African Journal of Biotechnology Vol. 11(37), pp. 9086-9093, 8 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1320
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Effect of iron deficiency on the localization of phosphoenolpyruvate carboxylase in common bean nodules

Tarek Slatni[1,2]*, Imen Ben Salah[1], Marta Dell'Orto[3], Gianpiero Vigani[3], Saber Kouas[1], Houda Gouia[2] Graziano Zocchi[3] and Chedly Abdelly[1]

[1]Laboratoire des Plantes Extrêmophiles, Centre de Biotechnologie, Technopark de Borj Cedria, BP 901, Hammam Lif 2050, Tunisie.
[2]Unité de Nutrition et Métabolisme Azotés et Protéines de Stress, Département de Biologie, Faculté des Sciences de Tunis, Campus Universitaire, 1060 Tunis, Tunisie.
[3]Università Degli Studi Di Milano, Dipartimento di produzione Vegetale, Sezione di Fisiologia delle Piante Coltivate e Chimica Agraria, Via Celoria 2 - 20133 Milano.

Accepted 11 August, 2011

Phosphoenolpyruvate carboxylase (PEPC) plays an important role in nodules, when there is an increase in the demand for energy. This enzyme provides carbon skeletons to sustain amino acid synthesis and malate to support energy required to fix nitrogen. Since PEPC is important for nodules, and there is lack of information about the effect of some nutrient deficiency in the expression and localization of this enzyme in legume nodules, this work focused on the localization of PEPC in nodules under iron deficiency of two common bean cultivars: Flamingo tolerant and Coco blanc sensitive to iron (Fe) deficiency. The results of immunolocalization using polyclonal antibody showed that this enzyme was detected in all regions of nodule sections; but the signal intensity was increased in Fe-deficient nodules as compared to Fe-sufficient ones in the tolerant cultivar, whereas the intensity was less pronounced in nodules of Fe-deficient plants than in those of Fe-sufficient plants for the sensitive cultivar Coco blanc. This work showed that the symbiotic tolerance of Flamingo to iron deficiency was linked to the increase of PEPC enzymes expression. However, the activity of these enzymes supported the energy required in bacteroids to maintain the nitrogenase activity.

Keywords: Common bean, immunolocalization, iron deficiency, nodules, phospho*enol* pyruvate carboxylase.

INTRODUCTION

Phospho*enol*pyruvate carboxylase (PEPC) plays an important role in N_2-fixation by root nodules. This enzyme is a tetramer of 110 kD and is localized to the cytosol (Chollet et al., 1996; Pathirana et al., 1997). It is encoded by a multigene family and, in legumes at least, one of the PEPC genes shows highly enhanced expression in root nodules (Vance, 2008). The PEPC catalyses the fixation of bicarbonate to phosphoenolpyruvate to produce oxaloacetate and inorganic phosphate (Pi) and consequently keep the glycolysis rate sufficiently high in the plant (Abadia et al., 2002), thus sustaining the demand for ATP and NAD(P)H and contributing to the cytosolic pH-stat. PEPC plays a very important role in N_2-fixing root nodules, providing both C for N assimilation into amino acids and malate as an energy source for bacteroid nitrogenase (Vance et al., 2000). Both the protein and the mRNA of PEPC are significantly more abundant in legume nodules as compared to roots and the PEPC protein represents at least 1% of the nodule soluble protein (Vance, 2000). Several studies showed that the carbon fixed via root-nodule PEPC provides some 30% of that needed for aspartate and asparagine biosynthesis in N_2-fixing nodules. In ineffective nodules, PEPC activity and mRNA abundance are frequently quite low (Vance et al., 2000). Regulation of root-nodule PEPC occurs at both

*Corresponding author. E-mail: slatni_tarek@yahoo.fr Tel: 00 216 79 325 848. Fax: 00 216 79 325 638.

the transcriptional and post-translational levels. Post-translational regulation of nodule PEPC activity is achieved through protein turnover, phosphorylation of the enzyme, and allosteric modifications due to malate and glucose-6-phosphate (Chollet et al., 1996). Upon phosphorylation, which is achieved through a nodule-enhanced PEPC serine/threonine kinase (Xu et al., 2003), nodule PEPC becomes less susceptible to inhibition by malate. Reduced sensitivity to malate is a necessity when one considers the high malate concentrations in nodules. The dependence of nodule function on PEPC activity has been shown through the effects of antisense expression of PEPC in nodules (Schulze et al., 1998). Inhibition of nodule PEPC via antisense resulted in a decrease of nodule enzyme activity and mRNA, decreased N accumulation and plant growth, and lower nitrogenase activity. In addition, treatments that decrease N_2 fixation, like shading and stem girdling, lower both nodule PEPC and PEPC-kinase activity. Re-supply of carbohydrate to nodules restores the activity of PEPC and PEPC-kinase, as well as that of N_2 fixation (Vance, 2008). Since PEPC is importance for the nodules, and there is lack of information about the effect of some nutrient deficiency in the expression and localization of these enzymes in nodules of legumes, this study focused on the localization of PEPC in nodules under Fe deficiency of two cultivars of common bean: Flamingo tolerant and Coco blanc sensitive to Fe deficiency.

MATERIALS AND METHODS

Plant materials and growth conditions

Two common bean cultivars: Coco blanc sensitive and Flamingo tolerant to iron deficiency (Slatni et al., 2008), were inoculated with *Rhizobium tropici* CIAT 899 from the International Center of Tropical Agriculture, Colombia, maintained in the "Laboratory of legumes" (CBBC, Hammam-Lif, Tunisia) were grown with the following N-free nutrient solution (Hewitt, 1996): KH_2PO_4 (1.60 mM), $MgSO_4$ (1.50 mM), K_2SO_4 (1.50 mM), $CaSO_4$ (3.50 mM), H_3BO_3 (4 µM), $MnSO_4$ (4 µM), $ZnSO_4$ (1 µM), $CuSO_4$ (1 µM), $CoCl_2$ (0.12 µM) and $(Na)_6(Mo)_7O_{24}$ (0.12 µM) added with 5 µM Fe as K-Fe-EDTA and 1 mM urea as starter N needed for growth during the first two weeks. The nutrient solution was aerated with a flow of 400 ml mn^{-1} of filtered air. After two weeks of pre-treatment (appearance of functional nodules), plants were separated into two plots: the first one received no iron (Fe-deficient plants), and the second one received 45 µM Fe(III)EDTA (Fe-sufficient plants). After three weeks of treatment, plants were harvested. Samples of fresh material were weighted and dried at 70°C for 72 h and ground to fine powder using a grinder with agate pots. Mineral concentration was determined by atomic absorption spectrophotometer (Varian Spectr AAS 220), while total nitrogen was determined by the Kjeldahl method. Quantities of N fixed were calculated from the differences of the whole-plant N content at the final and initial harvests.

Preparation of soluble fraction

Nodules of common bean plants were excised, rinsed in distilled water and homogenized in a mortar at 2 to 4°C in a buffer containing 50 mM Tris–HCl (pH 7.5) with 10 mg ml^{-1} leupeptin, 10% glycerol, 20% polyvinylpolypyrrolidone (PVPP) and 10 mM $MgCl_2$, 1 mM EDTA, 14 mM β-mercaptoethanol and 1 mM phenyl-methylsulfonyl fluoride (PMSF). After filtration through four layers of gauze, the homogenate was centrifuged at 13 000 *g* for 15 min and the supernatant was centrifuged at 80 000 *g* for 30 min to obtain a soluble fraction (supernatant) (De Nisi and Zocchi, 2000).

Enzyme assays

PEPC (EC 4.1.1.31) activity was determined as reported by De Nisi and Zocchi (2000). Reaction was started by adding aliquots of protein extracts and the enzymatic assay was performed at 25°C in 1.5 ml final volume. Oxidation of NADH was followed spectrophotometrically at 340 nm.

Organic acid assays

Organic acids, such as malate, were extracted from nodules grown in the presence or absence of Fe as reported by Rabotti et al. (1995). The nodules of each treatment were rinsed in distilled water, homogenized in the presence of 5 ml of 10% (v/v) perchloric acid and centrifuged for 15 min at 10 000 *g*. Supernatant pH was brought to 7.5 with 0.5 M K_2CO_3 to neutralize the acidity and precipitate the perchlorate. The extract was clarified with another centrifugation at 15 000 g for 15 min. Malic acid contents were determined enzymatically, according to Rabotti et al. (1995). The recovery of organic acids was more than 90% as determined by the use of an internal standard.

Fixing, embedding and sectioning

Nodules were detached from roots and fixed at 4°C either in ethanol-acetic acid (3:1 v/v, for immunolocalization) overnight or in 100 mM sodium phosphate buffer (pH 7.0) containing 2% paraformaldehyde (w/v) and 2.5% glutaraldehyde, then dehydrated through an ethanol-tertiary butanol series and embedded in paraffin (Paraplast plus, Sigma) as described by Villalba et al. (1991). Serial sections of 7 µm were cut with a microtome, after which sections were mounted on polylysine-treated slides, deparaffinized in xylene and rehydrated through an ethanol series.

Immunolocalization

Immunological detection in tissue sections fixed as described in the foregoing was performed as in Parets-Soler et al. (1990) with some modifications. Sections were incubated for 30 min at room temperature in 3% H_2O_2, then blocked for 1 h in Tris buffered saline (TBS) (150 mM NaCl, 25 mM Tris–HCl, pH 7.6) with 2% BSA (w/v). Polyclonal antibodies, raised against a PEPC isoform of sorghum (a kind gift from Dr. J. Vidal, Université de Paris-Sud), were used. Antibodies were diluted at 1:1000 in TBS with 0.5% bovine serum albumin (BSA) and incubated overnight at 4°C with the sections. After washing in TBS, the sections were incubated for 2 h at room temperature with a biotinylated secondary antibody (anti-rabbit IgG biotin conjugate developed against goat, Sigma) diluted at 1:1000 in TBS with 0.5% BSA. In the same way, after washing in TBS, the sections were incubated for 30 min in the ExtrAvidin-Peroxidase (ExtrAvidin Peroxidase Staining Kit, Sigma) and for 5 to 10 min in 0.05 M acetate buffer pH 5.0 containing 5% dimethylformamide, 0.04% 3-amino-9-ethylcarbazole (AEC) and 0.015% H_2O_2, before finally washing it in distilled water.

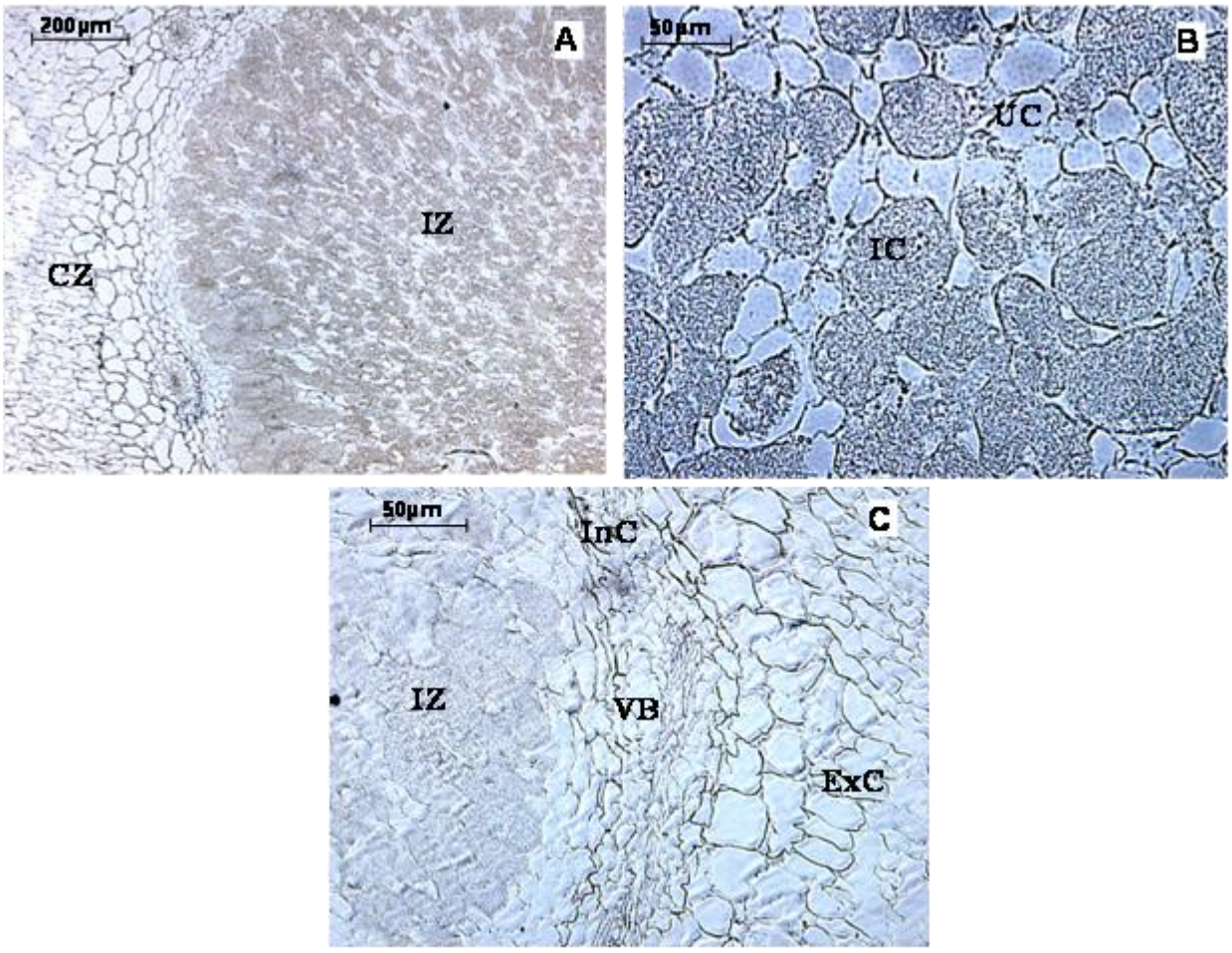

Figure 1. A-C, Control sections of common bean nodules tissues treated without polyclonal antibodies. CZ, Cortex zone; Ex C, external cortex; IC, infected cell; In C, internal cortex; IZ, infected zone; UC, uninfected cell; VB, vascular bundle.

Statistic analysis

A two-way analysis of variance (ANOVA), with the cultivars and treatments as factors, was performed for the data set using the SPSS 10.0 program. Means were compared using the Duncan's test at $P < 0.05$ level when significant differences were found (Slatni et al., 2011).

RESULTS

Immunolocalization

Two major parts were recognized at the histological level of the control section treated without primary antibodies, according to the microscopy observation in nodules (Figure 1) of the infected zone (IZ) and cortex zone (CZ) (Figure 1A). The central part of the nodules contained infected cells (IC) (Figure 1B) and interconnected aggregates of smaller, uninfected cells (UC) (Figure 1B). This central tissue was almost completely surrounded by the nodule parenchyma or internal cortex (InC) including the vascular bundles (VB) (Figure 1C) and external cortex (ExC) (Figure 1C). The control sections appear not to be stained, indicating that a specific reaction attributable to the secondary antibody and/or extravidin-peroxidase complex do not occur during the immuno-localization experiments. Iron-sufficient and Fe-deficient nodule sections from both Flamingo and Coco blanc were subjected to immunoreaction with primary antibodies against PEPC in order to localize the expression of these enzymes in nodule tissues.

In nodules of the tolerant cultivar, Flamingo (Figure 2), the results show that PEPC was detected in all regions of the nodule sections (Infected zone, vascular bundle and cortex zone); but the signal intensity was increased in Fe-deficient nodules (Figures 2A, B and C) as compared to Fe-sufficient ones (Figure 2D, E and F), especially in the infected and cortex zone (Figures 2D and F). On the contrary, PEPC intensity was the same or slightly less pronounced in nodules of Fe-deficient plants (Figures 3D, E and F) than in those of Fe-sufficient plants (Figures 3A, B and C) for the sensitive Coco blanc cv. In addition, the

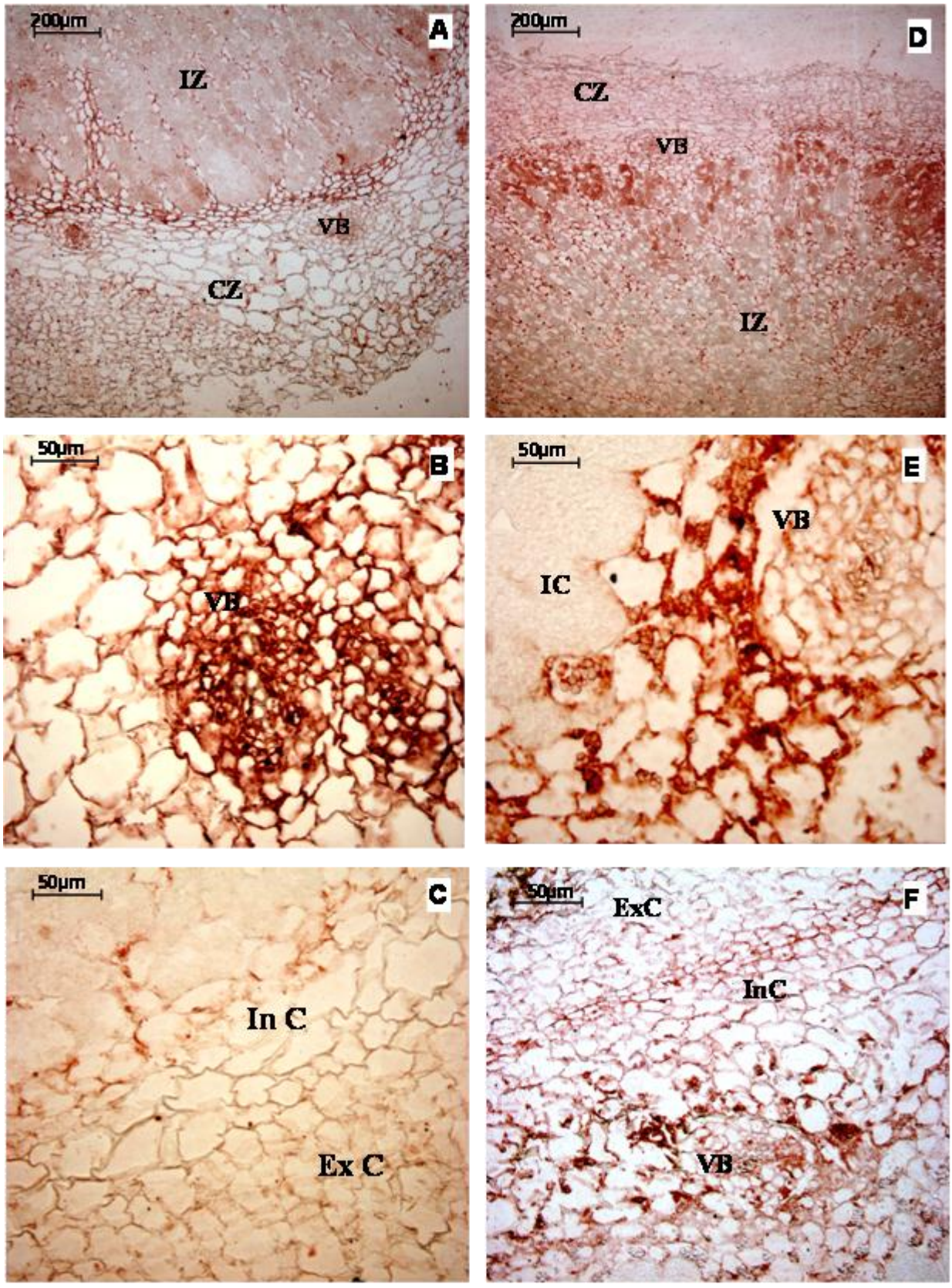

Figure 2. A-F, Immunolocalization of PEPC in common bean nodules tissues of Flamingo. A, B and C, sections of Fe sufficient nodules; D, E and F, sections of Fe deficient nodules treated with polyclonal antibody; Ex C, external cortex; IC, infected cell; In C, internal cortex; UC, uninfected cell; VB, vascular bundle; CZ, cortex zone; IZ, infected zone.

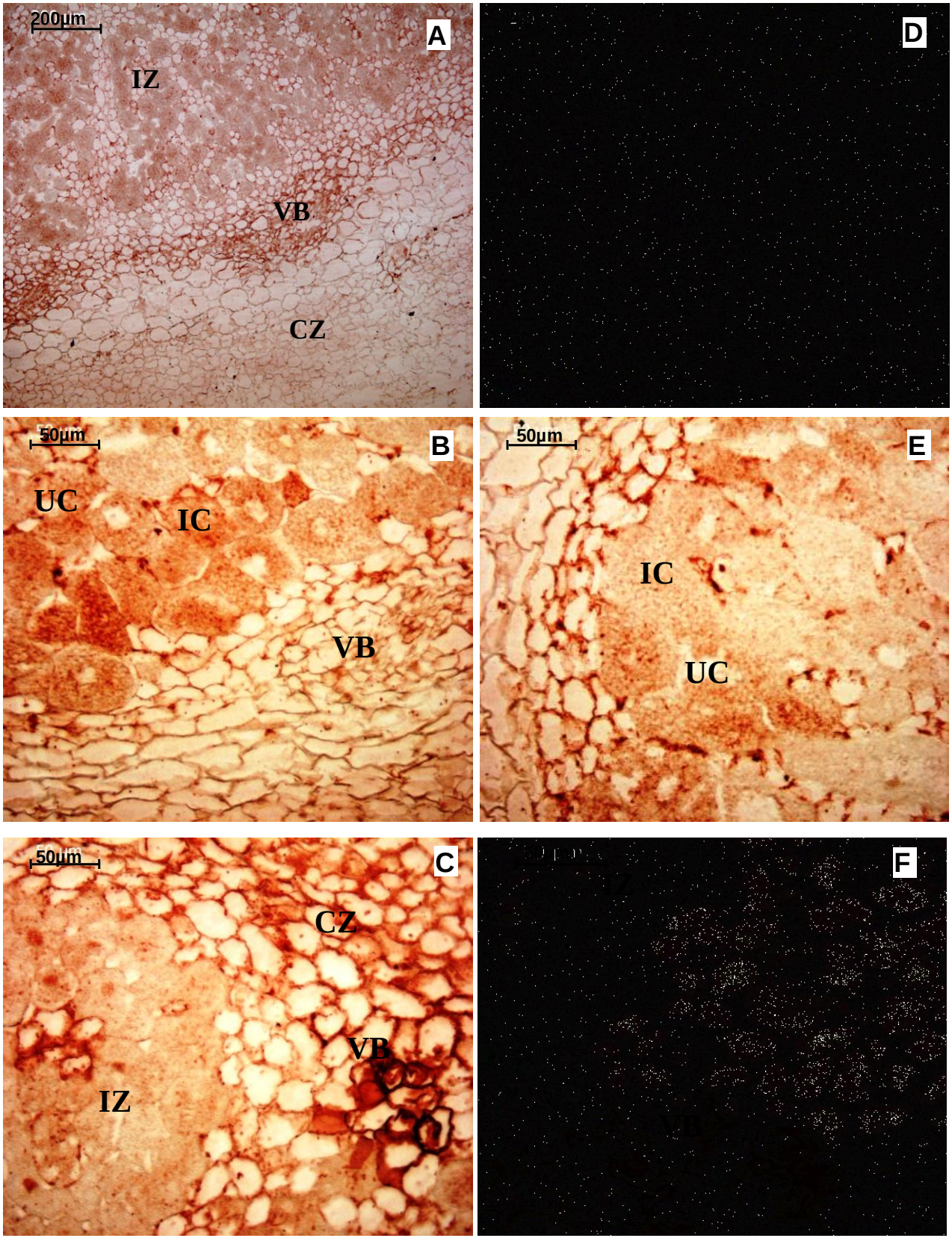

Figure 3. A-F: Immunolocalization of PEPC in common bean nodules tissues of Coco blanc. A, B and C, sections of Fe sufficient nodules; D, E and F, sections of Fe deficient nodules treated with polyclonal antibody; Ex C, external cortex; IC, infected cell; In C, nternal cortex; UC, uninfected cell; VB, vascular bundle; CZ, cortex zone; IZ, infected zone.

Table 1: PEPC activity (nmol NADH min^{-1} mg^{-1} Protein) determined in soluble fraction of nodules and quantities of fixed nitrogen (mmol.$plant^{-1}$) of common bean plants grown in the presence or absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at P ≤ 0.05

		Fixed N	PEPC activity
Flamingo	+Fe	1.61± 0.053d	465.3± 13a
	-Fe	1.27± 0.037b	883.3±17c
Coco blanc	+Fe	1.40± 0.042c	532.6± 16b
	-Fe	0.75± 0.036a	478.7± 12a

Table 2: Effect of iron deficiency on malate content (µg/ g FW) in nodules grown in the presence or in the absence of Fe. Data are the means ± standard error of 4 replicates. Mean values followed by the same letter are not significantly different at P ≤ 0.05

	Malate content in nodules (µg/ g FW)	
	+Fe	+Fe
Flamingo	30.27± 1.9ab	91.34± 6.2c
Coco blanc	30.27± 1.9ab	25.64± 6.6a

comparison between the sections in both cultivars showed that the intensity signal relative to this enzyme was less pronounced in the sensitive cultivar than in the tolerant ones.

PEPC activity

Results of Table 1 show the PEPC activity in the soluble fraction extracted from nodules of both cultivars grown in the presence and absence of Fe. The activity of this enzyme was higher in Fe-deficient plants than in Fe-sufficient plants for the tolerant cultivar, Flamingo (Table 1). Conversely, this activity was less important in Fe-deficient plants than in Fe-sufficient plants for Coco blanc.

N_2-fixation

The amount of N_2 fixed during treatment was estimated from the difference between nitrogen accumulation in all tissues at the final and initial harvest. The results (Table 1) show that N_2-fixing capacity was significantly inhibited under Fe deficiency treatment. In addition, this capacity was decreased to about 46 and 21% respectively in Flamingo and Coco blanc as compared to the control plants. Also, the capacity to fix nitrogen was more important in Flamingo than in Coco blanc.

Malate content

The data in Table 2 showed an increase in nodule malate content, mainly in Flamingo subjected to Fe deficiency: the content was about three times higher in Fe-deficient nodules than in Fe-sufficient ones. In Coco blanc (sensitive cultivar), the malate content decreased (about 33%) in nodules subjected to Fe deficiency.

DISCUSSION

Under Fe deficiency, several adaptive responses were developed and various metabolic pathways were changed to mobilize and increase Fe availability in order to support the Fe required in bacteroids (Day et al. 2001; Kaiser et al. 2003; Slatni et al. 2011). The Fe deficiency affects mainly the carbon and energy metabolism, which are indispensable for the two partners of symbiosis. In fact, bacteroids need to obtain organic compounds and energy from plant to fix more nitrogen. The increase in the PEPC activity was on the basis of this carbon and energy supply in microsymbiont. Since the PEPC catalyses the fixation of bicarbonate to phosphor-enolpyruvate to produce oxaloacetate, it provides both the carbon skeletons for the amino acid synthesis and sustains ATP and NAD(P)H demands (Vance et al., 2000; Prell and Poole, 2006).

In the same context, our results show that the intensity of PEPC proteins was detected in the vascular bundle, and the infected and cortex zone of Fe sufficient nodules. This intensity was increased in Fe deficient nodules particularly in tolerant cultivar, whereas in the sensitive one (Coco blanc), this protein was less pronounced in Fe deficient nodules than in Fe-sufficient ones. In addition, the determination of the PEPC activity in both cultivars confirmed the immunolocalization results, since the PEPC activity increased only in the tolerant cultivar (Flamingo) of Fe-deficient nodules, and decreased in Coco blanc nodules. A similar result was found in soybean, which confirms our results (Zocchi et al., 2007). In fact, the correlation between the increase of PEPC enzyme intensity and their PEPC activity in Fe-deficient nodules of Flamingo and the decrease of these two parameters in Coco blanc indicates that this protein, in Fe-deficient nodules, is in part under transcriptional regulation. On the other hand, the higher PEPC intensity in cortex zone of nodules sections of the tolerant cultivar suggest that under Fe deficiency, this region may need more energy like ATP for support of some metabolic pathway, such as proton extrusion via H^+-ATPase activity. Several studies supported this idea and it was shown in the cucumber root that the activities of PEPC were strictly correlated to the increase in proton extrusion (Rabotti et al., 1995; De Nisi and Zocchi, 2000).

The essential exchange between the two partners of symbiosis is (i) the flux of carbon skeletons from the plant to the bacteroid and (ii) the product of nitrogen fixation from the bacteroid to the plant. There is a general agreement that dicarboxylates, especially malate, are the

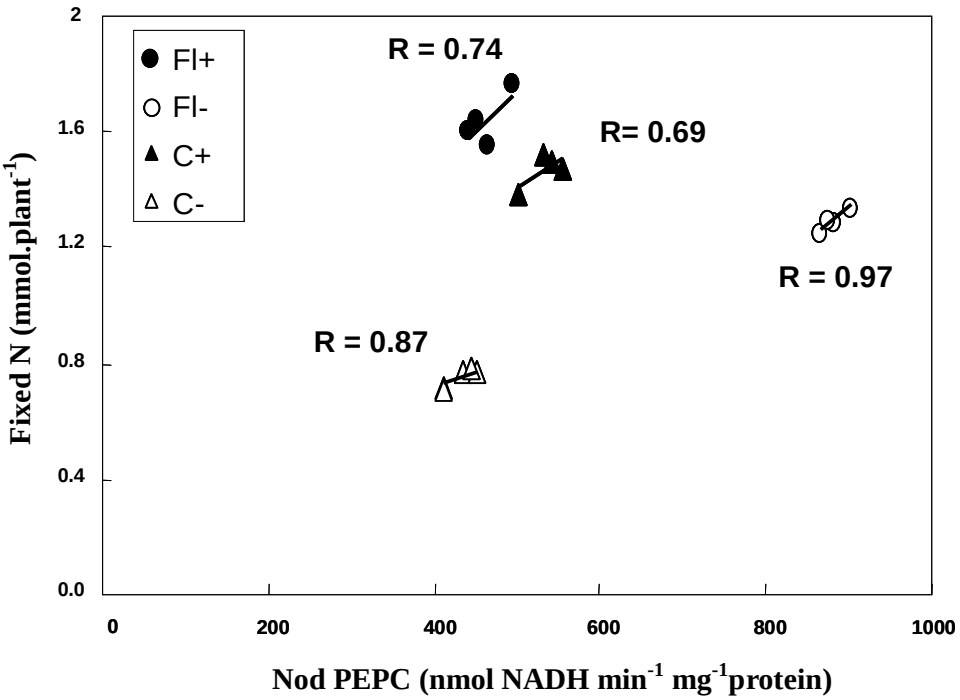

Figure 4. Correlation between fixed nitrogen (mmol.plant^{-1}) and PEPC activity (nmol NADH min^{-1} mg^{-1} protein) in nodules. Data are means ± standard error of four replicates.

main source of carbon supplied to the bacteroids and that ammonium is the form in which fixed nitrogen is supplied to the plant (Day and Copeland, 1991). Similar to PEPC activity, the malate production in Fe deficient nodules increased in Flamingo and decreased in Coco blanc as compared to the control nodules. Various studies show that under Fe starvation, the increase of PEPC activity results in a greater production of organic acids, in that malate mainly supports the increase demand of ATP (Abadia et al. 2002; Zocchi et al. 2006; Jiménez et al., 2011). On the other hand, several studies suggest that the enhancement of the ATP content is important to support the ATP required for the nitrogenase activity (Chollet et al., 1996; Vance, 2008). In fact, our results show that since the iron deficiency decreased the quantity of fixed nitrogen in both cultivars, Flamingo maintain higher capacity to fix N_2 than Coco blanc. The performance of Flamingo was linked to the increase in PEPC enzymes and their activities in nodules of Fe deficient plant and also to the production of malate, which is the major source of energy for nodules to fix nitrogen and maintain the plant in suitable condition. In order to explain more the relationship between the parameters studied, the correlation between fixed nitrogen and PEPC activity in nodules was performed. Figure 4 shows a positive correlation between the two parameters especially in nodules subjected to iron deficiency with a correlation coefficient of R= 0.97 and 0.87 for Flamingo and Coco blanc, respectively. Besides, our previous studies showed that in response to iron deficiency, some mechanisms implied in Fe solubilisation and reduction like H^+-ATPase and FC-R activity were developed in the tolerant cultivar, Flamingo (Slatni et al., 2009, 2011). Consequently, the performance of Flamingo as compared to Coco blanc was linked to these responses. In fact, the results obtained in this work suggested that the performance of Flamingo was linked to its capacity to maintain high energy pools, in which the tolerant cultivar kept the enhanced energy from the increase of PEPC activity and malate production to support: (i) the energy necessary for the nitrogenase activity and (ii) the ATP demand for H^+ expulsion via H^+-ATPase to balance the roots in Fe availability. While the sensitive cultivar, Coco blanc, was not able to sustain the high energy necessary for the nitrogenase activity, the energy source via PEPC activity was necessary not only for the nitrogenase activity, but also to support the H^+ expulsion and Fe reduction by the cortex zone of nodules, especially in Flamingo. This suggestion supports the idea concerning the implication of nodules in iron solubilisation and uptake from the nutrient solution proposed by our previous works (Slatni et al., 2009, 2011).

ACKNOWLEDGEMENT

This work was supported by grants from the Tunisian Ministry of Higher Education, Scientific Research

(LR10CBBC02).

REFERENCES

Abadia J, López-Millán AF, Rombolà A, Abadia A (2002). Organic acids and Fe deficiency: Rev. Plant Sci. 241: 75-86.

Chollet R, Vidal J, O'Leary MH (1996). Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants, Ann. Rev. Plant Physiol. Plant Mol. Biol. 47: 273- 298.

Day DA, Copeland L (1991). Carbon metabolism and compartmentation in nitrogen fixing legumes nodules. Plant Physiol. Biochem. 29: 185-201.

Day DA, Poole PS, Tyerman SD, Rosendahl L (2001). Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell Mol. Life Sci. 58: 61-71.

De Nisi P, Zocchi G (2000). Phosphoenolpyruvate carboxylase in cucumber (*cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. J. Exp. Bot. 352: 1903-1909.

Hewitt EJ (1966). Sand and water culture methods used in the study of plant nutrition, 2nd ed. Commonwealth Bureau. Horticult. Tech. Com. pp. 22.

Jiménez S, Ollat N, Deborde C, Maucourt M, Rellán-Álvarez R, Moreno MÁ, Gogorcena Y (2011). Metabolic response in roots of Prunus rootstocks submitted to iron chlorosis. J. Plant Physiol. 168: 415-423.

Kaiser BN, Moreau S, Castelli J, Thomson R, Lambert A, Bogliolo S, Puppo A, Day DA (2003). The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. Plant J. 35: 295-304.

Parets-Soler A, Pardo JM, Serrano S (1990). Immunocytolocalization of plasma membrane H^+-ATPase. Plant Physiol. 93: 1654-1658.

Pathirana MS, Samac DA, Roeven R, Yoshloka H, Vance CP, Gantt JS (1997). Analysis of phosphoenolpyruvate carboxylase gene structure and expression in alfalfa nodules. Plant J. 12: 293-304.

Prell J, Poole P (2006). Metabolic changes of rhizobia in legume nodules. Trends in microb. 14: 161-168.

Rabotti G, De Nisi P, Zocchi G (1995). Metabolic implications in the biochemical responses to iron deficiency in cucumber roots. Plant Physiol. 107: 1195-1199.

Schulze J, Shi LF, Blumenthal J, Samac DA, Gantt JS, Vance CP (1998). Inhibition of alfalfa root nodule phospho*enol*pyruvate carboxylase through an antisense strategy impacts nitrogen fixation and plant growth. Phytochem. 49: 341-346.

Slatni T, Krouma A, Aydi S, Chaiffi C, Gouia H, Abdelly C (2008). Growth, nitrogen fixation and ammonium assimilation in common bean (*Phaseolus vulgaris* L) subjected to iron deficiency. Plant Sci. 312: 49-57.

Slatni T, Krouma A, Gouia H, Abdelly C (2009). Importance of ferric-chelate reductase activity and acidification capacity in root nodules of N_2-fixing Common bean (*Phaseolus vulgaris* L.) subjected to iron deficiency. Symb. 47: 35-42.

Slatni T, Vigani G, Salah IB, Kouas S, Dell'Orto M, Gouia H, Zocchi G, Abdelly C (2011). Metabolic changes of iron uptake in N_2-fixing common bean nodules during iron deficiency. Plant Sci. 18: 151-158.

Vance CP, Graham PH, Allan DL (2000). Biological nitrogen fixation. Phosphorus: a critical future need. In FO Pedrosa, H Hungria, MG Yates, WE Newton, (eds), Nitrogen Fixation: From Molecules to Crop Productivity. Kluwer Acad. Pub. Dordrecht, Netherlands. pp. 506-514.

Vance CP (2008). Carbon and nitrogen metabolism in legumes nodules. M.J. Dilworth et al. (eds.), Nitrogen-fixing Leguminous Symbioses, pp. 293–320.

Villalba JM, Lützelschwab M, Serrano R (1991). Immunocytolocalization of plasma membrane H+-ATPase in maize coleoptiles and enclosed leaves. Planta, 185: 458-461.

Xu W, Zhou Y, Chollet R (2003). Identification and expression of a soybean nodule-enhanced PEP-carboxylase kinase gene (NE-PpcK) that shows striking up-/down-regulation *in vivo*. Plant. J. 34: 441–452.

Zocchi G (2006). Metabolic changes in iron-stressed dicotyledonous plants. In: Barton LL, Abadıà J, editors. Iron nutrition in plants and rhizospheric microorganisms. Dordrecht: Springer. pp. 359–370.

Zocchi G, De Nisi P, Dell'Orto M, Espen L, Marino Gallina P (2007). Iron deficiency differently affects metabolic responses in soybean roots. J. Exp. Bot. 58: 993-1000.