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Research article

Lipid production in aquatic plant Azolla at vegetative and reproductive stages and in response to abiotic stress

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ABSTRACT

The aquatic plant Azolla became increasingly popular as bioenergy feedstock because of its high growth rate, production of biomass with high levels of biofuel-producing molecules and ability to grow on marginal lands. In this study, we analysed the contribution of all organs of Azolla to the total yield of lipids at vegetative and reproductive stages and in response to stress. Triacylglycerol-containing lipid droplets were detected in all (vegetative and reproductive) organs with the highest level in the male microsporocarps and microspores. As a result, significantly higher total yields of lipids were detected in *Azolla filiculoides* and *Azolla pinnata* at the reproductive stage. Starving changed the yield and composition of the fatty acid as a result of re-direction of carbon flow from fatty acid to anthocyanin pathways. The composition of lipids, in regard the length and degree of unsaturation of fatty acids, in Azolla meets most of the important requirements for biodiesel standards. The ability of Azolla to grow on wastewaters, along with their high productivity rate, makes it an attractive feedstock for the production of biofuels.

1. Introduction

The goal of reducing our dependency on fossil fuels, and to prevent further deforestation and competition with agriculture, has triggered an extensive search for domestication of new bioenergy feedstock which (i) can generate substantial renewable biomass over a short period; (ii) can grow on marginal lands; and (iii) are rich in bioenergy molecules which can be converted into biofuels using a set of well-established technologies (Long et al., 2013; Miranda et al., 2016). Search for the energy crops which can use wastewater as a source of key nutrients represents one of the most globally researched areas and is becoming a subject of intense public and scientific interest (Miranda et al., 2014, 2016; Marmiroli et al., 2006; Salt et al., 1998; Dushenkov, 2003; Muradov et al., 2014). Most of the known terrestrial bioenergy crops cannot grow even in diluted wastewaters. Microalgae have been extensively investigated as the third generation of bioenergy feedstocks because of their high growth rates, substantial lipid concentration and ability to grow in wastewaters removing their primary pollutants (C, N, and P) (Borowitzka and Moheimani, 2013; Schenk et al., 2008; Rajkumar et al., 2014; Egede et al., 2016). However, the high cost of harvesting of microalgae (up to 30% of total cost) is still the major obstacle for the large-scale production of low-value products such as biofuels (Borowitzka and Moheimani, 2013; Schenk et al., 2008; Rajkumar et al., 2014; Aguirre et al., 2013).

Aquatic plants represented by submerged, emerged (rooted) and free-floating species colonise contaminated wetlands have attracted significant attention as a potential valuable feedstock for second and third generation biofuels because of their ability to produce a large amount of biomass, impressive bioremediation rates, as well as cheap and easy maintenance and harvesting (Miranda et al., 2014, 2016; Dhir et al., 2009; Cui and Cheng, 2015). Among these species, free-floating plants have obvious advantages because of their low harvesting cost. A number of free-floating aquatic species which have been evaluated for both wastewater treatment and biofuel production include: water hyacinth (*Eichhornia crassipes*) (Ruan et al., 2016; Zhao et al., 2012a), water lettuce (*Pistia stratiotes*), (Viger et al., 2015; Robles-Pliego et al., 2015; Mukherjee et al., 2015), water ferns: Salvinia (Chandanshive et al., 2016; Mubarak et al., 2016) and Azolla species (Miranda et al., 2016; Muradov et al., 2014; Costa et al., 1999, 2009; Pereira et al., 2011; Pereira and Carrapico, 2009; Brouwer et al., 2014, 2016), and

Abbreviations: *A. azollae*, *Anabaena azollae* Strasburger; *A. filiculoides*, *Azolla filiculoides*; *A. pinnata*, *Azolla pinnata*; dw, dry weight; GC, gas chromatography; *L. punctata*, *Landoltia punctata*

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representatives of the *Lemnaceae* or duckweed (Appenroth et al., 2015; Lam et al., 2014; Zhao et al., 2012b; Verma and Suthar, 2015).

Azolla (also known as mosquito fern, duckweed fern, fairy moss and water fern) has become increasing popular because of its biomass production and bioremediation potential (Miranda et al., 2016; Muradov et al., 2014; Costa et al., 1999, 2009; Pereira et al., 2011; Brouwer et al., 2014, 2016; Carrapiço, 2010). Unlike most of the terrestrial and aquatic plants, Azolla can grow efficiently even in the absence of nitrogen in media utilising the nitrogen-fixing capacity of its natural symbiont, the endophytic cyanobacterium, *Anabaena azollae* Strasburger (*A. azollae*) (Zheng et al., 2009; Pereira and Vasconcelos, 2014; Calvert and Peters, 1981). This symbiosis is associated with the fixation of up to 1.1 t/ha-yr of nitrogen, which is significantly higher than the nitrogen fixation rate of legumes (0.4 t N/ha-yr) (Costa et al., 1999; Hall et al., 1995; Kollah et al., 2016).

Doubling its biomass every 4–7 days Azolla is one of the fastest growing plants, with a productivity varying between 2.9 and 5.8 g dw/m^2-day (10.5–21.1 t dw/ha-yr) when grown on artificial media, wastewaters and maturation ponds (Fig. S1) (Kollah et al., 2016). Under optimal conditions in natural ecosystems, such as rivers, lagoons and irrigation channels Azolla can bloom with growth rates up to 300 g/m^2-day of fresh biomass (1095 t/ha-yr) (Van Hove et al., 1987) and 25.6–27.4 g dw/m^2-day of dry biomass (93.4–100 t dw/ha-yr) (Miranda et al., 2016; Costa et al., 1999; Peters et al., 1980a). Growth in wastewater is associated with the removal of key nutrients such as N and P with rates of up to 2.6 t N/ha-year and 0.434 t P/ha-yr, respectively (Muradov et al., 2014; Costa et al., 1999, 2009; Song et al., 2012).

Azolla is a relatively new feedstock for bioenergy production, and its promising potential is based on its unique chemical composition. Together with their evolutional symbiont, *A. azollae,* Azolla representatives contain three major types of energy molecules, starch (approx 6% dw) and cellulose/hemicellulose (up to 35% dw) and lipids (8% dw) which are found separately in known terrestrial feedstocks and microalgae (Miranda et al., 2016; Costa et al., 1999, 2009; Brouwer et al., 2016; Song et al., 2012; Peters et al., 1980b).

The energy accumulated in lipids/TAG is twice that of cellulose which makes them a desirable feedstock for bio-oil production (Winichayakul et al., 2013). In terrestrial plants, TAG are mainly accumulated in seeds, pericarps, and pollen, which can transfer carbon molecules from one generation to the next and allow seeds to germinate until photosynthesis becomes effective (Lin and Oliver, 2008; Baud and Lepiniec, 2010). The production of these oil-containing organs in terrestrial plants is seasonal. Moreover, in spite of the fact that oil plants can accumulate up to 50% dw of TAG in their seeds, just 0.06–0.5% dw of the TAG can be extracted from the vegetative organs, leaves and stems (Lin and Oliver, 2008; Yan et al., 2013). This triggers an intensive search for the plant species which can accumulate a substantial amount of lipids/TAG in their vegetative organs, such as leaves.

In this work, we continued research on the use of Azolla as the next generation bioenergy crop. To our knowledge, for the first time, we (i) analysed the contribution of each organ and cyanobacterial symbiont of *A. filiculoides* on its total lipid yield; (ii) showed developmental stage-specific changes in lipid's yield and composition triggered by the production of the lipid-rich male microsporocarps at the reproductive stage; (iii) showed changes in lipid yield and composition associated with up-regulation of the flavonoid pathway in stressed plants; (iv) assessed *A. pinnata* (the second most common Azolla species widely found in warm-temperate and tropical regions) as feedstock for lipid/bio-oil production; and (v) evaluated contributions of lipid composition, the length, and degree of unsaturation of fatty acids of Azolla for the key biodiesel properties, such as iodine number, cetane number, density, pour point and others. Duckweed representative, *Landoltia punctata (L. punctata)*, which lipid content was analysed earlier was used as a control in this study (Verma and Suthar, 2015; Yan et al., 2013; Zhong et al., 2016).

2. Materials and methods

2.1. Growing Azolla and duckweed

L. punctata, A. filiculoides, and *A. pinnata* were collected from RMIT University's collection of aquatic plants. Plants were grown in 10 L containers with Hoagland nutrient solution (for *L. punctata*) and H40 media (for Azolla species) (Pereira and Carrapico, 2009). The plants were grown in a glasshouse covered with shade cloth, with natural photoperiod and at 23-26 °C. The photosynthetic photon flux density was 50–70 μmol/m^2/s. The solution in each container was mixed every day. Three replicates were included for each treatment. Collected samples of whole plants and roots were rinsed in deionized water to remove unwanted debris. The filamentous cyanobacterium *A. azollae* was squeezed manually from the Azolla leaves and washed out of the Azolla debris using a few centrifugations. The collected filaments were checked for their intactness by microscopy. Sporulation of *A. filiculoides* was observed in September–November 2016 (Spring in Australia), and mature microsporocarps were plucked manually from the plants. To extract lipids from leaves at the reproductive stages mature microspores were removed manually. Because of the small size of female megasporocarps (0.3–0.5 mm), their contribution to the level of total lipids in Azolla was not analysed. All samples were freeze-dried and weighed before extraction of lipids. Starving conditions were achieved by growing the Azolla plants for an extended period (over one month) in the same medium.

2.2. Nile red staining

For Nile Red staining the Azolla and *A. azollae* were incubated in 1 mL of 20% DMSO containing 5 μL of Nile Red stock solution (0.10 mg/mL of Nile Red dissolved in acetone) for 5 min. The stained samples were then subjected to fluorescent microscopy analysis to observe the formation of lipid droplets using a Leica DM 2500 microscope with an attached camera Leica DFC 310 FX. Nile-Red filter: excitation at 543 nm, emission 555–650 nm.

2.3. Lipid extraction and fatty acid composition determination

Freeze-dried and ground plant materials (25 mg) were extracted in 4 mL of chloroform/methanol (2:1, v:v) overnight. After centrifugation, the supernatant was transferred to a pre-weighed 5-mL glass vial. After removal of the organic phase under a stream of nitrogen, the content of total crude lipids in each sample was determined by weight difference. To determine the fatty acid composition of the lipids, 2.4 mL of 6% H_2SO_4 in methanol was added to each vial, and the vial was sealed with a Teflon cap. Fatty acids were methylated by acid-catalysed transesterification at 80 °C for 3 h. After cooling to room temperature, the fatty acid methyl esters (FAMEs) formed were extracted into hexane containing an internal standard (octacosane, 15 mg/L) and analysed directly by GC-MS. The separation of the FAMEs was achieved by a BPX-70 column (50 m × 0.22 mm ID, 0.25 μm film thickness, SGE Analytical Science) with a constant flow of 1.0 mL/min helium as carrier gas and the following oven temperature program: 120 °C to 245 °C ramping at 3 °C/min, with a total run time of 42 min. The detection was by an Agilent 7000 GC/MS Triple Quad with the following settings: scanning mass range of 40–550 amu, transfer line temperature of 240 °C, source temperature of 280 °C, the quad temperature of 150 °C and a solvent delay of 4.1 min. A standard mix (C4-C24, Supelco) containing 37 FAMEs was used to provide absolute quantification of each fatty acid methyl ester.

2.4. Statistical analysis

All experiments in this study were conducted at least in triplicate. All data are expressed as a mean ± standard deviation. Data variance