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Pigment composition of putatively achlorophyllous angiosperms

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Key words: *Angiospermae, Lennoaceae, Monotropaceae, Orobanchaceae, Orchidaceae.* – Chlorophyll, carotenoid, pigment, high-performance liquid chromatography.

Abstract: Chlorophyll and carotenoid pigment composition was determined for ten species of putatively achlorophyllous angiosperms using high-performance liquid chromatography. Four families were represented: *Lennoaceae* (*Pholisma arenarium*); *Monotropaceae* (*Allotropa virgata, Monotropa uniflora, Pterospora andromedea, Sarcodes sanguinea); Orobanchaceae* (*Epifagus virginiana, Orobanche cooperi, O. uniflora*); *Orchidaceae* (*Cephalanthera austinae, Corallorhiza maculata).* Chlorophyll **a** was detected in all taxa, but chlorophyll **b** was only detected in *Corallorhiza maculata.* The relative amount of chlorophyll and chlorophyll-related pigments in these plants is greatly reduced compared to fully autotrophic angiosperms.

One of the most conspicuous features of plants is green coloration conferred by the presence of the pigment chlorophyll. However achlorophyllous plants, as their name implies, are thought to lack chlorophyll and other pigments associated with photosynthesis. This lack of chlorophyll is thought to be associated with the nonphotosynthetic habit, and hence the completely heterotrophic nature of holoparasites (KUIJT 1969; VISSER 1981, 1985) and some mycotrophic angiosperms (FURMAN & TRAPPE 1971, CULLEN 1978, MAAS 1986).

Putatively achlorophyllous plants occur throughout the angiosperms (CRONQUIST 1981) as shown in Table 1. The achlorophyllous habit has been used as a character to delineate taxa at several taxonomic ranks. For example, at the familial level lack of chlorophyll has been considered important in distinguishing *Orobanchaceae* from *Scrophulariaceae* (KUIJT 1969, HUTCHINSON 1973, CRONQUIST 1981), *Monotropaceae* from *Pyrolaceae* (HUTCHINSON 1973, CRONQUIST 1981), or *Monotropoideae* as a subfamily within *Ericaceae* (WALLACE 1975, CULLEN 1978), and *Cuscutaceae* from *Convolvulaceae* (KUIJT 1969, CRONQUIST 1981).

While many taxa are considered achlorophyllous (KUIJT 1969; FURMAN & TRAPPE 1971; HUTCHINSON 1973; WALLACE 1975; CULLEN 1978; MOORE 1978; STACE 1978; CRONQUIST 1981; VISSER 1981, 1985; MAAS 1986), there are contradictions in the literature about whether or not particular taxa have chlorophyll. For example VISSER (1985) considers dodder (*Cuscuta*) to be lacking chlorophyll. However,

Table 1. Angiosperm families with putatively achlorophyllous members. Data taken from CRONQUIST (1981)

Magnoliopsida		
Dilleniidae	*Ericales*	*Montropaceae*
Rosidae	*Santales*	*Balanphoraceae*
	Rafflesiales	*Hydnoraceae*
		Mitrastemmataceae (Syn. *Mitrastemonaceae*)
		Rafflesiaceae
	Polygales	*Polygalaceae*
Asteridae	*Gentianales*	*Gentianceae*
	Solanales	*Cuscutaceae*
	Lamiales	*Lennoaceae*
	Scrophulariales	*Scrophulariaceae*
		Orobanchaceae
Liliopsida		
Alismatidae	*Triuridales*	*Petrosaviaceae*
		Triuridaceae
Liliidae	*Orchidales*	*Geosiridaceae*
		Burmanniaceae
		Corsiaceae
		Orchidaceae

KUIJT (1969) cites several references that give evidence for chlorophyll in *Cuscuta*. Similarly, many authors state that members of *Orobanchaceae* are achlorophyllous (KUIJT 1969; HUTCHINSON 1973; STACE 1978; CRONQUIST 1981; VISSER 1981, 1985). One report for *Orobanche hederae* DUBY (BACCARINI & MELANDRI 1967) and one for *Q. fuliginosa* REUT. (KOLLMANN & al. 1969) noted no detectable chlorophyll, however earlier work showed the presence of chlorophyll in *Orobanche rubens* WALLR. (WIESNER 1872).

As pointed out by KUIJT (1969) examination of putatively achlorophyllous plants for the presence of chlorophyll would be a rewarding undertaking in light of advances in analytical techniques. High-performance liquid chromatography (HPLC) has proved to be an extremely sensitive technique for the detection of plant pigments (BRAUMANN & GRIMME 1981, GOODWIN & BRITTON 1988, RUDIGER & SCHOCH 1988, SHIOI 1990). The primary objective of the present study is to determine if evidence exists for the presence of chlorophyll and other pigments associated with photosynthesis in several putatively achlorophyllous angiosperms using HPLC.

Materials and methods

Plant material was collected from ten putatively achlorophyllous species in four families (Table 2), and stored at −70 °C until prepared for analysis. To avoid the possibility of contamination from extraneous sources of pigments such as algae, the plant material was

Table 2. Taxa, collection locality, and voucher information. *JVF* J. V. FREUDENSTEIN collections in the L. H. Bailey Hortorium (BH), Cornell University

Taxon	Locality	Voucher
Pholisma arenarium NUTT. ex HOOK.	San Diego Co., CA	no voucher
Allotropa virgata TORR. & A. GRAY	Josephine Co., OR	JVF 2297
Monotropa uniflora L.	Worchester Co., MA	no voucher
Pterospora andromedea NUTT.	Fresno Co., CA	JVF 2259
Sarcodes sanguinea TORR.	Butte Co., CA	JVF 2241
Epifagus virginiana (L.) BART.	Tompkins Co., NY	no voucher
Orobanche cooperi (A. GRAY) A. HELLER	San Diego Co., CA	no vouncer
O. uniflora L.	Middlesex Co., MA	no voucher
Cephalanthera austinae (A. GRAY) A. HELLER	Nevada Co., CA	JVF 2249
Corallorhiza maculata RAF.	Riverside Co., CA	no voucher

thoroughly washed with moderate scrubbing and rinsed in running sterile distilled H_2O. Because these plants do not possess well developed leaves the upper part of the shoot was used, and therefore the sample material analyzed consisted of some or all of the following; stems, leaves, scales, bracts, flowers, and flowerbuds.

To prepare the samples for pigment separation by reversed-phase HPLC, the plant material (3–8 g) was cut into small ($< 0.5\,cm^3$) pieces and the pigments were extracted in acetone solution (9:1, acetone:sterile distilled H_2O, v:v) overnight at 4 °C. The tissue was then macerated in sterile glass test tubes on ice using a Tekmar Tissumizer, and the extraction continued overnight at 4 °C. The total volume of acetone solution used was 8 ml per sample. The extraction mixture was centrifuged at 12000 × g and the supernatant filtered through sterile glass wool. A 2 ml aliquot of the supernatant was subsequently concentrated (2:1) using a C_{18} preparatory column. This was done by the washing the column with 2 ml of 100% acetone followed with 2 ml of sterile distilled H_2O, then adding the sample diluted 1:1 with sterile distilled H_2O, and eluting the absorbed pigments with 1 ml of 100% acetone. Just prior to HPLC injection, the sample extract was diluted 2:1 with sterile distilled H_2O. This last step reduced the sample solvent strength and prevented band spreading in the early eluting peaks. The injection volume was 250 µl. All supplies and equipment were washed with 100% acetone prior to coming into contact with the samples to prevent contamination. Additionally the syringe and injection port were cleaned with 100% acetone between injections.

The HPLC instrumentation consisted of a ternary gradient solvent delivery system (Spectra Physics 8700) with paired absorbance/fluorescence detectors plumbed inline (ISCO V-4 VIS absorbance detector set at 440 nm, and Kratos 950S filter fluorometer using Corning C-60/5-64 excitation/emission filter combinations and F4T4B lamp). Fluorescence detection provided a sensitive means of monitoring chlorophyll and its degradation products; these fluorescent pigments, when present in trace quantities, might otherwise be masked by more abundant, co-eluting carotenoids. A short (10 cm) C_{18} reverse phase column with 3 µm packing material (Rainin Microsorb) was used in all analyses. HPLC solvents consisted of 0.5 M aqueous ammonium acetate, methanol and acetone. Ammonium acetate solution and methanol were premixed (15:85, v:v) as the initial solvent in order to smooth the early baseline on the VIS detector. The programmed solvent delivery gradient is given in Table 3. Pigment identities were confirmed from elution times with authentic pigment standards.

Table 3. HPLC solvent gradient. Linear gradient profile between steps.* 85:15, vol:vol, ammonium acetate concentration 0.5 M

	Solvent		
Time (min)	Methanol-ammonium acetate*	Methanol	Acetone
0	100%	0	0
10	0%	85	15
14	0%	20	80
16	0%	20	80
18	100%	0	0

Results

Chlorophylls and chlorophyll-related pigments were separated and detected in the plant material analyzed by HPLC (Table 2). Chlorophyll **a** was detected in all plants examined, but chlorophyll **b** was only detected in *Corallorhiza maculata* RAF. The other chlorophyll-related pigments that were detected in some, but not all, of the taxa were pheophoribide **a** and pheophytin **a** (Table 4).

The amount of chlorophyll and related pigments is extremely low in these plants in comparision to leaves of fully autotrophic angiosperms. On a dry weight basis the proportion of chlorophyll in a green leaf may be about 1×10^{-2} (ROBINSON 1983). Assuming that representative angiosperm leaves are 13–20% dry weight (M. D. BOWERS, pers. comm.), then the plants examined in this study have a chlorophyll content on the order of 1×10^{-9}–10^{-7}. These estimates are concordant with the measured amount of chlorophyll and related pigments from *Cynodon dactylon* (L.) PERS. (*Poaceae*), which has been included in Table 4 for comparison.

In addition to chlorophyll, several carotenoid pigments were separated and detected in the plant material as shown in Table 5.

Table 4. Chlorophyll and chlorophyll-related pigments detected by HPLC. *N.D.* not detected. Mass: nanograms of pigment per gram fresh weight of tissue

	Chlorophyll				
Taxon	**a**	**b**	Pheophorbide **a**	Phenophytin **a**	Mass
Pholisma arenarium	×	N.D	N.D.	N.D.	1.26
Allotropa virgata	×	N.D.	×	N.D.	0.09
Monotropa uniflora	×	N.D.	×	N.D.	1.64
Pterospora andromedea	×	N.D.	N.D.	N.D.	1.24
Sarcodes sanguinea	×	N.D.	N.D.	N.D.	5.00
Epifagus virginiana	×	N.D.	×	N.D.	6.12
Orobanche cooperi	×	N.D.	×	N.D.	2.28
O. uniflora	×	N.D.	×	N.D.	1.80
Cephalanthera austinae	×	N.D.	N.D.	×	8.71
Corallorhiza maculata	×	×	×	×	26.75
Cynodon dactylon	×	×	×	×	521335.50

Table 5. Mass of carotenoid pigments detected by HPLC. *N. D.* not detected. Mass: nanograms of pigment per gram fresh weight of tissue. Lutein and zeaxanthin coelute and are therefore combined

Taxon	Unidentified	Neoxanthin	Violaxanthin	Antheraxanthin	Lutein/zeaxanthin
Pholisma arenarium	115.48	29.70	34.60	N.D.	0.43
Allotropa virgata	5.79	N.D.	4.54	51.71	46.81
Monotropa uniflora	1.02	N.D.	N.D.	2.91	3.65
Pterospora andromedea	21.58	12.58	6.45	129.72	231.06
Sarcodes sanguinea	12.75	5.30	N.D.	33.92	190.76
Epifagus virginiana	N.D.	N.D.	2.99	12.95	15.98
Orobanche cooperi	70.69	52.30	56.22	78.02	56.88
O. uniflora	271.23	53.98	150.82	111.22	96.28
Cephalanthera austinae	24.45	6.51	6.12	6.41	7.94
Corallorhiza maculata	226.08	100.55	59.60	48.64	754.11

Discussion

The results demonstrate that all of the species examined contained chlorophyll, and thus should not be considered achlorophyllous. It follows therefore, that the families to which these species belong should not be considered wholly comprised of achlorophyllous members, as has been the case for *Lennoaceae* (KUIJT 1969, HUTCHINSON 1973, MOORE 1978, CRONQUIST 1981), *Monotropaceae* (FURMAN & TRAPPE 1971, HUTCHINSON 1973, CRONQUIST 1981) and subfam. *Monotropoideae* within *Ericaceae* (WALLACE 1975, CULLEN 1978), and *Orobanchaceae* (KUIJT 1969; HUTCHINSON 1973; CRONQUIST 1981; VISSER 1981, 1985). However, these families may not be composed entirely of species that contain chlorophyll, other species not examined in this study may lack detectable chlorophyll. Other studies using less sensitive detection methods did not detect chlorophyll in *Monotropa hypopitys* L. (NEAMTU & BODEA 1971), *Orobanche hederae* (BACCARINI & MELANDRI 1967), and *O. fuliginosa* (KOLLMANN & al. 1969).

While both the low number of pigments and number of taxa in this study preclude extensive conclusions, a few comparisons based on life history can be made. *Monotropaceae* and members of *Orchidaceae* examined here are mycotrophic, and *Lennoaceae* and *Orobanchaceae* are root parasites, but no distinctions between these life history types is suggested based on pigment composition. From a systematic perspective *Orchidaceae* is distinct from the other families in the study due to the presence of pheophytin **a**. Within families all taxa display unique patterns of pigment composition with the exception of the two species of *Orobanche*, which show identical pigment profiles.

Because heterotrophic angiosperms are thought to have evolved from autotrophic progenitors (KUIJT 1969, FURMAN & TRAPPE 1971, CRONQUIST 1981) it is not surprising that they may have maintained some vestiges of their ancestry, including the presence of chlorophyll. Although the presence of chlorophyll alone does not mean that these taxa have the capability to photosynthesize, we are unaware of any other functional role ascribed to chlorophyll apart from the light reactions of photosynthesis. For example, while we detect chlorophyll in

Monotropa uniflora L., another study failed to detect evidence for photosynthetic carbon fixation in this species (MALIK & al. 1983). Similarly, other studies have also failed to demonstrate photosynthetic carbon fixation in *Orobanche hederae* (BACCARINI & MELANDRI 1967), and *O. lucorum* A. BR. (SOLDATINI & al. 1981). With the exception of *Monotropa uniflora*, we are unaware of reports on the photosynthetic capability of the species examined. However the loss of photosynthetic ability might conceivably occur by several means, and is not necessarily contemporaneous with loss of chlorophyll or other pigments. Biosynthetic constraints might be responsible for the continued presence of these pigments, as chlorophylls and related pigments might be precursors or byproducts during the course of synthesis of other biochemicals. In addition, other non-photosynthetic functions of chlorophyll, such as photodetection, cannot be excluded.

The presence of carotenoid pigments in these heterotrophs is not surprising, because carotenoid pigments are known to have non-photosynthetic functions, such as photoprotection (GOODWIN 1980). Even within fully photosynthetic plants carotenoid pigments are distributed in many non-photosyntheic tissues (GOODWIN 1980).

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