Research Article

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The effect of *Cladophora glomerata* exudates on the amino acid composition of *Cladophora fracta* and *Rhizoclonium* sp.

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Abstract: Filamentous green algae (FGA) frequently forms dense mats which can be either mono- or polyspecies. While various defense mechanisms of competition in algae are known, little is known about the interactions between different species of FGA. An experiment in controlled laboratory conditions was conducted to gather data on the changes in amino acids (AA) concentrations in FGA species in the presence of exudates from different other species. The aim of the present study was to identify the AA whose concentrations showed significant changes and to assess if the changes could be adaptation to stress conditions. The major constituents of the AA pool in *Cladophora glomerata*, *C. fracta* and *Rhizoclonium* sp. were Glutamic acid (Glu), Aspartic acid (Asp) and Leucine (Leu). In response to chemical stress, that is the increasing presence of exudates, a significant increase in the concentrations Proline (Pro) and Tryptophan (Trp) was noted. The increase in Proline levels was observed in *C. fracta* and *Rhizoclonium* in response to chemical stress induced by *C. glomerata* exudates. As the concentration of exudates increased in the medium, there was a progressive shift in the pattern of AA group in FGA.

Keywords: freshwater *Cladophora*; *Rhizoclonium*; biochemical changes; proline, amino acids; exudates.

1 Introduction

Cosmopolitan filamentous green algae (FGA) such as branched *Cladophora* and unbranched *Rhizoclonium* often experience massive growth in eutrophic water ecosystems, forming dense, seasonal mats [1-4]. The mats are varied in many ways and one of their characteristic features is the number of taxa forming them [5-7]. In some cases they are formed from only one taxon whose rapid growth monopolizes the space on the water; other species co-exist with the dominant one or disappear enhancing the dominant species [8,9]. According to Khanum [8] *Cladophora glomerata* (L.) Kützinger, the most common Chlorophyta in the world, forms mainly monoalgal mats but sometimes becomes dominant in an algal community. The *C. glomerata* algae may co-exist with other *Cladophora* species or with other FGA taxa such as *Rhizoclonium*, *Oedogonium* [3,10]. Filaments of *C. glomerata* formed monoalgal mats in the Samica Stęszewska river, Lake Oporzynskie; they co-existed with *Rhizoclonium* sp. Kützinger in Mogilnica river and with *Cladophora fracta* (L.) in Malta reservoir [11,12]. The wide ecological range and fast growth of *C. glomerata* is a strategy which allows it to establish dominance over other macroalgae in water ecosystems [13]. This fact, confirmed in previous studies, has shown that the species belonging to the genus *Cladophora* (*C. glomerata*, *C. rivularis*, *C. fracta*) well tolerates a wide range of habitat requirements [12,14]. Additionally, mat formation, morphological variability, and chemical defense strategies used by FGA are the components of its stress avoidance mechanisms.

Several types of interactions between algae are seen in nature, e.g. allelopathic, non-allelopathic interactions and responses to different factors like resource competition or environmental stresses [15]. In water environments, *Cladophora* displays a high level of interactions with other organisms (microalgae, macroalgae, macrophytes) related to competing for light exposure, nutrients, and attachment substrate [16]. Much of the available evidence demonstrates

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competition between macroalgae and phytoplankton including periphytic algae and macroalgae-macrophytes but not between macroalgae and macroalgae. *C. glomerata* can prevent overgrowth by periphytic diatoms because dense coverings of diatoms would interfere with nutrient uptake by *Cladophora* and therefore inhibit growth [16,17]. According to Tarmanowska [18] *C. glomerata* inhibits the growth of *Elodea canadensis* Michx. Moreover, *Cladophora* is an example of a freshwater chlorophytes species which is capable of mediating allelopathic interactions, causing an increase in allelochemical concentration in response to competitors [1,19]. The green alga *C. glomerata* synthesizes a wide variety of toxic fatty acids (capric and palmitoleic acids) which have been demonstrated to be allelopathic [1]. Extract from *C. glomerata* slightly reduces the photosynthetic rate of diatoms [17].

Although the morphological variability related to the effect of different biotic and abiotic factors is well-known, the changes in the contents of proteins synthesized in algae biomass are little known. According to literature, only changes in Proline accumulation in response to environmental stress have been studied. The defense of resources by FGA involves competition, which may influence biochemical and morphological changes in cells and filaments - which have not been yet sufficiently studied [20]. This study focuses on *C. glomerata*, the most common and dominant FGA in the world and other species interaction with *C. fracta* and *Rhizoclonium* sp. We hypothesize that the presence of *C. glomerata* exudates have a significant impact on the amino acid content in *C. fracta* and *Rhizoclonium* sp. The results of this study could provide first data for understanding the mechanisms of *C. glomerata* response to competition and its strategy of stress avoidance.

2 Materials and methods

2.1 Raw materials and their collection

The study was carried out in June 2015 when freshwater populations of FGA were in the optimal phase of development. Samples of *Cladophora glomerata* were taken from the shallow Lake Oporzynskie (N52°55'; E17°9'), *Cladophora fracta* from the surface of the Malta Reservoir in Poznań (N52°24'; E16°57') and *Rhizoclonium* sp. from a pond in Poznań (N52°28'; E16°55') in Wielkopolska Province in Poland. All samples were randomly selected from the mat at a depth of about 10 cm from the water surface. The filamentous green algae samples were collected

into a plastic container and transported in a refrigerated container (at 4°C) to a laboratory. Next, the filaments were rinsed repeatedly with distilled water.

2.2 Microscopic identification

Morphometric measurements of the length and width of cells, pyrenoids (stained with Lugol's) and nuclei (stained by 1% acetocarmine) were made using a light microscope (LM). Images of opportunistic *C. glomerata* were made using a transmission electron microscopy (TEM), scanning electron microscopy (SEM) and light microscopy (LM). TEM images were recorded of filaments in control samples and filaments in the presence of 100% exudates. For the TEM technique, the filaments of *C. glomerata* were fixed in a mixture of glutaraldehyde (3%) in 0.1 M phosphate buffer (pH= 7.2) for two hours, washed several times in a buffer and postfixed in 1% osmium tetroxide (OsO₄) in the same buffer. The cells were dehydrated by an ascending ethanol series, according to the method proposed by Farias et al. [21]. Finally, the samples were embedded in Spurr's resin (Low viscosity, ProScitech) [22] to produce a solid block, which was cut with and ultramicrotome (Leica EM UC7) at 60 nm, the ultrathin sections were contrasting in uranyl acetate, according to methods described by Reynolds [23]. Observations and photographs were carried out on a TESLA BS 500 transmission electron microscope.

2.3 Determination of total phenolic content

The total phenolic content of the filamentous green algae extract was determined by the Folin-Ciocalteu spectrophotometric method [24]. First, 30 µL of crude extract (10 mg/mL) were diluted with distilled water to achieve 1.8 mL, mixed thoroughly with 1.5 mL of Folin-Ciocalteu, followed by the addition of 4.5 mL of 20% (w/v) sodium carbonate and 5.7 mL of distilled water. The mixture was allowed to stand for further 2 hours in the dark, and absorbance was measured at 760 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight.

2.4 Determination of pigments

Photosynthetic pigments: chlorophyll a, chlorophyll b and carotenoids concentrations in the filaments were analyzed according to the methods proposed by Yoshii

et al. [25] and Dere et al. [26] and were determined spectrophotometrically using the equations proposed by Lichtenthaler and Welburn [27].

2.5 Experimental set-up

The first experiment was designed to observe the mutual impact of filamentous green algae (*C. glomerata*, *C. fracta*, *Rhizoclonium* sp.) exudates on each other and on the composition of their amino acids. For the laboratory experiments, ca. 500 g fresh weight (FW) of either *C. glomerata*, *C. fracta* and *Rhizoclonium* sp. were placed into 3 aquaria which were fully filled up with 10 L of filtered and sterilized freshwater. FGA were acclimated and maintained in aquaria for 24 h. Then, cultures of FGA species were incubated in Wang's medium at a temperature of 21°C and in a diurnal regime (16/8 h light/dark cycle, 250 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in phytothron (CONVIRON model CMP 6050). The cultures were left to rest for 7 days so that the green algae species secreted the exudates to the medium. Upon commencing the experiment, algal pieces of ca. 5.0 g FW (only filaments in good condition, without evidence of necrosis) were placed into 200 mL beakers. In the experiment the control sample contained Wang's medium (100 % medium) and three samples with the medium containing algae exudates at different concentrations (25, 50, 100% exudates in medium). Each level was studied in triplicate using glass containers with 200 mL of medium and 5 g of fresh algae biomass. Four series of experiments were performed; the first with *C. fracta* cultured in the medium with *C. glomerata* exudates; the second with *C. glomerata* cultured in the medium with *C. fracta* exudates; the third with *Rhizoclonium* sp. with *C. glomerata* exudates and the fourth with *C. glomerata* filaments in the presence of *Rhizoclonium* sp. exudates. Each time, after completion of the series of experiments, the FW of FGA filaments was determined, as well as their DW after drying in a laboratory oven for 2 hours at 105°C. Then, the filaments were put into plastic 100 mL containers.

2.6 Amino acids analysis in biomass

The algae samples were analyzed using the modified Kjeldahl procedure (AoAC 2007) for dry matter (method no. 934.01) and ash (method no. 942.05). Hydrolysis of total protein was performed according to the protocol described by Malmer and Schroeder [28]. The amino acid standards alanine (Ala), aspartic acid (Asp), arginine (Arg), cysteine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys),

methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), tyrosine (Tyr), threonine (Thr) and valine (Val) were from Sigma-Aldrich (Steinheim, Germany), and the amino acid standard tryptophan (Trp) were purchased from Merck (Darmstadt, Germany). The ground algae samples were subjected to acid hydrolysis with 6 M HCl at the temperature of 383.15 K for 23 hours. Amino acids were determined using a AAA 400 amino acid analyzer (INGOS, Czech Republic) with ion exchange chromatography. Post column ninhydrin-based detection and sodium citrate buffer were used. The ninhydrin amino acid derivatives were detected using packing of column OSTION Lg ANB (column height: 35 x 0.37 cm) at 570 nm for primary amino acids and at 440 nm for secondary amino acids. Amino acid concentrations in samples were calculated from calibration plots obtained by analysis of working solutions of different concentration. The contents were expressed as $\text{g} \cdot 100\text{g}^{-1}$ of total proteins.

2.7 Statistical analysis

The homogeneity of the variance was analyzed using Levene's test. A One-way ANOVA followed by Tukey's RIR post hoc test was used to determine significant differences between the control and treatment groups. Statistical tests were performed with the program STATISTICA (ver. 12). Differences were considered to be significant at $p < 0.05$.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results

3.1 Freshwater filamentous green algae: morphology and chemical composition

The studied FGA formed dense, monoalgal, free-floating mats in eutrophic freshwater ecosystems. The morphology of algae taxa was studied carefully, and the taxa were identified as *C. glomerata*, *C. fracta* and *Rhizoclonium* sp. classified as Order: Cladophorales, Class: Ulvophyceae and Phylum: Chlorophyta. The detailed taxonomic description is included in Table 1. The *C. glomerata* filaments were strongly branched and inhabited by large numbers of periphytic communities which were responsible for their roughness (Figure 1). Each cell in the branching filaments of *C. glomerata* is multinucleate and contains many discoid chloroplasts in peripheral arrangement. The pyrenoid is bilenticular and is divided into two hemispheres by a single thylakoid, and each hemisphere is covered by a

Figure 1: Details of the ultrastructure of *Cladophora glomerata* thalli: a – general view of branched filaments (LM); b - surface of filament inhabited by periphytic diatoms (SEM); c – TEM micrograph of mitochondrion (M) in the vicinity of chloroplast (Chl) and pyrenoid (P) surrounded by chloroplast; the arrangement of thylakoids (Th) in chloroplast; internal singular vacuole (V) well organized; d – cell wall (CW), external layer and internal division of two cells; lipids on the chloroplast (Chl); (c, d – scale bar = 0.5 μm).

Table 1: Morphology and chemical characteristic of freshwater green algae (values: minimum – maximum or mean $\pm$ SD, n=30 for morphology data, n=3 for chemical data).

Taxon Features		<i>Cladophora glomerata</i>	<i>Cladophora fracta</i>	<i>Rhizoclonium</i> sp.
MORPHOLOGY	Filaments characteristic	dark green, rough in touch, often trichotomies and subtending branching	yellow green, delicate, branching frequent	dark green, long, rough in touch, mainly without branched or only with short branched
	Cell shape	cylindrical, elongated	cylindrical	cylindrical
	Cell width (μm)	30 – 70	20 – 31	20 - 55
	Cell length (μm)	125 – 400	50 – 155	70 - 174
	Shape of chloroplasts	reticulate	parietal	reticulate/parietal
	Diameter of pyrenoids (μm)	4.5 - 7.2	2.8 -5.6	2.9-5.5
	Diameter of nuclei (μm)	3.8 - 8.5	1.9 - 4.1	2.2-4.6
CHEMICAL COMPOUNDS	Pigments ($\mu\text{g mL}^{-1}$):			
	Chl a	8.45 $\pm$ 0.18	3.13 $\pm$ 0.12	4.43 $\pm$ 0.10
	Chl b	6.36 $\pm$ 0.11	2.95 $\pm$ 0.07	2.81 $\pm$ 0.08
	Carotenoids	2.32 $\pm$ 0.03	0.72 $\pm$ 0.05	1.15 $\pm$ 0.03
	Amino Acid	18 AA	18 AA	18 AA
	(g $\cdot$ 100g $^{-1}$) of total protein)	91.17 $\pm$ 0.24	90.33 $\pm$ 0.28	90.30 $\pm$ 0.37
	Total phenolic content mg gallic acid equivalent (GAE)/g DW	17.32 $\pm$ 0.23	11.55 $\pm$ 0.21	12.28 $\pm$ 0.14

Figure 2: Amino acid profile in study algae (g·100g⁻¹ of total protein).

bowl-shaped starch grain. Each chloroplast is densely packed with tightly appressed thylakoids which are often paired. Grains of starch are distributed through the stroma of the chloroplast. The nucleus has a very large nucleolus. Each cell contains a single Golgi body, and two, sometimes three large, elongated mitochondria with very long cristae. In the cell cross-section, the greater part of the cell can be seen occupied by a central vacuole surrounded by a thin layer of cytoplasm. The cell walls are multilayered and composed of microfibrils (12-22 nm in diameter) arranged in parallel to each other. In contrast, *C. fracta* cells were more delicate, its filaments were thinner than those of the other FGA studied and were like cotton to the touch. Filaments of *Rhizoclonium sp.* were characterized by the same width close to 50 µm. Among the studied algae, *C. glomerata* had got the highest content of pigments, amino acids and total phenolic content (Table 1). The chl a, chl b and carotenoids concentration in *C. glomerata* biomass was almost twice higher than in *C. fracta* or in *Rhizoclonium sp.* In the studied FGA the same 18 amino acids have been identified (Figure 2). The relative abundance of AA in *C. fracta* decreases in the order: Glu> Asp> Leu> Arg> Val> Ala> Gly> Lys> Thr> Ser> Pro> Phe> Ile> Tyr> His> Cys> Met> Trp. The analogous order for *C. glomerata* was: Glu> Asp> Leu> Arg> Val> Ala> Gly> Lys> Thr> Pro> Ser> Phe> Ile> Tyr> His> Cys> Met> Trp. In *Rhizoclonium sp.* the distribution order from higher to lower was: Glu> Asp> Leu> Arg> Val> Ala> Gly> Lys> Ser> Thr> Pro> Phe> Ile> Tyr> His> Cys> Met> Trp. The abundance of AA in the studied FGA decreased in similar order and varied only in the sequence of Thr, Ser and Pro (underlined). Moreover, a considerable difference in phenols content was noted for the taxa studied and its highest concentration was in *C. glomerata*.

3.2 Amino acid composition

3.2.1 Impact of exudates from *C. glomerata* on *C. fracta* amino acids production

In *C. fracta* filaments the presence of 18 amino acids was identified. Glutamic acid (Glu), Aspartic acid (Asp) and Leucine (Leu) were the most frequent (>10% of total AA content) while the least frequent (<1% of total AA content) was Methionine (Met) and Tryptophan (Trp) (Table 2). Production of AA in *C. fracta* cells was significantly higher for the filaments growing in the medium with *C. glomerata* exudates than in the control sample. For 12 AA the changes were statistically significant (Table 2). The concentration of 6 AA in the *C. fracta* cells was increased in response to increasing the exudates content. The greatest changes were observed for Proline: from 4.497 g·100g⁻¹ of total protein in the control sample to 6.523 g·100g⁻¹ of total protein in the medium with 100% of algal exudates and Tryptophan (Trp) from 0.359 to 0.466 g·100g⁻¹, respectively. However the content of Asp, Glu significantly increased only in the medium with the highest exudates concentration (100%), in contrast to the contents of Ala, Lys and Phe which decreased (Table 2, S1).

3.2.2 Impact of exudates from *C. fracta* on *C. glomerata* amino acids production

The data in Table 3 show changes in the 18 amino acids levels in *Cladophora glomerata* thalli from the culture in the medium with *C. fracta* exudates. The most abundant amino acids in *C. glomerata* filaments were Glu, Asp, Leu and Arg. The results indicate a gradual increase in the

Table 2: The content of amino acids (AA) in *Cladophora fracta* biomass cultured in medium with exudates from *C. glomerata* in 3 series (g·100g⁻¹ of total proteins; df=3; bold - statistically significant, P<0.05; gray selection - fluctuation in the content of amino acid > 10%).

Impact of exudates extracted from <i>C. glomerata</i> on <i>C. fracta</i> AA production						
AA	Control	25% exudate	50% exudates	100% exudate	F _{3,8}	P
Glutamic acid (Glu)	12.690±0.008	12.706±0.007	12.704±0.002	12.731±0.003	8.939	0.000120
Aspartic acid (Asp)	10.527±0.002	10.528±0.004	10.532±0.002	10.567±0.004	1.561	0.000001
Leucine (Leu)	9.646±0.002	9.646±0.004	9.650±0.002	9.651±0.002	2.224	0.162901
Arginine (Arg)	7.412±0.001	7.409±0.002	7.413±0.002	7.414±0.001	5.333	0.026000
Alanine (Ala)	5.457±0.001	5.456±0.004	5.459±0.002	5.164±0.510	0.990	0.444947
Valine (Val)	5.512±0.001	5.510±0.002	5.512±0.002	5.517±0.005	4.444	0.040679
Glycine (Gly)	5.173±0.001	5.171±0.003	5.174±0.001	5.179±0.003	7.379	0.010840
Lysine (Lys)	4.824±0.001	4.825±0.001	4.827±0.002	4.799±0.003	1.186	0.000000
Proline (Pro)	4.496±0.002	4.553±0.005	5.464±0.004	6.523±0.03	5.290	0.000000
Serine (Ser)	4.502±0.001	4.504±0.002	4.506±0.002	4.506±0.003	2.119	0.176059
Threonine (Thr)	4.527±0.001	4.525±0.007	4.530±0.001	4.536±0.117	3.916	0.054420
Phenylalanine (Phe)	4.410±0.001	4.410±0.002	4.412±0.002	4.345±0.001	0.939	0.465777
Isoleucine (Ile)	3.401±0.002	3.392±0.005	3.402±0.001	3.407±0.003	17.194	0.000756
Tyrosine (Tyr)	2.873±0.002	2.871±0.002	2.870±0.005	2.876±0.002	2.733	0.113590
Histidine (His)	2.207±0.001	2.209±0.002	2.209±0.002	2.212±0.003	4.471	0.040118
Cysteine (Cys)	1.505±0.002	1.501±0.003	1.503±0.001	1.509±0.005	6.925	0.012961
Methionine (Met)	0.798±0.002	0.797±0.003	0.811±0.001	0.815±0.002	26.589	0.000164
Tryptophan (Trp)	0.357±0.002	0.364±0.002	0.382±0.001	0.466±0.001	30.667	0.000000

contribution of all AA in *C. glomerata*, but significant changes occurred only for the samples from the medium containing 50% or 100% of *C. fracta* exudates. The effect was the same for all of amino acids in *C. glomerata* (Table S2). The content of Proline was increased from 4.5 to 4.9 g·100g⁻¹ of total proteins and it was the highest change in the AA compositions.

3.2.3 Impact of exudates from *C. glomerata* on *Rhizoclonium* sp. amino acids production

Amino acids in *Rhizoclonium* sp. showed a different response to addition of exudates from *C. glomerata*. The effect of experiment was irregular: the level of AA increased or decreased in response to addition of the exudates (Table 4). The contents of Proline and Lysine increased in the sample with the maximal concentration of macroalgal exudates when compared to those in the control sample.

However, the contents of Asp, Ile and Tyr were reduced in the sample with 100% exudates relative to those in the control sample. The changes in the concentration of the other AA were less pronounced, although all changes in AA were statistically significant (Table S3).

3.2.4 Impact of exudates from *Rhizoclonium* sp. on *C. glomerata* amino acids production

The results shown in the Table 5 indicate a gradual increase in the contribution of all AAs in *C. glomerata* in response to the addition of *Rhizoclonium* sp. exudates. The results were statistically significant for all of the amino acids in *C. glomerata*. Changes in Proline content varied from 4.58 to 5.12 g·100g⁻¹ of total proteins and its increase was the highest from among all AA studied (Table S4).

Table 3: The content of amino acids (AA) in *Cladophora glomerata* biomass (g·100g⁻¹ of total proteins; df=3; bold - statistically significant, p<0.05; gray selection - fluctuation in the content of amino acid > 10%).

Impact of exudates extracted from <i>C. fracta</i> on <i>C. glomerata</i> AA production						
	Control	25% exudate	50% exudate	100% exudate	F _{3,8}	P
Glutamic acid (Glu)	12.781±0.001	12.787±0.002	12.789±0.001	13.149±0.003	24.74	0.000000
Aspartic acid (Asp)	10.648±0.002	10.652±0.003	10.657±0.001	11.006±0.003	16.27	0.000000
Leucine (Leu)	9.735±0.001	9.740±0.001	9.747±0.001	9.967±0.002	21.19	0.000000
Arginine (Arg)	7.302±0.002	7.307±0.001	7.311±0.001	7.633±0.002	30.29	0.000000
Alanine (Ala)	5.492±0.001	5.503±0.002	5.509±0.001	5.586±0.002	28.03	0.000000
Valine (Val)	5.507±0.002	5.509±0.001	5.513±0.001	5.532±0.003	1.79	0.000000
Glycine (Gly)	5.185±0.002	5.193±0.002	5.200±0.001	5.224±0.001	2.48	0.000000
Lysine (Lys)	4.862±0.002	4.867±0.002	4.903±0.001	4.939±0.002	1.38	0.000000
Proline (Pro)	4.581±0.002	4.594±0.003	4.609±0.001	4.996±0.010	3.14	0.000000
Serine (Ser)	4.512±0.002	4.513±0.001	4.518±0.001	4.554±0.004	2.74	0.000000
Threonine (Thr)	4.586±0.002	4.590±0.001	4.596±0.001	4.627±0.002	5.44	0.000000
Phenylalanine (Phe)	4.406±0.002	4.411±0.002	4.417±0.001	4.445±0.003	1.93	0.000000
Isoleucine (Ile)	3.392±0.002	3.404±0.003	3.743±0.001	3.767±0.578	0.77	0.544255
Tyrosine (Tyr)	2.875±0.002	2.878±0.004	2.886±0.001	2.917±0.001	2.69	0.000000
Histidine (His)	2.213±0.002	2.216±0.002	2.219±0.001	2.240±0.002	1.29	0.000000
Cysteine (Cys)	1.508±0.002	1.511±0.001	1.519±0.001	1.546±0.003	1.34	0.000000
Methionine (Met)	0.810±0.002	0.814±0.002	0.819±0.001	0.832±0.003	6.91	0.000000
Tryptophan (Trp)	0.765±0.002	0.836±0.001	0.861±0.001	0.943±0.002	6.89	0.000000

3.3 Changes in *Cladophora glomerata* cell structure

Cell components such as chloroplasts, mitochondria, starch grains, lipid bodies, nuclei, and small vacuole were well organized and visible in the control samples. Changes caused by the presence of exudates were also observed in morphological features, i.e. the width of cell wall, shape and diameter of pyrenoids and in chloroplast arrangement. We observed (using TEM) these changes in *C. glomerata* in the control samples (Figure 3a,c,e) relative to the analogous features in the samples cultured in the medium with the highest concentration of exudates (Figure 3b,d,f). High magnification image of the cell wall clearly shows the parallel organization of the cellulose fibrils and the cell wall thinner (3.5 µm) in the control medium (Figure 3a) than (4.3 µm) in the medium with 100% exudates (Figure 4). The difference in diameter of pyrenoids were up to 3µm and the external layer was fuzzy (Figure 3c,d). Mitochondria were well organized

in the control sample cells, and the parallel thylakoids membranes of the chloroplast were well preserved. Chloroplasts were in peripheral arrangement and were densely packed with tightly appressed thylakoids but under stress condition they were more rarely packed (Figure 3e,f).

4 Discussion

The results of our study have evidenced that filamentous green algae respond to the presence of other FGA species in the natural environment. The competition between different species of macroalgae may lead to the exchange of particular species in the community structure. We have shown for the first time the evidence of morphological changes at the cellular level in the filaments of *C. glomerata* in response to a biotic factor which is the appearance of exudates as a result of thalli accumulation. The effect of abiotic factors on morphological changes has been

Table 4: The content of amino acids (AA) in *Rhizoclonium* sp. biomass (g·100g⁻¹ of total proteins; df=3; bold - statistically significant, p<0.05; gray selection - fluctuation in the content of amino acid > 10%).

Impact of exudates extracted <i>C. glomerata</i> on <i>Rhizoclonium</i> sp. AA production						
	Control	25% exudate	50% exudate	100% exudate	F _{3,8}	p
Glutamic acid (Glu)	12.431±0.002	12.702±0.006	12.566±0.003	12.716±0.004	4.12	0.000000
Aspartic acid (Asp)	10.863±0.001	10.541±0.002	10.702±0.003	10.551±0.003	1.29	0.000000
Leucine (Leu)	9.731±0.002	9.697±0.002	9.714±0.003	9.721±0.002	1.64	0.000000
Arginine (Arg)	7.298±0.001	7.253±0.040	7.276±0.004	7.313±0.002	5.0	0.027661
Alanine (Ala)	5.469±0.001	5.470±0.002	5.469±0.004	5.481±0.002	1.3	0.002032
Valine (Val)	5.509±0.002	5.514±0.006	5.512±0.004	5.531±0.002	2.5	0.000192
Glycine (Gly)	5.178±0.001	5.182±0.002	5.180±0.001	5.207±0.002	1.57	0.000000
Lysine (Lys)	4.842±0.001	4.844±0.002	4.843±0.002	8.520±0.001	4.34	0.000000
Proline (Pro)	4.514±0.001	4.567±0.002	4.541±0.007	6.946±0.013	7.39	0.000000
Serine (Ser)	4.612±0.001	4.616±0.005	4.614±0.002	4.627±0.002	1.4	0.001374
Threonine (Thr)	4.536±0.001	4.538±0.002	4.537±0.001	4.547±0.002	3.9	0.000039
Phenylalanine (Phe)	4.402±0.001	3.387±0.002	3.895±0.002	3.406±0.004	12.88	0.000000
Isoleucine (Ile)	3.387±0.002	2.872±0.002	3.130±0.003	2.898±0.008	9.9	0.000000
Tyrosine (Tyr)	2.869±0.001	2.212±0.003	2.541±0.002	2.225±0.002	7.89	0.000000
Histidine (His)	2.211±0.001	2.218±0.001	2.215±0.001	2.223±0.002	6.7	0.000005
Cysteine (Cys)	1.356±0.001	1.359±0.001	1.357±0.001	1.370±0.002	7.2	0.000004
Methionine (Met)	0.824±0.001	0.818±0.008	0.821±0.002	0.837±0.003	1.0	0.004162
Tryptophan (Trp)	0.268±0.001	0.275±0.002	0.271±0.003	0.314±0.002	3.31	0.000000

Table 5: The content of amino acids (AA) in *Cladophora glomerata* biomass. (g·100g⁻¹ of total proteins; df=3; bold - statistically significant, p<0.05; gray selection - fluctuation in the content of amino acid > 10%).

Impact of exudates extracted <i>Rhizoclonium</i> sp. on <i>C. glomerata</i> AA production						
	Control	25% exudate	50% exudate	100% exudate	F _{3,8}	p
Glutamic acid (Glu)	12.774±0.002	12.778±0.002	12.783±0.002	12.896±0.002	2.64	0.000000
Aspartic acid (Asp)	10.639±0.001	10.643±0.002	10.646±0.001	10.970±0.003	2.84	0.000000
Leucine (Leu)	9.728±0.002	9.730±0.001	9.733±0.002	9.835±0.003	3.78	0.000000
Arginine (Arg)	7.302±0.001	7.306±0.002	7.309±0.002	7.431±0.002	4.60	0.000000
Alanine (Ala)	5.487±0.002	5.491±0.002	5.507±0.001	5.523±0.002	1.20	0.000001
Valine (Val)	5.501±0.001	5.504±0.002	5.518±0.001	5.521±0.002	3.50	0.000000
Glycine (Gly)	5.183±0.002	5.193±0.002	5.205±0.002	5.223±0.002	9.08	0.000000
Lysine (Lys)	4.861±0.001	4.871±0.002	4.895±0.001	4.925±0.002	41.47	0.000000
Proline (Pro)	4.579±0.001	4.599±0.001	4.637±0.002	5.123±0.004	4.47	0.000000
Serine (Ser)	4.508±0.001	4.511±0.001	4.519±0.003	4.548±0.001	7.05	0.000003
Threonine (Thr)	4.587±0.001	4.592±0.004	4.603±0.004	4.612±0.002	2.78	0.000000
Phenylalanine (Phe)	4.405±0.001	4.408±0.002	4.413±0.001	4.439±0.003	6.51	0.000000
Isoleucine (Ile)	3.390±0.001	3.393±0.002	3.402±0.001	3.427±0.001	2.11	0.000000
Tyrosine (Tyr)	2.872±0.001	2.875±0.002	2.881±0.002	2.913±0.004	1.63	0.000001
Histidine (His)	2.207±0.001	2.211±0.002	2.221±0.003	2.233±0.002	1.46	0.000000
Cysteine (Cys)	1.508±0.001	1.512±0.001	1.524±0.001	1.529±0.002	1.17	0.000000
Methionine (Met)	0.805±0.001	0.810±0.002	0.810±0.001	0.827±0.002	2.16	0.000000
Tryptophan (Trp)	0.767±0.001	0.825±0.002	0.825±0.003	0.915±0.003	2.64	0.000000

Figure 3: Ultrastructure analysis by transmission electron microscopy (TEM) of *Cladophora glomerata* with (right side) and without stress (left side): a – thin cell wall (CW) and normal chloroplast (Chl), b – multilayered cell walls (CW) and chloroplast (Chl); c, d – pyrenoid (P) surrounded by the chloroplast (Chl); e – multilayered cell wall (CW), chloroplast (Chl) and pyrenoid (P), f – cross section of filament showing chloroplast (Chl) (scale bar: 0.2 μm).

partly described by Whitton [16], Pikosz and Messyas [12]. Filamentous green algae often form monospecies mats in water ecosystems. Ecological characteristics of certain species may lead to their definite domination, as e.g. *C. glomerata* which is the most common FGA in the world [16]. Other species of filamentous green algae, as *Cladophora rivularis* (L) Kuntze and *Rhizoclonium* sp., do not occur so frequently or are less abundant [12]. The important outcome of our study is the evidence that the interactions between the macroalgae species can lead to changes in their biochemical structure. The green algae species selected for our study responded to the presence of exudates of other species recognized as competitors for the environment resources through changes in the quantitative composition of amino acids. In *C. fracta* and *Rhizoclonium* sp. the changes in AA profile were visible at the lower exudates concentration than in *C. glomerata*. It may explain, why *C. fracta* and *Rhizoclonium* sp. more

rarely formed monoalgal mats and are rarely dominant in algae structures. Furthermore, different responses in AA contents were observed for *C. rivularis* and *Rhizoclonium* sp. under *C. glomerata* pressure. In *C. fracta* the changes in the content of three AA were more pronounced in the sample having the highest concentration of exudates (100%) than in the control sample. Ala decreased from 5.4 to 5.1 $\text{g}\cdot 100\text{g}^{-1}$ of total proteins, Trp increased from 0.36 to 0.46 $\text{g}\cdot 100\text{g}^{-1}$ of total proteins (30% of fluctuation in the content of amino acid) and the most pronounced changes were observed for Pro whose content increased from 4.49 to 6.5 $\text{g}\cdot 100\text{g}^{-1}$ of total proteins (45%). However, in *Rhizoclonium* sp. the following changes in the contents of seven amino acids were observed, the concentrations of Pro (53% of fluctuation in the content of amino acid), Lys (75%), Trp (17%) and Glu increased, while those of Asp, Phe (22%) and Tyr (22%) decreased. The highest changes were noted for Lysine from 4.8 to 8.5 $\text{g}\cdot 100\text{g}^{-1}$ of

Figure 4: Diagram of Proline concentration in algae cell in control and in medium with 100% of exudates (*C. fracta* with 100% of *C. glomerata* exudates, *C. glomerata* I with 100% of exudates of *C. fracta*, *C. glomerata* II with 100% of exudates of *Rhizoclonium* sp. and *Rhizoclonium* sp. with 100% of *C. glomerata* exudates).

total proteins (30%) and Proline increased from 4.8 to 6.9 g·100g⁻¹ of total proteins (53%). An interesting observation is that the changes in the AA composition of *C. glomerata* in response to the presence of *C. rivularis* and *Rhizoclonium* sp. exudates are different. The changes in concentrations in AA in *C. glomerata* in response to the presence of *C. fracta* exudates were detected for seven amino acids (Leu, Glu, Asp, Arg, Pro – 11%, Ile, Trp – 19%), while in response to the *Rhizoclonium* sp. exudate only in one (Pro - 9% - 30% of fluctuation in the content of amino acid). This phenomenon confirms the *C. glomerata* plasticity and the fact that this species shows a wide ecological range [16].

The protein content of algae can vary according to the species, seasonal period, environmental condition and geographic area [29]. Amsler [30] determined the amino acid composition of 8 species of macroalgae and the most abundant AA were Asp, Glu, Lys, His, Val and Ala. *C. glomerata* contained 17 amino acids and Glutamic acid and Aspartic acid were detected in the highest contents in the filaments collected from the lake [11,31]. According to our results, the same AA were dominant, but we also observed the presence of Tryptophan. The major abundant amino acids (from 17 AA) identified in the freshwater *Rhizoclonium hieroglyphicum* (C. Agardh) Kützinger from Northern Thailand were Tryptophan, Tyrosine and Phenylalanine [32]. Their content was lower than that of AA in freshwater *Rhizoclonium* sp. from our study, in which also Arg was noted. Interestingly, protein concentration in marine *C. glomerata* range about 20% of dry basic [33], while total protein content was slightly

smaller and ranged from 12.75% to 14.45% in freshwater *Cladophora* [31].

Kaur and Asthir [34] suggest that accumulation of Proline is a common physiological response in plants exposed to various environmental stress, mainly high soil salinity, drought, oxidative stress, heavy metals presence. In the control samples for all individual taxa, the Proline content was at the same level (about 4.5 g·100g⁻¹ of total proteins). The Proline content increased as a result of increasing content of exudation, a biotic stressor, both in *C. glomerata*, *C. fracta* and *Rhizoclonium* sp. However in *C. fracta* and *Rhizoclonium* sp. there was a higher increase in Proline production than in *C. glomerata* (Figure 4). Slight differences were observed in the changes of Proline content in *C. glomerata* cultured in the medium with exudates of different macroalgae. The content of Proline in *C. glomerata* cultured with exudates of *Rhizoclonium* sp. (*C. glomerata* II in Figure 4) was higher than in *C. glomerata* cultured with exudates of *C. fracta* (*C. glomerata* I in Figure 4).

There are a lot of factors which influence the morphometric features of *C. glomerata*, including seasonality, stage of development, physicochemical parameters of water. The thickness of the *C. glomerata* cell wall may show seasonal variations, for example the samples from June had thinner cell walls than the samples from July [35]. However, results of this study indicate that also biotic factors may affect the morphological structure of the cell.

5 Conclusions

The results of the experiment with the three taxa of green algae *C. glomerata*, *C. fracta* and *Rhizoclonium* sp., show that the changes in amino acids production depend on FGA species and exudates concentration. Generally, the amount of some amino acids increased with increasing exudates concentrations. *C. fracta* and *Rhizoclonium* sp. react (by changes in AA profile) at the minimum *C. glomerata* exudates in medium, it was visible mainly in Proline concentration. We also observed, that *C. glomerata* produced more amino acids in response to exudates from the other FGA, but its response was noted only in the highest exudate concentration. Important are results which show that one species can have different strategies. To find out why *C. glomerata* monopolizes water surface, although this species occupies the same niche as the other filamentous green algae, a future study is needed to establish the composition of its secretions.

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