Aquatic Botany 106 (2013) 20–28

Contents lists available at SciVerse ScienceDirect

Aquatic Botany

journal homepage: www.elsevier.com/locate/aquabot

Responses of the *Azolla filiculoides* Stras.–*Anabaena azollae* Lam. association to elevated sodium chloride concentrations: Amino acids as indicators for salt stress and tipping point

Monique M.L. van Kempen[a,*], Alfons J.P. Smolders[a,b], Gerard M. Bögemann[c], Leon L.M. Lamers[a], Eric J.W. Visser[c], Jan G.M. Roelofs[a]

[a] Radboud University, Faculty of Science, Department of Aquatic Ecology and Environmental Biology, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
[b] B-WARE Research Centre, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands
[c] Radboud University, Faculty of Science, Department of Experimental Plant Ecology, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands

ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form
15 December 2012
Accepted 21 December 2012
Available online 14 January 2013

Keywords:
Azolla filiculoides
Anabaena azollae
Amino acid
Proline
Asparagine
Gamma amino butyric acid
Glutamine
Glutamate
Salt stress
Tipping point in salt tolerance
Eocene *Azolla* event

ABSTRACT

The water fern genus *Azolla* has been found in marine sediments, showing that ancestral species grew in marine ecosystems during the Eocene. Modern *Azolla* species, however, only live in freshwater. Besides aiming to elucidate experimentally the conditions that prevailed over the Arctic Ocean and adjacent Nordic seas during the Eocene, studies on tolerance of these plants to salt stress have applied potential, as *Azolla* may be used as a fertilizer, due to its symbiosis with N_2-fixing *Anabaena* cyanobacteria, and grown on salt-infiltrated agricultural fields. Here, the response of a temperate *Azolla filiculoides–Anabaena azollae* association to a wide salinity gradient (0–210 mM NaCl) was studied by measuring growth, nutrient content, nitrogenase activity and the accumulation of free amino acids in the association. The association was able to grow at salt concentrations up to 90 mM NaCl and appeared to acclimate in this range, but only after a period of 75 days. At concentrations exceeding 120 mM NaCl, however, roots were shed and impairment of water and nutrient uptake (including dinitrogen fixation) resulted in die off. Increased Na concentrations in the plants grown at external salt concentrations of 30 mM NaCl were to some extent reverted by decreased K concentrations, suggesting that K in the plants was replaced by Na as plant growth was not affected. Absolute nutrient concentrations in plant tissue were not correlated with plant growth and therefore not suitable as reliable indicators for salt stress. However, significant increases in the free amino acids proline and glutamate and significant decreases in asparagine, glutamine and gamma-amino-butyric-acid were found with increasing salinity. The constitutively high glutamine concentrations in the plants grown up to 90 mM NaCl may have contributed to the salt tolerance of the plants by providing osmotic adjustment. Proline concentrations and glutamine/glutamate ratios proved to be strong linear indicators for the level of salt stress and can therefore be used to predict the tipping point concentration. The salt resistance limit of 90 mM NaCl corresponds well to climate model predictions of salinity levels of ancestral habitats of *Azolla* in the Eocene Arctic Ocean.

1. Introduction

Azolla represents a free floating plant genus belonging to the Pteridophyta with a global distribution. Distinctive for *Azolla* is its symbiosis with the dinitrogen fixing cyanobacterium *Anabaena azollae* that lives inside the leaf cavities of the plant. *An. azollae* provides *Azolla* with ammonium, whereas *Azolla* provides the cyanobacterium with a fixed source of carbon (Peters and Mayne, 1974a, 1974b). Thanks to this symbiosis *Azolla* is usually very rich in nitrogen and therefore particularly well known for its use as a nitrogen fertilizer in rice paddies (Moore, 1969; Lumpkin and Plucknett, 1980, 1982; Wagner, 1997; Mandal et al., 1999; Hove and Lejeune, 2002). In addition, *Azolla* is widely used as a phytoremediation tool for contaminated surface water (Wagner, 1997; Rai, 2007; Ferdoushi et al., 2008) and as animal feed (Khatun et al., 1999; Abou et al., 2007; Leterme et al., 2009).

Present day *Azolla* spp. are only known from freshwater environments and can thus be expected to be sensitive to elevated salt concentrations. Ancestral *Azolla* spp. are, however, known to have also been associated with marine environments (Bujak and Mudge, 1994; Eldrett et al., 2004; Brinkhuis et al., 2006). Species such as *Azolla arctica* (Collinson et al., 2009) and *Azolla jutlandica* (Collinson et al., 2010) grew and reproduced in the central Arctic Ocean and

* Corresponding author. Tel.: +31 243652860; fax: +31 243652409.
E-mail addresses: moniquevankempen@home.nl, m.vankempen@science.ru.nl (M.M.L. van Kempen).

0304-3770/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquabot.2012.12.006

adjacent Nordic seas during the Eocene, some 50 million years ago. It has been suggested that the massive growth of these *Azolla* spp. significantly contributed in tilting the world's climate from greenhouse to icehouse via massive drawdown of atmospheric CO_2 (Brinkhuis et al., 2006; Speelman et al., 2009). Although the onset and termination of the *Azolla* blooms were mainly determined by changes in surface water salinities, the highest spore abundances occurring at times where the top surface water salinity was relatively low (Brinkhuis et al., 2006; Stickley et al., 2008), it is still very likely that the genus must have shown some degree of salinity tolerance.

Growth responses of *Azolla* spp. in relation to salt stress have been studied in considerable detail, mainly for subtropical and tropical species and strains. The interest in this topic largely originates from the fact that the utilization of *Azolla* in agriculture is increasingly affected by salinization, particularly in arid and semi-arid regions where irrigation is a contributory factor. The salt resistance limit of *Azolla* spp. roughly varies between 40 mM and 220 mM salt (Haller et al., 1974; Deng-hui et al., 1987; Johnson, 1986; Rai and Rai, 1999; Arora and Singh, 2003; Masood et al., 2006; Rai et al., 2006). It has been shown that in the lower ranges salt tolerance can be increased by pre-incubation at non-lethal salt concentrations (Abraham and Dhar, 2010; Rai et al., 2001; Rai and Rai, 2003). Next to phenotypical differences among *Azolla* spp. and their different strains (Johnson, 1986), this may partly explain the considerable variation that has been found in the salinity tolerance of the species, although differences in experimental design might also have played a significant role (Grattan and Grieve, 1992; Marschner, 1995; Munns and Tester, 2008).

Generally, the growth reduction that salt sensitive plants experience after exposure to elevated salt concentrations results from immediate osmotic stress due to a disrupted water uptake, often followed by ionic stress due to the accumulation of inorganic ions in the plant cells up to concentrations that are inhibitory to many plant physiological processes (Munns and Tester, 2008). The degree to which algae and plants are able to overcome such stress depends on their ability to sequester the ions in vacuoles and accumulate compatible osmotic compounds in the cytoplasm, including carbohydrates, polyhydric alcohols and amino acids and/or related soluble nitrogenous compounds (Flowers et al., 1977; Mansour, 2000; Bouché and Fromm, 2004; Burg and Ferraris, 2008). These compounds may not only contribute to the osmotic adjustment but also stabilize membranes and proteins and act as free-radical scavengers (Burg and Ferraris, 2008; Munns and Tester, 2008).

So far, studies addressing physiological changes in *Azolla* spp. subjected to salt stress have only been carried out using subtropical and tropical varieties of the species (Rai and Rai, 1999; Masood et al., 2006; Rai et al., 2006; Abraham, 2010; Abraham and Dhar, 2010; Singh et al., 2010). Here we studied a temperate *Az. filiculoides–An. azollae* strain grown at different salt concentrations (up to 210 mM NaCl) in a laboratory experiment by measuring the growth and examining the nutrient concentrations in the plants under the given conditions. *Az. filiculoides* is the northernmost occurring *Azolla* species (Lumpkin and Plucknett, 1980). Maritime climate, like in the Netherlands, may temper winter periods and incline salt influences in freshwater systems near the coast. As *Azolla* is rich in nitrogen compounds due to its symbiosis, we hypothesized that the accumulation of free amino acids is a likely strategy to implement as an adaptive response to salinity stress. We therefore studied the free amino acid composition, as well as the nitrogenase activity in the *Az. filiculoides–An. azollae* association, in relation to salt stress. We grew the association in a nitrogen free solution because glutamate and glutamine, which are the first essential amino acids in the nitrogen metabolism of the symbiosis, are then initially derived from the activity of the cyanobiont that fixes atmospheric dinitrogen. As a whole, the present study will generate a broader knowledge about the salt stress responses of the genus and about the physiological and biochemical advantages of the symbiosis in temperate brackish water systems, including novel indicators for salt stress.

2. Materials and methods

2.1. Experimental design

Azolla filiculoides, including the *An. azollae*, was collected from a field location in the Netherlands (N51°55′48″; E5°50′6″) and cultivated in the laboratory for approximately 4 months in a nutrient solution which was based on water quality data obtained in a field survey by de Lyon and Roelofs (1986) on the distribution of *Az. filiculoides*, among other water plants in the Netherlands. The solution contained 1.75 mM $NaHCO_3$, 1.75 mM $CaCl_2$, 25 μM NaH_2PO_4, 1 mM K_2SO_4, 1 mM $MgSO_4$, 10 μM Fe-EDTA, 1 μM $CuSO_4$, 20 μM $MnCl_2$, 10 μM $ZnSO_4$, 3 μM Na_2MoO_4, 20 μM H_3BO_3 and 4 μM $CoCl_2$ (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands) and was kept free of nitrogen. It was refreshed weekly. Containers with *Az. filiculoides* were placed in a water bath at 16 °C at an air temperature of 25 °C. The light regime was set to 16 h day and the light intensity at vegetation level was 230 μmol m^{-2} s^{-1} PAR (Quantum sensor, Skye Instruments Ltd., Wales, England), provided by six 400 W high pressure sodium lamps (Hortilux-Schréder, Monster, The Netherlands).

Az. filiculoides, including the *An. azollae*, was grown at 7 different salt concentrations and a control for 101 days. The different treatments were established by adding 0, 30, 60, 90, 120, 150, 180 or 210 mM NaCl to the nutrient solution using a 5 M NaCl (Sigma–Aldrich Chemie B.V., Zwijndrecht, The Netherlands) stock solution. Each treatment was replicated 3 times. The plants were grown in 6 L glass aquaria (25 cm length; 12.5 cm width; 30 cm height) which were randomly placed in the water bath to minimize the effect of microclimate differences. The underwater parts of the aquaria were kept dark using light impermeable foil to avoid light penetration from the sides. At the start of the experiment 4 g of fresh *Az. filiculoides* was introduced in each aquarium, covering approximately a quarter of the water surface.

2.2. Growth response measurements

Total fresh biomass in each aquarium was determined once a week and subsamples were taken for fresh weight and dry weight measurements. Fresh weight was determined by taking the plants out of the aquaria and carefully blotting them dry on tissue paper before weighing. The dry weight samples were washed in a sieve for 1 min with running demineralized water to remove adhering ions and then put in paper bags in a stove for 48 h at 70 °C.

After each harvest 4 g of fresh *Azolla* was returned into a cleaned aquarium containing new nutrient solution that was adjusted to the right NaCl concentration. When less than 4 g of fresh biomass was available, only a small subsample was harvested and the rest of the biomass was put back into the aquarium. Because biomass declined in the highest salt treatments (>120 mM NaCl), the starting biomasses for each sampling period differed among treatments. The relative growth rates were calculated on a dry weight basis to eliminate growth differences that may have arisen from the initial size differences: $RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$, where W_1 and W_2 represent the dry biomass at the beginning (t_1) and end (t_2) of the sampling period. Due to continuous low biomass production rates, the 150, 180 and 210 mM NaCl treatments had to be terminated after 48, 27 and 19 days respectively.

2.3. Plant nutrient analyses

Dried plant material was ground in liquid nitrogen after which 200 mg of the plant material was digested in 4 mL HNO_3 (65%) and 1 mL H_2O_2 (35%), using an Ethos D microwave (Milestone, Sorisole Lombardy, Italy) (Kingston and Haswell, 1997). Since for most species sodium appears to reach toxic concentrations before chloride does (Munns and Tester, 2008), in this report we focussed on the accumulation of sodium (Na) in the plant tissue. In addition to Na, the digestives were analyzed for elemental potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) concentrations using an inductively coupled plasma emission spectrophotometer (ICP-OES, model IRIS Intrepid II XDL, Thermo Fisher Scientific). For the analyses of total nitrogen (N) concentrations, the ground plant material was further homogenized using a ball mill (type Mixer Mill 301, Retsch GmbH, Germany). Ca. 2 mg of the plant material was weighed in pressed, ultra-light-weight tin capsules which were analyzed by an elemental CNS analyzer (EA 1110, Carlo Erba; Thermo Fisher Scientific). Both in the elemental composition analyses and in the plant total nitrogen analyses standard references were included. The plant total nitrogen concentrations could not be determined for the plants grown at the three highest salt concentrations due to the low biomass production.

2.4. Amino acid analysis

At the start and end of the experiment fresh plant biomass was collected for the purpose of amino acid analysis according to the method described by van Dijk and Roelofs (1988). Samples were stored in plastic bags at −80 °C until analysis. Amino acids were quantified by measuring fluorescence after pre-column derivation with 9-fluorenylmethyl-chloroformate (FMOC-Cl) and using HPLC (Star 9050 variable wavelength UV-VIS and Star 9070 fluorescence detector; Varian Liquid Chromatography, Palo Alto, USA) with norleucine as an internal standard. The plant material of the 120 mM NaCl treatment had to be pooled at the end of the experiment due to low biomass production, thereby leaving only 2 replicates. The amino acid analysis method did not allow us to measure glycine betaine (Rhodes and Hanson, 1993), which besides proline has been recognized as a compatible compound abundant in higher plants. However, the method did allow us to measure serine and ethanolamine, which are essential precursors in the biosynthesis of glycine betaine (Hitz et al., 1981; Rhodes and Hanson, 1993; Nuccio et al., 1998; Waditee et al., 2007).

2.5. Nitrogenase activity of the Az. filiculoides–An. azollae association

Nitrogenase activity was measured in duplicate using an acetylene reduction assay (Hardy et al., 1968) as described by Arora and Singh (2003) after 9 days. The activities were first averaged per replicate, after which the mean was calculated per treatment.

2.6. Nutrient composition analysis of the nutrient solution

The nutrient solution in each aquarium was refreshed once a week at the event of harvesting to keep the culture free of algae and to prevent nutrient depletion. Before refreshing the nutrient solution, samples were taken from the aquaria using 30 mL acid rinsed glass bottles, to determine the nutrient concentrations. Total concentrations of Al, Ca, Fe, K, Mg, Mn, Na, P, S, Si and Zn were determined using inductively coupled plasma emission spectrometry (ICP-OES), using standard references. During the experiment all nutrients in the solution were present in adequate amounts (data not shown).

2.7. Statistical analysis

Mixed Linear Models (MLM) in SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA) were used to analyze the growth responses and the nutrient concentrations in the plants. Time was specified as a repeated variable with plant as the subject variable to correct for the correlated residuals within the random effects. The repeated co-variance type was specified based on the smallest −2 Restricted Log Likelihood. Data were analyzed considering treatments effects, time effects and time/treatment interactions in order to study whether there was plant adaptation. As the absolute nutrient concentrations in the plant tissue were not correlated with plant growth it was decided to analyze the nutrient concentrations solely for treatment effects. Pairwise comparisons were made based on the estimated marginal means to determine the differences between the treatments and were adjusted for multiple comparisons using Bonferroni, the mean difference being significant at the 0.05 level. Missing data from the 150, 180 and 210 mM treatments were indicated as such, except in the analysis of the plant nitrogen concentrations, because these were missing from the start. Care was taken that the model's assumptions were met. For this purpose the plant sodium, calcium and magnesium concentrations were log transformed for the analyses.

Analyses of variances (ANOVA) were used to analyze the differences among the nitrogenase activities and the amino acid compositions of the association at the end of the experiment. Differences between treatments were determined using Bonferroni post hoc analyses. The missing replicate in the 120 mM NaCl treatment in the amino acid analysis was specified as such, whereas the three highest salt treatments were excluded because the plants had died before the end of the experiment. Care was taken that the model's assumptions were met. For this purpose total amino acid concentrations, glycine, serine, asparagine, glutamine, aspartate, glutamate, gamma amino butyric acid (GABA) and ethanolamine data were $\log(1+X)$ transformed.

3. Results

3.1. Growth responses

The relative growth rates (RGRs) of the *Az. filiculoides–An. azollae* association initially decreased with increasing NaCl in the nutrient solution (Fig. 1A, Table 1). During the experiment, however, the RGRs of the plants grown in treatments up to 90 mM NaCl became equally high (Fig 1A, Table 1). In the 120 mM NaCl treatment the RGR was highly variable, but overall, it was significantly lower than in the plants grown at lower salt concentrations. This was also true for the three highest salt treatments where RGRs were generally negative (Fig. 1A, Table 1). In the three highest salt concentrations (≥150 mM NaCl), a large part of the plants became necrotic and subsequently sank to the bottom of the aquaria. Hence, the treatments with 150, 180 and 210 mM NaCl already had to be terminated during the first half of the experiment after 48, 27 and 19 days respectively.

The water contents of *Az. filiculoides* initially decreased with increasing salt in the nutrient solution (Fig. 1B, Table 1). The plants grown at salt concentrations ≥60 mM NaCl became smaller and partially orange, and the length of the roots substantially decreased. In the treatments with ≥120 mM NaCl, the *Azolla* plants shed nearly all their roots and it was only in the 120 mM salt treatment that they occasionally regenerated during the experiment (personal observations). For the plants grown at salt concentrations ≤90 mM water contents increased again (Fig. 1B, Table 1), the roots lengthened and the plants returned to their original green colour during the second half of the experiment. In the 120 mM NaCl treatment, in

Table 1
Results of the Mixed Linear Model analyses and the Bonferroni adjusted pairwise comparisons based on the estimated marginal for treatment effects for the moisture contents and the relative growth rates (RGR) of the *Az. filiculoides–An. azollae* associations exposed to the different salt concentrations. The mean difference is significant at the 0.05 level.

Variable	Mixed Linear Models									Salt treatment (mM NaCl)							
	Covariance type	Treatment			Time			Treatment × time			0	30	60	90	120	150	180	210
		df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	Bonferroni adjusted pairwise comparisons							
Moisture content	FOAD	7	92.800	0.000	11	15.356	0.000	52	10.069	0.000	a	a	ab	b	c	d	d	d
RGR	SI	7	50.159	0.000	11	14.547	0.000	52	3.190	0.000	a	ab	bc	c	d	de	e	de

FOAD, first order ante dependence; SI, scaled identity.

contrast, the stress symptoms remained present until the end of the experiment.

3.2. Plant nutrient composition

Sodium concentrations in the plants increased with increasing salt concentrations in the nutrient solution, but only up to the 60 mM NaCl treatment (Table 2). The plants in the 30 mM NaCl treatment accumulated almost eight times more sodium than the controls, and the plants in the 60, 90 and 120 mM NaCl treatments also accumulated significantly more sodium than the plants in the 30 mM NaCl treatment. The sodium concentrations in the plants grown at the highest three salt concentrations, however, stayed relatively low due to the fact that these plants had lost their roots.

The biggest difference in the potassium concentrations of the plants occurred between the control plants and the plants grown at 30 mM NaCl (Table 2). In plants grown at 60, 90 and 120 mM NaCl potassium concentrations were lower than in plants grown at 30 mM NaCl whereas in the highest three treatments plant potassium concentrations were comparable to all other salt treatments. In contrast, calcium and magnesium concentrations in the plants decreased with increasing salt concentration in the nutrient solution (Table 2). Calcium concentrations decreased most in the plants grown at 90 mM NaCl, while at higher salt concentrations they stayed relatively high, especially at 120 mM NaCl. Magnesium concentrations became particularly low in the plants grown at salt concentrations ≥150 mM NaCl. Phosphorus and nitrogen concentrations were comparably high in the plants grown up to 90 mM NaCl (Table 2). At higher salt concentrations the plants contained significantly lower amounts. The phosphorus concentrations in the plants grown at ≥150 mM NaCl became significantly lower than those in the plants grown at 120 mM NaCl.

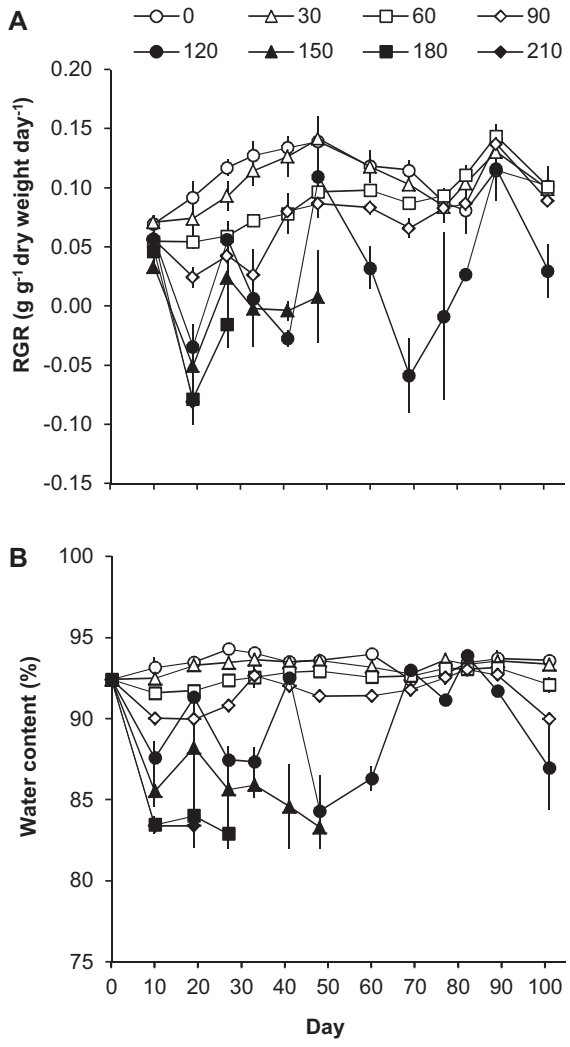

Fig. 1. (A) The relative growth rates (RGRs) ($g\,g^{-1}$ dry weight day^{-1}) ± SE and (B) the water contents (%; w/w) ± SE of the *Az. filiculoides–An. azollae* associations in the different salt treatments (0–210 mM NaCl; $n = 3$) at different times during the experiment. The results of the statistical analyses are presented in Table 1.

3.3. Amino acid composition

To study the accumulation of free amino acids as a possible adaptive response to salt stress we determined the amino acid composition in the association at the start of the experiment and at the end, when the growth rates of the plants grown at salt concentrations up to 90 mM NaCl had become equally high. A range of amino acids and some other soluble nitrogenous compounds were detected including glycine, alanine, valine, isoleucine, proline, phenylalanine, serine, threonine, asparagine, glutamine, arginine, aspartate, glutamate, GABA and ethanolamine (Table 3). In addition, small traces of leucine, tryptophan, methionine, tyrosine, cysteine, lysine, histidine, ornithine, carnithine and glucosamine were detected (data not shown). The analyses of the amino acid concentrations of the plants showed significant differences between treatments for proline, asparagine, glutamine, glutamate and GABA. Proline and glutamate concentrations in the plants increased, whereas asparagine, glutamate and GABA concentrations in the plants generally decreased with increasing salt concentration in the nutrient solution.

3.4. Acetylene reduction assay

We measured the nitrogenase activity of the cyanobiont in response to different salt concentrations, because in a nitrogen free environment the amino acid synthesis in *Azolla* strongly depends on the activity of *An. azollae*. For the *Az. filiculoides–An. azollae* associations grown at salt concentrations up to 90 mM NaCl equally high nitrogenase activities were measured. At higher concentrations the activities significantly decreased with increasing salt stress (ANOVA: $F(7) = 79.653$, $p < 0.000$) (Fig. 2).

Table 2
Results of the Mixed Linear Model analyses on treatment effects with mean differences ($n = 3$) being significant at the 0.05 level, and the mean sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P) and nitrogen (N) concentrations in the *Az. filiculoides–An. azollae* associations grown at the different salt concentrations ($\mu mol\ g^{-1}$ dry weight) ± SE. The total number of samples used for calculating the mean nutrient concentrations are given in the second row. Differences between the treatments, as determined with the Bonferroni adjusted pairwise comparisons, are indicated with different letters.

Treatment (mM NaCl)					0	30	60	90	120	150	180	210
Number of samples					36	36	36	36	33	15	6	3
Nutrient	Mixed Linear Models				Mean nutrient concentrations ($\mu mol\ g^{-1}$ dry weight) ± SE							
	Covariance type	Treatment effects										
		df	*F*	*p*								
Plant Na	SI	7	662.467	0.000	148 ± 7a	1142 ± 21b	1655 ± 24c	1765 ± 36c	1770 ± 96c	1550 ± 181b	1156 ± 60b	1042 ± 54b
Plant K	HFOAR	7	95.032	0.000	1086 ± 30a	576 ± 17b	449 ± 10c	420 ± 9c	419 ± 24c	502 ± 38bc	510 ± 37bc	376 ± 46bc
Plant Ca	SI	7	9.823	0.000	200 ± 12a	149 ± 9bc	117 ± 4c	116 ± 5c	183 ± 14ab	133 ± 14bc	127 ± 9ac	118 ± 18ac
Plant Mg	SI	7	40.796	0.000	165 ± 3a	143 ± 4b	131 ± 3bc	123 ± 3 cd	119 ± 6de	83 ± 3f	82 ± 2f	86 ± 2ef
Plant P	SI	7	46.061	0.000	264 ± 9a	273 ± 9a	268 ± 9a	266 ± 9a	227 ± 13b	134 ± 9c	81 ± 6c	71 ± 5c
Plant N	FOAD	4	6.505	0.002	2579 ± 73a	2445 ± 104a	2411 ± 82ab	2265 ± 150ab	2209 ± 212b			

SI, scaled identity; HFOAR, heterogeneous first order autoregressive; FOAD, first order ante dependence.

Table 3
The amino acid concentrations in the plants (±SE) at the start and at the end of the experiment. The results of the analyses of variances (ANOVA) are significant at the 0.05 level. Significant differences between treatments, as determined with Bonferroni post hoc analyses, are indicated with different letters.

Amino acid	ANOVA fixed effects			Treatment (mM NaCl)					
	df	*F*	*p*	– Start	0 Day 101	30	60	90	120
Glycine	4	1.210	ns	2.47 ± 0.36	1.56 ± 0.17	1.34 ± 0.17	0.99 ± 0.14	0.97 ± 0.05	1.58 ± 0.82
Alanine	4	0.527	ns	0.74 ± 0.11	1.56 ± 0.46	1.88 ± 0.22	1.79 ± 0.45	2.22 ± 1.15	2.95 ± 0.67
Valine	4	2.428	ns	0.51 ± 0.05	0.69 ± 0.10	0.50 ± 0.28	0.77 ± 0.08	0.98 ± 0.05	1.22 ± 0.25
Isoleucine	4	1.710	ns	0.36 ± 0.04	0.35 ± 0.35	0.18 ± 0.18	0.35 ± 0.19	0.59 ± 0.02	0.78 ± 0.25
Proline	4	14.983	0.001	0.17 ± 0.01	0.51 ± 0.13a	0.73 ± 0.19a	0.99 ± 0.17ab	1.53 ± 0.05bc	1.97 ± 0.13c
Phenylalanine	4	0.913	ns	1.05 ± 0.05	0.98 ± 0.17	0.78 ± 0.12	0.97 ± 0.11	1.03 ± 0.04	0.78 ± 0.09
Serine	4	2.660	ns	0.64 ± 0.04	1.31 ± 0.26	2.60 ± 0.76	1.87 ± 0.29	3.67 ± 1.11	3.83 ± 1.29
Threonine	4	1.659	ns	0.88 ± 0.19	0.78 ± 0.24	0.89 ± 0.12	0.85 ± 0.24	1.29 ± 0.11	1.30 ± 0.26
Asparagine	4	5.740	0.014	0.18 ± 0.08	0.87 ± 0.28ab	1.11 ± 0.26a	0.61 ± 0.15ab	0.34 ± 0.01ab	0.00 ± 0.00b
Glutamine	4	26.902	0.000	95.32 ± 8.36	77.96 ± 12.23a	76.23 ± 1.82a	66.20 ± 13.30a	62.64 ± 9.15a	7.44 ± 2.01b
Arginine	4	3.434	ns	0.26 ± 0.02	0.78 ± 0.21	0.80 ± 0.13	1.11 ± 0.03	1.51 ± 0.14	1.14 ± 0.32
Aspartate	4	2.280	ns	0.09 ± 0.06	0.84 ± 0.16	1.14 ± 0.11	1.27 ± 0.23	1.75 ± 0.39	1.63 ± 0.23
Glutamate	4	4.566	0.027	7.03 ± 1.00	6.92 ± 0.14a	11.07 ± 1.69a	16.52 ± 4.35b	15.80 ± 1.99b	14.51 ± 1.83ab
GABA	4	3.603	0.051	12.55 ± 2.72	14.35 ± 4.36a	9.91 ± 0.72ab	8.78 ± 0.73ab	8.08 ± 0.67ab	4.25 ± 0.84b
Ethanolamine	4	1.093	ns	0.73 ± 0.10	2.61 ± 0.62	1.01 ± 1.01	2.45 ± 1.23	2.56 ± 0.61	0.50 ± 0.50
Glutamine/glutamate ratio	4	23.876	0.000	15.45 ± 3.38	11.23 ± 1.64a	7.22 ± 1.08a	4.38 ± 1.09a	4.25 ± 1.14a	0.50 ± 0.07b
Total	4	7.026	0.008	130.25 ± 4.95	113.21 ± 19.23a	111.01 ± 3.85a	106.95 ± 17.53a	106.60 ± 7.86a	44.67 ± 2.48b

4. Discussion

4.1. Plants responses to salt stress

Initially, the *Az. filiculoides–An. azollae* association grown at salt concentrations up to 90 mM NaCl grew at slightly lower rates than the control plants. Their decreased moisture contents indicated that the plants suffered from osmotic stress whereas the signs of chlorosis and the shorter root lengths pointed to salt injury. After a period of approximately 75 days, however, these signs of stress and injury had gradually disappeared and the plants in the salt treatments up to 90 mM NaCl were able to grow at equally high rates as the plants in the control treatment. This shows that the temperate *Az. filiculoides–An. azollae* strain was plastic in its response to salt stress and seemed able to acclimate to relatively high salt concentrations even at immediate exposure. This important long term effect should be considered when interpreting the results of relatively short-term experiments. A comparable plasticity has been shown for subtropical strains of *Azolla pinnata* and *Azolla microphylla* (Rai and Rai, 1999; Abraham and Dhar, 2010) which were able to increase salt tolerance after pre-incubation at non-lethal NaCl concentrations for a specified time period.

For the four highest salt concentrations (120–210 mM NaCl) the osmotic stress and salt injury was more severe and remained present until the end of the experiment. We observed that the plants grown at high salt concentrations almost immediately shed their roots, with strong implications for their nutrient uptake. For *Az. filiculoides* shedding of roots is known to be caused by inhibitors

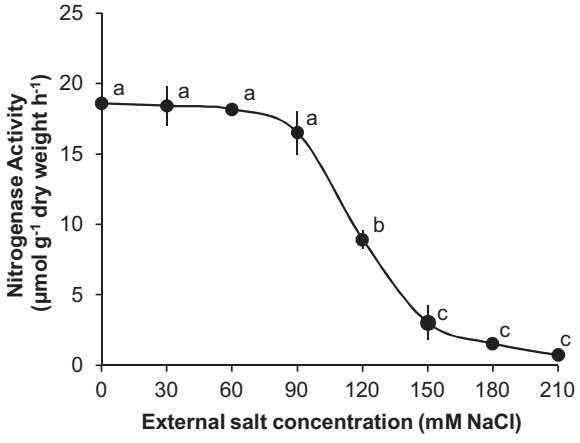

Fig. 2. The mean nitrogenase activity ($\mu mol\ g^{-1}$ dry weight $hour^{-1}$) ± SE in the *Az. filiculoides–An. azollae* association in the different salt treatments (0–210 mM NaCl; $n = 3$) after 9 days.

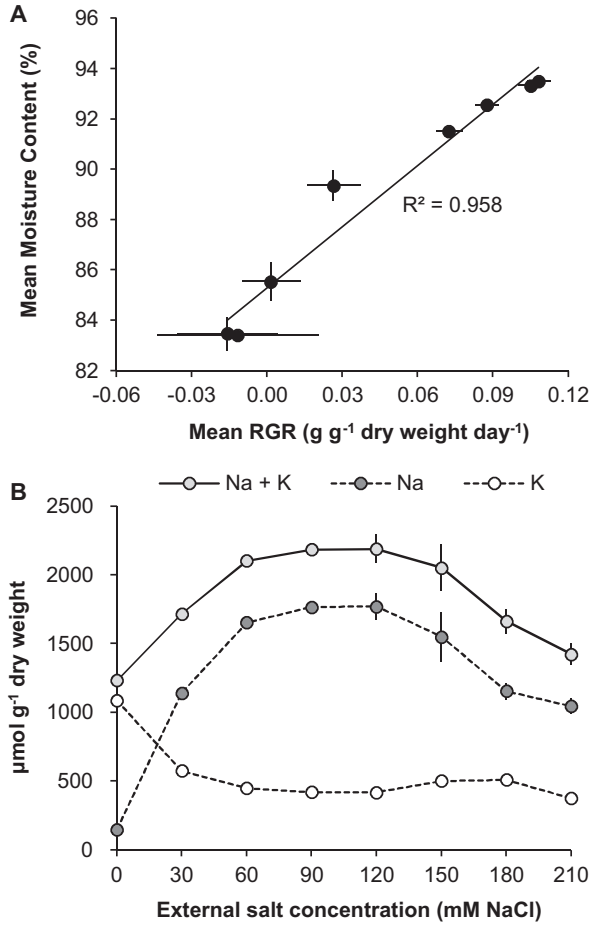

Fig. 3. (A) The correlation between the mean moisture contents (%) of the plants and the mean relative growth rates (g g^{-1} dry weight day^{-1}). (B) The mean sodium (Na) and potassium (K) concentrations in the plant in the different salt treatments (0–210 mM NaCl).

of respiration (Uheda and Kitoh, 1994) and for *Az. pinnata* this phenomenon has also been observed following salt stress (Rai et al., 2006). Although plants at 120 mM NaCl were able to re-grow their roots, their turnover rates were much higher than for the plants grown at lower salt concentrations. In addition, root re-growth in this treatment resulted in very marked fluctuations in RGRs and moisture contents, indicating physiological stress. Nevertheless, these responses probably contributed to their survival and we therefore suggest that the shedding of roots may provide an escape for *Azolla* to avoid salinity stress at fluctuating salt concentrations. At salt concentrations ≥150 mM NaCl, in contrast, plants were not able to re-grow their roots which ultimately resulted in the death of these plants due to desiccation and impairment of nutrient uptake.

4.2. Roles of Na and K in osmoregulation

Fig. 3A shows that the mean RGRs of the plants in the different treatments were strongly correlated with the mean moisture contents of the plants, as an indicator of osmotic stress. Osmotic effects of high external salt concentrations on plants result from the lowering of the external water potential as opposed to the internal water potential thereby interfering with the plants ability to extract ions and nutrients and maintain turgor. At moderate external salt concentrations the accumulation of the monovalent inorganic solute Na may have provided some osmotic adjustment to the plants by maintaining the potential gradient for the influx of water. The increased Na concentrations in the plants at elevated salt concentrations clearly show that *Az. filiculoides* is a salt includer (Marschner, 1995), at least up to external salt concentrations of 60 mM NaCl. The increased Na concentrations in the plants were to some extent reverted by decreased K concentrations (Fig. 3B). Although K concentrations in the plants grown at 30 mM NaCl were half as high as in the control plants, their growth rates were not affected suggesting that K in the plants was replaced by Na. Substitution of K by Na in various metabolic functions is a well-known feature in salt tolerance (Marschner, 1995) and for some species, for example *Lemna* spp., has been shown to even result in a growth stimulation (Haller et al., 1974).

Both Rai and Rai (1999) and Abraham and Dhar (2010) suggested that the increase in the salt tolerance of *Azolla* after pre-incubation at non-lethal salt concentrations involved the development of a capability in the plants to regulate their ion concentrations (Na, K and Ca). In the current experiment, where the *Az. filiculoides–An. azollae* association seemed able to acclimate at exposure up to 90 mM NaCl, we also found that the plants were able to soothe the uptake of Na (MLM, SI, Time × Treatment: $F(48) = 4.84$, $p < 0.000$) and improve the uptake of K (MLM, SI, Time × Treatment: $F(48) = 3.00$, $p < 0.000$) and Ca (MLM, SI, Time × Treatment: $F(48) = 4.06$, $p < 0.000$), especially in the last 30 days of the experiment (data not shown). The resulting absolute internal nutrient concentrations in the plants, however, cannot explain why the plants in the salt treatments up to 90 mM NaCl were able to grow and increase their salt tolerance, whereas the plants in the 120 mM NaCl treatment just about survived. In most cases the ion and nutrient concentrations were not significantly different between the plants grown in the 120 mM NaCl treatment and the plants grown at lower salt concentrations, even despite the fact that the plants in the 120 mM NaCl treatment on average were 1.7–3.2 times older than the plants grown at lower salt concentration and were thus subjected to the salt stress for a relatively longer time. Only the phosphorus concentrations were significantly lower in the plants grown at 120 mM NaCl than in the plants grown at lower salt concentrations, but the concentrations were still reasonably high. We therefore conclude that the differences in the ion and nutrient concentrations in the plants grown at the various salt concentrations were not the main cause for the observed differences in growth. Furthermore, we suggest that the changes in ion and nutrient uptake rates were more a consequence, rather than a contributory factor, to the increase in the salt tolerance of the *Az. filiculoides* plants grown up to 90 mM NaCl.

4.3. Roles of nitrogen compounds in osmoregulation

Since *Azolla* is known to be very rich in nitrogen we hypothesized that salt tolerance might be facilitated by the production of free amino acids. We found significant differences for proline, asparagine, glutamine, GABA and glutamate. The increased proline concentrations in the *Az. filiculoides* plants seemed to have been triggered by the decreasing moisture contents of the plants which showed a strong negative correlation with the proline concentrations (Fig. 4A). Although proline, which is the most widely distributed osmoprotectant (Delauney and Verma, 1993; Yoshiba et al., 1995; Verbruggen and Hermans, 2008), significantly increased in the plant tissue with increasing salt stress, the concentrations were rather low, as was also found in *Salvinia natans* (Jampeetong and Brix, 2009), which like *Azolla*, belongs to the family of heterosporous ferns. This makes it unlikely that proline played a significant role in the osmotic adjustment of the plants. However, other functions have been postulated for proline accumulation in stressed plant tissues. For instance, it may enhance stress tolerance by acting as a detoxificant of free radicals and a protectant for enzymes and membranes (Delauney and Verma, 1993).

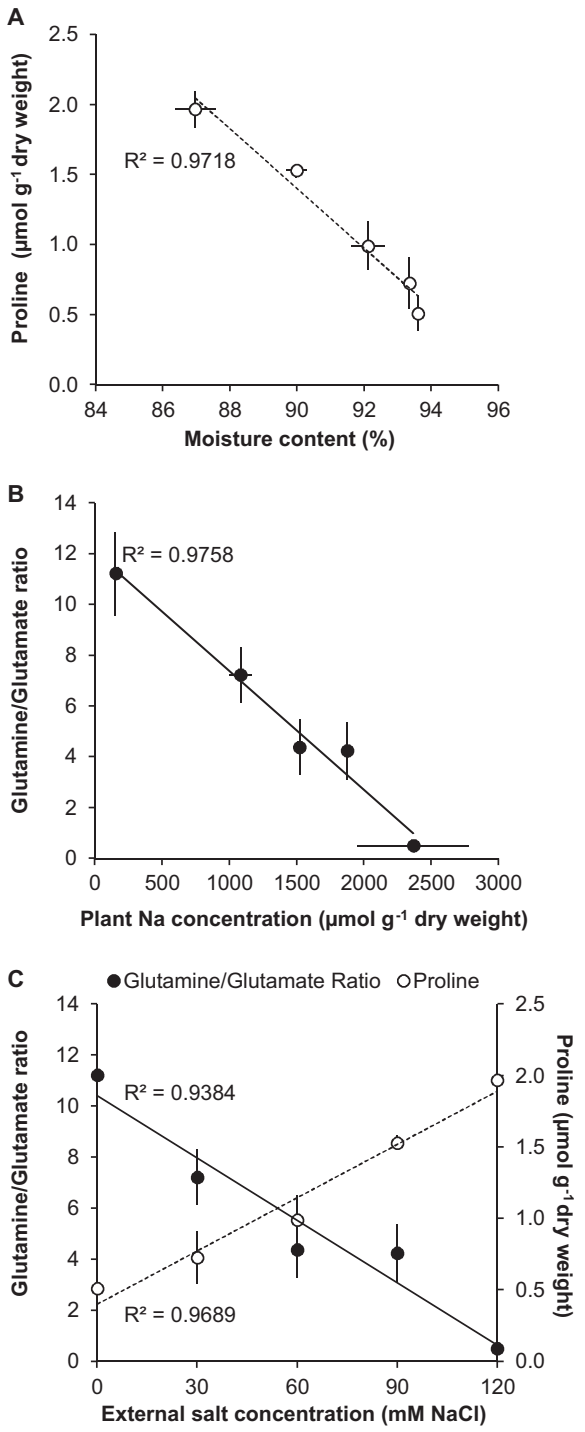

Fig. 4. (A) The correlation between the mean moisture content of the plants and the proline concentrations in the plants at day 101. (B) The correlation between the glutamine/glutamate ratio in the plants and the internal Na concentrations in the plants at day 101. (C) Proline and the glutamate/glutamine concentrations in the plants as indicators of salt stress in relation to the external salt concentration.

The glutamine/glutamate ratios in the plants were clearly altered due to the salt stress and strongly correlated with the Na concentrations in the plants (Table 3, Fig. 4B). The free glutamate concentrations in the plants increased with increasing salt stress up to the 60 mM NaCl treatment, whereas the glutamine concentrations in the plants decreased at high external salt concentrations (≥120 mM NaCl). Glutamate and glutamine play essential roles in the amino acid metabolism in plants. In a nitrogen free environment, like in the current experiment, the glutamine and glutamate in *Azolla* initially are derived from the activity of the cyanobiont that fixes atmospheric dinitrogen and converts it to ammonium that *Azolla* then processes in the glutamine synthase/glutamate synthase (GS/GOGAT) cycle (Bernard and Habash, 2009; Teixeira and Fidalgo, 2009; Ghanem et al., 2011).

The increases in the glutamate concentrations we found in the plants grown at elevated salt concentration are quite remarkable because glutamate concentrations in plants usually are fairly constant where many other amino acids may dramatically change in response to environmental changes or plant physiological changes (Forde and Lea, 2007). The high glutamine levels we observed at the beginning of the experiment and at the end of the experiment in the plants grown up to 90 mM NaCl are not uncommon for *Azolla* spp. (Newton and Cavins, 1976). The glutamine concentrations constituted about 65% of the total free amino acid pool in the plants and may have contributed to the increase in the salt tolerance of the plants grown in the salt treatments up to 90 mM NaCl by providing osmotic adjustment (Mansour, 2000; Bernard and Habash, 2009). They cannot be regarded as a fully functional adaptive response to salt stress though because at even higher salt concentrations the glutamine levels were severely reduced. This was probably due to the reduced activity of the cyanobionts in the associations grown at 120 mM NaCl which in turn is likely to have resulted from the reduced overall performance of the *Azolla* in this treatment (Rai et al., 2001).

No significant changes were found in the serine or ethanolamine concentrations in the plants. It remains worthwhile, though, to look into the potential role of glycine betaine directly in the salt tolerance of the *Az. filiculoides–An. azollae* association in future research. Especially since the species is known to be able of producing betaine lipids (Kunzler and Eichenberger, 1997).

4.4. Indicators for salt stress

Absolute nutrient concentrations in plant tissue were not correlated with plant growth and therefore not suitable as reliable indicators for salt stress. Although the absolute nitrogen concentrations in the plants in the 120 mM NaCl treatment were not even that different from the plants grown at lower salt concentrations due to the decreased growth of the plants, the total free amino acid concentrations were significantly reduced and the free amino acid composition was considerably altered. In the salt tolerance of the Dutch *Az. filiculoides–An. azollae* association thus a clear tipping point was found at 120 mM NaCl. The strongly impaired N metabolism in the plants at 120 mM NaCl may be related to decreased recycling of carbon skeletons where compounds such as GABA are often involved (Bouché and Fromm, 2004; Ghanem et al., 2011). Accordingly, we found significantly decreased GABA concentrations in the plants in the 120 mM NaCl treatment and no more asparagine, which is the most important amino acid being transported (Lea et al., 2007).

Although we have not been able to identify the accumulation of free amino acids as the facilitating mechanisms in the salt tolerance of the *Az. filiculoides–An. azollae* association, we did find important indicators based on which the salt resistance limit of the species might be predicted. The proline concentrations in the plants and the glutamine/glutamate ratios in the plants provide important novel indicators in the salt tolerance of the *Az. filiculoides–An. azollae* association, both showing linear responses in relation to external salt concentrations (Fig. 4C). They therefore can be used to predict the tipping point in the salt tolerance of the species accurately and instantly. In contrast, the RGRs and/or moisture contents of the plants show very marked fluctuations around the tipping point, and are not always possible to monitor.

4.5. Paleolimnological implications

The salt resistance limit of 90 mM NaCl we found here for the *Az. filiculoides–An. azollae* association is quite striking as this corresponds to a ~5‰ surface water salinity. Climate modelling experiments elucidating the circulation in the Eocene Arctic Ocean have shown that a surface water salinity of 5‰ in combination with a bottom water salinity of 21–25‰, as was proposed by Waddell and Moore (2008), offered an answerable scenario for the growth of the ancestral *Azolla* spp. in the Eocene Arctic Ocean (Speelman et al., 2010). There is strong evidence that dinitrogen fixation was already a persistent feature in the Eocene Arctic Ocean (Bauersachs et al., 2010). Ancestral *Azolla* spp. should have been able to survive surface water salinities around 5‰ and develop the high biomass as reported (Brinkhuis et al., 2006; Speelman et al., 2009).

Funding

Funding for this research was provided by Radboud University and Darwin Centre for Biogeosciences.

Acknowledgements

We thank StatoilHydro for general support. Also, we thank Jelle Eygensteyn, Rien van der Gaag, Germa Verheggen-Kleinheerenbrink and Roy Peters for their help in the chemical analyses and Iris Bakker for practical assistance.

References

Abou, Y., Fiogbé, E.D., Micha, J.C., 2007. Effects of stocking density on growth, yield and profitability of farming Nile tilapia, *Oreochromis niloticus* L., fed *Azolla* diet, in earthen ponds. Aquacult. Res. 38, 595–604, http://dx.doi.org/10.1111/j.1365-2109.2007.01700.x.

Abraham, G., 2010. Antioxidant enzyme status in *Azolla microphylla* in relation to salinity and possibilities of environmental monitoring. Thin Solid Films 519, 1240–1243, http://dx.doi.org/10.1016/j.tsf.2010.08.076.

Abraham, G., Dhar, D.W., 2010. Induction of salt tolerance in *Azolla microphylla* Kaulf through modulation of antioxidant enzymes and ion transport. Protoplasma 245, 105–111, http://dx.doi.org/10.1007/s00709-010-0147-3.

Arora, A., Singh, P.K., 2003. Comparison of biomass productivity and nitrogen fixing potential of *Azolla* spp. Biomass Bioenergy 24, 175–178, http://dx.doi.org/10.1016/S0961-9534.

Bauersachs, T., Speelman, E.N., Hopmans, E.C., Reichart, G.J., Schouten, S., Sinninghe Damsté, J., 2010. Fossilized glycolipids reveal past oceanic N_2 fixation by heterocystous cyanobacteria. Proc. Natl. Acad. Sci. U.S.A. 107, 19190–19194, http://dx.doi.org/10.1073/pnas.1007526107.

Bernard, S.M., Habash, D.Z., 2009. The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytol. 182, 608–620, http://dx.doi.org/10.1111/j.1469-8137.2009.02823.x.

Bouché, N., Fromm, H., 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9, 110–115, http://dx.doi.org/10.1016/j.tplants.2004.01.006.

Brinkhuis, H., Schouten, S., Collinson, M.E., Sluijs, A., Sinninghe Damsté, J.S., Dickens, G.R., Huber, M., Cronin, T.M., Onodera, J., Takahashi, K., Bujak, J.P., Stein, R., van der Burgh, J., Eldrett, J.S., Harding, I.C., Lotter, A.F., Sangiorgi, F., Cittert, H.V.V., de Leeuw, J.W., Matthiessen, J., Backman, J., Moran, K., 2006. Episodic fresh surface waters in the Eocene Arctic Ocean. Nature 441, 606–609, http://dx.doi.org/10.1038/nature04692.

Bujak, J., Mudge, D., 1994. A high-resolution North Sea Eocene dinocyst zonation. J. Geol. Soc. 151, 449–462, http://dx.doi.org/10.1144/gsjgs.151.3.0449.

Burg, M.B., Ferraris, J.D., 2008. Intracellular organic osmolytes: function and regulation. J. Biol. Chem. 283, 7309–7313, http://dx.doi.org/10.1074/jbc.R700042200.

Collinson, M.E., Barke, J., van der Burgh, J., van Konijnenburg-van Cittert, J.H.A., 2009. A new species of the freshwater fern *Azolla* (*Azollaceae*) from the Eocene Arctic Ocean. Rev. Palaeobot. Palynol. 155, 1–14, http://dx.doi.org/10.1016/j.revpalbo.2008.12.014.

Collinson, M.E., Barke, J., van der Burgh, J., van Konijnenburg-van Cittert, J.H.A., Heilmann-Clausen, C., Howard, L.E., Brinkhuis, H., 2010. Did a single species of Eocene *Azolla* spread from the Arctic Basin to the southern North Sea? Rev. Palaeobot. Palynol. 159, 152–165, http://dx.doi.org/10.1016/j.revpalbo.2009.12.001.

de Lyon, M.J.H., Roelofs, J.G.M., 1986. Waterplanten in relatie tot waterkwaliteit en bodemgesteldheid, Deel 1 en 2, Technisch Rapport. Katholieke Universiteit Nijmegen, Nijmegen.

Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plants. Plant J. 4, 215–223, http://dx.doi.org/10.1046/j.1365-313X.1993.04020215.x.

Deng-hui, S., Ho, W., Xi-pan, C., 1987. The cultivation of *Azolla filiculoides* for the reclamation and utilization of heavy saline soils. In: *Azolla* Utilization: Proceedings of the Workshop on *Azolla* Use. Fujian Academy of Agricultural Sciences, IRRI Publications, p. 274.

Eldrett, J.S., Harding, I.C., Firth, J.V., Roberts, A.P., 2004. Magnetostratigraphic calibration of Eocene-Oligocene dinoflagellate cyst biostratigraphy from the Norwegian-Greenland Sea. Mar. Geol. 204, 91–127, http://dx.doi.org/10.1016/S0025-3227(03)00357-8.

Ferdoushi, Z., Haque, F., Khan, S., Haque, M., 2008. The effects of two aquatic floating macrophytes (*Lemna* and *Azolla*) as biofilters of nitrogen and phosphate in fish ponds. Turk. J. Fish. Quat. Sci. 8, 253–258.

Flowers, T.J., Troke, P.F., Yeo, A.R., 1977. The mechanism of salt tolerance in halophytes. Annu. Rev. Plant Phys. 28, 89–121, http://dx.doi.org/10.1146/annurev.pp.28.060177.000513.

Forde, B.G., Lea, P.J., 2007. Glutamate in plants: metabolism, regulation, and signalling. J. Exp. Bot. 58, 2339–2358, http://dx.doi.org/10.1093/jxb/erm121.

Ghanem, M., Martínez-Andújar, C., Albacete, A., Pospíšilová, H., Dodd, I., Pérez-Alfocea, F., Lutts, S., 2011. Nitrogen form alters hormonal balance in salt-treated tomato (*Solanum lycopersicum* L.). J. Plant Growth Regul. 30, 144–157, http://dx.doi.org/10.1007/s00344-010-9178-4.

Grattan, S.R., Grieve, C.M., 1992. Mineral element acquisition and growth response of plants grown in saline environments. Agric. Ecosyst. Environ. 38, 275–300, http://dx.doi.org/10.1016/0167-8809(92)90151-Z.

Haller, W.T., Sutton, D.L., Barlowe, W.C., 1974. Effects of salinity on growth of several aquatic macrophytes. Ecology 55, 891–894, http://dx.doi.org/10.2307/1934427.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C., 1968. The acetylene–ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43, 1185–1207, http://dx.doi.org/10.1104/pp.43.8.1185.

Hitz, W.D., Rhodes, D., Hanson, A.D., 1981. Radiotracer evidence implicating phosphoryl and phosphatidyl bases as intermediates in betaine synthesis by water stressed Barley leaeves. Plant Physiol. 68, 814–822, http://dx.doi.org/10.1104/pp.68.4.814.

Hove, C., Lejeune, A., 2002. Applied aspects of *Azolla–Anabaena* symbiosis. In: Cyanobacteria in Symbiosis. Kluwer Academic Plublishers, Netherlands, pp. 179–193.

Jampeetong, A., Brix, H., 2009. Effects of NaCl salinity on growth, morphology, photosynthesis and proline accumulation of *Salvinia natans*. Aquat. Bot. 91, 181–186, http://dx.doi.org/10.1016/j.aquabot.2009.05.003.

Johnson, G.V., 1986. Evaluation of salt tolerance in *Azolla*. Technical Completion Report, project number 1423626. Water Resources Research Institute in cooperation with the Department of Biology, University of Mexico, New Mexico.

Khatun, A., Ali, M.A., Dingle, J.G., 1999. Comparison of the nutritive value for laying hens of diets containing *Azolla* (*Azolla pinnata*) based on formulation using digestible protein and digestible amino acid versus total protein and total amino acid. Anim. Feed Sci. Technol. 81, 43–56, http://dx.doi.org/10.1016/S0377-8401(99)00071-1.

Kingston, H.M., Haswell, S.J., 1997. Microwave Enhanced Chemistry: Fundamentals Sample Preparation and Applications. American Chemical Society, Washington, DC.

Kunzler, K., Eichenberger, W., 1997. Betaine lipids and zwitterionic phospholipids in plants and fungi. Phytochemistry 46, 883–892, http://dx.doi.org/10.1016/S0031-9422(97)81274-5.

Lea, P.J., Sodek, L., Parry, M.A.J., Shewry, R., Halford, N.G., 2007. Asparagine in plants. Ann. Appl. Biol. 150, 1–26, http://dx.doi.org/10.1111/j.1744-7348.2006.00104.x.

Leterme, P., Londono, A.M., Munoz, J.E., Suarez, J., Bedoya, C.A., Souffrant, W.B., Buldgen, A., 2009. Nutritional value of aquatic ferns (*Azolla filiculoides* Lam. and *Salvinia molesta* Mitchell) in pigs. Anim. Feed Sci. Technol. 149, 135–148, http://dx.doi.org/10.1016/j.anifeedsci.2008.04.013.

Lumpkin, T.A., Plucknett, D.L., 1980. *Azolla* – botany, physiology and use as a green manure. Econ. Bot. 34, 111–153, http://dx.doi.org/10.1007/BF02858627.

Lumpkin, T.A., Plucknett, D.L., 1982. *Azolla* as a Green Manure: Use and Management in Crop Production. Westview Press, Inc., Colorado.

Mandal, B., Vlek, P.L.G., Mandal, L.N., 1999. Beneficial effects of blue-green algae and *Azolla*, excluding supplying nitrogen, on wetland rice fields: a review. Biol. Fertil. Soils 28, 329–342, http://dx.doi.org/10.1007/s003740050501.

Mansour, M.M.F., 2000. Nitrogen containing compounds and adaptation of plants to salinity stress. Biol. Plant. 43, 491–500, http://dx.doi.org/10.1023/A:1002873531707.

Marschner, P., 1995. Mineral Nutrition of Higher Plants. Elsevier Academic Press, Amsterdam.

Masood, A., Shah, N.A., Zeeshan, M., Abraham, G., 2006. Differential response of antioxidant enzymes to salinity stress in two varieties of *Azolla* (*Azolla pinnata* and *Azolla filiculoides*). Environ. Exp. Bot. 58, 216–222, http://dx.doi.org/10.1016/j.envexpbot.2005.08.002.

Moore, A.W., 1969. *Azolla* – biology and agronomic significance. Bot. Rev. 35, 17–34, http://dx.doi.org/10.1007/BF02859886.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Ann. Rev. Plant Biol. 59, 651–681, http://dx.doi.org/10.1146/annurev.arplant.59.032607.092911.

Newton, J.W., Cavins, J.F., 1976. Altered nitrogenous pools induced by the *Azolla–Anabaena azollae* symbiosis. Plant Physiol. 58, 798–799, http://dx.doi.org/10.1104/pp.58.6.798.

Nuccio, M.L., Russell, B.L., Nolte, K.D., Rathinasabapathi, B., Gage, D.A., Hanson, A.D., 1998. The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. Plant J. 16, 487–496, http://dx.doi.org/10.1046/j.1365-313x.1998.00316.x.

Peters, G.A., Mayne, B.C., 1974a. *Azolla*, *Anabaene azollae* relationship. I. Initial characterisation of the association. Plant Physiol. 53, 813–819, http://dx.doi.org/10.1104/pp.53.6.813.

Peters, G.A., Mayne, B.C., 1974b. *Azolla*, *Anabaene azollae* relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction. Plant Physiol. 53, 820–824, http://dx.doi.org/10.1104/pp.53.6.820.

Rai, A.K., Rai, V., 2003. Effect of NaCl on growth, nitrate uptake and reduction and nitrogenase activity of *Azolla pinnata–Anabaena azollae*. Plant Sci. 164, 61–69, http://dx.doi.org/10.1016/S0168-9452(02)00335-7.

Rai, P.K., 2007. Wastewate management through biomass of *Azolla pinnata*: an eco-sustainable approach. Ambio 36, 426–428, http://dx.doi.org/10.1579/0044-7447(2007)36[426:WMTBOA]2.0.CO;2.

Rai, V., Rai, A.K., 1999. Growth behaviour of *Azolla pinnata* at various salinity levels and induction of high salt tolerance. Plant Soil 206, 79–84.

Rai, V., Sharma, N.K., Rai, A.K., 2006. Growth and cellular ion content of a salt-sensitive symbiotic system *Azolla pinnata–Anabaena azollae* under NaCl stress. J. Plant Physiol. 163, 937–944, http://dx.doi.org/10.1016/j.jplph.2005.08.005.

Rai, V., Tiwari, S.P., Rai, A.K., 2001. Effect of NaCl on nitrogen fixation of unadapted and NaCl-adapted *Azolla pinnata–Anabaena azollae*. Aquat. Bot. 71, 109–117, http://dx.doi.org/10.1016/S0304-3770(01)00174-7.

Rhodes, D., Hanson, A.D., 1993. Quaternary ammonium and tertiary sulfonium compounds in higher plants. Ann. Rev. Plant Phys. 44, 357–384, http://dx.doi.org/10.1146/annurev.pp.44.060193.002041.

Singh, S.S., Mishra, A.K., Upadhyay, R.S., 2010. Potentiality of *Azolla* as a suitable P-biofertilizer under salinity through acid phosphatase activity. Ecol. Eng. 36, 1076–1082, http://dx.doi.org/10.1016/j.ecoleng.2010.04.022.

Speelman, E.N., van Kempen, M.M.L., Barke, J., Brinkhuis, H., Reichart, G.J., Smolders, A.J.P., Roelofs, J.G.M., Sangiorgi, F., Leeuw, J.W.D., Lotter, A.F., Sinninghe Damsté, J., 2009. The Eocene Arctic *Azolla* bloom: environmental conditions, productivity and carbon drawdown. Geobiology 7, 155–170, http://dx.doi.org/10.1111/j.1472-4669.2009.00195.x.

Speelman, E.N., Sewall, J.O., Noone, D., Huber, M., von der Heydt, A., Sinninghe Damsté, J., Reichart, G.J., 2010. Modelling the influence of a reduced equator-to-pole sea surface temperature gradient on the distribution of water isotopes in the Early/Middle Eocene. Earth Planet. Sci. Lett. 298, 57–65, http://dx.doi.org/10.1016/j.epsl.2010.07.026.

Stickley, C.E., Koc, N., Brumsack, H.J., Jordan, R.W., Suto, I., 2008. A siliceous microfossil view of middle Eocene Arctic paleoenvironments: a window of biosilica production and preservation. Paleoceanography 23, 19, http://dx.doi.org/10.1029/2007PA001485.

Teixeira, J., Fidalgo, F., 2009. Salt stress affects glutamine synthetase activity and mRNA accumulation on potato plants in an organ-dependent manner. Plant Physiol. Biochem. 47, 807–813, http://dx.doi.org/10.1016/j.plaphy.2009.05.002.

Uheda, E., Kitoh, S., 1994. Rapid shedding of roots from *Azolla filiculoides* plants in response to inhibitors of respiration. Plant Cell Physiol. 35, 37–43.

van Dijk, H.F.G., Roelofs, J.G.M., 1988. Effects of excessive ammonium deposition on the nutritional stutus and condition of pine needles. Physiol. Plant. 73, 494–501.

Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759, http://dx.doi.org/10.1007/s00726-008-0061-6.

Waddell L.M., Moore T.C., 2008. Salinity of the Eocene Arctic Ocean from oxygen isotope analysis of fish bone carbonate. Paleoceanography 23: PA1S12, https://dx.doi.org/ 10.1029/2007PA001451

Waditee, R., Bhuiyan, N.H., Hirata, E., Hibino, T., Tanaka, Y., Shikata, M., Takabe, T., 2007. Metabolic engineering for betaine accumulation in microbes and plants. J. Biol. Chem. 282, 34185–34193, http://dx.doi.org/10.1074/jbc.M704939200.

Wagner, G.M., 1997. *Azolla*: a review of its biology and utilization. Bot. Rev. 63, 1–26, http://dx.doi.org/10.1007/BF02857915.

Yoshiba, Y., Kiyosue, T., Katagiri, T., Ueda, H., Mizoguchi, T., Yamaguchishinozaki, K., Wada, K., Harada, Y., Shinozaki, K., 1995. Correlation between the induction of a gene for delta(1)-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J. 7, 751–760, http://dx.doi.org/10.1046/j.1365-313X.1995.07050751.x.