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Sucrose Synthesis in Nitrate Fed Detached Maize and Wheat Leaves as Analyzed by ^{13}C-NMR

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Key words: Nitrate, phosphoenolpyruvate carboxylase, sucrose synthesis, *Triticum aestivum*, *Zea mays*, ^{13}C-NMR.

Summary

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Sucrose and the organic acids, which provide carbon skeletons for amino acid synthesis, have triose phosphate as common precursor. Different biological systems fed with a nitrogen source showed an increase in amino acid synthesis in parallel with a decrease in sucrose formation. The main objective of the present research was to study the interaction between carbon and nitrogen metabolism in maize, a C_4 type plant, in order to compare with the results previously published on wheat leaves.

Leaves detached from two weeks N-limited seedlings were fed high nitrate concentrations by the transpiration stream. Nitrate uptake and assimilation was proportional to time and NO_3^- concentration, showing that detached maize leaves represent a suitable system for the present study. Sucrose synthesis and phosphoenolpyruvate carboxylase (PEPCase) activity were the main parameters analyzed. Feeding 40 mM NO_3^- for more than four hours decreased sucrose synthesis in leaves of both species, although in maize at a lesser extent than in wheat. In C_3 as in C_4 plants, PEPCase activity is responsible for the anaplerotic reactions, which assure the equilibrium between carbon and nitrogen metabolism. In wheat, it has been previously shown that enzyme activity is activated by nitrate. Maize leaves showed an increase of more than 50% after one-hour uptake on 40 mM NO_3^-.

The aim of furnishing $^{13}C_1$-glucose to maize and wheat leaves was to identify by ^{13}C-NMR the ^{13}C-metabolites, particularly sucrose, synthesized within short times, and thus to compare the response of C_3 and C_4 plants to high NO_3^-. In wheat leaves the decrease in resonances corresponding to the direct synthesis of sucrose was more evident than in maize. No variation was noticed in the ^{13}C resonances of other metabolites for both types of leaves.

The results are discussed in terms of competition between carbon and nitrogen metabolism upon NO_3^- supply in wheat and maize leaves.

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Introduction

In photosynthetic tissues several points of interaction between carbon and nitrogen metabolism may assure the allocation of carbon skeletons and reducing power needed for the synthesis of amino acids and carbohydrates (CHAMPIGNY 1995). Sucrose and organic acids provide carbon chains for amino acid biosynthesis with triose-phosphate as common precursor. Short-term responses to nitrogen supply were similar in C_3 and C_4-type leaves, carbon being diverted to amino acid synthesis with a simultaneous decrease in sucrose synthesis (VAN QUY & al. 1991a, CHAMPIGNY & FOYER 1992). The amphibolic function of the citric acid cycle is associated with the synthesis of intermediate compounds through anaplerotic reactions. One such reaction is catalyzed by phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31). Expression and activity of this enzyme show an integrated modulation with nitrate reductase (NR, EC 1.6.6.1) and sucrose phosphate synthase (SPS, EC 2.4.1.14) (CHAMPIGNY 1995). In a C_4-type plant like maize (*Zea mays* L.), atmospheric photosynthetic CO_2 is fixed in the light by PEPCase while in wheat (*Triticum aestivum* L.), a species with C_3-type photosynthesis, the main role of PEPCase is to replenish the tricarboxylic acid cycle with carbon skeletons removed for amino acid synthesis (MELZER & O'LEARY 1987). PEPCase regulation by light is well established in C_4-type photosynthesis plants through reversible phosphorylation, resulting in a decrease of inhibition by malate (JIAO & CHOLLET 1991, JIAO & al. 1991). In wheat leaves, high-NO_3^- availability increased the activity of the enzyme induced by light as well as the phosphorylation state (VAN QUY & al. 1991a). SPS, the regulatory enzyme of sucrose synthesis, is activated by light (STITT & al. 1988) through a phosphorylation/dephosphorylation mechanism (HUBER & al. 1992). Nitrate reductase also presents short-term changes under light/dark regimes and NO_3^- availability: NR is activated in vivo by dephosphorylation after high light or high content NO_3^- treatments (KAISER & HUBER 1994).

Feeding NO_3^- to N-depleted detached leaves leads to modulation of carbon metabolism enzymes, PEPCase being activated in vivo by phosphorylation while SPS is activated in vivo by dephosphorylation. Protein phosphorylation of PEPCase and SPS in response to nitrogen nutrition is interpreted as the basic mechanism assuring the coordination of the regulation of C and N assimilation in leaves (CHAMPIGNY 1995).

The aim of the present research was to assess whether C_4-type metabolism responded like the C_3-type metabolism after N-supply to N-depleted leaves. Maize leaves detached from seedlings previously subjected to N-limitation were fed high NO_3^- concentrations through the transpiration stream. The experimental system and the parameters analyzed, namely sucrose content, nitrate uptake and assimilation, and in vitro PEPCase activity were as described for wheat (VAN QUY & al. 1991a). After supplying 1-^{13}C-glucose to maize and wheat detached leaves, the metabolites, particularly sucrose synthesized within short times (1 hour) were identified by ^{13}C-NMR, allowing comparing the responses to NO_3^- of a C_4 and a C_3-type plant.

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Materials and Methods

1-^{13}C-glucose enriched to 99% ^{13}C was purchased from CEA, Saclay, France. All enzymes were from Sigma, St Louis, USA. All other chemicals were of reagent grade.

Maize (*Zea mays* L., hybrid DEA) was grown in vermiculite in the green house under 16 h photoperiod assured by day light supplemented with 520 µmol m^{-2} s^{-1}, watered every second day and used 14 days after sowing. Wheat (*Triticum aestivum* L., cv. Fidel) was grown hydroponically as in CHAMPIGNY & al. 1992 and transferred onto an N-free solution 48 h before the experiment.

At the end of the night period the youngest fully expanded leaves were detached under the water and placed in 5 ml test tubes filled with 1 ml N-free nutrient solution (TALOUIZTE & al. 1984) supplemented with 0 (control), 20 40 or 80 mM nitrate. The tubes were immediately placed under the light (520 or 1000 µmol m^{-2} s^{-1} as referred in Results) and samples taken after 1 (or shorter periods as indicated in Results), 2, 4 or 8 hours in at least four repetitions for each point. After the time correspondent to each treatment, leaves were immediately frozen in liquid N_2 and the nutrient solution of each tube adjusted to 1 ml was frozen at -25° C. For recording the initial conditions of the plant material, four leaves were frozen at time zero.

Leaf samples frozen in liquid N_2 were reduced to powder in a mortar with pestle. After separation of the aliquot used for PEPCase activity (see below), the samples were extracted by the method of STITT & al. 1983 with 0.6 ml 20 mM Hepes-NaOH buffer, pH 8.5, 50 mM EGTA, 50 mM FNa, 1.2 ml chloroform and 3.0 ml methanol. After extraction in mortar the extract was kept for 40 min at ice temperature before 5 ml of H_2O were added. After strong mixing and centrifugation at 1,500 g for 10 min the supernatant was collected and the pellet used for chlorophyll determination.

The supernatant collected as referred above was adjusted to 8 ml, 1 ml aliquots were evaporated to dryness and the deposit resuspended in 1 ml or 0.25 ml H_2O, respectively for sucrose or nitrate determination. Sucrose was quantified by a coupled enzyme assay through the spectrophotometric measurement of NADPH formation, after adaptation of JONES & al. 1977 method. Sucrose was expressed in µmol mg^{-1} Chl. Nitrate was assayed by the Cataldo method (CATALDO & al. 1975) both in leaf feeding solution and in leaf extracts. Net nitrate uptake corresponds to NO_3^- decrease in feeding solution; nitrate accumulated is the amount of NO_3^- present in each detached leaf. NO_3^- assimilation corresponds to the difference between the sum (net uptake + NO_3^- present in leaves at time zero) and NO_3^- accumulated during the experiment. NO_3^- values were expressed in µmol. mg^{-1} Chl.

One fraction of the leaf tissue reduced to powder was used to measure phosphoenolpyruvate carboxylase (PEPCase) activity. The powder was resuspended in 1 ml extraction buffer (50 mM Hepes- KOH, pH 7.4, 12 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT and 10% (v/v) glycerol) in eppendorf tubes, Vortex mixed and frozen in liquid N_2. After thawing and centrifugation at 16,000g for 2 min, the supernatant was desalted by centrifugal filtration at 5,000g at 5° C through Sephadex G-25 as in VAN QUY & CHAMPIGNY 1992. The desalted extract was immediately assayed for enzymatic activity by the spectrometric measurement of NADH-oxidation mediated by malate dehydrogenase (MDH). The reaction mixture contained in 1 ml 50 mM Tricine buffer, pH 7.4, 25 µmol $NaHCO_3$, 125 nmol NADH, 1 unit MDH and 25 or 15 µl enzyme extract. Reactions at 30° C were initiated by the addition of 125 nmol phosphoenolpyruvate (PEP). Enzymatic activity was expressed in µmol NADH min^{-1} mg^{-1} Chl.

The pellets obtained after centrifugation of the crude extracts for PEPCase activity determination or sucrose and nitrate measurements were resuspended in ethanol 96 %, centrifuged and the absorbance read at 652 nm for chlorophyll quantification in mg. ml^{-1} (ARNON 1949).

To identify the metabolites synthesized by excised maize and wheat leaves in the presence (40 mM) or absence (0 mM) of NO_3^-, in short times (1 hour), extracts prepared by the same method as for sucrose determination were from 3 g of leaves after uptake of 21 mM 1-^{13}C-glucose added to the nutrient solution bathing the excised leaves. After evaporation of extracts, deposits were resuspended in 0.1 N HCl, pH 5.8 and cleared by centrifugation. ^{13}C-NMR analysis were performed on a Bruker AMX 500 spectrometer operating at 125.77 MHz and using a 10 mm broad band probehead, at 300 K. The spectra of the leaf samples were consecutively obtained with the following

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parameters: 30 kHz sweep width, 64 k acquisition data points, 65° flip angle (15 µs), broad band proton decoupling by WALTZ-16 composite pulse sequence, total recycle delay of 2.05 s and 1200 scans. Chemical shifts were quoted relative to ^{13}C-methanol resonance at 49.3 ppm. The assignment of resonances was made by comparison with ^{13}C-spectra described in the literature.

Results

Fig. 1 shows the variation along 8 h of sucrose content in detached maize leaves immersed in nutrient solution without (control) or with 20, 40 and 80 mM NO_3^-, and illuminated. Sucrose accumulation in leaves treated with 80 mM NO_3^- showed a slight decrease at the 4th h (Fig. 1 A). After 8 h the decrease in sucrose content was circa 30% for 40 mM and more than 40% for 80 mM, as compared to the control. During the first two hours sucrose synthesis rates were almost constant for 20 and 40 mM NO_3^- (Fig. 1 B). At the 4th and 8th h the rate of sucrose synthesis of control was 5.4 and 4.4 µmol. mg^{-1} Chl. h^{-1}, respectively. At the 4th h 80 mM NO_3^- lead to a decrease of 13% in sucrose synthesis; after 8 h feeding 40 or 80 mM NO_3^- the sucrose synthesis rates were 75% and 59% of the control, respectively.

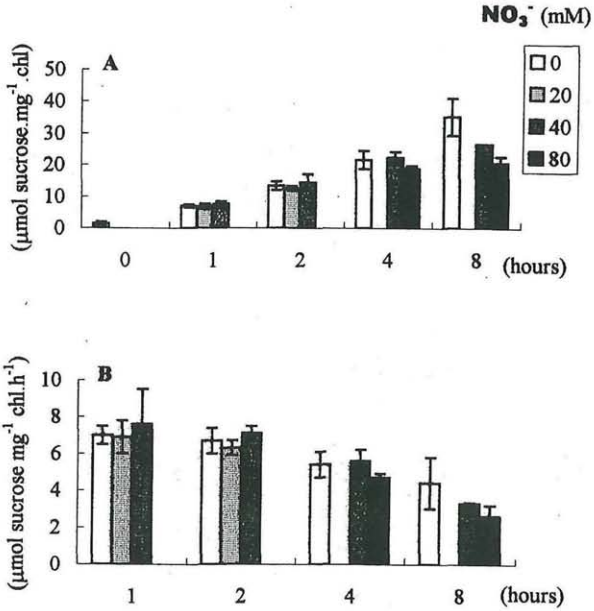

Fig. 1. Sucrose synthesis, net (A) and rate (B) in leaves excised from N-limited maize plants supplied with 0, 20, 40 or 80 mM KNO_3 in the light (520 µmol m^{-2} s^{-1} for up to 8 h. In A, zero time point represents leaves before treatments. Each point is the mean ± SE (n ≥ 4).

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Nitrate uptake and assimilation was proportional to time and NO_3^- concentration. In Fig. 2 A the net uptake and assimilation of 20, 40 and 80 mM NO_3^- are shown; in Fig. 2 B the respective uptake and assimilation rates. During the first hour of treatment with 20 or 40 mM NO_3^- maize leaves assimilated only vestigial amounts of the nitrate absorbed during that period. Between the 2nd and the 4th h, uptake and assimilation rates attained maximal values.

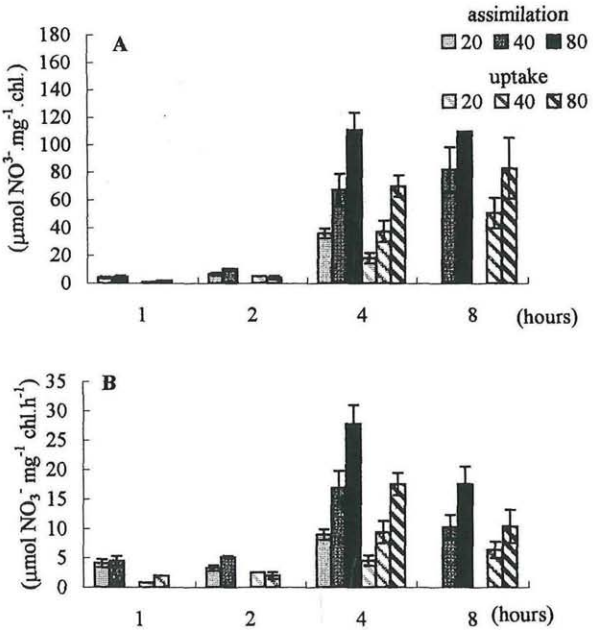

Fig. 2. Nitrate uptake and assimilation, net (A) and rates (B) in leaves excised from N-limited maize plants exposed to 20, 40 or 80 mM KNO_3 in the light (520 µmol m^2 s^{-1} for up to 8 h. Each point is the mean ± SE ($n \geq 4$).

PEPCase activity of detached maize leaves subjected to high light irradiance (1000 µmol m^{-2} s^{-1}) is shown in Fig. 3. PEPCase activity increased transiently during the first 15 min while in leaves treated with 40 mM, enzyme activity increased more than 50% as compared to control during the first hour, maintaining almost the same difference after two hours.

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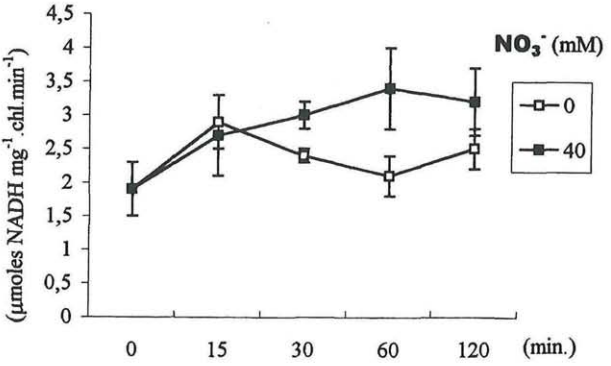

Fig. 3. Effect of 40 mM KNO_3 on PEPCase activity of detached maize leaves. The leaves were detached from N-limited plants after a 16 h period in darkness and transferred to 0 or 40 mM KNO_3 nutrient solutions in the light (1000 µmol m^{-2} s^{-1}), for up to 2 h. Each point is the mean ± SE ($n \geq 3$)

To study the metabolites formed either in maize or wheat leaves after a short period of time (one hour) detached leaves immersed in 0 (control) and 40 mM NO_3, and illuminated by 1000 µmol. m^{-2} s^{-1} (maize) and 520 µmol m^{-2} s^{-1} (wheat) received $^{13}C_1$-glucose in the nutrient solution. In $^{13}C_1$-NMR spectra the resonances between 59 and 107 ppm correspond to carbon atoms of sucrose, except for 65.3 and 70.5, assigned to the buffer mixture (Fig. 4 A, B). At time 0 of the experiment (natural abundance), the only sucrose carbon atom visible at 104 ppm in either maize or wheat leaves, was for 2-C-fructose into sucrose (Fig. 4 A, B, a). After one hour labelling, the positions of 61.9 and 95.5 ppm corresponding respectively to 1-^{13}C-fructose and 1-^{13}C-glucose into sucrose were strongly labelled. Comparing the spectra, it is noticeable that after NO_3^- feeding the labelling in sucrose carbon atoms from direct synthesis decreased in wheat (Fig. 4B, compare b to c) while ^{13}C atoms resulting from 1-^{13}C-glucose recycling were more abundant in spectra of both species. Other metabolites, pyruvate, PEP, tricarboxylic acids or amino acids were not $^{13}C_1$-labelled in either control or NO_3^- treatment.

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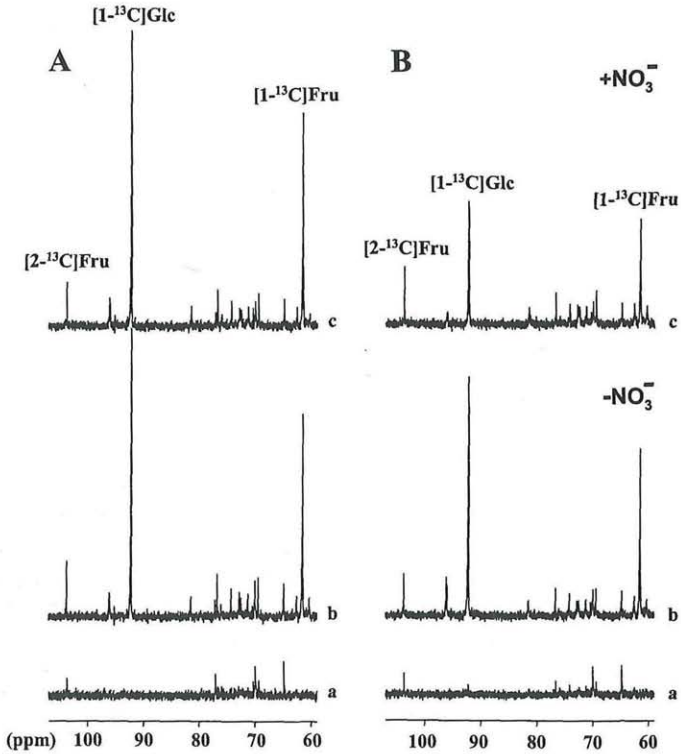

Fig. 4. ^{13}C-NMR spectra of extracts prepared from maize (A) and wheat (B) leaves fed KNO_3 0 mM (A,b and B,b) or 40 mM (A,c and B,c) in the light (wheat, 520 µmol m^{-2} s^{-1}; maize, 1000 µmol m^{-2} s^{-1}), after one hour labeling with 21 mM 1-^{13}C-glucose. In A,a and B,a is shown the natural abundance of ^{13}C-metabolites present in no-labeled leaves of maize and wheat, respectively. ^{13}C-NMR analysis as in Material and methods.

Discussion

The rate of net sucrose synthesis decreased in illuminated detached N-depleted maize leaves. At 8 h, it was 60% of the initial rate. The NO_3^--inhibition of sucrose synthesis was observed for concentrations of 40 mM or higher, from the h 4. During the 4th to 8th h interval, the rate of sucrose synthesis decreased to 75% or 59% the control when leaves were fed 40 or 80 mM NO_3^-, respectively (Fig. 1). Apparently, the response of maize leaves was delayed as compared to wheat exposed to the same NO_3^- treatment (CHAMPIGNY & al. 1992).

During the first h maize leaves supplied with 20 or 40 mM NO_3^- assimilated NO_3^- in vestigial amounts, uptake and assimilation rates attaining maximal rates at the 2nd or 4th while the maximal uptake rate of wheat leaves fed 40 mM was

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found at the first h, at values very close to that measured in maize at the 2nd h. (VAN QUY & al. 1991a,b). Wheat maintained a high uptake rate up to the 10th h of treatment (VAN QUY & al. 1991b), and maize NO_3^--uptake rate commenced to decrease at the 8th h. Considering a constant NO_3^--assimilation rate of 3.6 µmol mg $Chl^{-1}.h^{-1}$ in wheat fed 40 mM NO_3^- (VAN QUY & al. 1991b), the rate in maize was almost three times higher at the second and 4th h, decreasing to the double at the 8th h. It seems that immediately after feeding NO_3^- to illuminated maize leaves N-assimilation is less competitive with C-assimilation than in wheat. It results that for the same NO_3^- concentration, the increase in N-assimilation and the inhibition of sucrose synthesis occurs earlier in wheat than in maize. However, feeding NH_4^+ to maize leaves resulted in an approximate doubling of the total amino acid content, within 1 h. (FOYER & al. 1994).

Due to its anapleurotic function, PEPCase is a crucial enzyme in the interaction of C and N metabolism, both in C_3 and C_4 plants. The enzyme is activated by phosphorylation, dependent on a protein kinase activity, which is enhanced by N-supply, most probably through reduced N-compounds, namely Gln. However, the link between NO_3^- and PEPCase activation is not fully understood. In C_4-type plants, in vitro PEPCase activities are 10 times the values measured in C_3 plants (DAVIES 1979). The same relationship was found for PEPCase activity in detached maize and wheat leaves (VAN QUY & al. 1991a). Also, in both species PEPCase is activated by light and the light activation is enhanced by nitrate (CHAMPIGNY 1995). In fact, as in wheat (CHAMPIGNY & FOYER 1992), the effect of NO_3^- over detached maize leaves (Fig. 3) confirms the activation of maize PEPCase by NO_3^-, certainly through a kinase-modulated process.

In C_4-type plants most of PEPCase activity corresponds to the primary fixation of CO_2 while in C_3-type plants all the enzyme activity is devoted to anaplerotic reactions of PEP carboxylation. For that reason, changes in enzyme activity could be less evident in C_4-type plants. The present results show that in short-term treatments, PEPCase of detached maize leaves respond to NO_3^- as predicted in the above regulation model. On the other hand, PEPCase is known as being involved in the maintenance of cellular pH-stat (SMITH & RAVEN 1979). In vivo ^{31}P-NMR allowed to measure the cytoplasmic and vacuolar pH of maize *callus* tissue under different N-nutrition conditions. When NO_3^- was the sole N-source the cytoplasmic pH showed an increase of approximately 0.3 units, and in vitro PEPCase activity increased significantly of more than 25%, comparing to balanced N-supply (AMÂNCIO & al. 1993).

PEPCase activation was observed after one-hour NO_3^- feeding. The fate of carbon chains formed during that period by maize and wheat leaves fed 1-^{13}C-glucose was identified by ^{13}C-NMR spectroscopy. Natural abundance signals of most carbon atoms of sucrose and other carbon compounds were not detected (Fig. 4 A, B, a). Leaves receiving 1-^{13}C-glucose synthesised sucrose by incorporating directly 1-^{13}C-glucose and 1-^{13}C-fructose. In wheat, NO_3^- feeding decreased the direct synthesis of sucrose confirming the inhibition of SPS by short term NO_3^- nutrition (CHAMPIGNY 1995). The labelling in the remaining sucrose atoms including the 2-C carbon from fructose is the result of the scrambling of ^{13}C from 1-^{13}C-

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glucose. ^{13}C-acetate is recycled through TCA cycle and gluconeogenesis at higher rates under NO_3^- enrichment conditions. However, unlike the very rapid incorporation of ^{14}C from $^{14}CO_2$ (CHAMPIGNY & al. 1991) and the increase in absolute levels of amino acids and other metabolites after two hours 40 mM NO_3^- feeding of detached illuminated wheat leaves (CHAMPIGNY & al. 1992), the concentration of ^{13}C-labelled PEP, TCA intermediates or amino acids, was not detectable after $^{13}C_1$-glucose uptake for one or two (data not shown) hours.

Taken as a whole, the results point to the following evidences: In detached leaves of maize, as in C_3-type plants, the first effect observed after short-term NO_3^- enrichment is an increase in PEPCase activity, NO_3^- acting as a signal to enhancing light-dependent activation of protein kinases which modulate PEPCase. Secondly, in maize, the decrease in direct sucrose synthesis due to NO_3^- assimilation is delayed comparing to wheat although the recycling of carbon chains is also stimulated. Finally, prolonged treatments are equivalent to the long-term high N-nutritional conditions at the plant level, which give rise to balanced responses.

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