BIOLOGIA PLANTARUM 45 (4): 613-615, 2002

BRIEF COMMUNICATION

Does *Spirodela punctata* break P-C bonds?

K.M. JANAS and Z. ROMANOWSKA-DUDA

Department of Plant Growth Regulation, University of Łódź, Banacha 12/16, PL-90237 Łódź, Poland

Abstract

Spirodela punctata was cultivated on phosphate-deficient medium (-P_i) with racemic 1-amino-2-phenylethylphosphonic acid (PheP) as a source of P_i. The growth of duckweed was inversely correlated with PheP concentration. The growth of plants on medium -P_i with 0.1 M PheP was accelerated whereas with 0.001 mM PheP was slower than in -P_i control. PheP at low concentrations decreased loss of chlorophyll in comparison with -P_i plants. Content of anthocyanins decreased but activity of the extractable constitutive phosphatases of pH 6.0 and pH 7.5 increased along with increasing concentration of PheP in the medium. We suggest that *S. punctata* does not break P-C bonds but probably PheP interrupts processes involved in the regulation of P_i-starvation response.

Additional key words: aminophosphonic acids, anthocyanins, chlorophyll, duckweed, phosphatases, phosphate deficiency.

Spirodela punctata is a small, free floating monocotyledonous water plant belonging to the family *Lemnaceae*. This plant is an ideal object for biochemical studies on plant mineral nutrition as it is easily cultured under fully controlled environmental conditions (Bollard 1966). In response to phosphorus deficiency growth of this plant is almost immediately slowed down (Knypl and Sobolewska 1978) and alkaline phosphatase activity is induced (Reid and Bieleski 1970).

Aminoalkanephosphonic acids are amino acid analogues in which the carboxylic group is replaced by a phosphonic or related groups which have the same function (phosphonous, phosphinic, phosphine oxide, *etc.*). As inhibitors of metabolic processes, they are used as anticancer drugs, pesticides, *etc.* (Kafarski and Lejczak 1991). Some of these compounds and their derivatives are used as agrochemicals, not only as plant growth regulators or herbicides, but also as fungicides and insecticides. These compounds are characterised by the unique nature of the P-C bond which is resistant to chemical and biological cleavage. The ability to catabolise the P-C bond is widespread among bacteria (Harkness 1966).

The preliminary results show that addition of PheP to the nutritional medium, deprived of phosphate, significantly reduces symptoms of P_i deficiency. This phenomenon stimulated us to answer the question: Can *Spirodela punctata* cultivated on phosphate-deficient medium use PheP as a source of P_i?

A culture of *Spirodela punctata* (Kurz.) Hegelm. (= *S. oligorrhiza*) strain 05 was obtained from Prof. R. Kandeler (Botanisches Institut, Universität für Bodenkultur, Wien, Austria). The plants were grown on: *1*) control medium with P_i (C), *2*) phosphorus-deficient medium (-P_i), or *3*) medium -P_i with PheP (-P_i + PheP), under aseptic conditions in Erlenmeyer flasks. The inoculum was *ca.* 10 fronds per flask. Each flask contained 20 cm^3 of the nutrient medium. The mineral medium contained 4 mM ammonium sulphate as the sole nitrogen source and 1 % glucose (Janas *et al.* 1998). PheP (1 mM) was dissolved in deionized water and aliquots were added to autoclaved medium to give the desired concentrations. The content of P_i in medium was determined by the Janicke (1974) method. All plants were illuminated continuously by fluorescent tubes of the *Flora LF40W* (*Polam*, Poland; irradiance 6.8 W m^{-2}). The ambient air temperature was 25 ± 1 °C.

For determination of growth rate, fronds were counted daily over a period of 8 d in four replications. Plantlets homogenized in 80 % acetone and measured at 645, 652,

Received 24 July 2001, *accepted* 11 December 2001.
Abbreviations: Chl - chlorophyll; P_i - orthophosphate; PheP - 1-amino-2-phenylethylphosphonic acid; *p*-NP - *p*-nitrophenol.
Acknowledgements: We thank Prof. P. Kafarski for gift of PheP and Prof. R. Kandeler for culture *Spirodela punctata*.
Fax: (+48) 42 6354423; e-mail: kjanas@ biol.uni.lodz.pl

and 663 nm by the spectrophotometer *Spectronic 601* (*Milton Roy Company*, USA). Chlorophyll content was calculated according to Bruinsma (1963). Anthocyanins were extracted in 3 cm^3 of methanol containing 1 % HCl (v/v) for 24 h at room temperature and their content was determined according to Elliott (1977). The absorbance was read at A_{535} for anthocyanin and at A_{660} for chlorophyll *a* on the above mentioned spectrophotometer. Each determination was replicated three times. Constitutive (soluble) phosphatase was extracted by grinding plants (100 mg fresh mass) in a pre-chilled mortar and pestle with 200 mg of hydrated *Polyclar AT* (*Serva*, Germany) and 2 cm^3 aliquots of 0.05 M Tris-HCl buffer (pH 7.4). After centrifugation for 20 min at 3 800 *g* the pellet was extracted again with 2 cm^3 of this buffer. Activities of phosphatases (EC 3.1.3.1 and 3.1.3.2) were measured in pooled supernatants at 30 °C for 20 min, at pH 6.0 and 7.5, using *p*-nitro-phenylphosphate (*p*-NPP; *Merck*, Germany) as a substrate. The assay mixture contained 0.3 cm^3 *p*-NPP, 0.1 cm^3 of enzyme extract, 1 cm^3 of TCGM buffer brought to a desired pH with NaOH or HCl, and water to 1.5 cm^3. TCGM buffer contained 0.05 M Tris, 0.033 M citrate, 0.05 M glycine and 0.05 M maleate (Bieleski 1968). *p*-NPP was added to blank samples at the end of the incubation period. A_{404} was measured after adding 1 cm^3 of 0.3 M NaOH. A unit of phosphatase activity was defined as the amount of enzyme which produced 1 μmol of *p*-nitrophenol (*p*-NP) from *p*-NPP g^{-1}(d.m.) min^{-1} under conditions specified above.

The growth rate of *S. punctata* in P_i-deficient medium was slowed down after 2 d of cultivation (Fig. 1). The old fronds became yellow while the younger ones were dark green but the total content of chlorophyll (Chl) decreased in comparison to the controls. The progeny fronds on the downward side contained much more anthocyanin than the control ones. The roots were longer in duckweed grown in -P_i medium in comparison to the control (Knypl and Sobolewska 1978). These visible symptoms were caused by the exhaustion of the nutrients in the medium (Table 1). In *S. punctata* strain 05 the decrease in Chl in -P_i medium was faster than in *S. oligorrhiza* (Knypl and Sobolewska 1978). *S. punctata* grown on -P_i medium displayed symptoms as described by Reid and Bieleski (1970) and Knypl and Sobolewska (1978).

Visible symptoms of P_i deficiency in *Spirodela* were weaker when -P_i medium was supplemented with 0.1 mM PheP. During 8 d of culture on medium -P_i + PheP growth of plants was faster (Fig. 1) and small morphological changes were observed (data not shown). PheP in lower concentrations (0.01 and 0.001 mM) led to decreased growth rates in plants grown in -P_i + PheP in comparison to the control, however growth was slightly stimulated in comparison to -P_i plants (Fig. 1). However, we did not detect contamination with P_i in -P_i medium supplemented with PheP. A similar tendency of changes was observed in *Brassica nigra* seedlings when compared with plants cultivated with P_i-sufficient and P_i-deficient medium in the presence of fungicide phosphonate (Carswell *et al.* 1996). The effect of P_i-deficiency on Chl content was reduced when plants were grown in the presence of PheP. PheP at 0.001 mM concentration increased but at 0.1 mM decreased the content of Chl in comparison with plants grown on -P_i medium (Table 1).

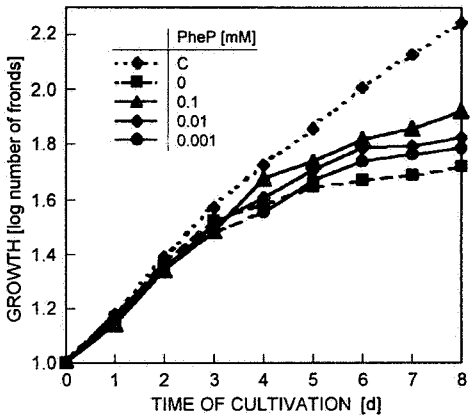

Fig. 1. The growth of *Spirodela punctata* cultivated on full medium (C), medium without P_i (0) and with different concentrations of PheP. Original inoculum: about 8 - 10 fronds per flask. Each value represents the mean of 4 parallel determinations from 3 independent experiments.

In *S. punctata* grown on -P_i medium accumulation of anthocyanin was observed (Table 1). Similar results in *S. oligorrhiza* cultivated on P_i-deficient medium described Reid and Bieleski (1970). We have presumed that if *S. punctata* breaks P-C bonds, antocyanin content in duckweeds grown on medium -P_i + PheP should be similar as in the control. However, the content of anthocyanins was lower in plants cultivated on medium -P_i + PheP than in control. PheP added to the -P_i medium reduced anthocyanin content in a dose-dependent manner (Table 1). However, the decrease in the content of anthocyanin may not be caused by the break of P-C bonds. PheP is an inhibitor of the L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). PAL is the key enzyme which catalyses deamination of L-phenylalanine to *trans*-cinnamic acid which is a precursor of phenylpropanoid-derived secondary products such as coumarins, lignin, anthocyanin, and many others. PheP also inhibited synthesis of anthocyanins in *S. punctata* (Knypl and Janas 1986) and buckwheat (Laber *et al.* 1986). Another characteristic feature of phosphorus deficiency is a manifold increase in alkaline phosphatase activity that was observed in axenic *S. oligorrhiza* (Reid and Bieleski 1970), tomatoes (Hewitt and Tatham 1960), *Euglena* (Blum 1965) and other plant species. Increased alkaline phosphatase with optimum pH 7.5 indicate that plants synthesised this enzyme *de novo* under P_i deficiency (Reid and Bieleski 1970). In *S. oligorrhiza* cultured on complete medium an increased activity of the

constitutive acid phosphatase was observed (Table 1) but when *S. punctata* was grown in deficient -P_i medium, phosphatase with optimum pH 7.5 appeared (Table 1). If *S. punctata* hydrolyses P-C bonds, alkaline phosphatase activity in plants cultivated on medium -P_i + PheP should be lower than in plants grown on medium -P_i. However, PheP added to the P_i-deficient medium increased both acid and alkaline phosphatase activity in a dose-dependent manner (Table 1). A manifold increase in alkaline phosphates in plants grown on medium -Pi + PheP can suggest that *S. punctata* does not hydrolyse P-C bonds (Reid and Bieleski 1970).

Table 1. Effect of different concentrations of PheP on chlorophyll (Chl) and anthocyanin contents in 6-d old *Spirodela punctata*, and on acid and alkaline phosphatase activities in 19-d old *S. punctata* cultivated on full medium (C), medium without phosphate (-P_i) and without P_i and with different concentrations of PheP. Means from at least 3 independent experiments ± SE.

	Chlorophyll *a*+*b* [mg g^{-1}(d.m.)]	Anthocyanins [% of control]	Acid phosphatase [μmol(*p*-NP) g^{-1}(d.m.)]	Alkaline phosphatase [μmol(*p*-NP) g^{-1}(d.m.)]
C	1.5 ± 0.7	100	7.3 ± 1.5	1.3 ± 0.2
-Pi	0.6 ± 0.4	306	13.1 ± 3.4	14.1 ± 1.5
-Pi + 0.001 mM PheP	1.0 ± 0.3	106	24.5 ± 4.9	29.2 ± 6.2
-Pi + 0.010 mM PheP	0.8 ± 0.3	79	21.3 ± 4.1	21.3 ± 5.7
-Pi + 0.100 mM PheP	0.8 ± 0.3	41	17.6 ± 8.4	20.0 ± 7.3

References

Bieleski, R.L.: Effect of phosphorus deficiency on levels of phosphorus compounds in *Spirodela*. - Plant Physiol. **43**: 1309-1316, 1968.

Blum, J.J.: Observations on the acid phosphatases of *Euglena gracilis*. - J. Cell Biol. **24**: 223-234, 1965.

Bollard, E.: A comparative study of the ability of organic nitrogenous compounds to serve as sole source of nitrogen for the growth of plants. - Plant Soil **25**: 153-166, 1966.

Bruinsma, J.: The quantitative analysis of chlorophylls *a* and *b* in plant extracts. - Photochem. Photobiol. **2**: 241-249, 1963.

Carswell, C., Grant, B.R., Theodorou, E., Harris, J., Niere, J.O., Plaxton, W.C.: The fungicide phosphonate disrupts the phosphate-starvation response in *Brassica nigra* seedlings. - Plant Physiol. **110**: 105-110, 1996.

Elliott, D.C.: Induction of anthocyanin synthesis in *Spirodela oligorrhiza*. - Aust. J. Plant Physiol. **4**: 39-49, 1977.

Harkness, D.R.: Bacterial growth on aminoalkylphosphonic acids. - J. Bacteriol. **2**: 623-627, 1966.

Hewitt, E.J., Tatham, P.: Interaction of mineral deficiency and nitrogen source on acid phosphatase activity in leaf extracts. - J. exp. Bot. **11**: 367-376, 1960.

Janas, K.M., Osiecka, R., Zoń, J.: Growth-retarding effect of 2-aminoindan-2-phosphonic acid on *Spirodela punctata*. - J. Plant Growth Regul. **17**: 169-172, 1998.

Janicke, L.: A rapid micromethod for the determination of nitrogen and phosphate in biological material. - Anal. Biochem. **61**: 623-627, 1974.

Kafarski, P., Lejczak, B.: Biological activity of aminophosphonic acids. - Phosphorus Sulfur Silicon **63**: 193-215, 1991.

Knypl, J.S., Janas, K.M.: Physiological activity of 1-amino-2-phenylethyl-phosphonic acid, a substrat analogue of phenylalanine. - Biol. Plant. **28**: 91-94, 1986.

Knypl, J.S., Sobolewska, J.: Age dependent changes of phosphatase activity in phosphate supplied and phosphate deficient *Spirodela*. - Physiol. vég. **16**: 73-84, 1978.

Laber, B., Kiltz, H.H., Amrhein, N.: Inhibition of phenylalanine ammonia-lyase *in vitro* and *in vivo* by (1-amino-2-phenylethyl)phosphonic analogue of phenylalanine. - Z. Naturforsch. **41c**: 49-55, 1986.

Reid, M.S., Bieleski, R.L.: Changes in phosphatase activity in phosphorus-deficient *Spirodela*. - Plant Physiol. **46**: 609-613, 1970.