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Changes in phosphatase activity during germination and in early phases of cucumber seedlings growth

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Abstract

In cotyledons and roots of *Cucumis sativus* seedlings, the presence of phosphatases was proved at two optimal pH values: 6.4 and 5.0. The phosphatases are characterized by low substrate specificity. Among the analysed substrates the following were hydrolyzed particularly preferentialy while fissuring: p-nitrophenylophosphate (pNPP), adenosinotriphosphate (ATP), pyrophosphate, β-naphthylophosphate and in case of the cotyledon extracts also calcium phytinate. Mo, Zn, Cu and Co present in an incubation mixture inhibited this activity. Analysis of the phosphatase activity in cotyledons and roots of cucumber seedlings showed that in early phases of cucumber growth (germination and the first week of growth in ligth), the activity remained at a relatively high level and had a few peaks connected with the vegetable growth phase whereas the frequency of their occurance was connected with the analysed organ. It was stated that changes of the phosphatase activity were visibly correlated to changes of the phosphorus content in the period under study. In roots similar correlations were not observed.

INTRODUCTION

Both the germination and further early growth phases of a young plant are characterized by a particulary dynamic metabolism. This is connected with the metabolic start of the reserve compounds wich are essential for sprout development. After seed germination and in the course of further seedling growth hydrolysis of the reserve materials stored either in dicotyledonous cotyledons or in caryopsis of monocotyledonous plants continues and, as reported by many authors, reaches its maximum only after a few days of seedling growth (Beevers, 1968; Firenzuoli et al., 1968; Palmiano, Juliano, 1973; Yo-

mo, Varner, 1973; Prisco et al., 1975; Basha, Beevers, 1975; and others). There are two reasons of the increasing hydrolytic activity: activation of the enzymes present in the seeds (Shain, Mayer, 1968; Eastowood et al., 1969; Mayer et al., 1971) and a *de novo* synthesis of the hydrolase series (Yung et al., 1960; Jacobson, Varner, 1967; Chrispeels, Varner, 1967). The first week of seedling growth is characterized by the greatest intensity of hydrolysis.

Most experiments on the degradation of reserve materials during germination and growth of young seedlings deal with proteins, sugars, fats and activity of hydrolases responsible for the degradation of these substances. Changes of phosphatase activity are less recognised. Also physiological role of enzymes taking part in phosphatase changes of plant remaines rather vaque. Due to this, in the present work investigations were undertaken on phosphatase activity kinetics during early phases of the growth of young seedlings. Level of phosphatase activity was examined beginning from the moment of seed germination and ending on the phase which preceeds the formation on the first leaf. Changes in the content of each phosphorus fraction were also observed.

MATERIAL AND METHODS

Plant material (cultures)

The experiments were performed on cucumber seedlings (*Cucumis sativus*, L. var. 'Wisconsin'). Imbibed seedlings were germinated on Petri dishes in a dark thermostate for 48 h, at 27°C. Uniform seedlings were transplanted to cheesecloth stretched over dishes filled with proper medium. Dishes were placed in glass crystallizers and left in the fotothermostat at 25°C untill the first leaf has developed. Seedlings grow in a medium under variable light conditions — for 16h in light (5000 lux), and for 8h in darkness, at 27°C.

Plants grew either in the full medium (medium I, according to Ingestad, 1973) containing the following macroelements (mg/l): KH_2PO_4 — 7.91, $CaCl_2 \times 6H_2O$ — 13.66, KNO_3 — 48.434, $MgSO_4 \times \times 7H_2O$ — 25.648, $NaNO_3$ — 129.046, ferric citrate — 0.7, or in nutrient solution without phosphorus (medium II): $CaCl_2 \times 6H_2O$ — 13.66, KNO_3 — 54.3, $NaNO_3$ — 124.4, $MgSO_4 \times 7H_2O$ — 25.648, ferric citrate — 0.7. Both mediums were suplemented with microelements (mg/l): $MnSO_4 \times 7H_2O$ — 0.4, H_3BO_3 — 0.2, $CuSO_4 \times 5H_2O$ — 0.03, $ZnSO_4 \times \times 7H_2O$ — 0.3, Na_2MoO_4 — 0.007. pH of the solutions was adjusted to 6.2.

Analytical methods

Enzyme extracts were prepared by grinding the roots or cotyledons in cold 0.1 M Tris-maleinate buffer (pH 6.4), or in 0.1 M acetate buffer (pH 5.0). For 1 g of fresh tissues 10 ml of buffer used. After 1h shaking, the samples were centrifuged at 15000 × g for 20 min. The supernatant fraction (crude enzyme extract) was used as the source of enzyme and for soluble protein determination. All these preparations were carried out at 0° to 4°C.

Phosphatase activity was determined after Lorenc-Kubis and Morawiecka (1973) at pH 5.0 and 6.4. In the first case incubation mixture contained 1 ml 0.1M acetate buffer, pH 5.0, 0.5 ml enzyme extract, and 0.5 ml (4 μM) pNPP as substrate. For determination of phosphatase activity at pH 6.4 incubation mixture was used which contained 1 ml 0.1M Tris-maleinate buffer, 0.5 ml crude enzymatic extracts, and 0.5 ml (4 μM) ATP as substrate. The mixture was incubated at 37°C for 30 min. and the reaction was stopped by an addition of 2 ml of 5% cold trichloroacetic acid. Proteins were removed by centrifugation and inorganic phosphate released from the substrate was determined by the method of Fiske and Subbarow (1925). One unit of acid phosphatase activity was expressed as μmoles of inorganic phosphate liberated per 1 min., at 37°C. Specific enzyme activity was expressed as unit of enzyme activity per mg of protein.

Phosphorus fraction was determined in fresh plant material after Fiske-Subbarow, according to Mejbaum-Katzenellenbogen and Mochnacka (1969) scheme. Protein content in crude enzyme extract was determined by Lowry et al. (1951) method. All experiments were made separately for the cotyledon and root extracts.

RESULTS

Initial experiment refered to measurements of phosphatase activity of the enzymatic extracts taken from four-day cucumber seedlings, at different pH values, in the presence of two substrates; pNPP and ATP. Fig. 1 shows that in the extracts two optima of phosphatase activity occured depending on the substrate used. In the presence of pNPP, the highest phosphatase activity was observed at 5.0 pH, wherease in case of ATP as the substrate, optimum pH value was 6.4. Consequently, further experiments were carried out at those two pH values.

Data presented in Tables 1 and 2 ilustrate substrate specificity of the cotyledon and root extracts. The experiments were carried out at two variants: the cucumber grew either on the medium I (the complete medium), or on the medium II (without phosphorus).

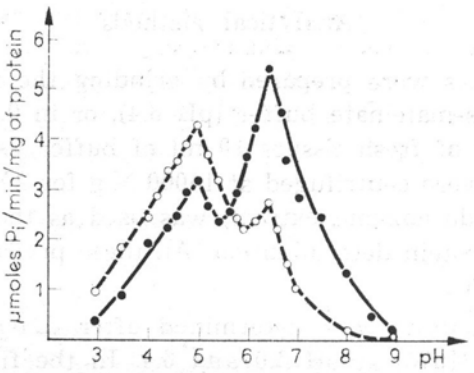

Fig. 1. Influence of pH on phosphatase activity in extracts of cucumber seedlings

O- - -O phosphatase activity towards pNPP as a substrate
●——● phosphatase activity towards ATP as a substrate

Table 1

Substrate specificity of phosphatase activity from cotyledons and roots of cucumber seedlings

Substrate	Cotyledon		Root	
	6.4*	5.0*	6.4*	5.0*
pNPP	75	100	65	100
ATP	100	80	100	89
GTP	29	28	43	81
AMP	9	33	20	72
Glycero-6-phosphate	56	28	27	56
β-naphtylophosphate	73	65	77	78
Pyrophosphate	149	68	153	154
Calcium phytinate	56	90	0	0

Cucumber seedlings grew for four days in the medium. Activity is expressed as per cent in reference to the activity determined towards ATP (pH 6.4) or pNPP (pH 5.0). Incubation mixture contained 4 µmoles of respective substrates.

* — pH.

In case of the cotyledon, apart from pNPP and ATP, also β-naphtylophosphate and pyrophosphate — the preferential natural substrate of acid plant phosphatase — yielded strong hydrolysis. In four-day-old seedlings there was a very high activity toward calcium phitynate which disapeared during further growth phases.

Root extracts also hydrolyzed a wide range of substrates. Symilary as the cotyledons, high activity was marked for pNPP, ATP, pyrophosphate, β — naphtylophosphate, and at 6.4 pH also for GTP and AMP.

Substrate specificity of phosphatases was similar both, for seedlings grown on complete medium, and on medium without phosphorus, but

the asbolute values of phosphatase activity were higher for plants grown on medium without inorganic phosphorus (Table 2). This data point to inhibiting influence on phosphatase activity of inorganic phosphorus present in the nutrient solution.

Table 2

Substrate specificity of acid phosphatase from cotyledon and root extracts of cucumber seedlings

Part of seedling	Substrate	−P		+P	
		6.4*	5.0*	6.4*	5.0*
		μmole $P_i \times min.^{-1} \times 100$ mg			
Cotyledon	pNPP	14.734	13.30	11.056	11.197
	ATP	18.417	11.330	16.023	9.293
Root	pNPP	3.749	3.967	3.510	3.350
	ATP	4.166	2.440	3.582	1.501

Cucumber seedlings grew for four days in the medium.
* — pH.

The next table (Table 3) shows the influence of ions present in the incubation medium on phosphatase activity of cotyledons and roots. It was found that Cu^{2+}, Co^{2+}, Zn^{2+} and Mo^{2+} ions in the incubation medium distinctly inhibited phosphatase activity towards pNPP and ATP as substrates. Calcium and megnesium ions also caused lowering of activity levels at 6.4 pH.

The analyses of the changes of phosphatase activity in cucumber seedlings showed that this activity had a few peaks during 14-day growth of seedlings.

Fig. 2a shows changes of phosphatase activity in the root extracts. Its first peak took place already during seed germination (2 days after imbibition). After the seedlings have been brougth to light, activity of phosphatases rapidly decreased. However, a day later it began to increase again. The third peak took place on the 6th day of seedling growth; later on the activity was going down, and from the 7th day it remained on a relatively constant level.

Specific phosphatase activity (Fig. 2b) was somewhat different; no peak activity was observed after 2 days of seed germination, and differences in the absolute values after 4th and 6th day of growth were considerably more distinctive.

Total phosphatase activity of the cotyledon extracts (Fig. 3a), marked at pH 5.0 towards pNPP as a substrate, reached the highest value after 2 days of seed germination. Further on, i.e. untill the 7th day of seedling growth, it continued to decrease. At pH 6.4 phosphatase activity had two peaks toward ATP: on the 4th and 6th day of cucumber growth. Since the 7th day till the moment of formation of the first leaf,

Table 3

The effect of various cations present in the incubation mixture on phospphatase activity of cotyledon and root extracts of cucumber seedlings

Cation (mM)		Cotyledon		Root	
		6.4*	5.0*	6.4*	5.0*
Ca^{2+}	2	79	110	121	105
	10	54	109	61	105
	20	38	105	38	85
Mg^{2+}	2	63	114	64	78
	10	23	104	23	56
	20	13	105	27	48
Zn^{2+}	2	95	68	96	83
	10	16	32	13	22
Mo^{2+}	1	93	8	43	8
	10	0	0	0	0
Co^{2+}	1	86	95	78	81
	10	23	56	7	40
Cu^{2+}	1	58	35	30	17
	10	12	15	0	0

Phosphatase activity is expressed as per cent of the control (sample without ions).

* — pH.

phosphatase activity remained at the same leavel, independently of pH. Specific phosphatase activity of cotyledon extracts (Fig. 3b) was similar as of roots. No peak was observed after two-days of seed germination, and maximum activity was noted after 6 days of seedling growth.

The data presented here showed also that during the growth period under study absolute activity of the cotyledon extracts was higher than of the root extracts. On the other hand, specific activity was much higher in root extracts.

The phosphatases consistitute a group of enzyms which are responsible for the phosphorus budget, and it would be interesting to examine the content of each phosphorus fraction on the background of activity changes taking place during seedling growth. Hance, further works were concentrated on this problem. Investigations were limited only to these phase of seedling growth in which the highest values of phosphatase activity were noted, i.e. to the 2nd (germination), 4th and 6th day of growth. Analyses were made for seedlings growing on complete medium, and on medium deprived of phosphorus. The results are given in Table 4. As it is seen, after 4 days of growth level of almost all phosphorus fractions in the cotyledons and roots was much lower than

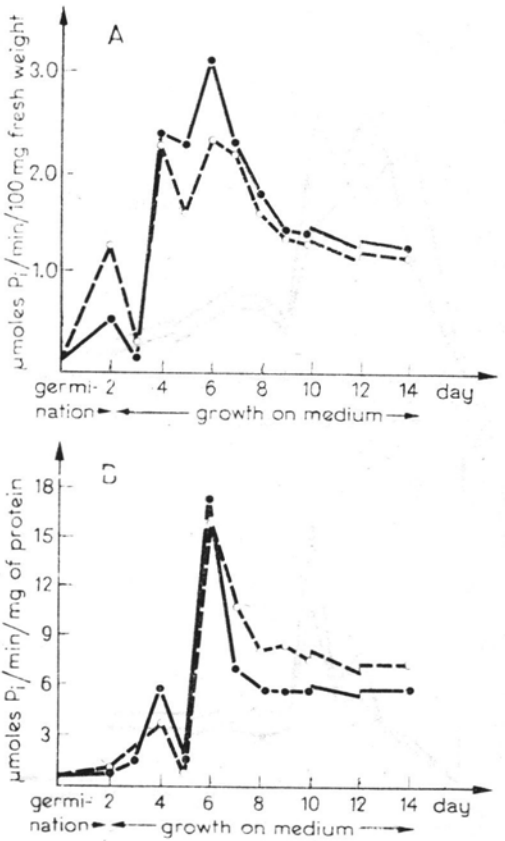

Fig. 2. Changes of total (A) and specific activity (B) of phosphatase in root extracts of cucumber seedlings

O– – –O phosphatase activity towards pNPP (pH 5.0) as a substrate
×——× phosphatase activity towards ATP (pH 6.4) as a substrate

the values received after 2 days of germination. This may have been caused by the following reason limitiation of the analysis to cotyledons and roots, with no attention being paid to hypocotyl, which was already well formed after 4 days of growth. After 6 days of plant growth on the medium, an increase of inorganic phosphorus was observed in cotyledons, whereas organic phosphorus level within easily and hardly hydrolyzing fraction decreased. Lipid phosphorus level slightly decreased, whereas of protein and nucleic acid phosphorus reached higher values in this phase of growth, than in the previous phase. Changes of each phosphorus fraction level in the cotyledons of seedling growing on the complete medium were similar.

Inorganic phosphorus fraction in root extracts prepared from 6-day seedlings remained at the same level, both in case of complete medium

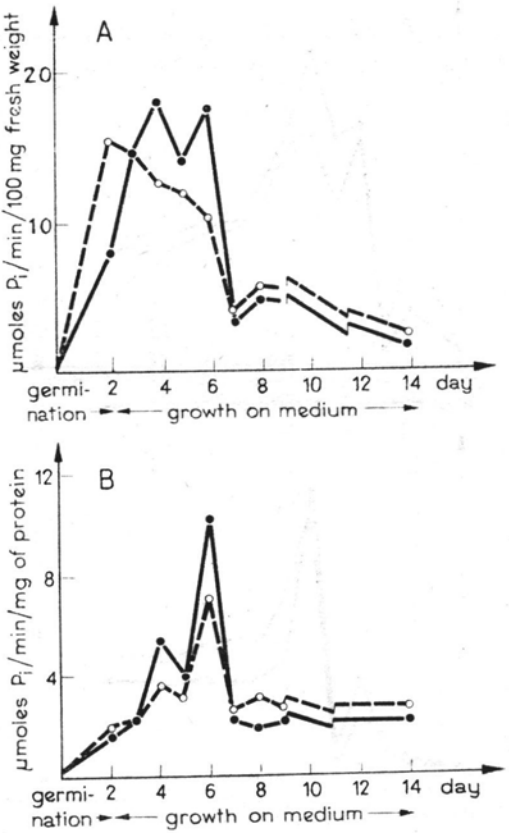

Fig. 3. Changes of total (A) and specific (B) phosphatase activity in cotyledon extracts of cucumber seedlings

O- - -O phosphatase activity towards pNPP (pH 5.0) as a substrate
X——X phosphatase activity towards ATP (pH 6.4) as a substrate

and on medium without phosphorus. Content of easily hydrolyzing, hardly hydrolyzing, and lipid organic phosphorus fraction decrease slightly.

DISCUSSION

Initial experiments, aimed at an establishment of optimum pH values for phosphatase activity, suggested the presence of two groups phosphatases, both in root and cotyledon extracts of cucumber seedlings. Both groups differed with respect to pH optimum. The first group had the peak of activity at pH 5.0, the second — at pH 6.4.

Investigations on substrate specificity supported these suggestions. Enzyme with the optimum of pH 5.0 showed the highest activity towards

Table 4

Changes of the level phosphorus fraction (μmole P_i/g fresh weight) in cotyledons and roots during growth phases of cucumber seedlings

Phosphorus fraction	Part of seedling	Day of measurement				
		2 th	4 th	6 th	4 th	6 th
			Growth non medium −P		Growtnh on medium +P	
		μmole P_i/g fresh weight				
Total-P	C	2780	2833	2146	3272	2700
	R	1380	560	614	575	520
Inorganic-P	C	233	237	1080	650	1280
	R	312	160	157	146	145
Organic-P	C	4585	1712	300	1902	560
	R	308	180	157	153	180
Protein and nucleic acid-P	C	1200	300	500	370	520
	R	142	183	197	180	200
Lipid-P	C	6000	320	280	350	300
	R	324	89	75	103	90
Hardly hydrolyzing-P	C	4520	1280	250	1812	480
	R	280	145	130	115	155
Easily hydrolyzing-P	C	390	120	50	90	80
	R	300	35	27	29	27

C — cotyledon
R — root

pNPP as a substrate, while with the optimum at pH of 6.4 towards ATP. In both cases relatively high activity was noted towards other substrates. This would point to unspecific character of the activities examined. Pyrophosphate was especially dynamically hydrolyzed; this substrate being preferred by plant acid phosphatases (K u b i c z, 1973). The same is true of β-naphtylophosphate, and also of GTP and AMP at pH 6.4 in extracts of 2-day old roots. In cotyledon extracts prepared from 2-day cucumber seeds at pH 6.4 high activity towards calcium phytinate was noted. This seems to be natural since phytine-calcium-magnesium salt of inositohexaphosphoric acid constitutes a reserve of inorganic phosphorus in seeds (K o l l e r et al., 1962). There is a number of works reporting an increase of phopshatase activity towards this substrate during germination (M a y e r, 1958; M u k h e r j i et al., 1971; P a u l et al., 1971; P a l m i a n o, J u l i a n o, 1972).

The optimal pH values, significant substrate unspecificity, and inhibiting effect of such elements as zinc, copper, molybdenum, calcium and magnesium, indicate that the activities described here belong to the group of acid phosphatases.

Substrate specificity of phosphatase was similar for seedlings grown on the complete medium, and on the medium without phosphorus, but the absolute values of activities were much higher for the letter (Table 2). This fact points to an inhibiting influence of phosphorus on the described activity. Hewitt and Tatham (1960) observed similar increase of phosphatase activity in leaves of tomato grown on a medium without phosphorus. Jonson et al. (1973) observed it in the leaves of other higher plants. According to Reid and Bieleski (1970) control of phosphatase activity by phosphorus level in the medium may take place in two ways: by inhibiting by the product of the reaction, or by the regulation of real enzyme concentration in the tissue.

Results concerning changes of phosphatase activity during the 1st week of cucumber seedling growth differ considerably from the literature data. Palmiano and Juliano (1973), Firenzuoli et al. (1968), Keswani and Upadhya (1969), and Jonson et al. (1973) observed only one peak of activity — after 4th or the 5th day, whereas in my experiments there were as many as 3 peaks of activity during the same period of time. This discrepancy may result both, from species differences between the examined plants or from different growth conditions. The mentioned authors carried out their experiments from the moment of soaking, either in continuous light or in constant darkness, whereas in my experiments growth of cucumber seedlings took place in the day/night conditions with the exception of two days of germination. Hence, it is possible that the breakdown of the whole phosphatase activity, noted in roots and cotyledons directly after the germed seeds were transformed to light, was caused by sudden, drastic change of light conditions.

Interpretation of the fluctuations of phosphatase activity during further phases of seedling growth seems to be more difficult. These fluctuation were expressed by a slight decrease of activity after the 5th day, and a new increase after the 6th day of growth. However if consideration is given to seedling metabolic changes with respect to chlorophyll synthesis, intensified cell mitosis in the region of root growth, and formation of epicotyl and leaves, it seems that the observed fluctuations of phosphatase activity may result from changes taking place in plant, and possibly also from the activation and inactivation, or synthesis *de novo* of different types of phosphatases.

Changes of phosphatase activity taking place in cotyledon extracts during early phase of cucumber growth, were plainly correlated with changes of phosphorus fraction content in the period under study. Increase of inorganic phosphorus with simultaneous drop of organic phosphorus was observed within such fractions — as easily and hardly hydrolyzing, and lipid ones.

In case of root extracts decrease of the content of organic phosphorus fraction was consistent with simultaneous increase of phosphatase activity. However, no increase of inorganic phosphorus was observed, probably as a result of inorganic phosphorus transport to overground part of plant (Lundegärdh, 1939; Nowotny-Mieczyńska, 1976). The fact, that there is an almost identical amount of total phosphorus in roots of the seedlings grown on the complete medium, as in case of medium without phosphorus, proves the validity of this statement, while the amount of total phosphorus in cotyledons of plants grown on the medium with phosphorus, exceeded the values obtained for cucumber grown on the medium deprived of prosphorus (Table 4).

Differences of the distribution of specific and total activity during the first week of cucumber growth, both in root as cotyledon extracts, consisting of a lack of peak of specific activity after 6 days of growth as compared to the activity of 4-day seedling extracts, probably result from changes in the content of proteins soluble in 1N NaOH in this time. Quantity of proteins visibly decreased until the 7th day of growth.

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Zmiany aktywności fosfatazowej podczas kiełkowania i wczesnych faz rozwoju Cucumis sativus

Streszczenie

W liścieniach i korzeniach siewek *Cucumis sativus* L. wykazano obecność fosfataz posiadających dwie optymalne wartości pH: 6,4 i 5,0. Charakteryzuje je niska specyficzność substratowa. Spośród analizowanych substratów szczególnie

preferencyjnie rozszczepiane były p-nitrofenylofosforan, adenozynotrójfosforan, β-naftylofosforan a także, w przypadku wyciągów liścieniowych, fitynian wapnia. Mo, Zn, Cu oraz Co obecnie w mieszaninie inkubacyjnej hamowały omawianą aktywność. Analiza zmian aktywności fosfatazowej w liścieniach i korzeniach siewek ogórków wykazała, że podczas wczesnych faz rozwoju ogórków (kiełkowanie i pierwszy tydzień wzrostu na świetle) aktywność ta utrzymywała się na stosunkowo wysokim poziomie i posiadała kilka szczytów, które były związane z fazą rozwoju rośliny a częstotliwość ich pojawiania się z analizowanym organem. Stwierdzono, że zmiany aktywności fosfatazowych podczas wczesnych faz rozwoju ogórków w wyciągach liścieniowych były wyraźnie skorelowane ze zmianami zawartości fosforu w badanym okresie. W korzeniach nie zaobserwowano podobnych korelacji.