

NITROGEN FIXATION BY AZOLLA CULTURED IN NUTRIENT ENRICHED WATERS¹

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ABSTRACT

Growth and nitrogen (N) fixation potential of *Azolla caroliniana* Willd, cultured in nutrient-enriched wastewaters, was evaluated under greenhouse conditions. Nitrogen fixation rates were determined by acetylene reduction assay (ARA), ¹⁵N₂ fixation, and ¹⁵N dilution methods. Specific growth rates were found to be in the range of 0.069 to 0.115 day⁻¹ for *A. caroliniana* cultured in various types of wastewaters. Nitrogen fixation by *A. caroliniana* was inversely related to NH₄⁺ concentration of the water. The molar ratio of C₂H₄ production/¹⁵N₂ fixation was 4.43 ± 0.34. The optimal temperature for *A. caroliniana* growth was 25 C, the rate of N₂ fixation (determined by ¹⁵N dilution) was found to be highest at 30 C. All three methods for measuring N₂ fixation produced satisfactory results. The use of ¹⁵N₂ produced direct documentation of N₂ fixation by *A. caroliniana*.

Key words: Water fern, Lake Apopka, specific growth rate, acetylene reductase, sewage effluent.

INTRODUCTION

Azolla spp., a floating aquatic macrophyte, is widely distributed throughout the tropical and temperate freshwater ecosystems of the world (8). There are about six species of

A. caroliniana, and all species usually contain an N₂-fixing cyanobacterium of the *Nostoc-Anabaena* group as a symbiont (9). The N₂-fixation potential of this association has been recognized as an N source in rice production (7,10,16,20). More recently, the potential use of *A. caroliniana* in wastewater treatment was evaluated (11).

In recent years, there has been significant interest in the use of aquatic macrophytes for treating nutrient-rich wastewaters. Treatment systems usually incorporate retention ponds or wetlands in which selected aquatic macrophytes such as water hyacinth (*Eichhornia crassipes* [Mart] Solms) (15), cattails (*Typha* spp.), and reeds (*Phragmites* spp.) (6,21) are cultured in wastewater pumped through the system at a predetermined flow rate. These plants have been found to be highly efficient in removing N from primary and secondary sewage effluents, but the effluent leaving these systems usually contains unacceptable high levels of P (12,17). *Azolla*, on the other hand has a high P-assimilation capacity (11) and an ability to grow in N-limiting waters. As a result, *Azolla* becomes a potentially good candidate for possible inclusion in these systems for increased efficiency and effectiveness.

To evaluate the potential of *Azolla* for use in either water treatment or rice production systems, it is important to develop a better understanding of the physiological processes involved, including N₂ fixation and environmental influences (9). The N₂-fixation potential of *Azolla* is usually measured by C₂H₂ reduction assay. Although this method is rapid and sensitive, it does not provide a direct measurement of the fixation process. Use of the stable isotope ¹⁵N

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in fixation studies is less common, because of the high cost of isotopes and frequent lack of necessary instruments and facilities. The purpose of this study was to compare three methods of N₂-fixation measurement, *i.e.*, 1) acetylene reduction assay (ARA); 2) isotopic (¹⁵N) dilution of *Azolla* cultured N-free medium; and 3) direct incorporation of ¹⁵N₂ into the plant tissue. These methods were tested on *A. caroliniana* cultured at varying temperatures and in different types of nutrient-enriched waters.

MATERIALS AND METHODS

Azolla used in the study was initially obtained from the St. Johns River marsh, near Sanford, Florida. The plants collected were tentatively identified as *Azolla caroliniana* Willd, the species most commonly found in freshwater ecosystems of the southeastern United States. Plants were cultured in N-free medium until used in the following experiments:

Experiment I

This study was conducted to compare two N₂-fixation methods, *i.e.*, ARA and ¹⁵N₂ incorporation, for *A. caroliniana* cultured in a variety of wastewaters as indicated below (Table 1):

1. Tap water
2. Nitrogen-free nutrient medium
3. Nitrogen-enriched medium
4. Primary sewage effluent (untreated)
5. Primary sewage effluent (treated using a water hyacinth system)
6. Secondary sewage effluent (untreated)
7. Secondary sewage effluent (treated using a water hyacinth system)
8. Eutrophic lake water (from Lake Apopka)

Primary sewage effluent (after solids were settled in a clarifier) was obtained from Walt Disney World sewage

treatment plant (14). A portion of this primary sewage effluent was allowed to flow through channels containing water hyacinths to achieve secondary treatment. Effluent leaving this system was used in *Azolla* experiments. Secondary sewage effluent was also obtained from the same sewage treatment plant. This effluent was allowed to flow through hyacinth channels to obtain advanced treatment (15).

Eutrophic lake water was obtained from Lake Apopka located in central Florida. This lake is highly eutrophic as a result of both external and internal nutrient loading (14).

To acclimatize *A. caroliniana* to wastewaters, bulk cultures were maintained for 4 weeks in 70-L plastic tubs containing the wastewaters, as described above under greenhouse conditions. One liter of wastewater was placed in plastic containers containing *A. caroliniana* (5 g fresh wt/ container) obtained from bulk cultures with each treatment was replicated three times. Plants were grown in wastewater for an additional period of 7 days under greenhouse conditions (October 25, 1984, to November 3, 1984). Average minimum and maximum temperatures in the greenhouse during the study period were 16.2 ± 0.9 C and 30.5 ± 1.8 C, respectively. At the end of 7 days, fresh weights of the plants were recorded. A subsample was dried at 70 C for a period of 48 hr to determine % dry weight in the tissue. A portion of the fresh plants were washed and then transferred into Mason jars containing 100 ml of culture medium. The head space containing air was enriched with 10% C₂H₂ or 10% ¹⁵N₂ gas enriched (99 atom % ¹⁵N excess). Total air pressure in the jar was about 1 atm. Mason jar caps were fitted with a rubber septum through which gas samples were withdrawn for analysis. All jars were placed in a water bath maintained at about 25 C for a period of 2 hr, after which 5 ml gas samples were drawn from the jars and analyzed for C₂H₄ using a gas chromatograph. Plant samples were analyzed for both total N and labeled N.

TABLE 1. SELECTED WATER QUALITY PARAMETERS OF THE WATER USED TO CULTURE *A. CAROLINIANA*.

Culture medium	Chemical parameters						BOD ₅ ¹
	pH	NO ₃ -N	NH ₄ -N	Org-N	Total P	Ortho-P	
1. Tap water	7.0	<0.1	ND ²	ND	ND	ND	ND
2. N-free nutrient medium	7.1	0.0	0.0	0.0	1.0	1.0	ND
3. N-enriched medium	7.2	0.0	2.0	0.0	1.0	1.0	ND
4. Primary sewage effluent (untreated)	7.5	<0.1	28.5	9.5	6.5	5.9	224
5. Primary sewage effluent (treated using a water hyacinth system)	6.8	<0.1	19.4	6.5	6.0	5.5	55
6. Secondary sewage effluent (untreated)	7.2	0.8	2.7	2.9	1.1	0.9	14
7. Secondary sewage effluent (treated using a water hyacinth system)	6.9	0.1	0.1	0.7	0.1	0.05	3
8. Eutrophic lake water (Lake Apopka)	8.3	0.2	0.1	3.1	0.4	0.1	15

¹BOD₅ = Biological oxygen demand (20 C).

²ND = Not detectable.

Experiment II

This study was conducted to determine the effect of temperature on growth and N₂ fixation of *A. caroliniana* cultured in N-free nutrient medium. *A. caroliniana* (50 g fresh wt) was placed in two 70-L plastic tubs containing 5%-strength modified Hoagland's nutrient medium enriched with 2 mg N l⁻¹ as ¹⁵NH₄Cl (5 atom % ¹⁵N excess), and allowed to grow for 15 days, with nutrients replaced with fresh medium after 7 days. This ¹⁵N-enriched *A. caroliniana* was used in the following experiment to determine N₂ fixation as measured by isotopic (¹⁵N) dilution in the plant tissue:

Six plastic containers, each containing one liter of nutrient medium were placed in each of the growth chambers maintained at constant temperatures of 10, 15, 20, 25, and 30 C, with 16 hr light (200 E m⁻² sec⁻¹) daily using four daylight 40-W fluorescent bulbs. Light transmission was measured with a Li-cor, Inc., Lincoln, NB) equipped with a photometric sensor. One set (three containers per each temperature) of containers was stocked with *A. caroliniana* (5 g fresh wt/container) previously cultured in N-free nutrient medium, while the second set was stocked with ¹⁵N-enriched *A. caroliniana*. At the end of a 7-day growth period, ¹⁵N-enriched *A. caroliniana* was exposed to C₂H₂ for a period of 2 hr as described in Experiment I, and the amount of C₂H₄ production was determined. Fresh weight of the plants was recorded at the end of ARA assay. Specific growth rates were calculated using the changes in biomass during the 7-day growth period (13). All plant samples were analyzed for total and labeled N content. Nitrogen fixation due to ¹⁵N dilution of the plant tissue was calculated as follows:

$$N_2 \text{ fixed} = [1 - ((AN_t - NA)/(AN_o - NA))] \text{TKN}$$

where

AN_t = Atom % ¹⁵N in the plant tissue of *Azolla* at the end of the growing period,

NA = Natural abundance of ¹⁵N in the air sample

AN_o = Atom % ¹⁵N in the plant tissue at the beginning of the growing period

TKN = Total Kjeldahl N in the plant tissue at the end of the growing period

Analytical Methods:

Plant samples were dried in an oven at 70 C for a period of 48 hr. Nitrogen in the plant tissue was determined using a micro Kjeldahl method (4). All samples were analyzed for ¹⁵N content on an isotope ratio mass spectrometer (Micromass 602E) using the method described by Hauck (5). Gas samples (5 ml) were drawn from Mason jars with a hypodermic needle and syringe and stored in evacuated tubes. At all times, gas storage period in the tubes was less than 24 hr. Gas samples were analyzed for C₂H₄ with a 5840 Hewlett Packard gas chromatograph equipped with a flame ionization detector and a 1.83 m stainless steel column packed with Porapak N material (80-100 mesh). The temperature settings were: 70 C for column temperature, and 150 C for interface temperature. Water samples were analyzed for selected parameters using standard methods (1).

TABLE 2. GROWTH RATES AND NITROGEN FIXATION BY *A. CAROLINIANA* CULTURED IN DIFFERENT TYPES OF NUTRIENT-ENRICHED WATERS.

Culture medium	Specific growth rate day ⁻¹	¹⁵ N ₂		
		ARA nmol C ₂ H ₄ g ⁻¹ h ⁻¹	fixation nmol N ₂ g ⁻¹ h ⁻¹	C ₂ H ₄ / ¹⁵ N ₂ molar ratio
Tap water	0.069 ±0.003 ¹	431.0 ±41.4	96.9 ±5.4	4.45
N-free nutrient medium	0.097 ±0.003	613.7 ±138.7	162.6 ±18.4	3.77
Primary sewage effluent (untreated)	0.112 ±0.008	257.0 ±16.3	53.7 ±2.4	4.79
Primary sewage effluent (treated using a water hyacinth system)	0.093 ±0.003	298.3 ±50.7	64.9 ±5.8	4.60
Secondary sewage effluent (untreated)	0.115 ±0.003	456.7 ±21.3	100.7 ±2.8	4.54
Secondary sewage effluent (treated using a water hyacinth system)	0.115 ±0.004	404.3 ±35.1	85.5 ±1.9	4.73
Eutrophic lake water (Lake Apopka)	0.105 ±0.006	531.0 ±110.5	128.7 ±1.4	4.12

¹± Standard deviation.

RESULTS

Growth rates of *Azolla* were not significantly influenced by the culture medium (Table 2) except for the plants cultured in tap water. Specific growth rate (SGR) was in the range of 0.069 to 0.115 day⁻¹ (doubling times = 6 to 10 days). Doubling times observed in this study were slightly lower than those reported by other researchers for different species of *Azolla*.

Growth rates of *A. caroliniana* were influenced by the ambient air temperature, with maximum SGR observed at 25 C (Fig. 1). A 10 C rise in temperature between 10-25 C resulted in a doubling of the SGR values. Growth rates, however, decreased when ambient air temperature was increased to 30 C.

Nitrogenase activity of *A. caroliniana* was found to be influenced by the culture medium (Table 2). In general, ARA was found to be inversely related to NH₄⁺ concentration of the culture medium. Plants cultured in primary sewage effluent and NH₄⁺-enriched nutrient medium exhibited lower ARA compared to other culture media evaluated. Ammonium N content of the untreated secondary sewage effluent was 2.7 mg N l⁻¹, but the ARA values of *A. caroliniana* cultured in this water were much higher than those observed for *A. caroliniana* cultured in ¹⁵NH₄-N-enriched nutrient medium (Table 3). These results suggest that NH₄⁺ may not be the sole inhibitor of N₂ fixation. Nitrogen fixation measured by exposure of the plants to ¹⁵N₂ showed a similar trend. The molar ratio of C₂H₄/N₂ was 4.43 ± 0.34 for *A. caroliniana*.

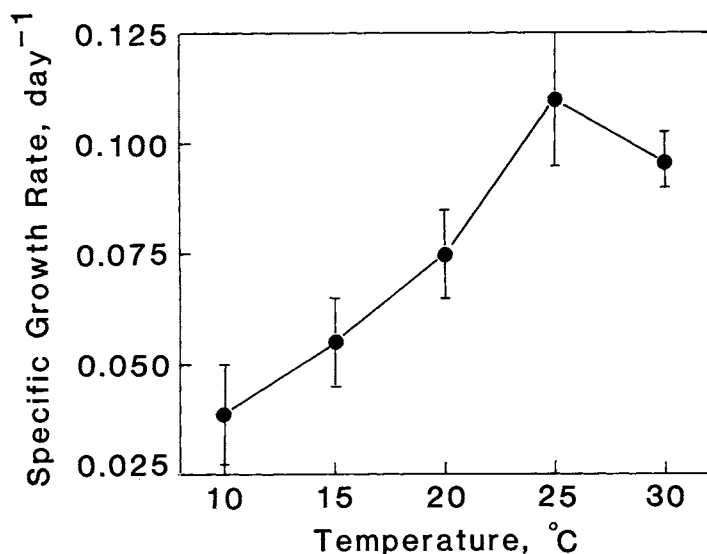


Figure 1. Specific growth rates of *A. caroliniana* as influenced by ambient air temperatures.

Data in Table 3 show a comparison among the three methods used to measure N_2 fixation. Growing *A. caroliniana* in ^{15}N -enriched medium resulted in a decrease in N_2 fixation, as compared to plants cultured in N-free medium. The molar ratio of C_2H_4/N_2 was found to be approximately the same for either of two ^{15}N methods, *i.e.*, ^{15}N dilution and $^{15}N_2$ incorporation.

Since growing *A. caroliniana* in ^{15}N -enriched medium affected N_2 fixation (Table 3), these plants were grown further in N-free medium for a period of 2 weeks before they were used in a growth chamber experiment where N_2 fixation activity was measured at varied temperatures. After 2 weeks of growth on N-free medium, the repression of nitrogenase activity due to growth on $^{15}NH_4^+$ enriched water was completely alleviated and the activity restored to the level of that present in plants grown in the absence of a combined N source (Table 4). Nitrogenase activity, however, was affected by temperature, with maximum activity observed between 20-30 C. Results presented in Table 5 show that maximum isotopic dilution in the plant tissue as a result of N_2 fixation at 30 C.

Data on the relationship between N_2 fixation estimated by isotopic method and ARA are shown in Fig. 2. *A. caroliniana* cultured under low light conditions (growth chambers) generally produced low N_2 fixation rates, as

TABLE 3. NITROGEN FIXATION BY *A. CAROLINIANA* AS DETERMINED BY THREE METHODS.

Method	Nutrient medium	
	Enriched with N	No N added
	nmol g ⁻¹ (fw) hr ⁻¹	
ARA	311.7 ± 9.7 ¹	613.7 ± 138.7
^{15}N dilution	91.4 ± 3.9	—
$^{15}N_2$ incorporation	—	162.6 ± 18.4
$C_2H_4/^{15}N_2$	3.41 ± 0.11	3.77 ± 0.49

¹ ± Standard deviation.

TABLE 4. RELATIVE RATES OF ACETYLENE REDUCTION (ARA) BY *A. CAROLINIANA* CULTURED AT VARIED AMBIENT AIR TEMPERATURES.

Temperature (C)	Non-labeled medium	^{15}N -enriched <i>Azolla</i>
	nmol C_2H_4 g ⁻¹ (fw) hr ⁻¹	
10	42.5 ± 12.8 ¹	36.9 ± 7.9
15	88.8 ± 11.2	92.2 ± 7.4
20	186.8 ± 4.6	200.6 ± 28.8
25	190.9 ± 6.1	186.1 ± 4.6
30	166.1 ± 51.1	185.5 ± 41.5

¹ ± Standard deviation.

TABLE 5. NITROGEN FIXATION BY *A. CAROLINIANA*, AS ESTIMATED BY USING ^{15}N DILUTION AND ARA, AT VARYING AMBIENT AIR TEMPERATURES.

Temperature (C)	^{15}N in <i>Azolla</i> ¹ after N_2 fixation	N_2 fixation after ^{15}N dilution	$C_2H_4/^{15}N_2$ molar ratio
	atom % ^{15}N	nmol N_2 g ⁻¹ (fw) hr ⁻¹	
10	1.999 ± 0.100 ³	34.4 ± 3.2	1.2
15	1.995 ± 0.105	35.4 ± 3.6	2.6
20	1.655 ± 0.110	65.3 ± 5.0	3.1
25	1.401 ± 0.005	78.0 ± 4.2	2.4
30	1.220 ± 0.110	105.2 ± 11.3	1.8

¹ ^{15}N content of the *A. caroliniana* before N_2 fixation = 2.435 ± 0.105 atom % ^{15}N .

²For ARA values, see Table 4.

³ ± Standard deviation.

compared to plants cultured under greenhouse conditions. Highest correlation ($r=0.934^{**}$) was observed between the ARA method and the $^{15}N_2$ fixation method evaluated on *A. caroliniana* cultured under greenhouse conditions using a variety of nutrient-enriched waters. Correlation between the ARA method and the ^{15}N dilution method was found

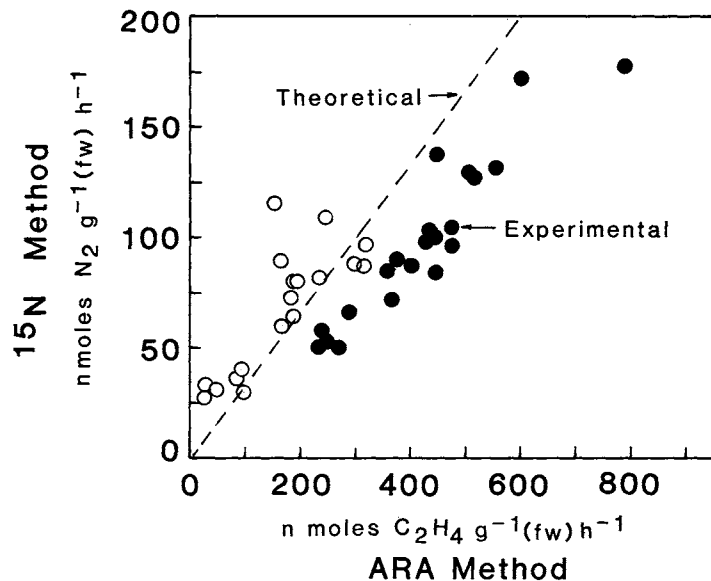


Figure 2. Relationship between N_2 fixation measured by ^{15}N and acetylene reduction methods. Open circles represent ^{15}N dilution method and closed circles represent $^{15}N_2$ fixation method.

to be lower ($r=0.747^{**}$), although the correlation coefficient was highly significant ($P \leq 0.01$).

DISCUSSION

The potential of using *Azolla* in rice culture as a source of N is fairly well documented (3,7,10,16,19). However, limited information is available on the ecological and environmental significance of this plant. Results presented in this study show that *A. caroliniana* can be cultured in a variety of nutrient-enriched wastewaters with varied chemical composition. *Azolla* growth rates observed in this study were generally lower than those reported in the literature. Under controlled conditions, several researchers (9,18,20) observed doubling times of less than 7 days for *A. filiculoides* Lam, *A. mexicana* Presl, and *A. caroliniana* as compared to 6-10 days observed in this study for *A. caroliniana* cultured in wastewaters.

Utilization of *Azolla* in water treatment also depends on its capability to adapt to a wide range of temperatures encountered under field conditions. Optimal temperature for growth of *A. caroliniana* was found to be about 25 C. Optimal temperature for *A. mexicana* was reported to be about 30 C (18), while 27.5 C was found to be optimal for *A. filiculoides* (2). Under central Florida conditions, Reddy and DeBusk (13) observed very little or no seasonal variability for *A. caroliniana* cultured in N-free medium.

The N_2 -fixing activity of *Azolla* is usually estimated by acetylene reduction activity (ARA) and rates are usually calculated by a conversion factor of 3 (3 moles of $C_2H_2/1$ mole of N_2). C_2H_2 reduction requires the transfer of 2 electrons while N_2 reduction requires 6. Direct evidence of N_2 fixation by *Azolla* is scarce, because of limitations involved in the use of ^{15}N tracer. Results reported in this paper indicate that the ratio of C_2H_4 production to $^{15}N_2$ fixation was in the range of 3.4 to 4.8. These values are in the same range as those presented by Peters et al. (9) and Watanabe et al. (20) for *A. caroliniana* and *A. pinnata*, respectively.

All three methods used to measure N_2 fixation produced satisfactory results. Low light levels under growth-chamber conditions resulted in low N_2 fixation by *A. caroliniana*, as compared to N_2 fixation by plants cultured under greenhouse conditions. The N_2 fixation rates reported in this study are in the same range as those reported for other *Azolla* species (3,9,18,20). Although the ^{15}N dilution technique produced satisfactory results, it has several limitations. This technique assumes N_2 fixation as the sole source of N for *Azolla*. This would be true if *Azolla* is cultured under N-free medium. However, when *Azolla* are cultured in wastewaters or in paddy fields, a significant amount of N will be derived by *Azolla* from wastewater or soil. Using the ^{15}N dilution technique, these sources are not differentiated from N_2 fixation. In basic physiological studies or screening studies where *Azolla* is cultured in N-free culture medium, this technique can be used quantitatively to estimate N_2 fixation rates.

In conclusion, this study has shown that *A. caroliniana* can be successfully cultured in nutrient-enriched wastewaters, without significantly influencing their N_2 -fixation rates. Optimal temperature for *A. caroliniana* growth and

N_2 fixation was found to be about 25-30 C. Nitrogen fixation rates were inversely related to the NH_4^+ concentration of the wastewater used to culture *Azolla*. Use of the ^{15}N technique provided direct documentation of the N_2 -fixation potential of *A. caroliniana*.

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