Pigment Studies in the Genus *Vaucheria*

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Abstract

Vegetative thalli of *Vaucheria dichotoma* and *Vaucheria geminata* were studied for their pigment content.

Chromatography and spectral absorption characteristics of the pigments from those 2 species revealed the presence of chlorophyll c as a minor fraction, in addition to chlorophyll a, β -carotene and 3 xanthophylls.

The fact of the presence of chlorophyll c in the genus *Vaucheria* offers a useful clue for discussing its systematic relationship.

The taxonomic position and the phylogenetic relationships of the genus *Vaucheria* have been much discussed. Whether one is considering the general morphology of this genus, its detailed cytology, the ultrastructure of the individual organelles, its way of reproduction, its chemical cell contents, or its pigment composition, there are always diagnostic features which do raise points of difficulty to give it with certainty a real taxonomic status.

In the past it has been placed in the Siphonales (Chlorophyceae, Euphycophyta)¹⁾. After it was found that several biochemical parameters were in contradiction with this point of view, its systematic position was revised.

Its cell walls do not contain native or mercerized cellulose as in other members of the Chlorophyceae, whilst the discoid chloroplasts, which lack pyrenoids, have an excess of β -carotene and starch is replaced by oil as the normal food storage material²⁻⁴⁾. Nevertheless Dangeard⁵⁾ and Descomps⁶⁾ claim the presence of pyrenoids in some *Vaucheria* species, although these structures seem to be questionable. Also the chemical nature of the major cell wall constituents gives cause for doubts⁷⁾. Analysis of the photosynthetic pigments revealed similarities to the Xanthophyceae⁸⁾.

On the basis of these biochemical characteristics it was concluded that *Vaucheria* should belong to the Heterosiphonales (Xanthophyceae, Chrysophycophyta), which, so far as general morphology is concerned, bear much resemblance to chlorophycean groups. So far as reproduction is concerned *Vaucheria* presents anomalies whether placed in the Siphonales or Heterosiphonales. Sexual reproduction in most of the Xanthophyceae is isogamous, while there is a well-developed oogamy in *Vaucheria*. Depending upon the stage of the life cycle the motile cells of *Vaucheria* are isokont or heterokont⁹⁾. The flagella of the spermatozooids correspond with those of the Xanthophyceae, the flagella of the zoospores with those of the Chlorophyceae.

Considering the very heterogeneous features of *Vaucheria*, Maekawa¹⁰⁾ proposed the taxon Vaucheriophyta, a division between Chlorophyta and Chrysophyta.

In a recent investigation of the cytology and ultrastructure of a number of

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species from several genera of Xanthophyceae, Hibberd and Leedale¹¹⁾ separated a new class, the Eustigmatophyceae. This study clearly emphasizes the taxonomic difficulties in the Xanthophyceae and the problem of the whole concept of heterokont organization.

Studies on the pigment content of *Vaucheria*, however, are not convincing, neither conclusive, and show even more discrepancies. According to Seybold and Egle¹²⁾ both chlorophylls a and b would be present in *Vaucheria*, while in further studies only chlorophyll a could be demonstrated^{8,13,14)}.

In *Vaucheria synandra* and *V. dichotoma* two types of chlorophylls were found¹⁵⁾: chlorophyll a and a "chlorophyll a like substance". Soma¹⁶⁾ showed that only at the zoospore stage there may exist "some other substance(s) different from chlorophyll a". Sagromsky¹⁷⁾ reexamined Soma's data and concluded that in zoospores phaeophytin a was present in addition to chlorophyll a.

The aim of the present work is to investigate the photosynthetic pigments of *Vaucheria* qualitatively and to work out this enigmatic problem with modern techniques.

Materials and Methods

Vaucheria species were collected from a natural stand. The plants were washed thoroughly in running tap water for several days to eliminate contaminating organisms. Only green thalli were selected for pigment analysis. The purity of the material was checked microscopically.

Fig. 1. Sex organs of *Vaucheria geminata*.

Fig. 2. *Vaucheria dichotoma*.
Upper: female filament with oogonium.
Lower: male filament with several antheridia.

The pigments were extracted with light petroleum plus 2% methanol by mixing in a Waring blender for 10 min. During extraction the mixture was cooled with ice.

By centrifugating the cell débris were precipitated and discarded. The supernatant was passed through a column of starch (3×100 cm). The column was washed with petroleum ether containing 50% benzene. In this way the pigments were roughly separated in a number of zones. Most of the chlorophyll c was absorbed on the upper part of the column. The large distance for washing the zones was very favorable for removing contaminating chlorophyll a in the trailing region of the chlorophyll c zone. After the different bands were dug out, the pigments of each zone were eluted in diethyl ether and concentrated under vacuum.

Further purification was carried out by thin layer chromatography. In order to characterize the pigment compounds several systems were chosen (Table 1). The use of various adsorbents with different activity grades and various mixtures of solvents of different polarity made it possible to obtain a successful separation of the applied substances.

For identification of chlorophyll c this pigment was prepared from the brown alga, *Fucus vesiculosus*, according to a modified method of Smith and Benitez¹⁸⁾. The spectral properties of chlorophyll c were then compared with the fraction isolated from the *Vaucheria* species.

Chopped thalli of *Fucus* were extracted two times with 1 liter methanol, each for 30 minutes. The methanol extracts were combined and treated with 200 ml of petroleum ether to remove most of the chlorophyll a.

A 2 liters portion of the methanolic solution was mixed with 1 liter diethyl ether and then shaken with 3 liters of saturated salt solution. The ether layer was removed and the aqueous alcohol layer extracted again with 800 ml of ether. The combined ether extracts were washed two times with 1 liter of saturated salt solution and 1 liter of distilled water. The emulsion formed during each washing contained an interfacial precipitate, rich in chlorophyll c. The emulsion was removed separately and dissolved in methanol. The methanol solution was mixed with ether and then shaken with saturated salt solution. This treatment transferred the chlorophyll c to ether.

All the ether extracts were combined, concentrated under reduced pressure and further purified by rechromatography on cellulose MN 300 plates, as described above (System I). The pigments were eluted with chloroform, benzene or petroleum ether.

Absorption spectra of intact thalli and of the pigments solutions were measured with a Shimadzu MPL 50 recording spectrophotometer.

Table 1.

System	Adsorbent	Solvent	Reference
I	cellulose (MN 300) impregnated with paraffin	paraffin-diethylether-acetone (20:20:1)	26)
II	kieselguhr impregnated with paraffin	acetone-methanol-water (50:47:3)	27)
III	idem	acetone-methanol-water (20:76:4)	27)
IV	cellulose	light petroleum-CCl ₄ (60:40)	28)
V	kieselgel	light petroleum-ethylacetate-dimethylamine (58:30:12)	29)

Results

Two species of the genus *Vaucheria* were used in this investigation, *V. geminata* (Fig. 1) and *V. dichotoma* (Fig. 2), as determined by means of their sex organs. Since the latter was abundantly found in the natural stand, most experiments were carried out with this organism.

From the absorption spectrum of thalli, measured *in vivo* (Fig. 3), it can only be concluded that chlorophyll a is present, according to the long wavelength absorption maximum at about 678 nm¹⁹⁾. The secondary and tertiary absorption bands of chlorophyll a are peaking higher, however, than normally would be expected. The absorption bands in the blue region reveal a mixture of several pigment components. After extraction of the pigments with light petroleum and 2% methanol, and chromatography on a starch column with the nonpolar solvent, benzene-light petroleum (1:1), several pigment zones were separated (Fig. 4a). We distinguished from bottom to top of the column: a light-yellow zone (fraction A), a yellow-orange band (fraction B), a concentrated, blue-green band (fraction C) and a pale, olive green band (fraction D). This last pigment zone of the column showed a very particular absorption spectrum, when eluted in ether (Fig. 5, A).

Next, each fraction was isolated, eluted in diethyl ether, concentrated and purified by thin layer chromatography (system I in Table 1). The results are shown in Fig. 4b. Fraction C was easily identified as chlorophyll a spectrophotometrically (Fig. 6). Fraction D, present as a very minor component, showed the spectral properties of chlorophyll c as compared to the standard preparation of chlorophyll c, obtained from *Fucus vesiculosus* (Figs. 5, B and 7). In fact

Fig. 3.

Fig. 4.

Fig. 3. Absorption spectrum (at 25°) of intact thalli.

Fig. 4. Chromatography of *Vaucheria* pigments.

- a) Different zones after column chromatography.
- b) Rechromatography on thin layer plates with System I (further explanation: see results).

we could observe two closely associated bands of chlorophyll c on the thin layer plates, but we were not able to separate them qualitatively for absorption spectrophotometry.

In Fig. 8 we present the absorption curve of fractions A and B. The identity of both yellow zones were tested for their polarity with different adsorbents and solvents (systems II, III and IV of Table 1). With systems II and III fraction A remained at the start line, while fraction B moved with the solvent and was sub-fractionated in 2 or 3 spots. Using system IV fraction A moved with the front line, while fraction B responded as an immobile spot. From this data we assumed that fraction A was one apolar substance, while fraction B has to be a more polar compound, being composed of several subfractions. The latter fraction was then chromatographed with system V. It spreads out in 3 bands. These components had very similar absorption curves (Fig. 9). We concluded that fraction A corresponds to β -carotene and that fraction B is a mixture of 3 xanthophylls, regarding their chromatographic behavior and their spectral properties. In an analogous way we could prove the presence of chlorophyll c in *Vaucheria geminata*.

Fig. 5.

Fig. 6.

Fig. 5. A. Absorption spectrum of the crude chlorophyll c fraction in diethyl ether after column chromatography.

B. Absorption spectrum of purified chlorophyll c in diethyl ether. This was prepared from the crude fraction by rechromatography on thin layers of cellulose MN 300, impregnated with paraffin and run in one dimension with paraffin-diethyl ether-acetone (20:20:1 v/v) as solvent.

Fig. 6. Absorption spectrum of purified chlorophyll a in chloroform.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 7. Absorption spectrum of purified chlorophyll c from *Vaucheria*(solid line), compared to that of chlorophyll c obtained from *Fucus vesiculosus* (dashed line). Chloroform was used as solvent.

Fig. 8. Absorption spectra of β -carotene (Fraction A) and of the sum of all the xanthophylls (Fraction B) in chloroform after thin layer chromatography (System I).

Fig. 9. Absorption spectra of the 3 xanthophylls, separated from fraction B with the use of System V. Solvent: ethanol.

Discussion

From our results, based upon chromatography and spectral absorption curves, we conclude that in *Vaucheria* chlorophyll c is present in addition to chlorophyll a, β -carotene and 3 unknown xanthophylls.

The presence of chlorophyll c in the genus *Vaucheria* does raise another point of real taxonomic difficulty, necessitating a review of the whole concept of its phylogenetic position. Studies on the photosynthetic pigments of many kinds of plants indicate a remarkable constancy of pigment distribution amongst the members of the same phylogenetic group²⁰⁻²².

Therefore, chlorophylls are generally accepted as an excellent parameter for phylogenetic relationship. In this view *Vaucheria* cannot be held in the class Xanthophyceae. Our finding gives thus support to the many other anomalies, found in the genus *Vaucheria* as mentioned in the introduction. The fact, that many authors^{8,12-17} did not succeed in isolating chlorophyll c from *Vaucheria* species, is primarily due to its scanty presence and to the difficulty for purifying this pigment from contaminating chlorophyll a. The crude fraction D obtained on the starch column was a "chlorophyll a like substance" as it was probably found by Sagermsky¹⁵ and Soma¹⁶ (Fig. 5, A).

Only by thoroughly washing the column over a large distance and by rechromatography on thin layer plates with appropriate solvents we could get rid of disturbing impurities.

The absorption maxima of the different pigments in *Vaucheria* (Table 2) are in good agreement with those reported in literature¹⁸.

Analyzing the absorption curves of different chlorophyll c preparations, dissolved in ether, we observed small shifts in the absorption maxima. The ratios of the Soret (445 nm) to red (629 nm) bands also showed slight differences.

These variations can be explained by the fact that there exist 2 spectrally distinct components in preparations of chlorophyll c in *Vaucheria*. Indeed, on the thin layer plates, 2 light green zones with very similar R_f-values were present (see results). This interpretation is supported by the work of Jeffrey²³ who could separate 2 chlorophyll c components from *Sargassum flavicans*.

Table 2. Absorption maxima (in nm) of *Vaucheria* pigments in different solvents.

<i>chlorophyll a</i> in chloroform	411 429		535 578	617	661
<i>chlorophyll c</i> in chloroform		456		586	635
in ether	445-449		579-582	628-629	
β -carotene in chloroform	434	461	489		
<i>xanthophylls</i> in ethanol					
X ₁	444	469			
X ₂	447	476			
X ₃	443	471			

The 3 unidentified xanthophylls show absorption maxima very close to those found by Strain *et al*²⁴⁾.

Comparing the ultrastructural organization of the plastids in *Vaucheria*⁶⁾ with other chlorophyll c containing algae, e.g. Phaeophyta, Bacillariophyceae and Chrysophyceae²⁵⁾ there is a striking similarity in their photosynthetic membrane arrangement: they are characterized by parallel series of 3 thylacoids. Hence, it would be very tempting to think that some chlorophyll form (c?) could act as a cementing agent in the partitions. However, some Xanthophyceae also have normal three-thylacoid lamellae¹¹⁾.

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References

- 1) Heering, W., Die Süßwasser-Flora Deutschlands, Oesterreichs und der Schweiz, p. 69 (Pascher, A., Ed., 1921).
- 2) Chadeaud, M., Bull. Soc. Bot. France 92: 47 (1945).
- 3) ———, Bull. Soc. Bot. France 98: 210 (1951).
- 4) Egerod, L.E., Univ. Calif. Publ. Bot. 25: 325 (1952).
- 5) Dangeard, P., Compt. Rend. Acad. Sc. Paris 205: 1429 (1937).
- 6) Descomps, S., Compt. Rend. Acad. Sc. Paris 256: 1333 (1963).
- 7) Maeda, M., Kuroda, K., Iriki, Y., Chihara, M., Nisizawa, K. and Miwa, T., Bot. Mag. Tokyo 79: 634 (1966).
- 8) Strain, H.H., Carneg. Inst. Washington Year Book 47: 97 (1948).
- 9) Koch, W.J., Elisha Mit. Sci. Soc. 67: 123 (1951).
- 10) Maekawa, F., Jour. Jap. Bot. 28: 105 (1953).
- 11) Hibberd, D.J. and Leedale, G.F., Nature 225: 758 (1970).
- 12) Seybold, A. und Egle, K., Planta 26: 491 (1937).
- 13) Montfort, C. und Küsters, G., Bot. Archiv. 40: 571 (1940).
- 14) Seybold, A., Egle, K. und Hülsbruch, W., Bot. Archiv. 42: 239 (1941).
- 15) Sagromsky, H., Zeitschr. Naturforschg. 12b: 684 (1957).
- 16) Soma, S., J. Fac. Sci. Tokyo 7: 535 (1960).
- 17) Sagromsky, H., Ber. Deutsch. Bot. Ges. 75: 345 (1962).
- 18) Smith, J.H.C. and Benitez, A., Modern Methods of Plant Analysis, p. 142 (Peach, K. and Tracey, M.V., Ed. Springer Verlag, Berlin 1955).
- 19) Shibata, K., J. Biochem. 44: 147 (1957).
- 20) Allen, M.B., French, C.S. and Brown, J.S., Comparative Biochemistry of Photoreactive Systems, p. 33 (Allen, M.B., Ed., Academic Press, New York, 1960).
- 21) Holt, A.S., Chemistry and Biochemistry of Plant Pigments, p. 3 (Goodwin, T.W., Ed., Academic Press, London, 1965).
- 22) Strain, H.H., Biochemistry of Chloroplasts, Vol. I, p. 387 (Goodwin, T.W., Ed., Academic Press, New York, 1966).
- 23) Jeffrey, S.W., Nature 220: 1032 (1968).
- 24) Strain, H.H., Svec, W.A., Aitzetmüller, K., Grandolfo, M. and Katz, J.J., Phytochem. 7: 1417 (1968).
- 25) Manton, I., Biochemistry of Chloroplasts Vol I, p. 23 (Goodwin, T.W. Ed., Academic Press, New York, 1966).
- 26) Randerath, K., Thin-layer Chromatography (Academic Press, New York, 1966).
- 27) Egger, K., Planta 58: 664 (1962).
- 28) ———, Chromatographie Symposium II, Brussel 1962, p. 75 (1965).
- 29) Riley, J.P. and Wilson, T.R.S., J. Mar. Biol. Assoc. U.K. 45: 583 (1965).