Plant Physiol. (1976) 58, 798-799

Short Communication

Altered Nitrogenous Pools Induced by the *Azolla-Anabaena Azolla* Symbiosis

Received for publication June 28, 1976 and in revised form August 17, 1976

JACK W. NEWTON AND JAMES F. CAVINS
Northern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 1815 North University, Peoria, Illinois 61604

ABSTRACT

The free amino acid and ammonia pools of *Azolla caroliniana* were analyzed by quantitative column chromatography on columns capable of separating all of the nitrogenous constituents normally found in physiological fluids. Comparisons were made of plants containing symbiotic algae and grown on nitrogen-free media, plants grown on media containing nitrate, and algae-free plants also grown on nitrate media. The major feature of the data was a very high level of intracellular ammonia found in plants which contain N_2-fixing algal symbionts. In addition to the more usual amino acids, serine and cystathionine were found in the free amino acid pool.

The genus *Azolla*, small aquatic ferns of worldwide distribution, generally contains symbiotic algae resembling *Anabaena* sp. within their leaf cavities (3). These algae are capable of fixing N_2 and consequently can supply all of the nitrogenous nutrient requirements of the fern. The rapid growth of this fern and its ease in handling for laboratory studies make this plant particularly attractive as experimental material for study of plant nutrition in a symbiotic system. Relatively little work had been done with this system until Peters and Mayne (4, 5) recently made some initial characterizations of the relationship and showed the essential nature of the algae for growth of plants on N-free media.

To provide a basis for more extensive studies of the N nutrition of this symbiosis, we have recently separated and quantitatively analyzed the components of the free nitrogenous pools in these ferns with and without algal symbionts, and the results are presented in this communication.

MATERIALS AND METHODS

Plant Material. *Azolla caroliniana* was a strain originally obtained from Dr. Gerald Peters, C. F. Kettering Laboratories, Yellow Springs, Ohio. All plants were grown aseptically in shallow layers of modified Hoaglands salts (4), with or without nitrate, in 3-liter Fernbach flasks under constant illumination (about 100 ft-c white light) in air at 25 C. Plant material was collected by decantation, washed with water, and the wet plant tissue immediately treated with boiling water for 5 to 10 min. The hot extract was expressed from the plant material, centrifuged at 25,000*g* for 10 min, frozen, thawed, and recentrifuged to remove coagulated material, and extracts were then made to volume with water. Extracts were generally derived from 10 g wet plant material and contained approximately 1 μmol total ninhydrin reactive material/g. Appropriate samples were taken for amino acid and ammonia analyses on columns using an amino acid analyzer and the lithium buffer system for physiological fluids (1); the ammonia values obtained this way were also compared by microdiffusion and microcolorimetric analyses performed directly on the extracts.

RESULTS AND DISCUSSION

To provide as broad a base as possible for our comparisons, we have analyzed several dozen samples of different plant tissues of different ages. Plants were usually collected and immediately treated with boiling water to avoid deterioration which might alter pool contents or composition, and samples were either analyzed directly or stored frozen for subsequent analysis. In general, we found the pool size and composition to be rather uniform from one age of plant culture to another, provided the plants were grown under similar conditions.

Data in Table I summarize the major effect we have noted in the pools and compares ammonia analyses obtained by microdiffusion and colorimetry and the chromatographic method. As expected, ammonia content determined by direct analysis of extracts was higher, probably due to decomposition of amides during analysis. It should be noted that the levels of ammonia found in N_2-fixing plants are too high to be accounted for by hydrolysis of glutamine or any other pool constituent during sample preparation or analysis. The pool of intracellular ammonia in N_2-fixing plants can rise to as much as five to 10 times that found in plants grown on media containing a N source, and can constitute as much as half of the total pool N. This effect is greatest when one compares algae-containing and algae-free plants. It should be noted that the growth rates of plants on nitrate and N_2 are similar, except for the algae-free strain which has a slower growth rate than our other plant material. However, this algae-free strain was obtained by prolonged cultivation in media containing antibiotics, and the reason for its slower growth rate is unknown at present (4). Our data also show substantial levels of free glutamine and glutamate in all plants. This finding was of particular interest in view of the postulated

Table I. Comparison of Analyses of Plants for Ammonia

Plant Sample	Total Ninhydrin Reactive	NH_3 by Micro-diffusion[1]	Analysis on Columns		
			NH_3	Glutamic Acid	Glutamine
	nmol/gm Wet Tissue				
N_2 grown 3 weeks	980	528	423	84	224
N_2 grown 4 weeks	1090	576	438	90	254
NO^-_3 grown 3 weeks	580	106	84	150	150
NO^-_3 grown 3 weeks	460	100	94	144	140
Algae free 12 weeks	760	120	72	204	285
Algae free 8 weeks	680	80	48	180	300

[1] Determined by method of Ternberg and Hershey (8).

Table II. Components of Free Amino Acid Pools in Azolla caroliniana

All data are nmol/gram wet tissue, average of triplicate samples; relative standard deviation ± 20%. Differences among minor constituent amino acids are not considered significant.

Plant Material Medium	Algae Containing N Free	Algae Containing With NO_3^-	Algae Free With NO_3^-
Asp	20	20	40
Thr	10	10	10
Ser	40	20	30
Asn	10	10	10
Glu	80	100	180
Gln	240	140	280
Ala	30	30	30
Cyth[1]	70	50	70
Phe	20	10	20
γ Ab[2]	30	30	20
NH_3	420	80	60
Total	970	500	730

[1] Cystathionine.
[2] γ-Amino butyrate.

role of glutamine synthetase as a positive control element for N_2 fixation in microbial systems (7), and will require further study.

Data in Table II show all of the nitrogenous constituents detected in the pool. Among the free amino acids, two not typically found in pools are present. Peaks in the elution positions corresponding to cystathionine and serine were consistently observed in these extracts. These amino acids, which are metabolically interrelated and involved in methionine synthesis, could be accumulating because of some aberration in the metabolism of sulfur-containing amino acids in the fern. However, since the component free amino acids in plants can vary greatly, it is not possible to make an unambiguous interpretation of such a finding. For example, Steward and co-workers earlier found the fern *Adiantum* to contain high levels of γ-hydroxy-γ methyl glutamic acid in its free amino acid pool (2, 6). Thus, the presence of unusual pool constituents in ferns may not be atypical.

CONCLUSION

Relatively high levels of free ammonia are found in the intracellular nitrogenous pool of *Azolla* when these plants contain symbiotic blue-green algae fixing N_2. This level of internal ammonia can far exceed the concentration of any other pool constituent and constitute as much as half of the total pool N. This free ammonia, produced by the algal nitrogenase and utilized by the plant, can therefore serve as the major source of N for the symbiotic relationship.

LITERATURE CITED

1. Benson, J. V., Jr., M. J. Gordon, and J. A. Patterson. 1967. Accelerated chromatographic analysis of amino acids in physiological fluids containing glutamine and asparagine. Anal. Biochem. 18: 228-240.
2. Grobbelaar, N., J. K. Pollard, and F. C. Steward. 1955. New soluble nitrogen compounds (amino and amino acids and amides) in plants. Nature 175: 703-708.
3. Moore, A. W. 1969. *Azolla:* biology and agronomic significance. Bot. Rev. 35: 17-34.
4. Peters, G. A. and B. C. Mayne. 1974. The *Azolla, Anabaena Azollae* relationship. I. Initial characterization of the association. Plant Physiol. 53: 813-819.
5. Peters, G. A. and B. C. Mayne. 1974. The *Azolla, Anabaena Azollae* relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction. Plant Physiol. 53: 820-824.
6. Steward, F. C., R. H. Wetmore, J. F. Thompson, and J. P. Nitsch. 1954. A quantitative chromatographic study of nitrogenous components of shoot apices. Am. J. Bot. 41: 123-134.
7. Streicher, S. L., K. T. Shanmugam, F. Ausubel, C. Morandi, and R. B. Goldberg. 1974. Regulation of nitrogen fixation in *Klebsiella pneumoniae:* evidence for a role of glutamine synthetase as a regulator of nitrogenase synthesis. J. Bacteriol. 120: 815-821.
8. Ternberg, J. and F. B. Hershey. 1960. Colorimetric determination of blood ammonia. J. Lab. Clin. Med. 56: 766-776.