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The influence of phosphate deficiency on photosynthesis, respiration and adenine nucleotide pool in bean leaves

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Abstract

The decrease in inorganic phosphate (P_i) content of 10-d-old *Phaseolus vulgaris* L. plants did not affect rates of photosynthesis (P_N) and respiration (R_D), leaf growth, and adenylate concentration. Two weeks of phosphate starvation influenced the ATP content and leaf growth more than P_N and R_D. The ATP concentration in the leaves of 15- and 18-d-old phosphate deficient (-P) plants after a light or dark period was at least half of that in phosphate sufficient (+P, control) plants. Similar differences were found in fresh and dry matter of leaves. However, P_N declined to 50 % of control in 18-d-old plants only. Though the R_D of -P plants (determined as both CO_2 evolution and O_2 uptake) did not change, an increased resistance of respiration to KCN and higher inhibition by SHAM (salicylhydroxamic acid) suggested a higher engagement of alternative pathway in respiration and a lower ATP production. The lower demand for ATP connected with inhibition of leaf growth may influence the ATP producing processes and ATP concentration. Thus, the ATP concentration in the leaves depends stronger on P_i content than on P_N and R_D.

Additional key words: adenylate; ADP; AMP; ATP; CO_2 compensation concentration; dry matter; KCN; *Phaseolus vulgaris*; plant age; salicylhydroxamic acid.

Introduction

Plants grown on a phosphate depleted medium develop morphological and metabolic adaptations to a low P_i concentration in the enviroment. They may decrease their shoot matter but root matter remains the same or may be higher when compared to

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Abbreviations: AEC - adenylate energy charge; DMSO - dimethyl sulfoxide; +P - phosphate sufficient plants; -P - phosphate deficient plants; P_i - inorganic phosphate; P_N - net photosynthetic rate; PAR - photosynthetically active radiation; R_D - dark respiration rate; SHAM - salicylhydroxamic acid; Γ - CO_2 compensation concentration.
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plants grown on a complete medium (Sicher and Kremer 1988, Rao and Terry 1989, Rychter and Mikulska 1990, Rychter and Randall 1994). A low phosphate level in the tissue may decrease P_N (Sicher and Kremer 1988, Freeden *et al.* 1990) but according to some authors the depletion of phosphate in nutrient solution does not affect CO_2 assimilation (Foyer and Spencer 1986). Growth limitations often do not correlate with decreased P_N (Rao and Terry 1989, Kondracka and Rychter 1997).

The availability of P_i may regulate intracellular adenylate level. Usually, under a phosphate starvation ATP and the total adenylate concentration decrease in a similar way as P_N (Miginiac-Maslow and Hoarau 1982, Dietz and Heilos 1990) but in some plants there is no correlation between P_N and ATP level (Thorsteinsson and Tillberg 1987). In photosynthetic carbon metabolism, P_i can be liberated during synthesis of saccharides, organic acids, and also during photorespiration. Thus, under phosphate deficiency some above mentioned reactions may enhance P_i recirculation (Harley and Sharkey 1991, Kondracka and Rychter 1997).

Previously, we observed that respiration of roots of the phosphate deficient plants was slightly lower than that of phosphate sufficient roots, and was carried out mainly by alternative nonphosphorylating cyanide resistant pathway (Rychter and Mikulska 1990). The participation of alternative pathway in root respiration resulted in a lower ATP production. Estimated concentrations of ATP and ADP, and adenylate energy charge (AEC) values in the phosphate deficient roots were also lower (Rychter *et al.* 1992). The AEC decreases in tissues under conditions limiting the ATP production (Pradet and Raymond 1983). However, a low ATP concentration in the roots does not affect their growth (Rychter and Mikulska 1990) but lowers other energy driven processes such as nitrate uptake (Rufty *et al.* 1993). Since the growth of the shoot of phosphate deficient plants is considerably reduced, the purpose of this study was to investigate the effect of phosphate deficiency on P_N, R_D, and the contents of adenine nucleotides in the leaf tissue.

Materials and methods

Plants: Seeds of *Phaseolus vulgaris* L., cv. Zlota Saxa were germinated in darkness for 4 d at room temperature, and then transferred to small (2 000 cm^3) containers filled with complete Knop nutrient medium (control, +P plants) or with P_i deficient (-P plants) Knop medium. The complete nutrient solution contained: 4.5 mM $Ca(NO_3)_2$, 3 mM $MgSO_4$, 1.5 mM KNO_3, 1 mM KH_2PO_4, 76 μM H_2BO_3, 9 μM $MnCl_3$, 0.3 μM $CuSO_4$, 0.8 μM $ZnSO_4$, and 1.1 μM H_2MoO_4. In P_i deficient Knop medium, KH_2PO_4 was substituted by KCl. The culture medium was adjusted to a pH of 5.7, continuously aerated, and changed every 4 d. The cotyledons were removed after 5 d of cultivation, then the seedlings were transferred to 4 000 cm^3 containers (8 seedlings in each), and grown as described by Rychter and Mikulska (1990). The plants were harvested at the age of 10, 15, and 18 d (after 4 d of germination, and additional 6, 11, and 14 d on +P or -P medium) at the end of 8 h of the night period (R_D measurements) or after 4 h of the light period (P_N measurements).

Gas exchange of the shoots was measured on intact bean plants. The plants were transferred to a photosynthetic chamber (volume 1 500 cm^3), and left to acclimate for 1 h before measurements started. During the P_N measurements, PAR of 150 μmol m^{-2} s^{-1} at plant height (saturating irradiance, Kondracka and Rychter 1997) was provided by *PR* 250 W tungstene photolamps (*Narva*). Temperature was kept at 22 °C, the ambient CO_2 concentration was 350 cm^3 m^{-3}, and the relative air humidity was 80 %. In the course of measuring the roots were immersed in an aerated nutrient solution. CO_2 gas exchange was determined in a leaf chamber within a closed system with an infrared gas analyser (*ADC 225 MK 3*, England).

O_2 uptake measurements: R_D of leaf slices was measured in the darkness using an O_2 Clark electrode (type *Yellow Springs*) as described by Rychter *et al.* (1988). The effect of cutting injury was eliminated by rinsing the slices 3 times with 0.2 mM $CaCl_2$ solution as described by Azcón-Bieto *et al.* (1983). R_D was measured after 1 h of steady-state rate. Plants were taken from the growth chamber after 4 h of the light period or after 8 h of the dark period. The respiratory inhibitors KCN (adjusted to a pH of 7) and SHAM dissolved in dimethyl sulfoxide from stock solutions were added to the final concentrations of 1.0 and 2.5 mM, respectively. The concentrations of inhibitors were estimated from titration curves as described by Möller *et al.* (1988).

Leaf area and dry matter: The leaf area measurements were carried out using an *A 4 SCAN* scanner with the respective software (*Witra*, Poland). The leaves were dried at 70 °C to a constant matter.

Inorganic phosphate concentration was measured by the method of Fiske-Subbarow (1925) after homogenization and extraction of leaf P_i with cold 10 % trichloroacetic acid as described by Rychter and Mikulska (1990).

Nucleotide determinations: The adenine nucleotides concentration in leaf extracts was determined not considering their intracellular compartmentation. Having determined their mass, the leaves were ground in a precooled mortar containing sand, 10 % perchloric acid (PCA), small amounts of concentrated PCA, and polyvinyl-pyrrolidone. All chores were performed in a growth chamber under light or in the darkness. The ground material was centrifuged, and the supernatant neutralized with trioctylamine (*Sigma*) and 1,1,2-trichloro-1,2,2-trifluoroethane (*Sigma*, 1:1, v/v). After centrifugation, the top layer containing neutralized extract was decanted and stored at -20 °C. ATP, ADP, and AMP contents were determined using the luciferin-luciferase reaction according to Pradet (1967) as described by Rychter *et al.* (1992). The AEC was calculated using the formula:

$$AEC = (ATP)\ 0.5\ (ADP)/(ATP)\ (ADP)\ (AMP)$$

Results

Inorganic phosphate content of bean leaves decreased rapidly during the cultivation of -P plants while in control leaves it remained almost constant (Fig.1*A*). In 10-d-old

plants grown for 6 d without phosphate in the medium, the P_i content in the leaves dropped by more than 50 %, as compared to the control. P_i content in the leaves of -P plants at the age of 15 and 18 d was about 80 and 90 % lower, respectively, than in the +P plants.

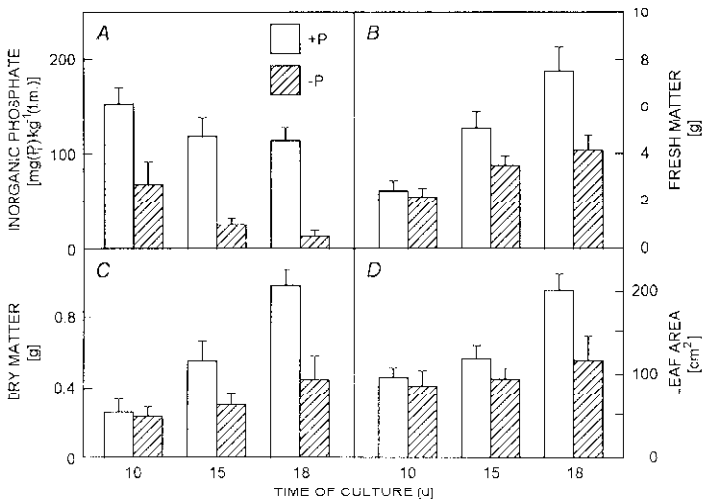

Fig. 1. Inorganic phosphate content (*A*), fresh (*B*) and dry (*C*) matters of leaves, and leaf area (*D*) of bean plants grown on phosphate sufficient (+P) and phosphate deficient (-P) medium.

Fresh and dry matters of leaves and leaf area of 10-d-old +P and -P plants did not differ (Fig.1*B,C,D*). During the next 8 d these parameters increased both in +P and -P plants, but in the -P plants this increase was lower than in +P plants. In 18-d-old -P plants, the accumulation of fresh and dry matters was lowered to 55 and 45 %, respectively, as compared to the +P plants. Leaf area was also reduced to 60 % of the control (Fig. 1*D*).

During 18 d of cultivation P_N of the leaves of +P and -P plants, expressed per both fresh matter and leaf area, declined (Fig. 2*A,B*). Phosphate deficiency had no effect on the P_N expressed per fresh matter in 10- or 15-d-old plants, but in 18-d-old plants the value was half of that in the control (Fig. 2*A*). Changes in P_N per dry matter were nearly the same (results are not shown). When P_N was expressed per leaf area, the decrease in -P plants was observed earlier than in +P plants (Fig. 2*B*). In 15-d-old -P plants P_N was already about 25 % lower than in +P plants, whereas at the age of 18 d it was about 40 % of the control.

The CO_2 compensation concentration (Γ) of the control leaves was constant during 18 d cultivation (40 $cm^3\ m^{-3}$) while in the -P plants it increased by 40-50 % at the age of 15 and 18 d, as compared to the +P plants (Fig. 3). The increase in Γ may be related to an enhanced photorespiration.

R_D expressed per fresh and dry matter of the leaves declined during cultivation in both +P and -P plants, and there was no substantial difference between these plant groups (Fig. 2*C,D*). The R_D was also measured by the Clark oxygen electrode as O_2

uptake by leaf slices. In the slices of +P leaves after the light and dark period this rate was nearly the same in 15- and 18-d-old plants (Table 1). In the -P leaf slices it did not depend either on the age of the plants or on the light/dark conditions, and it was similar to the control values.

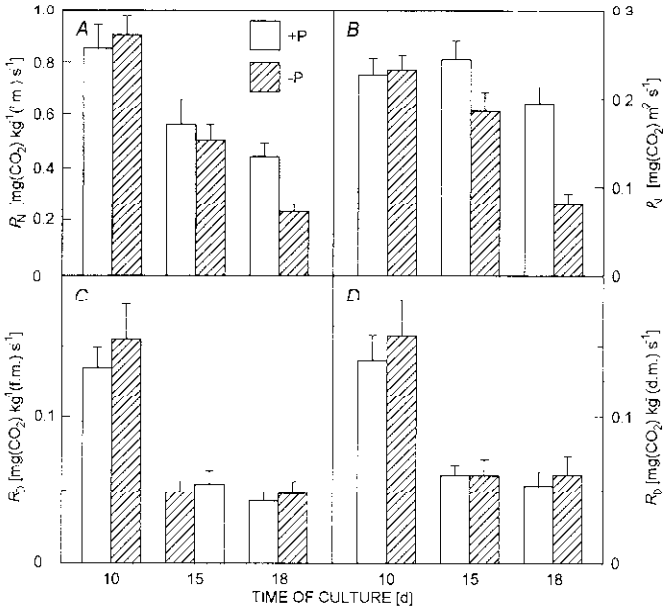

Fig. 2. The rates of photosynthesis (P_N, *A,B*) and respiration (R_D, *C,D*) expressed per leaf fresh (*A,C*) or dry (*D*) matter or per leaf area (*B*) of bean plants grown on phosphate sufficient (+P) and phosphate deficient (-P) medium.

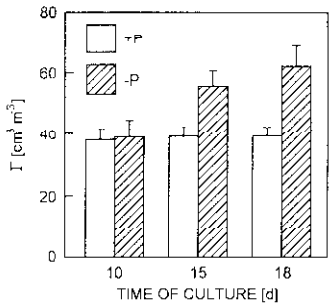

Fig. 3. CO_2 compensation concentration of bean plants grown on phosphate sufficient (+P) and phosphate deficient (-P) medium.

KCN inhibition of oxygen uptake by leaf slices was the same, independent on the age and light/dark conditions of plants (Table 1). Generally, respiration of +P leaf slices was inhibited by cyanide more than that of the -P leaf slices. Respiration which was not inhibited by KCN was completely inhibited after the addition of 2.5 mM SHAM. The SHAM used in the absence of KCN inhibited R_D of -P leaf slices more

than R_D of the +P slices. In +P slices after the dark period, the SHAM inhibited R_D by about 25 %, and after irradiation by 30-35 %, but in -P leaf slices by about 33 and 45 % after the dark and light periods, respectively.

Table 1. Respiration rate (R_D) [μmol(O_2) m^{-2} s^{-1}] and its inhibition by KCN and SHAM in leaf slices of bean plants grown on phosphate sufficient (+P) and phosphate deficient (-P) medium. Measurements in darkness after 8 h of dark period or 4 h of light period. Means ± S.D.

Time of culture [d]	After	R_D		+1 mM KCN [%]		+2.5 mM SHAM [%]	
		+P	-P	+P	-P	+P	-P
15	dark	0.50±0.05	0.60±0.15	51±5	40±5	23±4	33±4
	light	0.60±0.10	0.70±0.05	51±4	43±4	34±3	42±5
18	dark	0.80±0.15	0.60±0.05	60±5	44±8	25±2	32±2
	light	1.00±0.15	0.70±0.05	52±8	44±4	30±3	45±6

The ATP concentration in +P and -P leaves of 10-d-old beans was nearly the same both after the light and dark periods (Fig. 4*A,B*) but in leaves of 15- and 18-d-old -P plants after irradiation it declined to 50 and 30 % of control values, respectively (Fig. 4*A*). The decrease in ATP concentration was observed also after the dark period. In 18-d-old plants the concentration of ATP was half of that in the control (Fig. 4*B*).

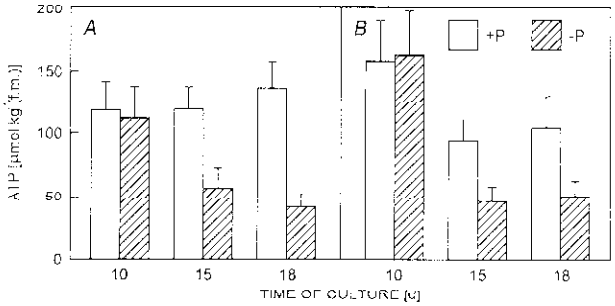

Fig. 4. ATP concentration in the leaves of bean plants grown on phosphate sufficient (+P) and phosphate deficient (-P) medium measured in the light (*A*) and in the dark (*B*).

The ADP and AMP concentrations as well as AEC did not change during 18 d of growing the bean plants on -P and +P media both in the light and in darkness (values not shown). The ADP and AMP concentrations were 60 and 20 μmol kg^{-1}(f.m.), respectively, and AEC was 0.8. Since ADP and AMP concentrations did not change, the decrease in total adenylates reflected the changes of ATP concentration in the leaves. The total adenylate content in the leaves of +P plants was nearly the same during 18 d of culturing both after the light and dark period, and it remained at 200-250 μmol kg^{-1}(f.m.), but in the leaves of 15- and 18-d-old -P plants it was reduced to 100-150 μmol kg^{-1}(f.m.) (results are not shown).

Discussion

The ATP concentration in a leaf cell depends on the efficiency of processes producing and consuming ATP. During irradiation, the ATP is produced by photosynthetic and oxidative phosphorylation, but in the dark, mainly mitochondrial oxidative phosphorylation can play the role. The decrease in P_i content of leaves may influence photophosphorylation (Heber and Heldt 1981, Walker and Sivak 1985, Furbank *et al.* 1987, Giersch and Robinson 1987) and oxidative phosphorylation rate (Rychter *et al.* 1992).

We have found that the decrease in P_i content of bean leaves greatly reduced ATP concentration after the light or dark periods (Fig. 4*A,B*). However, differences in the ATP concentrations between the light and dark periods were not significant in the leaves of both +P and -P plants. A higher ATP concentration during irradiation was observed by Rao *et al.* (1989) in phosphate sufficient sugar beet leaves and by Fredeen *et al.* (1990) in phosphate sufficient soybean leaves, but in phosphate deficient plants the ATP content was only slightly higher in the light than in the dark. The ATP determinations in extracts from green tissues in the light may reflect the chloroplastic, mitochondrial, and cytosolic pools of ATP, whereas the extracts prepared after the dark period reflect mainly cytosolic and mitochondrial ATP. The part of ATP which is produced by photophosphorylation may be immediately utilized in the chloroplasts to support CO_2 fixation and synthesis of starch, fatty acids, and amino acids (Krömer 1995). ATP synthesized in mitochondria can be transported to cytosol to support cytosolic reactions connected with sucrose synthesis during photosynthesis, *e.g.*, glucose triphosphate needed for uridinediphosphate-glucose formation (Krömer 1995). Hence, in our experiments we might determine mainly the cytosolic pool of ATP, more dependent on the efficiency of mitochondrial oxidative phosphorylation. This may explain the lacking differences of ATP content in the light and darkness.

The efficiency of ATP production by oxidative phosphorylation in plant respiration depends on the rate of electron transport through cytochrome pathway and on the engagement of an alternative pathway (Wagner and Krab 1995). In leaves of most plant species a decrease in R_D during phosphate starvation was observed (Thorsteinsson and Tillberg 1987, Usuda and Shimogawara 1993). A low phosphate concentration in bean leaves had no effect on R_D determined as CO_2 evolution by intact leaves or O_2 uptake by leaf slices after dark and light periods (Fig. 2*C,D* and Table 1). However, the increased resistance of respiration to KCN and the increased inhibition by SHAM may indicate that in -P leaves the nonphosphorylating, alternative pathway perhaps operates to a higher extent than in +P leaves (Table 1). The larger engagement of alternative pathway, which is not involved in the ATP production and therefore does not depend on P_i concentration, may keep R_D value similar to the control one.

The alternative pathway may operate when the cytochrome pathway is unsaturated and both pathways can compete for the electrons from reduced ubiqinone pool (Wagner and Krab 1995). Thus, the inhibition of respiration by SHAM can not be used for the estimation of alternative pathway activity because the electrons may be

diverted to the cytochrome path, and this may lead to underestimation of alternative pathway activity (Atkin *et al.* 1995, Hoefnagel *et al.* 1995). However, the comparison of KCN resistance and SHAM inhibition of respiration in +P and -P leaves indicates that although we can not calculate the rate of involvement of the alternative path in respiration, it is certainly different and possibly higher in -P leaves. Since the involvement of alternative path considerably lowers the ATP production by plant respiratory chain (from 3 ATP to 1 ATP per electron pair), the lower ATP content in -P leaves may be partially accounted for a lower ATP production by the respiratory chain.

P_N of 15-d-old -P beans (determined at the same irradiance as in the growth chamber) did not differ from that of +P plants but it dropped by about 50 % in 18-d-old -P plants (Fig. 2*A,B*). ATP content in the leaves of 15- and 18-d-old -P plants was half of that in control plants (Fig. 4*A*). Low phosphate concentration in the tissue may affect P_N in different species differently: in -P soybean plants a 40 % drop of P_N measured at the growth irradiance has been observed (Fredeen *et al.* 1990). This decrease of P_N in -P plants was accompanied by a 50 % decrease in the ATP concentration. Rao and Terry (1989) and Rao *et al.* (1989) observed a 40 % decrease in ATP concentration in the leaves of -P sugar beet plants, although P_N (determined at an irradiance similar to that for growth) was nearly the same as in control plants. Under phosphate deficiency, the ATP produced during photophosphorylation under a low irradiance may be insufficient, and in order to support the CO_2 fixation by the Calvin cycle and other processes that occur in the light the ATP may be transported from cytosol to chloroplasts (Krömer 1995). This might explain the fairly small effect of phosphate deficiency on photosynthesis and the large decrease of ATP content in cytosol. The possibility of ATP transport from cytosol into chloroplasts was suggested by Krömer (1995). It takes place when the ATP/ADP ratio in cytosol is higher than in stroma (Heber and Heldt 1981).

The ATP consumption may involve ATP utilization for growth and other metabolic processes utilizing cytosolic ATP pool and occurring both in the light and darkness. We found that the production of fresh and dry matter as well as leaf area of the -P leaves were more limited than in +P plants (Fig. 1*B,C,D*). Therefore the demand for ATP in growth processes of the -P leaves may also be lower, and thus it may change the efficiency of energy producing processes. This change may involve the lower efficiency of photosynthetic phosphorylation but also the changes in efficiency of oxidative phosphorylation. The effect of a low P treatment on growth processes of the leaves may be also mediated by growth hormones (Horgan and Wareing 1980). The inhibition of shoot growth in bean plants may be the result of a large increase in content of abscisic acid in -P leaves (results are not shown).

We have found that phosphate starvation affected the leaf fresh and dry matter production stronger than P_N (Figs. 1*B,C* and 2). Under a limited P_i deficiency, Kondracka and Rychter (1997) observed a substantial reduction of bean leaf growth rate while P_N decreased only slightly, because of enhancement of P_i recirculation during glycolic and phosphoenolopyruvate metabolism. The increase of Γ in the leaves of -P plants (Fig. 3) suggests that some CO_2 evolving processes, such as

photorespiration, may be stimulated (Jacob and Lawlor 1993, Kondracka and Rychter 1997), and they mask the CO_2 uptake in photosynthesis of 15-d-old -P plants.

We also found that during phosphate deficiency the ATP content in leaves dropped significantly, whereas the P_N and R_D were only slightly affected. This result is similar to that of Hauschild *et al.* (1996). The decrease in ATP concentration may reflect the lowering of ATP synthesis both by photosynthesis and respiration, a lower ATP demand for growth, and possibly higher ATP degradation rates, as suggested by Miginiac-Maslow *et al.* (1986).

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