

Article

# Differential Production of Phenolics, Lipids, Carbohydrates and Proteins in Stressed and Unstressed Aquatic Plants, *Azolla filiculoides* and *Azolla pinnata*

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**Simple Summary:** Crops are the primary feedstock for the production of food/feed and energy, and our dependency on them has been increasing over the last decade. Climate change, reduced land for active agriculture, increased population, reduced amount of clean water, and high costs of chemical fertilizers and energy make modern agriculture unsustainable. Demands are rising for the next generation of cheap and sustainable feedstock, which could be productive under existing conditions. Aquatic plants, such as the free-floating species duckweed and *Azolla*, have started attracting attention because of their unique features, presenting advantages over terrestrial plants. Terrestrial plants grow on the land or need to be on dry land to survive. On the other hand, aquatic plants have the ability to thrive in ponds under controlled conditions using wastewaters as a source of nutrients. Over the last decade, *Azolla* species became an attractive feedstock for livestock because of their accumulation of valuable products, phenolic compounds, proteins, lipids, and carbohydrates. Stress-triggered changes in these nutritional components could have a significant impact on the nutritional value of *Azolla*, which is used as a sustainable food supplement for livestock, poultry, and fish industries. This paper aims to assess the effects of environmental and nutrient stresses on the biosynthesis of valuable metabolites in *Azolla*.

**Abstract:** The metabolic plasticity of shikimate and phenylpropanoid pathways redirects carbon flow to different sink products in order to protect sessile plants from environmental stresses. This study assessed the biochemical responses of two *Azolla* species, *A. filiculoides* and *A. pinnata*, to the combined effects of environmental and nutritional stresses experienced while growing outdoors under Australian summer conditions. These stresses triggered a more than 2-fold increase in the production of total phenols and their representatives, anthocyanins (up to 18-fold), flavonoids (up to 4.7-fold), and condensed tannins (up to 2.7-fold), which led to intense red coloration of the leaves. These changes were also associated with an increase in the concentration of carbohydrates and a decrease in concentrations of lipids and total proteins. Changes in lipid biosynthesis did not cause significant changes in concentrations of palmitoleic acid (C16:0), linolenic acid (C18:3), and linoleic acid (C18:2), the fatty acid signatures of *Azolla* species. However, a reduction in protein production triggered changes in biosynthesis of alanine, arginine, leucine, tyrosine, threonine, valine, and methionine amino acids. Stress-triggered changes in key nutritional components, phenolics, lipids, proteins, and carbohydrates could have a significant impact on the nutritional value of both

Azolla species, which are widely used as a sustainable food supplement for livestock, poultry, and fish industries.

**Keywords:** anthocyanins; condensed tannins; fatty acid methyl esters (FAMES); flavonoids; nutrients

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## 1. Introduction

Crops are the primary feedstock for the production of food/feed and energy, and our dependency on them has increased over the last decade. Climate change, reduced land for active agriculture, increased population, reduced amount of clean water, along with high costs of chemical fertilizers and energy, make modern agriculture unsustainable [1,2]. Demands are rising for the next generation of cheap and sustainable feedstock which could be productive under existing conditions. Microalgae tick all the boxes for producing a broad spectrum of nutrients and showing the capacity to grow rapidly on wastewater nutrients [3–5]. However, challenges with harvesting under large scale production make their technology expensive [6,7]. Aquatic plants, such as the free-floating species duckweed and Azolla, have started attracting attention because of their unique features, which present the advantages of microalgae with easy and cheap harvesting technologies [8–13]. Azolla species grow in a symbiotic association with *Nostoc (Anabaena) azollae*, a nitrogen-fixing cyanobacterium that allows its host plant to grow rapidly in nitrogen-free solutions [14–16]. The application of this species for wastewater treatment and the production of biomass for food/feed and as feedstock for bioenergy has been broadly investigated [9,13,17–20]. Azolla thrives in tropical to temperate regions of the world [15,21]. When grown under optimal conditions, this species can double its biomass every two to four days, with average annual productivity of 39 t/hectare [11]. Over the last decade, Azolla species became an attractive feedstock for livestock because of the accumulation of valuable products, such as proteins (up to 400 g/kg DW), lipids (160 g/kg DW), and phenolics comprising antioxidants such as flavonoids, tannins, and anthocyanins (50 g/kg DW in total) [11,13,20,22–24].

Fresh Azolla can replace up to 50% of feed concentrate for cows, decreasing feed and labor costs by 16.5% and the cost of milk production by 18.5% [25]. The addition of fresh Azolla to the diet of native chickens, laying hens, and grazing ducks has also been shown to reduce the feeding costs significantly without affecting egg weight and other quality parameters [26]. The use of dried Azolla at 7.5% resulted in a 2.6% increase in the bodyweight of broilers and contributed to a better immune response [27]. Moreover, Azolla has been widely used as a low-cost and a high nutritional supplement in fish meal [28].

Azolla's protein level and composition of amino acids are the main nutritional components that make it a valuable feed supplement [11,29,30]. An average yield of dry Azolla biomass of 35 t DW/ha-year correlates with an annual protein production of 7–9 t DW/ha-year [29]. This is up to 3-fold higher than the soybean, which yields up to 2 t protein/ha-year [11,31]. An additional advantage of Azolla-based protein production is related to the fact that under favorable conditions these species can be grown and collected throughout the year, whereas the production of soybean grains is seasonal (once per year). Azolla can grow without nitrogen due to its nitrogen-fixing capacity, while commercial-scale soybean production still requires at least 150 kg of N fertilizer ha<sup>-1</sup> per year [11,32]. However, Azolla has a lower protein content compared to Spirulina, a cyanobacterium commonly used as a food source. Spirulina (*Arthrospira* sp.) contains around 48.9% protein and can produce in semi-continuous cultures up to 25 t protein/ha-year [33]. However, harvesting and concentration of cyanobacteria and algae still account for 30–40% of the product's cost [7].

Phenolic compounds, the products of shikimate and phenylpropanoids pathways, comprise more than 8000 compounds, including flavonoids, flavonols, flavones, anthocyanins, tannins and condensed tannins (proanthocyanins), and other benzene (C6) ring-containing molecules [34,35]. The most abundant phenolic compounds identified in Azolla include anthocyanidins, coumarins, flavonols,

flavones, and condensed tannins [23,35]. The antioxidation mechanism triggered by plant phenolics has been shown to benefit human health, including a reduced risk of developing cardiovascular diseases, cancer, diabetes type 2, and neurological diseases [36–38]. Condensed tannins are the most abundant phenolic component extracted from *Azolla* species [10,17]. Because of their health benefits, which include anticancer, antidiabetic, neuroprotective, and antimicrobial properties, they became popular as a food/feed supplement [39]. As livestock feed, low concentrations of these polymers can non-specifically bind food proteins, reducing their fermentation in gastrointestinal tracts and reducing ruminal bloats [17]. A high concentration of phenols and condensed tannins, however, can have a negative impact on the nutritional value of *Azolla* plants, reducing their digestibility [11,29,30,40–42]. Phenolics from *Azolla* have been extensively characterized previously [10,35,43].

The metabolic plasticity of shikimate and phenylpropanoid pathways redirecting carbon flow to the biosynthesis of different phenolic compounds and other carbon sinks, such as carbohydrates, lipids, and proteins, is a crucial evolutionary factor developed by sessile plants to protect them against biotic and abiotic stresses [44–46]. Stressed *Azolla* plants show the intense red coloration of their leaves, indicating enhanced production of colored phenolic pigments (Figure S1). Earlier, we showed that this triggered differential accumulation of metabolites from the phenylpropanoid and fatty acid biosynthesis pathways when plants were grown indoors, in glasshouse conditions [20,47]. In this study, we assessed the biochemical responses of two *Azolla* species, *A. filiculoides* and *A. pinnata*, to nutrient and environmental stresses experienced while growing outdoors under summer conditions in Australia. These two species differ in habitat, growth rates, and genetic makeup (i.e., *A. filiculoides* grows in temperate climates in Europe and *A. pinnata* grows in tropical climates in Asia and Australia) [48,49]. To our knowledge, this is the first comprehensive study of biochemical responses of two *Azolla* species, showing differential accumulation of the key nutrients (anthocyanins, flavonoids, condensed tannins, carbohydrates, lipids, and proteins) in response to stressed conditions.

## 2. Materials and Methods

### 2.1. Growth Conditions

Two aquatic plants, *A. filiculoides* and *A. pinnata*, were obtained from the RMIT collection of aquatic plants and algal species. As a stock, both plants were grown in 250-mL plastic boxes (11 cm × 18 cm) in half strength (1/2SH) Schenk and Hildebrandt's medium [49] in a glasshouse with a 16-h photoperiod, 25 °C, and a photosynthetic photon flux density of 100  $\mu\text{m}^2\text{-s}$  provided by fluorescent tubes. In further experiments, *A. filiculoides* and *A. pinnata* plants were grown in 1.3 m (length) × 0.7 m (width) × 0.50 m (depth) tanks in 1/2SH medium without nitrogen. Plants were stressed by starving and growing under direct exposure to sunlight during Australian summer conditions (light intensity 5–10 Klux daytime). In control experiments, plants were regularly fed and grown under 70% shade cloth conditions, reducing light intensity to 0.5 Klux.

### 2.2. Biochemical Analysis

#### 2.2.1. Lipid Extraction of Fatty Acid Methyl Esters

Lipid extraction and fatty acid methyl esters (FAMES) analysis was conducted according to Miranda et al. [20], with minor modifications. Briefly, 5 mL of solvent (chloroform/methanol 2:1) was added to a tube containing 50 mg of ground *Azolla* and was left to shake at 200 rpm overnight. Samples were then vortexed at the maximum speed for 1 min and centrifuged at 3000 rpm for 30 min. The resulting supernatant was transferred to a new tube, and 2 mL of NaCl was added before vortexing for 5 s. After that, the tubes were centrifuged at 3000 rpm for 3 min. The lower layer was transferred into the pre-weighed tube and dried under nitrogen flow at 70 °C.

### 2.2.2. Total Carbohydrates

Total carbohydrates were measured according to Chandran et al. [12], with minor modifications. Briefly, 2.5 mL of 2.5 N hydrochloric acid was added to 50 mg of sample. The sample was then hydrolyzed by boiling in a water bath for three hours. After cooling down, solid sodium carbonate was added to neutralize the reaction, and distilled water was added up to 50 mL. Samples were then centrifuged at  $3000\times g$  for 10 min at room temperature. The supernatant was then collected and 500  $\mu$ L was added to a test tube. One milliliter of Milli-Q and 4 mL of anthrone reagent was added to each test tube, including the blank. After mixing well, the samples were kept in a boiling water bath for 8 min. After cooling, the absorbance of a blue-green solution was measured at 630 nm using a spectrophotometer (POLARstar Omega, BMG Labtech, Offenburg, Germany) and compared with a standard curve prepared with known amounts of glucose. The amount of total carbohydrates in the sample was calculated and expressed as g glucose equivalents/100 g sample.

### 2.2.3. Total Protein

The total protein content was measured using the Kjeldahl method by using Foss™ Kjeldahl Digestion Systems. The nitrogen content of the samples was measured and converted to protein content by multiplying by 6.25, 4.59, and 5.0 [50].

### 2.2.4. Total Phenolics

Total phenolic concentration was estimated using the Folin–Ciocalteu method, according to Makkar [51], with minor modifications. To 1 g of powdered sample, 7 mL of extract buffer (80% methanol) was added. The mixture was kept shaking for 12 h at 4 °C in the dark before being centrifuged at  $3000\times g$  for 10 min at room temperature. The supernatant was collected, and 0.5 mL of Folin–Ciocalteu reagent (1 N) was added to each sample, including a blank. After mixing using a vortex, the samples were rested for 5 min, and 2.5 mL of 5% sodium carbonate was added to all tubes, except the blank. Finally, the samples were shaken and incubated for 40 min at room temperature. The absorbance of the blue color developed against the reagent blank at 725 nm was determined using a spectrophotometer (POLARstar Omega, BMG Labtech, Offenburg, Germany).

### 2.2.5. Total Flavonoids

The total amount of flavonoid was estimated according to Zhishen et al. [52] by using the aluminum chloride method. Briefly, 500  $\mu$ L of extract (supernatant in 2.4) was diluted to 1 mL with Milli-Q water, and 150  $\mu$ L of 5% sodium nitrite was added to all samples, including a blank. The tubes were then mixed and incubated at room temperature for 5 min before adding 150  $\mu$ L of 10% aluminum chloride to all the test tubes, including the blank. The mixture was mixed and further incubated for 6 min before adding 2 mL of 4% sodium hydroxide to all the test tubes. The contents of the tubes were made up to 5 mL using Milli-Q water, and the contents were mixed well using a vortex. The samples were rested for 15 min at room temperature, and the absorbance of the pink color that had developed was measured against the blank at 510 nm using a spectrophotometer (POLARstar Omega, BMG Labtech, Offenburg, Germany) and estimated based on the amounts of rutin as standard and expressed as mg/standard equivalent per gram dry powder for the samples.

### 2.2.6. Total Condensed Tannins

The amount of total condensed tannins was measured as described by Ray et al. [53], with minor modifications. To each gram of sample powder, 3 mL of extract buffer (80% methanol and 5.3 mM sodium bisulfite) was added in the dark. The mixture was kept shaking for 12 h at room temperature and centrifuged at  $3000\times g$  for 10 min at room temperature. The supernatant containing the methanol-soluble fractions was collected and analyzed according to Ray et al. [53] and using catechin as standard.

### 2.2.7. DMACA Staining of Condensed Tannins

The stain 4-dimethylaminocinnamaldehyde (DMACA) was used to detect condensed tannins in aquatic plants using the method described in Abeynayake et al. [54]. Briefly, plants were decolorized in absolute ethanol for 3 h and stained for the presence of proanthocyanidins and flavan-3-ols using 0.01% (*w/v*) 4-dimethylaminocinnamaldehyde (DMACA) in absolute ethanol containing 0.8% *w/v* hydrochloric acid for 2 h [55]. After this, plants were transferred to 100% ethanol and further vacuum-infiltrated for 1 min with fixative (6% *w/v* glutaraldehyde, 4% *w/v* paraformaldehyde in 50 mM sodium phosphate buffer, pH 7.4) before being incubated for 2 h at 4 °C. Finally, plants were washed three times (5 min) in 50 mM sodium phosphate buffer, pH 7.4. For making sections, tissues were embedded in LR White or paraffin matrices, followed by DMACA staining [55]. Transverse and longitudinal sections of embedded samples in LR White resin (6–10 µm) and paraffin (8 µm) were generated using a microtome. Cell walls were stained with 0.05% toluidine blue (ProSciTech, Kirwan, QLD, Australia, Cat. # C078). Images were captured using a Leica MZFLIII light microscope (Leica, Wetzlar, Germany).

### 2.2.8. Anthocyanin Levels

Anthocyanin levels were measured as in Neff et al. [56], with minor modifications. To 1 g of powdered sample, 3 mL of methanol 1% HCl was added in the dark. The mixture was shaken overnight in a dark room at 4 °C. After that, 2 mL of Milli-Q water and 5 mL of chloroform was added to the samples. The samples were centrifuged at 8000× *g* for 5 min at 4 °C. The supernatant was collected, and anthocyanin levels were estimated following [56] and by measuring the absorbance at 530 and 657 nm using a spectrophotometer (POLARstar Omega, BMG Labtech, Offenburg, Germany). The relative amount of anthocyanin was calculated by subtracting the A657 from the A530.

### 2.3. Statistical Analysis

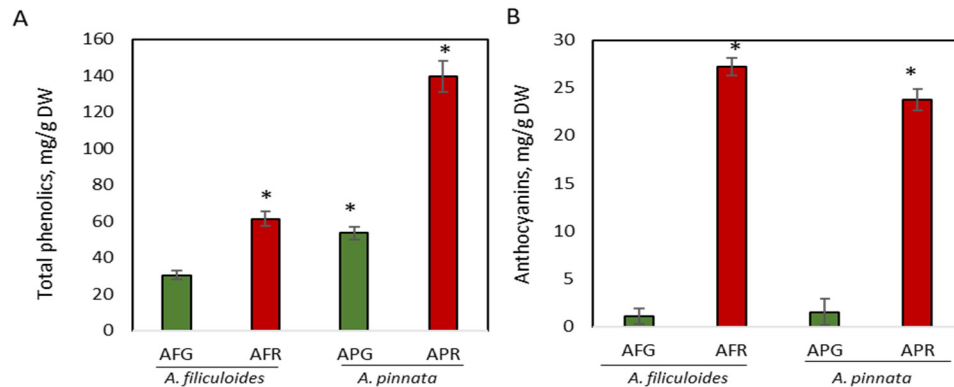
All experiments in this study were conducted in triplicate. All data are expressed as a mean ± standard deviation. The experimental data were subjected to the one-way analysis of variance (ANOVA) as implemented in the GraphPad InStat 3 statistics platform, San Diego, CA, USA. Tukey simultaneous tests were conducted to determine the statistical differences between treatments. To ascertain that the observed variations were statistically significant, the probability (P) values were determined. A 95% confidence level ( $p < 0.05$ ) was applied for all analyses.

## 3. Results and Discussion

### 3.1. Accumulation of Phenolic Compounds in Green and Red *A. filiculoides* and *A. pinnata* Plants

*A. filiculoides* and *A. pinnata* plants growing outdoors under normal and stressed conditions (Material and Methods) were assessed for the concentration of phenolic compounds, total phenols, anthocyanins, flavonoids, and condensed tannins, which have a complex effect on the nutritional value of these plants [10,11]. In unstressed plants, the concentration of total phenols in *A. pinnata* was 1.7-fold higher than in *A. filiculoides*, producing 53.5 mg/g DW (5.1% DW) compared to 30.4 mg/g DW (3.1% DW) in *A. filiculoides* ( $p < 0.05$ ) (Figure 1A). Stress increased the accumulation of phenols up to 2.6-fold in *A. pinnata*, and 2-fold in *A. filiculoides*, producing 139.5 mg/g DW (14.2% DW) and 61.4 mg/g DW (6.0% DW), respectively (Figure 1A). A higher concentration of phenolics in *A. pinnata* than in *A. filiculoides* was also shown for the same species by Brouwer et al. [11]. The enhancement of total phenolic compounds exposed to different abiotic stresses has also been shown for other *Azolla* species [57–60]. Unstressed *A. filiculoides* and *A. pinnata* showed similar very low concentrations of anthocyanins (Figure 1B). Stress significantly increased the concentration of anthocyanins in red *A. filiculoides* by up to 18-fold, with the concentration rising from 1.5 mg/g DW to 27.18 mg/g DW (Figure 1B). In red *A. pinnata*, the concentrations of anthocyanins showed a 15-fold increase (up to 23.8 mg/g DW) when compared to green plants. In terrestrial plants, an increased

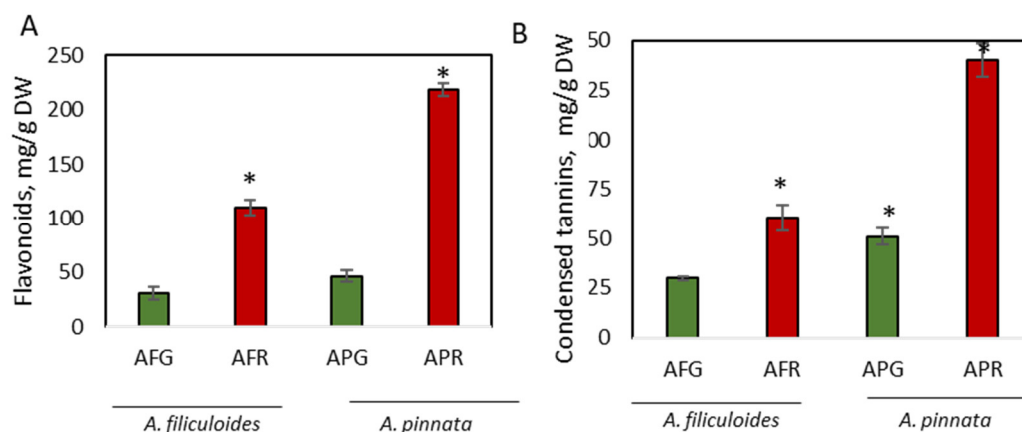
concentration of anthocyanins in response to biotic/abiotic stress conditions, such as high light intensity, cold, and pathogen infections, in order to protect from oxidative damage is a well-investigated phenomenon [61,62]. A much lower increase in the concentration of anthocyanins was observed in *Azolla imbricata* after nine days of exposure to 0.05–0.1 mg/L of cadmium [60].



**Figure 1.** Accumulation of total phenolics (A) and anthocyanins (B) in green and red *A. filiculoides* and *A. pinnata*. AFG: *A. filiculoides*\_Green; AFR: *A. filiculoides*\_Red; APG: *A. pinnata*\_Green; APR: *A. pinnata*\_Red. (ANOVA; \*  $p < 0.05$  compared to AFG).

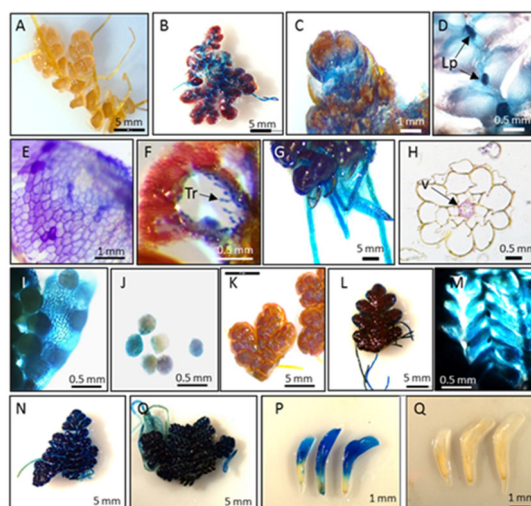
It was shown that anthocyanins in frond extracts of *Azolla* are mainly represented by 3-deoxyanthocyanins and their acetylated glycosides, such as luteolinidin ( $m/z$  475) and apigeninidin ( $m/z$  459) [63,64]. High levels of deoxyanthocyanins in *Azolla* fronds negatively correlated with their palatability for adult *Lymnaea swinhoei* snails and *Polypedates leucomystax* tadpoles [65].

The concentration of another representative of phenolic compounds, flavonoids, were increased 3.6-fold and 4.7-fold, reaching concentrations of 109 mg/g DW and 218 mg/g DW, respectively, in stressed *A. filiculoides* and *A. pinnata* plants (Figure 2A). Both stressed and green *A. pinnata* plants contained a higher concentration of flavonoids than *A. filiculoides*. Earlier, we showed a detailed composition of flavonoids in *Azolla* grown on swine wastewater during the summer and winter in Australia [47]. An extensive list of the flavonoids identified in *A. filiculoides* and *A. pinnata* was previously shown [17,66]. Similarly to *Azolla*, the increase in the concentrations of flavonoid compounds was triggered in duckweed (*Landoltia punctata*) by nutrient starvation [67]. In terrestrial plants, the composition of flavonoids and their metabolic plasticity is mostly dependent on the plant species, their developmental stage, and environmental conditions, including both biotic and abiotic stresses [35].



**Figure 2.** Accumulation of flavonoids (A) and condensed tannins (B) in green and red *A. filiculoides* and *A. pinnata*. AFG: *A. filiculoides*\_Green; AFR: *A. filiculoides*\_Red; APG: *A. pinnata*\_Green; APR: *A. pinnata*\_Red. (ANOVA; \*  $p < 0.05$  compared to AFG).

Condensed tannins were identified as the most abundant soluble phenolic compounds in both *Azolla* species, accounting for 30.2 mg/g DW (3.1% DW) and 51.1 mg/g DW (5.2% DW) of the biomasses of *A. filiculoides* and *A. pinnata*, respectively (Figure 2B). Their concentrations were further increased, up to 2.7-fold, in response to stress (Figure 2A). Staining with DMCA showed a blue coloration ubiquitously accumulating in vegetative and reproductive organs (Figure 3). In leaves, blue staining was observed mainly in dorsal compared to ventral lobes (Figure 3B,C; Figure S2). Intense blue staining was detected in leaf primordia (Figure 3D). At the cellular level, blue staining was mainly observed in cell walls (Figure 3E). Within leaf cavities, strong staining was detected in trichomes (Figure 3F). Accumulation of condensed tannins in trichomes was also shown by Pereira et al. [68]. In *Azolla* roots, DMACA staining was detected in the vascular cells around the xylem (Figure 3G,H). The accumulation of insoluble (poly)phenols around the vascular tissue of *A. filiculoides* was previously observed, using confocal imaging, in stems and leaves of *A. filiculoides* stained with propidium periodide [17]. In reproductive organs, a well-detectable level of condensed tannins was observed in microsporocarps and microspores (Figure 3I,J). In both species, stronger coloration was detected in stressed plants compared to green plants. White clover flowers were used as a positive control for DMACA activity, since they have a high level of condensed tannins [69]. Accumulation of condensed tannins in root vasculature cells resembles accumulation profiles of lignin in the roots of terrestrial plants. Existing data describing lignin content in *Azolla* species is controversial, with a broad range of concentrations observed at different developmental stages, from no lignin until 41% in mature plants [23,42,70,71].



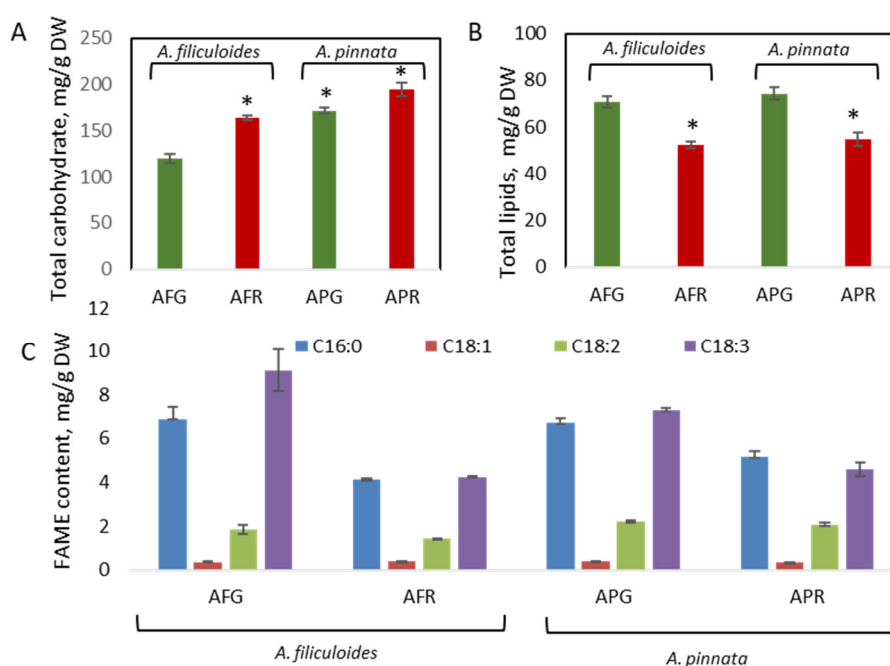
**Figure 3.** Staining of condensed tannins in *A. filiculoides* and *A. pinnata* with 4-dimethylaminocinnamaldehyde (DMACA). *A. filiculoides* green, non-stained (A) and stained by DMACA (B–J); leaf (E); leaf cavity (F); roots (G–H); microsporocarps (I) microspores (J); *A. filiculoides* red, non-stained (K) and stained with DMACA (L,M); *L. pinnata* green, stained (N); *L. pinnata* red, stained (O); white clover flowers stained with DMACA (P), positive control); white clover flowers not stained with DMACA (Q), negative control). Lp: leaf primordia; Tr: trichomes; V: vasculature cells.

Based on the molecular analysis of flash pyrolysis and thermally assisted hydrolysis and methylation, the production of lignin was not predicted in *Azolla caroliniana* [23]. However, it is very likely that these strategies were not sensitive enough, since low levels of G-lignin-specific pyrolysis products, such as 4-(1-propenyl)guaiacol, cis-4-(2-propenyl)guaiacol, and trans-4-(2-propenyl)guaiacol, were identified in *A. filiculoides* [17]. Recent studies indicate that high concentrations of phenolic compounds, caffeoylquinic acids, and condensed tannins are responsible for reducing the nutritional value and digestibility of *Azolla* species [10].

However, low concentrations of condensed tannins (<3% DW) in a common forage plant, white clover, showed a beneficial effect on animal health, preventing pasture bloat and increasing nutrient uptake in ruminant livestock as a result of protection of proteins from fermentation [35,72]. Inconsistent data reflecting animal responses to the concentration of condensed tannins in the diet also correlates with their structures [73]. In terrestrial plants, the accumulation of protein-bound or fiber-bound condensed tannins protects them from vertebrates and insects [11,74]. Condensed tannins are built by two main monomers: 2,3-trans-flavan 3-ols (afzelechin, catechin, and gallocatechin) and 2,3-cis-flavan 3-ols (epiafzelechin, epicatechin, and epigallocatechin) [10,69].

### 3.2. Accumulation of Proteins, Lipids, and Carbohydrates in Green and Red *A. filiculoides* and *A. pinnata*

Green *A. pinnata* showed a significantly higher content of total carbohydrates than *A. filiculoides*, with 171 mg/g DW and 119 mg/g DW (12% DW and 17% DW), respectively (Figure 4A). Stress conditions increased the concentration of carbohydrates in both plants, with up to 164 mg/g DW and 195 mg/g DW (16% DW and 19% DW) in *A. filiculoides* and *A. pinnata*, respectively. These levels are similar to the sum of polysaccharides (19.8% DW) and soluble sugars (2.1% DW) but lower than the levels of carbohydrates observed for *A. filiculoides* (40% DW) collected from a lake in Iran [17,18].



**Figure 4.** Accumulation of carbohydrates (A) and total lipids (B) in green and red *A. filiculoides* and *A. pinnata*. Fatty acid methyl ester (FAME) composition of lipids (C). AFG: *A. filiculoides*\_Green; AFR: *A. filiculoides*\_Red; APG: *A. pinnata*\_Green; APR: *A. pinnata*\_Red. (ANOVA; \*  $p < 0.05$  compared to AFG).

The lipid content in green plants was around 7% DW (72 mg/g DW) and was significantly increased when plants were stressed (5% DW, 52 mg/g DW) (Figure 4B). High concentrations of palmitoleic acid (C16:0), linolenic acid (C18:3), and linoleic acid (C18:2) with lower levels of oleic acid (18:1) are FAME signatures for *Azolla* species (Figure 4C) [20]. In both *Azolla* species, stress conditions have not been shown to contribute to significant changes in the FAME profiles. Earlier, it was shown that, based on its FAME composition, *Azolla* could be a high-quality feedstock for biodiesel if an additional fractionation step could be used to decrease the cold filter plugging point [11,20].



Total nitrogen, protein, and amino acid contents of *A. filiculoides* and *A. pinnata* are shown in Tables 1 and 2. Using the Kjeldahl analysis, stressed *A. filiculoides* and *A. pinnata* showed 1.4- and 1.6-fold lower concentrations of nitrogen than green plants, respectively. Table 1 shows total protein concentrations based on three known nitrogen-to-protein conversion factors: a standard nitrogen-to-protein conversion factor (6.25) based on albumin protein, as well as updated conversion factors, 4.59 and 5.0, used for marine and aquatic plants respectively [11,75,76].

Based on our results, the sum of amino acids in green *A. filiculoides* and *A. pinnata* ranged from 205 to 265 mg/g DW, which is lower than the total protein value based on the 6.25 conversion factor, but higher than for conversion factors 4.59 and 5.0 and can be averaged as 5.3. Stressed plants showed lower protein concentrations than green plants, with 141 mg/g DW and 134 mg/g DW for *A. filiculoides* and *A. pinnata*, respectively, reflecting the differences in concentrations of total nitrogen. The fact that the 6.25 conversion factor is not accurate for nitrogen-fixing plants can be explained by the accumulation of nitrogen in the form of different nitrogen-containing compounds [11]. Conversion levels for nitrogen-to-protein lower than 6.25 were observed for other aquatic plants such as duckweed (4.8), water hyacinth (4.59), and *Ulva lactuca* (4.59) [11]. The FAME composition of *Azolla* plants is shown in Table 2. Stress led to changes in the profiles of some amino acids in both plants. In *A. filiculoides*, it caused an increase in levels of alanine, arginine, leucine, tyrosine, threonine, and valine ( $p \leq 0.05$ ), while the percentage of glycine was decreased. In *A. pinnata*, stress increased the contents of asparagine and glycine. In both species, the methionine biosynthesis was reduced. In terrestrial plants, the increased levels in some of these amino acids have previously been shown to be triggered by abiotic stress [77–82].

**Table 1.** Concentrations of nitrogen, total amino acids (AA) and proteins in *A. filiculoides* and *A. pinnata*.

	Total N mg/g DW	Total Protein *, mg/g DW	Total Protein **, mg/g DW	Total Protein ***, mg/g DW	Total AA, mg/g	N-Prot Factor
AFG	38.1 ± 2.5 #	237.5 ± 22.2	174.42 ± 12.1	190 ± 9.1	205.3 ± 8.1 #	5.40
AFR	26.4 ± 3.2 #	165 ± 8.3	121.176 ± 10.1	132 ± 10.8	140.9 ± 7.4 #	5.34
APG	46.0 ± 4.1 #	287.5 ± 15.4	211.14 ± 14.4	230 ± 18.5	265.0 ± 10.4 #	5.76
APR	27.5 ± 2.3 #	171.8 ± 10.0	126.225 ± 8.8	137.5 ± 6.6	134.0 ± 4.3 #	4.87

AFG: *A. filiculoides*\_Green; AFR: *A. filiculoides*\_Red; APG: *A. pinnata*\_Green; APR: *A. pinnata*\_Red. Nitrogen-to-protein conversion factors: \* 6.25 [11]; \*\* 4.59 [75]; \*\*\* 5.0 [76]. AA: amino acids. # Significance levels:  $p < 0.05$ .

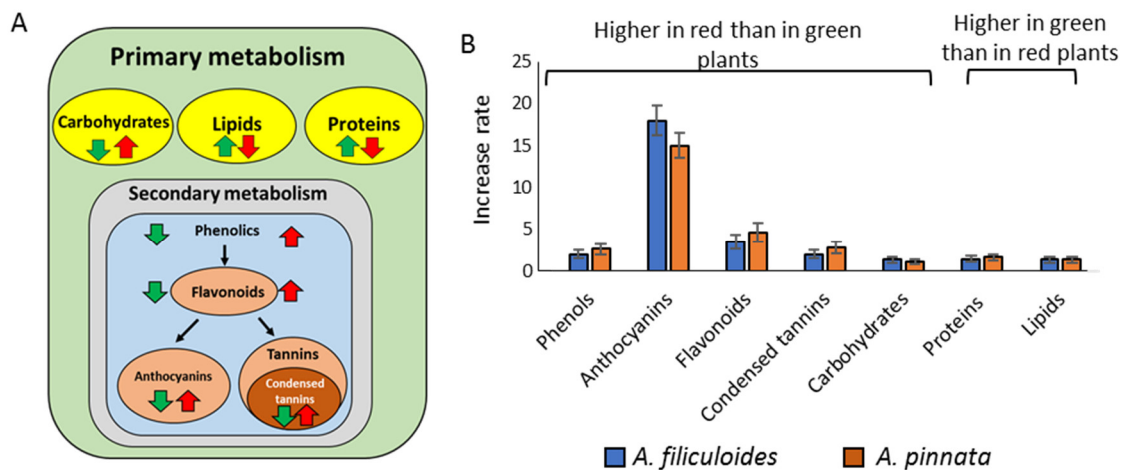
**Table 2.** Amino acid concentrations in biomass of *A. filiculoides* and *A. pinnata* grown under normal and stress conditions.

AA	AA Content, % DW				AA Content, mg/g				AA Content, % in Total AA			
	AFG	AFR	APG	APR	AFG	AFR	APG	APR	AFG	AFR	APG	APR
Ala	0.46 ± 0.01	0.44 ± 0.1	0.62 ± 0.1	0.27 ± 0.01	4.6 ± 0.1	4.35 ± 0.1	6.22 ± 0.02	2.705 ± 0.1	2.26 ± 0.02	3.07 ± 0.02	2.32 ± 0.01	2.32 ± 0.02
Arg	2.3 ± 0.05	2.28 ± 0.07	3.1 ± 0.02	1.59 ± 0.08	22.97 ± 0.5	22.81 ± 0.65	31.03 ± 0.19	15.93 ± 0.8	11.21 ± 0.01	16.12 ± 0.02	11.55 ± 0.03	11.51 ± 0.1
Asp	2.08 ± 0.07	1.55 ± 0.1	2.47 ± 0.1	1.45 ± 0.06	20.8 ± 0.66	15.46 ± 0.47	24.5 ± 0.11	14.46 ± 0.6	10.15 ± 0.11	10.92 ± 0.12	9.19 ± 0.06	10.45 ± 0.02
Cys	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glu	4.51 ± 0.09	2.09 ± 0.06	6.94 ± 0.04	4.29 ± 0.02	45.1 ± 0.9	20.89 ± 0.64	69.37 ± 0.36	38.92 ± 2.1	22.00 ± 0.05	14.76 ± 0.10	25.83 ± 0.04	31.3 ± 0.16
Gly	1.27 ± 0.02	0.75 ± 0.02	1.56 ± 0.02	0.85 ± 0.03	12.67 ± 0.16	7.50 ± 0.20	15.55 ± 0.16	8.50 ± 0.3	6.18 ± 0.06	5.30 ± 0.06	5.79 ± 0.04	5.79 ± 0.02
His *	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ile *	1.06 ± 0.03	0.76 ± 0.02	1.29 ± 0.02	0.59 ± 0.02	10.57 ± 0.25	7.59 ± 0.21	12.89 ± 0.03	5.87 ± 0.27	5.16 ± 0.01	5.37 ± 0.02	4.80 ± 0.02	4.25 ± 0.02
Leu *	1.8 ± 0.03	1.34 ± 0.03	2.26 ± 0.02	1.31 ± 0.04	17.95 ± 0.32	13.35 ± 0.37	22.61 ± 0.017	11.5 ± 0.49	8.76 ± 0.04	9.43 ± 0.04	8.42 ± 0.03	7.99 ± 0.06
Lys *	1.43 ± 0.02	0.96 ± 0.03	1.64 ± 0.02	0.53 ± 0.03	14.34 ± 0.2	9.59 ± 0.32	16.38 ± 0.21	5.34 ± 0.32	7.00 ± 0.11	6.78 ± 0.13	6.10 ± 0.06	6.03 ± 0.1
Met *	1.22 ± 0.04	0.62 ± 0.02	1.58 ± 0.02	0.37 ± 0.01	12.25 ± 0.35	6.18 ± 0.20	15.75 ± 0.21	4.85 ± 0.19	5.96 ± 0.05	4.37 ± 0.03	5.87 ± 0.08	3.51 ± 0.08
Phe *	0.92 ± 0.02	0.62 ± 0.02	1.14 ± 0.00	0.54 ± 0.002	9.15 ± 0.22	6.24 ± 0.18	11.36 ± 0.03	5.35 ± 0.24	4.47 ± 0.01	4.41 ± 0.02	4.23 ± 0.02	3.87 ± 0.01
Pro	0.09 ± 0.02	0.06 ± 0.01	0.11 ± 0.02	0.06 ± 0.01	0.91 ± 0.16	0.61 ± 0.11	1.09 ± 0.18	ND	0.44 ± 0.07	0.43 ± 0.08	0.41 ± 0.07	ND
Ser	0.94 ± 0.02	0.65 ± 0.02	1.15 ± 0.01	0.6 ± 0.02	9.4 ± 0.16	6.49 ± 0.17	11.54 ± 0.08	6.05 ± 0.2	4.6 ± 0.03	4.59 ± 0.03	4.3 ± 0.02	4.37 ± 0.00
Thr *	0.9 ± 0.02	0.73 ± 0.02	1.05 ± 0.01	0.44 ± 0.02	9.01 ± 0.24	7.25 ± 0.20	10.52 ± 0.06	4.45 ± 0.22	4.40 ± 0.02	5.12 ± 0.02	3.92 ± 0.04	3.22 ± 0.01
Tyr	0.33 ± 0.01	0.33 ± 0.01	0.51 ± 0.00	0.12 ± 0.02	3.2 ± 0.05	3.29 ± 0.08	5.12 ± 0.04	1.17 ± 0.06	1.59 ± 0.02	2.33 ± 0.02	1.91 ± 0.01	1.91 ± 0.02
Val *	1.23 ± 0.03	0.93 ± 0.03	1.48 ± 0.00	0.72 ± 0.01	12.26 ± 0.34	9.35 ± 0.27	14.84 ± 0.04	7.19 ± 0.35	5.98 ± 0.03	6.60 ± 0.05	5.53 ± 0.03	5.20 ± 0.01
Total	20.5 ± 0.4	14.1 ± 0.2	26.5 ± 0.4	14.0 ± 0.5	205.3 ± 8.01	140.9 ± 7.4	265.9 ± 10.4	134 ± 4.3	100.1 ± 0.9	99.6 ± 0.8	100.1 ± 0.6	99.3 ± 0.1

\* Essential amino acids; AFG: *A. filiculoides*\_Green; AFR: *A. filiculoides*\_Red; APG: *A. pinnata*\_Green; APR: *A. pinnata*\_Red. AA: amino acids. Significance levels:  $p < 0.05$ ; ND-Not detected; Alanine (Ala); Arginine (Arg); Aspartic acid (Asp); Cysteine (Cys); Glutamic acid (Glu); Glycine (Gly); Histidine (His); Isoleucine (Ile); Leucine (Leu); Lysine (Lys); Methionine (Met); Phenylalanine (Phe); Proline (Pro); Serine (Ser); Threonine (Thr); Tyrosine (Tyr); Valine (Val).

#### 4. Conclusions

Azolla is a novel sustainable feed supplement, producing compounds that can be broadly assessed under fully controlled indoor conditions. However, globally, a large majority of farmers are growing plants outdoors, and as a result, the plant's nutritional value can be affected by environmental and nutrient conditions. The changes in key components, phenolics, proteins, carbohydrates, and proteins were summarized in Figure 5. Anthocyanins, flavonoids, and condensed tannins were observed to be the most induced nutrients in response to stresses. Increased levels of these molecules, decreased concentrations of lipids and proteins, along with changes in amino acid profiles could have a complex impact on the nutritional value of these plants.



**Figure 5.** (A) Representation of differential accumulation of cellular primary (carbohydrates, lipids, and proteins) and secondary (phenolics, anthocyanins, flavonoids, and condensed tannins) metabolites in red (red arrow) and green (green arrow) plants; (B) comparative increase rates (fold increase,  $p < 0.05$ ) in production of metabolites between red and green Azolla plants.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2079-7737/9/10/342/s1>, Figure S1: (A) *A. filiculoides* green (a) and red (b) grown outdoors. (B) *A. pinnata* green (a) and red (b) grown outdoors. Figure S2: (A) Longitudinal sections of *A. filiculoides* plants. (B) *Nostoc azollae* in leaf cavities shown by arrow.

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#### References

- Ritchie, H.; Roser, M. Environmental impacts of food production. *Our World Data*. 2020. Published online at [OurWorldInData.org](https://ourworldindata.org). Available online: <https://ourworldindata.org/environmental-impacts-of-food> (accessed on 5 September 2020).
- Popp, J.; Lakner, Z.; Harangi-Rákos, M.; Fári, M. The effect of bioenergy expansion: Food, energy, and environment. *Renew. Sustain. Energy Rev.* **2014**, *32*, 559–578. [[CrossRef](#)]
- Siddiqui, M.R.; Miranda, A.; Mouradov, A. Microalgae as Bio-Converters of Wastewater into Biofuel and Food. In *Water Scarcity and Ways to Reduce the Impact: Management Strategies and Technologies for Zero Liquid Discharge and Future Smart Cities*; Pannirselvam, M., Shu, L., Griffin, G., Philip, L., Natarajan, A., Hussain, S., Eds.; Springer International Publishing: Cham, Switzerland, 2019; pp. 75–94.

4. Miranda, A.F.; Ramkumar, N.; Andriotis, C.; Höltkemeier, T.; Yasmin, A.; Rochfort, S.; Wlodkovic, D.; Morrison, P.; Roddick, F.; Spangenberg, G. Applications of microalgal biofilms for wastewater treatment and bioenergy production. *Biotechnol. Biofuels* **2017**, *10*, 120. [[CrossRef](#)] [[PubMed](#)]
5. Pulz, O.; Gross, W. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol.* **2004**, *65*, 635–648. [[CrossRef](#)] [[PubMed](#)]
6. Borowitzka, M.A.; Moheimani, N.R. Sustainable biofuels from algae. *Mitiga. Adapt. Strat. Glob. Chang.* **2013**, *18*, 13–25. [[CrossRef](#)]
7. Muradov, N.; Taha, M.; Miranda, A.F.; Wrede, D.; Kadali, K.; Gujar, A.; Stevenson, T.; Ball, A.S.; Mouradov, A. Fungal-assisted algal flocculation: Application in wastewater treatment and biofuel production. *Biotechnol. Biofuels* **2015**, *8*, 24. [[CrossRef](#)] [[PubMed](#)]
8. Pereira, A.L. The Unique Symbiotic System Between a Fern and a Cyanobacterium, *Azolla-Anabaena azollae*: Their potential as biofertilizer, feed, and remediation. In *Symbiosis*; IntechOpen: London, UK, 2017.
9. Costa, M.; Santos, M.; Carrapiço, F.; Pereira, A. *Azolla-Anabaena's* behaviour in urban wastewater and artificial media—Influence of combined nitrogen. *Water Res.* **2009**, *43*, 3743–3750. [[CrossRef](#)] [[PubMed](#)]
10. Brouwer, P.; Nierop, K.G.; Huijgen, W.J.; Schluempmann, H. Aquatic weeds as novel protein sources: Alkaline extraction of tannin-rich *Azolla*. *Biotechnol. Rep.* **2019**, *24*, e00368. [[CrossRef](#)]
11. Brouwer, P.; Schluempmann, H.; Nierop, K.G.; Elderson, J.; Bijl, P.K.; van der Meer, I.; de Visser, W.; Reichart, G.-J.; Smeekens, S.; van der Werf, A. Growing *Azolla* to produce sustainable protein feed: The effect of differing species and CO<sub>2</sub> concentrations on biomass productivity and chemical composition. *J. Sci. Food Agric.* **2018**, *98*, 4759–4768. [[CrossRef](#)]
12. Chandran, R.; Nivedhini, V.; Parimelazhagan, T. Nutritional composition and antioxidant properties of *Cucumis dipsaceus* ehrenb. ex spach leaf. *Sci. World J.* **2013**, *2013*. [[CrossRef](#)]
13. Miranda, A.F.; Biswas, B.; Ramkumar, N.; Singh, R.; Kumar, J.; James, A.; Roddick, F.; Lal, B.; Subudhi, S.; Bhaskar, T.; et al. Aquatic plant *Azolla* as the universal feedstock for biofuel production. *Biotechnol. Biofuels* **2016**, *9*, 221. [[CrossRef](#)]
14. Watanabe, I.; Liu, C.C. Improving nitrogen-fixing systems and integrating them into sustainable rice farming. *Plant Soil* **1992**, *141*, 57–67. [[CrossRef](#)]
15. van Hove, C.; Lejeune, A. The *Azolla*: *Anabaena* Symbiosis. *Biol. Environ. Proceed. R. Ir. Acad.* **2002**, *102B*, 23–26. [[CrossRef](#)]
16. Carrapico, F. The *Azolla*–*Anabaena*–Bacteria association: A case of symbiotic abduction? In *Algal and Cyanobacteria Symbioses*; World Scientific: Hackensack, NJ, USA, 2017; pp. 329–345.
17. Brouwer, P. Turning the Aquatic Weed *Azolla* Into a Sustainable Crop. Ph.D. Thesis, Universiteit Utrecht, Utrecht, The Netherlands, 7 July 2017.
18. Dohaie, M.; Karimi, K.; Rahimmalek, M.; Satari, B. Integrated biorefinery of aquatic fern *Azolla filiculoides* for enhanced extraction of phenolics, protein, and lipid and methane production from the residues. *J. Clean. Prod.* **2020**, *276*, 123175. [[CrossRef](#)]
19. Miranda, A.F.; Kumar, N.R.; Spangenberg, G.; Subudhi, S.; Lal, B.; Mouradov, A. Aquatic Plants, *Landoltia punctata*, and *Azolla filiculoides* as Bio-Converters of Wastewater to Biofuel. *Plants* **2020**, *9*, 437. [[CrossRef](#)]
20. Miranda, A.F.; 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*, 117–125. [[CrossRef](#)] [[PubMed](#)]
21. Lumpkin, T.A.; Plucknett, D.L. *Azolla*: Botany, physiology, and use as a green manure. *Econ. Bot.* **1980**, *34*, 111–153. [[CrossRef](#)]
22. Anitha, K.C.; Rajeshwari, Y.B.; Prasanna, S.B.; Shilpashree, J. Nutritive evaluation of *azolla* as livestock feed. *J. Exp. Biol. Agric. Sci.* **2016**, *4*, 670–674. [[CrossRef](#)]
23. Nierop, K.G.J.; Speelman, E.N.; de Leeuw, J.W.; Reichart, G.-J. The omnipresent water fern *Azolla caroliniana* does not contain lignin. *Org. Geochem.* **2011**, *42*, 846–850. [[CrossRef](#)]
24. Kadir, A.A.; Abdullah, S.R.S.; Othman, B.A.; Hasan, H.A.; Othman, A.R.; Imron, M.F.; Ismail, N.I.; Kurniawan, S.B. Dual function of *Lemna minor* and *Azolla pinnata* as phytoremediator for Palm Oil Mill Effluent and as feedstock. *Chemosphere* **2020**, *259*, 127468. [[CrossRef](#)]
25. Murthy, T.; Ashok, M.; Thirumalesh, T.; Umesh, B.; Nataraju, O. Effect of partial replacement of *Azolla* for concentrate supplement on lactating crossbred cows. *Environ. Ecol.* **2013**, *31*, 415–417.

26. Sujatha, T.; Kundu, A.; Jeyakumar, S.; Kundu, M. Azolla supplementation: Feed cost benefit in duck ration in andaman islands. *Tamilnadu J. Vet. Anim. Sci.* **2013**, *9*, 130–136.
27. Prabina, B.J.; Kumar, K. Dried Azolla as a nutritionally rich cost effective and immuno-modulatory feed supplement for broilers. *Asian J. Anim. Sci.* **2010**, *5*, 20–22.
28. Sithara, K.; Kamalaveni, K. Formulation of low-cost feed using azolla as a protein supplement and its influence on feed utilization in fishes. *Curr. Biot.* **2008**, *2*, 212–219.
29. Becerra, M.; Murgueitio, E.; Reyes, G.; Preston, T.R. *Azolla filiculoides* as partial replacement for traditional protein supplements in diets for growing-fattening pigs based on sugar cane juice. *Livest. Res. Rural Dev.* **1990**, *2*, 15–22.
30. Leterme, P.; Londoño, A.M.; Ordoñez, D.C.; Rosales, A.; Estrada, F.; Bindelle, J.; Buldgen, A. Nutritional value and intake of aquatic ferns (*Azolla filiculoides* Lam. and *Salvinia molesta* Mitchell.) in sows. *Anim. Feed Sci. Technol.* **2010**, *155*, 55–64. [[CrossRef](#)]
31. Pugesgaard, S.; Schelde, K.; Larsen, S.U.; Lærke, P.E.; Jørgensen, U. Comparing annual and perennial crops for bioenergy production—influence on nitrate leaching and energy balance. *GCB Bioenerg.* **2015**, *7*, 1136–1149. [[CrossRef](#)]
32. Anglade, J.; Billen, G.; Garnier, J. Relationships for estimating N<sub>2</sub> fixation in legumes: Incidence for N balance of legume-based cropping systems in Europe. *Ecosphere* **2015**, *6*, 1–24. [[CrossRef](#)]
33. Olguín, E.J.; Galicia, S.; Mercado, G.; Pérez, T. Annual productivity of *Spirulina* (*Arthrospira*) and nutrient removal in a pig wastewater recycling process under tropical conditions. *J. Appl. Phycol.* **2003**, *15*, 249–257. [[CrossRef](#)]
34. Bhattacharya, A.; Sood, P.; Citovsky, V. The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. *Mol. Plant. Pathol.* **2010**, *11*, 705–719. [[CrossRef](#)]
35. Mouradov, A.; Spangenberg, G. Flavonoids: A metabolic network mediating plants adaptation to their real estate. *Front. Plant Sci.* **2014**, *5*, 620. [[CrossRef](#)]
36. Rupasinghe, H.V.; Arumuggam, N. Health Benefits of Anthocyanins. In *Anthocyanins from Natural Sources*; Royal Society of Chemistry: Cambridge, UK, 2019; pp. 121–158.
37. Ballard, C.R.; Junior, M.R.M. Health Benefits of Flavonoids. In *Bioactive Compounds*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 185–201.
38. Perez-Vizcaino, F.; Fraga, C.G. Research trends in flavonoids and health. *Arch. Biochem. Biophys.* **2018**, *646*, 107–112. [[CrossRef](#)] [[PubMed](#)]
39. Rauf, A.; Imran, M.; Abu-Izneid, T.; Iahtisham Ul, H.; Patel, S.; Pan, X.; Naz, S.; Sanches Silva, A.; Saeed, F.; Rasul Suleria, H.A. Proanthocyanidins: A comprehensive review. *Biomed. Pharm.* **2019**, *116*, 108999. [[CrossRef](#)] [[PubMed](#)]
40. Basak, B.; Pramanik, M.A.H.; Rahman, M.S.; Tarafdar, S.U.; Roy, B.C. Azolla (*Azolla pinnata*) as a feed ingredient in broiler ration. *Int. J. Poult. Sci.* **2002**, *1*, 29–34.
41. Naghshi, H.; Khojasteh, S.; Jafari, M. Investigation the Effect of Different Levels of Azolla (*Azolla pinnata*) on Performance and Carcass Characteristics of Cobb Broiler Chicks. *Int. J. Farm. Allied Sci.* **2014**, *1*, 45–49.
42. Alalade, O.A.; Iyayi, E.A. Chemical composition and the feeding value of Azolla (*Azolla pinnata*) meal for egg-type chicks. *Int. J. Poult. Sci.* **2006**, *2*, 137–141.
43. Güngör, E.; Brouwer, P.; Dijkhuizen, L.W.; Shaffar, D.C.; Nierop, K.G.J.; de Vos, R.C.H.; Sastre Toraño, J.; van der Meer, I.M.; Schlupepman, H. Azolla ferns testify: Seed plants and ferns share a common ancestor for leucoanthocyanidin reductase enzymes. *New Phytol.* **2020**. [[CrossRef](#)]
44. Rivero, R.M.; Ruiz, J.M.; García, P.C.; López-Lefebvre, L.R.; Sánchez, E.; Romero, L. Resistance to cold and heat stress: Accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci.* **2001**, *160*, 315–321. [[CrossRef](#)]
45. Isah, T. Stress and defense responses in plant secondary metabolites production. *Biol. Res.* **2019**, *52*, 39. [[CrossRef](#)]
46. Pérez-López, U.; Sgherri, C.; Miranda-Apodaca, J.; Micaelli, F.; Lacuesta, M.; Mena-Petite, A.; Quartacci, M.F.; Muñoz-Rueda, A. Concentration of phenolic compounds is increased in lettuce grown under high light intensity and elevated CO<sub>2</sub>. *Plant Physiol. Biochem.* **2018**, *123*, 233–241. [[CrossRef](#)]
47. Muradov, N.; Taha, M.; Miranda, A.F.; Kadali, K.; Gujar, A.; Rochfort, S.; Stevenson, T.; Ball, A.S.; Mouradov, A. Dual application of duckweed and azolla plants for wastewater treatment and renewable fuels and petrochemicals production. *Biotechnol. Biofuels* **2014**, *7*, 30. [[CrossRef](#)]

48. Moretti, A.; Siniscalco Gigliano, G. Influence of light and pH on growth and nitrogenase activity on temperate-grown *Azolla*. *Biol. Fertil. Soils* **1988**, *6*, 131–136. [[CrossRef](#)]
49. Schenk, R.U.; Hildebrandt, A. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* **1972**, *50*, 199–204. [[CrossRef](#)]
50. Podpora, B.; Świdorski, F.; Sadowska, A.; Rakowska, R.; Wasiak-Zys, G. Spent brewer's yeast extracts as a new component of functional food. *Czech J. Food Sci.* **2016**, *34*, 554–563. [[CrossRef](#)]
51. Makkar, H.P. *Quantification of Tannins in Tree and Shrub Foliage: A Laboratory Manual*; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2003.
52. Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555–559. [[CrossRef](#)]
53. Ray, H.; Yu, M.; Auser, P.; Blahut-Beatty, L.; McKersie, B.; Bowley, S.; Westcott, N.; Coulman, B.; Lloyd, A.; Gruber, M.Y. Expression of anthocyanins and proanthocyanidins after transformation of alfalfa with maize Lc. *Plant Physiol.* **2003**, *132*, 1448–1463. [[CrossRef](#)] [[PubMed](#)]
54. Abeynayake, S.W.; Panter, S.; Mouradov, A.; Spangenberg, G. A high-resolution method for the localization of proanthocyanidins in plant tissues. *Plant Meth.* **2011**, *7*, 13. [[CrossRef](#)]
55. Li, Y.G.; Tanner, G.; Larkin, P. The DMACA–HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J. Sci. Food Agric.* **1996**, *70*, 89–101. [[CrossRef](#)]
56. Neff, M.M.; Chory, J. Genetic interactions between phytochrome A, phytochrome B, and cryptochrome 1 during *Arabidopsis* development. *Plant Physiol.* **1998**, *118*, 27–35. [[CrossRef](#)] [[PubMed](#)]
57. Forni, C.; Braglia, R.; Harren, F.; Cristescu, S. Stress responses of duckweed (*Lemna minor* L.) and water velvet (*Azolla filiculoides* Lam.) to anionic surfactant sodium-dodecyl-sulphate (SDS). *Aquat. Toxicol.* **2012**, *110*, 107–113. [[CrossRef](#)]
58. Sánchez-Viveros, G.; Ferrera-Cerrato, R.; Alarcón, A. Short-term effects of arsenate-induced toxicity on growth, chlorophyll and carotenoid contents, and total content of phenolic compounds of *Azolla filiculoides*. *Water Air Soil Pollut.* **2011**, *217*, 455–462. [[CrossRef](#)]
59. Sánchez-Viveros, G.; Gonzalez-Mendoza, D.; Alarcon, A.; Ferrera-Cerrato, R. Copper effects on photosynthetic activity and membrane leakage of *Azolla filiculoides* and *A. caroliniana*. *Int. J. Agric. Biol.* **2010**, *12*, 365–368.
60. Dai, L.P.; Xiong, Z.T.; Huang, Y.; Li, M.J. Cadmium-induced changes in pigments, total phenolics, and phenylalanine ammonia-lyase activity in fronds of *Azolla imbricata*. *Environ. Toxicol.* **2006**, *21*, 505–512. [[CrossRef](#)]
61. Saigo, T.; Wang, T.; Watanabe, M.; Tohge, T. Diversity of anthocyanin and proanthocyanin biosynthesis in land plants. *Curr. Opin. Plant Biol.* **2020**, *55*, 93–99. [[CrossRef](#)] [[PubMed](#)]
62. Tohge, T.; Fernie, A.R. Leveraging Natural Variance towards Enhanced Understanding of Phytochemical Sunscreens. *Trends Plant Sci.* **2017**, *22*, 308–315. [[CrossRef](#)]
63. Ishikura, N. 3-Desoxyanthocyanin and other phenolics in the water fern *Azolla*. *Bot. Mag. Shokubutsu-Gaku-Zasshi* **1982**, *95*, 303–308. [[CrossRef](#)]
64. Cohen, M.F.; Sakihama, Y.; Takagi, Y.C.; Ichiba, T.; Yamasaki, H. Synergistic effect of deoxyanthocyanins from symbiotic fern *Azolla* spp. on *hrmA* gene induction in the cyanobacterium *Nostoc punctiforme*. *Mol. Plant Microbe Interact.* **2002**, *15*, 875–882. [[CrossRef](#)] [[PubMed](#)]
65. Cohen, M.F.; Meziane, T.; Tsuchiya, M.; Yamasaki, H. Feeding deterrence of *Azolla* in relation to deoxyanthocyanin and fatty acid composition. *Aquat. Bot.* **2002**, *74*, 181–187. [[CrossRef](#)]
66. Kunjappan, S.; Chowdhury, R.; Bhattacharjee, C. Isolation and structural elucidation of flavonoids from aquatic fern *Azolla microphylla* and evaluation of free radical scavenging activity. *Int. J. Pharm. Pharm. Sci.* **2013**, *5*, 743–749.
67. Tao, X.; Fang, Y.; Huang, M.J.; Xiao, Y.; Liu, Y.; Ma, X.R.; Zhao, H. High flavonoid accompanied with high starch accumulation triggered by nutrient starvation in bioenergy crop duckweed (*Landoltia punctata*). *BMC Genom.* **2017**, *18*, 166. [[CrossRef](#)]
68. Pereira, A.L.; Carrapiço, F. Histochemistry of simple hairs from the foliar cavities of *Azolla filiculoides*. *Plant Biosyst. Int. J. Deal. All Asp. Plant Biol.* **2007**, *141*, 323–328. [[CrossRef](#)]
69. Abeynayake, S.W.; Panter, S.; Chapman, R.; Webster, T.; Rochfort, S.; Mouradov, A.; Spangenberg, G. Biosynthesis of proanthocyanidins in white clover flowers: Cross talk within the flavonoid pathway. *Plant Physiol.* **2012**, *158*, 666–678. [[CrossRef](#)] [[PubMed](#)]

70. Becker, M.; Ladha, J.K.; Simpson, I.C.; Ottow, J.C.G. Parameters Affecting Residue Nitrogen Mineralization in Flooded Soils. *Soil Sci. Soc. Am. J.* **1994**, *58*, 1666–1671. [[CrossRef](#)]
71. Costa, M.L.; Santos, M.C.; Carrapiço, F. Biomass characterization of *Azolla filiculoides* grown in natural ecosystems and wastewater. *Hydrobiologia* **1999**, *415*, 323–327. [[CrossRef](#)]
72. Wang, Y.; Douglas, G.; Waghorn, G.; Barry, T.; Foote, A. Effect of condensed tannins in *Lotus corniculatus* upon lactation performance in ewes. *J. Agric. Sci.* **1996**, *126*, 353–362. [[CrossRef](#)]
73. Mueller-Harvey, I.; Bee, G.; Dohme-Meier, F.; Hoste, H.; Karonen, M.; Kölliker, R.; Lüscher, A.; Niderkorn, V.; Pellikaan, W.F.; Salminen, J.-P.; et al. Benefits of Condensed Tannins in Forage Legumes Fed to Ruminants: Importance of Structure, Concentration, and Diet Composition. *Crop Sci.* **2019**, *59*, 861–885. [[CrossRef](#)]
74. Barbehenn, R.V.; Peter Constabel, C. Tannins in plant-herbivore interactions. *Phytochemistry* **2011**, *72*, 1551–1565. [[CrossRef](#)]
75. Lourenço, S.O.; Barbarino, E.; Lavín, P.L.; Lanfer Marquez, U.M.; Aidar, E. Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors. *Eur. J. Phycol.* **2004**, *39*, 17–32. [[CrossRef](#)]
76. Mariotti, F.; Tomé, D.; Mirand, P.P. Converting nitrogen into protein—Beyond 6.25 and Jones' factors. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 177–184. [[CrossRef](#)]
77. Shi, H.; Ye, T.; Chen, F.; Cheng, Z.; Wang, Y.; Yang, P.; Zhang, Y.; Chan, Z. Manipulation of arginase expression modulates abiotic stress tolerance in *Arabidopsis*: Effect on arginine metabolism and ROS accumulation. *J. Exp. Bot.* **2013**, *64*, 1367–1379. [[CrossRef](#)]
78. Winter, G.; Todd, C.D.; Trovato, M.; Forlani, G.; Funck, D. Physiological implications of arginine metabolism in plants. *Front. Plant Sci.* **2015**, *6*. [[CrossRef](#)]
79. Joshi, V.; Joung, J.-G.; Fei, Z.; Jander, G. Interdependence of threonine, methionine and isoleucine metabolism in plants: Accumulation and transcriptional regulation under abiotic stress. *Amino Acids* **2010**, *39*, 933–947. [[CrossRef](#)] [[PubMed](#)]
80. Parthasarathy, A.; Savka, M.A.; Hudson, A.O. The Synthesis and Role of  $\beta$ -Alanine in Plants. *Front. Plant Sci.* **2019**, *10*. [[CrossRef](#)] [[PubMed](#)]
81. Yuan, N.; Yuan, S.; Li, Z.; Zhou, M.; Wu, P.; Hu, Q.; Mendu, V.; Wang, L.; Luo, H. STRESS INDUCED FACTOR 2, a leucine-rich repeat kinase regulates basal plant pathogen defense. *Plant Physiol.* **2018**, *176*, 3062–3080. [[CrossRef](#)]
82. Qiu, X.-M.; Sun, Y.-Y.; Ye, X.-Y.; Li, Z.-G. Signaling role of glutamate in plants. *Front. Plant. Sci.* **2019**, *10*. [[CrossRef](#)] [[PubMed](#)]

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