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Growth-Retarding Effect of 2-Aminoindan-2-phosphonic Acid on *Spirodela punctata*

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Abstract. 2-Aminoindan-2-phosphonic acid (AIP) retarded the growth of duckweed, *Spirodela punctata*, and increased its dry mass. The accumulation of starch was observed at all concentrations of AIP at 8 days after treatment. The increase in starch was inversely proportional to the growth. The retarded growth of *Spirodela* by AIP was not limited only by excessive starch accumulation.

Key Words. Aminophosphonic acids—Growth—Starch—*Spirodela*

The pathway of aromatic amino acid biosynthesis depends upon carbohydrate metabolism as the source of initial substrates for the shikimate pathway, erythrose 4-phosphate (an intermediate of the pentose phosphate pathway) and phosphoenolpyruvate (intermediate of glycolysis pathway). Both of these are involved in the primary metabolism of sugars and play key roles in the carbon assimilation cycle of photosynthesis. The Phe produced is converted to *trans*-cinnamic acid in a reaction catalyzed by L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). Cinnamic acid flows through the pathway to yield phenolic acids, e.g. *p*-coumaric, caffeic, and ferulic acids, which are the precursors of secondary metabolites such as phenylpropanoids (coumarins, flavonoids, lignins). Derivatives of cinnamic acid, for example ferulic and *p*-coumaric acids, are known to be potent plant growth inhibitors (Kefeli and Kadyrov 1971). Because of the important role of PAL as a bridge between primary and secondary metabolism, there was a strong incentive to develop inhibitors of this enzyme, most of which are derivatives of phenylalanine, e.g. aminooxy and phosphonic, L-α-aminooxy-β-phenylpropionic acid (AOPP), 2-aminoindan-2-phosphonic acid (AIP) (Fig. 1), 1-amino-2-phenylethylphosphonic acid (PheP), and 1-amino-3-phenylpropylphosphonic acid (PhPP) (Amrhein and Gödecke 1977, Janas et al. 1985, Janas and Olechnowicz 1994, Zoń and Amrhein 1992). Specific enzyme inhibitors are valuable tools in the study of in vivo metabolic pathways.

Using AIP, a potent PAL inhibitor ($K_i = 0.08$ μM for buckwheat PAL), phenylpropanoid inhibition of buckwheat and rye seedlings was observed (Reuber et al. 1993, Zoń and Amrhein 1992). This compound had a negative effect on growth, fresh weight, and root development, and it reduced the content of phenylpropanoids in primary leaves of rye (*Secale cereale* L.) and suspension culture of *Lithospermum erythrorhizon* Sieb. et Zucc. (Gaisser and Heide 1995, Reuber et al. 1993) and inhibited lignification in single cells isolated from the mesophyll of *Zinnia elegans* L. (Sato et al. 1993). Growth reduction of plants by different compounds may be caused not only by disturbing phenylpropanoid metabolism, but also, for example, by excessive starch accumulation (Tasseron-De Jong and Veldstra 1971). Inhibition of growth in *Lemna minor* L. was caused by excessive starch accumulation when the plants were grown in the presence of ABA and BA (McLaren and Smith 1975, Tasseron-De Jong and Veldstra 1971). We observed a reduction in the growth of *Spirodela punctata*, which belongs to family Lemnaceae, when grown

Abbreviations: ABA, abscisic acid; AIP, 2-aminoindan-2-phosphonic acid; AOPP, L-α-aminooxy-β-phenylpropionic acid; BA, 6-benzylaminopurine; MR, multiplication rate; Phe, phenylalanine; PheP, 1-amino-2-phenylethylphosphonic acid; PhPP, 1-amino-3-phenylpropylphosphonic acid; PVP, polyvinylpyrrolidone.

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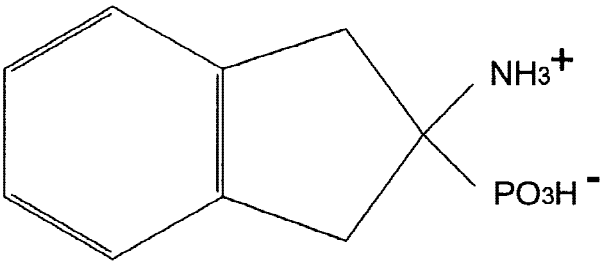

Fig. 1. Structure of AIP.

Table 1. Effect of different concentrations of AIP on multiplication rate after 3, 5, and 7 days of cultivation of cultures of *S. punctata.* Inoculum was 10 fronds/flask. Each value is the mean of three replicates.

AIP concentrations (μM)	Multiplication rate		
	3 days	5 days	7 days
0	176 ± 8.6	170 ± 7.5	166 ± 9.7
1	181 ± 6.0	164 ± 6.7	132 ± 9.0
10	171 ± 7.5	152 ± 11.3	116 ± 8.7
100	155 ± 1.0	138 ± 13.4	102 ± 6.4

in the presence of AIP. We propose that AIP inhibited the growth of *Spirodela,* in part, by stimulation of starch accumulation.

Materials and Methods

Plants

A culture of *S. punctata* (=*oligorrhiza* (Kurz.) Hegelm.) strain 05 was obtained from Prof. R. Kandeler (Botanisches Institut, Universität für Bodenkultur, Wien, Austria). The plants were grown under sterile conditions in Erlenmeyer flasks containing 20 cm^3 of the nutrient medium. The mineral medium contained 4 mM ammonium sulfate as the sole nitrogen source and 1% glucose (Knypl et al. 1986). AIP (1 mM) was dissolved in deionized water, and aliquots were added to autoclaved medium to give the desired concentrations. The inoculum was about 10 fronds/flask. All plants were illuminated continuously by fluorescent tubes of the Flora type (intensity 5.8 W m^{-2}). The ambient air temperature was 25 ± 1°C.

Growth Parameters

For determination of growth rate, fronds were counted daily over a period of 8 days. Growth was assessed by counting the number of fronds and expressed as a multiplication rate (MR) (Knypl 1976). After cultivating for 8 days, fronds were removed from the flasks, washed with distilled water, and the fresh weights were recorded. The fronds were dried for 24 h at 80°C for determination of the dry weight. All experiments were repeated four times.

Extraction and Determination of PAL Activity

Samples of fresh matter were homogenized with chilled Tris-HCl (0.05 M, pH 8.8, 4 cm^3/g fresh weight) supplemented with β-mercaptoethanol and PVP (polyvinylpyrrolidone) and centrifuged (5,000 × *g*, for 10 min followed by 15,000 × *g* for 30 min). The final supernatant was used as a source of crude enzyme (Knypl et al. 1986).

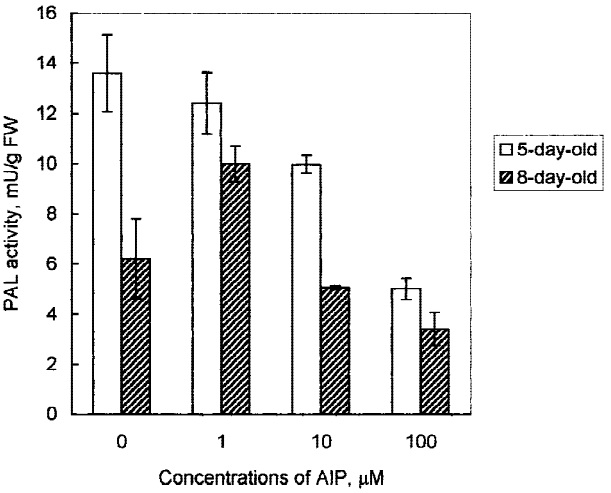

Fig. 2. PAL activity extractable from *S. punctata* after 5 and 8 days of cultivation in the presence of AIP at different concentrations. Each value represents the mean of three parallel determinations ± S.E. in all experiments.

Table 2. Effect of AIP on growth of *Spirodela.* Measurements were taken after 8 days of cultivation. Original inoculum was about 8–10 fronds/flask. Each value represents the mean of four parallel determinations ± S.E. in one experiment.

AIP concentrations (μM)	Fresh weight (mg $flask^{-1}$)	Dry mass (mg)
0	245.0 ± 14.0 (100%)	20.5 ± 3.40 (100%)
1	184.0 ± 11.1 (88%)	22.0 ± 0.51 (109%)
10	81.7 ± 13.8 (38%)	36.0 ± 2.05 (176%)
100	57.7 ± 15.5 (33%)	44.6 ± 1.63 (218%)

Extraction and Determination of Starch

Extraction of starch was carried out according to McCombs and Ralph (1972). The plantlets were homogenized in 3 cm^3 of 0.01 M Tris-HCl, pH 7.6, with 0.01 M $MgCl_2$ in a prechilled mortar and pestle. The homogenate was centrifuged at 12,000 × *g* for 10 min, and the pellet was extracted twice with 10 cm^3 of the buffer containing 2% Triton X-100 (to free starch grains from chloroplasts). The remaining insoluble material was dehydrated with 95% (w/v) ethanol and twice with acetone. The starch in the dry residue was hydrolyzed with 50% (v/v) $HClO_4$, and the resulting sugars were determined by the anthrone-sulfuric acid method (McCready et al. 1950).

Statistical Analyses

Each assay was performed three times, and the experiments were repeated at least twice. The S.E. was calculated between experiments.

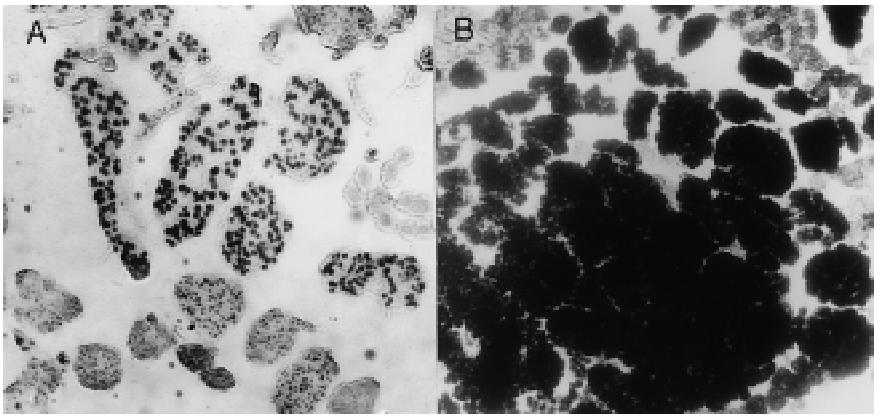

Fig. 3. Squashed parenchyma cells from fronds of *S. punctata* after 7 days of cultivation in control (*A*) and in the presence of AIP (*B*, 100 μM) seen under a light microscope. The preparations are stained with potassium iodide (JKJ).

Results and Discussion

Members of the family Lemnaceae are suitable for investigating physiological processes and the effects of different chemical substances. The growth rate of Lemanaceae cultures might be expressed as the difference of the logarithms of the final frond number (F_d) and the initial number (F_o) divided by the number of days (d) of growth: MR = $\log_{10}(F_d) - \log_{10}(F_o) \times 1{,}000/d$, as MR means a multiplication rate.

AIP treatments caused a virtually linear decrease of MR. The effect was age and concentration dependent. The MR over 7 days decreased by 176 in the control compared with 116 and 102 in 10 μM and 100 μM AIP, respectively (Table 1). This compound caused morphological malformation in *Spirodela.* Old fronds became dark green compared with the controls, whereas progeny fronds were yellow-green at the bases. The plantlets contained the same quantity of chlorophyll/unit fresh weight in the controls (without AIP) and in the presence of AIP. Fronds were smaller in the AIP plantlets than those in the controls. In plants growing in AIP the roots were inhibited. Because of shortened and tough interfrond connections, the progeny fronds did not separate from the mother fronds; the AIP-treated cultures formed clusters comprised of almost all the progeny fronds. PheP was found to have a similar effect on growth (Knypl et al. 1986).

In controls the *Spirodela* PAL activity was decreased in older plants (8 days old) compared with younger (5 days old). AIP inhibited PAL activity in vivo in a dose-dependent manner when *Spirodela* were grown for 5 and 8 days except plants that were cultivated in the presence of 1 μM AIP for 8 days (Fig. 2).

After 8 days, cultures of *Spirodela* with increased concentrations of AIP had a dramatic decrease in fresh weight, from 245 mg/flask in controls to about 82 mg and 60 mg/flask in the presence of 10 μM and 100 μM AIP, respectively (Table 2). In D,L-PhPP- and D,L-PheP-grown plants their fresh weight decreased significantly less compared with those in the presence of AIP (data not shown). The dry mass of AIP-treated *Spirodela* increased about 176% and 218% after 8 days in 10 μM and 100 μM AIP, respectively (Table 2). Cytochemical analyses showed that plants grown in the presence of AIP accumulated a large amount of starch (Fig. 3). In the presence of PheP and PhPP, a lower accumulation of starch was observed (Janas and Osiecka 1995). AIP at all concentrations caused an increase of starch after 8 days (Fig. 3). In AIP- (100 μM) treated duckweed, the amount of starch rose by 130% during the first 5 days, but during the next 3 days it increased by about 370% compared with the control plants (Fig. 4). In *L. minor* L., which were growing for 6 days in the presence of 10^{-6} M ABA, the fresh weight decreased by about 60%, the dry mass increased 220%, but the starch content was higher by about 600% compared with the controls (McLaren and Smith 1976). The results indicate that the growth of *Spirodela* was only in part decreased by the accumulation of starch. We think that AIP might retard the growth of *Spirodela* by other effects. (1) There may be a decrease in mitotic activity as was observed in root meristems of *Vicia faba* (in preparation). (2) It may block the production of some secondary substances integral to the activity of the differentiated plants (Jones 1984). We observed that the degree of inhibition of growth in *Spirodela* was connected to the inhibition of PAL activity. More starch was accumulated in *Spirodela* that were cultivated in the presence of a strong inhibitor of PAL activity (AIP) compared with a weak PAL inhibitor (PhPP) (Janas and Osiecka 1995). (3) AIP can disturb a mechanism for the maintenance of homeostasis between primary and secondary metabolism. Accumulating starch in a high concentration could decrease the growth of plants. Van der

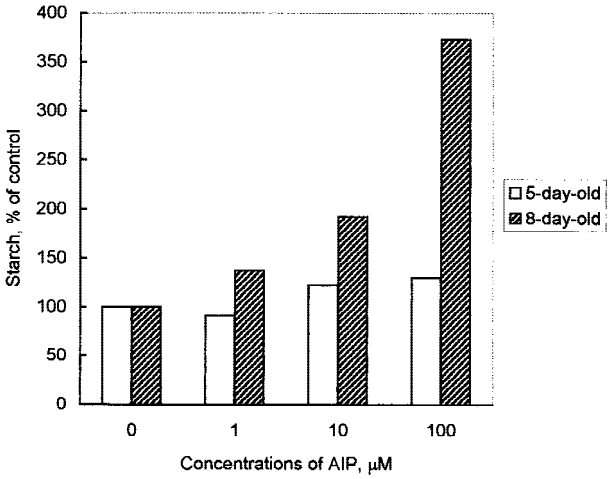

Fig. 4. Changes in starch levels after the addition of different concentrations of AIP in 5-day-old and 8-day-old plants of *Spirodela.* Each value is the mean of three replicates.

Plas et al. (1995) observed that there is a close relationship between primary and secondary metabolism in a cell suspension culture of *Morinda citrifolia.* These authors showed that the cell division rate as well as the metabolic activity were low, but the endogenous sugar levels were high in suspension cells of *Morinda.* However, more information is needed to explain more exactly the effects and influence of AIP on carbohydrate metabolism.

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