Environmental and Experimental Biology (2023) 21: 53–60
http://doi.org/10.22364/eeb.21.07

Original Paper

Association between *Spirodela polyrrhiza* and blue green algae in aquatic habitats and *in vitro* conditions in relation to metabolite concentrations under monoculture and co-culture

ISSN 2255-9582

Santanu Gupta[1]*, Sudipta Kumar Sil[2]

[1]Department of Botany, Malda College, Malda 732101, West Bengal, India
[2]Department of Botany, The University of Gour Banga, Malda 732103, West Bengal, India

*Corresponding author, E-mail: santanubotanist@gmail.com

Abstract

Spirodela polyrrhiza samples were collected from various locations within the geographical periphery of Malda district in West Bengal, India. Samples collected from all sites were associated with algal colonies comprised of diatoms and blue green algae. Such association of different algal groups and greater duckweed in their natural habitat occurred in the lower epidermis of the fronds. Among the members of Cyanophyceae, *Oscillatoria sancta* was found to be abundantly associated with the fronds under *in vivo* conditions. To mimic the in vivo association, axenic monocultures of both *S. polyrrhiza* and *O. sancta* were maintained separately or by co-cultivation. Association with *O. sancta* under *in vitro* growth conditions prolonged the stationary growth phase of greater duckweed significantly. A growth-promoting association between the two species was shown by increased concentration of some metabolites of *S. polyrrhiza*. Correlation between growth of the two species were confirmed.

Key words: blue green algae; diatoms, duckweed, fronds, Lemnaceae, nitrogen, *Spirodela*.

Introduction

Duckweeds are gregariously growing, free floating, minute aquatic monocotyledonous plants abundantly present in nutrient rich water bodies of tropical parts of the world. These plants belong to the order Alismatelis, family Lemnaceae which is further subdivided into two subfamilies Lemnoideae and Wollfiodeae (Tippery et al. 2015). Duckweeds are represented by five genera (*Lemna, Wolffia, Spirodela, Wolfiella, Landoltia*) consisting of 37 species (Bog et al. 2010). Duckweeds consist of two or more poorly differentiated fronds separated by large intercellular vacuoles that help to maintain buoyancy. They are made up of chlorenchymatous cells. Owing to the small size of the fronds ranging from 1.5 to 4 mm, they are difficult to identify. The diploid chromosome number of duckweeds varies from 2n = 20 to 2n = 126 (Landolt et al. 1986). Such variation is mainly attributed due to the generation of asexual clones by many species through vegetative propagation (Hastwell et al 2008). Epigenetic modification in duckweeds is quite prominent, which significantly influences gene transcription and translation (Cao et al 2015). Generally, the C_3 mode of carbon fixation is preferred by duckweeds and they can double their biomass within 48 h of growth (Driever et al 2005). Duckweeds grow in wide geographical habitats, but they cannot tolerate temperatures below 0 °C. Generally warm and sunny conditions support the growth of duckweeds (Goppy, Murray 2003). Duckweeds prefer stagnant fresh water bodies such as ponds, ditches, lakes etc for their luxuriant growth. Duckweeds can accumulate phosphorus and nitrogen within their vacuolar sap in the form of oligo-, cyclic and high-molecular compounds (Khondkar et al. 1994). Urban development and eutrophication of water bodies has resulted in decline of many aquatic plants, but duckweeds have survived under these changing conditions by rapidly accumulating nutrients from the surrounding water (Ghosh 2005). The duckweeds can multiply within 48 h by vegetative reproduction and their growth can be compared with microbial growth (Beppu, Takimotto 1981).

Spirodela polyrrhiza (L.) Schleid., commonly known as greater duckweed or giant duckweed, is cosmopolitan in occurrence in eutrophic water bodies of tropical countries. The plant body is composed of a stem and leaf, known as a frond. The lower surface of the frond bears numerous roots of varying length. Multiple roots develop from the lower surface of the fronds. The fronds of *S. polyrrhiza* represent a high degree of structural reduction (Kim, Kim 2000). The

upper surface appears deep green as compared to the lower surface that appears violet at maturity. It reproduces by vegetative propagation where the daughter frond remains attached to the mother frond by a connective stalk. *S. polyrrhiza* is known to produce hibernating structures called turions, which on return of favourable conditions develop into new plants (McLay 1974). Turions are vegetative structures and therefore are often compared with seeds of higher plants (Landolt 1986). Turion formation is also enhanced under deficiency of phosphate in the water bodies (Schwalbe 1999). The lower epidermis of duckweed species like *Lemna minor* and *Lemna gibba* are often found to be infested with cyanobacterial colonies (Zuberer 1982). Sometimes they are associated in the reproductive pockets or in the attached roots of *Lemna* (Rao 1953). Previous reports have suggested occurrence of heterocystous blue green algae like *Gloeotrichia* spp., *Nostoc* spp., *Cylindrospermum* spp., *Calothrix* spp. and *Anabaena* spp. (Coler, Gunner 1969). Such colonies thrive on the organic debris released from the duckweeds and exist as epiphytes on the fronds of duckweeds, creating a microenvironment favoring their growth (Fay 1976). The aim of the present study was to assess the association between algae and greater duckweed, *S. polyrrhiza*, in natural and laboratory conditions.

Materials and methods

Plant material and microscopy

To examine the association between greater duckweed and algae, *Spirodela polyrrhiza* (L.) Schleid. samples were collected randomly from 10 different sites (CSI to CSX) in Malda district (West Bengal, India) from November to December 2020 (Table 1). The samples were kept in sterile plastic 100 mL containers. The specimens were identified with the help of Central National Herbarium Shibpur Howrah. To study the algal association with fronds of *S. polyrrhiza*, the fronds were surface sterilized using 0.1% mercuric chloride solution for 30 s to 1 min followed by washing in double distilled water. The surface sterilized fronds were dehydrated following chemical fixation with 2.5% glutaraldehyde overnight. This was followed by dehydration in alcohol (50, 70, 80, and 95%). The dehydrated plant material was subjected to scanning electron microscopy. The scanning electron microscopy was performed in USIC, The University of North Bengal, West Bengal using a JSM-IT 100 (JEOL), which was operated using a secondary electron detector of 5.0 kV and working distance 10 nm, probe current (PC #30) and accelerating voltage 5.0 kV.

Cultivation conditions

To study the *in vitro* association between algae and greater duckweed, fronds of *S. polyrrhiza* were collected from pure liquid culture maintained in tissue culture laboratory maintained in pH 5.8, relative humidity 100%, photoperiod 16 h, photon flux density of photosynthetically active radiation 22.2 µmol m^{-2} s^{-1} and temperature 28 ± 2 °C in SH basal medium (HIMEDIA PT059). The fronds from the pure culture were freed from cyanobacteria infestation. The fronds were then surface sterilized using 0.1% mercuric chloride solution for 1 min. The sterilized fronds were separately placed on liquid culture of SH basal medium in Petri dishes (diameter 100 mm, depth 17 mm). *O. sancta*, attached with fronds of *S. polyrrhiza* in natural conditions, were separately cultured in BG11 broth medium (HIMEDIA M1958) maintained at pH 7.10, temperature 22 to 26 °C, photoperiod 12 h and photon flux density of photosynthetically active radiation 37. 2 µmol m^{-2} s^{-1} in Petri dishes (diameter 100 mm, depth 17 mm).

Then, fronds of *S. polyrrhiza* from pure culture were infested with *O. sancta* from pure culture and the coculture of the organisms was maintained in SH basal medium (Himedia PT059) supplemented with BG11 broth in 2:1 v/v proportion, pH was adjusted within the range 5.8 to 6.2, relative humidity 100%, photoperiod 16 h, photon flux density of photosynthetically active radiation 22.2 µmol m^{-2} s^{-1} and temperature 28 ± 2 °C in Petri dishes (diameter 100 mm, depth 17 mm). The entire setup was established in three replicates. A similar setup was used in three replicates without *O. santa*. The pH was adjusted to 5.8, relative humidity 100%, photoperiod 16 h, photon flux density of

Table 1. Collection sites of duckweeds from Malda District along with their geographical coordinates

Site	Location	Date of collection	Latitude	Longitude
CSI	English Bazar	November 19, 2019	25.004101	88.136797
CSII	English Bazar	November 19, 2019	25.017052	88.126833
CSIII	Old Malda	November 24, 2019	25.012480	88.153755
CSIV	Old Malda	November 26, 2019	25.065583	88.143261
CSV	Mangalbari (Old Malda)	November 26, 2019	25.025516	88.146586
CSVI	Kaliachak I	December 2, 2019	24.882428	88.013306
CSVII	Kaliachak I	December 2, 2019	24.880258	88.028318
CSVIII	Kaliachak II (Paglaghat)	December 12, 2019	24.92355	87.975536
CSIX	Gazole	December 26, 2019	25.211174	88.179049
CSX	Gazole	December 26, 2019	25.200940	88.181436

photosynthetically active radiation 22.2 μmol m^{-2} s^{-1} and temperature 28 ± 2 °C. Culture-related data were observed and recorded from the starting day (day 0) to the 18th day of culture (day 18) at an interval of three days. The number of new fronds regenerating in each of the Petri dishes, both in co-culture and single culture was recorded.

Molecular identification

For identification of cyanobacteria, sequencing of 16S rDNA was used. Genomic DNA was isolated from the sample. Around 0.1 g of culture was placed in a mortar and homogenized with 1 mL of extraction buffer and the homogenate was transferred to a 2 mL microfuge tube. Then an equal volume of phenol/chloroform/isoamlyalcohol (25:24:1, *v*/*v*) was added to the tubes and mixed well by gently shaking the tubes. The tubes were centrifuged at room temperature for 15 min at 14 000 rpm. The upper aqueous phase was collected in a new tube and an equal volume of chloroform/isoamly alcohol (24:1, *v*/*v*) was added and mixed. The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14000 rpm was transferred to a new tube. The DNA was precipitated from the solution by adding 0.1 volume of 3 M sodium acetate pH 7.0 and 0.7 volume of isopropanol. After 15 min of incubation at room temperature the tubes were centrifuged at 4 °C for 15 min at 14000 rpm. The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried. The DNA was dissolved in TE (Tris-HCl 10 mM pH 8.0, EDTA 1 mM). To remove RNA, 5 μL of DNAse free RNAse A (10 mg mL^{-1}) was added to the DNA.

The 700 bp RBCL-rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was sequenced bi-directionally. The forward primer had sequence ATGTCACCACAAACAGAGACTAAAGC whereas the reverse primer had sequence GTAAAAT CAAGTCCACCRCG. The sequence data was aligned and analyzed to identify the cyanobacteria and its closest neighbours. PCR was conducted for 25 cycles with initial denaturation at 96 °C for 5 min, followed by proper denaturation at 96 °C for 30 s. Hybridization temperature was maintained at 50 °C for 30 s and the elongation step was conducted at 60 °C for 1 min 30 s. Sequencing was done with a ABI 3130 Genetic Analyzer Chemistry Cycle sequencing kit: Big Dye Terminator version 3.1"Polymer & Capillary Array: POP_7 pol Capillary Array with analysis protocol BDTv3-KB-Denovo_v 5.2; data Analysis Seq Scape_ v 5.2 Software; and Applied Biosystem Micro Amp Optical 96-Well Reaction plate. The software used was Phylogentic Tree Builder that uses sequences aligned with System Software aligner. A distance matrix was generated using the Jukes-Cantor corrected distance model. When generating the distance matrix, only alignment model positions were used, alignment inserts were ignored and the minimum comparable position was 200. The tree was created using Weighbor with alphabet size 4 and length size 1000.

Lipid extraction

Dried plant material (only duckweed, only blue green algal biomass and co-cultured biomass separately) were wrapped in filter paper, tied with string to keep it together, placed in a Soxhlet extractor with *n*-hexane (25 mL g^{-1} dry weight) and extracted for 3 h. The solvent was removed from the lipid extract with a rotary evaporator. The dry weight of the final separated lipids was estimated as proportion of dry weight of the initial biomass (g per g dry biomass). The remaining biomass was used for the extraction of protein.

Protein extraction

The residual biomass after lipid extraction was dried in an oven at 40 °C for 30 min to remove residual solvent, dissolved in 5% sodium chloride (20 mL per g dry weight) and the pH of the resulting suspension was raised to pH 10 with 1 M caustic soda. The suspension was then stirred and heated on a hotplate for 30 min at 40 °C, cooled to room temperature and left undisturbed overnight, during which time the suspension separated into a supernatant phase and

Table 2. Association between algae and *Spirodela polyrrhiza* in all collection sites of Malda district of West Bengal, India (+ and ++ indicate presence in low frequency and high frequency, respectively; – indicates absence of the individual algae in that collection site

Collection site	*Nitzschia sigmoidea*	*Anomoeoneis sphaerophora*	*Navicula gottlandica*	*Gomphonema olivaceum*	*Oscillatoria autumnalis*	*Oscillatoria jenensis*	*Oscillatoria sancta*
CSI	–	++	–	++	–	–	++
CSII	++	+	++	++	–	++	++
CSIII	–	–	++	–	+	–	++
CSIV	–	–	++	–	++	–	++
CSV	–	–	–	–	++	–	++
CSVI	–	–	–	–	++	–	++
CSVII	–	–	–	–	–	–	++
CSVIII	+	++	–	+	++	++	++
CSIX	++	–	++	–	–	++	++
CSX	+	–	++	–	–	++	++

Table 3. Taxonomy and morphological characterization of diatoms and blue green algae associated with *Spirodela polyrrhriza*

Species	Taxonomic position	Morphological characteristics
Nitzschia sigmoidea (Nitzsch) Smith	Class Bacillariophyceae Order Bacillariales Family Bacillariaceae	Frustules linear, sigmoid with rounded ends, raphe thin central, striation not clearly visible, 81.9 – 90.7 µm long and 7.2- 8.5 µm broad.
Anomoeoneis sphaerophora Pfitzer	Class Bacillariophyceae Order Cymbelales Family Anomoeoneidaceae	Valve broadly lanceolate or elliptical with produced end, apices acutely rounded, striation not clearly visible; valve 17.7 – 20.8 µm long and 7.7 – 9.3 µm broad.
Navicula gottlandica Grunow	Class Bacillariophyceae Order Naviculales Family Naviculaceae	Elongated lanceolate frustules, median raphe clear, central area wide, narrowed to the end to acutely rounded end, striation clear, radiate throughout the valve, 8 – 11 µm, frustules 50 – 55 µm long and 10 – 15 µm broad
Gomphonema olivaceum (Hornemann) Brebisson	Class Bacillariophyceae Order Cymbelales Family Gomphonemataceae	Linear, lanceolate, clavate cell with 20 –50 µm length and 4 – 6 µm width. Raphe lateral, undulated, proximal end dilated.
Oscillatoria autumnalis C. Agardth	Class Cyanophyceae Order Oscillatoriales Family Oscillatoriaceae	Filaments straight, sheaths thin, bright blue green, 6.2 – 7.0 µm wide, crosswalls slightly constricted and granulated, cells isodiametric, 6.2 – 6.8 µm long, apical cell elongate, capitate, with rounded calyptras.
Oscillatoria jenensis Schmid	Class Cyanophyceae Order Oscillatoriales Family Oscillatoriaceae	Thallus dark bluish green, trichome without sheath, cells 18 – 20 µm broad, 4.5 – 5.5 µm long, cell wall thick, filament not constricted, cross walls indistinctly granulated, trichome shortly attenuated at the ends, hook like bent, 11 – 13 µm wide near the ends, apical cell convex, rounded, not capitate.
Oscillatoria sancta Kutzing and Gomont	Class Cyanophyceae Order Oscillatoriales Family Oscillatoriaceae	Thallus straight but with apical bending dark bluish green, trichome without sheath, cells 10 – 15 µm broad, 4 – 6 µm long, cell wall thick, filament not constricted, cross walls indistinctly granulated, trichome shortly attenuated at the ends, hook like bent, 11 – 13 µm wide near the ends, apical cell convex, rounded, capitate. This species was found firmly attached on the undersurface of the leafy biomass of the greater duckweed collected from most of the collection spots.

a residual biomass phase. The supernatant was collected, its pH adjusted to pH 4 with 1 M citric acid and stirred thoroughly while heating on a hotplate for 15 min at 40 °C. The supernatant was allowed to cool to room temperature and was then left undisturbed for 12 h to precipitate out the protein mass. The precipitated proteins were collected

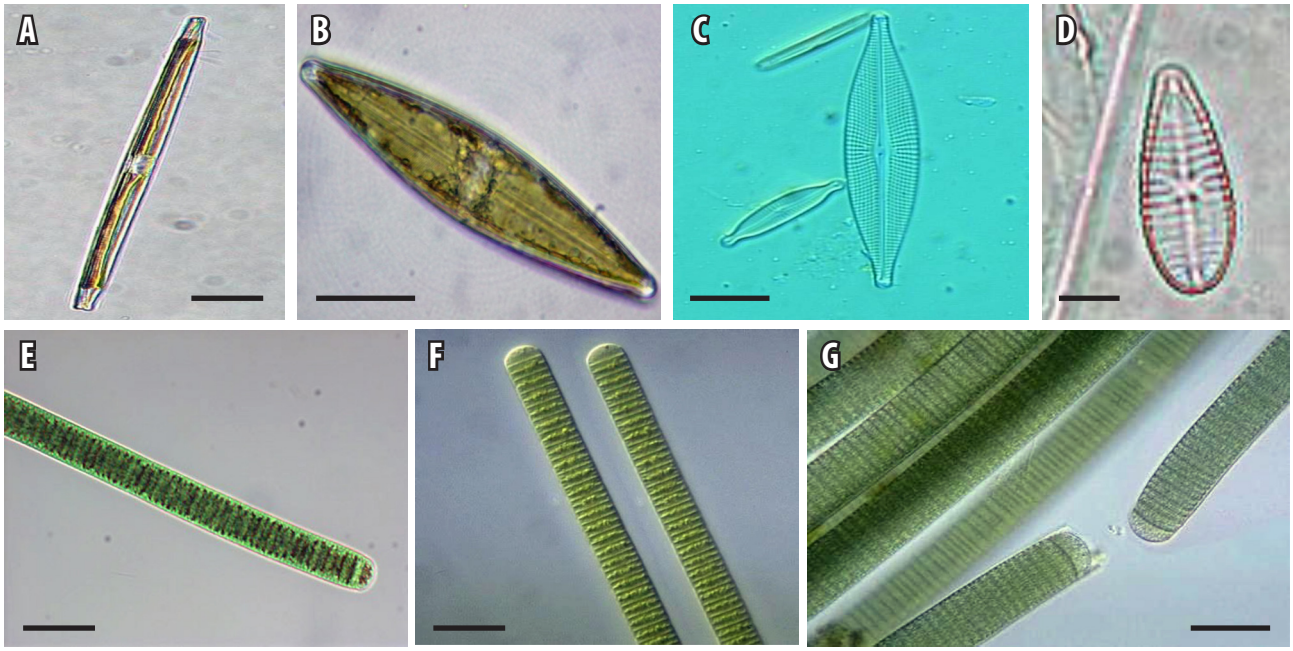


Fig. 1. Algae associated with *Spirodela polyrrhiza* under light microscope. A, *Nitzschia sigmoidea* (scale bar 10 µm); B, *Anomoeoneis sphaerophora* (scale bar 10 µm), C, *Navicula gottlandica* (scale bar 10 µm), D, *Gomphonema olivaceum* (scale bar 10 µm), E, *Oscillatoria autumnalis* (scale bar 20 µm), F, *Oscillatoria jenensis* (scale bar 20 µm), G, *Oscillatoria sancta* (scale bar 25 µm).

by centrifugation for 10 min at 5000 rpm at 4 °C. The dry weight of the precipitated protein was estimated as proportion of dry weight of the initial biomass (g per g dry biomass).

Determination of fatty acids

Lipid samples (10 mg) were mixed with 0.5 mL of 0.5 N NaOH and heated in a water bath at 60 °C for 20 min, after which 1 mL of 10% boron trifluoride was added. The solution was reheated for 20 min and cooled to room temperature. Next, 2 mL of saturated saline iso-octane (2,2,4-trimethylpentane) (2:1, v/v) solution was added and mixed until it was homogenous. This addition results in the formation of two phases. One gram of anhydrous sodium sulphate was added to the upper phase, which was then left undisturbed for 30 min. The organic phase from this mixture was separated and injected into a gas chromatograph (GC-17V3 Shimadzu, Japan). The results from the analysis were compared to the fatty acid methyl ester standard and determined as a percentage of total fatty acids.

Determination of amino acids

Protein samples (2 mg) were mixed with 1 mL 6 N hydrochloric acid, placed in an oven at 60 °C for 24 h, dried with a rotary evaporator and mixed with 6 mL 0.01 N HCl. The solution was filtered with Millipore paper (0.22 µm MF-MilliporeTM, Merck, NJ, USA). Aliquots (100 µL) of this mixture were reacted with 300 µL of *o*-phthaldialdehyde reagent for 1 min before being injected into an HPLC (CBM-20A Shimadzu, Japan) with a Hypersil ODS-2 column (Thermo Fisher Scientific, USA). The mobile phase constituted phosphate saline buffer at pH 7.4.

Data analysis

The data were statistically analysed using SPSS v.24.0 using mean of triplicates followed by standard deviation.

Results

The location of the 10 collection sites along with the geographical coordinates are given in Table 1. All of the collected *S. polyrrhiza* fronds from the sites were associated with algae on their lower surface. The algal association with *S. polyrrhiza* in natural habitats is shown in Table 2. The morphological characteristics of the associated diatoms and blue green algae with *S. polyrrhiza* are shown in Table 3. The predominant algal association included diatoms and blue green algae either alone or in combination, when observed under a light microscope (Fig. 1). The associations are indicated in Table 2. Similar associations with *S. polyrrhiza* under *in vivo* conditions were shown by scanning electron microscopy (Fig. 2). Among the members of blue green algae, *O. sancta* was found to be the dominant species associated with fronds of greater duckweed collected from

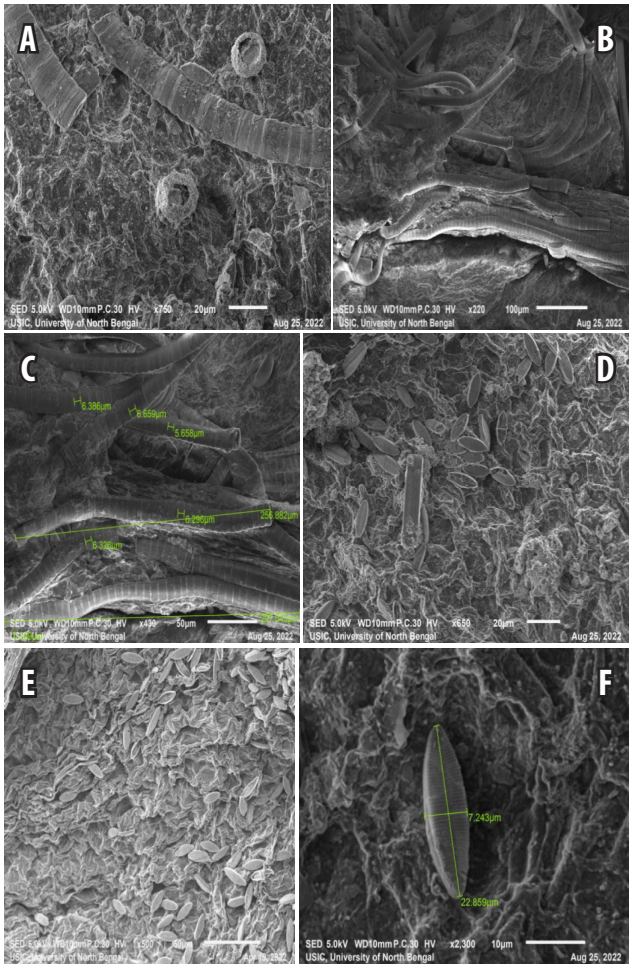


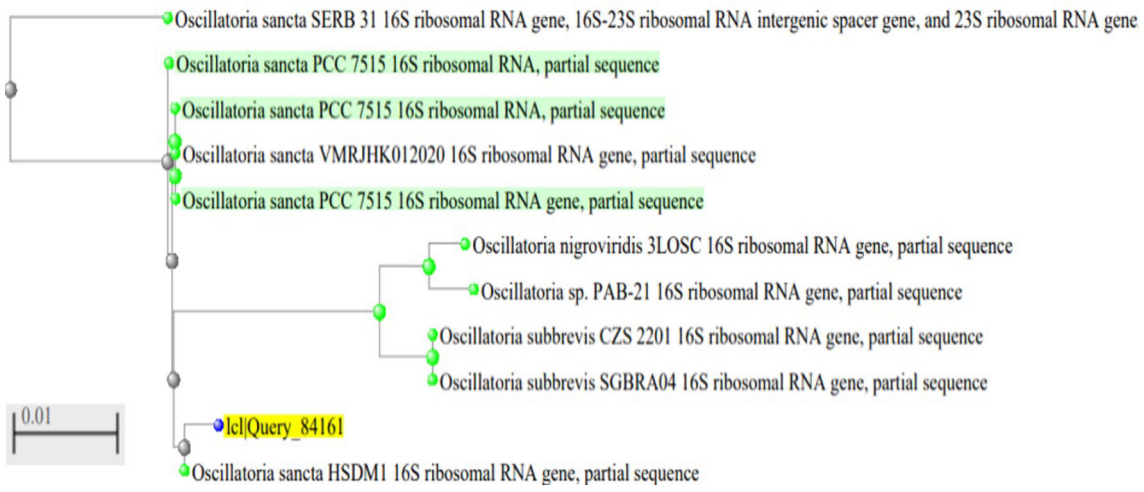
Fig. 2. Scanning electron micrographs of algae associated with the lower surface of the fronds of *Spirodela polyrrhiza*. A, *Oscillatoria autumnalis*; B, *Oscillatoria jenensis*; C, *Oscillatoria sancta*; D, *Nitzschia sigmoidea* E, *Navicula gottlandica*; F, *Gomphonema olivaceum*.

the 10 collection sites, which was confirmed by microscopic images and molecular 16S rDNA sequence analysis (Fig. 3).

The i*n vitro* growth of *S. polyrrhiza* on SH basal medium in association with *O. sancta* and in its absence is shown in Fig. 4. The *in vitro* growth of greater duckweed *S. polyrrhiza* inoculated with *O. sancta* was significantly higher than that of *S. polyrrhiza* in monoculture condition. The growth kinetics of co-culture systems were significantly improved over the axenic duckweed monoculture system (Table 4). The divergent nature of the moving average between the two sets of columns, one representing the co-culture and the other representing the duckweed monoculture indicate significant impact of algal association on duckweed growth.

The protein and fatty acid content was found to differ in the dried biomass of greater duckweed *S. polyrrhiza* between monoculture and co-culture conditions. In monoculture, *S. polyrrhiza* had lower accumulation of ω-3 and ω-6 fatty acids, whereas *in vitro* co-culture of *S. polyrrhiza* with *O. sancta* had greater accumulation of ω-3 and ω-6 fatty acids

A



B

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Oscillatoria sancta VMRJHK012020 16S ribosomal RNA gene, partial sequence	Oscillatoria sanct...	2512	2512	100%	0.0	99.49%	1411	MW364271.1
Oscillatoria sancta PCC 7515 16S ribosomal RNA, partial sequence	Oscillatoria sanct...	2512	2512	100%	0.0	99.49%	1409	NR_114511.1
Oscillatoria sancta PCC 7515 16S ribosomal RNA, partial sequence	Oscillatoria sanct...	2460	2460	100%	0.0	98.91%	1433	NR_112112.1
Oscillatoria sancta PCC 7515 16S ribosomal RNA gene, partial sequence	Oscillatoria sanct...	2379	2379	99%	0.0	98.18%	1373	OQ600802.1
Oscillatoria sancta HSDM1 16S ribosomal RNA gene, partial sequence	Oscillatoria sanct...	2311	2311	95%	0.0	98.56%	1325	MZ366482.1
Oscillatoria sancta SERB 31 16S ribosomal RNA gene, 16S-23S ribosomal RNA intergenic spacer gene, and 23...	Oscillatoria sanct...	2215	2215	100%	0.0	95.73%	1430	KM982580.1
Oscillatoria nigroviridis 3LOSC 16S ribosomal RNA gene, partial sequence	Oscillatoria nigro...	2154	2154	93%	0.0	96.89%	1378	EU244875.1
Oscillatoria sp. PAB-21 16S ribosomal RNA gene, partial sequence	Oscillatoria sp. P...	2137	2137	93%	0.0	96.66%	1378	EU253967.1
Oscillatoria subbrevis CZS 2201 16S ribosomal RNA gene, partial sequence	Oscillatoria subb...	2132	2132	92%	0.0	96.73%	1329	OQ627016.1
Oscillatoria subbrevis SGBRA04 16S ribosomal RNA gene, partial sequence	Oscillatoria subb...	2132	2132	92%	0.0	96.73%	1329	JQ083642.1

Fig. 3. A, identification of 16s r DNA sequence of *Oscillatoria sancta* associated with fronds of *Spirodela polyrrhiza*. B, similarity matrix corresponding to the tree.

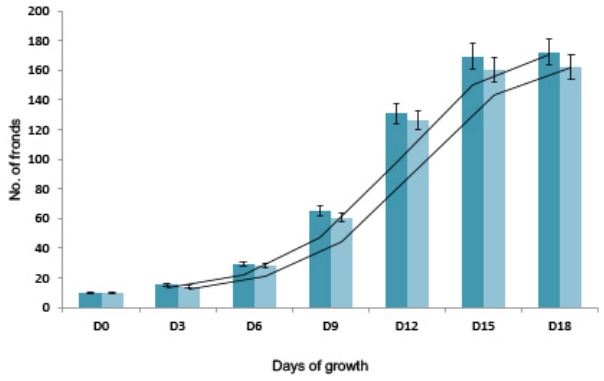


Fig. 4. Comparative growth pattern of *Spirodela polyrrhiza* in presence of blue green algal association with *Oscillatoria sancta* (darker columns) and in absence of association (lighter columns).

(Table 5). Not only was the accumulation of fatty acids enhanced, but also the ω-6 / ω-3 fatty acid ratio increased in co-culture. A higher value of the ratio indicates utilization of associated biomass from the perspective of nutraceutical importance. It was found that the amino acid profile of the proteins extracted from *S. polyrrhiza* fronds with algal association was improved, in comparison to proteins that were extracted from the dried biomass of *S. polyrrhiza* without *O. sancta* (Table 6).

Discussion

Under *in vitro* conditions, the growth curve of liquid cultures of *S. polyrrhiza* maintained in SH basal medium showed lag, exponential, stationary and decline phases

Table 4. Statistical assessment of data by one-way ANOVA ($p < 0.05$) among the data sets viz. monoculture of greater duckweed *Spirodela polyrrhiza* and co-culture of *Spirodela polyrrhiza* with blue green alga *Oscillatoria sancta*

Culture		Sum of squares	*df*	Mean square	*F*	Significance
Spirodela polyrrhiza	Between groups	83132.586	6	13855.431	6.928 E7	0.000
	Within groups	0.003	14	0.000		
	Total	83132.589	20			
Spirodela polyrrhiza + *Oscillatoria sancta*	Between groups	92473.051	6	15412.175	9.808 E7	0.000
	Within groups	0.002	14	0.000		
	Total	92473.053	20			

Table 5. Fatty acid content (in % from total fatty acids) and its characteristics in greater duckweed *Spirodela polyrrhiza* under *in vitro* growth conditions in presence and in absence of *Oscillatoria sancta*. Values are means followed by SD

Type of fatty acids	*S. polyrrhiza* in absence of *O. sancta*	*S. polyrrhiza* in presence of *O. sancta*
Saturated fatty acids	31.44 ± 4.43	39.19 ± 5.12
Mono unsaturated fatty acids	10.18 ± 0.65	16.35 ± 1.24
Polyunsaturated fatty acids	58.38 ± 0.45	60.46 ± 0.76
ω-3	44.93 ± 3.23	22.17 ± 0.14
ω-6	10.36 ± 0.87	16.04 ± 0.43
ω-9	4.87 ± 0.70	8.02 ± 1.23
Medium-chain fatty acids	1.30 ± 0.07	1.32 ± 0.42
Long-chain fatty acids	95.44 ± 2.13	94.49 ± 3.76
Very long-chain fatty acids	3.26 ± 0.15	4.29 ± 0.05
ω-6/ω-3 fatty acids	0.23 ± 0.01	0.72 ± 0.03

within the observation period of 18 days. Liquid cultures inoculated with *O. sancta* had a higher frond multiplication rate with lower doubling time and plants remained in the stationary phase for a longer period of time as compared to liquid cultures of *S. polyrrhiza* in SH basal medium without *O. sancta*, which had a lower frond multiplication rate with shorter stationary phase. This can be associated with higher accumulation of essential amino acids in the protein fraction, which can supplement the Krebs cycle at different points. Fronds of *S. polyrrhiza* without *O. sancta* started yellowing from day 12 in the axenic culture, whereas cultures supplied with *O. sancta* remained fresh for a longer period of time with a lower rate of chlorophyll destruction. Thus, it may be concluded that higher vegetative propagation of greater duckweed under co-culture with *O. sancta* is mainly due to an increased respiration rate and hence aggravated supply of energy for supporting a number of cell cycles up to the stationary phase. The up scaling in essential fatty acid synthesis in response to algal association is supposed to provide a higher concentration of acetyl-CoA, which is a pivotal molecule in shifting protein metabolism to fatty acid metabolism. As a result, higher concentration of polyunsaturated fatty acids occurred in response to association between the two species. Therefore, as a logical consequence of our study, it is presumed that duckweed-algal associated biomass upon purification and after proper trials may be prescribed as a value added feed for both fish and poultry birds.

Table 6. Characteristics of amino acids in proteins (in % from total) from *Spirodela polyrrhiza* under *in vitro* growth conditions in absence and in presence of *Oscillatoria sancta*. Values are means followed by SD

Amino acid	*S. polyrrhiza* in absence of *O. sancta*	*S. polyrrhiza* in presence of *O. sancta*
Alanine	5.6 ± 0.3	6.2 ± 1.7
Arginine	4.2 ± 1.1	4.4 ± 1.2
Aspartate	8.0 ± 2.1	8.2 ± 1.7
Glutamate	8.9 ± 1.3	9.1 ± 2.3
Glycine	3.7 ± 0.2	4.1 ± 1.0
Histidine	1.4 ± 0.3	1.5 ± 1.6
Isoleucine	6.9 ± 0. 7	7.5 ± 1.5
Lysine	5.5 ± 0.3	5.9 ± 1.7
Leucine	2.0 ± 0.5	2.4 ± 0.8
Methionine	6.9 ± 0.7	7.6 ± 0.2
Phenylalanine	3.4 ± 0.8	3.6 ± 1.2
Serine	4.6 ± 1.5	5.1 ± 1.6
Threonine	4.5 ± 1.7	4.8 ± 1.4
Tyrosine	3.5 ± 1.0	4.0 ± 0.5
Valine	5.4 ± 0.9	5.8 ± 0.7

The co-culture of *O. sancta* and *S. polyrrhiza* resulted in an increase of ω-3 and ω-6 fatty acids concentration in *S. polyrrhiza*, compared to that of monoculture. This is again clearly reflected by the higher ω-6 / ω-3 fatty acid ratio. ω-3 fatty acids are usually found in blue green algae in higher proportion. ω-6 fatty acids are pro-inflammatory, whereas ω-3 fatty acids are anti-inflammatory (Fang et al. 2007). Similar upscaling of fatty acids and amino acids was also found in other duckweeds like *Wolffia* spp. and *Landoltia punctata* (Sembada, Faizal 2022). Such fatty acid accumulation may directly protect the plant from oxidative damage and can activate certain enzymes that can scavenge reactive oxygen species. This association may protect greater duckweed from oxidative stress and aid to withstand oxidative damage under stress, mainly under pathogen attack (Lu et al. 2021). Algal colonies of *Gleotrichia, Calothrix, Nostoc, Anabaena* etc. are common in occurrence as epiphytes on a large number of aquatic macrophytes (Finkler, Seyley 1978). Under direct sunlight, the nitrogen fixing ability of cyanobacteria decreases (Daubs 1965). Thus, it may be concluded that the cyanobacteria obtains a favourable environment for N_2 fixation under the lower epidermis of the fronds of *S. polyrrhiza*; they were not found to be attached in the upper epidermis of the fronds in any of the collection sites. Diatoms species found attached with the fronds may be for their shelter, as they do not influence the growth rate of the greater duckweed under liquid culture conditions. The close association of *O. sancta* with fronds of *S. polyrrhiza* in

all collection spots may be for the supply of nitrogen under natural conditions, which is required for luxurious growth of duckweed. Higher accumulation of amino acids as well as protein can justify its value as food supplement for fishes and poultry birds (Appenroth 2017). The bioavailability of protein naturally growing other duckweed species like *Wolffia* spp. also increases when grown associated with cyanobacteria (Kaplan et al. 2019). Observations between duckweeds and blue green algae were reported in a few cases from aquatic ecosystems of other tropical countries like Malaysia and Indonesian islands (Guiry 2020). This paper possibly explored the duckweed-algal association in aquatic ecosystems of West Bengal for the first time.

Acknowledgements

The authors are grateful to USIC, The University of North Bengal, to provide us support regarding the SEM of the specimens collected from 10 different sites.

References

Appenroth K.J., Sree K.S., Böhm V., Hammann S., Vetter W., Leiterer M., Jahreis G. 2017. Nutritional value of duckweeds (Lemnaceae) as human food. *Food Chem.* 217: 266–273.

Beppu T., Takimoto A. 1981. Geographical distribution and cytological variation of *Lemna paucicostata* Hegelm. in Japan. *Bot. Mag.* 94: 11–20.

Bog M., Baumbach H., Schween U., Hellwig F., Landolt E,. Appenroth K.J. 2010. Genetic structure of the genus *Lemna* (Lemnaceae) as revealed by amplified fragment length polymorphism. *Planta* 232: 609–619.

Cao H.X., Vu G.T., Wang W., Messing J., Schubert I. 2015. Chromatin organisation in duckweed interphase nuclei in relation to the nuclear DNA content. *Plant Biol.* 17: 120–124.

Coler R.A., Gunner H.B. 1969. The rhizosphere of an aquatic plant (*Lemna minor*). *Can. J. Microbiol.* 15: 964–966.

Daubs E.H. 1965. *A monograph of Lemnaceae.* Biology Monograph No. 34. The University of Illinois Press, Urbana.

Driever S.M., Vannes E.H., Roijackers R.M.M. 2005. Growth limitation of *Lemna minor* due to high plant density. *Aquat. Bot.* 81: 245–251.

Fang Y.Y., Babourina O., Rengel Z., Yang X.E., Pu P.M. 2007. Ammonium and nitrate uptake by the floating plant *Landoltia punctata*. *Ann. Bot.* 99: 365–370.

Fay P. 1976. Factors influencing dark nitrogen fixation in a blue-green alga. *Appl. Environ. Microbiol.* 31: 376–379.

Finkel R., Seeley H.W. 1978. Nitrogen fixation (acetylene reduction) by epiphytes of freshwater macrophytes. *Appl. Environ. Microbiol.* 36: 129–138.

Ghosh S.K. 2005. *Illustrated Aquatic and Wetland Plants in Harmony with Mankind*. Standard Literature, India.

Goopy J.P., Murray P.J. 2003. A review on the role of duckweed in nutrient reclamation and as a source of animal feed. *Asian Austr. J. Anim. Sci.* 16: 297–305.

Guiry M.D., Guiry G.M. 2020. Algae Base. World-wide electronic Publication, National University of Ireland, Galway. https://www.algaebase.org.

Hastwell G.T., Daniel A.J., Vivian-Smith G. 2008. Predicting invasiveness in exotic species: do subtropical native and invasive exotic aquatic plants differ in their growth responses to macronutrients. *Divers. Distrib.* 14: 243–251.

Kaplan A., Zelicha H., Tsaban G., Meir A.Y., Rinott E. 2019. Protein bioavailability of *Wolffia globosa* duckweed, a novel aquatic plant-a randomized controlled trial. *Clin. Nutr.* 38: 2576–2582.

Khondker M., Nurul Islam A.K.M., Dur-E-Makhnun A. 1994. *Lemna perpusilla*: Screening on habitat limnology. *Bangladesh J. Bot.* 23: 99–106.

Kim K., Kim I. 2000. Structural aspects of the reduced free-floating hydrophyte, *Spirodela polyrhiza*. *Korean J. Microsc.* 30: 233–240.

Landolt E. 1986. *The Family Lemnaceae, a Monographic Study.* Stiftung Rubel, Zurich.

Liu Y., Xu H., Yu C., Zhou G. 2021. Multifaceted roles of duckweed in aquatic phytoremediation and bioproducts synthesis. *GCB Bioenergy* 13: 70–82.

Mc Lay J. 1974. The distribution of duckweed *Lemna perpusilla* in a small southern Californian lake: an experimental approach. *Ecology* 55:262–267.

Rao C.B. 1953. On the distribution of algae in a group of six small ponds. *J. Ecol.* 41: 62–71.

Schwalbe M., Teller S., Oelmuller R., Appenroth K.J. 1999. Influence of UVB irradiation on nitrate and ammonium assimilating enzymes in *Spirodela polyrhiza*. *Aquat. Bot.* 64: 205–211.

Sembada A.A., Faizal A. 2022. Protein and lipid composition of duckweeds (*Landoltia punctata* and *Wolffia arrhiza*) grown in a controlled cultivation system. *Asian J. Plant Sci.* 21: 637–642.

Tippery N.P., Les D.H., Crawford D.J. 2015. Evaluation of phylogenetic relationships in Lemnaceae using nuclear ribosomal data. *Plant Biol.* 17: 50–58.

Zuberer D.A. 1982. Nitrogen fixation (acetylene reduction) associated with duckweed (Lemnaceae) mats. *Appl. Environ. Microbiol.* 43: 344–352.

Received 24 April 2022; received in revised form 20 May 2023; accepted 30 June 2023