Transactions of the Illinois State Academy of Science
(1995), Volume 88, 3 and 4, pp. 97-107

Factors Affecting Nitrogen Fixation in *Azolla caroliniana*

William D. Hechler and Jeffrey O. Dawson
Forestry Department
University of Illinois
Urbana, IL 61801

ABSTRACT

The effects of environmental variables on nitrogen fixation and growth of *Azolla caroliniana* were studied. Nitrogenase activity was highest at ~25°C. Nitrogenase activity was highest at photosynthetic photon flux densities of 400-1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and decreased sharply at lower levels. *Azolla* showed great sensitivity to drying in that nitrogenase activity was approximately halved as moisture concentration decreased from 95% to 87% of total fresh plant mass. A strong diurnal pattern of nitrogenase activity was detected; this pattern was not due to an endogenous rhythm. High plant density decreased specific nitrogenase activity per unit plant mass and per unit area; the optimal plant density for nitrogen fixation on an area basis is approximately 50-100 $g_{dry} \cdot m^{-2}$. Two or 10.0 mg NH_4-$N \cdot l^{-1}$ decreased nitrogenase activity; 10.0 mg NO_3-$N \cdot l^{-1}$ decreased nitrogenase activity while 10.0 mg urea-$N \cdot l^{-1}$ was associated with an increase in nitrogenase activity. Ten mg NH_4-$N \cdot l^{-1}$ caused a decline in Azolla growth; 10.0 mg NO_3-$N \cdot l^{-1}$ resulted in an increased growth rate.

INTRODUCTION

The genus *Azolla* comprises seven species of symbiotic nitrogen-fixing aquatic ferns. *Azolla caroliniana* is found in rivers and cypress swamps of southern Illinois. The symbiosis consists of an association between the fern *Azolla* and the cyanobacterium (blue-green alga) *Anabaena azollae*, which inhabits cavities in the leaves of the fern (Lin and Watanabe 1988, Hill 1975). The cyanobacterium carries out the process of nitrogen fixation, i.e., the enzymatic reduction of atmospheric dinitrogen to ammonia (Oes 1913, Olson 1970, Venkataraman 1962). *Azolla* is economically important as a natural source of nitrogen fertilizer for agriculture in many areas of the world where industrially manufactured nitrogen fertilizers are expensive or unavailable (Lumpkin and Plucknett 1982). *Azolla* has the potential to become important in the industrialized countries for nitrogen fertilization and soil organic matter management. Its role in the nitrogen economy of natural wetlands in North America has not been established. Physiologically, *Azolla* species and strains differ in their responses to environmental variables (Vu et al. 1986, Subudhi and Watanabe 1981). The purpose of this study was to determine the effects of temperature, photosynthetic photon flux density (PPFD), time of day, moisture, plant density, and combined nitrogen on nitrogen fixation by *Azolla caroliniana* of southern Illinois. This information could be useful in the cultivation of *Azolla* for use in

Illinois agriculture and in understanding the impacts of high nitrogen levels from pollution and other environmental factors on nitrogen fixation by this plant in wetlands.

METHODS AND MATERIALS

Azolla caroliniana in southern Illinois inhabits ponds, cypress swamps and slowly-flowing rivers. The *Azolla* studied in these experiments was collected at Cache River, Pulaski County, Illinois (37°03'30"N, 89°03'30"W) from a cypress and tupelo swamp. A single clone of *Azolla* derived from one frond was propagated vegetatively in a greenhouse on the N-free medium (pH 5.2) described in Table 1.

Nitrogen fixation by *Azolla* was estimated by the acetylene reduction assay (Stewart et al. 1967, Cornaby and Waide 1973). This assay exposes the symbiont of nitrogen fixing plants or plant parts to 10% acetylene; the nitrogenase enzyme complex breaks the triple bond of acetylene, producing ethylene, just as it ordinarily breaks the triple bond of dinitrogen as the first step in producing ammonia. Experimental plants were placed in 240 ml jars fitted with airtight lids and rubber septa. Except in the case of the moisture experiment, 40 ml of the culture medium was placed in the assay vessel along with the plants. Ten percent of the air was withdrawn by syringe from each vessel and replaced with an equal volume of acetylene. Incubation was conducted for 1.00 h; then a gas sample was withdrawn, 1.0 ml for immediate analysis or 10.0 ml for storage in vaccuum tubes for later analysis. Samples were analyzed for ethylene content by gas chromatography using a flame ionization detector.

Temperature Experiment

Three to five fronds of *Azolla caroliniana* from greenhouse stock were placed in each of five 240 ml jars open to the atmosphere in a growth chamber (Fisher Scientific #626, .75 m^3) 22 h prior to measurements on each of 10 consecutive days. Photosynthetically active radiation was provided on a 0400-2000 h schedule by a fluorescent lamp providing a photosynthetic photon flux density (PPFD) of ~125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height. The ten temperature treatments consisted of a series ranging from 0°C to 45°C in 5C° increments. The acetylene reduction assays were conducted at noon. Mean values and standard errors were calculated to determine magnitude of effect and statistically significant differences where error bars do not overlap.

Photosynthetic Photon Flux Density (PPFD) Experiment

Seven shade treatments (100, 67, 43, 27, 10, 3.0 and 1.7% full solar irradiance) were imposed using shadecloth in a greenhouse and 3-5 *Azolla* fronds per cup in 100 ml cups (4 replications per treatment) were placed under the shadecloth 24 h prior to measurements. Shading decreased the PPFD measured with a quantum meter (Li-Cor, Lincoln NE) allowing us to measure the effects of reduced solar irradiance on nitrogenase activity. The acetylene reduction assay for nitrogenase (nitrogen fixation) activity was conducted at midday on a sunny, cloudless day under ambient greenhouse conditions with PPFD values of 1500±100 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Moisture Experiment

In the laboratory 21 different *Azolla* fronds collected at midday from full light conditions in a greenhouse were dried to various degrees by placing the plants on absorbent paper towels for periods ranging from 1 to 20 minutes. The acetylene reduction assay was conducted immediately after drying at 25°C under fluorescent lamps providing a PPFD of 125 μmol·m^{-2}·s^{-1} in the laboratory. Moisture content was determined by comparing fresh mass (immediately after assay) to oven-dry (70°C) mass by the equation:

$$H_2O \text{ content } (\%) = ((\text{mass}_{\text{fresh}} - \text{mass}_{\text{dry}}) \div \text{mass}_{\text{fresh}}) \text{ X } 100 \qquad \text{(Equation 1)}$$

The best fit of a regression line was determined by comparing r^2 values for linear and curvilinear regression equations, and the equation yielding the highest r^2 value was plotted together with the data points.

Diurnal Nitrogenase Activity Experiment

The acetylene reduction assay was conducted on five sets of *Azolla* plants every three h from 0000 to 2100 in the greenhouse under natural illumination only on a sunny, cloudless day, photoperiod ~14 h, under the temperature ranges described previously. Maximum PPFD was 1500 μmol·m^{-2}·s^{-1} and occured at noon. The purposes of this study were to confirm that that midday sample on cloudless days had the highest daily nitrogenase activity values and to confirm the probability that nitrogenase activity is closely linked with photosynthesis. Also, four other experiments were conducted to determine whether the diurnal pattern depends on an endogenous circadian rhythm of nitrogenase. The plants were subjected to various treatments and assayed for nitrogenase activity at 3, 4, or 12 (day-night) intervals. Treatments were 1) complete darkness for 3 d, assay every 3 h; 2) low PPFD (20 μmol·m^{-2}·s^{-1}) for 3 d, assay every 4 h; 3) low PPFD for 3 d, moderate PPFD (150 μmol·m^{-2}·s^{-1}) during assay every 4 h; 4) moderate PPFD for 3 d, assay noon and midnight. Means and standard errors were calculated for each sample treatment.

Plant Density Experiment

Azolla fronds were placed in 100 ml cups 22.8 cm^2 in surface area at various densities (1-20 fronds) in the greenhouse, photoperiod extended in the evenings to 14 h by high-pressure sodium-vapor lamps. Then they were allowed to grow further until about half of the samples were visibly crowded (2 weeks). Nutrient solution (Table 1) was changed midperiod. The acetylene reduction assay was conducted at midday in the greenhouse under ambient temperature and PPFD conditions (26°C and 1400-1500 μmol·m^{-2}·s^{-1}). Acetylene reduction values were expressed per g dry mass. The best fit of a regression line was determined by comparing r^2 values for linear and curvilinear regression equations. The regression equation yielding the highest r^2 value was plotted together with the data points.

Combined Nitrogen Experiment

Stock solutions of KNO_3, NH_4Cl, and urea were made up at a concentration of 1.00 g N·l^{-1}. *Azolla* fronds were weighed after a 1.0 h blot on absorbent paper towels and placed in 100 ml cups containing 3 levels of nitrogen in the standard nutrient solution (Table 1) for each source: 0, 2.0 and 10 mg N·l^{-1}. The addition of nitrogen compounds to the nutrient solution resulted in no discernible change in pH. After 16 d, each cup was

assayed for nitrogenase activity at 29°C and 1400-1500 μmol·m^{-2}·s^{-1}, then weighed. Mean relative growth rate was determined by the equation

$$RGR_{1-2} = (\ln mass_2 - \ln mass_1) \div (T_2 - T_1) \qquad \text{(Equation 2)}$$

where RGR_{1-2} is the mean relative growth rate from time 1 to time 2;

$mass_2$ and $mass_1$ are mass at the end and mass at the beginning of the experiment, respectively; and

$T_2 - T_1$ is the time interval of the experiment; in this case, expressed as days.

RESULTS AND DISCUSSION

Azolla caroliniana nitrogenase activity was strongly dependent on temperature (Figure 1). No activity occured at 0°C, and only a very small amount could be detected at 5°C. Activity rose sharply above 15°C, and reached its maximum at 25°C. Nitrogenase activity declined precipitously from 35°C to 40°C and could not be detected at 45°C.

Azolla caroliniana carries out nitrogenase activity over a wide range of PPFD levels with maximum values occuring between PPFDs of 400 and 1000 μmol·m^{-2}·s^{-1} (Figure 2). Full solar irradiance (1500 μmol·m^{-2}·s^{-1}) was slightly but significantly ($p<0.05$) inhibitory to nitrogenase activity compared to 1000 μmol·m^{-2}·s^{-1}. Nitrogenase activity declined significantly ($p<0.05$) between 400 and 150 μmol·m^{-2}·s^{-1}, between 150 and 50 μmol·m^{-2}·s^{-1}, and between 50 and 25 μmol·m^{-2}·s^{-1}. *Azolla caroliniana* seems to be capable of fixing nitrogen at high or moderate levels over a wide range of light conditions characteristic of the understory of swamp forests.

Azolla caroliniana was extremely sensitive to moisture deficiency (Figure 3). Maximum nitrogenase activity occured when moisture content of *Azolla* tissue was about 88% to 95% of fresh mass and decreased to less than one fifth of the maximum at around 80% moisture content. Nitrogenase activity below 80% moisture content was negligible.

Azolla caroliniana showed a definite diurnal pattern of nitrogenase activity (Figure 4). It was very low early in the morning, climbed sharply in midmorning, and reached its peak from about 1200 to 1500 h. It then declined to a low level in the evening. In the circadian rhythm experiments there were no significant differences between day and night acetylene reduction rates under constant conditions. Nitrogenase activity went to zero within three hours and never increased in darkness, and remained constant throughout the light treatments at a level proportional to light intensity. Results indicate that *Azolla* nitrogenase activity is strictly under the control of environmental factors, especially temperature and light, and does not show endogenous patterns of activity.

Plant density markedly affected nitrogenase activity (Figure 5). A definite linear response occured over the range from 20 to 125 g·m^{-2} (dry mass basis). At 125 g·m^{-2}, nitrogenase activity declined to about one sixth of the maximum value. However, on a per-area basis, the optimum stocking density of *Azolla* appears to be about 50-100 g_{dry}·m^{-2} (Figure 6).

Response of *Azolla caroliniana* differed with each source of nitrogen. Ammonium was the most inhibitory to *Azolla* growth; nitrate treatments caused a slight increase in growth (Table 2). *Azolla* growth was not sensitive to urea. Two mg NH_4-N·l^{-1} was sufficient to cause a decrease in nitrogenase activity; 10.0 mg NH_4-N or NO_3-N·l^{-1} caused decreased nitrogenase activity. Nitrogenase activity at the 10.0 mg urea-N·l^{-1} level was nearly 50% higher than that of the control. It is possible that the effects may have been due to pH changes caused by uptake of different forms of nitrogen. However, this is unlikely, because *Azolla* species are relatively insensitive to changes in pH between 5.0 and 6.0 with respect to nitrogen fixation, and have some capacity to buffer their own environments (Vu et al. 1986).

Maximum acetylene reduction values between some experiments differed due to experimental conditions. In the temperature and moisture studies, the acetylene reduction assay was conducted under fluorescent lights at PPFDs of about 125 μmol·m^{-2}·s^{-1} and yielded relatively low maximum nitrogenase activity values; in the other experiments, the assay was conducted under ambient greenhouse conditions. Some variation in maximum nitrogenase activity values, probably due to seasonal factors, was also found between the greenhouse assays in different experiments. These differences do not influence the response of plants within an experiment relative to the maximum value with a changing environmental factor.

The growth and nitrogenase activity response of *Azolla caroliniana* of southern Illinois is typical for most *Azolla* species worldwide. The temperature response of *Azolla caroliniana* nitrogenase is characteristic of nitrogenase in general (Alexander et al. 1974, Heath et al. 1988, Kallio 1973) and temperate species of *Azolla* in particular (Vu et al. 1986). The response of southern Illinois *Azolla* nitrogenase to PPFD does not differ markedly from that recorded for *Azolla* elsewhere (Peters et a1. 1980). *Azolla caroliniana* is highly sensitive to drying as quantified in this study. The response of *Azolla* nitrogenase activity to drying has not been previously quantified. Similarly, the effects of specific *Azolla* densities on nitrogenase activity, and optimum densities for nitrogen fixation, had not previously been determined. The finding that crowding (>100 g_{dry}·m^{-2}) adversely affects nitrogen fixation suggests that a sound aquacultural management technique for nitrogen production by *Azolla* is to thin overcrowded ponds, possibly composting or incorporating into soil the harvested excess material. *Azolla* is highly sensitive to ammonium; the presence of this ion in, for instance, farm ponds or other bodies of water subject to ammonium-rich agricultural runoff could preclude the growth of *Azolla*. Although nitrate at concentrations up to 10.0 mg N·l^{-1} does not seem to affect the growth of *Azolla* adversely, the reduction of nitrogenase activity associated with it indicates that in nitrate-rich waters, a large portion of the nitrogen content of *Azolla* will be nitrogen taken up from the water, not enzymatically fixed nitrogen derived from the air.

Results clearly indicate the potential of *Azolla* to fix significant amounts of nitrogen under a range of environmental conditions possible in Illinois wetland ecosystems and that ammonium is particularly inhibitory to *Azolla* nitrogen fixation. The importance of *Azolla* to the nitrogen economy of southern Illinois native wetlands, and its potential for improving wildlife habitat and as a beneficial crop in aquaculture and agriculture, merit further attention.

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Table 1. Composition of *Azolla* culture medium.

Compound	Concentration	Compound	Concentration
KCl	74.5 mg/l	H_3BO_3	1.13 mg/l
$CaCl_2 \cdot 2H_2O$	147.0 mg/l	$MnSO_4 \cdot H_2O$	0.78 mg/l
KH_2PO_4	27.1 mg/l	$ZnSO_4 \cdot 7H_2O$	0.12 mg/l
$MgSO_4 \cdot 7H_2O$	98.5 mg/l	MoO_3	0.14 mg/l
Fe as EDTA	2.5 mg/l	$CuSO_4 \cdot 5H_2O$	0.06 mg/l

Table 2. Effect of three nitrogen sources at two nitrogen concentrations on growth and acetylene reduction (ethylene evolution) of *Azolla caroliniana*.

treatment	RGR[1]	C_2H_4 evolved[2]	treatment	RGR	C_2H_4 evolved	treatment	RGR	C_2H_4 evolved
control -N	.194[a]	49.6[a]	control -N	.194[a]	49.6[a]	control -N	.194[a]	49.6[a]
NO_3-N 2mg/l	.221[ab]	63.0[a]	NH_4-N 2mg/l	.178[a]	33.2[b]	urea-N 2mg/l	.200[a]	58.2[a]
NO_3-N 10mg/l	.227[b]	29.8[b]	NH_4-N 10mg/l	.117[a]	4.6[c]	urea-N 10mg/l	.199[a]	73.8[b]

Means within a column followed by the same superscript are not significantly different ($p<0.05$).

[1] RGR is expressed as $g \cdot g^{-1} \cdot d^{-1}$.

[2] C_2H_4 evolution is expressed as $\mu mol \cdot g^{-1} \cdot h^{-1}$.

Figure 1. Effect of temperature on nitrogenase activity (acetylene reduction) in clonal *Azolla caroliniana*. (Points represent means, vertical bars represent standard errors, n=5.)

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Figure 2. Effect of photosynthetis photon flux density (PPFD) on nitrogenase activity in clonal *Azolla caroliniana*. (Points represent means, vertical bars represent standard errors, n=4.)

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Figure 3. Effect of moisture content on nitrogenase activity in clonal *Azolla caroliniana.* (Points represent nitrogenase activities for 21 individual *Azolla* fronds at varying degrees of dessication. Regression line yielding highest r^2 value, $y = (0.24x - 16.17)^2$, shown in figure.)

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Figure 4. Diurnal pattern of nitrogenase activity in clonal *Azolla caroliniana.* (Points represent means, vertical bars represent standard errors, n=5.)

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Figure 5. Effect of plant density on nitrogenase activity in clonal *Azolla caroliniana* per g dry mass of plant tissue. (Points represent nitrogenase activities for 31 samples of *Azolla* fronds at varying densities. Linear regression line yielding highest r^2 value, y = (-0.29x - 43.8), shown in figure.)

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Figure 6. Effect of plant density on nitrogenase activity in clonal *Azolla caroliniana* per square meter of surface area. (Points represent nitrogenase activities for 31 samples of *Azolla* fronds at varying densities. Regression line yielding highest r^2 value, $y = -77.068 + 54.548x - 0.49535x^2 + 0.001268x^3$, shown in figure.)

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