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Comparative Biochemical Studies on the Vegetative and Reproductive Stages of *Chara vulgaris*

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Abstract. Vegetative and reproductive stages of *Chara vulgaris* L. have been analysed for the major chemical constituents; ash, crude fibre, protein, lipid and carbohydrate. Distribution of sugars (free and combined), free amino acids, fatty acids, sterols and hydrocarbons in the two stages was determined. The vegetative stage was found to have considerably higher amounts of ash, carbohydrate and protein than the reproductive stage indicating that this stage is potentially a better source of these constituents. Reproductive stage was distinctive for its high content of lipids.

Significant variations were observed in distribution patterns of most chemical constituents examined and appeared to indicate differences with stage of growth. The distribution of combined sugars did not reveal any striking differences between the vegetative and reproductive stages. High levels of hydrocarbons and saturated fatty acids were observed in both stages.

Introduction

The Characeae is a family of highly-differentiated thalloid algae. Characean plants are of current interest since their long, filamentous internodal cells facilitate biophysical [1], physiological [2-4], cytological [5] and metabolic [6-8] experiments. Information on the chemical composition of Characeae, however, is very limited [9-13]. Most of these studies have ignored plant age (stage of growth). Consequently, some information upon the effect of stage of growth on the chemical nature of the alga seemed appropriate.

In this study, the sugar, amino acid, sterol, hydrocarbon and fatty acid composition of two stages of *Chara vulgaris* L. (vegetative and reproductive) were examined in order to make comparisons between the chemical composition of these stages, and to determine the relative value of these constituents for taxonomic purposes.

As far as the author is aware, this is the first study concerning the chemical composition of different stages of *C. vulgaris* collected from Egypt.

Material and Methods

Chara vulgaris L. was collected from a freshwater streamlet near Abu Hatab Village, El-Sharkeia Governorate, Egypt, in April, 1992 and was cultured in plastic tanks containing tap water and a mixture of sand and garden loam (2:3). The tanks were placed in an air-conditioned room under a 14 h light: 10 h dark cycle at 26 ± 1°C. Light was provided by white fluorescent lamps (50 $\mu E\ m^{-2}\ sec^{-1}$). This technique is a modification of many methods developed for cultivation of Characeans [6; 12; 14].

After 4 weeks from cultivation date, the plants showed active vegetative growth. These plants reached their reproductive (sexual) stage after 2 to 3 weeks period. Samples of vegatative stage were obtained from the plants that showed active vegetative growth. For reproductive stage, the plants bearing the sexual organs only were selected. The latter appears easy because these plants have distinctive form of sexual organs. pH values of water in tanks changed little (7.6-8.2) during the period of cultivation.

The samples of each stage (vegetative and reproductive) were thoroughly cleaned with running tap water to remove epiphytes, loam and sand particles and then rinsed many times in distilled water. The cleaned samples were spread on string nets and allowed to dry in air. Air-dried samples were ground and stored in stoppered bottles at room temperature.

Major chemical constituents

Samples of both stages were analysed for their ash, crude fibre, lipid, protein and carbonhydrate content. Ash content was determined following the method of Rosell and Srivastava [15]. Crude fibre content was determined according to the method described by El-Naggar [16]. Lipid was extracted in chloroform - methanol mixture (2:1 v/v) following the method of Holme and Hazel [17, p. 424]. Protein and carbohydrate were determined spectrophotometrically according to Coombs *et al.* [18, p. 298] and Dubois *et al.* [19] respectively. Results are means of three replicates.

Free and combined sugars

Free sugars were extracted in 80% (v/v) ethanol following the method described by El-Naggar [16]. As regards combined sugars, samples of air-dried algal materials pre-extracted with 80% ethanol were completely hydrolyzed with H_2SO_4 as described by Fisher and Dörfel [20], and El-Naggar [16]. Free and combined sugars were identified using thin layer chromatography with the following solvent systems.

n-butanol-acetic acid-water (4:1:5 v/v)
n-butanol-acetic acid-water (12:3:5 v/v)
n-butanol-ethanol-water (40:11:19 v/v)

Detection of sugars was affected with aniline hydrogen phthalate [21, p. 288]. Analyses of free and combined sugars were done in duplicate.

Free amino acids

Free amino acids were extracted in 70% (v/v) ethanol following the method described by El-Naggar [16]. The identification of amino acids was carried out using Amino Acid Analyzer Beckman 118 Cl. Analyses of amino acid were carried out in duplicate.

Lipoid constituents

The lipid samples were saponified with ethanolic KoH solution. The unsaponifiable matter (sterols and hydrocarbons) was isolated from the saponified neutral lipid fraction by extraction with ether. After removal of unsaponifiable matter, the aqueous solution left was acidified with 2.5% H_2SO_4 to liberate the fatty acids from their salts. The liberated fatty acids were extracted with ether. The methyl ester derivatives of the fatty acids were then prepared using the procedure of Moussa [22].

Analysis of fatty acid methyl esters was carried out using Pye Unicam Pu 4550 gas-liquid chromatograph on 1.5 m × 4 mm glass column packed with 10% polyethylene glycol adipate (PEGA). The carrier gas was nitrogen with flow rate of 30 ml/min. Column temperature programme was 70-190°C. Detector temperature was 300°C.

Analysis of unsaponifiable matter (sterols and hydrocarbons) was carried out using Pye Unicam GCV gas chromatograph on 1.5 m × 4 mm 3% OV - 17 glass column at 70-270°C. Detector temperature was 300°C with nitrogen flow rate of 30 ml/min.

Identification of fatty acids, sterols and hydrocarbons was carried out by comparing retention times and co-chromatography with those of authentic samples. The quantitative analysis was carried out by measuring peak areas. All analyses were carried out in triplicate.

Results

Major chemical constituents

Data recorded in Table 1 indicated that amounts of major chemical constituents in the two stages of *C. vulgaris* were quite variable. Ash, carbohydrate and protein were found to be more concentrated in the vegetative stage than in the reproductive stage. In contrast, lipids were higher in reproductive stage.

Table 1. Major chemical constituents of vegetative and reproductive stages of *C. vulgaris* (% dry weight)

Constituent	Vegetative stage	Reproductive stage
Ash	40.0 ± 2.6	33.0 ± 3.0
Crude fibre	21.0 ± 1.5	19.3 ± 1.0
Lipids	1.0 ± 0.1	2.8 ± 0.2
Protein	11.4 ± 0.8	9.1 ± 1.0
Carbohydrate	16.7 ± 1.7	14.1 ± 1.4

Values are means ± s.d. (n = 3)

Free and combined sugars

The present study has demonstrated that the distribution pattern of free sugars (Table 2) in the vegetative and reproductive stages of *C. vulgaris* was quite different. Sucrose, arabinose and rhamnose were detected in the vegetative stage while in the reproductive stage they were absent. Glucose, galacturonic acid and galactose were common in both stages.

The results also indicate that combined surgars, in contrast to free sugars, showed no remarkable difference between the vegetative and reproductive stages (Table 2). Both stages examined contained galacturonic acid, galactose and glucose in combined stage. However, fructose was further detected in the reproductive stage.

Table 2. Distribution of free and combined sugars in vegetative and reproductive stages of *C. vulgaris*

Sugar	Vegetative stage	Reproductive stage
a) *Free sugar*		
Glucose	+	+
Galactose	+	+
Galacturonic acid	+	+
Arabinose	+	−
Rhaminose	+	−
Sucrose	+	−
b) *Combined sugars*		
Glucose	+	+
Galactose	+	+
Galacturonic acid	+	+
Fructose	−	+

Free amino acids

Distribution patterns of free amino acids were found to vary with stage of growth (Table 3). Fifteen amino acids were found in the reproductive stage compared to thirteen in the vegetative stage. Cysteine, methionine, phenylalanine and arginine were detected in the reproductive stage while in vegetative stage they were replaced by proline and alanine. Aspartic acid, threonine, serine, glutamic acid, glycine, valine, isoleucine, leucine, ornithine, lysine and histidine were detected in both stages.

Lipoid constituents

Fatty acids

Distribution pattern of fatty acids indicated that fatty acid composition of the two stages had different characteristics according to the stage of growth (Table 4). Quality of fatty acids appeared to indicate a difference with the stage. Also, the levels of individual fatty acid showed considerable variation from stage to stage. 13:0, 14:0, 17:0, 18:0 and 20:0 were found to be more concentrated in the vegetative stage than in the reproductive stage. In contrast, 15:0, 16:0 and 18:3 were higher in the reproductive stage. Concentrations of 18:2 in both stages were relatively constant. In addition 6:0 and 18:1 were only detected in the reproductive stage.

Table 3. Distribution of free amino acids in vegetative and reproductive stages of *C. vulgaris*

Amino acid	Vegetative stage	Reproductive stage
Aspartic acid	+	+
Threonine	+	+
Serine	+	+
Proline	+	–
Glutamic acid	+	+
Glycine	+	+
Alanine	+	–
Valine	+	+
Cysteine	–	+
Methionine	–	+
Isoleucine	+	+
Leucine	+	+
Phenylalanine	–	+
Ornithine	+	+
Lysine	+	+
Histidine	+	+
Arginine	–	+

Table 4. Fatty acid composition of vegetative and reproductive stages of *C. vulgaris* (% total fatty acids)

Fatty acid	Vegetative stage	Reproductive stage
6:0	–	0.1 ± (<0.05%)
10:0	0.4 ± (<0.05%)	0.2 ± (<0.05%)
12:0	0.5 ± (<0.05%)	1.0 ± 0.2
13:0	25.1 ± 0.6	11.2 ± 0.3
14:0	2.3 ± 0.1	0.3 ± (<0.05%)
15:0	1.8 ± 0.2	9.5 ± 0.4
16:0	15.8 ± 0.4	23.8 ± 0.7
17:0	2.7 ± 0.3	1.5 ± 0.2
18:0	21.2 ± 0.9	11.4 ± 0.6
18:1	–	7.0 ± 0.3
18:2	8.2 ± 0.4	8.3 ± 0.3
18:3	12.9 ± 0.4	20.4 ± 0.6
20:0	9.2 ± 0.3	5.4 ± 0.3

Values are means ± S.D. (n = 3)

As indicated in Table 4, the fatty acid composition of the vegetative stage was apparent as follows: 13:0 and 18:0 were the most prominent, 16:0 and 18:3 were of moderate level, while 10:0 and 12:0 were detected in trace amounts. As regards reproductive stage, the major constituent fatty acids were 16:0 and 18:30. 13:0 and 18:0 were of moderate level. 6:0, 10:0, 12:0 and 14:0 were detected in trace amounts.

Sterols and hydrocarbons

GLC analysis of unsaponifiable lipid fraction showed that both stages were characterized by their higher content of hydrocarbons (Table 5). In contrast, concentrations of sterols were lower in the reproductive stage as well as vegetative stage.

The data recorded in Table 5 indicated that concentrations of hydrocarbons were greater in the reproductive stage than in the vegetative stage. As regards sterols, it was evident that the vegetative stage had much higher amount of sterols than reproductive stage.

A comparison of sterols occurring in vegetative stage with those occurring in reproductive stage showed that 28-isofucosterol and cholesterol were found in vegetative stage whereas the reproductive stage was found to contain only clionasterol. The proportion of 28-isofucosterol was higher representing 11.6% of total sterols and hydrocarbons detected.

Furthermore, the data indicated that significant variations were observed in the types of hydrocarbons in the two stages. It was found that a larger number of hydrocarbons was found in the vegetative stage (Table 5) with 20:0, 24:0 and 25:0 being restricted to this stage. In addition, concentrations of individual hydrocarbon in the two stages differed markedly. Concentrations of 18:0 and 28:0 were much higher in the reproductive stage than in the vegetative stage. Other hydrocarbons such as 16:0 and 32:0 also were relatively abundant in the reproductive stage. In contrast, the amount of 23:0 tended to be much higher in the vegetative stage.

The data in this study clearly indicated that only hydrocarbon 22:0 in the two stages was present in nearly equal amounts. It is also interesting to note that the vegetative stage was characterized by having high amounts of 25:0 and 24:0. These two hydrocarbons were particularly absent in the reproductive stage (Table 5).

Table 5. Hydrocarbon and sterol composition of vegetative and reproductive stages of *C. vulgaris* (% total hydrocarbons and sterols)

Constituent	Vegetative stage	Reproductive stage
1) *Hydrocarbon*		
16:0	0.6 ± 0.1	4.1 ± 0.2
18:0	3.3 ± 0.3	32.4 ± 0.4
20:0	1.3 ± 0.1	–
22:0	7.0 ± 0.2	7.3 ± 0.4
23:0	11.5 ± 0.6	8.4 ± 0.5
24:0	17.7 ± 0.4	–
25:0	22.2 ± 0.7	–
28:0	15.8 ± 0.8	29.4 ± 1.1
32:0	1.5 ± 0.1	9.9 ± 0.9
2) *Sterol*		
28-isofucosterol	11.6 ± 0.5	–
Clionasterol	–	8.5 ± 0.2
Cholesterol	7.3 ± 0.3	–

Values are means ± s.d. (n = 3)

Discussion

The present study has demonstrated that the total content of major chemical constituents of *C. vulgaris* is so strongly affected by the stage of growth. Ash, carbohydrate and protein were found to be more concentrated in the vegetative stage than in the reproductive stage. In contrast, accumulation of lipids has been noted in the reproductive stage. Early literature [23, pp. 131-201] has indicated that two phases of metabolic activity are distinguishable in many organisms: a phase of growth and a phase of fat accumulation. The extent of such variation suggests that the vegatative stage is a suitable material for extracting the majority of chemical constituents of *C. vulgaris*.

Significant variations were also observed in distribution of free sugars in *C. vulgaris* and appeared to indicate differences with the stage of growth. As shown in the results, the vegetative stage appear to have a larger number of free sugars than the reproductive stage. These results suggest that the vegetative stage may have the ability to maintain a large number of sugars in the free state.

A study of free sugars of *C. vulgaris* revealed that sucrose was detected in the vegetative stage whilst it was absent in the reproductive stage. There is a possible explanation for this variation. Sucrose was reported to be one of the main photoassimilates transported from leaf to non-photosynthetic tissues in higher plants [24, pp. 395-431]. In *C. vulgaris* Kirst *et al.* [25] found that the concentration of sucrose in the vacuolar sap increased from about 2 $mol.m^{-3}$ during the vegetative growth to 110 $mol.m^{-3}$ at the initiation of sexual reproduction. According to Ding *et al.* [8], intercelluarly – transported sucrose may be first incorporated as starch and stored during the vegetative growth and then used for the formation of the reproductive organs. Consequently, the plant after formation of the reproductive organs may be free from sucrose.

Distribution of combined sugars in *C. vulgaris* did not reveal any striking differences between the vegetative and reproductive stages with the exception of fructose found only in the reproductive stage.

Based on the results of the present study, one may reach to the conclusion that glucose, galacturonic acid, galactose, fructose, arabinose, rhamnose and sucrose seemed to be the sugar constituents of the carbohydrate of *C. vulgaris*. In this connection, it should be mentioned that all sugars detected in *C. vulgaris* are those commonly found in Characeae [9;10;26] and green algae [16; 27-30].

With regard to the free amino acid composition, the results showed the presence of 17 amino acids in the vegetative and reproductive stages (Table 3). The presence of the above mentioned free amino acids in many Characean species has been demonstrated by Sakano *et al.* [31;32] and Sakano and Tazawa [12]. Furthermore, the distribution pattern of the free amino acids in *C. vulgaris* was affected by the stage of growth. Cysteine, methionine, phenylalanine and arginine were only detected in the reproductive stage. At the same time, proline and alanine were only observed in the vegetative stage.

In both stages of *C. vulgaris,* 13 fatty acids were detected. Fatty acid composition was generally in agreement with the known distribution of fatty acids in green algae [33, pp. 236-265; 34, pp. 433-525; 35]. In the present study, the fatty acid composition of the examined species was also influenced by the stage of growth. The stage of growth appear to affect it in many ways; the number of fatty acids was promoted when *Chara* in reproductive stage; the ratio of unsaturated to saturated fatty acids was greater in the reproductive stage than in the vegetative stage; the proportion of saturated fatty acids was greater in the vegetative stage than in the reproductive stage. The higher content of saturated fatty acid in the vegetative stage may

suggest possible uses of these products as food supplements if sufficient quantities of the alga are available. The predominant fatty acids in the two stages varied considerably. 13:0 and 18:0 were the predominant acids in the vegetative stage whereas 16:0 and 18:3 were particularly abundant in the reproductive stage. Finally, the concentrations of the individual fatty acid changed according to the stage of growth.

The hydrocarbon composition of the two stages of *C. vulgaris* is different. The results indicate that the amount of hydrocarbons varied with the stage of growth. Hydrocarbons were found to be more concentrated in the reproductive stage than in the vegetative stage. A similar variation has been reported previously for the green alga *Botryococcus braunii* [36-39].

The idea of utilizing solar energy through photosynthesis in algae for the production of renewable sources of energy has been considered in a few recent publications [40-42]. It is interesting to note that the capability of algae to produce large amounts of hydrocarbons has led to utilization as a biosolar energy converter for the production of liquid fuel [42]. In the present study, the hydrocarbons were the major fraction of the unsaponifiable matter of reproductive (91.5%) and vegetative (81.1%) stages of *C. vulgaris*. Furthermore, the high production of hydrocarbons in *C. vulgaris* was made under normal growth conditions. In this connection, it should be mentioned that in microalgae the high production of hydrocarbons occurs under limited growth condition [42]. Consequently, the present results suggest that *C. vulgaris* may be utilized as a biosolar energy converter for the production of liquid fuel.

Marked variations were also evident in quality and quantity of sterols in both stages of *C. vulgaris*. Sterols were found to be more concentrated in the vegetative stage than in the reproductive stage. 28-isofucosterol and cholesterol were detected in the vegetative stage while in the reproductive stage clionasterol only was observed. Patterson [11] reported the sterol composition of *Chara vulgaris* and *Nitella flexilis* and his results are, in general, similar to those in the present investigation. Clionasterol and 28-isofucosterol have been identified in members of the chlorophyta, but they have not been previously identified together in the same organisms [11].

The presence of clionasterol in the reproductive stage only is a distinctive finding. However, it is difficult to give an accurate explanation for presence of clionasterol in the reproductive stage and its absence in the vegetative stage. There is a possible explanation which may be illustrated as follows: 28-isofucosterol which is determined in the vegetative stage of *C. vulgaris,* was reported to be a biosynthetic precursor of sitosterol [43]. According to Patterson [11] the enzyme systems of algae, which

always seem to produce sterols with the 24 β configuration, convert 28-isofucosterol to clionasterol, rather than sitosterol, as occurs in higher plants [43]. Therefore, based on the present results, it can be said that this conversion occurred during the formation of reproductive organs. This suggestion may explain why clionasterol was detected only in the reproductive stage. This aspect deserves further investigation.

In conclusion, two generalizations can be formulated with respect to the chemical composition of *C. vulgaris:*

1) Each stage of growth has its own chemical constituents which may or may not coincide with that of other stages. Similar observation has been reported previously for the production of antimicrobial compounds by marine algae. Hornsey and Hide [44] reported that there is a variation in the antibacterial content of different life-cycle phases. Consequently, to give a complete picture of the chemical nature of the alga, the different stages of growth should be analysed.

2) Use of any chemical constituent as a tool in the chemotaxonomy of the alga should be common to the different stages of growth. This is a distinctive finding in view of the difficulties in classifying the Characean plants. These algae have been placed in the Chlorophyta by some phycologists [45;46, pp. 29-72; 47, p. 645] and in a separate division (Charophyta) by others [48, p. 632].

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دراسات كيموحيوية مقارنة على مرحلتي التكوين الخضري والتكاثر لطحلب *كارافولجارس*

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ملخص البحث. تمّت دراسة المكوّنات الكيميائيّة الكبرى لمرحلتيْ التكوين الخضري والتكاثر لطحلب *كارافولجارس* وشملت كُلًّا من الرماد والألياف والبروتين والليبيدات والكربوهيدات. بالإضافة إلى ذلك، تمّت دراسة توزيع كُلٍّ من السكريات الحرة والمعقدة، والأحماض الأمينية الحرة، والاستيرودات والأحماض الدهنية والهيدروكربونات في هاتين المرحلتين.

أوضحت هذه الدراسة أنّ كمية كُلًّا من الرماد والكربوهيدرات والبروتين كانت أكثر تركيزًا في المرحلة الخضرية عما هي في المرحلة التكاثرية مما يعني أنّ هذه المرحلة يمكن اعتبارها مصدرًا جيّدا لهذه المكوّنات الكيميائية. في الوقت نفسه تميزت المرحلة التكاثرية بمحتواها العالي من الليبيدات.

دلّت هذه الدراسة أيضًا على أنّ هناك تباينًا واضحًا في توزيع معظم المكوّنات الكيميائية التي دُرست خلال هذه الدراسة وأنّ هذه الاختلافات مرتبطة بمرحلة النمو. الاستثناء الوحيد أنّه لم يكن هناك اختلاف واضح في توزيع السكريّات المعقدة بين هاتين المرحلتين.

بصفة عامة، وجدت كميات عالية من الهيدروكربونات والأحماض الدهنية المشبعة في هذا الطحلب.