Delpinoa, n.s. **33-34**: 99-110. 1991-1992

Enzyme activities in young barley roots after ammonium or methylammonium supply

SERGIO ESPOSITO , PETRONIA CARILLO , SIMONA CARFAGNA

Dipartimento di Biologia vegetale, Facoltà di Scienze, Università di Napoli Federico II, Via Foria, 223 - I-80139 Napoli, Italy.

Riassunto

Gli effetti dell'aggiunta di ammonio sul metabolismo del carbonio in radici di plantule d'orzo (*Hordeum vulgare* var. *distica penice*) sono stati misurati attraverso l'analisi delle variazioni delle attività enzimatiche .

Un aumento nelle attività specifiche sia della fosfo*enol*piruvato carbossilasi (+300%) che della glucosio-6-fosfato deidrogenasi (+40%) è stato misurato, mentre non si sono osservate variazioni apprezzabili negli enzimi glicolitici durante l'assimilazione dell'ammonio, suggerendo che la sintesi degli (iso)enzimi del metabolismo del carbonio è differentemente regolata dallo stato azotato delle cellule della radice.

I risultati ottenuti sembrano indicare che la regolazione a lungo termine di questi processi coinvolge direttamente la fissazione buia della anidride carbonica e la via del pentoso fosfato, che sintetizzano i precursori metabolici per la sintesi degli amminoacidi mediante l'azione delle transaminasi.

INTRODUCTION

The relationship between carbon metabolism and nitrogen assimilation in plants occupies a central role in plant metabolism (DENNIS & MIERNYK, 1982; OAKS & HIREL, 1985; EMES & BOWSHER, 1991).

The stimulation of respiration upon ammonium supply has been studied in algae (DI MARTINO RIGANO *et al.*, 1990) and in plants (FARRAR, 1981; EMES & FOWLER, 1983).

The main regulatory enzymes of glycolysis are phosphofructokinases: one enzyme (PFK - EC 2.7.1.11) uses ATP to phosphorylate fructose-6P; another form is named phosphofructo-pyrophosphorylase (PFP, EC 2.7.1.90), and

Key words: Ammonium assimilation, Barley roots, Glucose-6P dehydrogenase, Methylammonium, PEP carboxylase.

utilizes inorganic pyrophosphate (SABULARSE & ANDERSON 1981; KOMBRINK *et al.*,1984; BOTHA & SMALL, 1987); both these enzymes are cytosolic, but there is also a plastidial PFK that plays a central role in fructose-6P metabolism (DENNIS & GREYSON, 1987), altough a complete glycolytic pathway is not complete in some plastids (STITT & REES, 1979): the PFP plays an important role in metabolically active tissues, where the PFK activity is not sufficient to maintain the required rate of metabolism.

The oxidative pentose phosphate pathway (OPPP) seems to regulate the flux of reducing power for the enzymes of nitrogen metabolism (e.g. nitrate reductase, glutamate synthase); this pathway operates both the cytosol and the plastid and it is regulated by glucose-6-phosphate dehydrogenase (G6PDH - EC 1.1.1.49) (EMES & FOWLER, 1979).

Dark CO_2 fixation by phospho*enol*pyruvate carboxylase (PEPC - EC 4.1.1.31) also plays a central role in the interactions between nitrogen assimilation and carbon metabolism because of refurnishing of carbon skeletons for amino acid synthesis (CHAMPIGNY & FOYER, 1992).

The carbon skeletons produced during carbon oxidative pathways were used to synthetize aminoacids through the action of transaminases, mainly the glutamate oxalacetate transaminase (GOT - EC 2.6.1.1) and the glutamate piruvate transaminase (GPT - EC 2.6.1.2).

In this paper respiration and variations in levels of key enzymes of carbon metabolism pathways were investigated after the ammonium supply to young barley roots; control experiments were done using an ammonia-analogue, methylammonium, which is absorbed but not assimilated in carbon compounds by the plants.

MATERIALS AND METHODS

Cultivation of plants

Seeds of barley (*Hordeum vulgare* L., var. *distica penice*), supplied by Istituto Sperimentale di Cerealicoltura, Fiorenzuola D'Arda, were germinated on filter paper moistened with de-ionised water in darkness at 21 °C. On the fifth day, when the first leaf was emerging from the coleoptile, individual

seedlings were transferred to a nitrogen-free hydroponic culture.

The culture vessel consisted of a Plexiglass container, with a capacity of 5 litre, placed in a controlled cabinet at 20 °C, 60% r.h. By means of soft foam strips, groups of 4-6 seedlings were positioned in thin cuts in the foam cover, with the roots immersed into the culture medium inside the vessel. The medium was aerated continuously with air. The shoots were illuminated with a photon fluence rate of 300 μmol · m^{-2} · s^{-1} with a photoperiod of 16 h light/8 h dark.

A nitrogen-free modified Long Ashton medium (HEWITT, 1966) was used. Ammonium phosphate or methylammonium chloride, 2 mM, were supplied at the time indicated. The nutrient solutions were adjusted every day to maintain constant the pH and NH_4^+ (or $CH_3NH_3^+$) concentrations.

Enzyme extraction

Barley seedling groups (4-6 plants) were collected 2 hours after the beginning of the light period and the roots were separated, blotted, weighed and frozen with liquid nitrogen. All the operations were carried out at 4 °C. Cold extraction buffer (see later) was added (W/V 1:2) and the roots were ground in a mortar. The slurry was squeezed through three layers of muslin and centrifuged for 20 min at 20,000 x *g*. The clear supernatant obtained was defined as crude extract and used for the enzymatic assays.

Extraction buffers.

For G6PDH, PGlcM, PEPC, and HK the extraction buffer was 75 mM Tris-HCl , pH 8.0, containing 1 mM $MgCl_2$, 5 mM EDTA, 1 mM PMSF, 10 mM mercaptoethanol , 0,1% Triton X100 .

The extraction buffer for ASNase, GOT, and GPT, was 50 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM EDTA , 1 mM $CaCl_2$.

During the PFK and the PFP extraction 100 mM HEPES-KOH pH 8.0, 10 mM $MgCl_2$, 2 mM EDTA, 2 mM DTE, 30 mM mercaptoethanol and 0,1% Triton X100 were used.

Enzyme assays

All enzymes were assayed at 25 °C in 1 cm light path cuvette by monitoring NADH oxidation or $NADP^+$ reduction

(Beckman DU-65 spectrophotometer with Kinetics Pack Module). The coupling enzymes were always desalted before use. All the activities were measured against blanks without substrates and optimized for buffer, coupling enzymes and substrate compositions and concentrations.

Glucose-6-phosphate dehydrogenase assay (G6PDH-EC 1.1.1.49).

The reaction mixture for G6PDH contained 50 mM Tris-HCl pH 8.0, 0.15 mM $NADP^+$, 18 mM $MgSO_4$, extract and glucose-6P to a final concentration of 1.5 mM.

Phosphoglucomutase assay (PGlcM - EC 5.4.2.2).

The reaction mixture for PGlcM contained 50 mM imidazole-HCl pH 7.4, 0.15 $NADP^+$, 2 mM $MgCl_2$, 1 U/ml glucose-6P dehydrogenase, extract and 1mM glucose-1P.

Phosphoenolpyruvate carboxylase assay (PEPC - EC 4.1.1.31)

The enzyme was routinely assayed in a mixture of 50 mM Tris-HCl, 2mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$, 2 U/ml malic dehydrogenase, extract and 1 mM phospho*enol*pyruvate .

Hexokinase assay (HK - EC 2.7.1.1.)

The assay mixture contained 50 mM Tris-HCl, pH 7.6, 50 mM KCl, 25 mM $MgCl_2$, 0.2 mM $NADP^+$, 10 mM EDTA, 0.2 U/ml glucose 6P dehydrogenase, 5 mM ATP, extract and 15 mM glucose.

Asparaginase assay (ASNase - EC 3.5.1.1)

The enzyme was assayed by a coupled enzymatic assay with 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM NADH, 1 mM 2-ketoglutaric acid, 1 mM asparagine, 5 U/ml glutamate-oxalacetate transaminase, 5 U/ml malic dehydrogenase and extract.

Glutamate oxalacetate transaminase (GOT - EC 2.6.1.1) *and glutamate piruvate transaminase* (GPT - EC 2.6.1.2).

The transaminase activities were measured by the standard assays in 50 mM Tris-HCl pH 8.0, 0.2 mM NADH and extract; in the GOT assay, 1.5 U/ml malic dehydrogenase and a mixture of 1 aspartic acid - 1 mM 2-ketoglutarate were added; in the GPT

assay, 6 U/ml lactic dehydrogenase were added, and the reaction started with a mixture of 1 mM alanine - 1 mM 2-ketoglutarate.

Phosphofructokinase (PFK - EC 2.7.1.11) *and phosphofructo-pyrophosphorylase* (PFP - EC 2.7.1.90) *assays,*

The mixture contained 100 mM HEPES-KOH, pH 8.0, 1 mM $MgCl_2$, 0.2 mM NADH, 10 mM fructose-6P, extract and the enzyme mixture composed by aldolase (1U/ml), triose-phosphate-isomerase (10 U/ml) and glicerol-3 phosphate-dehydrogenase (0.1U/ml); PFK activity was measured in presence of ATP 0.5 mM; PFP activity was measured with Na-pyrophosphate 1 mM in presence of fructose-2,6 bis-phosphate 1 µM.

Protein Determination

Protein concentrations in the crude extract were determined using the Bio-Rad assay based on the Coomassie-blue G-250 dye-binding protocol (BRADFORD, 1976) with bovine serum albumine as standard.

RESULTS

Changes in enzyme activities

The activity of phosphoglucomutase remained constant in the first 10 days of hydroculture (50-75 $nmol \cdot min^{-1} \cdot mg^{-1}$ prot.) in N-deplete plants, as shown in table 1; if 2 mM ammonium or methylammonium were supplied at fifth day these values didn't change appreciably (Tab. I).

The HK activity in N free grown plants was low (about 20 $nmol \cdot min^{-1} \cdot mg^{-1}$ prot.); five days after the NH_4^+ supply the activity of the enzyme increased up to an activity of 45 $nmol \cdot min^{-1} \cdot mg^{-1}$ prot. The methylammonium supply caused a little increase in hexokinase activity (Tab. I).

Also the phosphofructokinases activities did not appreciably change both without and with nitrogen supply. The reaction rate of these enzymes remained constant with time: 55 - 75 $nmol \cdot min^{-1} \cdot mg^{-1}$ prot. for the PFK, whereas the PFP activity was about 300-350 $nmol \cdot min^{-1} \cdot mg^{-1}$ prot. (Tab. I).

Tab. I - Changes in glycolytic enzyme activities during the first days of germination. For every enzyme, in the first row is shown the activity in absence of nitrogen supply; in the second row, the activities changes after 2 mM ammonium supply at fifth day; in the third row, the activity changes after 2 mm methylammonium supply at fifth day. The activities are expressed as nmol · min^{-1} · mg^{-1} prot. ± SE (n=5).

Enzyme	Conditions	Day of Hydroculture			
		1	5	8	10
Phosphoglucomutase	-N			66±8	54±20
(EC. 5.4.2.2)	+NH_4^+	74.5±5.8	58±7	56.6±3.7	58.6±7
	+$CH_3NH_3^+$			50±8	60±15
Hexokinase	-N			24±3	21.3±8
(EC.2.7.1.1)	+NH_4^+	19.1±1.9	24.4±1.2	29.8±3.8	45±13
	+$CH_3NH_3^+$			32.6±2	31.6±1
Phosphofructokinase	-N			70±20	45±10
(EC 2.7.1.11)	+NH_4^+	56.4±2	73±6	69±12	71.8±1
	+$CH_3NH_3^+$			58.8±15	62.5±1.5
Phosphofructo-	-N			347±13	275±70
pyrophosphorilase	+NH_4^+	313±2.5	336±24	340±11	265±38
(EC. 2.7.1.90)	+$CH_3NH_3^+$			339±73	290± 9

The activity of PEP carboxylase reaction doubled soon after the ammonium supply, reaching values of 300 nmol·min^{-1}·mg^{-1} prot. at 7th day (two days from the nitrogen refurnishing), and remaining high until the end of the experiments (10th day, five days after NH_4^+ supply). The methylammonium supply provoked a lower stimulation in the activity of the enzyme, reaching a level of 200 nmol·min^{-1}·mg^{-1} prot. (Fig. 1a).

The G6PDH activity (Fig. 1b) decreased from 240 nmol · min^{-1} · mg^{-1} prot., one day after the beginning of

hydroculture to a minimum activity 115 nmol·min^{-1}·mg^{-1} prot., at the 5th day of hydroculture then remained constant in the control plants (not shown). The ammonium refurnishing caused a slight increase in enzyme activity, reaching a new high value, 155 nmol · min^{-1} · mg^{-1} prot., three days after ammonium supply (8th day). Experiments with methylammonium showed an immediate strong increase in the enzyme activity, from 120 to a maximum activity of 320 nmol·min^{-1}·mg^{-1} prot. at 8th day (about three times the normal value without nitrogen supply).

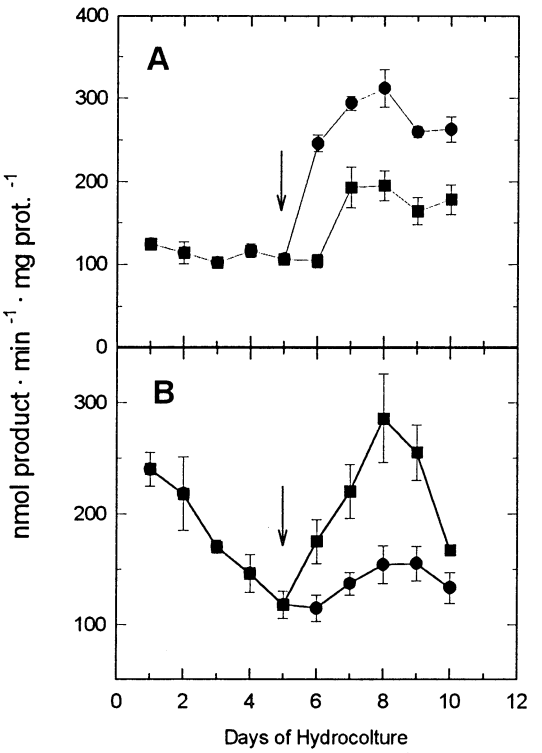

Fig. 1 - Variations in enzyme activities in the roots of barley plants during the first 10 days of hydroculture; the arrows indicate the ammonium (●) or methylammonium (■) 2 mM supply; control experiments without N-providing at fifth day were always carried out: A - Phospho*enol*pyruvate carboxylase; B - Glucose-6-phosphate deydrogenase. Error bars indicate standard error (n= 7).

The GOT activity (Fig. 2a), which remained constant after the initial peak at the hydroculture beginning (340 nmol·min^{-1}·mg^{-1} prot.), had a significant increase only 3-4 days after the ammonium supply, from 200 to 340 nmol · min^{-1} · mg^{-1} prot. (+70 %); similary GPT activity (Fig. 2b) increased at 8th day (3 days after ammonium supply) to 300 nmol · min^{-1} · mg^{-1} prot. (over two times the initial value, 140 nmol · min^{-1} · mg^{-1} prot.)

The asparaginase activity, after the peak at the beginning of the hydroculture, remained constant (50 nmol · min^{-1} · mg^{-1} prot.); only after the ammonium supply an esponential increase in activity was measured, reaching an higher value at 9th day (4 days after N-supply) to 110 nmol·min^{-1} · mg^{-1} prot. (Fig. 2c).

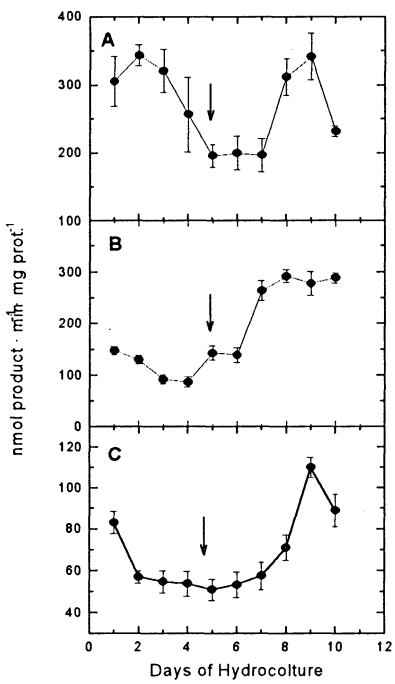

Fig. 2 - Variations in enzyme activities in the roots of barley plants during the first 10 days of hydroculture; the arrows indicate the ammonium 2 mM supply; control experiments without N-providing at fifth day were always carried out: A - Glutamate - Oxalacetate Transaminase (GOT); B- Glutamate - Pyruvate Transaminase (GPT); C - Asparaginase. Error bars indicate standard error (n=5).

DISCUSSION

The activities of glycolytic enzymes did not change within five days after ammonium or methylammonium supply.

Thus the phosphoglucomutase reaction was not increased during ammonium assimilation, suggesting the involvement of starch degradation by amylases in barley roots. The low activity of hexokinase measured throughout the experiments seems to confirm that in these plants glycolysis is not stimulated by ammonium uptake.

Phosphofructokinases, both PFK and PFP, which are the key enzymes of the glycolytic pathway, remained quite the same throughout the experiments before and after nitrogen supply; so, glycolysis seems to be subjected to a short term regulation by relative levels of metabolites. The differences in the reaction rates between the "true" PFK and PFP confirm the hypothesis that in active growing tissues (such as the seedling roots) the PFK reaction, ATP dependent, may be insufficient to refurnish carbon skeletons for active metabolism, and the PFP reaction, using the large amount of PPi realased by active cell wall biosynthesis, equilibrates the carbon demand by the cell (DENNIS & GREYSON, 1987). So, the constitutive high levels of PFP activity indicate that under experimental conditions the "normal" glycolytic pathway was insufficient for metabolism energy requirement, and in the roots the PFP reaction has been utilized to increase the glycolytic yield.

On the contrary, the changes in PEP-carboxylase and glucose-6P dehydrogenase seem to indicate that the alternative pathways to the glycolysis are stimulated by the ammonium uptake; the increase in the activities of these enzyme also after methylammonium supply seems to confirm this hypothesys.

The anaplerotic reaction of dark CO_2 fixation and oxidative pentose phosphate pathway were used to refurnish metabolites for nitrogen assimilation.

The use of methylammonium, an ammonium analogue which is not assimilated by the plant cells, could clear some aspects of these processes of regulation. For example, the stronger stimulation of glucose-6P dehydrogenase in presence of the ammonia-analogue than NH_4^+, demonstrates the increase in energy demand to store the methylammonium in the root vacuoles; this behaviour could indicate that in physiological conditions the excess of ammonium could be stored in the vacuoles in the first uptake phase before assimilation; the

energy to mantain the NH_4^+ inside the cell could be supplied only in part by glycolysis, wich is weakly increased by methylammonium supply.

The physiological significance of PEP carboxylase increase may be attributed to the refurnishing of oxalacetic acid for the Krebs cycle, that could be stopped by large consumption of ketoglutaric acid due to the shunt of this ketoacid through GOGAT reaction; or the supply of oxalacetic acid for GOT reaction, to synthetize aspartic acid to be later transformed in asparagine by asparagine synthetase.

The increase measured in the activity of glucose-6P dehydrogenase (about 40% two days after N-supply) may suggest that this enzyme could be the main site of reducing power for GOGAT; this hypotesis has been demonstrated in pea root plastids where the existance of a complete OPPP has been found (EMES & FOWLER, 1979) and the source of reductant for GOGAT is generated by pentose phosphate cycle (BOWSHER *et al.*1992).

The stimulation by ammonium of oxidative pentose phospate pathway may suggest the existence of a feedback regulation between ammonium uptake system(s) and carbon oxidative pathways in the root cell; the sharp and strong increase in the activity of the glucose-6P dehydrogenase after the methylammonium supply seems to confirm this hypothesis.

After the first phase of assimilation, the amino groups were shunted towards the synthesis of other amino compunds through transaminases reactions: glutamate-oxalacetate transaminase and glutamate-pyruvate transaminase levels were strongly increased by N-assimilation. The asparaginase action (LEA *et al.*, 1978; SODEK *et al.*, 1980; CHANG & FARNDEN, 1981) could avoid the excess of asparagine in the root cells and may release the amino-group from asparagine, bringing about a new ammonium incorporation via GS-GOGAT cycle in the following days; the explanation of this apparently futile reactions could be due to the fact that the intracellular asparagine pool, stored in the vacuole, is the main regulator of ammonium uptake by the cells for its inhibition of ammonium permease system (SLAUGHTER *et al.*, 1990).

Abstract

The effect of ammonium supply on carbon metabolism in young barley roots (*Hordeum vulgare* var. *distica penice*) was investigated measuring the variation in enzyme activities.

The increase in phospho*enol*pyruvate carboxylase (+300%) and glucose-6P dehydrogenase (+40%) activities were measured while little or no variations in glycolytic enzymes occurred during the ammonium assimilation, suggesting the synthesis of (iso)enzymes of carbon metabolism is differently regulated by nitrogen status of root cell.

The results obtained seems to indicate the long term regulation of this processes directly involved the dark CO_2 fixation and the pentose phosphate pathway, which synthetize metabolic precursors for the amino acid synthesis through the action of transaminases.

REFERENCES

BOTHA F.C. AND SMALL C.J.G., 1987. *Comparison of the activities and some properties of pyrophosphate and ATP dependent Fructose-6-phosphate 1-phosphotransferase of* Phaseolus vulgaris *seeds.* Plant Physiol., 83: 772-777.

BOWSHER C.G., BOULTON E.L., ROSE J., NAYAGAM S., AND EMES M.J., 1992. *Reductant for glutamate synthase is generated by the oxidative pentose phosphate pathway in non-photosynthetic root plastids.* Plant J., 2 (6): 893-898.

BRADFORD M.M., 1976. *A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal. Biochem., 72: 248-254.

CHAMPIGNY M.L. AND FOYER C., 1992. *Nitrate activation of cytosolic protein kinases diverts photosynthetic carbon from sucrose to aminoacid biosynthesis. Basis for a new concept.* Plant Physiol., 100: 7-12.

CHANG K.S. AND FARNDEN K.J.F., 1981. *Purification and properties of asparaginase from* Lupinus arboreus *and* Lupinus angustifolius. Arch. Bioch. Biophys., 208: 49-58.

DENNIS D.T. AND GREYSON M.F., 1987. *Fructose 6-phosphate metabolism in plants.* Physiol. Plant., 69: 395-404.

DENNIS D.T. AND MIERNYK J.A., 1982. *Compartmentation of nonphotosynthetic carbohydrate metabolism.* Ann. Rev. Plant. Physiol., 33: 27-50.

DI MARTINO RIGANO V., VONA V., ESPOSITO S., DI MARTINO C. AND RIGANO C., 1990. *Light dependent decline of NH_4^+ assimilation under CO_2 deprivation in N-limited* Chlorella sorokiniana *(Chlorophyceae).* J. Phycol., 26: 69-72.

EMES M.J. AND BOWSHER C.G., 1991. *Integration and compartmentation of carbon and nitrogen metabolism in roots.* Semin. Ser. Soc. Exp. Botany, 42: 147-165.

EMES M.J. AND FOWLER M.W., 1979. *Intracellular interactions between the pathways of carbohydrate oxidation and nitrate assimilation in plant roots.* Planta, 145: 287-292.

EMES M.J. AND FOWLER M.W., 1983. *The supply of reducing power for nitrite reduction in plastids of seedling pea roots* (Pisum sativum *L.*). Planta, 158: 97-102.

FARRAR J.F., 1981. *Respiration rate of barley roots: its relation to growth, substrate supply and the illumination of the shoot.* Ann. Bot., 48: 53-63.

HEWITT E.J., 1966. *Sand and water culture methods used in the study of plant nutrition.* In: Commonw. Bureau Hort., tech. comm., 2nd ed, Vol. 22, Farnham Royal, England.

KOMBRINK E., KRUGER N.J., AND BEEVERS H., 1984. *Kinetic properties of pyrophosphate: fructose 6-phosphate phosphotransferase from germinating castor bean endosperm.* Plant Physiol., 74: 395-401.

LEA P.J., FOWDEN L. AND MIFLIN B.J., 1978. *The purification and properties of asparaginase from* Lupinus *species.* Phytochem., 17: 217-222.

OAKS A. AND HIREL B., 1985. *Nitrogen metabolism in roots.* Ann. Rev. Plant Physiol., 36: 345-365.

SABULARSE D. C. AND ANDERSON R.L., 1981. *Inorganic pyrophosphate: D-fructose-6-phosphate 1-phosphotransferase in mung beans and its activation by D-fructose-1,6-bisphosphate and glucose-1,6-bisphosphate.* Bioch. Biophys. Res. Comm., 100: 1423-1429.

SLAUGHTER C.J., MCKERNAN G. AND SAITA M., 1990. *Intracellular asparagine pool as a factor in control of ammonium uptake by* Saccharomices cerevisiae. Mycol. Res., 49: 1009-1012.

SODEK L., LEA P.J., AND MIFLIN B.J., 1980. *Distribution and properties of a potassium dependent asparaginase isolated from developing seeds of* Pisum sativum *and other plants.* Plant Physiol., 65: 22-26.

STITT M. AND REES T., 1979. *Capacities of pea chloroplasts to catalyse oxidative pentose phosphate pathway and glycolysis.* Phytochem., 18: 1905-1911.

Finito di stampare nel marzo 1996.