Arch Biol Sci. 2019;71(3):475-482 https://doi.org/10.2298/ABS190131030K

Growth performance and biochemical profile of *Azolla pinnata* and *Azolla caroliniana* grown under greenhouse conditions

Taylan Kösesakal[1,*] and Mustafa Yıldız[2]

[1]*Department of Botany, Faculty of Science, Istanbul University*, Istanbul, Turkey
[2]*Department of Aquaculture, Faculty of Aquatic Sciences, Istanbul University*, Istanbul, Turkey

***Corresponding author**: taylank@istanbul.edu.tr

Received: January 31, 2019; **Revised**: March 22, 2019; **Accepted**: April 25, 2019; **Published online**: May 10, 2019

Abstract: This study aimed to evaluate the growth performance, pigment content changes, essential amino acids (EAAs), fatty acids (FAs), and proximate composition of *Azolla pinnata* and *Azolla caroliniana* grown in a greenhouse. Plants were grown in nitrogen-free Hoagland's solution at 28±2°C/21±2°C, day/night temperature and 60-70% humidity and examined on the 3rd, 5th, 10th and 15th days. The mean percentage of plant growth and relative growth rate for *A. pinnata* were 119% and 0.148 $gg^{-1}day^{-1}$, respectively, while for *A. caroliniana* these values were 94% and 0.120 $gg^{-1}day^{-1}$, respectively. Compared to day 3, the amount of total chlorophyll obtained on day 15 decreased significantly ($p<0.05$) for *A. pinnata* while the total phenolic and flavonoid contents increased significantly ($p<0.05$) from the 3rd to the 15th day. However, the total phenolic and flavonoid contents did not differ ($p>0.0.5$) in *A. caroliniana.* The crude protein, lipid, cellulose, ash values and the amounts of EAAs were higher in *A. pinnata* than *A. caroliniana*. Palmitic acid, oleic acid, and lignoceric acid were found to be predominant in *A. pinnata* and *A. caroliniana*. From the plant growth and pigment contents, we concluded that *A. pinnata* grew faster than *A. caroliniana* and its photosynthetic efficiency was more effective.

Keywords: *Azolla*; chlorophyll; fatty acids; phenolics; essential amino acids

INTRODUCTION

Azolla is a floating fern that can grow in the absence of nitrogen in freshwater because of the symbiotic relationship between the heterocyst-forming, filamentous, nitrogen-fixing cyanobacteria *Anabaena azollae,* which lives in the dorsal lobe cavity of the leaves [1]. This symbiotic association has recently gained considerable importance due to its potential for use as an alternative to nitrogenous chemical fertilizers and animal feeding [2-4]. *Azolla* is very important for the agricultural activities of developed and developing countries [5]. *Azolla* has great potential for biological N fixation (30-100 kg N ha^{-1}) and thus *Azolla* species can be used effectively as a biofertilizer for paddy fields [6]. Furthermore, the use of *Azolla* species as a biofertilizer in rice fields improves soil fertility by increasing the organic matter in the soil, thus improving soil structure and environmental safety [7,8]. In addition, *Azolla* species are rich in proteins, essential amino acids, minerals, vitamins, carotenoids and growth promoter intermediaries. Therefore, with these nutritional values, *Azolla* species are a good source of feed for livestock [9].

Industrial development and agricultural practices have negative climate and environmental impacts. To meet the requirements for sustainable agriculture there is a need for novel crops that require less or no nitrogen fertilizer, use nonarable land with high biomass yields, and provide for both the food and chemical industries [10]. The use of wastewater as a source of reclaimed water would significantly reduce the cost and impact on the environment. Since the utilization of wastewater is very limited for most terrestrial crops, attention has shifted towards the use of aquatic plants [11]. *Azolla* species are one of the world's most economically important macrophytes [12] because of their high growth rates, high biomass production, bioremediation capacity, easy maintenance and easy harvest [13]. These plants can be used to improve water quality in view of their phytoremediation potentials.

In addition, *Azolla* species have been proposed as good candidates for phytoremediation of polluted freshwater areas [14-17]. Because of the multifaceted uses of *Azolla* species, especially in food, feed, biofuel production, agriculture and phytoremediation, it would be an ideal and environmentally-friendly factor in sustainable agriculture [2].

The main purpose of this study was to elucidate growth performance, pigment content changes, proximate composition, fatty acids and essential amino acids of *Azolla pinnata* and *Azolla caroliniana* grown under greenhouse conditions. Also, the potential use of *A. pinnata* and *A. caroliniana* for further experimental studies has also been evaluated.

MATERIALS AND METHODS

Azolla pinnata and *Azolla caroliniana* plants were used, which were grown in modified Hoagland's nutrient solution containing (in mg L^{-1}): KCl, 74.55; KH_2PO_4, 136.08; $CaCl_2 \cdot 2H_2O$, 147.02; $MgSO_4 \cdot 7H_2O$, 246.08; $ZnSO_4 \cdot 7H_2O$, 0.22; H_3BO_3, 2.86; $Na_2MoO_4 \cdot 2H_2O$, 0.09; $CuSO_4 \cdot 5H_2O$, 0.09; $MnCl_2 \cdot 4H_2O$, 1.82; $FeCl_3 \cdot 6H_2O$, 4.84 and Na_2EDTA, 15. The pH value of the nutrient solution was adjusted to 6.0. Plants were grown at 28±2°C/21±2°C, day/night temperature and 60-70% humidity under greenhouse conditions. To ensure the transfer of only healthy and young plantlets, the procedure described in [18] was applied. The percentage of plant growth, relative growth rate (RGR), total chlorophyll, carotenoid, total phenolic and flavonoid content changes were examined on the 3rd, 5th, 10th and 15th days.

Plant growth

Prior to application, the plants were weighed. The growth rate was measured by comparing the weight of the plants before and after the experimental times. Also relative growth rate (RGR) (g g^{-1} d^{-1}) of the plants was calculated using the formula:

$$RGR = (\ln W2 - \ln W1)/t,$$

where W1 and W2 are the initial and final fresh weights, respectively, and t is the experimental time [19].

Determination of chlorophyll and carotenoid contents

To determine the chlorophyll and carotenoid contents, 200 mg of leaves were extracted in 80% acetone (Merck) and the samples were centrifuged (Heraeus Labofuge 400 R) at 3000 *x* g (4°C) for 15 min. The pigment contents (chlorophyll a and b, total chlorophyll, and carotenoid) were measured using a Shimadzu 1601 UV-Visible Spectrophotometer) and expressed in µg/g fresh weight [20].

Analysis of total phenolics and flavonoids

Total phenolic and flavonoid contents of *Azolla* were measured as described [21]. The leaves were extracted with 1% HCl-methanol (5 mL), the extract was filtered and the filtrate was diluted with 1% HCl-methanol to 10 mL. Absorbance of the solution was measured at 280 nm for total phenolic and at 325 nm for the flavonoid contents. The total phenolic content was calculated from a standard curve made with gallic acid as a standard and expressed in GAeq. The flavonoid content was expressed as the absorbance at 325 nm/g fresh weight of *Azolla*.

Crude protein, lipid and cellulose analysis

The crude protein, lipid, cellulose and ash contents were determined according to the standard methodology [22]. Crude protein was determined as total nitrogen (N) using a semi-automatic Kjeldahl (Gerhardt VAPODEST, 45s) technique (N×6.25). The lipid content was determined by ether extraction using a Soxtherm Multistat/SX PC (Gerhardt, Germany). The ash content was obtained from the weight loss after incineration of dried samples in a muffle furnace. Cellulose was determined using sulfuric acid, then sodium hydroxide (12.5%, w/w), and the final residue was washed with 5% HCl and water, then filtered, dried, and weighed. All samples were analyzed in triplicate.

Analysis of amino acids

Amino acids were determined as described [23]. One hundred mg of freeze-dried plant samples (Alpha 1-2 LD plus, Christ, Germany) were digested in sealed glass tubes under nitrogen with 6 N HCl for 24 h at 110°C.

The samples were then filtered and the excess acid from the hydrolysate was removed by flash evaporation under reduced pressure and resuspended in 0.02 N HCl. Amino acid analysis was performed using HPLC (Agilent 1100) [24].

Fatty acid (FA) analysis

Fatty acid methyl esters were transmethylated with 2 M potassium hydroxide (KOH) (Merck, Germany) in methanol and n-hexane (Sigma-Aldrich, Germany) [25], with minor modification. Ten mg of extracted oil were dissolved in 2 mL of hexane followed by the addition of 4 mL of 2 M methanolic KOH. By vortexing the tube for 2 min at room temperature and a centrifugation at 4000 *x* g for 10 min, the resulting hexane layer was taken for GC analyses. By means of a gas chromatograph (Auto System XL Perkin Elmer, FID detector), using a 30 m x 0.25 mm x 0.25 µm capillary column (CP-2380 Supelco, USA), the FA composition was analyzed. The conditions of the method were as follows: carrier gas, helium; flame ionization detection temperature, 260°C; split rate was 1/0, oven temperature programmed to rise from 120°C/2 min to 220°C/15 min at a rate of 5°C min^{-1}; injector temperature was 240°C. Fatty acid methyl esters were identified by comparison to external standards (Sigma, USA). All FA analyses were performed on triplicate samples.

Statistical analysis

All the experimental data were obtained in 3 replicates. The experimental results are expressed as the mean±standard deviation. Statistical analysis was performed using GraphPad Prism version 5.2 for windows (GraphPad Software, San Diego, CA, USA). Statistically significant differences between the means were determined by *post-hoc* Tukey's multiple comparison test.

RESULTS

Plant Growth

Fig. 1. shows plant growth percentages and RGR's of *A. pinnata* and *A. caroliniana* respectively. Plant growth percentages of *A. pinnata* on the 3rd, 5th, 10th and 15th days were 44%, 107%, 129% and 194%, respectively. The RGR's of the *A. pinnata* plants at the experimental times were determined as 0.137, 0.180, 0.139 and 0.135 $gg^{-1}day^{-1}$, respectively. The average percentage growth and RGR for *A. pinnata* were 119% and 0.148 $gg^{-1}day^{-1}$, respectively. According to the plant growth percentage and RGR values, the biomass-doubling time of *A. pinnata* is 5.6 days (Fig. 1A). The percentage growth of *A. caroliniana* on days 3, 5, 10, and 15 was 36%, 82%, 104% and 154%, respectively, whereas the RGRs were 0.111, 0.146, 0.113 and 0.109 $gg^{-1}day^{-1}$ (Fig. 1B). The mean percentage growth and RGR for *A. caroliniana* were 94% and 0.120 $gg^{-1}day^{-1}$, respectively. The biomass-doubling time of *A. caroliniana* was 6.8 days.

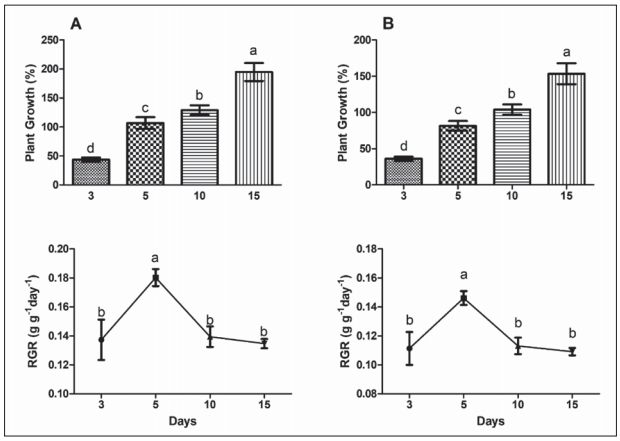

Fig. 1. Plant growth percentages and RGRs of *A. pinnata* (**A**) and *A. caroliniana* (**B**) on days 3, 5, 10 and 15. The bars represent the standard deviation. Significant differences determined by Tukey's multiple comparison test ($p<0.05$) are indicated by different letters.

Photosynthetic pigment contents

Table 1 shows the changes in the photosynthetic pigment contents of *Azolla pinnata* and *Azolla caroliniana* on days 3, 5, 10, and 15. For *A. pinnata*, the amount of chlorophyll a decreased significantly ($p<0.05$) from day 3 to day 15. The chlorophyll a/b ratio did not differ ($p>0.05$), even though the chlorophyll/carotenoid ratio decreased (Table 1). In addition, the total chlorophyll and carotenoid amounts on the 15th day decreased significantly ($p<0.05$) compared to the 3rd day (Table 1, Fig. 2A). The pigment contents of *Azolla caroliniana* on days 3, 5, 10 and 15 are given in Table 1. The photosynthetic pigment contents for *A. caroliniana* were slightly different from day 3 to day 15 (Table 1, Fig. 2B).

Table 1. Photosynthetic pigment contents of *A. pinnata* and *A. caroliniana* on days 3, 5, 10 and 15.

	Days	3	5	10	15
A. pinnata	Chlorophyll a (µg/g FW)	325.8±6.7	317.0±1.4	318.3±5.0	307.2±10.8
	Chlorophyll b(µg/g FW)	74.4±6.6	67.8±4.9	67.0 ±3.7	71.5±11.8
	Chlorophyll a/b	4.40±0.45	4.69±0.32	4.76±0.30	4.38±0.77
	Total Chlorophyll/Carotenoid	1.77±0.03	1.69±0.06	1.75±0.04	1.82±0.04
A. caroliniana	Chlorophyll a (µg/g FW)	289.7±5.8	292.5±15.6	292.5±11.2	266.4±9.3
	Chlorophyll b (µg/g FW)	62.2±1.2	61.8±0.5	62.2±3.0	58.0±3.3
	Chlorophyll a/b	4.66±0.16	4.73±0.27	4.71±0.13	4.60±0.10
	Total Chlorophyll/Carotenoid	1.90±0.03	1.84±0.03	1.90±0.13	1.96±0.14

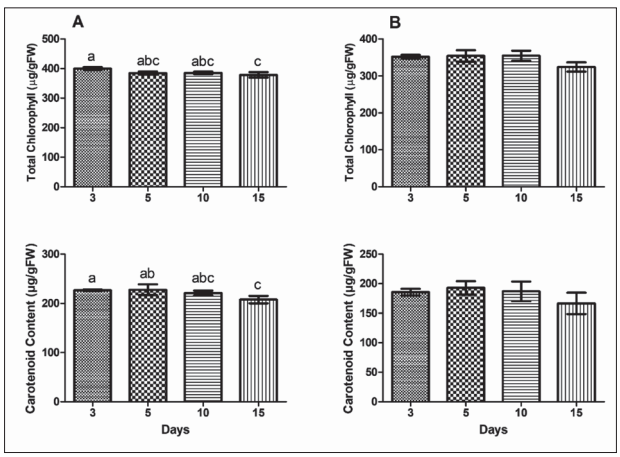

Fig. 2. Total chlorophyll and carotenoid contents of *A. pinnata* (**A**) and *A. caroliniana* (**B**) on days 3, 5, 10 and 15. Bars represent the standard deviation. Significant differences determined by the Tukey's multiple comparison test ($p<0.05$) are indicated by different letters.

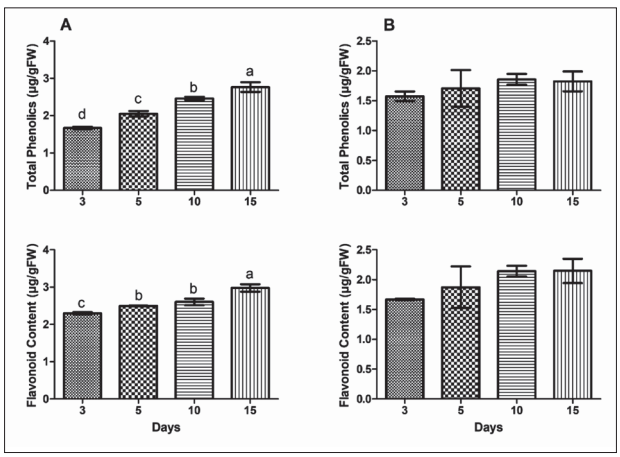

Fig. 3. Total phenolics and flavonoid contents of *A. pinnata* (**A**) and *A. caroliniana* (**B**) on days 3, 5, 10 and 15. Bars represent the standard deviation. Significant differences determined by the Tukey's multiple comparison test ($p<0.05$) are indicated by different letters.

Total phenolic and flavonoid contents

The amounts of total phenolics and flavonoids of *A. pinnata* on days 3, 5, 10 and 15 are given in Fig. 3A. The total phenolic content increased significantly ($p<0.05$) from day 3 to 15 (Fig. 3A). The mean total phenolic amount was 2.24 µg/g FW on the 15th day. The amount of total flavonoid content increased significantly from day 3 to 15 (Fig. 3A). However, the values obtained between day 5 and day 10 did not exhibit any significant difference ($p>0.05$). The mean total flavonoid content was 2.59 µg/g FW (Fig. 3A). The total phenolic content of *A. caroliniana* increased with time; however, this increase was not significant ($p>0.05$). The mean total phenolic content was 1.74 µg/g FW, while the total flavonoid content was 1.96 µg/g FW for the 15-day experimental period (Fig. 3B).

The proximate composition, essential amino acids and FA

The amounts of crude protein, lipid, ash and cellulose of *A. pinnata* and *A. caroliniana* plants are given in Table 2 and the amounts of EAAs are given in Table 3. Crude protein, lipid, cellulose and ash amounts were higher in *A. pinnata* than in *A. caroliniana* (Table 2). Analysis of the amino acid concentrations of *A. pinnata* showed higher amounts of EAAs than *A. caroliniana* (Table 3). On the other hand, arginine and leucine concentrations in *A. pinnata* and *A. caroliniana* were higher whereas histidine and methionine concentrations were lower at the end of the 15-day growth period (Table 3).

The main FA composition shows quantitative variations in *Azolla* species (Table 4). Oleic acid (C18:1),

Table 2. Proximate composition of *A. pinnata* and *A. caroliniana* after 15 days.

	Crude Protein (% dry weight)	Crude Lipid (% dry weight)	Crude Cellulose (% dry weight)	Crude Ash (% dry weight)
A. pinnata	22.8±1.56***	4.4±0.35	17.6±1.69	19.6±1.47
A. caroliniana	19.7±0.93	4.1±0.17	16.2±0.25	18.2±0.14

Table 3. The amounts of essential amino acids of *A. pinnata* and *A. caroliniana* after 15 days.

Essential amino acids (% dry matter)	*A. pinnata*	*A. caroliniana*
Arginine	1.32±0.11	1.23±0.15
Histidine	0.34±0.04	0.31±0.06
Isoleucine	0.79±0.08	0.68±0.09
Leucine	1.78±0.15	1.62±0.18
Lysine	1.21±0.09	1.11±0.14
Methionine	0.26±0.02	0.21±0.02
Tryptophan	1.25±0.09	1.15±0.15
Threonine	0.91±0.07	0.86±0.09
Valine	0.86±0.09	0.74±0.08

Table 4. Fatty acid composition of *A. pinnata* and *A. caroliniana* after 15 days.

Fatty Acids	*A. pinnata*	*A. caroliniana*
C16:0	19.83±2.08	35.61±3.74***
C18:0	3.10±0.33	1.74±0.18
C18:1	25.01±2.63***	14.18±1.22
C18:2	6.50±0.68	9.41±0.99
C18:3n3	6.11±0.64	8.31±0.87
C18:3n6	21.68±2.28***	8.83±0.93
C20:3n6	2.80±0.29	0.40±0.04
C22:0	ND	1.06±0.09
C22:1	ND	2.24±0.19
C22:2	ND	1.47±0.13
C20:5	ND	1.11±0.09
C24:0	12.56 ± 1.32	9.90±1.04
C22:6	ND	3.37±0.29*

ND – not detected; * and *** – $p<0.05$ and $p<0.001$, respectively

gamma-linolenic acid (C18:3n6, GLA), docosahexaenoic acid (C22:6, DHA), lignoceric acid (C24:0), and palmitic acid (C16:0), linoleic acid (C18:2, LA), alpha-linolenic acid (C18:3n3, ALA) were dominant FAs in *A. pinnata* and *A. caroliniana*, respectively. Oleic acid and gamma-linolenic acid amounts were higher in *A. pinnata* than in *A. caroliniana*. In addition, palmitic acid amounts were significantly higher in *A. caroliniana*.

DISCUSSION

Azolla is one of the fastest growing plants capable of doubling its biomass in 5-6 days [11]. In the present study, *A. pinnata* showed higher growth performance than *A. caroliniana* considering the average plant growth percentage and RGR values and the biomass-doubling time. In [25] it was reported that the RGR in control *Azolla filiculoides* plants was 0.148 $gg^{-1}day^{-1}$. In [26] it was stated that the RGR for *A. microphylla* and *A. caroliniana* was 0.085 and 0.087 $gg^{-1}day^{-1}$, respectively, whereas biomass-doubling time for the same plants were 8.29 and 7.98 days, respectively. In [27] it was demonstrated that in control *Azolla microphylla* plants, the RGR was 0.133 $gg^{-1}day^{-1}$ and the biomass-doubling time was 8.6 days. Also, it was reported that the RGR values obtained on day 14 for *A. filiculoides*, *A. microphylla*, *A. pinnata*, *A. rubra*, *A. mexicana* and *A. caroliniana* grown under greenhouse conditions were 0.11, 0.13, 0.06, 0.11, 0.10, and 0.11 $gg^{-1}day^{-1}$, respectively [28]. In the same study, the biomass-doubling times of *A. filiculoides*, *A. microphylla*, *A. pinnata*, *A. rubra*, *A. mexicana* and *A. caroliniana* plants were 6.3, 5.4, 11.1, 6.1, 6.6, and 6.1 days, respectively. The RGR is one of the important components of plant health and theoretically, plant RGR is closely related to biomass [29]. RGR values obtained from the current study for *A. pinnata* and *A. caroliniana* show that plants grow rapidly under greenhouse conditions as compared to other studies [28,30].

In this study, the amount of chlorophyll a was 317 µg/g FW and the amount of chlorophyll b was 67.8 µg/g FW on the 5th day for *A. pinnata*. On the other hand, chlorophyll a and b amounts for *A. caroliniana* on day 5 were 292.5 µg/g FW and 61.8 µg/g FW, respectively. The total chlorophyll content of *A. pinnata* was 400.2 µg/g FW on day 3, and 378.7 µg/g FW on day 15. The total chlorophyll content of *A. caroliniana* was 351.9 µg/g FW on day 3 and 324.3 µg/g FW on day 15. The amounts of chlorophyll a and b for *A. filiculoides* in the control medium were about 9 and 4 mg/g FW, respectively, whereas the total amount of chlorophyll was approximately 14 mg/g FW at the end of 7 days [31]. It was reported that the amounts of chlorophyll in the control medium of *A. pinnata* plant were about 6 and 7 mg/g FW on the 6th and 12th days, respectively [32]. The amounts of chlorophyll a, chlorophyll b and total

chlorophyll for the control *A. caroliniana* plant were approximately 130, 90, and 40 μg/g FW, respectively [14]. Plants regulate the chlorophyll concentration to balance the absorption, utilization and distribution capacities of light energy. This arrangement is considered to be an adaptation of plants to seasonal fluctuations under environmental stress [33]. According to the total chlorophyll values obtained from this study, it could be stated that, although the photosynthetic efficiency was more effective up to 10th day in *A. pinnata*, it was elevated up to the 15th day in *A. caroliniana*. Thus, the adaptation of *A. pinnata* to greenhouse conditions was faster than that of *A. caroliniana*.

Carotenoids have central functions in plants and are essential for photosynthesis and photoprotection [35]. Furthermore, carotenoids influence many plant processes and as antioxidants they can protect photosynthetic organisms against oxidative stress [36]. The amounts of carotenoids in the control *A. imbricata* plant were 0.281 mg/g FW on day 1, and 0.373 mg/g FW on day 9 [37]; the amount of carotenoids in the control *A. caroliniana* plant was 26.7 μg/g FW [14]. In the present study, the highest amounts of carotenoids measured in *A. pinnata* and *A. caroliniana* were 227.5 and 193.0 μg/g FW, respectively, on day 5. Carotenoids act as an auxiliary pigment in photosynthesis and also protect the photosynthetic apparatus from photooxidative damage by quenching triplet chlorophyll molecules and scavenging reactive oxygen species (ROS) such as singlet oxygen [38]. We hypothesized that the increase in the carotenoid contents in both *A. pinnata* and *A. caroliniana* during the first five days in the greenhouse were the result of the adaptation of plants to changing growth conditions.

Phenolics protect plants from adverse conditions, diseases, ROS, wounding, and from UV radiation [39]. The phenylpropanoid biosynthesis pathway is responsible for the synthesis of various secondary metabolites, including phenolic esters, coumarins, flavonoids and lignin [40]. It was observed that the total phenolic content in the control medium of *Azolla filiculoides* did not show significant differences between days 3 and 7 and it was about 2.5 mg/g FW [41]. Furthermore the total phenolic and total flavonoid contents of *A. pinnata* and *A. rubra* were 95.25 and 92.16 μg GAE/mg and 41.13 and 39.66 μg CE/mg, respectively [42]. In the present study, the total phenolic and flavonoid contents of *A. pinnata* increased significantly ($p<0.05$) from day 3 to day 15. Although there was an increase in total phenolics and flavonoids from day 3 to day 15 in *A. caroliniana*, it was not statistically significant ($p>0.05$). It is likely that the increase in total phenolics and flavonoids in *A pinnata* and *A. caroliniana* was a protective reaction in the adaptation of plants to greenhouse conditions.

It was reported that the amounts of crude protein, lipid, ash and cellulose for *A. pinnata* were 275, 41, 200, and 116 g/kg, respectively, per DW [3]. According to [43], the crude protein and ash values of *A. filiculoides* were 232 and 112 g/kg, respectively. In the current study, crude protein, lipid, cellulose and ash per DW were 22.8%, 4.4%, 17.6% and 19.6% for *A. pinnata*, and 19.7%, 4.1%, 16.2% and 18.2%, for *A. caroliniana* respectively. The amounts of EAAs obtained from *A. pinnata* and *A. caroliniana* in this study are similar to the findings presented in other studies [44,45]. Examination of the EAA levels of *A. pinnata* and *A. caroliniana* showed that these plants can be processed and used in human diet. In addition, these plants can be used in fish feeds due to their EAA values. The quantitative composition of fatty acids of *A. pinnata* and *A. caroliniana* is characterized by a high content of palmitic acid, oleic acid, alpha-linolenic acid and lignoceric acids. In the present study, the FA contents in *A. pinnata* and *A. caroliniana* species were similar to those obtained in [46-49]. Furthermore, the concentration of palmitic acid (C16:0) in *A. pinnata* and *A. caroliniana* species are relatively high compared to soybean, the main oilseed crop of the world [50]. Very few plant protein sources are known to contain all EAAs found in nature. As mentioned above, *A. pinnata* and *A. caroliniana* are rich sources of essential FAs due to their contents of C18:3n3, C18:1, C18:2, C18:3n6, C20:5 and C22:6 FA, which are very important for human nutrition and health. These plants will also occupy an important place in fish nutrition. Therefore, it is important to increase the production of these plants both for human nutrition and use in aquaculture.

The present findings suggest that *A. pinnata* and *A. caroliniana* could be grown more efficiently at 28±2°C/21±2°C day/night temperatures and 60-70% humidity under greenhouse conditions. From the plant growth and pigment content results, we conclude that *A. pinnata* grew faster than *A. caroliniana* and

its photosynthetic efficiency was better. Considering the EAAs and essential FAs contained in these plants, their uses in human nutrition and aquafeed possess an important economic value. In addition, the data about the percentage of plant growth, RGR, photosynthetic pigment, total phenolic and flavonoid contents, proximate composition, EAA and FA composition indicated that in studies of organic and inorganic pollution, phytoremediation and animal feeding applied under different conditions, *A. pinnata* and *A. caroliniana* plants grown for at least 15 days in nutrient solution will be more efficient and healthier.

Funding: This work was supported by the Istanbul University under Grant ID: FDP-2016-20651.

Acknowledgments: We are grateful to Samuel Ofori-Mensah, Institute of Graduate Studies in Science and Technology, Istanbul University, Turkey, for the help in English editing.

Author contributions: Taylan Kösesakal performed the experiments, analyzed the data and wrote the manuscript. Mustafa Yıldız contributed to the experimental design and manuscript editing. All authors reviewed and approved the final manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

REFERENCES

1. Costa ML, Santos MC, Carrapico F, Pereira AL. *Azolla-Anabaena*'s behaviour in urban wastewater and artificial media – Influence of combined nitrogen. Water Res. 2009;43:3743-50.
2. Yadav RK, Abraham G, Singh YV, Singh PK. Advancements in the utilization of *Azolla-anabaena* system in relation to sustainable agricultural practices. Proc Indian Natn Sci Acad. 2014;80:301.
3. Gangadhar B, Sridhar N, Saurabh S, Raghavendra CH, Hemaprasanth KP, Raghunath MR, Jayasankar P. Effect of *Azolla*-incorporated diets on the growth and survival of *Labeo fimbriatus* during fry-to-fingerling rearing. Cogent Food Agric. 2015;1:1055539.
4. Yao Y, Zhang M, Tian Y, Zhao M, Zhang B, Zeng K, Zhao M, Yin B. Urea deep placement in combination with *Azolla* for reducing nitrogen loss and improving fertilizer nitrogen recovery in rice field. Field Crops Res. 2018a;218:141-9.
5. Bocchi S, Malgioglio A. *Azolla-Anabaena* as a biofertilizer for rice paddy fields in the Po Valley, a temperate rice area in Northern Italy. Int J Agron. 2010;2010:152158.
6. Yao Y, Zhang M, Tian Y, Zhao M, Zeng K, Zhang B, Zhao M, Yin B. *Azolla* biofertilizer for improving low nitrogen use efficiency in an intensive rice cropping system. Field Crops Res. 2018b;216:158-64.
7. Carrapiço F. *Azolla* as a superorganism. Its implication in symbiotic studies. In: Seckbach J, Grube M, editors. Symbioses and stress: joint ventures in biology. Dordrecht: Springer; 2010. p. 225-41.
8. Subedi P, Shrestha J. Improving soil fertility through *Azolla* application in low land rice: A review. Azarian J Agric. 2015;2(2):35-9.
9. Gouri MD, Sanganal JS, Gopinath CR, Kalibavi1 CM. Importance of *Azolla* as a sustainable feed for livestock and poultry – A Review. Agric Review. 2012;33(2):93-103.
10. Brouwer P, Brautigam A, Kulahoglu C, Tazelaar AO, Kurz S, Nierop KG, van der Werf A, Weber AP, Schluepmann H. *Azolla* domestication towards a biobased economy? New Phytol. 2014;202:1069-82.
11. Miranda AF, Biswas B, Ramkumar N, Singh R, Kumar J, James A, Roddick F, Lal B, Subudhi S, Bhaskar T, Mouradov A. Aquatic plant *Azolla* as the universal feedstock for biofuel production. Biotechnol Biofuels. 2016;9:221.
12. Kollah B, Patra AK, Mohanty SR. Aquatic microphylla *Azolla*: a perspective paradigm for sustainable agriculture, environment and global climate change. Environ Sci Pollut Res Int. 2016;23:4358-69.
13. Muradov N, Taha M, Miranda AF, Kadali K, Gujar A, Rochfort S, Stevenson T, Ball AS, Mouradov A. Dual application of duckweed and *Azolla* plants for wastewater treatment and renewable fuels and petrochemicals production. Biotechnol Biofuels. 2014;7:30.
14. Roberts AE, Boylen CW, Nierzwicki-Bauer SA. Effects of lead accumulation on the *Azolla caroliniana-Anabaena* association. Ecotoxicol Environ Saf. 2014;102:100-4.
15. Kosesakal T. Effects of seasonal changes on pigment composition of *Azolla filiculoides* Lam. Am Fern J. 2014;104(2):58-66.
16. Kosesakal T. Assessment of the biodegradation capacity of *Azolla* on polycyclic aromatic hydrocarbons in crude oil. Global NEST J. 2018;20(3):27-32
17. Gomes MP, de Brito JCM, Carvalho Carneiro MML, Ribeiro da Cunha MR, Garcia QS, Figueredo CC. Responses of the nitrogen-fixing aquatic fern *Azolla* to water contaminated with ciprofloxacin: Impacts on biofertilization. Environ Pollut. 2018;232:293-9.
18. Kosesakal T, Unal M, Kulen O, Memon A, Yuksel B. Phytoremediation of petroleum hydrocarbons by using a freshwater fern species *Azolla filiculoides* Lam. Int J Phytoremediation. 2016;18:467-76.
19. Jampeetong A, Brix H. Effects of NH_4^+ concentration on growth, morphology, and NH_4^+ uptake kinetics of *Salvinia natans*. Ecol Eng. 2009;35:695-702.
20. Lichtenthaler HK, Wellburn AR. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem Soc Trans. 1983;11:591-2.
21. Zhang D, Quantick PC. Effects of chitosan coating on enzymatic browning and decay during postharvest storage of litchi (*Litchi chinensis* Sonn.) fruit. Postharvest Biol Tecnol. 1997;12:195-202.
22. AOAC. Official methods of analysis of AOAC International. 16th ed. Arlington, VA, USA: AOAC International; 1995.
23. AOAC. Official methods of analysis of AOAC International. 17th ed. Maryland, USA: AOAC International; 2000.
24. Antoine FR, Wei CI, Littell RC, Marshall MR. HPLC method for analysis of free amino acids in fish using *o*-phthaldi-

aldehyde precolumn derivatization. J Agr Food Chem. 1999;47(12):5100-7.
25. Ichihara K, Shibahara A, Yamamoto K, Nakayama T. An improved method for rapid analysis of the fatty acids of glycerolipids. Lipids. 1996;31(5):535-9.
26. Pereira AL, Monteiro B, Azevedo J, Campos A, Osorio H, Vasconcelos V. Effects of the naturally-occurring contaminant microcystins on the *Azolla filiculoides-Anabaena azollae* symbiosis. Ecotoxicol Environ Saf. 2015;118:11-20.
27. Kannaiyan S. Effect of Benlate and *Rhizoctonia* interactions on the growth, chlorophyll contents and nitrogen fixation in three species of *Azolla*. S Afr J Bot. 1992;58:292-5.
28. Abraham G. Antioxidant enzyme status in *Azolla microphylla* in relation to salinity and possibilities of environmental monitoring. Thin Solid Films. 2010;519:1240-3.
29. Arora A, Saxena S. Cultivation of *Azolla microphylla* biomass on secondary-treated Delhi municipal effluents. Biomass Bioenergy. 2005;29:60-4.
30. Li YY, Lü XT, Wang ZW, Zhou C, Han XG. Linking relative growth rate to biomass allocation: the responses of a grass (*Leymus chinensis*) to nitrogen addition. Phyton Int J Exp Bot. 2014;83:283-9.
31. Mostafa EM, Tammam AA. The oxidative stress caused by NaCl in *Azolla caroliniana* is mitigated by nitrate. J Plant Interact. 2011;7:356-66.
32. Vafaei F, Khataee AR, Movafeghi A, Salehi Lisar SY, Zarei M. Bioremoval of an azo dye by *Azolla filiculoides*: Study of growth, photosynthetic pigments and antioxidant enzymes status. Int Biodeterior Biodegradation. 2012;75:194-200.
33. Rai AK, Rai V. Effect of NaCl on growth, nitrate uptake and reduction and nitrogenase activity of *Azolla pinnata/Anabaena azollae*. Plant Sci. 2003;164:61-9.
34. Close DC, Davidson NJ, Davies NW. Seasonal fluctuations in pigment chemistry of co-occurring plant hemi-parasites of distinct form and function. Environ Exp Bot. 2006;58:41-6.
35. Sun T, Yuan H, Cao H, Yazdani M, Tadmor Y, Li L. Carotenoid metabolism in plants: The role of plastids. Mol Plant. 2018;11:58-74.
36. Savicka M, Petjukevičs A, Batjuka A, Škute N. Impact of moderate heat stress on the biochemical and physiological responses of the invasive waterweed *Elodea canadensis* (Michx. 1803). Arch Biol Sci. 2018;70(3):551-7.
37. Dai LP, Xiong ZT, Huang Y, Li MJ. Cadmium-induced changes in pigments, total phenolics, and phenylalanine ammonia-lyase activity in fronds of *Azolla imbricata*. Environ Toxicol. 2006;21:505-12.
38. Candan N, Tarhan L. Relationship among chlorophyll-carotenoid content, antioxidant enzyme activities and lipid peroxidation levels by Mg2+ deficiency in the *Mentha pulegium* leaves. Plant Physiol Biochem. 2003;41:35-40.
39. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. Plant Cell. 1995;7(7):1085.
40. Zhang X, Liu CJ. Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol Plant. 2015;8:17-27.
41. Forni C, Braglia R, Harren FJ, Cristescu SM. Stress responses of duckweed (*Lemna minor* L.) and water velvet (*Azolla filiculoides* Lam.) to anionic surfactant sodium-dodecyl-sulphate (SDS). Aquat Toxicol. 2012;110-111:107-13.
42. Noor Nawaz AS, Syed J, Dileep N, Rakesh KN, Prashith Kekuda TR. Antioxidant activity of *Azolla pinnata* and *Azolla rubra* –A comparative study. Sch Acad J Biosci. 2014;2(10):719-23.
43. Leterme P, Londoño AM, Muñoz JE, Súarez J, Bedoya CA, Souffrant WB, Buldgen A. Nutritional value of aquatic ferns (*Azolla filiculoides* Lam. and *Salvinia molesta* Mitchell) in pigs. Anim Feed Sci Technol. 2009;149:135-48.
44. Datta SN. Culture of *Azolla* and its efficacy in diet of *Labeo rohita*. Aquaculture. 2011;310:376-9.
45. van Kempen MML, Smolders AJP, Bögemann GM, Lamers LLM, Visser EJW, Roelofs JGM. Responses of the *Azolla filiculoides* Stras.–*Anabaena azollae* Lam. association to elevated sodium chloride concentrations: Amino acids as indicators for salt stress and tipping point. Aquat Bot. 2013;106:20-8.
46. Paoletti C, Bocci F, Lercker G, Capella P, Materassi R. Lipid composition of *Azolla caroliniana* biomass and its seasonal variation. Phytochemistry. 1987;26:1045-7.
47. Cohen MF, Meziane T, Tsuchiya M, Yamasaki H. Feeding deterrence of *Azolla* in relation to deoxyanthocyanin and fatty acid composition. Aquat Bot. 2002;74 (2):181-7.
48. Brouwer P, van der Werf A, Schluepmann H, Reichart G-J, Nierop KGJ. Lipid yield and composition of *Azolla filiculoides* and the implications for biodiesel production. BioEnergy Res. 2016;9(1):369-77.
49. Miranda AF, Liu Z, Rochfort S, Mouradov A. Lipid production in aquatic plant *Azolla* at vegetative and reproductive stages and in response to abiotic stress. Plant Physiol Biochem. 2018;124:117-25.
50. Matoša Kočar M, Sudarić A, Sudar R, Duvnjak T, Zdunić Z. Screening of early maturing soybean genotypes for production of high quality edible oil. Zemdirbyste-Agric. 2018;105(1):55-62.