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# Diurnal variations in the activity of phosphoenolpyruvate carboxylase and NADP-malic enzyme during the early steps of interaction between *Glycine max* and *Bradyrhizobium japonicum*

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## Abstract

Two important enzyme in organic acid metabolism, phosphoenolpyruvate carboxylase (PEPC) and NADP-malic enzyme (NADP-ME), show marked diurnal rhythms in their activities during the establishment of the soybean - *B. japonicum* symbiosis. The pH of the nutrient solution changes in parallel with NADP-ME activity, being maximal during the night periods, whereas activity of PEPC was highest during the day periods. The results from the experiments with stem girdled plants indicated that the activity of root PEPC is modulated to a great extent by the supply of photosynthates from the shoots. It was also established that succinate application in the nutrient solution during inoculation altered significantly the pattern of assayed enzyme activities. Although our experiments did not reveal the precise mechanism of the involvement of root PEPC and NADP-ME in soybean response to inoculation with *B. japonicum*, they indicated the pattern of their activity during the first 72 h postinoculation which are critical for establishment and functioning of the symbiosis.

*Additional key words*: inoculation, medium pH, soybean roots, stem girdling, succinate.

## Introduction

Symbiotic nitrogen fixation takes place in nodules which form when legume roots are colonized by specific soil bacteria from the genera *Rhizobium*, *Azorhizobium*, *Sinorhizobium*, *Bradyrhizobium* or *Mesorhizobium*. In return for providing the bacteria with a source of carbon, the host received reduced nitrogen in the form of ammonia for amino acid and protein syntheses.

Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme in the interaction of carbon and nitrogen metabolism in legume nodules which contributes substantially to providing the energy required for symbiotic nitrogen fixation (King *et al.* 1986). Four different aspects of the physiological and metabolic roles of PEPC have been established: *1*) as a mechanism for recovery of some respired $CO_2$ (Vance *et al.* 1983); *2*) as part of a pathway for synthesis of dicarboxylic acids used as respiratory substrates (mainly malate and succinate) by the bacteroids (King *et al.* 1986, Rosendahl *et al.* 1990); *3*) as a source of carbon skeletons for amino acid biosynthesis (Christeller *et al.* 1977, Rosendahl *et al.* 1990); and *4*) as a mechanism involved in cytosolic and xylem fluid pH regulation by buffering $OH^-$ formation during nitrate reduction (Kaiser and Brendle-Behnisch 1995). In tissue from nitrate-grown pea plants, PEP carboxylation varied diurnally, showing an increase upon illumination and a decrease upon darkening (Leport *et al.* 1996). Nitrate uptake by roots of soybean depends on the availability of malate produced in the shoot and its translocation to the roots (Touraine *et al.* 1992), where it is decarboxylated by malic enzyme (ME), yielding pyruvate and $HCO_3^-$ ions (Kirkby and Knight 1977). During the day, nitrate assimilation and the accompanying synthesis of malate proceed more rapidly than malate export and malate accumulates to high concentrations (Müller *et al.* 2001). The soybean symbiont, *B. japonicum* produce two distinct malic

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*Abbreviations*: ME - malic enzyme; PEPC - phosphoenolpyruvate carboxylase
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enzymes, one specific for $NADP^+$ and with high affinity for malate (NADP-ME) and the other $NAD^+$-dependent enzyme with a lower affinity for malate (NAD-ME) (Day and Copeland 1991). Our previous investigations have shown that NADP-ME activity in the inoculated soybean roots is significantly higher than NAD-ME activity (unpublished data). NADP-ME appears to be constitutive and may be important in biosynthetic reactions as well as in supplying reducing power for nitrogenase (Day and Copeland 1991).

The aim of the present investigation was to trace the contents and diurnal variations of the PEPC and NADP-ME activities, and the accompanying changes of the medium pH during the early stages of interaction between soybean and *B. japonicum* strain 273. To examine whether an interruption in carbon supply from the shoots caused any changes in the activity of the root enzymes tested, experiments with stem girdled plants were performed. In addition, we provide data demonstrating that application of succinate during inoculation altered significantly the pattern of assayed enzyme activities.

## Materials and methods

Soybean seeds (*Glycine max* L. Merr. cv. Hodgson) were sterilized with 70 % ethanol and germinated in the dark at 25 °C. Five-day-old seedlings were selected for use and transferred into aerated quarter strength nutrient solution (Hellriegel 1898) containing 750 μM $NO_3$ and micronutrients (Hoagland and Arnon 1950). Initial pH of the nutrient solution was adjusted to 5.0 by the addition of 1 M NaOH and measured every 2 h with pH meter type *Radelkis OP 211*, (Budapest, Hungary). Twenty days after planting, soybean plants were inoculated with bacterial suspension of *Bradyrhizobium japonicum* strain 273 at approximately $10^8$ viable cells per $cm^3$. Where noted, plants were steam-girdled 1 cm above the root junction in order to interrupt phloem translocation to the root (Anderson *et al.* 1987). For the experiments implicating succinate, the 200 μM sodium succinate (*BDH Chemicals Ltd,* Poole, England) was added directly in the solution during inoculation. The plants were cultivated in a naturally illuminated greenhouse with photoperiod of 15-h and day/night temperature of 30/25 °C. The following treatment groups were tested: *1*) uninoculated control plants, *2*) inoculated control plants, *3*) plants treated with succinate during inoculation, *4*) girdled uninoculated plants, *5*) girdled inoculated plants, and *6*) girdled plants treated with succinate during inoculation.

In order to prepare crude extracts for the determinations of PEPC and NADP-ME activities, the roots were rinsed in distilled water, blotted with paper tissue and quenched in liquid nitrogen. After grinding the frozen root material, 5 $cm^3$ of the extraction buffer (50 mM Tris-HCl, pH 7.8, 400 mM sorbitol, 30 mM ascorbate, 2 mM EDTA, 10 mM dithiothreitol and 20 g $dm^{-3}$ polyvinylpyrrolidone-40) was added to 1 g fresh biomass of the tissue. After further grinding till thawing, the suspension was centrifuged (16 000 *g*, 20 min, 4 °C). The two enzymes were assayed spectrophotometrically by tracing the change in absorbance of a pyridine nucleotide at 340 nm, 27 °C, using spectrophotometer (*Shimadzu Co. UV-1601*, Tokyo, Japan). PEPC (EC 4.1.1.31) was assayed according to Guralnick and Ting (1987), and NADP-ME (EC 1.1.1.40) according to Garnier-Dardart and Queiroz (1974). Protein content was measured by the method of Lowry *et al.* (1951) with BSA as a standard. All chemicals were purchased from *Sigma Chemical Co.* (St. Louis, USA), unless noted otherwise. Data calculation and statistical analyses were conducted using *SYSTAT* software (*SYSTAT, Inc.*, Davis, USA).

## Results and discussion

Although the early stages of the interaction between soybean and *B. japonicum* have been well studied at the histological and ultrastructural levels (Turgeon and Bauer 1986, Eskew *et al.* 1993, Green and Emerich 1999), not too much is known about alteration in root metabolism after plant inoculation. In the present study, attention has been focused on the relationship between the activity and diurnal fluctuations of root PEPC and NADP-ME, and the accompanying changes in pH of the nutrient solution during the first 72 h post-inoculation. External pH is one of the most important determinants for the efficient achievement of symbiosis (Caetano-Anolles and Favelukes 1986). The activities of PEPC and NADP-ME in the roots of uninoculated soybean plants were low (Fig. 1*A*). After inoculation 3.6- to 4.8-fold increase of NADP-ME activity was observed (Fig. 1*B*). The activity of PEPC during the first 24 h post-inoculation was not significantly changed, but rose over 1.5-fold after 36 h and later. This is consistent with the findings of Dolgikh *et al.* (1998), who observed an increase of the root PEPC activity during the early stages of establishment of the pea-*Rhizobium leguminosarum* symbiosis. Pathirana *et al.* (1992) reported that maximum expression of PEPC in alfalfa nodules was related to two distinct signals; the first associated with nodule initiation and the second with the effectiveness of nodules.

In the roots of uninoculated and inoculated control plants the activity of both enzymes varied diurnally showing an increase of PEPC and decline of NADP-ME during the day periods. These changes were reversed during the night periods (Fig. 1*A*,*B*). Leport *et al.* (1996) suggested that diurnal modulation of PEP carboxylation in leaves and roots of nitrate grown pea is not caused by protein phosphorylation, but rather is allosterically regulated by malate and other organic acids. Scheible *et al.* (2000) have proposed that high PEPC activity during the day period facilitate preferential synthesis of malate to act as a counter-anion for pH regulation.

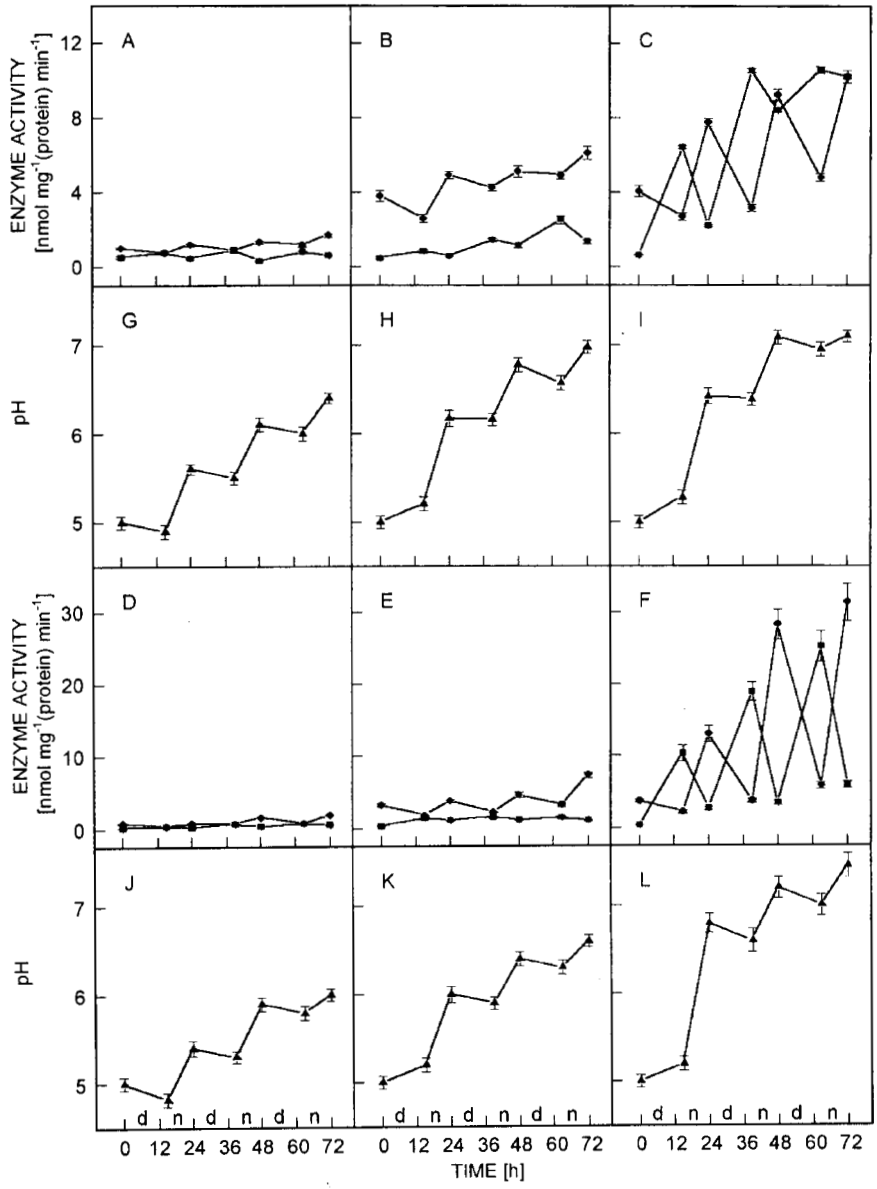


Fig. 1. Diurnal variations in the activities of soybean root PEPC (*squares*) and NADP-ME (*circles*) in uninoculated plants (*A*), during the first 72 h after inoculation of soybean plants with *B. japonicum* strain 273 (*B*), and so on (*C* - *F*), and in the accompanying changes of the medium pH (*triangles*) (*G* - *L*). The day periods are indicate with *d* and the night periods with *n*. *Vertical bars* represent SE of the mean of 10 replicates.

It should be noted that levels of NADP-ME activity were higher than these of PEPC, especially in the inoculated roots (Fig. 1*A*,*B*). According to El-Shora and Rees (1991) in the photosynthetic and non-photosynthetic tissues of $C_3$ plants, NADP-ME is a cytosolic enzyme with a great importance for carbohydrate oxidation, lipid synthesis and assimilation of inorganic nitrogen providing pyruvate for the synthesis of pyruvate-based amino acids. On the whole, the activity of NADP-ME is the highest in tissues where biosynthesis is most marked (El-Shora and Rees 1991), that is typical for inoculated roots of legumes.

The pattern of the medium pH variations before plant inoculation (Fig. 1*G*) was different compared to that observed after inoculation (Fig. 1*H*). An immediate consequence of the inoculation was rapid increase in external pH during the first 24 h (Fig. 1*H*). Legumes are known to alkalinize the rhizosphere in the dark but caused acidification with exposure of the shoot to light (Rao *et al.* 2002). The same authors have found that light-induced acidification of cowpea seedlings rhizosphere is regulated by photosynthetic activity. Supplement of succinate at the time of inoculation gave higher activities of PEPC and NADP-ME as well as greatly increased the amplitudes of durnal changes for both activities (Fig. 1*C*). The observed enzyme activation under succinate treatment could be possibly responsible for a more rapid alkalinization of the external medium (Fig. 1*H*).

Dependence of nitrogen fixation on photosynthetic products is a concept supported by data of many investigations (Day and Copeland 1991, de Veau *et al.* 1992). To examine the influence of the interruption of carbon supply from the host plant leaves to the roots, hypocotyls were steam girdled in some treatment groups (Fig. 1*D-F*). We established high rates and pronounced diurnal variations of NADP-ME activity in girdled uninoculated (Fig. 1*D*) and inoculated (Fig. 1*E*) plants. These results suggest the participation of this enzyme in degradation of the reserve saccharides upon interruption of phloem translocation to the roots. In contrast, the activity and diurnal variations of PEPC were substantially reduced or almost abolished especially in girdled inoculated plants (Fig. 1E). Zhang *et al.* (1995) have found that pretreatment of soybean plants by stem girdling causes a significant decrease in the apparent phosphorylation state of nodule PEPC. Enzyme phosphorylation is involved in the upregulation of PEPC activity in soybean and the phosphorylation state of PEPC is modulated by photosynthate supply to the nodules *in vivo* (Wadham *et al.* 1996). Succinate application in the nutrient solution induced a great increase of PEPC activity and its much higher diurnal changes in girdled plants (Fig. 1F). These findings suggest that PEPC activity depends on a continuous supply of shoot-born carbohydrates to the roots, and that it can be restored in roots of girdled plants by succinate addition to the external solution. The fact that all variants investigated showed a strongly negative correlation between the activity of PEPC and NADP-ME in the day/night periods, allows us to propose that NADP-linked ME decarboxylation is also subject of strict allosteric regulation in order to avoid "futile" cycle.

It should also be noted that in all treatment groups, the pattern of the diurnal changes of NADP-ME correlated with the diurnal changes of the medium pH. Douce and Neuburger (1989) have reported that malate accumulated during the day may be used as respiratory energy during the night. Malate synthesis and degradation are components of the pH-state mechanism. The $OH^-$ equivalents excreted by the roots originated from the decarboxylation of malate by ME (Martinoia and Rentsch 1994). Thus, NADP-ME activity in soybean roots may be coordinately regulated by fluctuations in malate concentration which permits the parallel diurnal variations of medium pH.

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