Article title: *Azolla* along a phosphorus gradient: biphasic growth response linked to diazotroph traits and phosphorus-induced iron chlorosis

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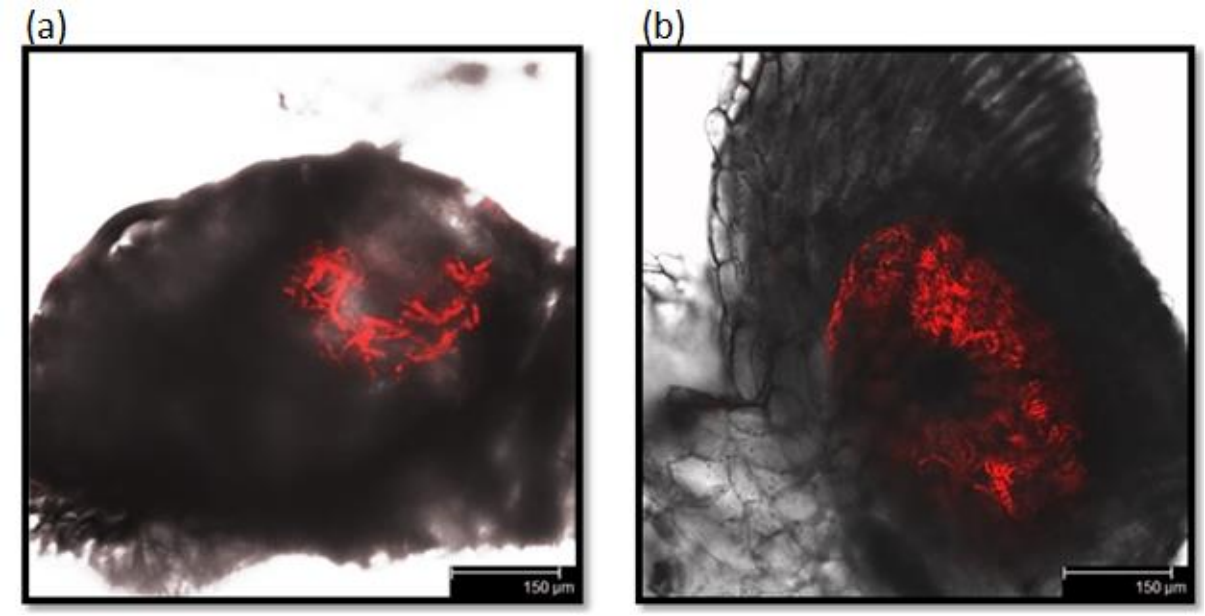
Supplementary Information



Supplementary Figure S1. CLSM image (overlay) of the lowest leaf (dorsal lobe) of *A. filiculoides* visualizing its diazotrophic cyanobacteria (red autofluorescence), grown at (a) 0.5 and (b) 100 µmol P l^{-1}. Grey cells represent photosynthetic cells. Diazotroph distribution in the *Azolla* leaf cavity was visualized using a Confocal Laser Scanning Microscope (CLSM; TCS SP2 AOBS, Leica-microsystems, Mannheim, Germany). The lowest leaf at the main axis was used, as this is known to host the highest number of diazotrophs (REF). A complete leaf was placed under a cover slip in Kaisers Glyceringelatine medium, and a 20x objective was used to create the images (excitation 488 nm at 51%, 496 nm at 41%, 514 nm at 40%, 561 nm at 55%; emission 577-671 nm; intensification 529 volt; pinhole 50-250 µm). Images were taken at different depths within the sample (z-stacks). The most detailed transmission image of the *Azolla* leaf and the most detailed autofluorescence image of the cyanobionts were combined using Adobe Photoshop CS6 (Adobe Systems Inc., San Jos, CA, U.S.A.). Picture: R. Temmink and L. Pierson.

***Supplementary Table S1**. Chemical composition of the N-free nutrient solution, based on field conditions of natural stands (de Lyon & Roelofs, 1986). Different P treatments were created by adding 0.5, 2, 10, 50 or 100 µmol P l^{-1} (NaH_2PO_4 * $2H_2O$) to the medium.*

Element	**Concentration (µmol l^{-1})**
$NaHCO_3$	1000
$CaCl_2$*$2H_2O$	1000
K_2SO_4	500
$MgSO_4$*$7H_2O$	500
Fe-EDTA	10
$CuSO_4$*$5H_2O$	1
$MnCl_2$*$4H_20$	20
$ZnSO_4$*$7H_2O$	10
$NaMoO_4$*$2H_2O$	3
H_3BO_3	20
$CoCl_2$*$6H_2O$	4
NaCl	10

***Supplementary Table S2.** Statistical results of the effects of 0.5, 2, 10, 50 and 100 µmol P l^{-1} (n=5) treatments for the relative growth rate (RGR; g g^{-1} DW d^{-1}), N : P ratio (mol mol^{-1}), N content (µmol N g^{-1} DW), P content (µmol P g^{-1} DW), P sequestration rate (µmol P m^{-2} d^{-1}), N sequestration rate (mmol N m^{-2} d^{-1}), chlorophyll a + b (mg g^{-1} DW; experiment 2) and N content (µmol N g^{-1} DW; experiment 2); ±SE. Significant differences are indicated by different letters.*

Relative growth rate (g g^{-1} DW d^{-1})						
	df	*F value*	*P*	Phosphorus	Tukey HSD pairwise comparisons	
ANOVA	4	12.072	<0.001	0.5	0.06 ± 0.005	a
				2	0.06 ± 0.003	a
				10	0.1 ± 0.01	b
				50	0.1 ± 0.01	b
				100	0.1 ± 0.009	b
N : P ratio (mol mol^{-1})						
	df	*F value*	*p*	Phosphorus	Bonferroni adjusted pairwise comparisons	
LMM (Covariance	4	733.53	<0.001	0.5	36.9 ± 0.6	a
				2	30.6 ± 0.5	b

type: unstructured)				10	16.1 ± 0.5	c
				50	10.5 ± 0.5	d
				100	9.3 ± 0.5	d
N content (µmol N g^{-1} DW)						
	df	*F value*	*P*	Phosphorus	Bonferroni adjusted pairwise comparisons	
LMM plant response (Covariance type: unstructured)	4	167.82	<0.001	0.5	1983 ± 19.4	a
				2	2040 ± 15.3	a
				10	2155 ± 15.3	b
				50	2510 ± 15.3	d
				100	2271 ± 15.3	c
P content (µmol N g^{-1} DW)						
	df	*F value*	*p*	Phosphorus	Bonferroni adjusted pairwise comparisons	
LMM plant response (Covariance type: unstructured)	4	69.273	<0.001	0.5	45.6 ± 3.3	a
				2	55.4 ± 3	b
				10	121 ± 3	c
				50	220.8 ± 3	d
				100	234.9 ± 3	d
P sequestration rate (µmol P m^{-2} d^{-1})						
	df	*F value*	*p*	Phosphorus	Tukey HSD pairwise comparisons	
ANOVA	4	38.549	<0.001	0.5	32.3 ± 20	a
				2	48.9 ± 16	b
				10	361.7 ± 39	c
				50	778.4 ± 151	d
				100	747.0 ± 90[c]	d
N sequestration rate (mmol N m^{-2} d^{-1})						
	df	*F value*	*p*	Phosphorus	Tukey HSD pairwise comparisons	
ANOVA	4	13.729	<0.001	0.5	2.3 ± 1	a
				2	4.4 ± 0.5	a
				10	11.1 ± 1	b

				50	10.8 ± 1.6	b
				100	10.6 ± 1.4	b
Chlorophyll a + b (mg g^{-1} DW) in experiment 2						
	df	*F value*	*P*	Treatment	Tukey HSD pairwise comparisons	
ANOVA	2	6.747	<0.02	C	10.8 ± 2	a
				Fe	15.2 ± 2.3	b
				N	9.5 ± 2.7	a
N content (µmol N g^{-1} DW) in experiment 2						
	df	*F value*	*P*	Treatment	Tukey HSD pairwise comparisons	
ANOVA	2	678.127	<0.001	C	2125 ± 20	a
				Fe	3000 ± 11	c
				N	2529 ± 18	b

Supplementary Table S3. *Results of the 0.5, 2, 10, 50 and 100 µmol P l^{-1} treatment (n=5) on the elemental content of A. filiculoides on day 21 (µmol g^{-1} DW) ±SE. Analyses of Variances (ANOVAs) were used to analyze potential differences in plant elemental contents. Differences between treatments were determined using Tukey HSD post hoc tests. Different letters indicate significant differences.*

		Phosphorus treatment (µmol P l^{-1})				
Elemental content (µmol g^{-1} DW)	*F value*	0.5	2	10	50	100
Al	19.01	1.8 ± 0.1^{b}	1.2 ± 0.1^{a}	0.9 ± 0.1^{a}	1 ± 0.1^{a}	1.04 ± 0.1^{a}
Ca	22.81	241 ± 3^{c}	199 ± 3^{b}	159 ± 7^{a}	159 ± 8^{a}	166 ± 12^{a}
Fe	0.982	26.3 ± 2^{a}	23.6 ± 1^{a}	25.1 ± 5^{a}	18.5 ± 2^{a}	21.9 ± 3^{a}
K	23.702	807 ± 15^{a}	965 ± 29^{b}	1252 ± 75^{c}	1179 ± 44^{c}	1132 ± 48^{bc}
Mg	8.786	142 ± 2^{c}	132 ± 3^{bc}	119 ± 4^{a}	129 ± 3^{ab}	130 ± 2^{ab}
Mn	3.016	145 ± 5^{b}	116 ± 5^{ab}	86 ± 10^{a}	115 ± 19^{ab}	106 ± 16^{ab}
Na	9.155	49.3 ± 1^{a}	55.5 ± 2^{a}	79.6 ± 7^{b}	103 ± 8^{bc}	87.6 ± 7^{b}
P	363.485	34.2 ± 1^{a}	45 ± 1^{b}	109 ± 6^{c}	196 ± 6^{d}	207 ± 3^{d}
S	7.298	139 ± 2^{a}	136 ± 2^{a}	162 ± 7^{b}	159 ± 5^{b}	154 ± 4^{ab}
Si	2.165	16.5 ± 2^{a}	18 ± 2^{a}	13 ± 2^{a}	11.1 ± 1^{a}	17.9 ± 3^{a}
Zn	41.706	37.4 ± 2^{b}	30.8 ± 2^{b}	18.6 ± 1^{a}	13.2 ± 1^{a}	15.6 ± 2^{a}
N	11.621	1890 ± 22^{a}	1875 ± 33^{a}	2270 ± 78^{b}	2436 ± 116^{b}	2254 ± 76^{b}

Supplementary Table S4. *Results of the Fe, N or no addition (n=4) on the elemental content of A. filiculoides grown at 100 µmol P l^{-1} after 2 weeks of nutrient addition (µmol g^{-1} DW) ±SE. Analyses of Variances (ANOVAs) were used to analyze potential differences in plant elemental contents. Differences between treatments were determined using Tukey HSD post hoc tests. Different letters indicate significant differences.*

Elemental content (µmol g^{-1} DW)	*F value*	Control	Iron	Nitrogen
Al	0.943	1.2 ± 0.1^{a}	1 ± 0.1^{a}	1 ± 0.04^{a}
Ca	5.683	150 ± 7^{a}	181 ± 8^{b}	161 ± 5^{ab}
Fe	4.598	35.8 ± 4^{a}	55.8 ± 8^{b}	37.9 ± 2^{ab}
K	3.381	1309 ± 86^{a}	1243 ± 34^{a}	1095 ± 46^{a}
Mg	2.039	112 ± 5^{a}	111 ± 2^{a}	103 ± 2^{a}
Mn	2.349	107 ± 4^{a}	133 ± 15^{a}	149 ± 18^{a}
Na	16.637	107 ± 5^{b}	84 ± 1^{a}	83 ± 3^{a}
P	9.492	253 ± 12^{a}	298 ± 4^{b}	270 ± 6^{b}
S	9.005	123 ± 8^{a}	149 ± 3^{b}	121 ± 4^{a}
Si	1.334	5.6 ± 0.7^{a}	5 ± 0.5^{a}	4.5 ± 0.2^{a}
Zn	1.569	27.2 ± 2^{a}	25 ± 2^{a}	30 ± 2^{a}
N	678.127	2125 ± 20^{a}	3000 ± 11^{c}	2529 ± 18^{b}