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EFFECT OF INOCULATION DENSITY OF *AZOLLA* FRONDS ON NITROGENASE ACTIVITY AND HETEROCYST FREQUENCY OF THE *AZOLLA-ANABAENA AZOLLAE* SYMBIOSIS

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Abstract

The effect of inoculation density level for three *Azolla* species namely *A. microphylla*, *A. filiculoides* and *A. pinnata* was investigated under greenhouse conditions in term of senescence associated with *Azolla* fronds overcrowding. In this respect three inoculum levels, i.e., 0.2 kg, 1.0 kg and 2.0 kg m^{-2} were tested.

Overcrowding influence on *Azolla* was assessed in terms of frond yellowing, nitrogenase activity and heterocyst frequency. Frond discoloration of *A. microphylla*, *A. filiculoides* was observed after only 7 days in higher inocula level (1.0 and 2.0 kg m^{-2}). However, in the lower level of inocula 0.2 kg m^{-2} the discoloration was observed after 15 days. However, *A. pinnata* exhibited frond discoloration on day 10 for 0.2 kg and on day 5 for higher inoculum levels. The nitrogenase activity increased significantly on 7 days at less crowded condition in the cultures inoculated with 0.2 kg m^{-2} for all the tested species. At higher inoculum levels, the nitrogenase activity declined on the third week, after which very low enzyme activity, overcrowding, and extensive senescence were noticed at all inoculurn levels. *Azolla* plants maintained in the dark for 12 hrs prior to beginning of dark incubation period of 3 hrs showed an almost zero nitrogen-fixing activity compared to plants exposed to continuous light. Heterocyst formation on the $12^{\underline{th}}$ leaf from the tip increased up to the seventh day in the three species at 0.2 kg $m^{-2}$and in *A. pinnata* only at 1.0 kg m^{-2} inoculum level. There was an apparent gradually decline in the heterocyst count in the three species at higher inoculum levels throughout the culture period.

Introduction

Azolla is a genus of small aquatic ferns which, under natural conditions, invariably contains the heterocystous blue-green alga, *Anabaena Azollae*, as a symbiont in an enclosed chamber in the dorsal leaf lobes (Peters *et al*., 1976 and Peters and Mayne, 1974). In the intact association, the alga can provide the *Azolla* plant with its total nitrogen requirement. Previous studies showed that the symbiont contained nitrogenase and was capable of C_2H_2 reduction, ATP dependent H_2 evolution, and excretion ammonia (Peters, 1976). Although C_2H_2 reduction is a simple and sensitive assay of nitrogenase activity, it is not the biologically important substrate, and the assay is an indirect measurement of nitrogen fixation (Peters *et al*., 1977).The heterocysts found in the symbiont

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Anabaena azollae are responsible for the nitrogen fixing activity of the association, (Moore, 1969). The development of *Anabaena* is synchronized with leaf development in *Azolla*. As leaves mature and cavities are differentiated, cell division in the symbiont appears to diminish. Likewise the vegetative cells enlarge and differentiation into heterocysts increased, (Hill, 1975). Environmental factors determine to a certain extent the growth and development of *Azolla-Anabaena* complex. Among the most important factors are nutrients and light. *Azolla* can readily survive and grow well in nitrogen-free inorganic nutrient solution (Yoshida *et al*., 1976). Only few studies and reports have been done on the physiology of senescence in *Azolla* due to its fronds density and crowding. Such studies describe the effect of senescence associated with *Azolla* frond crowding on biomass, nitrogen content, protein content, carbohydrate content, nitrogenase activity and heterocysts frequency of the *Azolla-Anabaena* complex (Cohn and Thimann, 1972; Cohn and Thimann, 1975 and Ghazal and Herzalla, 1997). This work aims to study the effect of *Azolla* crowding density on the nitrogenase activity and heterocysts frequency of three *Azolla* species namely *A. microphylla, A. fuliculoides and A. pinnata* at 3 different levels of inoculum, i.e., 0.2, 1.0 and 2.0 kg m^{-2}.

Materials and Methods

A greenhouse experiment was conducted to study the effect of overcrowded growth of *Azolla* fronds on the nitrogenase activity and heterocyst frequency of the symbiont *Anabaena azollae*. For this purpose, three species of *Azolla* namely: *A. filiculoides, A. pinnata and A. microphylla* were allowed to grow at three biomass inoculum levels (0.2, 1 and 2 kg m^{-2}) in polyethylene trays (25 cm x 20 cm) filled with one litter of nitrogen-free medium (Yoshida et al., 1976). Excess plants were constantly removed from each tray to maintain a relatively uniform frond growth pattern. This assured that the cultures were of comparable ages. The culture solution was replenished to a liter twice a week to prevent the drying of *Azolla* plants. After two weeks each tray was completely drained through a small net covered hole made at one corner of each tray and then rinsed and filled with fresh culture solution. The trays were arranged in three block design resorting three replicates for each *Azolla* species and each inoculum level at any period culture. Data were subjected to the analysis of variance (ANOVA) according to Gomez and Gomez (1984).

Nitrogenase activity was measured as acetylene reduction activity (ARA) according to the method described by Hardy *et al*. (1973) at intervals of 0, 7, 15, 22 and 29 days. By the same method (ARA) was measured under dark and light at periods of 0, 5, 12, 18 and 25 days. This was done after exposure of 2 set of samples to 12 hrs darkness, then one set of each species was exposed for 3 hrs to continuous light (40 - 50 K lux) and the other set was kept in the dark for the

same period. Generally nitrogenase activity was expressed as µmoles and nmoles C_2H_4 /g fresh weight/hr. as described by Yoshida *et al*. (1976).

Heterocyst count:

The algal symbiont was isolated from the basal cavities of the 12th leaf from the tip of *Azolla* frond. Anabaena filaments were teased out with needles under stereomicroscope and heterocysts frequency was estimated under compound microscope as described by Vaishampayan (1982). The schedule of the counting of heterocysts followed that of the acetylene reduction assay namely, at days 0 (initial),
7, 15, 22 and 29.

Results and Discussion

Nitrogenase activity:

During the 29 period, nitrogenase activity as measured by the acetylene reductions assay (ARA) was consistently higher in the three *Azolla* species when the inoculurn level was 0. 2 Kg m^{-2} (Table 1). Apparently higher inoculum levels of 1.0 and 2.0 kg m^{-2} significantly decreased nitrogenase activity (Table 1 a). Among the three *Azolla* species, *A. microphylla* at all inoculum levels had a higher nitrogenase activity than either *A. fuliculoids* or *A. pinnata* throughout the 29-culture period (Tables 1 and lb), *A. pinnata* had the lowest capacity to reduce acetylene.

Variations in the occurrence of nitrogenase maxima activity during the culture period were also noticed along the three *Azolla* species (Table 1). At an inoculurn level of 0.2 kg m^{-2}, the highest value of ARA in all the three *Azolla* species was recorded on the seventh day and at 1kg m^{-2}, then after two days in A. *microphylla* and five days in both *A. filiculiodes* and *A. pinnata*. At the highest inoculum level, highest nitrogenase activity was measured on the second day in *A. microphylla* and *A. fihiculoids* and initially in *A. pinnata*.

Dark conditions significantly reduced nitrogenase activity in the three symbionts at all inoculum levels (Table 2). It is also evident that the acetylene reduction capacity of A. *microphylla* was also greater than either of two other species at all inoculum levels in the absence of light.

Little information is available towards the effect of *Azolla* crowding due to levels of inoculation on its growth and nitrogenase activity. However, the nitrogenase activity in the three *Azolla* species is associated with the presence of the blue-green alga *Anabaena Azollae* within the fronds of the host plant (Moore, 1969; and Ashton and Walmsley, 1976 and Ghazal and Herzalla, 1997). Rather low levels of acetylene reduced by each species at the start of the culture period could be due to the disturbance of the inoculum during transfer. A decrease in

nitrogenase activity had also has been noticed in *A. filiculoides* (Ashton and Walmsley, 1976)

Table (1): The influence of inoculum level on the acetylene reduction assay of *A. microphylla, A. filiculoides and A. pinnata*

Inoculums level (kg m^{-2})	Culture period (days)	µ.moles C_2H_4 gfw h^{-1}*		
		A. microphylla	*A. filiculoides*	*A. pinnata*
0.2	0	2.93 ± 0.53	1.57± 0.41	2.89 ± 1.22
	2	6.21± 2.34	3.24 ± 0.27	1.85 ± 0.56
	7	6.57 ± 0.30	4.60 ± 1.10	3.59 ± 0.73
	15	3.80 ± 2.27	3.25 ± 1.42	2.47 ± 0.22
	22	1.38 ± 0.15	1.09 ± 0.36	0.74 ± 0.11
	29	0.56 ± 0.09	0.23 ± 0.07	0.44 ± 0.09
1.0	0	2.42 ± 0.85	1.93 ± 0.94	2.82 ± 0.92
	2	5.28 ± 1.38	2.22 ± 0.52	1.69 ± 0.65
	7	4.06 ± 0.70	2.55 ± 0.50	2.50 ± 0.88
	15	3.00 ± 0.44	2.35 ± 0.74	1.22 ± 0.23
	22	1.01 ± 0.26	0.63 ± 0.03	0.32 ± 0.11
	29	0.75 ± 0.17	0.21± 0.12	0.17 ± 0.09
2.0	0	3.59.± 0.99	1.14 ± 0.42	2.59 ± 0.42
	2	4.17.± 0.84	2.04 ± 0.79	1.65 ± 0.36
	7	2.13 ± 0.24	1.31± 0.32	1.82 ± 0.45
	15	0.62 ± 0.09	0.94 ± 0.18	0.70 ± 0.13
	22	0.90 ±1.16	0.66 ± 0.18	0.49 ± 0.05
	29	0.35 ± 0.28	0.19 ± 0.10	0.37 ± 0.19

Mean of 3 replicates ± stander deviation

Table (1a): Observed F value derived from analysis of variance of acetylene reduction assay (nmoles/g fresh weight /h) of 3 *Azolla* species in response to inoculums levels

	F		
	ARA (0.2 Kg m^{-2})	ARA (1.0 Kg m^{-2})	ARA (2.0 Kg m^{-2})
Inoculums (Main plot)			
Level	18.63**	36.23**	30.57**
Species (sub-plot)	16.29**	20.23**	17.85**
Inoculums level x species	3.347 n.s	3.85 n.s.	5.35 n.s.

** Significant at 1% level; n.s. Not significant.

Table (1b): Observed F value derived from analysis of variance of acetylene reduction assay (µmoles/g fresh weight /h) of 3 *Azolla* species in response to time

	F					
	Day 0	**Day 2**	**Day 7**	**Day 15**	**Day 22**	**Day 29**
Time (Main plot)	**10.11****	**31.95****	**16.66****	**3.74***	**6.22***	**19.82****
Species (sub-plot)	**2.22 n.s.**	**3.17n.s**	**53.95****	**20.66****	**3.59n.s**	**7.15****
Time x species	**1.46**	**7.44****	**3.19**	**1.11**	**1.13**	**3.70****

** Significant at 1% level; * Significant at 5 % level; n.s. Not significant.

That the three *Azolla* species used in the experiments differ in their nitrogenase activity had been previously reported by (IRRI Annual Report, 1985). *A. microphyll*a, for instance, has higher acetylene reducing power than other species when exposed to different temperatures. Since the three species used were acclimatized under greenhouse conditions (temperature range 28-35°C and light intensity of 30-35 K lux) for two months before the experiment, as a requisite for studies (Tung and Watanabe, 1983), the observed inter-specific variations in nitrogenase activity could not be attributed to temperature or light intensity shock but would be more a reflection of differences in their innate capacity to reduce ethylene.

The better performance of *A. fihiculoides* when compared with *A. pinnata* in terms of ARA at all inoculum levels does not support the finding of Watanabe and Berja (1983) and Tung and Watanabe (1983). This discrepancy may be explained in terms of better growth of *A. fihiculoides* under the temperature and light conditions or ranges in the greenhouse as elicited by the ability of their stem to grow upward, which was not observed in *A. pinnata*. The onset of early senescence in *A. pinnata* in comparison to both other species may also have resulted to its low performance. That crowding as a result of the higher inoculurn levels of 1.0 and 2.0 Kgm^{-2} depressed nitrogenase activity was quite evident in the three species. Using a decline in nitrogenase activity as an index of senescence, it can be inferred from that crowding can induce earlier senescence in *Azolla*.

While, the peak of acetylene reduction occurred on the seventh day in the three species at an inoculurn level of 0.2 Kg m^{-2}, this was noticed after only two days in *A. microphylla* and five days in the other two species when the inoculum level' was higher at 1.0 and 2 Kgm^{-2}. At an inoculum level of 0.2 Kgm^{-2}, crowding, apparently occurs at a latter period, which in effect, would allow individual plants to develop better because of less competition for space, nutrients and light when compared with the extent conditions at 1.0 and 2.0 Kgm^{-2} inoculum levels. The onset of senescence, as indicated by frond discoloration, was recorded on the 15^{th} day in both *A. microphylla* and *A. filiculoides* when the

inoculum was only 0.2 Kgm^{-2} and as early as the 7^{th} day at inoculum levels of 1.0 and 2.0 Kgm^{-2}.

Table (2): The influence of light and dark conditions on acetylene reduction of *A. microphylla, A. filiculoides and A. pinnata* at different inoculum levels (nmoles C_2H_4/g fresh weight/hr.)

Inoculum level (kg m^{-2})	Culture period (days)	*A. microphylla*		*A. filiculoides*		*A. pinnata*	
		Light	Dark	Light	Dark	Light	Dark
0.2	0	1700	13.1	1720	8.5	1470	7.1
	5	4260	36.8	2420	14.8	3840	8.4
	12	2580	24.3	1820	14.0	1020	6.8
	18	2440	17.5	1170	8.3	841	3.4
	25	1440	11.0	386	5.7	336	2.2
1.0	0	1700	20.0	1620	13.7	1400	15.6
	5	5400	28.6	3030	11.20.	3660	16.6
	12	2620	19.1	1800	10.9	636	12.2
	18	1650	12.8	1000	7.8	486	8.6
	25	1210	8.2	358	4.9	240	5.2
2.0	0	1590	15.2	1430	10.7	1330	8.6
	5	3570	24.1	2610	14.0	2460	10.0
	12	1170	21.1	1070	13.7	397	7.2
	18	974	17.1	476	5.6	335	5.2
	25	636	12.9	233	3.0	88	2.1

For *A. pinnata*, frond discoloration was exhibited on day 10 for 0.2 Kgm^{-2} and on day 5 for the higher inoculurn levels. The higher acetylene reduction noticed when *Azolla* plants were exposed to light for three hrs before the assay would confirm the observation that nitrogenase activity is light dependent (Peters, 1975). Apparently, the energy or substrate needed for nitrogen fixation is provided by prior photosynthesis stored during the light period is utilized during the night or the dark period (Lex and Stewart, 1973 and Beceking, 1976). Hence, when the three *Azolla* species were previously exposed to 12 hrs darkness prior to dark incubation period of the three hrs assay, the levels of photosynthates might have decreased further in the dark to drastically reduce the available energy or substrate for nitrogenase activity. Bar et al. (1991) and Bar and Tel-Or (1994) postulated that nitrogenase activity decreased when either *Azolla* plants or the cyanobiont Anabaena were transferred from light to dark. This occurred immediately independent of the length or intensity of the preceding light phase.

Heterocyst frequency and N_2 fixing activity:

Heterocyst count was done simultaneously with the acetylene reduction assay. The 12th leaf counting from the tip was used in the estimation of heterocyst numbers since it had been reported to exhibit maximum nitrogenase activity (Hill, 1975). Only reddish cells that were enlarged and with thick yellowish cell wall, polar bodies, and homogenous cellular content were counted.

It is quite evident from (Fig. 1) that there is an apparent direct relationship between the number of heterocyst and the level of nitrogenase activity in the three species at an inoculum level of 0.2 kg m^{-2} and in *A. pinnata* at an inoculum level of 1.0 kg m^{-2}. In *A. microphylla* and *A. filiculoides* at an inoculum level of 1.0 kg m^{-2} and in all three *Azolla* species at 2.0 Kg m^{-2} inoculurn level, the number of heterocysts generally declined as the culture aged. The lowest heterocyst count in three *Azolla* species and at all inoculum levels was noticed after 25 days when the plants were rapidly senescing. As stated by Moore (1969) and Hill (1975), the heterocysts of *Anabaena Azollae* are responsible for the nitrogen fixing activity in *Azolla-Anabaena* symbiotic association.

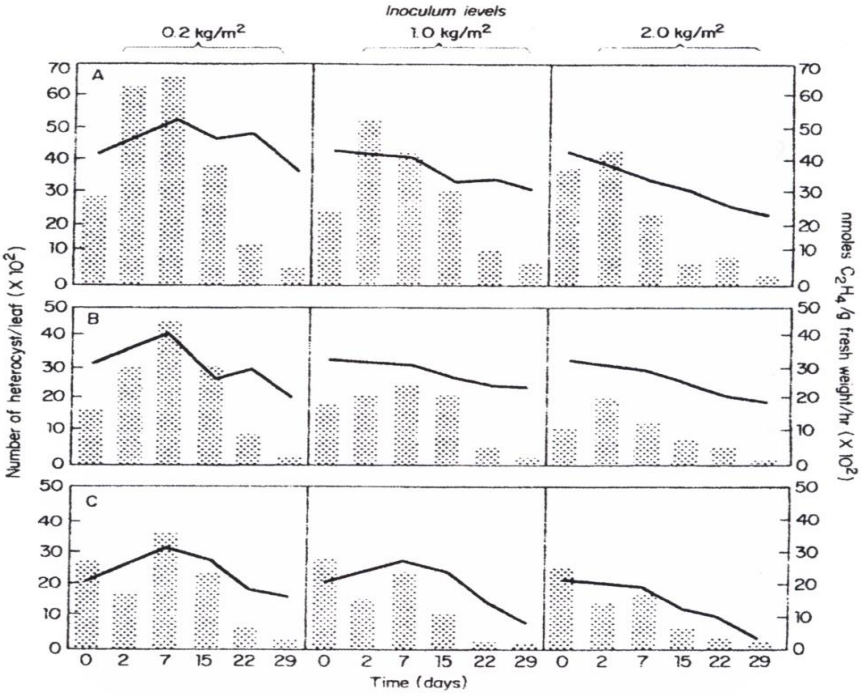

Figure (1): A correlation of the acetylene reduction assay (ARA) and heterocyst count of the three *Azolla* species at different inoculums levels (ARA ; heterocyst count --------; A- *A. microphylla*; B- *A. filiculoides*; C- *A. pinnata*

At an inoculum level of 0.2 kg m^{-2}, high heterocyst counts corresponded with high nitrogenase activity in the three *Azolla* species. At higher inoculum levels, heterocyst count declined as the cultures aged and failed to correlate directly with peaks of nitrogenase activity. This observation may reflect an influence of crowding on heterocyst differentiation and function since it has been reported that the peaks of nitrogenase activity are associated with recently different heterocysts (Fogg *et al*., 1973). Hence crowding as result of high inoculum levels would inhibit further heterocyst differentiation. When *Azolla* fronds were still green during the early culture stages, *Anabaena* filaments were extremely difficult to release or tease out from the frond cavities due to the presence of thick mucilage enclosing the filaments. But as frond discoloration set in, *A. microphylla* and *A. filiculoides*, the filaments were easily teased out and easily fragmentable due perhaps to the thinning out of the enclosing mucilage and the lysis of both vegetative and heterocyst cells as observed under the microscope. At the end of the culture period, the heterocysts appeared to be no longer functional as indicated by the highly vaculted or lysed yellowish vegetative and heterocyst cells in the filaments. In *A. pinnata*, however, the filaments were more difficult to release from senescing fronds because of the persistence of the enclosing thick mucilage and the presence of thin membrane partly coating the packed *Anabaena* filaments, which is referred to as algal packets (Uheda, 1986).

References

Ashton, P. J. and Walmsley. (1976). The aquatic fern *Azolla* and its *Anabaena* symbiont. *Endeavor*, **35:39-47**.

Bar, E.; Kulasooriya, A. and Tel-Or, E. (1991). Regulation of nitrogenase activity in the *Azolla-anabaen*a symbiosis. *Bioresource Technology*, **38: (2 and 3), 171-177**.

Bar, E. and Tel-Or, E. (1994). Effect of light and oxygen on nitrogenase and dinitrogenase reductase (Fe-protein) content in *Azolla-anabaena* association. *Journal of Plant Physiol*., **144: (4-5): 438-443**.

Becking, J. H. (1976). Contribution of plant-algal association In WE Newton, CJ Nyman, eds, Proceedings of the first international symposium on nitrogen fixation. Vol.2. Washington State University Press, Pullman **pp. 556-558**.

Cohn, M. and Thimann, K. (1972). The role of protein synthesis in the senescence of leaves. 1- The formation of protease. *Plant Physiol*., **49: 64-71**.

Chon, H. T. and Thimann, K. (1975). The metabolism of oat leaves during senescence. 3- The senescence of isolated chloroplast. *Plant Physiol*., **55: 828-834**.

Fogg, G. E.; Stewart, W. D. P.; Fay, P. and Walsby, A. E. (1973). The blue-green algae. 235, (Academic Press, London, New York).

Ghazal, F. M. and Herzalla, Nagat A. (1997). Effect of Senescence of *Azolla* fronds on nitrogenase activity and heterocyst frequency of the *Azolla-Anabaena Azolla* Symbioses. *Al-Azhar J Agric. Res*., **25, 98 – 114**.

Gomez, K. A. and Gomez, A. A. (1984). Statistical Procedures For Agricultural Research. 2nd ed. pp. **20-29 & 3 59-487**.

Hardy, R.F.; Burns R. C. and Hoisten, R. D. (1973). Application of the acetylene-ethylene assay for measurement of nitrogen - fixation. *Soil Biol. Biochem*., **5:47-81**.

Hill, D. J. (1975). The pattern of development of *Anabaena* in the *Azolla-Anabaena* symbiosis. *Planta*, **122: 179-184**.

International Rice Research Institute (IRRI) (1985). Nitrogen Fixation and *Azolla* Management. Annual Report, **P. 340-253**.

Lex, M. and Stewart, W. D. P. (1973). Algal nitrogenase reductant pools and photosystem I. activity. *Biochiem. Biophys. Acta*., **292: 436-443**.

Moore, A.W. (1969). *Azolla*: Biology and agronomic significance.Bot. Rev. 35 17-35. Munchen, F.R., Sheeny, J.E. and Witty, J.F.(1986). Further errorsin the acetylene reduction assay. Effect of plant disturbance. *J. Expt. Bot*., **37: 1581-1591**.

Peters, G. A. (1975). Studies on the *Azolla-Anabaena Azollae* relationship. III. Studies on metabolic capabilities and further characterization of the symbiont. *Arch. Micrbiol*., **103: 112-113**.

Peters, G. A. (1976). Studies on the *Azolla-Anabaena Azollae* symbiosis. In WE Newton, CJ Nyman, eds, Proceedings of the first international sympsoium on nitrogen fixation. Vol. 2. Washington State University Press, Pullman **pp. 295-6 10**.

Peters, G. A. and Mayne, B. C. (1974). *Azolla-Anabaena Azollae* relationship. I-Initial characterization on the association. *Plant Physiol*., **53, 813-819**.

Peters, G. A.; Evans, W. R and Toia, J. R. (1976). *Azolla-Anabaena Azollae* relationship. IV-Photosynthetically driven, nitrogenase catalyzed H2 production. *Plant Physiol*., **58: 118-126**.

Peters, G. A.; Robert, E.; Toia, J. R. and Samuel, M. L. (1977). *Azolla-Anabaena Azollae* relationship. V- $^{15}N_2$ Fixation, Acetylene reduction, and H_2 production. *Plant Physiol*., **59: 1021- 1025**.

Tung, H. F. and Watanabe, L. (1983). Accumulation of ammonium-N and Amino-N in the *Azolla-Anab*aena association. *Plant and Soil*, **73: 413-419**.

Uheda, F. (1986). Isolation of empty packet from *Anabaena*-free *Azolla*. *Plant Physiol*., **81: 1187-1 190**.

Yoshida, S.; Forno, D. A.; Cock, J. H. and Gomez, K.A. (1976). Laboratory Manual for Physiological studies of rice. The International Rice Research Institute, Manila, Philippines.

Vaishampayan, A. (1982). Cu-Fe interaction in the N_2 -fixing cyano bacterium *Nostoc muscorum*. *Microbios Letters*, **21: 17-23**.

Watanabe, I. and Berja, S. (1983). The growth of four species of *Azolla* as affected by temperature. *Aquatic Botany*, **15: 175-185**.

تأثير كثافة التلقيح لنباتات الأزولا على نشاط انزيم النيتروجينيز وأعداد خلايا الهيتيروسيست فى الـــ *Azolla-Anabaena azollae* symbiosis

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لقد أجريت تجربة تحت ظروف الصوبة وذلك لدراسة أثر كثافة أو معدل التلقيح لنباتات الأزولا على قدرتها على تثبيت النيتروجين الجوى وكذا أعداد خلايا الهيتيروسيست المسئولة عن تثبيت النيتروجين الجوى. وقد استخدمت فى هذه الدراسة ثلاثة سلالات من الأزولا هى *A. pinnata, A. filiculoides and A. microphylla* تحت ظروف الصوبة وبمعدلات تلقيح 0.2 , 1.0 , 2.0 كجم/ م^2.

وقد أوضحت النتائج مايلى:-

1- حدث اصفرارا واضحا لأوراق نباتات الأزولا فى اليوم السابع للنباتات الملقحة بمعدل 1.0 كجم/ م^2 و 2.0 كجم/م2 .

2- ظهر هذا الاصفرار بعد 15 يوما فى النباتات الملقحة بمعدل 0.2 كجم/ م^2 وذلك لكل من *A. filiculoides* و*A. Microphylla* أما فى حالة الـ *A. pinnata* فقد ظهر الاصفرار بعد 10 أيام عند معدل تلقيح 0.2 كجم/ م^2 وفى اليوم الخامس عند مستويات التلقيح الأعلى.

3- زاد نشاط انزيم النيتروجينيز عند اليوم السابع وذلك عند مستوى التلقيح الأقل ازدحاما (0.2 كجم/ م^2) فى كل سلالات الأزولا المستخدمة.

4- أدت مستويات التلقيح العالية (1,2 كجم/م^2) إلى انخفاض نشاط انزيم النيتروجينيز انخفاضا كبيرا وذلك فى الأسبوع الثالث.

5- أدى تحضين الأزولا فى الظلام الى انعدام نشاط انزيم النيتروجينيز تقريبا.

6- زادت أعداد الهيتروسيست لجميع السلالات عند اليوم السابع عند مستوى تلقيح 0.2 كجم / م^2.