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Nutrient Starvation Enhances the Phenolic Compounds and Antioxidant Activity in *Azolla caroliniana* Plant

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THE AIM of this study is evaluating the effects of nutrients deprivation in growth media of *Azolla caroliniana* plant on growth and production of various phenolic components and antioxidant activity. Nutrient-deficient cultures (0.2, 0.1, 0.05 strength Hoagland solution) of Azolla plants 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 significantly affected 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 phenylalanine ammonia lyase (PAL) and antioxidant activities in starved *Azolla*. The results indicated that, the antioxidant activity might depend mainly on phenolic compounds.

Keyword: Anthocyanins, Antioxidant activity, *Azolla caroliniana*, Flavonoids, Phenolics, Phenolic acids.

Introduction

One of the most abiotic stresses is the deficient of nutrients available for plant growth (Ramakrishna & Ravishankar, 2011; Yang et al., 2018). Tewari et al. (2007) and Khavari-Nejad 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. (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 been 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., 2005; Tyburski et al., 2009). Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet-}$), hydroxyl (OH^-), peroxyl (ROO^-) 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 and homeostasis (Gill & Tuteja, 2010; Li et al., 2018).

Plants have various defense mechanisms against the oxidative stress caused by nutrient deficiency including enzymatic and non-enzymatic antioxidants and secondary metabolites (Angaji et al., 2012). The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPx), polyphenol oxidase (PPO) and ascorbic peroxidase (APx) can scavenge the generated toxic ROS (Ahmad et al., 2014). Phenolic components are secondary metabolites including several derivatives such as flavonoids, polyphenols, anthocyanins and phenolic acids that act as non-enzymatic antioxidants (Guo et al., 2008; Cingöz & Karakas, 2016) and have a mechanism in suppression of the growth (Caretto 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., 2012; Angaji 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., 2008; Li et al., 2008; Ramakrishna & Ravishankar, 2011; Cingöz & Karakas, 2016).

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Phenylalanine ammonia lyase (PAL) is a key enzyme in diverting primary metabolism to secondary metabolism in response to stress. It has been reported that the accumulation of phenolics and flavonoids in plants under stress is accompanied with enhanced PAL activities (Kováˇcik et al., 2007; Guo et al., 2008). Recently, Darmanti 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 stresses.

Azolla, a free-floating water fernis 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., 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 such as biofertilizer in botanical gardens and green manure in rice fields, bioremediation through biofiltration of toxic elements and nutrients from pollutants or sewage water, food supplement for variety of animals and production of methane gas which can be utilized as biofuel (Raja et al., 2012). Thus, it is used as target in this research for the production of phytochemicals of bioactive functions.

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 activity of the key enzyme, phenylalanine ammonia lyase, that involves in the biosynthesis of these compounds.

Materials and Methods

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, Belgium, it 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 University (Hassan & Mostafa, 2016). The cultures were grown in a growth chamber under 16hrs photoperiod at irradiance of 1200µmol m^{-2} s^{-1} with cool white fluorescent tubes and light/dark temperature of 28-30/20-25°C (Stock culture).

Treatments

An aliquot of 5g 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 250cm^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). After10 days from starting the experiment, samples were collected for estimation of some growth parameters such as the number of generations and doubling time according to Peters et al. (1979) as follow:

$$n \text{ (final mass)} = n^o \times 2G,$$

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 using the formula of Subudhi & Watanabe (1981) as follows:

$$RGR\ [kg\ kg^{-1}d^{-1}] = 0.693\ DT^{-1}.$$

Determination of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents

Hydrogen peroxide was determined as described by Velikova et al. (2000) and malondialdehyde content was estimated according to Zhange et al. (2007).

Determination of anthocyanins

Total anthocyanins content was determined according to Mancinelli et al. (1988).

Extraction of total flavonoids and phenolics

Extraction of total flavonoid and phenolic compounds was carried out according to the method described by Proestos et al. (2005).

Determination of total flavonoids

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Meda et al., 2005). The total flavonoid content was expressed as mg of QE/g dry mass.

Determination of total phenolic contents

The total phenolic compounds content of the aqueous methanol extract was performed

according to the Folin Ciocalteau's method (Zheng & Wang, 2001). Tannic acid was used as a standard and 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 & Liu (2002) and subjected to RP-HPLC analysis.

Chromatographic analysis was performed by Ultimate 3000 HPLC coupled with a UV-VIS multiwave length detector. The flow rate was 0.5mL/min and injection volume was 25µL. The monitoring wavelength was 280nm (Proestos 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 according to the method described by Koracevic et al. (2001).

Determination of L-phenylalanine ammonia-lyase activity (PAL, EC 4.3.1.5)

A Fresh sample was homogenized and extracted according to Devi et al. (2012). PAL activity was measured by monitoring the reaction product *t*-cinnamate at 290nm and calculated as a difference in the optical density for 30min. The amount of *t*-cinnamic acid synthesized was calculated using its extinction coefficient (9630 M^{-1}) (Kagalea et al., 2004) and the results were expressed as µmol cinnamic acid g^{-1}f.m h^{-1}.

Statistical analysis

The statistical analysis was carried out 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≤ 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 10d was about 39%, compared to the control. The doubling time (DT) was significantly increased, while the relative growth rate (RGR) was significantly decreased 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, revealing reduction of growth.

There was a significant accumulation of $H_2O_2$and MDA in *Azolla* plants grown in nutrient deficient media compared to non-deficient ones. At 0.2 strength media, the H_2O_2 and MDA contents were 1.8- and 1.4-fold, respectively of those in the control plants**,** where they reached to 5.6- and 4.4-fold in case of 0.05 Hoagland strength (Fig. 1 A, B).

Anthocyanins content in starved-*Azolla* plants was significantly increased with the deprivation of nutrients in the growth media up to 0.2 strength then steady decreased (Fig. 2 C). The highest anthocyanins content was detected in starved-*Azolla* grown on 0.2 strength, in comparison to nutrients sufficient control.

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

Treatments	Biomass [g culture^{-1}]	Doubling Time (DT) [d]	Relative growth rate (RGR) [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

- Values are means of 3 replica± SD
- Different letter means significant at P≤ 0.05

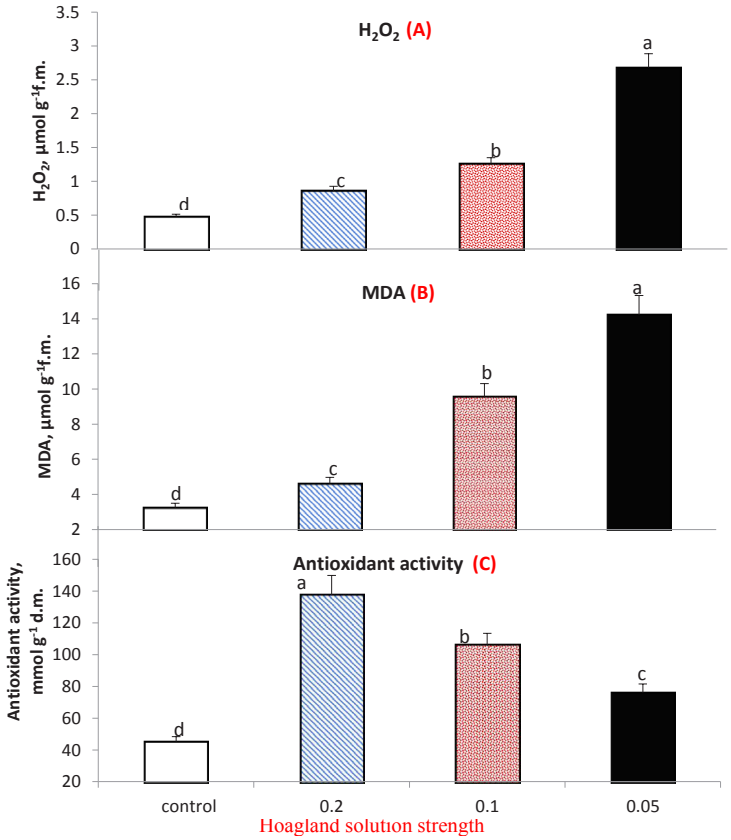

Fig. 1. Hydrogen peroxide (H_2O_2), Malondialdehyde (MDA) and antioxidant activity of *Azolla caroliniana* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days [Values are means of 3 replica ± SD, different letters means significant at P≤ 0.05].

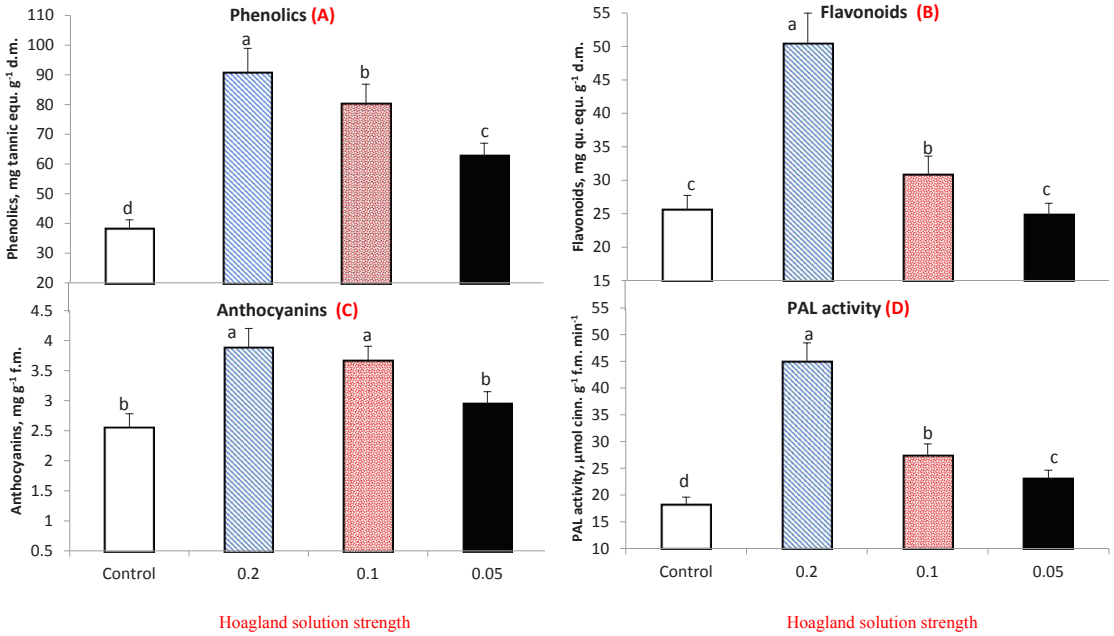

Fig. 2. Total phenolics, flavonoids, anthocyanins contents and PAL activity of *Azolla caroliniana* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) after 10 days [Values are means of 3 replica± SD, different letters means significant at P≤ 0.05].

Total flavonoids and total phenolics were significantly increased in 0.2 starved *Azolla* plants then markedly declined with increasing starvation stress (Fig. 2 B). The increase in total flavonoids and total phenolics in 0.2 starved *Azolla* were 2 and 2.4 fold, respectively of those grown on nutrient-sufficient control (Fig. 2 A, B).

Generally, there was a significant decrease in total phenolic acids content in *Azolla* grown on deficient nutrient media. 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, the phenolic acids content showed 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 significant decrease in phenolic acids (tannic, gallic, ferulic, *t*-cinnamic and vanillic acids) in *Azolla* grown on severely deficient nutrient media.

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 D). The PAL activity of 0.2 and 0.05 starved *Azolla* was 2.4 and 1.2 fold of control, respectively.

Parallel to increase of phenolics, 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 C).

Discussion

Nutrient-starved *Azolla* showed a significant reduction in the measured growth criteria, compared to control. In agreement with these results, it has been reported that nutrients deficiency suppressed the growth of several plants (Christin et al., 2009; Sadeghi et al., 2013). This reduction could be attributed to the inhibition of physiological and biochemical reactions (Khavari-Nejad et al., 2009; Caretto et al., 2015). Thus, the suppression of growth in *Azolla* in this study might be related to decrease of macro and micronutrients (Yu & Rengel, 1999; Biswas et al., 2005) in the supplemented nutrient cultures. Moreover, Shin et al. (2005) and Ahmad et al. (2014) concluded that nutrients deficiency induces a marked oxidative stress resulting in a suppression of plant growth. In accordance with these views, there was a significant increase in H_2O_2 and MDA contents in starved-*Azolla* in response to nutrient stress and that might induce the oxidative stress resulting in an inhibition of *Azolla* growth.

Caretto 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 instead of consuming in growth. Ramakrishna & 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. (2008) reported that Fe deficiency resulted in an enhancement of phenolics secretion of some monocots and dicots roots. In addition, Stewart et al. (2001) and Khavari-Nejad et al. (2009) mentioned that the increase of anthocyanins and flavonoids in tomato plants was related to N and P deficiency in growth medium. Therefore, the increase of phenolic components (Fig. 2 A) in starved *Azolla* might be attributed to decrease the concentrations of several elements of growth solution.

Many investigators reported that phenolic components including phenolic acids (such as coumaric, caffeic, gallic and chlorogenic) participate as reducing agents for eliminating generated ROS (Saxena et al., 2012; Cingöz & Karakas; 2016; Darmanti et al., 2018; Omede 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 insufficient scavenging of generated ROS and oxidative stress by synthesized phenolic and/or leakage of phenolic acids to surrounding media due to disturbance of plasma membranes, as indicated by increasing MDA content, as well as exert a toxic effect, hence inhibit *Azolla* growth.

Beckman (2000) suggested that increasing of phenolic acids accumulation could result in an acceleration of cell death. 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., 2007; Lattanzio 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 & Karakas (2016) found that nutrient stress such as 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. (2018) and Sarker & 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 PAL activity, which involve in phenylpropanoids and flavonoids biosynthesis as defense mechanisms against oxidative stress. However, the growth was suppressed, where Caretto 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. (2018) concluded that there was a positive correlation between antioxidant activity and phenolics in Chinese medicinal herbs. In agreement with this view, the significant increase in antioxidant activity of starved *Azolla* was accompanied with increase of phenolics content and decrease of growth. These findings might reveal that the induction of ROS and oxidative stress in nutrient starved *Azolla* was higher than the enhancement of phenolics biosynthesis and antioxidant activity, resulting in reduction of growth. Gill & Tuteja (2010) reported that the effect of abiotic stress on growth related to the imbalance between generation of ROS and eliminating or scavenging activities of antioxidant systems.

TABLE 2. Phenolic acids of *Azolla caroliniana* plants grown on 0.4 strength nutrient-sufficient (control) and nutrient-deficient media (0.2, 0.1 and 0.05 strength) for10 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	0.0001	0.0021	0.001		0.003		0.019	0.041	0.031	0.001
LSD	20.0	22.5	1.00	0.021	3.2	0.025	0.051	0.100		22.5

- Means of 3 replica± SD.
- 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
- Different letter means significant at P≤ 0.05.

Conclusion

It is concluded that the growth of *Azolla* plants on nutrients deficient media of different strength enhanced generation of ROS and PAL activity which induce the biosynthesis of phenolics including anthocyanins, flavonoids, and certain phenolic acids (caffeic, o-coumaric and *t*-cinnamic acids). These findings were accompanied with increase the antioxidant activity as a strategy for defense mechanism against nutrient stress, however, the growth was declined under nutrient deficiency.

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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التجويع الغذائى يحفز المركبات الفينولية والنشاط المضاد للأكسده فى نبات الأزولا

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تهدف هذه الدراسة إلى تقييم تأثير نقص العناصر الغذائية فى المحلول المغذى (تجويع) لنبات الأزولا على النمو والمركبات الفينوليه المختلفة والأنزيم الرئيسى المسؤل عن تخليقها وكذلك النشاط المضاد للأكسدة.

تم تجويع نبات الأزولا بزراعته على محلول هوجلاند المغذى بتركيزات منخفضة القوة (0.1،0.2، 0.05) مقارنة بالمحلول الكامل القوة (0.4) لمدة 10 أيام.

أوضحت النتائج نقص معنوى فى معدل النمو و زياده معنوية فى تراكم فوق أكسيد الهيدروجين والمالون داى ألديهايد مع انخفاض قوة تركيز المحلول المغذى وصاحبت هذه التغيرات زيادة معنوية فى الأنثوسيانين والمركبات الفينولية المختلفة وخاصة حمض الكفيك والكيوماريك والسينماميك وانزيم الفينيل ألانين أمونيا ليز. وكان مصاحب لذلك زيادة معنوية فى النشاط المضاد للأكسده خاصة مع تركيز 0.2.

بينت النتائج أن النشاط المضاد للأكسدة قد يرجع أساسا إلى الأنثوسيانين والمركبات الفينوليه فى نبات الأزولا النامى تحت نقص العناصر المختلفة.