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Enhancement of Growth and Endogenous Phytohormones of *Azolla pinnata* in Response to Tryptophan

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Abstract: Application of tryptophan at different concentrations (0, 5, 15, 30 ppm) to the growth media of *Azolla pinnata* enhanced growth, N- fixation and endogenous phytohormone levels. Increases of fresh and dry weights as well as doubling time of *A. pinnata* fronds were generally highly significant with respect to all tryptophan treatments, as compared to corresponding control values at different incubation periods (0, 10, 20, 30, 40 days). Dinitrogenase activity showed an inconsistent increase with elevating tryptophan concentration. The increase in *Azolla* growth was also concomitant with a noticeable increase in the endogenous content of IAA from zero time up to 40 days. GA_3 and ABA were highly increased after 10 days incubation, followed by a gradual decrease afterwards .Generally, IAA, GA_3, and ABA hormone levels were higher in response to tryptophan treatments, as compared with those of corresponding controls.

Key words: *Azolla*, dinitrogenase activity, tryptophan, phytohormones, IAA, GA_3, ABA.

INTRODUCTION

Azolla is a genus of free small aquatic heterosporous pteridophytes. *Azolla* sporophyte consists of a horizontal to a vertical main rhizome bearing individual roots or root bundles at branch points and alternately arranged bilobed leaves. The dorsal lobes are chlorophyllous and contain the symbiotic cyanobacterium *Anabaena Azollae*, within an ovoid cavity connected to the atmosphere by a pore. The translucent ventral lobes resting on the water surface support the frond and are nearly chlorophyllous (Peters *et al*., 1976 and Wagner, 1997). *Azolla-Anabaena* has many uses. The host- symbiont combination is exploited as a biofertilizer for rice and many other crops (Sharma *et al*., 1999, Pabby *et al*., 2003 and Ripley *et al*., 2003). It can be used for animal feeding, human food, in medicine, and as water purifier. It may also be used for the production of biogas, the control of weeds, the control of mosquitoes, and the reduction of ammonia volatilization that accompanies the application of chemical nitrogen fertilizers (Wagner, 1997). The aquatic fern *Azolla* appears also important because of its potentiality for heavy metal accumulation (Stepeniewska *et al*., 2005 and Zhang *et al* ., 2008). Katayama *et al*. (2008) added that nutritional value of *Azolla* is similar to that of *Alfalfa* sprouts or typical marine macro- algae. After an investigation for several years, they found that *Azolla* is also a promising plant to be applied in controlled ecological life support systems (Xiaofeng, *et al*., 2008).

Several micro-organisms exert a marked influence on the growth of plants by producing plant growth regulators (Forni *et al*., 1992). Stirk and Staden (2003) added that the symbiotic cyanobacterium *Anabaenaa Azollae* as well as the arthrobacter bacteria found in *Azolla filiculoides* do produce plant growth regulators. Extraction of auxins, gibberellic acid and cytokinins was recorded in *Azolla pinnata, Anabaena– Azolla* associations (Serdyuk *et al*., 1992), the aquatic fern *Salvinia molesta* (Geargina *et al*., 2007) and from sea weeds (Strik *et al*., 2004). *Arthrobacter* species, isolated from the leaf cavities and the microsporocarps of *A. pinnata* and *A. filiculoids* produced IAA in culture when the precursor tryptophan was added to the medium, (Forni *et al*. 1992) .

Koshy *et al*. (2001) reported that tryptophan and methionine application enhanced maximum germination percentage of *Azolla filiculoides* spores. On the other hand, indoleacetic acid (IAA) application significantly increased the sporulation frequency (Forni *et al*. , 1992), megasporocarp number (Kar *et al*., 2001) or microsporocarp and megasporocarp numbers of *Azolla* (Kar and Singh, 2002). Treatment with gibberellic acid (GA_3) enhanced germination of *Azolla caroliniana* sporocarps (Singh *et al*., 1990) and also induced higher

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sporocarp numbers of *Azolla caroliniana* and *Azolla pinnata* (Kar and Singh, 1999). In addition, sporulation frequency and microsporocarp number of *Azolla* were significantly higher on gibberellic acid application (Kar and Singh ,2002). Abscisic acid (ABA) is found universally in angiosperms, gymnosperms, ferns, mosses, liverworts and algae (Srivastava, 2002) but not in bacteria (Salisbury and Ross, 1992).

ABA at higher concentrations significantly inhibited *Azolla* germination, however, at relatively low concentrations, ABA increased megasporocarps germination (Singh *et al*., 1990).

Thus, the aim of the present work was to investigate the effect of tryptophan application on *Azolla* growth and the ability of *Azolla* to enhance the production of endogenous phytohormones, namely IAA, GA_3, and ABA.

MATERIALS AND METHODS

Materials:

Azolla pinnata used in the present investigation was kindly provided by the Agricultural Microbial Department, Soils, Water and Environment Research Institute (SWERI), Agric. Res. Center, Giza, Egypt.

Methods:

The present experiments were carried out in the Botanical Garden, Faculty of Science, Ain Shams university, Cairo, Egypt.

Ten g of *Azolla* was grown in plastic pots, 30 cm diameter and 15 cm depth, containing 1 Kg soil in 3 liters tap water according to El- Shahat (1988). These pots were kept in a greenhouse till *Azolla* covered the entire water surface.

Azolla was collected and incorporated in 0.01 mercuric chloride for 1 min. and washed gently in running tap water for several times, using screen of 0.2 mesh and then air dried on tissue paper for 30 min. The collected *Azolla* was used as an inoclum for the present experiment.

Four sets of plastic Pots (14 cm diam. and 7cm dep.) were prepared. Each pot contained 750 ml Yoshida medium (Yoshida *et al*., 1976), one concentration of tryptophan (0, 5, 15, and 30 ppm) and inoculated by one gram fresh *Azolla*. The experiment was carried out under green house conditions at a photoperiod of 18/6 hr, temperature of 25ºC± 2 at day time and 18ºC± 2 at night and air humidity of about 70%. Three samples (replicates) from each treatment were harvested after 0, 10, 20, 30, and 40 days incubation periods. *Azolla* fronds were washed by deionized water and placed under shade between two thick layers blotting papers for approximately 1h. before different measurements. The effect of increased concentration of tryptophan on biomass production, doubling time, dinitrogenase activity, and phytohormone production were studied. Dry weight was determined by drying *Azolla* fronds at 70ºC to a constant weight (to the nearest mg).

Doubling Time Calculation:

Growth rate of *Azolla* in terms of doubling time was calculated using the following equation according to Aziz and Watanabe (1983).

Doubling time = t/r ,whereas :

t = the duration of *Azolla* growth.

r = log wt /w_0 / 0.301

wt = weight of *Azolla* at time t,

W_0 = weight of *Azolla* at zero time i.e. weight of inoculum.

Acetylene Reduction Assay:

For Acetylene reduction assay, about 0.5 g fresh weight of *Azolla* fronds grown under different treatments was incubated under 10% C_2H_2 in air of 500 ml. flask, fitted with serum caps, containing 100 ml amended culture media. The incubation conditions and analysis of ethylene produced by *Azolla* were adopted as described by Kitoh *et al*. (1993).

Extraction, Separation and Estimation of Growth Regulating Substances:

Azolla fronds were collected and ground in cold 80% methanol and then the followed extraction method was essentially similar to that adopted by Shindy and Smith (1975).

The plant hormone fractions and standard ones were methylated according to Vogel (1975). Flame ionization detector was used for identification and determination of IAA, GA_3 and ABA, using Helwett packered GC (5890).

Statistical Analysis:

The individual data sets were subjected to the least significant differences at $p< 0.05$ (Gomez and Gomez, 1984).

RESULTS AND DISCUSION

Growth Density:

The data in Table (1) represent the fresh and dry weights as well as the doubling time of *Azolla pinnata* as influenced by different concentrations of tryptophan. Fresh and dry weights significantly increased with increasing the concentration of tryptophan from 5 to 15 ppm with time up to 30 days and then, slightly decreased (Table: 1). Maximum fresh and dry weights were observed at the concentration 15 ppm of tryptophan after 30 days incubation. It was obvious that values of fresh and dry weights were highly significant in all tryptophan treatments, as compared with corresponding control values at different incubation periods. Doubling time was generally decreased with increasing tryptophan concentration up to 30 ppm (Table: 1). The lowest doubling time was clearly demonstrated after 10 days in treatment with 15 ppm tryptophan. In this respect, Xiaofeng *et al*. (2008) showed that *Azolla* biomass increased and its doubling time was clearly shortened when grown on artificial controlled environmental conditions containing tryptophan. These results agree with our results showing highly significant effects on *Azolla* growth (Fresh and dry weights and doubling time), with different concentrations of tryptophan, as compared with the corresponding control values at all the applied conditions used.

Table 1: Effect of different concentrations of tryptophan on *Azolla pinnata* growth [fresh , dry weight (g/m^2) and Doubling time (days)].

Treatment / Period	Tryptophan concentration (ppm)											
	Control			5			15			30		
	F.wt.	D.wt.	D.t	F.wt.	D.wt.	D.t	F.wt.	D.wt.	D.t	F.wt.	D.wt.	D.t
0	90.90± 0.00	4.82± 0.00	00.0± 0.00	90.90± 0.00	4.82± 0.00	0.00 ± 0.00	90.90± 0.00	4.82± 0.00	0.00± 0.00	90.90± 0.00	4.82± 0.00	0± 0.00
10	563.64± 39.63	0.40	30.0± 0.15	3.8± 45.88	864.55± 3.21	46.36± 0.15	3.1± 111.72	1518.18± 4.81	80.00± 0.06	2.5± 80.68	1384.55± 3.17	72.73± 0.06	2.6±
20	2060.9± 31.93	0.90	109.09± 0.06	4.4± 80.80	2036.36± 3.94	108.18± 0.06	4.5± 131.92	3082.73± 6.10	162.73± 0.06	3.9± 168.94	3296.36± 8.95	147.55± 0.06	3.8±
30	2539.09± 108.48	0.53	134.55± 0.06	6.3± 146.42	3154.55± 8.08	167.27± 0.10	5.9± 131.92	4107.27± 6.50	217.27± 0.06	5.5± 120.26	3754.54± 6.70	198.18± 0.00	5.6±
40	2311.82 ±64.48	0.93	117.27± 0.86	8.6± 141.84	2741.82± 6.74	144.54± 0.10	8.2± 89.54	3209.09± 4.81	170.00± 0.06	7.8± 63.59	2954.54± 3.44	156.36± 0.06	7.9±
F.wt L.S.D at 0.05 Conc. x period			23.798	D.wt			L.S.D at 0.05	D.wt		-- L.S.D at 0.05 n.s			

Dinitrogenase Activity:

Dinitogenase activity showed an inconsistent increase with elevating the concentration of tryptophan and also increasing the incubation period from 15 up to 30 days (Table: 2). Dinitrogenase is the main enzyme responsible of atmospheric nitrogen fixation, where N_2 is reduced into ammonia (Church *et al*., 2005). Thus, understanding the factors affecting dinitrogenase activity is necessary for devising strategies to increase the amount of ammonium synthesized with the potential to be used in agriculture (Kennedy *et al*., 2004). Therefore, the data obtained in the present work showing enhancement of dinitrogenase activity in response to tryptophan application agree to a wide extents with the conclusions of other authors. These authers revealed firm evidence that IAA from tryptophan precursor enhances the nitrogen fixing and nitrogen assimilation enzymes (Hayat *et al*., 2009). Hayat *et al*. (2009) also showed that auxins significantly improved the nitrogen metabolism and yield of chickpea (*Cicer arietinum* L.). Tryptophan application with *Azotobacter* inoculation was also recorded to elevate NPK uptake and tuber as well as straw yield in potato (Zahir *et al*., 1997).

Endogenous Phytohormones:

The rate of growth of plants and integration of the different physiological processes to produce a recognized form is known to be controlled by phytohormones (Salisbury& Ross, 1992 and Davies, 2004), where extraction of auxins, gibberellic acid and cytokinins from *Azolla pinnata, Anabaena– Azolla* association, were recorded by Serdyuk *et al*. (1992) and Srivastava (2002). IAA production was greatly increased when tryptophan was added to the growth media of *Azolla* (Tharwat *et al*., 2002). Forni *et al*. (1992) reported that IAA content in the growth medium of *Azolla* varied according to the concentration of tryptophan added to the culture. These conclusions agreed, to a wide extent, with our results since *Azolla* fronds, at zero time, contained the lowest content of IAA and this content was afterwards increased with increasing tryptophan concentration to 15 ppm, then decreased with elevating the concentration to 30 ppm but was still higher than thecorresponding media free from tryptophan (Figure 1). The changes in auxins, in the present work, were

Table 2: Effect of different concentrations of tryptophan on dinitrogenase activity (µ mol C_2H_4 /g/dry wt./hr^{-1}) of *Azolla . pinnata*

Treatment Period	Tryptophan concentration (ppm)			
	Control	5	15	30
	N-ase	N-ase	N-ase	N-ase
0	2.45	2.45	2.45	2.45
10	6.70	17.70	21.75	22.92
20	5.80	11.60	15.65	13.36
30	8.50	15.80	18.38	10.72
40	2.09	7.68	13.00	8.07

L.S.D at 0.05
Conc. x period 1.56

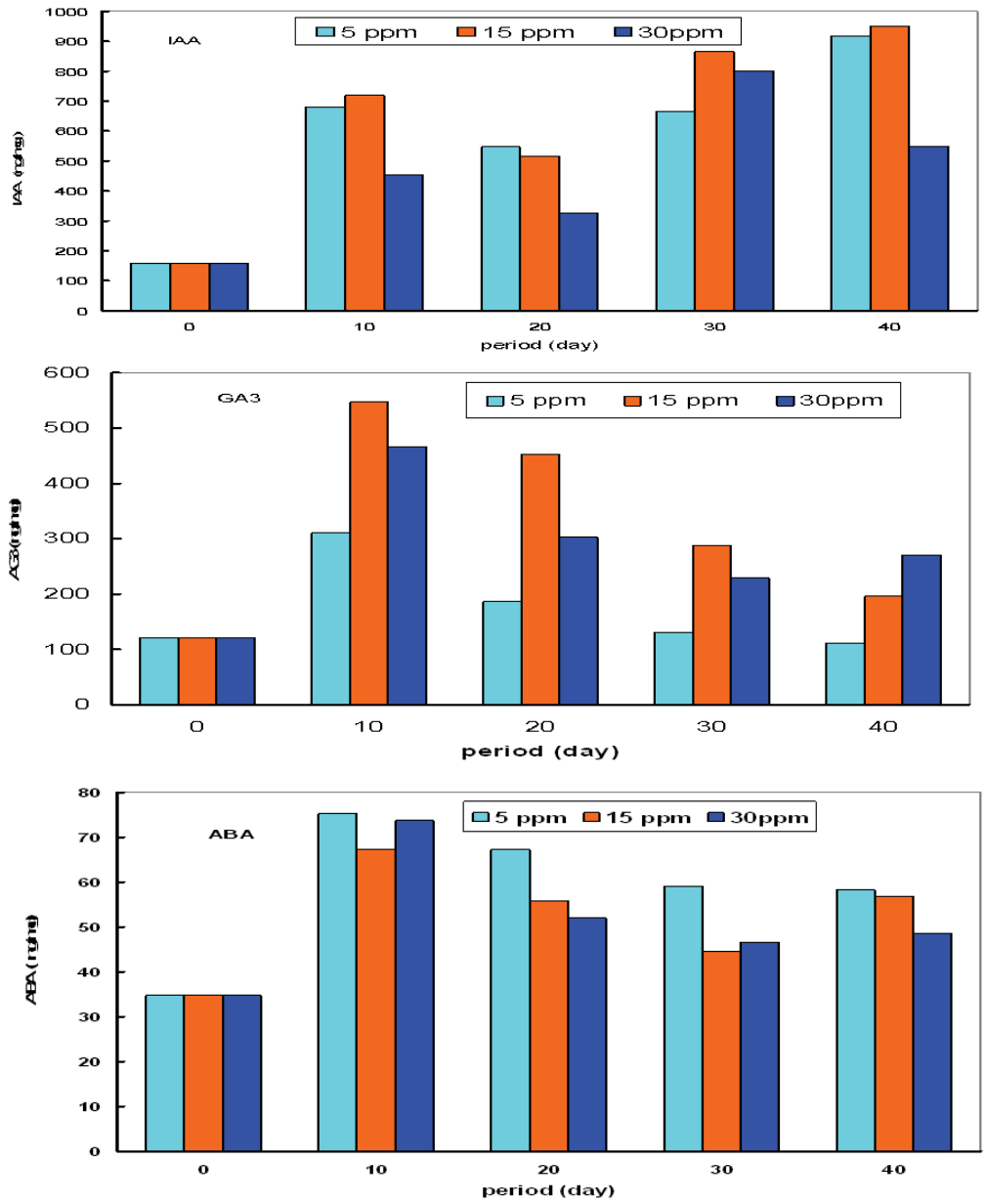

Fig. 1: Effect of different concentrations of tryptophan (ng/mg) on endogenous concentration of indole acetic acid (IAA), gibberellin (GA3) and abscisic acid (ABA) of Azolla pinnata.

Table 3: Effect of different concentrations of tryptophane on endogenous concentration of IAA, GA_3 , ABA and (ng/mg) of *A. pinnata.*

Treatmen Period	Tryptophane concentration (ppm)								
	5			15			30		
	IAA	GA_3	ABA	IAA	GA_3	ABA	IAA	GA_3	ABA
0	158.2	121.2	34.7	158.2	121.2	34.7	158.2	121.2	34.7
10	681.1	310.3	75.24	718.8	546.8	67.2	453.5	466.2	73.7
20	5467.0	186.0	67.2	515.2	452.1	55.8	327.4	302.5	51.9
30	664.4	130.5	59.08	864.1	288.0	44.5	801.5	228.5	46.5
40	917.5	111.9	58.2	949.1	195.8	56.7	548.4	270.4	48.6

tentatively assumed to control meristematic activity according to Howell (1998) and Srivastava (2002) and were predicted to interfere with GA_3 at different steps of extension growth (Westhoff, 1998; Hopkins, 1998 and Srivastava, 2002). Auxins also influence other developmental processes, including the control of cell elongation, cell division, root initiation, vascular deferentiation, etc., mainly through auxin-induced gene expression (Davies, 2004 and Hayat *et al*., 2009), sporulation frequency and microsporocarps number of *Azolla* (Kar and Singh, 2002).

Figure (1) also shows that maximum values of GA_3 and ABA were noticed at 5, 15 and 30 ppm tryptophan after 10 days of incubation, and then a drop in their concentration took place afterwards up to 40 days. The highest concentrations of IAA and GA_3 were recorded, at different incubation periods, with *Azolla* fronds grown in media supplemented by 15 ppm tryptophan, whereas the lowest value of ABA was obtained at the same concentration after 30 days. The recorded reduction in GA_3 and ABA is assumed to play a role in adjustment of the rate of growth (Davies, 2004).

ABA is generally known to be antagonistic to both auxins and gibberellins with respect to their effect on the arrangement of cytoskeleton microtubules and cell growth (Baluska *et al*., 1999). ABA seems to have three major effects, depending on the tissue involved: (1) effects on the plasma membrane of root, (2) inhibition of protein synthesis, and (3) specific activation and deactivation of certain genes. (Wittenmayer and Merbash, 2005 and Christmann *et al*., 2006). However, the beneficial effect of *Azolla* is generally attributed to their relatively high contents of auxins, gibberellins and cytokinins which enhance plant growth (Serdyuk *et al*., 1992 and Stirk and Staden, 2003). The presence of such phytohormones in *Azolla* encourages agriculturists to use it as a biofertilizer that influences the crop yield.

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