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Enzymic hydrolysis of adenosine triphospahtes by tobacco and tomato tissue at various stages of their development

A. KOZŁOWSKA, K. DUSTANOWSKA

INTRODUCTION

Enzymes hydrolizing ATP have been found in nearly all plant tissues (Fruton and Simmonds 1959). The specific phosphatase which causes the removal of the terminal phospate group of ATP forming ADP and inorganic phosphate, was discovered by Meyerhof (1949) in yeasts. It was termed like the muscle myosin and actin — adenosine triphosphatase (ATPase). During fermentation by the yeast extract, ATP accumulates and the inorganic phosphate disappears. The presence of ATP-ase in yeast cells maintains a balance between the phosphorylation of glucose, for which ATP is required, and the phosphorylation of glyceraldehyde-3--phosphate, for which inorganic phosphate is required.

The enzyme preparation from potatoes catalyzes the hydrolysis of both pyro--phosphate bonds of ATP with the formation of AMP and 2 equivalents of inorganic phosphate (Kalckar 1943, Krishman 1949). To distinguish these enzymes from the ATP-ases of muscle and yeasts, they were named "apyrases" (Kwan Hua and others 1951). Kotelnikowa (1951) has shown, that the activity of apyrase changes in various stages of germination of the tuber. Webster (1959) Young and Varner (1959) described in the cotyledons of germinating peas a crude phosphatase extract highly specific for adenosine triphosphate-ATP and adenosine diphosphate-ADP. The activity of apyrase was also investigated by Esanu (1962) in healthy and virus infected tobacco leaves.

The present study was undertaken to examine tobacco leaf and root tissues with respect to their ability to hydrolyse adenosine triphosphate at various stages of their development.

MATERIAL AND METHODS

Plant material

In all experiments seedlings of *Nicotiana tabacum* v. White Burley and *Lycopersicum esculentum* v. Selandia were used. Tobacco and tomato seedlings were cultivated in greenhouse conditions from spring to autumn in garden soil as well as in water cultures with full nutrient (according to Hewitt 1952).

Sampling

For analyses discs 2 mm in diameter were punched out with a cork borer from the green areas of the leaves. For every analysis samples of 100 discs each were taken. For dry weight determination samples were dried at 105°C to about 4 mg d.w.

From tomato seedlings cultivated in water cultures, 8 mm long root apices were dissected. Each sample for analysis consisted of 25 root apices (about 20 mg fresh weight).

Analytical procedure

Fresh samples of leaves and roots instantly after cutting off were minced with 0.5 ml of cold Tris buffer, pH 7,2—7,3. Subsequently 1,5 ml of the same Tris buffer (0°C) was added. The mixture was taken for enzyme assays. Apyrase activity was measured by incubating the enzyme preparation with 1 ml of 0.001 M ATP (623,19 μg ATP containing 81, 42 μg P) in a total volume of 3 ml, at 37°C for 20 min. The reaction was stopped by addition of 3%$HClO_4$ at pH 5,5. After centrifuging, the inorganic phosphate released was determined by the method of Martin and Doty (1949). The enzymic activity was calculated from the difference of inorganic phosphate in the crude plant extract before and after incubation with ATP. For each experiment, control of ATP hydrolysed during incubation without plant extract, was performed.

DEGREE OF SPECIFICITY OF ENZYMATIC HYDROLYSIS OF ATP IN TOBACCO AND TOMATO TISSUE

In order to reveal the degree of specificity and characteristics of the enzyme, hydrolysing ATP in fresh extracts from leaves and roots of tobacco and tomato plants, the following preliminary experiments were carried out:

1. Extracts from 100 leaf discs were boiled and afterwards incubated with the additon of ATP. The amount of inorganic phosphorus in the extract did not change after incubation.

2. The sap extracted from 5 g of fresh leaf tissue, and dialysed for 1 or 2 days in a cellophane tube, revealed only minute amounts of inorganic phosphorus. After incubation with ATP there appeared large quantities of phosphorus which was split off from this compounds as a result of enzymic activity.

3. Strophantine G — known as a specific inhibitor of muscle ATP-ase (Hokin and others 1966), did not show any effect when added to the extracts and incubated together with ATP. Similarly the addition of magnesium ions, 2-nitrophenol, hydroxylamine or EDTA in 0.001 and 0.01 M concentrations, before incubation with ATP, did not provoke any changes in the dephosphorylation process of the latter.

4. Contrary to animal ATP-ase, the investigated plant extracts are capable to dephosphorylate both ATP and ADP. As was shown in our experiments the phos-

phorus is separated in this case almost 1/3 times more intensively from ATP than from ADP.

5. As in the above described experiments the dephosphorylation of ATP took place in weakly alkaline medium (pH 7,3), the contribution of acid phosphatases to the hydrolysis of ATP must be excluded.

One of the best substrates for a range of different alkaline phosphatases is glycerophosphate (Summer 1950). The incubation of the investigated plant extracts was therefore carried out with the addition of 0.001 M sodium glycerophosphate instead of ATP. However in this case as well the content of inorganic phosphorus did not change after incubation. It may be therefore affirmed, that the investigated enzymatic processes in extracts from leaves and root tips of tobacco and tomato plants, are highly specific and point to the activity of "apyrase", already detected in potato tubers (Meyerhof 1945) and pea germs (Young and Varner 1959).

ENZYMATIC ACTIVITY OF APYRASE IN YOUNG TOBACCO LEAVES

From the beginning of March till the end of September the development of tobacco seedlings in greenhouse conditions is approximately similar. The juvenile phase of the plant lasts for about a month, the leaves — 5 or 6 in number — grow low above the ground. When the 7th mature leaf develops, the juvenile leaves of the plant are still in the stage of full growth. The moment they attain their full development and stop growing, the development of the mature leaves becomes accentuated.

Investigations on apyrase activity in young tobacco leaves were carried out in the greenhouse in the period from June 20 to July 9, starting from young seedlings in the three-leaves stage, up to the moment when the seventh leaf began to develop. Analyses were carried out every 2 or 3 day, always on the 4-th leaf, the surface area of which was estimated (length × width × 0,7).

Table 1

Inorganic P and quantity of hydrolysed ATP in leaves of one tobacco seedling

Date	Leaf surface area (cm^2)	Inorganic P		Hydrolysed ATP
		µg	After incub. µg	µ
17.VI	40	5,5	14,2	66,5
18.VI	54	5,2	14,8	73,4
19.VI	65	6,2	17.6	87,2

The results are summarised in fig. 1. The surface area of the investigated leaves increased in the period from June 20 to July 9 about sixteen times. Nevertheless the inorganic phosphorus level in the leaf tissue did not change apparently. However at the same time a close correlation could be observed between the increasing acti-

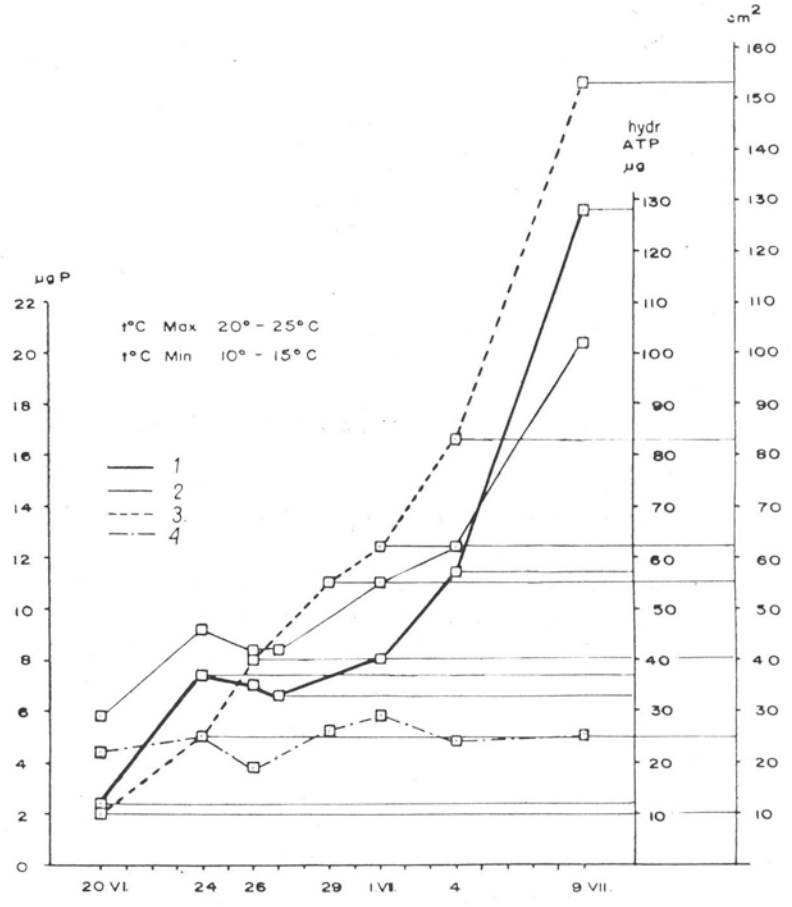

Fig. 1. Correlation between apyrase activity and leaf development in summer.

1 — Amount of hydrolysed ATP during incubation; 2 — Inorganic phosphorus content after incubation; 3 — Surface of leaves examined; 4 — Inorganic phosphorius content before incubation

vity of apyrase and the augmentation of the leaf blades. A second experiment, carried out on June 17th — on a separate tobacco seedling — confirmed the above given results. The content of apyrase was estimated here in three subsequent leaves of different size (Table 1).

Previous investigations, carried out in the same greenhouse conditions with young tobacco seedlings of the v. White Burley, have shown, that parallely to the growing leaf size, also the intensity of respiration increases (Kozłowska 1955), as well as the concentration of free amino acids in the leaf tissue. In the stage of the intensive growth of young leaves protein synthesis exceeds considerably their hydrolysis (Steward and Street 1947), and parallely increases also the requirement for free energy which is also obtained from the desintegration of ATP.

In winter time, under short day and at temperature ranging from 13°C to 20°C the development of tobacco seedlings in the greenhouse is inhibited. The size of

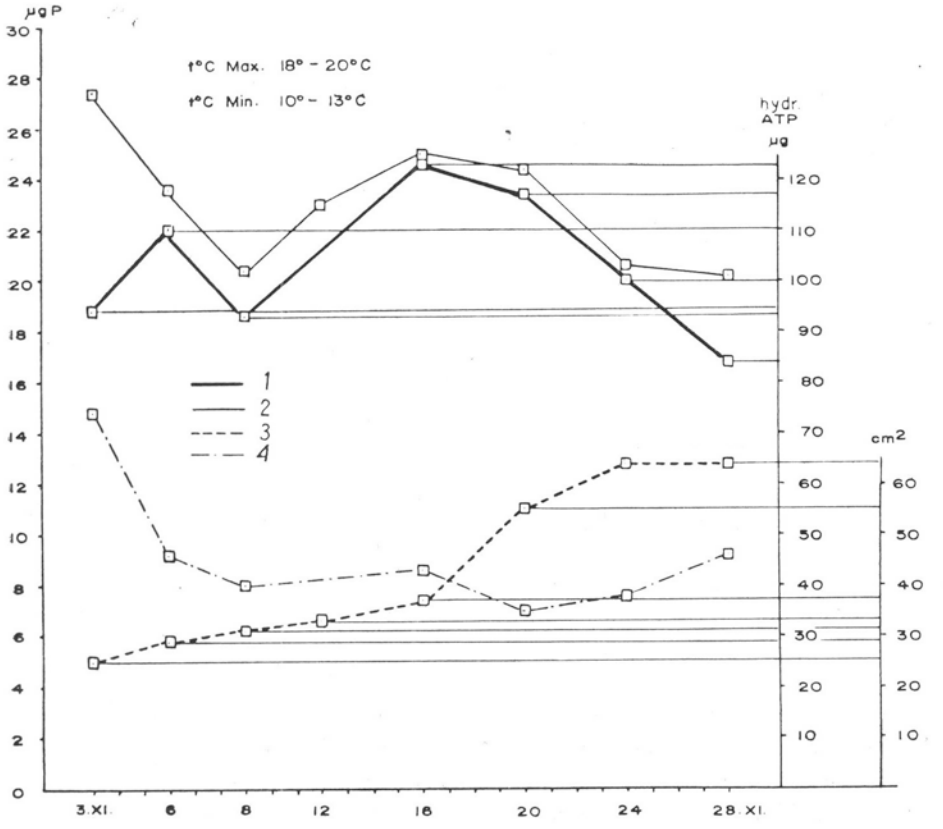

Fig. 2. Apyrase activity in tobacco leaves in winter.

1 — Amount of hydrolysed ATP during incubation; 2 — Inorganic phosphorus content after incubation; 3 — Surface of leaves examined; 4 — Inorganic phosphorus content before incubation.

leaves changes only insignificantly during the whole month. The respiration rate of the leaf tissue considerably decreases and also the concentration of free amino acids attains its lowest level (Kozłowska 1955). Similarly as in summer time, investigations on apyrase activity were carried out in the period between November 3 and 29 with tobacco seedlings in the six-leaves stage. Samples for analyses were taken every 2 or 3 days from the 4th leaf. The results are presented in fig. 2.

In comparison with the spring and summer, the content of inorganic phosphorus in winter remained at a slightly higher level, the leaf growth progressing very slow. However, strikingly enough, the activity of apyrase was very high during the whole period of the experiment.

In winter, the leaves inhibited in growth and development, become prematurely senescent, when the protein hydrolysis processes exceed those of resynthesis. Since in such leaves the respiration rate is very low, it may be expected, that the requirements of living energy are partly compensated by the enhanced ATP dephosphorylation.

COMPARISON OF APYRASE ACTIVITY IN TOBACCO AND TOMATO LEAVES

Investigations were carried out in March in the greenhouse with tobacco and tomato seedlings, in a similar stage of development, — tobaccos with 7 fully developed leaves and tomato plants with 7—9 leaves. Samples for analyses were taken from the 7th leaf on tobacco and from the 5th leaf on tomato plants by cutting out the middle part of the blade. For each estimation about 30 mg fresh weight of the leaf tissue were taken. Inorganic phosphorus content and apyrase activity were determined in each sample.

Table 2

Inorganic P and quantity of hydrolysed ATP in tobacco and tomato leaves

Plants	Inorganic P leaf extracts		Hydrolysed ATP
	µg	After incub. µg	µg
tobacco	3,6	21,9	140,3
tomato	30,6	49,3	145.0

As seen from table 2, the level of inorganic phosphate was about three times higher in tomato leaves than in young tobacco leaves. Nevertheless the rate of ATP dephosphorylation was about the same in both plants.

Similarly as in the investigated tobacco seedlings, the activity of apyrase in older tomato leaves inhibited in development in the winter period, was about two times higher than in spring, when the leaves developed most rapidly.

ENZYMATIC ACTIVITY OF APYRASE IN ROOT TIPS

In order to investigate the apyrase activity independently of the processes of photosynthesis and senescence in the leaf tissue, the concentration of this enzyme was also analysed in the tomato root tips. Appropriate experiments were carried out in the greenhouse during the period from November to May. Young tomato seedlings in the 3—4 leaves stage were transferred to a water culture, containing a full nutrient solution according to Hewitt (1952) of pH 6. The water was constantly maintained at the same level to keep the root system immersed in 2/3 in the nutrient. After 20—30 days, when the developing roots attained about a 30 cm length, their 8 mm long tips were cut out. They contained the meristematic tissue and the growth zone of the root. 25 such segments were taken for each analysis. Inorganic phosphorus content as well as apyrase activity were estimated on the basis of 15 mg fresh weight of the root tissue.

A total of six experiments were carried out independently: three in spring in March and April and three in winter — in December and February. In each expe-

riment investigations were carried out for 4—6 consecutive days. It was found that the differences in the results of analyses of young roots, made in March and May, were not statistically significant. All the investigated tissues were exactly in the same stage of development.

The increase of apyrase activity occurred similarly as in the leaf tissue, — in winter time, in the experiments carried out during December and February. In the latter experiment samples were taken from young plantlets in the 6—7 leaves stage, while in December the analysed plants were much older, attaining a height of about 60 cm. In these plants the main roots already ceased to grow and were replaced by slowly growing side roots. No significant differences in enzyme activity were found between young roots of both old and young tomatoes, grown in the winter period. Such differences appeared however, when comparing the main old roots with the young rootlets of the same plant, — the former exhibiting always a considerable increase in enzyme concentration. Apart from the observed fluctuations, the main differences in apyrase activity were found between plants analysed in spring and in winter. The results are summarized below:

Time of analysis (months)	Hydrolysed ATP μg
III and V	119,7
II and XII	194,3

This difference was found to be statistically significant at the 0,95 probability level. If we compare the same data obtained only in the winter experiments from young and old roots, the average values were as follows:

	Hydrolysed ATP (μg)
1. Young roots (December-February)	174.0
2. Old roots (December)	234.8

As compared with the leaf tissue, the activity of apyrase was higher in the root tips — both in the spring and the winter period. The average values, found to be significantly different at 0,95 and 0,99 probability levels, are summarized below:

	Hydrolysed ATP (μg)
Roots	164.0
Leaves	96.0

DISCUSSION

The enzymatic activity, connected with ATP hydrolysis, and characteristic of apyrase, as described for the potato tuber and pea germ tissues, has been found to be much higher in the root tips containing besides the meristem also the growth zone, than in the leaves of the tomato plant. The increase of the activity of this enzyme in plant tissue is therefore not connected with photosynthesis. Simultaneously with the pro-

ceeding development, tobacco leaves exhibit a gradual increase in apyrase activity connected with the liberation of corresponding quantities of energy. In the juvenile stage of leaf development, the increase in the activity of this enzyme is accompanied by a similar increase in respiration rate and protein synthesis dominates over their hydrolysis. There is therefore a correlation between apyrase activity and protein metabolism in the plant.

It is very interesting that the apyrase activity remains at about the same level both in the leaves of tomato and tobacco plants, although they contain different amounts of inorganic phosphorus. Investigations on the behaviour of this enzyme in plant tissues, in roots and leaves which have ceased to grow owing to their age or the winter season, have given unexpected results. In mature and senescent plants, in which the respiration rate considerably decreases and the hydrolysis of proteins exceeds their resynthesis, the dephosphorylation of ATP attains a constant high level. It may be assumed on this basis, that the maintenance of living equilibrium, when the basic metabolic processes are inhibited, occurrs mainly by way of ATP dephosphorylation.

Virus Laboratory
Institute of Plant Physiology
Polish Academy of Science
Kraków, Al. Mickiewicza 21

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Enzymatyczna hydroliza ATP w tkankach tytoni i pomidorów w zależności od ich stadiów rozwojowych

Streszczenie

Enzym apyraza, opisany po raz pierwszy u roślin w bulwach ziemniaków i kiełkach grochu, wykazał w naszych badaniach w wierzchołkach korzeni pomidorów dwukrotnie wyższą aktywność niż w odpowiadających tym korzeniom liściach. Wzrost aktywności apyrazy nie jest tym samym związany z fotosyntezą.

W miarę wzrostu i rozwoju młodocianych liści tytoniu aktywność apyrazy rośnie, dochodząc do najwyższego poziomu w liściach w pełni rozwiniętych. Rosnąca aktywność apyrazy, przebiegająca z równoczesnym wzrostem procesu oddechowego jest w tym wypadku związana z rosnącą syntezą białkową. W czasie rozwoju i wzrostu liści synteza białek góruje nad ich hydrolizą. Odwrotnie zjawisko spotykamy w tkankach liści, które w porze zimowej, w warunkach szklarniowych wykazują przez dłuższy okres czasu zahamowany wzrost, będący przedwczesnym procesem starzenia się. Defosforylacja ATP utrzymuje się w liściach stale na wysokim poziomie mimo, że proces oddechowy w porównaniu z okresem letnim jest silnie zahamowany.

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Further investigations on the relationship between soil fungi and the macroflora

M. BADUROWA and L. BADURA

INTRODUCTION

Numerous investigations prove unequivocally that there exists a positive correlation between the number of fungi occurring in the given habitat and the ecological conditions. According to W.G.E. Eggleton (1934), M. Witkamp and J. van der Drift (1961) and A. Burges and D. P. Nichelson (1962) mycological associations and their activities are controlled by the temperature and moisture of the soil as well as by the amount of available organic matter. There exists also a negative correlation between the number of isolated fungi and the depth of the soil sampled for mycological analysis (M. J. Timonin 1935; H. Krzemieniewska and L. Badura 1954; L. Badura 1960).

In so far as the relationship between ecological conditions and the number of fungi is concerned, the controlling effect of ecological conditions can be shown, but the dependence of myco-associations on the associations of higher plants is very little known. This problem has been dealt with by B. Peyronel and G. dal Vesco (1955) and by A. E. Apinis (1960, 1964). The former authors investigated the occurrence of soil fungi in cultivated soils in order to find a relationship between the mycoflora and cultivated plants. The latter attempted to establish a relation between the soil fungi and the associations of higher plants on various alluvial soils. The above investigations permitted to state that a relationship does exist. The question arises, however, whether the occurrence of soil fungi is conditioned by the ecological factors of the habitat, or by the organic substrate derived from the associations of higher plants. This problem has attracted the attention of L. Badura (1964) who by means of diagrams made according to B. Peyronel's method (1955, 1956) showed that there exists an analogy in the percentage of individual fungal groups occurring in beech forests, differing with the ecological conditions. In view of the above results L. Badura (1964) assumed that the dominance of strictly determined groups of fungi is conditioned by the litter because of its trophic character. Since the chemical composition varies with the litter, different groups of fungi will be more or less priviledged.

The purpose of the present paper was to obtain further information on the relations between the mycoflora and the vegetal cover.