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Propagation, taxonomy and ecophysiological characteristics of the *Azolla-Anabaena* symbiosis in freshwater habitats of Beni-Suef Governorate (Egypt).

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Abstract

A new ecotype of *Azolla* fern has established in freshwater habitats at Beni-Suef Governorate. The fern propagates in shallow irrigation canals and drains with low-velocity water. *Azolla* fern is circular (fan-like) in shape with a diameter of 2-3 cm; the surface of the fronds is covered with trichomes and contains stomata with annular guard cells and central pores. *Azolla* is a sterile hybrid, only forming microsporocarps, never megasporocarps: the microsporocarp contains about 64 microsporangia. Each microsporangium consists of 4 massula, each characterized by the presence of anchor-like multiseptate glochidia covering the whole surface of the massula. This ecotype probably belongs to *Azolla caroliniana* on the basis of vegetative and reproductive characteristics. Growth (biomass yield) is seasonal, with higher biomass (3.0-4.5 kg m^{-2}) and shorter doubling time in summer, reducing to about 1.0-1.5 kg m^{-2} in winter. *Azolla* plants accumulated the minerals Fe, Zn, Cu, Ni and Pb when growing in freshwater canals, drains and waste water. Salt stress treatment inhibited growth, nitrogen fixation and protein content. Iinhibitory levels of salinity were about 0.6-0.8 % NaCl. Growth and nitrogen fixation were inhibited at higher levels (2.4 mol m^{-3} and 5.1 mol m^{-3}) of combined nitrogen (ammonium sulphate and urea). The ecophysiological significance of *Azolla* in the freshwater habitats of Beni-Suef Governorate is discussed.

Introduction

Azolla plants were introduced into Egypt 20 years ago as a green manure. The high cost of mineral nitrogen has encouraged substitution of *Azolla*-nitrogen in rice fields (El-Bassel *et al*. 1994, Yanni *et al*. 1994). It is believed that at least five species of *Azolla* had invaded freshwater bodies of the northern governorates of the Nile Delta by 1995 (El-Hadidi & Fayed 1995), and the fern continued at a fast pace so that it became widespread to the extent that it is now to be observed floating on most stagnant water bodies of the drainage canals of the Nile Delta (Abd-Alla *et al*. 1994, Elhadidi & Fayed 1995, Yanni *et al*. 1994). In drainage canals a thick mat forms so that 2-5 kg of fresh *Azolla* can be collected up from a square meter of water surface (Yanni *et al*. 1994). However, this large biomass creates serious problems due to water loss by evapotranspiration and the mechanical obstruction of irrigation and drainage canals. An extensive survey of its occurrence in freshwater habitats of the Beni-Suef district started in 1994, when the fern was first observed. Farmers collect it to feed to their birds, and sometimes for other uses. *Azolla* forms thick mats which disrupt irrigation and clog pumps in summer.

The thick mats can cause changes which affect other organisms (Kroek *et al*. 1988): reducing relative light intensity to about 10 %, maximum dissolved oxygen concentration by 3-8 ppm, and pH by 1.4 points. Its geographic distribution clearly indicates that the genus is adapted to very wide-ranging temperature conditions. The optimum temperature for growth is between 20 and 30 °C. Some strains can tolerate frost, while others grow at temperatures higher than 45 °C (Lumpkin & Plucknett 1980, Watanabe & Berja 1983). *Azolla* plants are largely tolerant to pH variation, surviving between 3.5 to 10, and growing well between 4.5 to 8.0 (Nickell 1967). Salinity has rarely been mentioned as a problem in cultivation, but it is a factor which should be studied whenever the introduction of *Azolla* is being considered (Plazinski 1990).

Like most plants, *Azolla* is sensitive to changes and deficiencies in the supply of plant nutrients. The fern requires all the macro-and micronutrients essential for normal plant growth (Moore 1969). Macronutrients such as phosphorus, nitrogen, calcium, potassium and

magnesium are especially important, and produce marked effects on growth if present in too high or too low concentrations (Lumpkin & Plucknett 1980, Plazinski 1990, Yatazawa *et al*.1980). Micronutrients such as iron, cobalt and molybdenum, shown to be essential for the nitrogen-fixing process, must also be present in adequate supply in waters where the fern grows (Moore 1969, Plaziski 1990).

The most remarkable characteristic of *Azolla* is its symbiotic relationship with the nitrogen-fixing cyanobacterium *Anabaena azollae*. The agronomic importance of *Azolla* is related to its ability to grow very successfully in habitats where little or no combined nitrogen is available (Macale & Vlek 2004). Watanabe *et al*. (1977) estimated a daily rate of 1.1 kg N ha^{-1} day^{-1} as a potential capacity of *Azolla pinnata* nitrogen fixation in the field. The nitrogen fixation of the *Azolla-Anabaena* symbiosis is influenced to a large extent by various environmental factors, such as temperature, light, pH, salinity, and the nutrients iron and phosphorus (Kathiresan 2007, Lumpkin & Plucknett 1980, Petere *et al*. 1976, Wataanbe & Berja 1983).

Here the ecology, distribution and propagation of *Azolla* in the freshwater habitats of Beni-Suef district were studied. Vegetative and reproductive characteristics were examined for taxonomic characters, and the biomass yield determined as an indication of seasonal variation. Some other ecophysiological characteristics were also studied, such as the ability of the fern to take up minerals from fresh and waste waters. The relationship between *Azolla* growth, nitrogen fixation, salt stress and combined nitrogen was also studied.

Materials & Methods

Specimens of *Azolla* plants were collected from freshwater of an irrigation drain and kept in their natural water or in a fixative composed of 4 % glutaraldehyde in 0.1 M phosphate buffer, pH 7 for laboratory examination. Growth was determined as plant vigor, measured as the length of the fem and roots, and frond diameter. Biomass was measured as fresh weight of blot-dried plants, and oven-dried (85°C) material per surface area.

The internal structure of hand-cut sections of fresh *Azolla* plants was examined and photographed using Olympus Japan PK-10 AK microscopy equipped with a camera (C-35 AD-4). Sections in the dorsal and ventral sides of fronds were examined. The leaf cavities harbouring the endophyte cyanobacterium *Anabaena azollae*, and also the reproductive organs (microsporocarps) were examined by light microscopy. Specimens were prepared for scanning electron microscopy (SEM) examination following the method described by Lin & Watanabe (1988) with slight modifications. Samples in glutaraldehyde fixative were postfixed in 2% osmium tetroxide in the same buffer for 1-2 h, and dehydrated in an ethanol-water series. The fixed and dehydrated specimens were dried in a critical-point dryer (Tousimis Research Corporation, Rockville, MD, USA). Dried specimens were mounted on aluminium stubs with silver paste, coated with gold using an Ion Sputter (Jeol-JFC-1100E), and observed and photographed under scanning electron microscopy (JEM-100 S, Jeol, London, UK) at 40 kV.

In the field, water temperature and concentration of dissolved O_2 was measured monthly 10 cm from the surface in an irrigation drain under or near *Azolla* plants using an aquameter (Oxygen meter, N 5011 T, Electronic Works TEL-EKO, Poland). The pH was measured monthly using a combined pH electrode (Electronic Works TEL-EKO, Poland). Air temperature (day and night) was recorded in a meteorological station in the Beni-Suef district.

The concentration of the macro- (Ca, Na, Mg, K) and micro-elements (minerals Fe, Cu, Zn, Pb, Mn, Ni) were measured in fresh- and wastewater, following "Standard Methods 1985", by using an atomic-absorption spectrophotometer (Perkin-Elemer 403), and some were measured in plants as well. Available phosphorus was determined by the molybdenate-stannous chloride procedure according to Chapmann & Partt (1961). Protein content was

determined in oven-dried (85 °C) and ground plant material, following the method of Lowry *et al* (1951): bovine serum albumin was used as a protein standard.

Growth, nitrogen fixation and protein content were determined under salt-stress treatment. The *Azolla* plants were cultured in a nitrogen-free nutrient solution (Shiomi & Kitoh 1993) with the following composition (in mol m^{-3}): $CaCl_2 \cdot 2H_2O$ 1.0, $MgSO_4 \cdot 7H_2O$ 1.65, K_2SO_4 0.5, $NaH_2PO_4 \cdot 2H_2O$ 0.65, and in m mol m^{-3}, $FeSO_4 \cdot 7H_2O$ 27, $MnCl_2 \cdot 4H_2O$ 1.1, $CuSO_4 \cdot 5H_2O$ 0.08, $ZnSO_4 \cdot 7H_2O$ 0.19, $NaMoO_4 \cdot 2H_2O$ 0.05, H_3BO_3 5.8. The pH was adjusted to 7.0. The nutrient medium was supplemented with four levels (0.2 %, 0.4 %, 0.6 and 0.8 %) of NaCl, equivalent to about 35, 70, 100 and 120 mol m^{-3} NaCl. The salt-treated plants were compared with controls (no salt added to the medium). Five to ten plants (ca. 7 gms) were cultured in small (7-cm diameter) plastic pots, left at room temperature (26-28 °C) for up to 7 days. Ten replicates per treatment were kept in randomized blocks. Growth was measured by weighing blot-dried fresh plants harvested after 1 to 7 d of treatment.

The nitrogen-fixing activity (nitrogenase activity) of the *Azolla-Anabaena* symbiosis was determined by using the acetylene reduction assay according to the method described by Tung & Shen (1981). Plants were collected from pots after growth for 18 h or 7 days under salt treatment; young and mature plants (sporulated) were used. About 3-5 plants (ca. 1 gm) were placed in small serum bottles (30-ml capacity) fitted with subaseals to allow for addition and withdrawal of gas samples. Each bottle was injected with 3 ml acetylene (approximately equal to 10 % of the total gas volume) using a 5-ml plastic syringe. Before injection, 3 ml of the gas was withdrawn from each bottle in order to adjust the internal pressure of gases. The injected bottles were incubated for two hr at 27 °C, and then the reaction was terminated by injecting 2-3 ml of 6 N HC1 to each bottle. The gas was thoroughly mixed before assay. For the acetylene reduction assay, a 1-ml gas sample was withdrawn from each bottle and injected into an ATI Unicome 610 Series Gas Chromatograph with a flame ionization detector and a 5 ft. x 1/8 inch glass column of activated alumina (80-100 mesh) at a temperature of 150 °C. The carrier gas was nitrogen at a flow rate of 10 psi. The protein content was determined for plants cultured for 7 d under the same conditions of salt stress, using oven-dried (85 °C) plant material. Growth (fresh weight) and nitrogenase activity (acetylene reduction) were determined for plants cultivated under treatment with combined nitrogen (ammonium sulphate and urea) in nitrogen-free nutrient medium. Plants were cultivated in plastic pots under the same conditions as those used for the salt-stress experiment. Three levels (100, 200 and 300 ppm) equal to 0.8, 1.6, 2.4 mol m^{-3} (ammonium sulphate) and 1.7, 3.4, 5.1 mol m^{-3} (urea) were tested. The plants were left under treatment for 7 d.

Results & Discussion

Taxonomy: The ecotype of *Azolla* established in freshwater habitats of Beni-Suef Govemorate is fan-shaped (circular) in appearance with a diameter of about 2.5 cm. The length of the shoot system is about 2.3 cm and that of root about 3.7 cm. In bulk the fern forms mats 2-3 cms thick and the fronds are usually dark-green in color. The surface of the dorsal lobe has an epidermis covered with vertical rows of single-cell stomata and with two-celled trichomes (Fig. lA). The *Azolla* fem is unique in having a single annular guard cell surrounding each stoma, and the stomata have central pores (Konar & Kapoor 1972, Lumpkin & Plucknett 1980). The morphology of the individual plant and the type and distribution of hairs (trichomes) on the leaf surfaces and stems are important vegetational characteristics for identification (Lumpkin & Plucknett 1982, Perkins *et al*. 1985). In the dorsal lobe, the cavities containing the endophyte cyanobacterium symbiont (*Anabaena azollae*) were observed.

Anabaena grows as a filament or trichome (thread) of cells (Fig. 1B), unbranched and uniseriate. Two types of cells were recognized, the vegetative cells and the heterocysts (Fig. 1B).

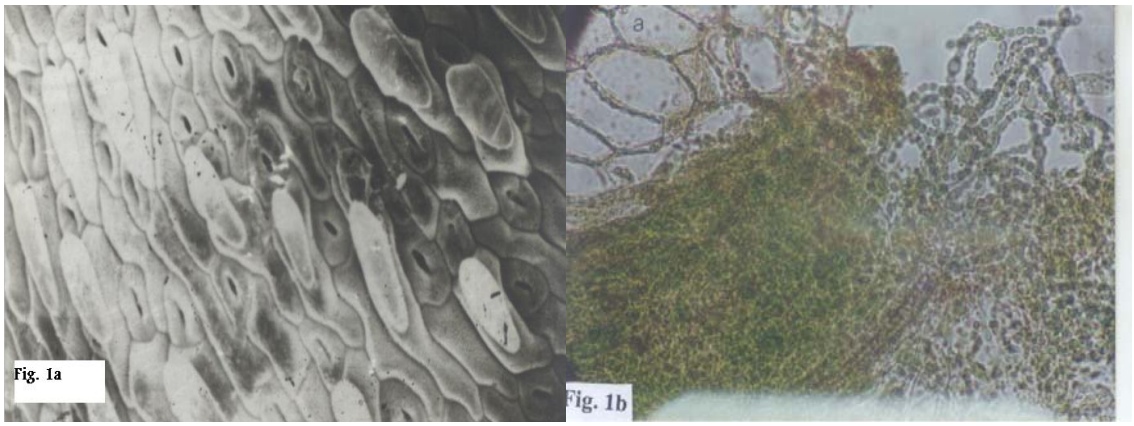

Fig. 1: (a) Scanning electron microscopy micrographs (X *350)* of the external morphology of *Azolla* fronds showing hairs (trichomes) and stomata with the annular guard cell and central pore. (b) Light microscopy micrograph (x1200) of the dorsal lobe of *Azolla* fronds showing the cavity harboring the cyanobacterium *Anabaena azollae.*

After 3 years of monitoring and examination, we conclude that this *Azolla* ecotype only forms microsporocarps, never megasporocarps. The globular microsporocarps occur in pairs on the ventral lobe, and contain about 64 microsporangia at maturity. Each microsporangium (Fig. 2A) consists of four massula (Fig. 2B), which in turn contain four microspores. The whole surface of the massulae (Fig. 2C) are covered with anchor-like glochidia characterized by the presence of 3-4 internal septa near to the tip (Fig.2D).

The formation of fruiting bodies (micro- or megasporocarps), and the presence or location of glochidia on the massulae of the microsporangium are important reproductive characteristics (Konar & Kapoor 1974). The vegetational and reproductive characteristics of the *Azolla* ecotype are basic criteria for classifying of the fern as one of the species of the section *Euazolla*, more particularly close to the species *Azolla caroliniana*, *A. mexicana*, and *A. microphylla*; it also shares a few characteristics with *A. filiculoides*. However, the absence of the megaspores and the presence of multiseptate glochidia suggests that the species is related to *A. caroliniana*, which also apparently never forms megasporocarps and thus is suggested to be a sterile hybrid (Tryon & Tryon 1982), since the massulae of these species are covered by multiseptate glochidia (Sevenson 1944). The frond morphology and other vegetative characteristics of the *Azolla* ecotype in this study are very similar to those of *A. mexicana* and *A. microphylla*, the two species also with multiseptate glochidia with a terminal anchor-shaped structure (Tan et al. 1986). Furthermore, this *Azolla* ecotype is found to propagate actively at temperatures between 30-37 °C, a character distinctive to *A. microphylla* (Tung & Watanabe 1983). The similarity between the *Azolla* ecotype here and each of *A. microphylla* and *A. mexicana* in some vegetative, reproductive and physiological characteristics suggests that our species may be a sterile hybrid of these two other species. Hybrids of *Azolla* have been reported to be sterile, forming only microsporocarps and showing new vegetative characteristics not present in the parents (Van Cat *et al*. 1989).

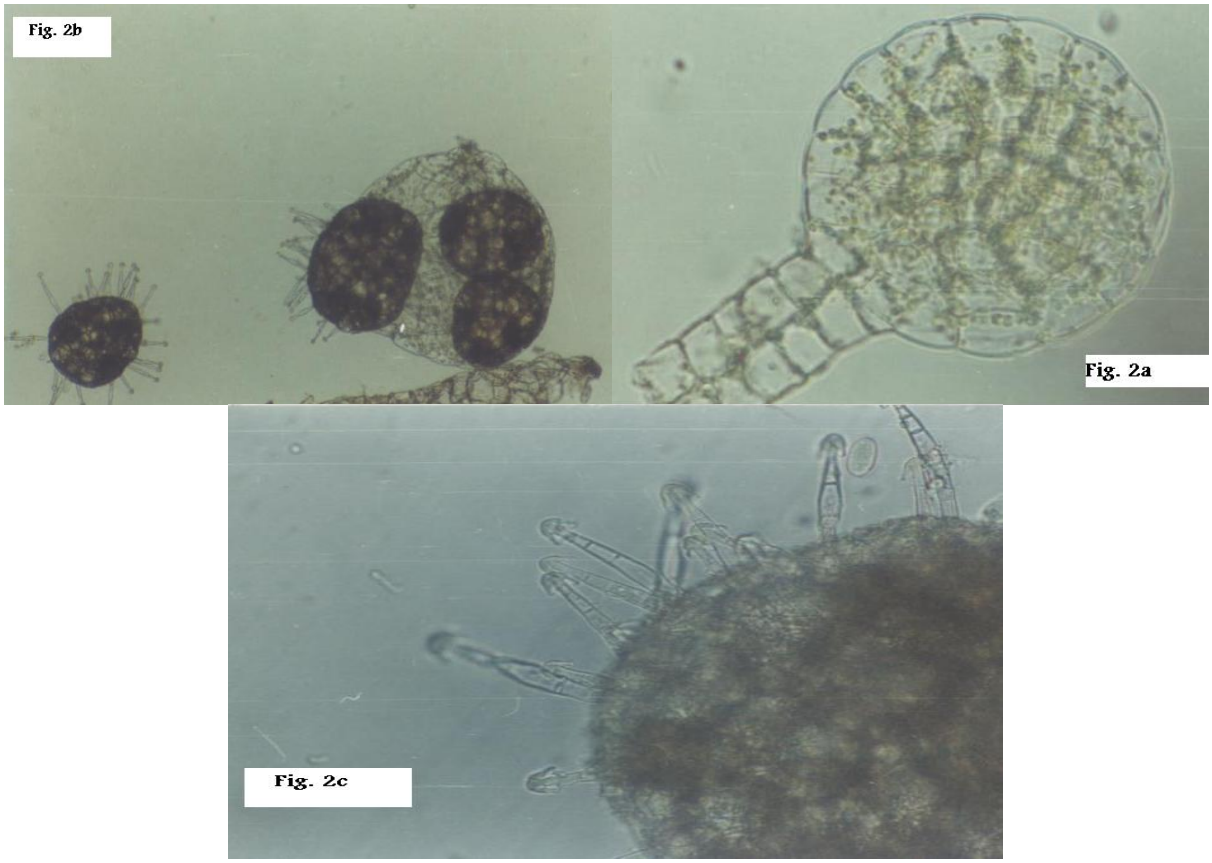

Fig. 2: Light microscopy micrographs (X 200) showing the reproductive structures of *Azolla,* (a) intact microsporangium containing four massula. (b) four massula released from the microsporangium (c) glochidia covered the whole surface of the massula (d) well-developed anchor-like shaped multiseptate glochidia (X 1200).

Biomass production: The *Azolla* plants yielded a biomass equal to about 1-4.5 kg fresh weight m^{-2} (Table 1). The biomass yield obviously had seasonal fluctuations: changes in temperature appear to be the most effective factor (Table 1). The doubling time of *Azolla* plants (data not shown) was about 5 and 6 days in spring and summer seasons, respectively. Propagation in winter was greatly reduced or completely ceased.

Seasonal variation may constitute a major factor affecting growth and biomass yield. It has been reported (Lumpkin & Plucknett 1982) that high temperature is considered a problem for the use of *Azolla* in the tropics, but temperature-tolerant strains of *Azolla* have been found among *A. microphylla* and *A. pinnata*; these plants have shown good growth for 6 weeks at 37/29 °C (Tung & Watanabe 1983). The ecotype examined in this study must therefore be temperature-tolerant.

Biomass production in *Azolla* is directly related to the rate of growth and nitrogen fixation of the *Azolla-Anabaena* symbiosis (Thung & Watanabe 1983). Biomass is usually influenced by a range of environmental factors, temperature and low phosphorus concentration being probably the most important. Since *Azolla* plants live in association with an endophyte cyanobacterium, tolerance to high temperature is determined both in the host fem and the symbiont *Anabaena* (Watanabe *et al*. 1989).

The diurnal O_2 cycle of water where *Azolla* grow was determined (Table 1). High biomass of *Azolla* appears to be related to high levels of dissolved O_2. However, the increase in O_2 concentration may be a direct result of the high rate of *Azolla* growth. The leaf cavity in the dorsal lobe of *Azolla* is surrounded by photosynthetic mesophyll that photoevolves O_2 in the light, thereby increasing the O_2 concentration in the environment (Mutuskin & Kolesnikov 1991). Therefore, *Azolla* plants may play an important role in the freshwater environment since

they change the levels of dissolved O_2, either increasing (as in this study) or decreasing it (by 3-8 ppm, as in a previous report: Krock *et al*. 1988).

Table 1. Monthly variation in biomass (fresh or dry weight m^{-2}) of *Azolla* plants growing naturally in an irrigation drain in relation to variation in temperature and dissolved oxygen.

Months	Biomass		Dissolved O_2(mg L^{-1})	Water Temp (°C)	Average Air Temp (°C)
	(Kg F.W. m^{-2})	(Kg D.W. m^{-2})			
January[a]	1.2 ± 0.08[b]	0.096 ± 0.007	8.0	17.0	12.7
February	1.4 ± 0.13	0.105 ± 0.007	10.0	20.0	14.9
March	1.0 ± 0.02	0.085 ± 0.007	9.5	21.5	17.3
April	3.7 ± 0.85	0.296 ± 0.028	7.5	27.0	20.6
May	3.1 ± 0.74	0.197 ± 0.016	8.0	27.5	27.2
June	4.0 ± 0.15	0.300 ± 0.007	10.0	28.0	28.1
July	3.2 ± 0.04	0.203 ± 0.003	7.1	30.7	29.1
August	3.8 ± 0.05	0.342 ± 0.004	9.5	32.0	29.4
September	4.2 ± 0.04	0.344 ± 0.003	8.5	28.0	28.6
October	4.5 ± 0.05	0.360 ± 0.005	10.0	27.0	23.2
November	2.1 ± 0.09	0.156 ± 0.009	9.6	21.5	19.7
December	2.6 ± 0.07	0.192 ± 0.007	8.5	19.5	14.0

a- Measurements were taken in the year 1996.
b- Mean ± SE, LSD 3.27 at the 5% level
c- Air temperature is an average of day and night temperatures.

Biomass production was determined together with the drainwater content of chlorides, carbonates, bicarbonates, sulphates, total soluble salts and pH (every month; data not shown). The higher biomass yield of *Azolla* in warm seasons coincided with higher total soluble salts (0.06-0.08 %), Cl^- (0.007-0.11 %), HCO_3^- (0.018-0.034 %), SO_4^{--} (0.020-0.027), and with high pH (7.37-8.01). *Azolla* was tolerant to different salts or ions (e.g. SO_4^{--}, HCO_3^- and Cl^-) in drain water, since the higher biomass yield coincided with the highest levels of these salts. High biomass yield and high concentration of anions and salts were in warm seasons, and the increase in salt and anion concentration may be attributed to the high evaporation rate in warm seasons. Higher concentrations of the cations Na^+ and K^+, and also of phosphorus were found in drain water (data not shown), with values ranging between 27-52 ppm, 7-8 ppm and 6-7 ppm, respectively.

The concentration of some minerals in the water was determined (Table 2). The minerals Cu, Fe and Zn were present in water at low concentrations, with the concentration of Ni being higher. With the exception of Cu, the *Azolla* plants accumulated high levels of the minerals in their tissues (Table 2), even though the concentrations of these elements were very low. Cu, Fe and Zn were increased greatly but variably in plant tissue by about 33 %, 99 % and 97 %, respectively. Rother & Whitton (1988) found no relationship between the concentration of elements in *Azolla* plants and those in water.

The higher biomass yield of *Azolla* plants in this study may be explained partly on the basis of higher mineral content. Low biomass production by some *Azolla* species (e.g. *A. pinnata*) has been attributed to phosphorus deficiency (Ali & Watanabe 1986, Arora *et al*. 2006), suggesting that the critical level of P in water which might affect growth is about 0.1 ppm. The P level here (6-7 ppm) therefore appears not to be a limiting factor for growth. On the other hand, *Azolla* plants may use the accumulated minerals as growth regulators, since it has been reported (Ali & Watanabe 1986) that aquatic plants grown in flooded soils may use the elements Zn, K, P, and Ca as "growth regulating" elements. These findings may explain why *Azolla* plants absorb high amounts of these minerals, significant because the fern is usually used as a green manure in many countries of the world. These minerals and also N (accumulated after symbiotic N_2 fixation) become available to other plants (e.g. rice in flooded

soils) only after *Azolla* mineralization. The rate of decomposition and mineralization of *Azolla* may affect its efficiency as a bio-fertilizer.

Table 2. Element analysis (%) of *Azolla* and freshwater (irrigation drain), where plants were grown.

Element	Fresh water (%)	Azolla plants (%)
Cu	0.003	0.004
Fe	0.004	1.156
Ni	0.138	0.022
Pb	---	0.029
Zn	0.003	0.112

The ability of *Azolla* plants to uptake minerals from waste water was determined by analysing waste water before and after cultivation of *Azolla* (Table 3). The Fe, Zn and P contents all decreased, whilst those of Cu and Mn was not changed. In a further experiment, the growth of *Azolla* in waste water decreased from 5 to 3.66 g after 7 d, whereas the same amount increased slightly to 5.16 g in N-free nutrient culture.

Current research in pollution control has been directed toward removing nutrients (e.g. N and P) and minerals (e.g. Zn, Ni, Cu, etc.) discharged into streams from sewage treatment plants (Shiomi & Kitoh 1987, Upadhyay *et al*. 2007). Various aquatic plants have been used to remove nutrients and minerals from contaminated freshwater and also waste water, among them *Eichhornia* (Cornwell *et al*. 1977), *Lemna* (Abdel-Hammeed 1993, Harvey & Fox 1973), *Ipomea* (Hashimoto 1983), and *Azolla* (Kitoh *et al*. 1993, Mishra *et al*. 2007). *Azolla* plants may need the absorbed minerals (e.g., iron) for growth and other activities, and it has been found (Rains & Talley 1979) that the critical iron concentration in water for *Azolla* growth is about 20 mg L^{-1}. The levels of iron determined in freshwater in this study was about 40 mg L^{-1} and waste water 90 mg L^{-1}. Although *Azolla* plants were tolerant to waste water treatment for one week, growth was reduced by about 30%. Waste or polluted water treatment was found to reduce growth and nitrogen fixation of *A. filiculoides*, and Kitoh *et al*. (1993) attributed the reduction in growth and nitrogen fixation to phosphorus deficiency. In this study, the concentration of phosphorus in waste water was about 0.650 %, and therefore was not limiting. The reduction in growth may be attributed to the accumulation of toxic minerals. The results underline that *Azolla* plants may be used to purify polluted water (fresh or waste) for recycling.

Table 3. Uptake of minerals from waste water by *Azolla* after one week of treatment.

Mineral	Control (%) (No plants)	Treatment (*Azolla* present) (%)
Cu	0.002	0.002
Fe	0.009	0.007
Mn	0.002	0.002
Ni	---	---
Zn	0.029	0.009
P	0.650	0.550

Effects of salt stress and combined nitrogen: Growth (measured as fresh weight) of *Azolla* was significantly inhibited by about 20-40 %, 35-55 %, and 50-63 % after one, two and three days of salt treatment, respectively (Table 4), with higher levels of salt (0.6 % and 0.8 %) being more inhibitory to growth than moderate levels (0.2 % and 0.4 %). The nitrogen-fixing activity (acetylene reduction) was also determined for under the same conditions of salinity. Nitrogenase activity increased after exposure to salt treatment for 18 h (data not shown), but long-term treatment with salt (7 d) significantly inhibited nitrogenase activity by about 90 % at 0.6 % NaCl (Table 5), and completely at 0.8 %. Young plants exhibited higher nitrogenase

activity than mature (sporulated) plants (Table 5). The effect of salt stress on growth and nitrogen fixation is reflected in their protein content; the salt stress treatments significantly inhibited the protein content in *Azolla* plants by up to 50 % (Table 5).

Table 4. Effect of salt stress on growth (Fresh weight) of *Azolla* grown in nitrogen – free nutrient solution.

Treatment Days	Fresh weight[a] (g) 1st	2nd	3rd	4th
Control (No salts)	7.3 ± 0.1[b]	7.3 ± 0.1	8.3 ± 0.2	5.9 ± 0.1
0.2 % (35 mol m^{-3})	7.1 ± 0.1	6.7 ± 0.1	6.5 ± 0.03	4.0 ± 0.02
0.4 % (70 mol m^{-3})	5.8 ± 0.1	4.6 ± 0.1	3.6 ± 0.2	2.7 ± 0.1
0.6 % (100 mol m^{-3})	4.7 ± 0.1	3.3 ± 0.4	2.9 ± 0.07	1.6 ± 0.1
0.8 % (120molm^{-3})	4.6 ± 0.3	3.1 ± 0.04	2.8 ± 0.1	1.3 ± 0.3
LSD at 5 %	0.50	0.96	0.59	0.74
LSD at 1 %	0.71	1.37	0.85	1.05

a - Initial fresh weight was about 7 grams
b - Mean ± SE

Table 5. Effect of salt stress on nitrogenase activity (acetylene reduction) and protein content of the *Azolla-Anabaena* symbiosis grown in nitrogen-free nutrient solution for 5 days.

Measurement	Salt stress (% NaCl) 0.0	0.2	0.4	0.6	0.8	L.S.D. at 5 %	1%
Acetylene reduction (nmoles C_2H_4 g fresh $weight^{-1}$ hr^{-1})							
Young Plants	790±87[a]	50 ±7.9	35±11.4	24±3.3	00	144	21.4
Mature Plants	253±9.9	16±2.3	13± 4.5	00	00	209	32.5
Protein Content (%)[b]	32±2.6	27±0.95	20±1.3	16±0.3	Nd	10	15.4

a- Mean ± SE
b- Protein content determined after 7 days of salt treatment, (Nd) Not determined

The plants here were able to withstand levels of salt higher than those causing significant reductions in other *Azolla* species. For example, Herz-Alla (1991) found that growth of *A. pinnata* was reduced after treatment with NaCl (0.10 %) and Na_2 SO_4 (0.15 %). Similarly, Moore (1969) reported that *Azolla* plants died in rice fields when salt concentrations reached 0.15 - 0.19 % during the summer, and FAO (1978) suggest that the maximum salt level for growing *Azolla* is 0.10 %. It appears therefore that the *Azolla* ecotype under investigation is salt-tolerant. This might explain its successful growth in drain water, which normally contains up to 0.10 %. Nitrogen fixation of the *Azolla-Anabaena* symbiosis is also clearly sensitive to salt stress, also reported (Herz-Alla 1991) in *A. pinnata*. The N content of *A. filiculoides* was reduced when salt concentration was increased from 0.3 % to 0.9 % (Geshian *et al*. 1980). The sensitivity of growth and nitrogen fixation to salt stress may be attributed to Na^+ toxicity, because Rahoma (1985) found that the total N content of *Azolla* increased with increasing dilution of drainage water to reduce Na^+ concentration.

The salt-tolerance of *Azolla* plants was studied further by testing the ability of the fern to absorb nutrients or salts from saline water and salty soil (data not shown). When grown in nutrient solution containing about 0.6 % NaCl, the plants were able to remove about 24 % of salts after 5 d of growth. When *Azolla* plants were cultivated in salty soils with salinity up to 1.8% and 3.3 %, the fern managed to withstand the first soil (1.8 % salinity) for one week and recovered about 27 % of salts from the soil, but plants in the highly saline soil (3.3 % salt) died after 2 days. These results show that the *Azolla* ecotype has better salt tolerance and ability to withstand saline environments than other *Azolla* species. Haller *et al*. (1974) found that *Azolla*

plants died when cultured in soil containing about 1.3% salts (about 33 % of sea water). Other species of *Azolla* reduced water salt content by about 0.012-0.049 % and soil salt content by about 0.014-0.485 % (see Lumpkin & Plucknutt 1980). The ecotype of *Azolla* under investigation may be used to purify salt-contaminated water and soil, and we suggest that it can be cultivated in habitats with moderate salinity (e.g. irrigation drains, flooded rice fields).

The growth and nitrogenase activity of the *Azolla-Anabaena* symbiosis were determined for plants grown in N-free nutrient solution in the presence of ammonium sulphate and urea (Table 6). Generally, growth was reduced as the concentration of combined nitrogen increased. The application of ammonium sulphate and urea at 100 ppm reduced growth slightly, but higher levels inhibited growth by up to 42%. Nitrogenase activity was significantly reduced as the concentration of combined nitrogen increased. *Azolla* failed completely to fix nitrogen at higher levels of combined nitrogen. The nitrogen-fixing *Azolla-Anabaena* symbiosis is much more tolerant to combined nitrogen in the medium than free-living *Anabaena* (Ito & Watanabe 1983). In symbiosis the cyanobacterium is enclosed in a leaf cavity, and therefore is not in direct contact with exogenous growth medium (Kitoh & Shiomi 1995). The presence of ammonium in the medium, however, is unfavourable for growth and nitrogen fixation, except when the concentrations are very low (ca. 10 mol m^{-3}: Kitoh & Shiomi 1991, Meeks *et al*. 1987, Shiomi & Kitoh 1993). *Azolla* plants completely lost their nitrogen-fixing ability and practically ceased to grow in a medium containing 20 mol m^{-3} ammonia (Kitoh & Shiomi 1995). In this investigation, the *Azolla-Anahaena* symbiosis is tolerant to treatment with ammonium sulphate and urea, presumably because the concentrations used were slightly lower (about 2.4 and 5.1 mol m^{-3}, respectively). *Azolla* species may vary in their tolerance to combined nitrogen: tolerant strains of *A. caroliniana*, *A. mexicana*, *A. microphylla* and *A. pinnata* have been selected (Kitoh & Shiomi 1995). The ecotype of *Azolla* studied here can be considered as tolerant to combined nitrogen; the levels used might equal the levels of nitrogen fertilizers sometimes added to rice fields.

Table 6. Effect of combined nitrogen (ammonium sulphate and urea) on growth (gm fresh weight) and nitrogenase activity (acetylene reduction, n moles C_2H_4 / g fresh weight / hr) of *Azolla* after one week of treatment in nitrogen - free culture medium.

Treatment	Combined nitrogen (ppm)				LSD	
	Control	100	200	300	At 5 %	At 1 %
Growth (gram fresh weight)						
Ammonium Sulphate	1.9±0.11[a]	1.7±0.05	1.4±0.05	1.3±0.26	None	None
Urea	1.9±0.11[b]	1.6±0.15	1.3±0.11	1.1±0.05	0.37	0.54
Nitrogenase activity						
Ammonium sulphate[c]	463±3.59	220±10.2	83±7.80	None	26.65	40.40
Urea	463±3.59	150±6.27	66±2.04	None	9.79	4.84

a- Mean ± SE.
b- Initial fresh weight was two grams.
c- Molar concentrations of ammonium are 0.8, 1.6 and 2.4 mol m^{-3}, and of urea 1.7, 3.4 and 5.1 mol m^{-3}, which correspond to 100, 200, and 300 ppm, respectively.

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الملخص العربى

انتشار و تصنيف و الصفات البيئيه الفسيولوجيه لتكافل أزولا – أنابينا فى بيئات المياه العذبه بمحافظه بنى سويف (مصر).

نوع جديد من سرخس الأزولا و جد و استقر فى بيئه المياه العذبه بمحافظه بنى سويف. لقد انتشر السرخس فى قنوات الرى الضحله و المصارف ذات سرعه المياه البطيئه.الأزولا عبارة عن سرخس دائرى (يشبه المروحه) الشكل و قطره يتراوح بين 2-3 سم. سطح الاوراق مغطى بشعيرات و يحتوى على ثغور بخلايا حارثه حلقيه و ثقب وسطى. الأزولا تحت الدراسع هى نوع عقيم, يكون الجراثيم الذكريه فقط و لم يلاحظ ابدا تكوين الجراثيم الأنثويه, تحتوى الحوصله الجرثوميه الذكريه على 64 جرثومه صغيره. كل جرثومه صغيرة تتكون من 4 ماسيولا و التى تتميز بوجود زوائد سهميه الشكل مجزأة. هذا النوع اقترح انه يخص أزولا كالوريانا على اساس الصفات الخضريه و التناسلية. لقد وجد أن النمو يتأثر بالتغيرات الموسميه. لقد أعطى السرخس أعلى انتاجيه (حوى 3-4.5 كجم/مP^{2-}) و أقصر زمن للتضاعف فى الصيف. فى فصل الشتاء كان انتاج السرخس يتراوح بين 1-1.5 كجم/ م-2 فقط. نبات الأزولا قام بتجميع كميات عاليه من المعادن Fe, Zn, Cu, Ni و Pb عند نموه بقنوات المياه العذبه و المصارف و المياه الراكدة. لقد تثبط النمو و تثبيت النيتلروجين و محتوى البروتين عند معاملته بالاملاح. و مستوى التأثير ظهر عند 0.6-0.8 % NaCl . و تأثر النمو و تثبيت النيتروجين عند مستويات عاليه (2.4 و 5.1 مول م-3) للنيتروجين المرتبط (كبريتات الامونيا و اليوريا). و لقد تم مناقشه الاهميه البيئيه للأزولا الموجودة فى بيئات المياه العذبه بمحافظه بنى سويف.