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Changes in Soluble Amino Acid and Polyamine Composition Associated with Increasing Plant Density and the Onset of Sporulation in *Azolla*

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Abstract

High plant density and diminished growth rate often are associated with sporulation in *Azolla* but the causal factors are unknown. As an initial approach to defining aspects of altered metabolism which might have a causal relationship to sporulation, soluble amino acids and polyamines were analyzed in selected populations of three *Azolla* species. Analyses were conducted weekly over a four week period as plant density increased in cultures grown under a 16/8 h, 28/16°C light-dark regime as well as under continuous light at a constant 24°C. Prior studies had established that the *Azolla caroliniana* population used did not sporulate under either light regime, while the *Azolla mexicana* population sporulated under both regimes and an *Azolla* sp. sporulated only under continuous light. Total soluble amino acids were higher under continuous light than under the light-dark cycle and increased with culture age and plant density under both treatments. Glutamine levels increased markedly in all species. On a percentage basis the other amino acids examined remained constant or decreased slightly. Except for the occurrence of a dipeptide unique to *A. caroliniana*, the constituents of the soluble amino acid pools in the three species grown under the same regime were comparable. Although substantial changes occurred in the levels of individual constituents of the pool in

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each species under the two photoperiods over time, these alterations in the composition of the pool appeared to be associated directly with culture age and plant density but not with the induction of sporulation. In contrast to the soluble amino acid profiles, soluble polyamines varied among the three species grown under the same regime and within the same species grown under the two regimes. Levels of spermine and homospermidine did not change appreciably in the individual species but putrescine and spermidine levels were generally higher in plants grown under continuous light than in plants grown under the light-dark cycle. In *A. caroliniana*, which did not sporulate, the putrescine levels increased steadily throughout the four week period under continuous light. In contrast, putrescine levels remained low in the two species which did sporulate under the continuous light regime. An analysis of putrescine:spermidine ratios in plants grown under the two regimes revealed a statistically significant difference between the light treatments for the *Azolla* sp; the species induced to sporulate under continuous light. Differences in the ratio were not significant for *A. caroliniana*, the species which did not sporulate under either regime or for *A. mexicana*, the species which sporulated under both conditions. These findings suggest that the onset of sporulation in this *Azolla* species is correlated with a significant alteration of polyamine metabolism and that these compounds may be involved in the induction of sporulation.

Keywords: *Azolla*, soluble amino acids, polyamines, sporulation, symbiosis

1. Introduction

Azolla is heterosporous, producing both microsporocarps containing microsporangia which yield microspores and megasporocarps, each of which contains a megasporangium which yields a single megaspore. The endophyte is partitioned into developing sporocarps (Campbell, 1893; Perkins and Peters, 1993) and, following gametogenesis and embryogenesis, the endophyte in the megasporocarps re-establishes the symbiosis (Campbell, 1893; Peters and Perkins, 1993). As the continuity of the symbiosis is maintained through the sexual cycle as well as during vegetative propagation, there is no obvious need for a free-living form of the cyanobiont. In fact, the cyanobiont may well be an obligate symbiont.

Although dried sporocarps can be stored and used successfully as an inoculum (Kannaiyan, 1993), the specific factor(s) which control the initiation of the sexual cycle in *Azolla* remain unknown. In most instances the inability to control the process of sporulation has necessitated vegetative maintenance of the sporophytes, as opposed to storage of spores, during the off-season. This and other labor intensive aspects of *Azolla* use as a green manure for rice, along with readily available fertilizer nitrogen in many regions, have contributed to

markedly lessened interest in *Azolla* as an alternative nitrogen source. In addition, the inability to control sporulation has precluded a reliable source of material for use in breeding programs and for the long-term germplasm storage.

Sporulation in field grown *A. filiculoides* has been attributed to a combination of environmental factors including light intensity, temperature, and nutrient availability (Ashton, 1977) as well as to a high plant density and confluence of frond material (Talley and Rains, 1980). Our observations of cultures of several other species which sporulated in outdoor pots clearly suggest that both specific environmental factors and high plant density are associated with the onset of sporulation. However, while such physical parameters clearly appear to have an effect in initiating the process of sporulation, the question remained as to what was casual. High plant density has been shown to be associated with a significant reduction in growth and with diminished nitrogenase activity (Becking, 1979; Peters et al., 1980; Tung and Watanabe, 1983) and soluble polyamines are recognized as regulators of plant development (Evans and Malmberg, 1989). Therefore we elected to determine whether or not the soluble amino acid pools and/or soluble polyamine levels were altered as a consequence of crowding and diminished nitrogenase activity in both sporulating and non-sporulating *Azolla* species.

2. Materials and Methods

Plant material and growth conditions

A. caroliniana Willd. (wtv in the collection of G.A.P; CA3001 in the collection at the International Rice Research Institute), *A. mexicana* Presl (BRGL in the collection of G.A.P.; see Toia et al., 1987 for details) and an isolate originally considered to be *A. filiculoides* but designated *Azolla* sp. based on the perine ultrastructure of the sporocarp (Perkins et al., 1985) were cultured aseptically on an N-free liquid (IRRI) medium (Peters et al., 1980) under two culture regimes. One regime consisted of a 26/15°C, 16/8 hr light/dark cycle with a photon flux density of 110–120 μmoles m^{-2} sec^{-1} provided by fluorescent and incandescent bulbs at a 3:1 energy mixture. The other regime consisted of a constant 24°C under continuous light at 230–275 μmoles m^{-2} sec^{-1} provided by fluorescent and incandescent bulbs at a 5:1 energy mixture. For each regime and species, three 125 ml Erlenmeyer flasks, each containing 50 ml of sterilized medium and capped with cotton sandwiched between several layers of cheesecloth secured to the top of the flask with a rubber band, were inoculated with 0.1 g blotted fw of frond material. Stock cultures were maintained under the 16/8 hr light-dark regime.

Sampling, extraction and HPLC analysis

Flasks were harvested at the times indicated in the Results and extracted for amino acid or polyamine (PA) analysis. For amino acid analysis, one or more samples of plant material (0.1–0.2 g fw) was removed from each of the three flasks, blotted, weighed, and extracted for 10 min in 2 ml of water in a boiling water bath. Each study was repeated two to four times. The soluble amino acids were analyzed as their o-phthalaldehyde derivatives as in Jones and Gilligan (1983). Plant material harvested at the same time as that for the amino acid analysis was extracted in perchloric acid and analyzed for polyamines as their benzolated derivatives according to Flores and Galston (1982) as detailed in Corbin et al. (1989).

3. Results

Sporulation

After attaining full cover of the liquid surface in the culture flask, *A. mexicana* consistently sporulated under both the 16/8 hr light/dark regime and the continuous light regime. When sporulating fronds were used as an inoculum, sporocarps were not detectable on the new growth until the end of the second or beginning of the third week; full cover was attained during this period and developing sporocarps were detectable under the microscope once again on the ventral surface of the young branches near the apices. After 3–4 weeks virtually every frond examined was fertile. The *Azolla* species did not sporulate under the 16/8 hr light/dark regime but sporocarps were detectable microscopically by the third week of culture under continuous light; after four weeks more than half (56%) of the fronds were fertile. There was no indication of sporulation in the cultures of *A. caroliniana* under either light regime during the duration of the study.

Amino acid composition

Analyses were conducted weekly over the four week period on each of the three species of *Azolla* under both light regimes to ascertain any effect of increasing culture age/plant density and initiation of sporulation on the soluble amino acid composition. The results obtained for *A. mexicana*, which consistently sporulated, and for *A. caroliniana*, which never sporulated, are presented in Tables 1 and 2, respectively. The data for the *Azolla* species, which sporulated only under continuous light, was consistent with that for the other two species and is not shown. While the total amino acid pool size

Table 1. Amino acid profiles of *A. mexicana* grown under 16 hour and 24 hour photoperiods

Wks in culture	Light treat	ASP	GLU	ASN	SER	GLN	GLY	THR	ARG	ALA	G-P	VAL	PHE	ILE	ORN	TOT
		mol %														nmol/gfw
1	16 h	7±1	22±0	3±0	11±2	18±3	3±1	3±0	4±0	7±0	–	10±1	9±2	1±0	3±1	3.3
	24 h	4±1	9±1	2±1	10±1	57±8	4±2	1±0	1±0	2±0	–	3±0	3±0	–	2±1	5.3
2	16 h	5±1	12±1	2±0	9±1	47±5	6±1	2±0	2±0	3±0	–	5±0	4±0	1±0	2±0	6.1
	24 h	3±1	10±1	1±0	10±0	54±7	5±1	2±1	4±2	2±0	–	3±1	4±0	1±0	1±0	6.7
3	16 h	2±0	7±1	1±0	7±1	62±16	1±0	1±0	5±3	2±0	–	2±0	2±0	–	8±1	8.1
	24 h	1±0	6±1	–	6±1	65±24	2±1	1±0	9±1	1±0	–	3±0	3±0	1±0	1±0	11.3
4	16 h	2±1	6±1	1±0	6±1	62±11	1±0	1±0	8±5	2±1	–	3±1	2±0	–	6±0	8.5
	24 h	1±0	4±1	–	4±0	75±5	1±0	1±0	7±3	1±0	–	3±0	2±0	1±0	1±0	14.6

Table 2. Amino acid profiles of *A. caroliniana* grown under 16 hour and 24 hour photoperiods

Wks in culture	Light treat	ASP	GLU	ASN	SER	GLN	GLY	THR	ARG	ALA	G-P	VAL	PHE	ILE	ORN	TOT
		mol %														nmol/gfw
1	16 h	7±0	16±0	2±0	8±1	12±3	–	3±0	–	4±0	24±2	10±0	5±0	–	6±2	2.4
	24 h	2±0	8±1	1±0	7±1	66±2	–	1±0	–	2±0	7±1	1±0	3±0	–	1±0	5.3
2	16 h	6±0	13±0	2±0	11±2	38±4	2±0	2±1	–	2±0	14±1	6±0	4±0	–	2±0	3.7
	24 h	3±1	9±1	1±0	7±1	59±22	–	1±0	1±1	2±0	7±1	2±0	5±1	–	1±0	6.3
3	16 h	3±0	10±2	1±0	11±1	47±3	–	2±1	–	2±1	12±0	6±0	3±0	–	2±0	4.5
	24 h	2±1	8±2	–	5±1	67±19	1±0	1±0	2±0	2±1	5±1	2±0	4±0	–	1±0	8.4
4	16 h	3±1	7±2	1±0	11±2	54±12	–	2±1	–	2±1	13±1	4±1	3±1	–	–	3.8
	24 h	2±0	5±1	–	3±1	75±6	–	1±0	2±1	1±0	4±0	2±0	3±1	–	1±0	12.1

Values are the mean ± standard deviation of triplicate samples from one experiment. Replicate experiments were similar.

Table 3. Polyamine composition in three *Azolla* species. The three species were grown under a 16 h light period and analyzed for soluble polyamines after one week of growth. Values are the mean ± the standard deviation of triplicate samples. Replicate experiments yielded similar results.

	A. caroliniana nmoles/gfw	*A. mexicana* nmoles/gfw	*Azolla* sp nmoles/gfw
Put	25 ± 6	17 ± 3	11 ± 0
Spd	69 ± 12	89 ± 25	35 ± 6
hSpd	21 ± 4	31 ± 11	14 ± 1
Spm	5 ± 0	10 ± 3	8 ± 1

increased with plant density and culture age in all three species under both culture regimes, the total pool size was larger in each species under the continuous light regime than it was under the 16/8 hr light/dark regime. Individual amino acid levels remained constant or decreased as a percent of the total during the four weeks of growth due primarily to a massive increase in the levels of glutamine (Tables 1 and 2). In the *Azolla* species, glutamine accounted for 22±9% and 74 ±33% of the total amino acids in plants grown under the 16/8 hr light/dark regime after one and four weeks, respectively, and under continuous light for 60±3% and 89±2% of the total amino acids in plants after one and four weeks growth, respectively. The only obvious difference in the composition of the soluble amino acids in plants which sporulated in this study and those which did not was the occurrence of a previously characterized novel dipetide, gama-glutamyl-beta-phenylalanine (Corbin et al., 1986) in *A. caroliniana*. This compound appears to be restricted to *A. caroliniana*. In addition to the other two species used here, analyses of the other recognized species of the genus for this compound were negative (data not shown). Its absence in the other species, especially under non-sporulating conditions, precludes assigning it any significance with respect to the absence of sporulation *A. caroliniana*.

Polyamine composition

The polyamines spermidine (Spd), spermine (Spm), the rather unusual sym-homospermidine (h-Spd) and the diamine putrescine (Put) were detected in all species examined (Table 3). A trace of what appeared to be cadaverine (Cad) was detected in some of the *A. mexicana* extracts and verification was obtained using another method for analysis in which the polyamines were derivatized with o-phthalaldehyde and mercaptoethanol (OPT) instead of

benzoyl chloride (Corbin et al., 1989). Although the levels of polyamines in extracts of other plant tissues (pea internodes, alfalfa sprouts, soybean leaves and oat leaves) were significantly higher with the OPT derivatization procedure than with the benzoyl chloride derivatization (Corbin et al., 1989), the two methods gave very comparable results with the *Azolla* extracts.

Changes in the levels of putrescine and spermidine in each of the three Azolla species during the four weeks of growth under the two light regimes are shown in Fig. 1. Homospermidine and Spm levels varied little from those detected after one week as shown in Table 3. Each of the three species contained higher levels of Put and Spd under continuous light than under the 16:8 light/dark regime (Fig. 1). During the four weeks of growth under the light-dark regime, the Put content remained constant while the Spd level decreased slightly in all species. Under continuous light, the Spd levels remained steady for the first three weeks and then dropped markedly in all species; Put levels remained virtually constant throughout the four weeks in *A. mexicana* and the *Azolla* sp. but increased steadily in *A. caroliniana*.

As the results obtained with *A. carolininia* under continuous light were in contrast to those obtained for the other two species and it was the only species which did not sporulate, we examined the Put to Spd ratio in each species under both regimes in all repetitions of this study. After the first and second

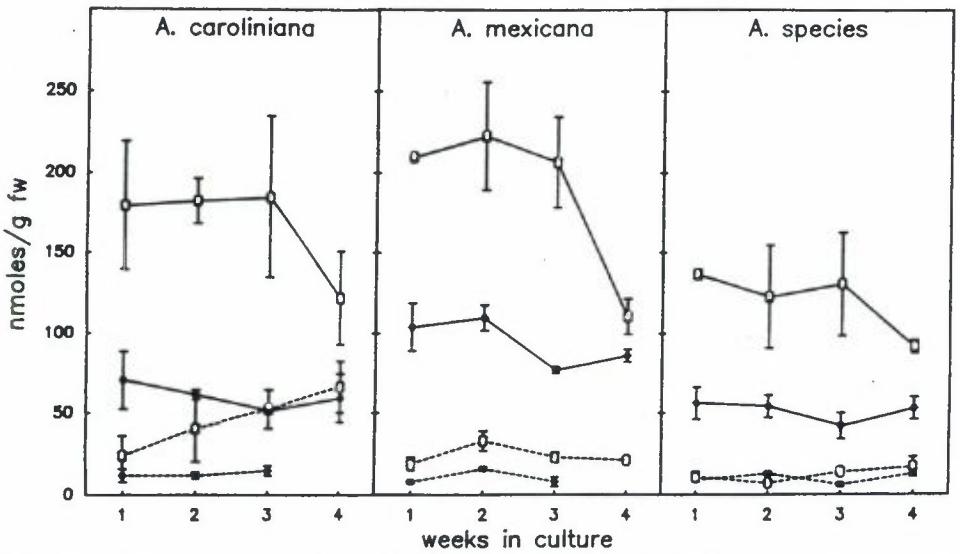

Figure 1. Time course of soluble putrescine and spermidine levels in *Azolla* grown under a 16 hour and under a 24 hour photoperiod. Putrescine is indicated by the dashed lines, spermidine by the solid lines. The closed symbols represent the 16 h and open symbols the 24 h light period. Values are the mean of triplicate samples. Error bars represent the standard deviation of the mean.

Table 4. Putrescine to spermidine ratios in three *Azolla* species. Levels of PCA-soluble PA were determined weekly for 4 weeks in 3 species of *Azolla* grown under 16 h and 24 h photoperiods. The PA were analyzed as their benzoylated derivatives and separated by HPLC. The data are the mean ± standard deviation of nmoles/gfw ratios from 2–4 experiments with 2–3 replicates/experiment.

	A. caroliniana		*A. mexicana*		*Azolla* species	
	16 h	24 h	16 h	24 h	16 h	24 h
0 wk	0.30±0.18		0.18±0.13		0.24±0.21	
1 wk	0.26±0.10	0.16±0.03	0.17±0.06	0.12±0.03	0.24±0.07	0.10±0.02**
2 wk	0.21±0.10	0.23±0.08	0.16±0.06	0.12±0.03	0.21±0.08	0.12±0.04*
3 wk	0.30±0.06	0.30±0.02	0.17±0.07	0.29±0.16	0.14±0.04	0.15±0.03
4 wk	0.28±0.09	0.56±0.35	0.13±0.04	0.32±0.13	0.23±0.05	0.18±0.01

**$p < 0.01$ (two-tailed Student's t-test). *$p < 0.05$ (two-tailed Student's t-test).

week of growth the difference between the Put to Spd ratio under the two light regimes was highly significant in the *Azolla* sp.. There was no significant difference in this ratio between the two regimes in either *A. caroliniana* or *A. mexicana* at any interval (Table 4). This finding suggests that an alteration of PA metabolism was associated with the induction of sporulation in the *Azolla* sp. under continuous light. There was no indication of an altered PA metabolism in either *A. caroliniana*, which did not sporulate under either light regime, or in *A. mexicana*, which sporulated under both light regimes.

4. Discussion

There was a significant alteration of the soluble amino acid composition with increasing culture age and plant density in the three *Azolla* species grown under both light regimes. Although the size of the total amino acid pool increased, the levels of most of the individual amino acids actually decreased as a percent of the total during the four week period due primarily to a massive increase in glutamine. The finding that the pool size increased with plant age and density is consistent with the report of a large accumulation of amino-N (and ammonium-N) in five *Azolla* species, including *A. caroliniana* and *A. mexicana*, under conditions of both high plant density and phosphate deficiency (Tung and Watanabe, 1983). The approximate doubling of soluble amino acids in *A. mexicana* from week one to week two under the 16:8 hr light-dark cycle (Table 1) in the present study agrees well with the reported

doubling of amino nitrogen in *A. mexicana* grown for two weeks under a 12:12 hr light-dark cycle and the same temperature regime (Tung and Watanabe, 1983). Our results are in contrast to a more recent study by Sanginga and Van Hove (1989) which compared the total N and amino acid composition of seven *Azolla* strains at four different phases (termed exponential, linear, slowing down, and constant) of growth/culture density. They reported that the total nitrogen content was not significantly influenced by growth rate or population density; and, that while the total amino acid content, and most individual amino acids, increased through the linear phase, it declined sharply at the constant phase (Sanginga and Van Hove, 1989). Unfortunately glutamine was not included in their analyses of amino acids and they did not specifically look at ammonium N. Newton and Cavins (1976) also stated that levels of free ammonium, and the size and composition of the amino acid pool, in *A. caroliniana* were quite uniform with culture age but they grew the plants at the rather low light intensity of 100 ft-c. They did show, however, that free ammonia accounted for as much as half of the total N pool and that glutamine accounted for about 25% of the total pool N (about 40–50% of the amino N) under their culture conditions. Neither Newton and Canvins (1976) nor Sanginga and Van Hove (1989) noted the occurrence of the novel dipeptide gama-glutamyl-beta-phenyalanine (Corbin et al., 1986) in *A. caroliniana* but this dipeptide clearly appears to be the third most abundant and developmentally regulated nitrogenous compound in this species of *Azolla*. The massive increase in glutamine levels detected in the present study, with this single amino acid accounting for 54–74% and for 75–89% of the soluble amino acid pool after four weeks of growth under a 16:8 hr light:dark cycle and under continuous light, respectively, has not been reported previously. Prior studies suggested that, as with asparagine in peanuts (Peoples et al., 1986), glutamine may be a transport form of nitrogen in *Azolla*. In actively growing cultures of *A. caroliniana* maintained at a low plant density, the glutamine pool size was higher in the forming leaves and stem apices than in the remainder of the stem axes (Marsh et al., 1984) and studies in which stem axes were pulsed with $[^{13}N]\ N_2$ followed by an air chase showed an enrichment of glutamine in, and its probable transport to, the apical portion of the segments (Peters et al., 1985). The present study suggests that as plant density increases and the growth rate declines, *Azolla* stores excess nitrogen fixed by the cyanobiont primarily in the form of this single amino acid.

From the results presented in Tables 1 and 2, along with the data obtained but not shown for the *Azolla* sp., it appears unlikely that the alteration of amino acid metabolism found to be associated with increased culture age and plant density has anything to do with the triggering of sporulation. The changes in both the individual soluble amino acids and in the pool sizes in the three species grown under the two light regimes were found to be similar even

though *A. mexicana* sporulated under both, *A. caroliniana* under neither, and the *Azolla* sp. only under the continuous light regime.

As with a number of other plant sources, the major soluble polyamines of the *Azolla-Anabaena* associations are Put, Spd, and Spm and they contain little or no Dap, Agm, or Cad (Birecka et al., 1985; Smith, 1985; Hamana et al., 1988; Evans and Malmberg, 1989; Corbin et al., 1989). The detection of the unusual sym-homospermidine in the three *Azolla* species is consistent with an earlier report. Although only sporadically detected in other ferns (Hamana and Matsuzaki, 1985), Hamana et al. (1988) reported its occurrence in the roots and leaves of two other *Azolla* species.

In contrast to changes in the soluble amino acid pools, our findings suggest that an alteration of polyamine metabolism, specifically a reduction of the Put/Spd ratio, may well be associated with the onset of sporulation in the *Azolla* sp. (Fig. 1, Table 4). In regard to this we note the following. Developing sporocarps were discernable microscopically by the third week of culture in all cases where sporulation was observed. Any alteration of polyamine metabolism associated with their induction would necessarily precede their detection, occurring in the critical first two weeks of culture. In *A. caroliniana*, which did not sporulate under either light regime, the Put/Spd ratio is high during the first two weeks in plants cultured under both light regimes. In *A. mexicana*, which sporulated consistently under both light regimes, developing sporocarps were detectable again in the apical portions of young branches after two weeks in freshly transferred cultures and during the first two weeks the Put/Spd ratio is low in plants cultured under both light regimes. In the *Azolla* species, which sporulated only under the continuous light regime, the Put/Spd ratio is high under the 16/8 hr light-dark regime and low under the continuous light regime. Moreover during the critical first two weeks the Put/Spd ratio present in the *Azolla* species cultured under continuous light is significantly different from the Put/Spd ratio in the *Azolla* species cultured under the light-dark regime (Table 4).

Polyamine metabolism and fluctuations in the levels of specific polyamines, especially putrescine, appear to have a role in the reproductive biology of at least some angiosperms. Temperature induced changes in auxin and polyamines, especially fluctuations in putrescine, have been studied in relationship to flowering in an orchid (*Phalaenopsis*) (Fouche et al., 1991). In chrysanthemum (*Chrysanthemum morifolium* Ramat.) it has been suggested that putrescine catabolism may be required for floral initiation and/or floral development (Martin-Tanguy et al., 1996). In hazel (*Corylus avellana* L.) polyamine levels (mostly free putrescine) were higher in juvenile tissues and it was suggested that the putrescine to spermidine plus spermine ratio may reflect a balance between vegetative growth and reproductive processes (Rey et al., 1994). While we are not aware of any studies showing a possible role of polyamine

levels in the reproductive biology of ferns, it does not seem implausible that the changes in polyamine levels reported here are involved with the process of sporulation in *Azolla*. Nevertheless, in order to show that the changes in polyamine levels are actually casual, it will be necessary to conduct additional studies including, but not limited to, the use of both exogenous polyamines and inhibitors of polyamine synthesis.

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