throughout Florida between June 16-30, 1998 (Figure 2). Sites were selected based on geography and photoenvironment. There was at least 100 miles between sites to increase chances of sampling distinct populations and areas were selected in full sun environments. Because *B. alba* grows in disturbed habitats, sites were along roadsides, parking lots, sidewalks or empty lots. Sampled leaves were restricted to the second leaf from the top of a flowering shoot to minimize variations in PHT levels due to leaf position and development.

Leaves were extracted for PHT by soaking three leaf discs, punched using a 6.3mm hole punch, in 6ml methanol (MeOH) for 14 days. In a preliminary study, MeOH extracts maintained the same levels of PHT for at least 60 days. Three additional leaf discs were collected from each leaf for dry weight determinations. Sampling was repeated for at least three sites, sites 8, 9 and 12, in October, January and April (October 1-3, 1998; January 8-10, 1999; April 9-10, 1999) to monitor the influence of season on PHT levels and dry weight. Extracts were analyzed for PHT by high-pressure liquid chromatography (HPLC).

Figure 2: Sample site locations, including longitude and latitude positions, throughout Florida.



Site	longitude	latitude
1	30°26.77	87°14.05
2	30°44.22	86°08.30
3	30°35.59	84°34.60
4	30°16.61	82°07.20
5	29°40.41	83°23.70
6	28°55.43	81°40.27
7	28°33.36	80°50.83
8	28°06.84	82°26.92
9	27°30.70	81°29.51
10	27°22.26	80°22.93
11	26°41.32	81°54.13
12	25°65	80°30
13	24°65	81°05

Protocol for Photoenvironment Experiments

Seeds were collected in Miami, FL and surface sterilized with 0.05% sodium hypochlorite as described by Reddy & Singh, 1992. Seedlings were randomly transplanted into 10.1cm round pots (VJ Grower Supply, Homestead, FL 33031) on October 15, 1998, 6 days after sterilization.

The experiment was set up in a partial factorial design with two levels for each of three light factors, quantity (high PPFD vs. low PPFD), UV radiation (normal UV vs. filtered UV), and red far-red ratios (R/FR) (normal R/FR vs. low R/FR) (Table 1). There were five treatments and five replications.

Table 1: Partial factorial design of photoenvironment experiment.

		+ UV	- UV
High light	R/FR normal	A, B	C
	R/FR low		
Low light	R/FR normal		D
	R/FR low		E

The five treatments were created using energy films or plastics. Treatment A was an open control and did not have any film or plastic. Treatment B (B) used a 4mil plastic (Home Depot brand) that did not significantly alter radiation; B was used as a control for the plastic and film effects. Treatment C used a clear 6mil POLY plastic (B&K Installations, INC. Homestead, FL 33030) that filtered UV radiation without decreasing PPFD. Treatments D and E used energy films that filtered UV radiation and reduced PPFD by about 80% but altered R/FR differently. Treatment D used a metal sputter-coated film (REAL20) that did not alter normal R/FR and treatment E used a dye-

impregnated film (NEARL20) which reduced R/FR to about 0.23 of normal. Both energy films were supplied by the 3M Corporation, St. Paul, Minnesota 55144. Light treatments were quantified using a LI-1800 spectroradiometer (Li-Cor Instruments, Lincoln, NE 68505, USA) at the beginning of the experiment (Table 2).

Table 2: Percent radiation at two bandwidths, UV-A (320-400nm) and PAR (400-700nm), and R/FR (656-664/726-734) for each light treatment.

Light treatments	% UV-A	% PAR	R/FR
A	100 (45.91 $W \cdot m^{-2}$)	100 (1700 $\mu mol \cdot m^{-2} \cdot s^{-1}$)	1.215
B	87.8	95.0	1.200
C	42.8	95.0	1.186
D	4.9	19.9	1.100
E	5.6	20.1	0.276

* All values are based on a single measurement (mean of 3 consecutive scans) made on October 15, 1998

The experiment was set up outside at University of Florida's Tropical Research Education Center in Homestead, FL. Twenty-five, 59x75x54cm, enclosures were made from 2.52cm PVC pipe and set up in a Latin square design. Energy films or plastics were taped across the top of the enclosure with duct tape. The sides of the enclosures were left open because preliminary studies with *B. alba* revealed that seedlings did not grow well when the sides of the enclosures were covered. Enclosures were tilted about 5cm towards the south to allow for rain runoff. Nine pots were placed in 10.1cm pot trays (VJ Grower Supply, Homestead, FL 33031) and rested on two cinder blocks underneath each treatment enclosure. This set-up gave plants about eight hours of treated light each day.

A few individuals died after transplantation but all treatment enclosures had a minimum of 8 or 9 plants. Plants were treated to get rid of aphids with a soap spray (5ml dove dish detergent in 896g water) after 2 weeks. All plants were treated even if they did not have aphids. In the third week almost all plants were infected with leaf-minors. Leaf-minors were manually removed from each infected leaf.

After four weeks, the top-most leaf and the second leaf from the bottom (leaf 2) from each plant were extracted for PHT by soaking 3 leaf discs in 5ml MeOH. One additional leaf disc was combined with other discs from each leaf position in each enclosure to determine an average dry weight. Extracts were analyzed by HPLC after 14 days.

Quantification of PHT

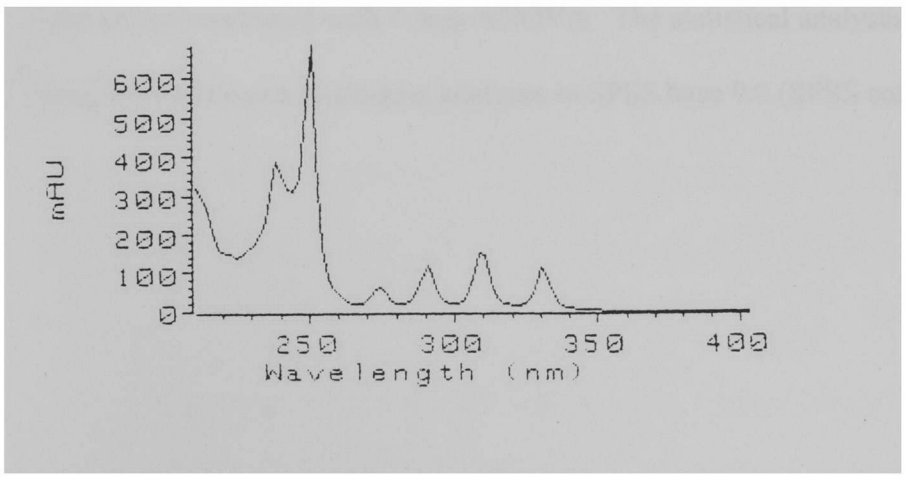
PHT for standard solutions was prepared by extracting PHT from fresh leaf material using HPLC grade MeOH. The methanolic extract was filtered through a Whatman 1 filter, diluted 1:1 with deionized water and partitioned against an equal volume of petroleum ether (bp 30°C-60°C). The PE extract was evaporated to dryness by rotary evaporation at 40°C, redissolved in HPLC grade MeOH and stored at 4°C. PHT was the predominant acetylenic compound in *B. alba* extracts and was easily identified by its unique UV absorption spectrum (Figure 3).

PHT concentrations were determined by UV spectroscopy at 250nm using dilutions of the PHT standard. The extinction coefficient of PHT at 250nm is 148000 (Bohlmann et al., 1973). Calibration curves were created to standardize PHT

concentrations with HPLC peak area units. Calibration curves of R^2=0.99 or better were used. Curves were updated at the beginning and end of each study to ensure accurate calculations.

PHT in field and experimental extracts were quantified by reverse-phase HPLC on a Hewlett-Packard Model 1090M high-pressure liquid chromatograph with diode array detection. Separations were accomplished on a 5µl Hypersil octaldecylsilica (ODS) microbore column (100x2.1mm) with an ODS guard column (Hewlett-Packard) at a flow rate of 0.5ml min^{-1} and a column temperature of 40° C. A water (H_2O)-acetonitrile (MeCN) solvent system was used to elute compounds from the column. The solvent program was linear and increased from 0% to 100% MeCN over 6.5 minutes and remained at 100% MeCN for 0.5 minutes. Elutions of compounds were monitored at 250nm. All extracts were filtered through a 0.2 micron filter before HPLC injection. Injection volumes were 4µl for geographic extracts and 7µl for photoexperiment extracts.

Figure 3: Absorption spectrum for PHT.



Statistical Analysis

PHT concentrations from plants (PHTc), PHT levels in three leaf discs (PHTl) and dry weights of three leaf discs (DW) from the different sites were independently compared by a one-way analysis of variance (ANOVA), with site as main effect. Means and standard errors for PHTc, PHTl and DW were calculated using descriptive statistics. Sites were distinguished into homogenous subsets with Tukey's post-hoc analysis. The correlations of PHTl with DW for all sites combined and for each site separately were tested with a 1-way ANOVA. Patterns for geographic influence were investigated using linear regression of PHTc by site location. Seasonal patterns were tested using two-way ANOVA with site and month as the main effects.

Photoexperiment data were separated into three subsets distinguished by light factor and analyzed independently. The influence of quantity was examined using data from treatments C and D, UV used treatments B and C and R/FR used treatments D and E. PHTc, PHTl and DW differences for light factor levels were tested with a 1-way ANOVA, with light factor as main effect. A correlation between PHTl and DW for each light factor was tested with 1-way ANOVA. The statistical analyses were performed using univariate and regression analyses in SPSS base 9.0 (SPSS software, Chicago, IL.).

RESULTS

Data Sets

There are three data sets referred to in the results. PHTl designates PHT levels extracted from three leaf discs (nmol in three leaf discs). Dry weight (DW) refers to dry weights of three leaf discs (g) and PHTc is the concentration of PHT in plants, calculated by PHTl / DW (μmol/g).

Geographic Variation in PHT Accumulation and Dry Weight

Sites were restricted to open canopy locations but they varied in geography and disturbed habitat type. Habitat descriptions and the means and standard errors of PHTl, DW and PHTc for sites are shown in Table 3.

PHTc was different between sites at $p<0.001$. Post-hoc analysis distinguished sites into 5 subsets (Table 4). Geographic patterns of PHTc showed a weak correlation with latitude going from North to South ($p=0.002$, $R^2=0.024$) and was not significantly related to longitude.

PHTl and DW were also significantly different between sites ($p<0.001$). PHTl was weakly correlated with latitude going from North to South ($p=0.000$, $R^2=0.033$) and longitude, from East to West ($p=0.001$, $R^2=0.022$). DW showed a significant but weak increase ($p=0.001$, $R^2=0.029$) going towards the West and no difference due to latitudinal variation.

Dry weights of three leaf discs ranged from 0.002-0.008g. The majority of samples were within 0.003-0.007g with sample sizes for these weights between 11 and

141 for all sites combined; there were only two samples for weights 0.002g and 0.008g. The complete data set (DW=0.002-0.008g) showed a weak correlation between DW and PHTl ($p<0.001$, $R^2=0.070$), while the 0.003-0.007g subset did not express a significant relationship between PHTl and DW ($p=0.083$, $R^2=0.008$). When sites were analyzed separately, only two sites, sites 6 and 13, showed a significant correlation between PHTl and DW (alpha=0.05) with $R^2=0.382$ and 0.386 respectively, yet when 0.002g and 0.008g weights were excluded from analyses their significance was retracted. Other sites demonstrated either a positive, negative or no trend in the relationship of DW and PHTl.

Table 3: Habitat description of sites and the means and standard errors for PHTc, PHTl and DW for each site.

Site	Habitat description	Mean PHTl (SE) (nmol in 3 leaf discs)	Mean DW for 3 leaf discs (SE) (g)	Mean PHTc (SE) (μmol/g)
1	parking lot gully	93.65 (6.69)	0.005 (0.0002)	21.49 (1.69)
2	roadside	71.04 (3.76)	0.004 (0.0002)	18.87 (1.02)
3	edge of building	66.43 (4.49)	0.005 (0.0002)	13.64 (0.98)
4	sidewalk cracks	80.72 (5.00)	0.005 (0.0002)	15.51 (1.04)
5	open field	125.10 (10.48)	0.005 (0.0002)	25.38 (2.37)
6	roadside	52.35 (7.36)	0.005 (0.0002)	11.43 (2.05)
7	roadside	91.13 (6.70)	0.006 (0.0002)	16.31 (1.07)
8	open field	100.24 (6.08)	0.005 (0.0002)	21.74 (1.19)
9	parking lot edge	97.93 (9.72)	0.005 (0.0002)	19.04 (1.89)
10	roadside	130.67 (7.17)	0.004 (0.0002)	30.40 (1.86)
11	roadside	84.69 (4.42)	0.004 (0.0002)	19.85 (1.16)
12	roadside	115.44 (6.74)	0.005 (0.0002)	24.29 (1.33)
13	roadside	90.71 (4.86)	0.005 (0.0002)	18.74 (0.95)

N = 30 for all sites.

Table 4: Tukey's post-hoc analysis using PHTc to distinguish homogeneous subsets of sites.

	Subset A	Subset B	Subset C	Subset D	Subset E
Site					
	6				
	3	3			
	4	4	4		
	7	7	7		
		13	13	13	
		2	2	2	
		9	9	9	
		11	11	11	
			1	1	
			8	8	
				12	12
				5	5
					10
p-value	0.522	0.151	0.147	0.089	0.170

Uses Harmonic Mean Sample Size = 30.000.
Alpha = .05.

Seasonal Variation in PHT Accumulation and Dry Weight

There were significant differences detected throughout the year for PHTc, PHTl ($p<0.001$) and DW ($p<0.05$) but they did not follow the same pattern. Variations across months are shown for PHTc (Figure 4), PHTl (Figure 5) and DW (Figure 6). June plants had significantly higher PHTc, PHTl and DW than January and April plants while January and April were not different from each other for all analyses. October plants had significantly more PHTc than plants from the other months. DW was positively correlated with PHTl ($p<0.001$; $R^2 = 0.103$) throughout the year, increasing in correlation when October was excluded from analyses ($R^2=0.239$).

Figure 4: Means and 95% confidence intervals for PHTc across months.

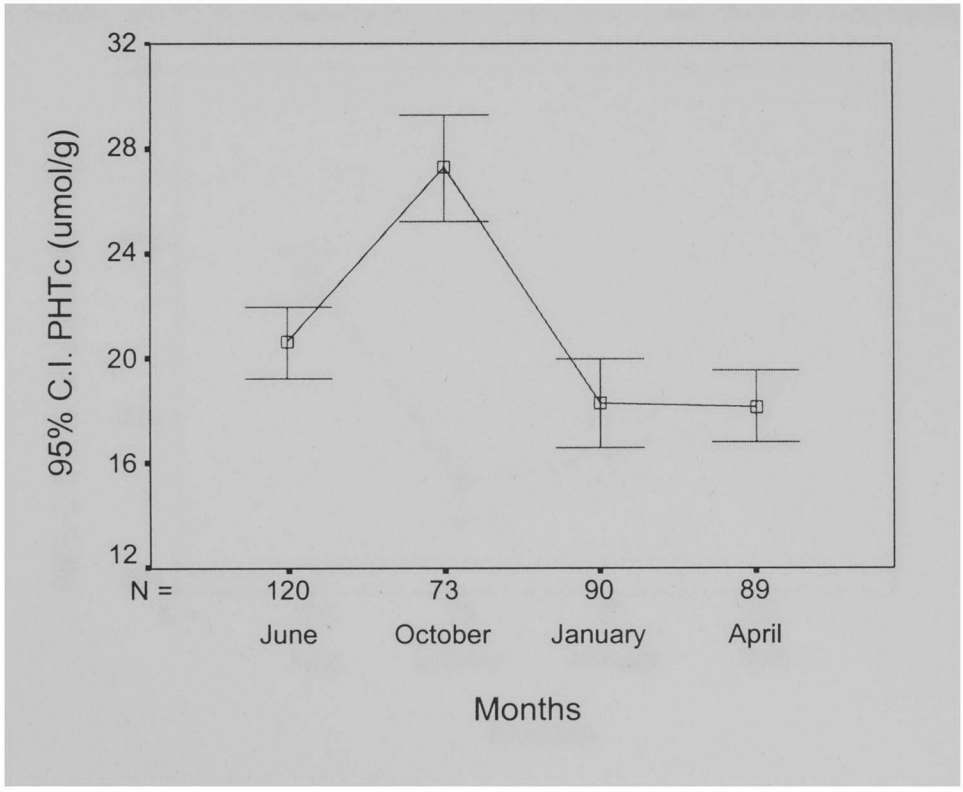


Figure 5: Means and 95% confidence intervals for PHTl across months.

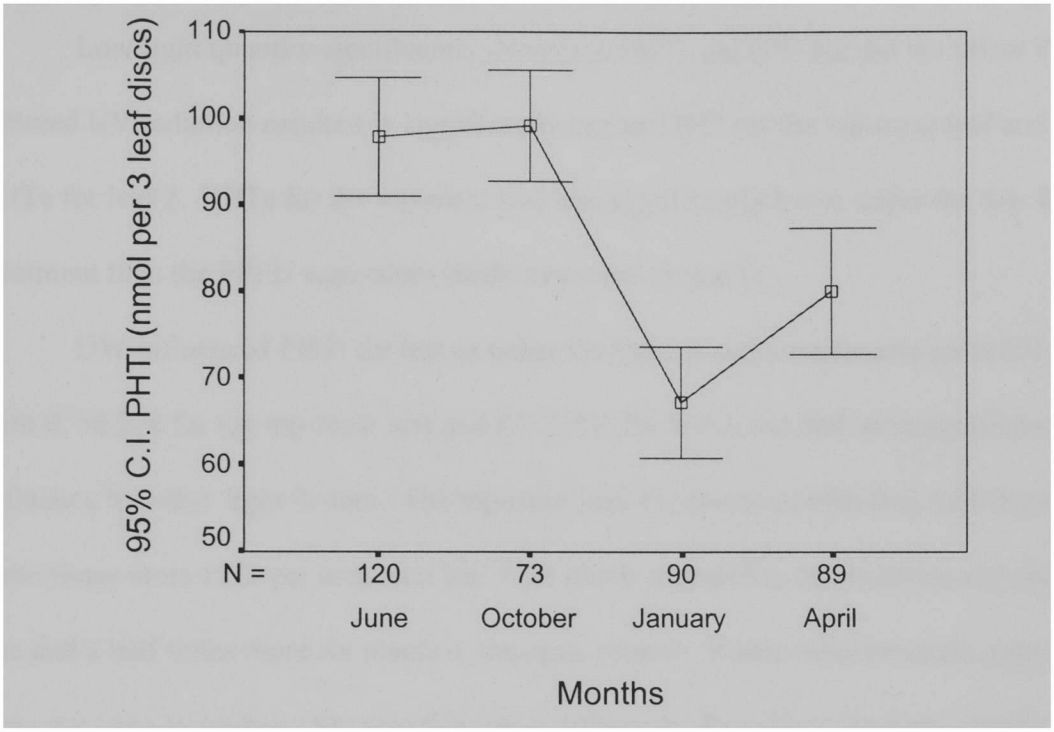
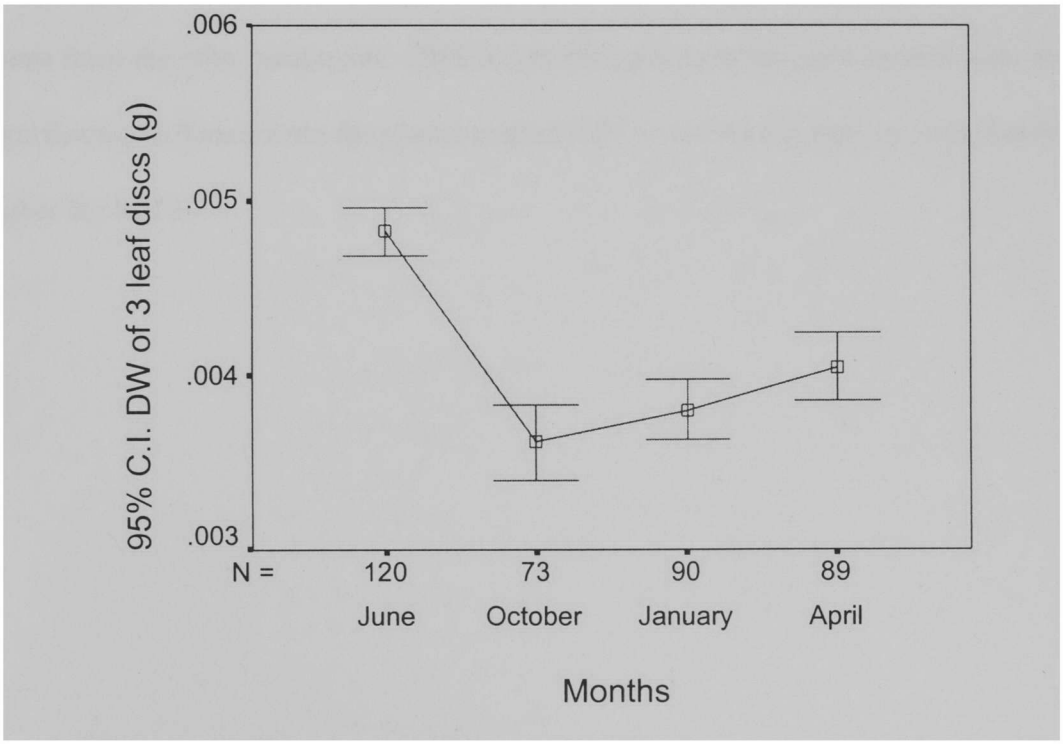


Figure 6: Means and 95% confidence intervals for DW of 3 leaf discs across months.



Effects of Photoenvironment on PHT Accumulation and Dry Weight

Low light quantity significantly decreased PHTl and DW but did not effect PHTc. Filtered UV radiation resulted in significantly higher PHTl for the top-most leaf and PHTc for leaf 2. PHTc for the top-most leaf was significantly lower under the low R/FR treatment than the PPFD equivalent shade treatment (Table 5).

DW influenced PHTl for leaves under the light quantity treatments ($p<0.001$), with $R^2=0.224$ for the top-most leaf and $R^2=0.204$ for leaf 2, but had an insignificant influence for other light factors. The top-most leaf, the fourth or fifth leaf, had about three times more PHT per area than leaf 2 for plants included in the analyses and about one and a half times more for plants in the open control. Plants from the open control were not used in analyses because they grew differently than plants from the plastic and film treatments. They were significantly shorter and had no lower node branching unlike plants from the other treatments. PHT levels from plants in the open control were not significantly different from the plastic control (UV +) for the top leaf, yet significantly higher for leaf 2.

Table 5: Mean and standard error (SE) for PHTc, PHTl and DW by light factor levels and leaf positions and p-values for light factor treatments.

Light Factor	Leaf position	Factor level	PHTl (nmol in 3ld)	DW (g)	PHTc (μmol/g)
Quant.	Top	↑	75.76 (4.47)	0.003 (0.0002)	23.85 (1.46)
		↓	47.11 (2.69)	0.002 (0.0003)	26.14 (2.00)
			p=0.000*	p=0.012*	p=0.356
	Leaf 2	↑	24.08 (1.73)	0.003 (0.0002)	8.68 (0.59)
		↓	14.20 (1.18)	0.002 (0.0000)	7.10 (0.59)
			p=0.000*	p=0.004*	p=0.061
UV	Top	↑	65.76 (3.20)	0.003 (0.0000)	21.92 (1.07)
		↓	75.76 (4.47)	0.003 (0.0002)	23.85 (1.46)
			p=0.038*	p=0.347	p=0.291
	Leaf 2	↑	21.86 (1.58)	0.003 (0.0002)	6.95 (0.55)
		↓	24.08 (1.73)	0.003 (0.0002)	8.68 (0.59)
			p=0.318	p=0.195	p=0.034*
R/FR	Top	↑	47.11 (2.69)	0.002 (0.0003)	26.14 (2.00)
		↓	43.63 (2.96)	0.002 (0.0002)	18.88 (1.40)
			p=0.385	p=0.347	p=0.004*
	Leaf 2	↑	14.20 (1.18)	0.002 (0.0000)	7.99 (0.59)
		↓	14.36 (1.12)	0.002 (0.0000)	7.18 (0.56)
			p=0.923	_____	p=0.923

DISCUSSION

Although there is no evidence that *Bidens alba* plants from different sites are reproductively isolated, the distances between sites, approximately 100 miles, suggests that it may be enough to restrict gene flow. In addition, B. alba flowers do not self pollinate (Ballard, 1986), increasing the possibility of divergent populations by distance. For this reason, the sites were treated as distinct populations for the purpose of this discussion.

PHT concentrations (PHTc) from plant leaves varied within and between populations throughout Florida. Almost none of the interpopulational variation detected in PHTc was explained by the variables examined in this study, latitude or longitude. Instead, concentrations were scattered throughout the state in no particular order. Though we restricted populations to open canopy locations, they were variable in soil type, water availability and level of disturbance. Environmental factors such as nutrients and available water have been shown to influence secondary compound production (Gershenzon, 1984) but these variables were not measured in this study.

Though the study controlled for within plant differences due to developmental stage and leaf position on the plant, results from the photoenvironment experiment suggest that branching is another factor that affects within plant variation. The presence of branching in the photoenvironment experiment significantly decreased PHTc in leaves at a branching node. We did not control for branching in our survey and therefore may have picked up additional within plant variation.

PHT levels in plant leaves (PHTl) and leaf biomass (DW) also varied within and between populations. For the majority of samples (DW in the range of 0.003-0.007g), PHTl was not correlated with DW. This suggests that the intra and interpopulational variation of PHTc in plant leaves were due to differences in allocation of resources to PHT over DW. Either some plants have a higher production level of PHT per DW than other plants due to genetic or environmental factors or there are differences in the quantity of PHT stored in leaf cuticles versus endodermal layers.

This multi leaf layer dynamic of secondary compound accumulation has been shown for phototoxic furanocoumarins in response to environmental factors, such as light, temperature and acid and salt sprays (Zobel et al., 1991; Zobel and Brown, 1990; Zobel and Brown, 1993). It is not known if PHT is stored in different leaf layers like furanocoumarins but the correlation of PHTl with DW for some studies (seasonal and light quantity) and lack of correlation for other studies (geographic and light quality) support the possibility of multi layer storage. In addition, PHT has been detected in leaf cuticles of *B. alba* (Towers, unpublished) and was suggested to be in all cells from *B. pilosa* leaves (Wat et al., 1979). Further investigation of PHT accumulation in leaf layers is necessary to understand the ecology of PHT in *B. alba* leaves.

Differences in PHTc from plant leaves sampled throughout the year were recorded. PHTc was high in October followed by June and low in January and April. Significantly higher PHTc in June and October may be a result of greater defense requirements in these months because of stronger herbivore pressures during this time of year. An alternative hypothesis is that PHTc was high in late June and October because

there was less carbon allocated to reproduction and more available for PHT synthesis. Although *B. alba* flowers year round, there is evidence that closely related *B. pilosa* plants require at least 9 hours of low light (night) to induce flowering (Kirszenzaft and Felippe, 1978). Day length is longest in late June and may have not been short enough by mid October to stimulate intensive reproduction. In addition, *B. alba* appears to flower more in South Florida in the winter and spring (personal observation).

PHTl and DW were also different in plant leaves throughout the year. They were correlated in June, January and April, but differed in October. In October, PHTl remand similar to June levels but DW significantly decreased, resulting in the highest PHTc levels recorded from the months sampled. The decrease in DW from October leaves without influencing PHTl, may be a mechanism to increase PHTc in response to October pressures either by allocating carbon to PHT over biomass or by extruding greater quantities of PHT to leaf cuticles.

The hypothesis that PHT levels would decrease as light quantity decreased was accepted. PHTl levels were influenced by light quantity but there was no difference for PHTc between light quantity treatments. This was true in part because PHTl and DW were correlated with each other, R^2=0.224 for the top-most leaf and R^2=0.204 for leaf 2. Because of this relationship, it appears as though PHTl and DW were similarly influenced by low PPFD, thus suggesting that carbon may not have been assimilated into growth significantly more than PHT synthesis which Bryant's model proposes. The correlation between PHTl and DW suggests that PHTl production might be linked in part to leaf tissue biomass rather than restricted by limited light.

Low R/FR significantly decreased PHTc for the top-most leaf but did not effect leaf 2. The lack of response from leaf 2 may be because the lower leaves were shaded by top leaves and surrounding plants. Shading reduces R/FR and therefor the treatments may have not been as different from each other for leaf 2. The response of PHTc to low R/FR is similar to what has been shown for flavonoids. Flavonoid biosynthesis is induced by R and reversed by FR, resulting in less biosynthesis of flavonoids when R/FR is low (Piringer and Heinze, 1954; Siegelman and Hendricks, 1957; Beggs and Wellmann, 1985). Therefore, low R/FR may inhibit PHT biosynthesis by a phytochrome mediated mechanism similarly to flavonoids. More research is needed to answer this question.

Filtered UV radiation significantly increased PHTl for the top-most leaf and PHTc for leaf 2. UV appears to have an influence on PHT levels, most likely affecting cuticle stored PHT, since epidermal leaf layers absorb most of the UV radiation reaching the leaf (Alenius et al., 1995). There are three possible modes of action suggested that may explain the response of PHTl and PHTc to UV. First, more PHT may have been photodegraded by higher levels of UV compared to filtered UV treatments, resulting in higher PHT levels. Photodegradation of PHT occurs when PHT is excited by UV (Marchant, 1987). Second, PHT accumulation may have increased in leaves to compensate for low UV levels to maintain defensive protection. Downum (1992) suggested that the high concentrations of PA commonly found in roots may be in response to low UV availability for bioactivity. And third, low UV might allow plants to accumulate higher levels of PHT with less autotoxicity. There is not much known about

polyacetylene autotoxicity but it has been shown that plant cells are susceptible to toxicity by PHT (Campbell et al., 1982). The last two hypotheses could influence PHT levels by UV mediated mechanisms. More research is needed to address this question.

Did photoenvironment explain any of the natural variation seen in the geographic and seasonal studies? For geographic studies, the variation detected within and between populations does not appear to be caused by light differences across geographic range. This is not surprising since the sampled populations were not that different in elevation or geographic position and probably did not influence light quantity or quality by much. Some of the variation detected in PHTc could be due to R/FR or UV effects from vegetative shading by neighbor plants. Otherwise we predict the variation seen was due to experimental error, populational differences (environmental or genetic) or differences in PHT allocation to the cuticle.

Variations in PHTl and DW detected in plants throughout the year can be explained in part by seasonal light quantity fluctuations for all months except for October. PPFD is highest in the summer and lowest in the winter (Lee and Downum, 1991), and plants in June, January and April appear to have responded to this variation much like the light quantity experiment, low PHTl and DW when PPFD is low, and high PHTl and DW when PPFD is high. However, October plants did not respond like plants from the other months and it is suggested that an alternative factor in response to greater herbivory pressures or flowering stage is a likely explanation.

CONCLUSION

This study supports that PHT concentrations in *B. alba* leaves are variable. There were significant concentration differences between populations but geographic positions of sites did not explain much of the variation detected. Populational variation of PHT concentrations could be due to genetic or environmental differences not measured in this study. PHT concentrations in plant leaves were highest in the fall and suggest that the plants defend themselves to the greatest extent in the fall. Light quantity did not influence PHT concentrations in leaves but did affect PHT levels and leaf biomass. Light quality did influence PHT concentrations in leaves and may be a regulatory factor in the biosynthesis of PHT. More research is necessary to demonstrate the effects of R/FR, UV and light quantity on PHT biosynthesis.

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APPENDIX I

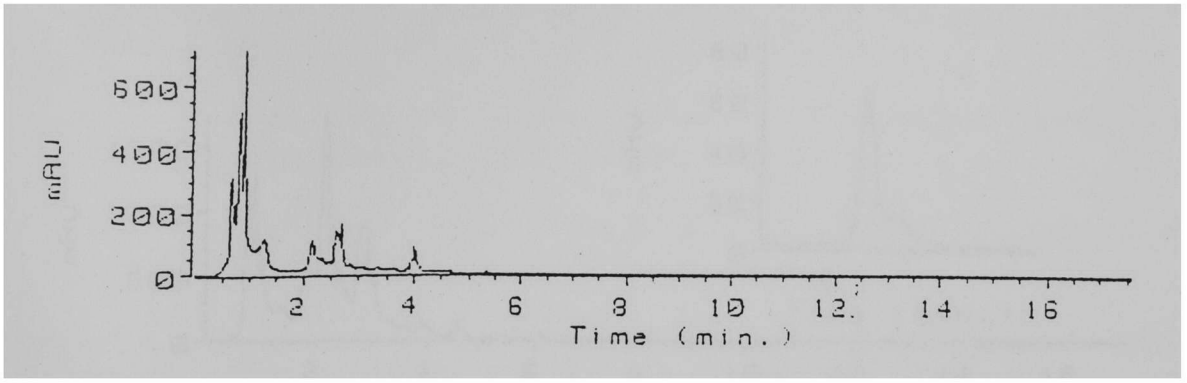
Medicinal extractions of *Bidens alba* leaves were prepared and investigated to look for the presence or absence of PHT which is believed to be the biologically active constituent in the leaves. Three traditional extracts, retrieved from the ethnobotanical literature, were tested.

1. A tea of fresh leaves (e.g. Watt & Breyer-Brandwijk, 1962; Eldridge, 1975). Prepared by adding 10g fresh leaves to 50ml of boiling distilled water and allowed to cool.
2. Fresh leaf juice (e.g. Bushnell et al., 1950; Asprey & Thorton, 1953; Burkhill, 1966; Von Reis Altshul, 1973). Prepared by crushing leaves with a mortar and pestle and squeezing material through cheesecloth.
3. A paste of crushed leaves and oil (Elderidge, 1975). Prepared by crushing 10g fresh leaves with 10ml vegetable oil (Publix brand) using a mortar and pestle. The extracted oil was partitioned with equal parts MeOH. The MeOH extract was tested with HPLC.

All extracts were filtered through a 2micron filter prior to HPLC injection.

HPLC chromatograms for extracts are shown below. Peaks representing PHT (retention time (rt)=6.1min) or other polyacetylenes are identified.

Figure 7: HPLC chromatogram of tea extract.



The tea extract did not have PHT but it did include other more polar PAs (Figures 8 and 9).

Figure 8: Absorption spectra for the PA with rt=2.6min.

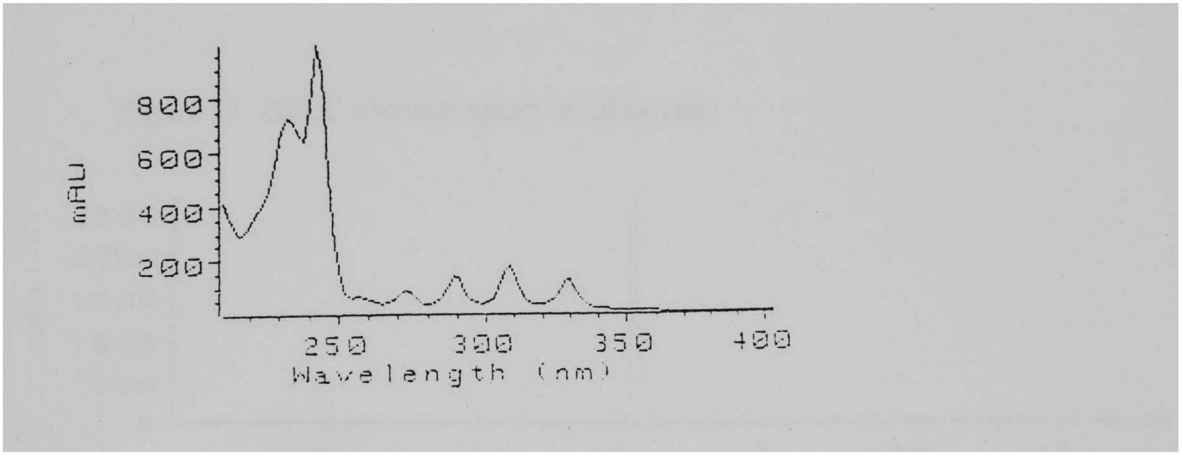


Figure 9: Absorption spectra for the PA with rt=4.0

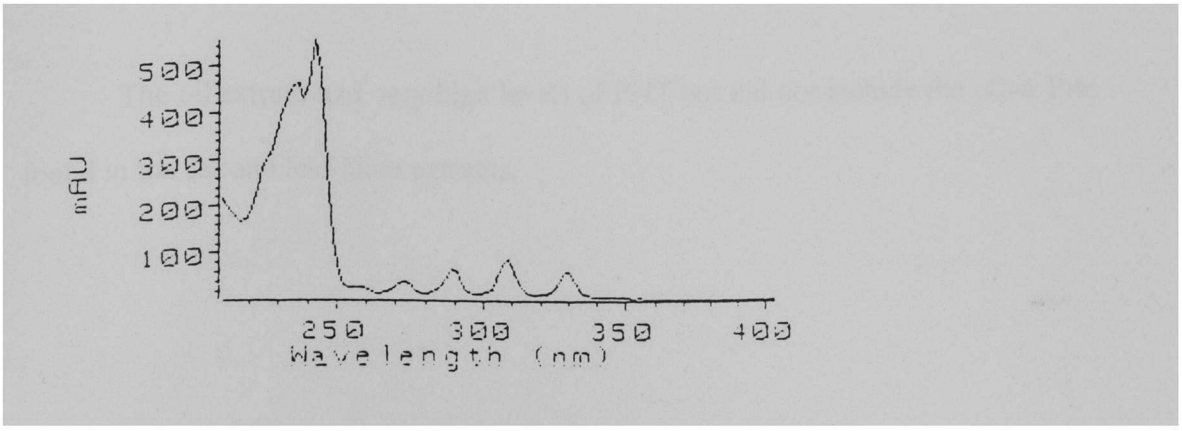
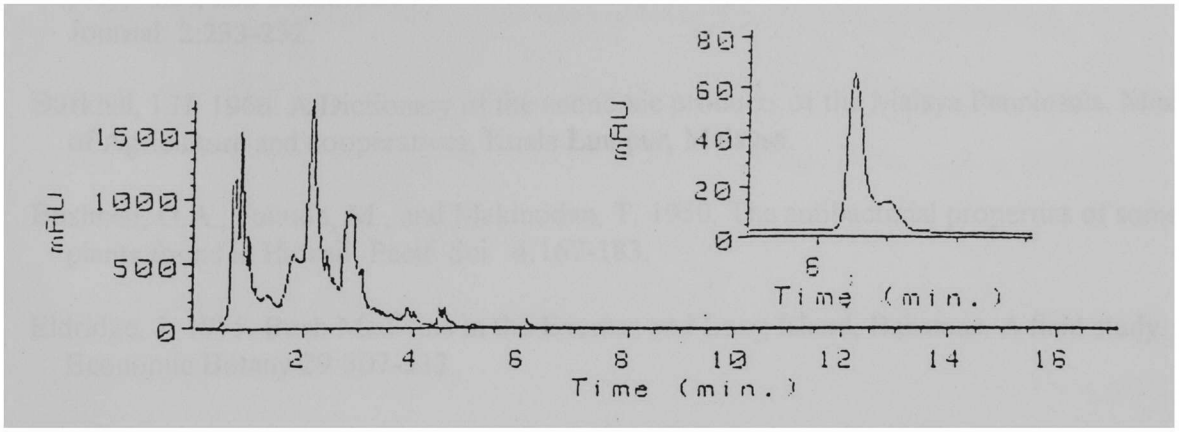
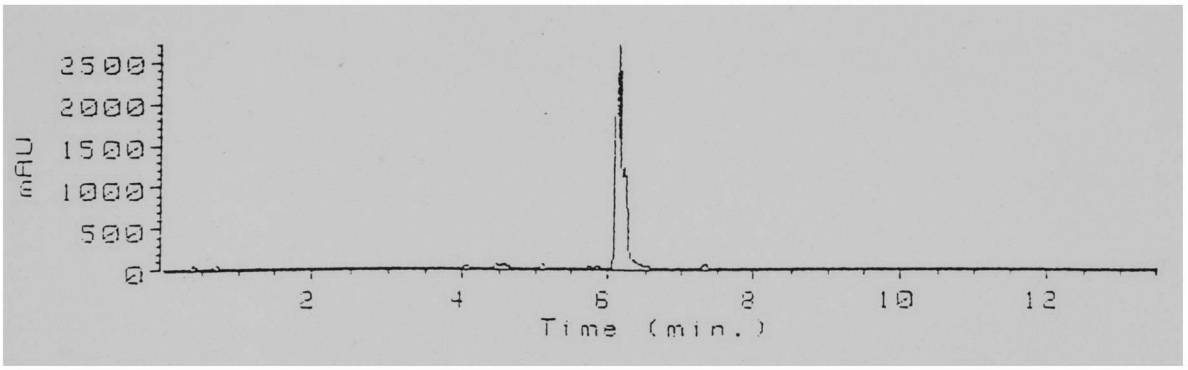


Figure 10: HPLC chromatogram of leaf juice extract.



The leaf juice extract included the PAs found in the tea extract but also had low levels of PHT.

Figure 11: HPLC chromatogram of oil extract.



The oil extract had very high levels of PHT but did not include the other PAs found in the tea and leaf juice extracts.

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APPENDIX II

Bidens alba var. *radiata* was distinguished from *B. pilosa* by Ballard in 1986. Some of the distinguishing characteristics include differences in ploidy level, flavonoid leaf chemisty, length of ray ligules and number of awns on achenes. According to Ballard, the achenes from *B. pilosa* have 3-5 awns while there are only 2 awns on *B. alba* achenes. Not all taxonomists have accepted Ballards revisions to the taxa (personal observation) and because of this it was especially important to take voucher specimens of the plants investigated in this thesis. In addition, seeds were collected at each sample site in Florida and the number of awns on the achenes were recorded.

The achenes collected from Florida sites included both 2 awned achenes and 3 awned achenes for most of the populations (Table 6).

Table 6: Number of awns on achenes collected from each Florida site sampled.

Sites	# awns
1	2
2	2
3	2&3
4	2
5	2&3
6	2&3
7	2
8	2&3
9	2&3
10	2
11	2&3
12	2&3
13	2&3

There was no relationship between mean PHT concentrations for sites and number of awns on achenes from sites (Table 7).

Table 7: Tukey's post-hoc analysis using PHTc to distinguish homogeneous subsets. * indicate sites with 2 and 3 awned achenes.

	Subset A	Subset B	Subset C	Subset D	Subset E
Site					
	6*				
	3*	3*			
	4	4	4		
	7	7	7		
		13*	13*	13*	
		2	2	2	
		9*	9*	9*	
		11*	11*	11*	
			1	1	
			8*	8*	
				12*	12*
				5*	5*
					10
p-value	0.522	0.151	0.147	0.089	0.170

Uses Harmonic Mean Sample Size = 30.000.
Alpha = .05.

The presence of 3 awned achenes from sampled sites suggest that either 1) populations sampled included both *B. alba* and *B. pilosa*, 2) *B. alba* achenes can be 2-3 awned or 3) *B. alba* and *B. pilosa* are not distinct species.

Investigation of the other distinguishing characteristics, particularly ploidy level, *B. alba* (n=24, tetraploid) and *B. pilosa* (n=36, hexaploid), would help explain the achene inconsistency observed.