Nutrient starvation enhances the phenolic compounds and antioxidant activity in *Azolla caroliniana* plant

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1 ABSTRACT

Nutrient deficiency is one of the most abiotic stress to plant growth and development. The main purpose of this study ~~is~~ was to evaluate the effects of nutrients deprivation in growth media of *Azolla* ~~*caroliniana*~~ *caroliniana* (add common name here) plant on growth and production of various phenolic components and antioxidant activity. The growth of *Azolla* plants on nutrient-deficient culture (0.2, 0.1, 0.05 strength Hoagland solution) resulted in a significant reduction in *Azolla* biomass, particularly with 0.05 strength compared to those grown on nutrient-sufficient culture (control). The doubling time (DT) and relative growth rate (RGR) were changed markedly with different Hoagland strength, and that was accompanied with a significant accumulation of H_2O_2 and MDA (????). Anthocyanins, total flavonoids and total phenolics were enhanced in starved-*Azolla*. HPLC analysis of phenolic acids revealed a significant increase in caffeic, o-coumaric and *t*-cinnamic acids in *Azolla* grown on 0.2 strength Hoagland culture, whereas coumaric acid was markedly accumulated in *Azolla* plants grown on 0.1 strength media. There was a significant increase in PAL (????) and antioxidant activities in starved *Azolla*. The results indicated that the antioxidant activity might depend mainly on phenolic compounds under the deficiency of nutrients in *Azolla* growth media.

Keyword: anthocyanins, antioxidant activity, *Azolla caroliniana*, flavonoids, phenolics

2 INTRODUCTION

One of the most abiotic stress is the ~~deficient~~ deficiency of nutrients available for plant growth (Ramakrishna and Ravishankar, 2011; Yang ~~et al.~~*et al*~~,~~, 2018). Tewari ~~et al.~~*et al.* (2007) and Khavari-Nejad ~~et al.~~*et al.* (2009) showed that decrease of N, K and P caused a great reduction in growth of mulberry and tomato plants, respectively. Similarly, Christin ~~et al.~~*et al.* (2009) mentioned that decrease of K, Mg and Fe resulted in a marked reduction of growth and development of sorghum and sunflower seedlings. It has concluded that the suppression of plant growth subjected to nutrient deficiency might be attributed to enhancement the generation of ROS and oxidative stress (Shin ~~et al.~~*et al*~~,~~., 2005; Tyburski ~~et al.~~*et al*~~,~~., 2009). Reactive oxygen species (ROS) such as superoxide ($O2^{\bullet-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$) radicals as well as non-free radical species such as hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are generated in chloroplast, mitochondria and peroxisome as a natural by-product of the normal metabolism and have vital roles in cell ~~signaling~~signalling and homeostasis (Gill and Tuteja, 2010; Li ~~et al.~~*et al.* , 2018). Plants have various defense mechanisms against the oxidative stress caused by nutrient deficiency including enzymatic and non-enzymatic antioxidants secondary metabolites (Angaji ~~et al.~~*et al*~~,~~., 2012). Antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), guaiacol~~gauaiacol~~ peroxidase (GPx), polyphenol oxidase (PPO) and ascorbic peroxidase (APx) which ~~scavenging~~scavenge the generated toxic ROS (Ahmad ~~et al.~~*et al.* , 2014). Phenolic components are secondary metabolites including several derivatives such as flavonoids, polyphenols, anthocyanins and phenolic acids, showed a similar defense mechanism (Guo ~~et al.~~*et al.* , 2008; Cingöz and Karakas, 2016) and/or mechanism of action in suppressing the growth (Caretto ~~et al.~~*et al.* , 2015). The antioxidant activity of phenolics is related to their

ability to donate H^+ to generated ROS, singlet oxygen quenchers and inhibit lipid peroxidation (Sharma ~~et al.~~*et al*~~,~~., 2012; Angaji ~~et al.~~*et al.* , 2012). Many authors have been suggested that deficiencies of essential elements such as N, P, K, S, Ca, Mg and Fe increase the amount of phenolics in plant tissues either from existing pools or by *de novo* synthesis (Jin ~~et al.~~*et al.* , 2008; Li ~~et al.~~*et al.* , 2008; Ramakrishna and Ravishankar, 2011; Cingöz and Karakas, 2016). Phenylalanine ammonia lyase (PAL) is a key enzyme in diverting primary metabolism to secondary metabolism in response to stress. It has been reported that accumulation of phenolics and flavonoids in plants under stress accompanied with enhanced PAL activities (Kováˇcik ~~et al.~~*et al.* , 2007; Guo ~~et al.~~*et al.* , 2008). Recently, Darmanti ~~et al.~~*et al.* (2018) suggested that increasing of phenolic components, due to stimulation of PAL activity, resulted in enhancement of tolerance mechanism in soybean plants under multiple stress. *Azolla,* a free-floating water fern is one of the world's fastest growing aquatic macrophytes (high growth rates), with a doubling time of only 2-5 days (Taghi-Ganji ~~et al.~~*et al.* , 2005) which can grow in the absence of nitrogen because of a symbiotic association with the nitrogen-fixing endophytic blue-green cyanobacterium. *Azolla* species have various benefits so, it is used as target in this research. This study aims to evaluate the effects of nutrients deprivation in growth media of *Azolla caroliniana* plant on growth and production of various phenolic components and antioxidant activity as well as the key enzyme, phenylalanine ammonia lyase, activity involves in biosynthesis of these compounds.

3 MATERIALS AND METHODS

3.1 Plant material and growth conditions: *Azolla caroliniana* Wild (~~Known as~~ water velvet) was provided by Prof. Weam El-Aggan in year 1982 from Catholic University of ~~louvain~~Louvain, Belgium. ~~;~~ I~~i~~t was identified by Prof. Peters G.A., Kettering laboratory Yellow Springs, Ohio 45387. The plants were acclimated in the green-house of the Faculty of Science, Alexandria, in 2500 cm^3 polyethylene vessels which were filled with a nitrogen free, modified [KNO_3 and $Ca(NO_3)_2$ were replaced by KCl and $CaCl_2$, respectively] 2/5 strength Hoagland solution (pH 5.1). About 5 g (fresh mass) of *Azolla* from the stock material were inoculated in each vessel to make a new subculture, and so on. The plants were freed from epiphytic microorganisms by thorough washing with distilled water. The cultures were grown in a growth chamber under 16-h photoperiod at irradiance of 1200 µmol m^{-2} s^{-1} with cool white fluorescent tubes and light/dark temperature of 28 - 30/20 – 25 °C (Stock culture).

3.2 Treatments: An aliquot of 5 g healthy and green-colored *Azolla* fronds were surface sterilized with 0.2 % Clorox (El-Aggan 1982), then thoroughly washed with distilled water and cultured in 250 cm^3 vessels containing N-free 2/5 Hoagland solution as nutrient-sufficient (0.4 strength, control) and nutrient-deficient (starved) employed by changing the strength of Hoagland solution (0.2, 0.1, 0.05 strength). At 10 days from starting the experiment, samples were collected for estimation the following growth parameters. The number of generations and doubling time were calculated from the fresh mass and duration of experiment employing the expression given by Peters ~~et al.~~*et al.* (1979): n (final mass) = $n^o \times 2G$, where G = number of generations, n^o = initial mass of *Azolla* plants (mass of inoculum). Doubling time (DT) = duration of experiment per one generation. Relative growth rate (RGR) was calculated by using the formula of Subudhi and Watanabe (1981): RGR [kg $kg^{-1}d^{-1}$] = 0.693 DT^{-1}.

Determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents: Hydrogen peroxide was determined as described by Velikova ~~et al.~~*et al.* (2000) and malondialdehyde content was estimated according to Zhange ~~et al.~~*et al.* (2007).

Determination of anthocyanins: Total anthocyanins content was determined using Mancinelli ~~et al.~~*et al.* (1988) method ~~with~~. Plant samples were extracted with 5 ml of acidified methanol (1% HCl, v/v) for 24 h at 4 °C in darkness with occasional shaking. The extract was centrifuged for 10 min at 6000 g. The absorbance was measured at 530 nm using UV-VIS spectrophotometer (JENWAY, 6305, UK).

Determination of total flavonoids: Total flavonoid content was measured by the aluminum chloride colorimetric assay (Meda ~~et al.~~*et al.* , 2005). One ml plant extract mixed with 0.30 ml of 5% sodium nitrite (for 5 minutes) and 0.30 ml of 10 % aluminum chloride followed by adding 2 ml of 1M sodium hydroxide and diluted to 10 ml with distilled water. The absorbance for test and standard (quercin, Q) solutions were determined against the reagent blank at 510 nm with an UV/Visible spectrophotometer (JENWAY, 6305, UK). The total flavonoid content was expressed as mg of QE/g dry mass.

Determination of total phenolic contents: Extraction of phenolic compounds was done as the method described by Proestos ~~et al.~~*et al.* (2005), and the total phenolic compounds content of the aqueous methanol extract was performed according to the Folin Ciocalteau's method (Zheng and Wang, 2001). Absorbance was measured at 760 nm using UV-VIS spectrophotometer (JENWAY, 6305, UK). Tannic acid was used as a standard. The total phenolic content was expressed in mg of tannic acid equivalents (TAE)/g dry matter.

Detection of phenolic acids: Phenolic acids were extracted according the method described by Adom and Liu (2002). Dried samples were extracted with 80% methanol for 24 h on the shaker, the supernatants were separated by centrifugation (5000 rpm for 15 min) and subjected to RP-HPLC analysis.

HPLC analysis: Chromatographic analysis was performed by Ultimate 3000 HPLC coupled with a UV-VIS

multiwave length detector. The separation was achieved on a Waters Spherisorb 5 µm ODS2 4.6 x 250 mm column at ambient temperature (35°C). The mobile phase consisted of 1% glacial acetic acid (solvent A), 6% glacial acetic acid (solvent B) and water/acetonitrite (65:30 v/v) with 5% glacial acetic acid (solvent C) were used. The flow rate was 0.5 mL/min, and injection volume was 25 µL. The monitoring wave length was 280 nm (Proestos ~~et al.~~*et al.* , 2005). The identification of each compound was based on a combination of retention time and spectral matching.

Determination of total antioxidant activity: The total antioxidant capacity (TAC) was measured in the previous tissues according to the method described by Koracevic ~~et al.~~*et al.* (2001).

Determination of L-phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.5): A Fresh sample was homogenized in chilled 50 mM Tris-HCl (pH 8.0) supplemented with 0.5 mM EDTA and 1% polyvinylpyrrolidone. The reaction mixture contained 50 mM Tris–HCl (pH 8.0), 20 mM L-phenylalanine, and 100 µL of the enzyme extract (Devi ~~et al.~~*et al*~~,~~*.,* 2012). PAL activity was measured by monitoring the reaction product *t*-cinnamate at 290 nm and calculated as a difference in the optical density for 30 min. The amount of *t*-cinnamic acid synthesized was calculated using its extinction coefficient (9630 M^{-1}) (Kagalea ~~et al.~~*et al*~~,~~*.,* 2004) and the results were expressed as µmol cinnamic acid g^{-1} f.m h^{-1}.

Statistical analysis: Statistical analysis was done using Statistical Package for Social Sciences (SPSS/version 20; SPSS Inc., Chicago, IL, USA) software. ANOVA was utilized for mean, standard deviation. The level of significance was $P \leq 0.05$. All treatments were replicated three times and the results are presented as means. Data for all attributes were subjected to one-way analysis of variance and the mean values were compared with least significance difference (LSD).

RESULTS

Starved-*Azolla* plants grown on nutrient-deficient cultures (0.2, 0.1, 0.05 strength Hoagland solutions) showed a significant reduction in biomass (Table 1). At 0.05 strength Hoagland solution the reduction in *Azolla* biomass after 10 d was about 39% compared to that grown on nutrient-sufficient media (control). The doubling time (DT) and relative growth rate (RGR) were changed markedly with the decrease in Hoagland strength (nutrient concentrations). The highest DT was detected in *Azolla* grown on 0.05 strength nutrient-deficient media (starved-*Azolla*) compared to control ones revealing reduction of growth.

Table 1: Biomass, doubling time (DT) and relative growth rate (RGR) of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days.

Treatments	Biomass [g culture^{-1}]	Doubling time [d]	Relative growth rate [Kg Kg^{-1}d^{-1}]
Control	20.84 ± 2.08^{a}	4.8 ± 0.48^{b}	0.14 ± 0.011a
0.2	16.25 ± 2.17^{ab}	6.15 ± 0.82^{ab}	0.11 ± 0.010^{b}
0.1	14.03 ± 1.71^{b}	7.13±0.87^{a}	0.09 ± 0.0087^{c}
0.05	12.78 ± 1.35^{b}	7.82 ± 0.82^{a}	0.09 ± 0.0092^{c}
p	0.001	0.016	0.042
LSD	5.01	2.11	0.002

Different letter means significant at $p \leq 0.05$ Values are mean ± SD (n = 3).

There was a significant accumulation of H_2O_2 and MDA in *Azolla* plants grown in nutrient deficient media. At 0.2 strength media, the H_2O_2 and MDA contents in *Azolla* plants were 1.8- and 1.4-fold respectively of control plants. These reached to 5.6- and 4.4-fold of 0.05 H strength (Fig 1). Anthocyanins content in starved-*Azolla* plants was significantly increased with the deprivation of nutrients in the growth media (Fig 2). The highest anthocyanins content was detected in starved-*Azolla* grown on 0.2 strength, in comparison to nutrients sufficient control. Total flavonoids and total phenolics were significantly increased when *Azolla* plants exposed to nutrient stress (Fig 2). The increase in total flavonoids and total phenolics in nutrient-starved *Azolla* grown on 0.2 strength were 2- and 2.4-fold respectively of those grown on nutrient-sufficient control.

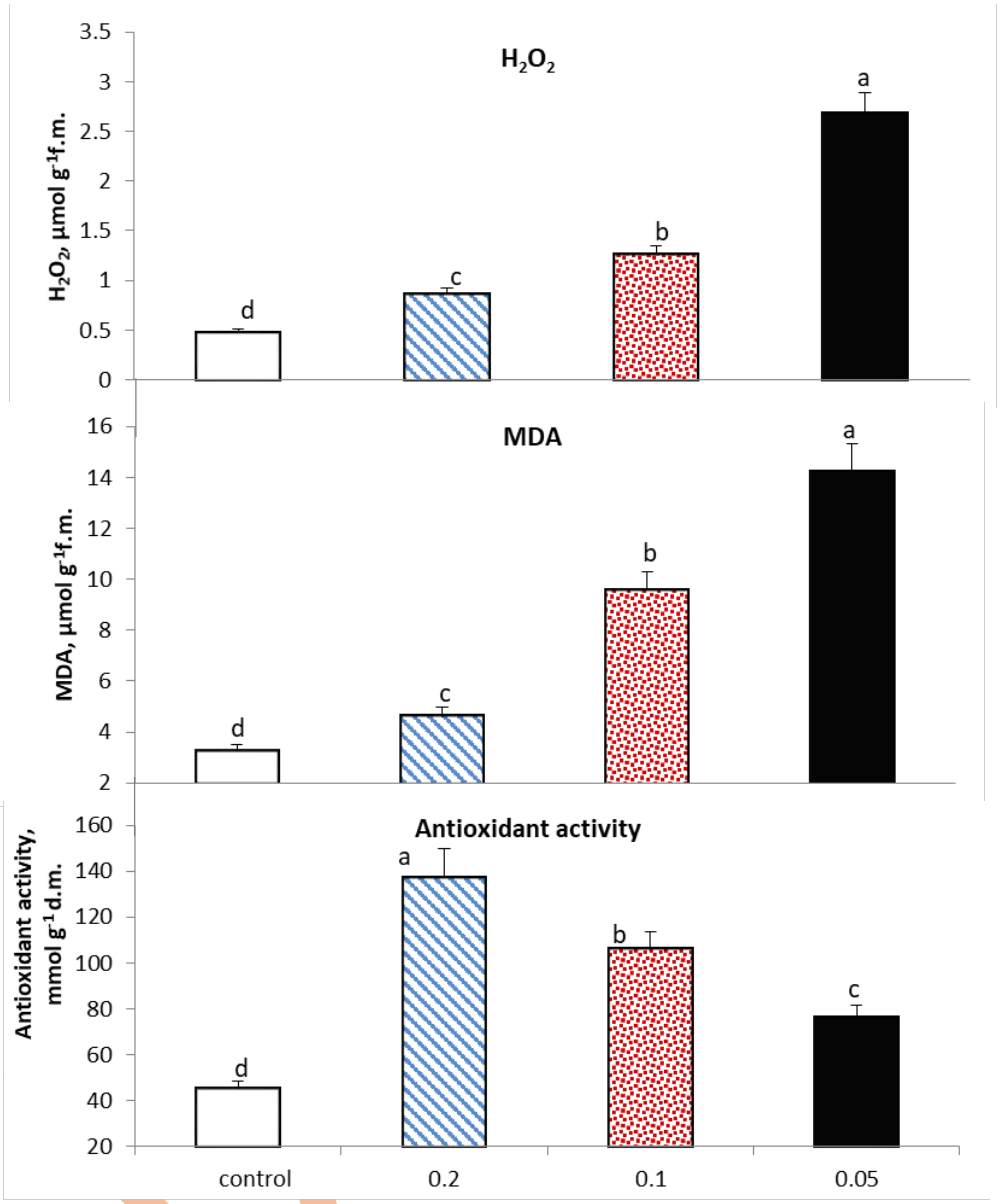

Fig 1: Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA) and antioxidant activity of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3). Different letter means significant at $p \leq 0.05$.

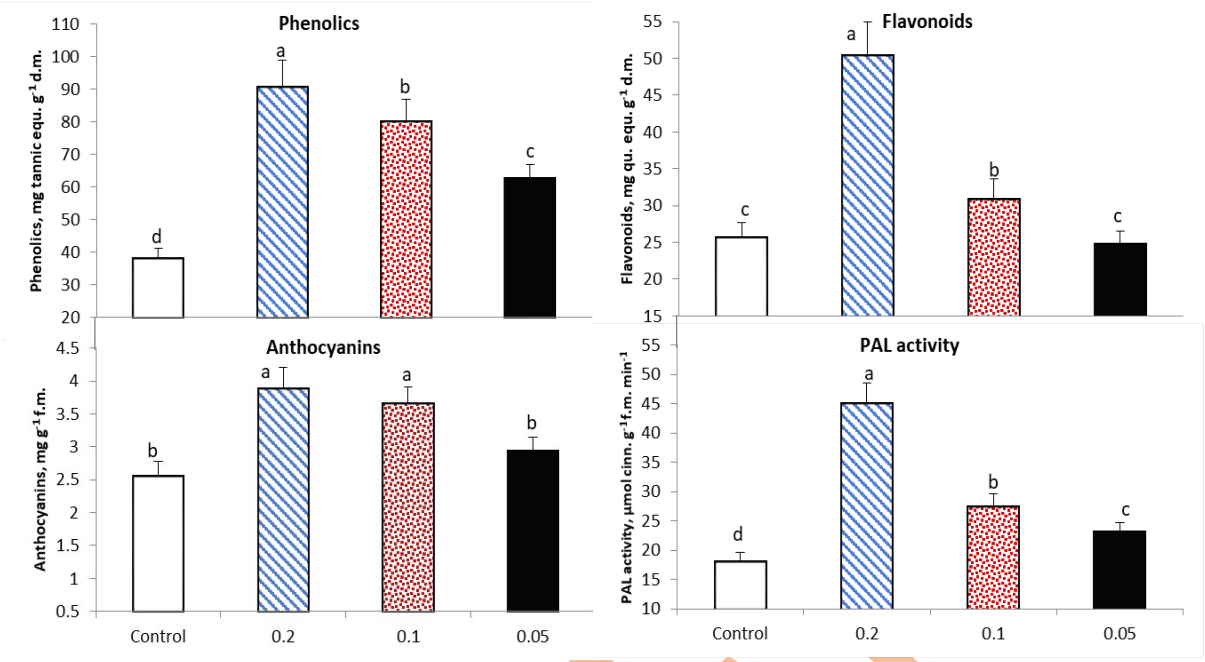

Fig 2: Total phenolics, flavonoids, anthocyanins contents and PAL activity of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3). Different letter means significant at $p \leq 0.05$

HPLC analysis of phenolic acids revealed that the major acids in *Azolla* grown on nutrients sufficient medium (control) were tannic, gallic and *t*-cinnamic acids (Table 2). After 10 d, phenolic acids appeared a different pattern in response to nutrient stress. There was an increase in caffeic, o-coumaric and *t*-cinnamic acids in *Azolla* grown on 0.2 strength Hoagland culture, whereas coumaric acid was markedly accumulated in *Azolla* plants grown on 0.1 strength media. There was a marked decrease in all tested phenolic acids in *Azolla* grown on severely deficient nutrient media.

Table 2: Phenolic acids of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days.

Treatments	Tan	Gal	Caf	Fer	*t*-Cin	Chlo	Sin	Van	O-Cou	Total
	µg g^{-1} d.m.									
Control	240.46 ±21.86^{a}	252.05 ±19.39^{a}	0.499 ±0.03^{b}	0.021 ±0.00	18.39 ±1.41^{b}	ND	0.025 ±0.00^{d}	0.054 ±0.00^{c}	ND	511.78 ±36.56^{a}
0.2	214.21 ±15.30^{b}	160.04 ±11.43^{c}	48.17 ±4.38^{a}	ND	51.95 ±4.72^{a}	ND	0.08± 0.01^{c}	0.16± 0.01^{b}	33.75 ±2.60	508.36 ±33.89^{a}
0.1	204.88 ±15.76^{b}	223.76 ±14.92^{b}	0.66± 0.05^{b}	0.51± 0.05	2.49± 0.19^{d}	0.53 ±0.04	0.13± 0.01^{b}	0.48± 0.04^{a}	52.50 ±4.38	485.07 ±40.42^{b}
0.05	10.16± 0.85^{c}	26.84± 1.79^{d}	0.68± 0.06^{b}	ND	8.81± 0.63^{c}	0.13 ±0.01	0.32± 0.02^{a}	0.02± 0.00^{c}	ND	46.96± 3.13^{c}

p LSD	0.0001 20.0	0.0021 22.5	0.001 1.00	0.021	0.003 3.2	0.025	0.019 0.051	0.041 0.100	0.031	0.001 22.5

Different letter means significant at $p \leq 0.05$. Values are mean ± SD (n = 3). Tan, tannic acid; Gal, gallic; Caf, caffeic acid; Fer, ferulic; *t*-cin, *trans*- cinnamic; Chlo, chlorogenic acid; Sin, sinapic acid; Van, Vanillic acid; O-Cou, O-Coumaric. ND; not detected.

In concomitant with the enhancement of total flavonoids and phenolics contents, in plants grown on nutrients deficient media, there was a significant increase in PAL activity (Fig 2). The increase in PAL activity was ranged from 18.9 µmol cinn. g^{-1} f.m min^{-1} in *Azolla* plants grown on nutrient sufficient control to 45 and 23 µmol cinn. g^{-1} f.m min^{-1} in plants grown on 0.2 and 0.05 strength Hoagland nutrient media, respectively. There was a significant increase in antioxidant activity in nutrient-deficient *Azolla* plants compared to control. The increase of antioxidant activity of *Azolla* grown on 0.2 and 0.05 strength cultures was 3- and 1.7-fold respectively compared to those grown on nutrient-sufficient control (Fig 1)

DISCUSSION

Nutrient-starved *Azolla* showed a significant reduction in biomass, relative growth rate (RGR) and increase in doubling time (DT) compared to control. In agreement with these results, it have been reported that deficiency of various nutrients suppressed the growth of several plants (Tewari ~~et al.~~*et al.* , 2007; Christin ~~et al.~~*et al.* , 2009; Sadeghi ~~et al.~~*et al.* , 2013) and this reduction could be attributed to the inhibition of physiological and biochemical reactions (Khavari-Nejad ~~et al.~~*et al.* , 2009; Caretto ~~et al.~~*et al.* , 2015). Thus, the suppression of growth in *Azolla* might be related to decrease of macro and micronutrient (Yu and Rengel, 1999; Biswas ~~et al.~~*et al.*, 2005) in the supplemented nutrient cultures. Moreover, Shin ~~et al.~~*et al.* (2005) and Ahmad ~~et al.~~*et al.* (2014) concluded that nutrients deficiency induces a marked oxidative stress resulting in a suppression of plant growth. In accordance with these views, a significant increase in H_2O_2 and MDA contents in starved-*Azolla* in response to nutrient stress might indicate the enhancement of oxidative stress and that reflect the inhibition of *Azolla* growth. Caretto ~~et al.~~*et al.* (2015) reported that the primary metabolites (synthesized from photosynthesis and consumed in growth) and secondary metabolites (used as a defense mechanism) are mostly influenced by the concentration of nutrients. In this study, the significant increase in secondary metabolites accumulation, including anthocyanins, flavonoids and total phenolics, in *Azolla* plants grown on nutrients deficient growth media indicated the conversion of primary metabolites to secondary metabolites for evolution defense mechanism under nutrients deficiency. Ramakrishna and Ravishankar (2011) stated that deficiencies of essential nutrients such as N, K and P may induce the accumulation of phenolics in plants. While, Jin ~~et al.~~*et al.* (2008) reported that Fe deficiency resulted in an enhancement of phenolics secretion of some monocots and dicots roots. In addition, Stewart ~~et al.~~*et al.* (2001) and Khavari-Nejad ~~et al.~~*et al.* (2009) concluded that the increase of anthocyanins and flavonoids in tomato plants was related to N and P deficiency in growth medium. Recently, Omede ~~et al.~~*et al.* (2018) reported that increase of phenolics, anthocyanins and flavonoids play an important role for reducing the oxidative damage during the generation ROS under various stresses. Many investigators reported that phenolic acids (such as coumaric, caffeic, gallic and chlorogenic) participate as reducing agents for reducing generated ROS (Saxena ~~et al.~~*et al.*, 2012; Cingöz and Karakas, 2016; Darmanti ~~et al.~~*et al.*, 2018). However, there was a marked variation in concentration of individual phenolic acids in *Azolla* plants, the total phenolic acids were markedly decreased with increasing nutrient limitation of growth media. These observations might be attributed to leakage of phenolic acids to surrounding media due to disturbance of plasma membranes, as indicated by increasing MDA content, and hence inhibit

Azolla growth. Beckman (2000) suggested that increasing of phenolic acids accumulation could result in an acceleration of cell death. Moreover, Morcuende ~~et al.~~*et al.* (2007) concluded that transcription of MYB factors resulted in an increase of anthocyanin biosynthesis in *Arabidopsis* plant. Saengnil ~~et al.~~*et al.* (2011) state that light induced anthocyanin content in mango plant *via* enhancing gene expression of key enzyme of anthocyanin pathway, particularly PAL enzyme. It has been concluded that the increase of phenolics under various stresses is accompanied with enhancing the enzymes activities of biosynthesis of these components (PAL and chalcone synthase) as well as phosphoenol pyruvic carboxylase (PEPC) which reflect the conversion of primary metabolites to defensive metabolites (Kováˇcik ~~et al.~~*et al.* , 2007; Lattanzio ~~et al.~~*et al.* , 2009). In this study, the increase of PAL activity in nutrient-deficient *Azolla* was accompanied with a significant accumulation of phenolic components. Alturki et al (2013) mentioned that decreased of nutrient strength of date palm cultures resulted in a marked increase of phenolics biosynthesis and antioxidant activity. Similarly, Cingöz and Karakas (2016) found that nutrient stress or Ca- and Mg-deficient media induced PAL activity and phenolics content in *Bellis perennis* cultures, and there was positive relationship between accumulated phenolics and antioxidant activity. Darmanti ~~et al.~~*et al.* (2018) and Sarker and Oba (2018) reported that, under drought stress, the increased PAL activity and phenolics content could participate in scavenging ROS in soybean and *Amaranthus* plants, respectively. Thus, the decline of nutrients concentration, such as N, K, P, Ca, Mg and Fe, in the nutrient-deficient media of *Azolla*, under this study, might result in an induction of the enzymes activities, including PAL, responsible for phenylpropanoids and flavonoids biosynthesis diverting carbon skeleton from primary production for synthesis of phenolic components, secondary metabolites, as defense mechanisms against oxidative stress, and hence suppress the growth. Caretto ~~et al.~~*et al.* (2015) suggested that, under various stresses e.g. mineral deficiency, secondary metabolites utilize available plant primary resources or carbon skeleton resulting in a more reduction of plant growth. Li ~~et al.~~*et al.* (2018) concluded that there was a positive correlation between antioxidant activity and phenolics in Chinese medicinal herbs. Thus, the significant increase in antioxidant activity of starved-*Azolla* was depend mainly upon phenolic compounds for defense mechanism against nutrient stress.

CONCLUSIONS

It is concluded that the growth of *Azolla* plants on nutrients deficient media of different strength enhanced some secondary metabolites such as anthocyanins, flavonoids, total phenolics as well as certain phenolic acids (caffeic, o-coumaric and *t*-cinnamic acids) and this increase was associated with induction of PAL and antioxidant activities as a strategy for defense mechanism against nutrient stress. The results indicated that the culture of *Azolla* on 0.2 strength Hoagland is the most effective for phenolic biosynthesis and high antioxidant activity.

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Table and Figure captions

Table 1: Biomass, doubling time (DT) and relative growth rate (RGR) of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3).

Table 2: Phenolic acids of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3). Tan, tannic acid; Gal, gallic; Caf, caffeic acid; Fer, ferulic; *t*-cin, *trans*-cinnamic; Chlo, chlorogenic acid; Sin, sinapic acid; Van, Vanillic acid; O-Cou, O-Coumaric. ND; not detected.

Fig 1: Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA) and antioxidant activity of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient- deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3). Different letter means significant at $p \leq 0.05$.

Fig 2: Total phenolics, flavonoids, anthocyanins contents and PAL activity of *Azolla* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days. Values are mean ± SD (n = 3)**.** Different letter means significant at $p \leq 0.05$.