

Influence of nitrogen fertilization on K^+ , Mg^{2+} and Ca^{2+} concentrations and on its bioindicators in roots and leaves of green bean plants

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Abstract

The pyruvate kinase (PK) and ATPase activities taking part in nitrogen (N) assimilation is essential for the growth and development of plants. Studies on the kinetics of these enzymes reveal that its activities are dependent of the cofactors K^+ , Ca^{2+} , and Mg^{2+} . Therefore, the objective of the present work was to determine the effect of different doses of N on enzymatic activities of ATPase and PK as potentials biochemical indicators of the levels of K^+ , Mg^{2+} , and Ca^{2+} in the roots and leaves of green bean plants. The N was applied to the nutrient solution as NH_4NO_3 at the following rates: 1.5, 3.0, 6.0, 12.0, 18.0, and 24.0 mM of N. These results indicate that deficient conditions of N (N1 and N2) were characterized by the lowest accumulation of K^+ , Mg^{2+} and Ca^{2+} in both total and soluble forms, and also minimum activities of PK and ATPase induced by K^+ , Mg^{2+} and Ca^{2+} , with respect to the activity of basal PK and ATPase; this could mean near optimum conditions for these cations. On the contrary, high-N treatments (N4, N5 and N6) were characterized by presenting decreasing concentrations of total and soluble K^+ , Mg^{2+} and Ca^{2+} in roots and leaves of green bean plants; however, the activities of PK and ATPase induced with K^+ , Mg^{2+} and Ca^{2+} were increased reaching their maximum activity with respect to basal PK and ATPase, both enzymes reflecting the level of cations in roots and leaves, hence being considered as good physiological bioindicators of these cations.

Additional key words: ATPase; cations; *Phaseolus vulgaris*; pyruvate kinase.

Resumen

Influencia de la fertilización nitrogenada sobre las concentraciones de K^+ , Mg^{2+} y Ca^{2+} y sus bioindicadores en raíces y hojas de plantas de judías

Las actividades piruvato kinasa (PK) y ATPasa participan en la asimilación de nitrógeno (N), la cual es esencial para el crecimiento y desarrollo de las plantas. Estudios sobre cinéticas de estas enzimas revelan que sus actividades son dependientes de los cofactores K^+ , Ca^{2+} y Mg^{2+} . Por lo tanto, el objetivo del presente trabajo fue determinar el efecto de diferentes dosis de N sobre las actividades de la ATPasa y PK como posibles bioindicadores de los niveles de K^+ ,

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Abbreviations used: ADP (adenosinediphosphate), ATP (adenosinetriphosphate), BSA (bovine serum albumin), BTP (1,3-bis-tris(hydroxymethyl)methylamino-propane), DTT (dithiothreitol), DW (dry weight), EDDHA (ethylenediamine-di(o-hydroxy-phenylacetic acid), EGTA (ethyleneglycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid), FW (fresh weight), MES (2-(N-morpholino)ethane-sulfonic acid), PEP (phosphoenolpyruvate), PK (pyruvate kinase), PMSF (phenylmethane-sulfonyl fluoride).

Mg²⁺ y Ca²⁺ en raíces y hojas de plantas de judía (*Phaseolus vulgaris* L. cv. Strike). Se aplicó N a la solución nutritiva como NH₄NO₃ en las siguientes dosis: N1 = 1,5 mM, N2 = 3,0 mM, N3 = 6,0 mM, N4 = 12,0 mM, N5 = 18,0 mM y N6 = 24,0 mM. Los resultados indican que bajo condiciones deficientes de N (N1 y N2), las plantas presentaron menor acumulación de K⁺, Mg²⁺ y Ca²⁺ en su forma total y soluble, así como mínimas actividades PK y ATPasa inducidas por K⁺, Mg²⁺ y Ca²⁺ respecto a la actividad PK y ATPasa basal; lo cual indica condiciones cercanas a las óptimas de estos cationes. Por el contrario, en los tratamientos elevados de N (N4, N5 y N6) las plantas presentaron concentraciones decrecientes de K⁺, Mg²⁺ y Ca²⁺ total y soluble tanto en raíces como en hojas; sin embargo, las actividades PK y ATPasa inducidas con K⁺, Mg²⁺ y Ca²⁺ se incrementaron alcanzando sus máximas actividades con respecto a la PK y ATPasa basal, lo que indica una mayor necesidad fisiológica de estos cationes en los tratamientos elevados de N. Finalmente, la actividad ATPasa basal y la inducida con K⁺, Mg²⁺ y Ca²⁺ se comportaron de forma similar a la actividad PK, lo que refleja el nivel de cationes en raíces y en hojas, por lo que se consideran buenos bioindicadores fisiológicos de estos cationes.

Palabras clave adicionales: ATPasa; cationes; *Phaseolus vulgaris*; piruvato kinasa.

Introduction

Beans (*Phaseolus vulgaris* L.) are grown and consumed in nearly all the world. In many developing countries, 20% of the available protein is provided by beans. Beans represent also integral part of dietary protein for 50% of the world's population (Broughton *et al.*, 2003). Beans are produced in large quantities in the American Continent and East Africa (Miklas *et al.*, 2006).

Nitrogen (N) assimilation is essential to the growth and development of plants. Because of the strong influence of N utilization efficiency on plant productivity, a vast amount of N fertilizers is poured onto fields to maximize crop yields (Nosengo, 2003). Nitrogen assimilation requires not only inorganic N but also the carbon (C) skeleton 2-oxoglutarate that is produced through sequential reactions from photoassimilated carbohydrates. The levels of C and N metabolites mutually influence each other, implying the intimate link between C and N metabolisms (Yanagisawa *et al.*, 2004).

Pyruvate kinase (PK), and thus glycolysis, is essential to the growth and development of plants (Muñoz and Ponce, 2003). Detailed studies on the interactions between C and N metabolism have revealed that the N assimilation rate in this system is critically dependent on the regulation of PK (Ambasht and Kayastha, 2002; Baud *et al.*, 2007). Work on the kinetics of this enzyme has revealed that its activation depends on the levels of phosphoenolpyruvate (PEP) and adenosinediphosphate (ADP), in addition to the presence of cations, principally Mg²⁺ (Baud *et al.*, 2007).

The elucidation of the metabolic regulation of glycolysis in plants requires a study of the key control enzymes, principally cytosolic PK (Plaxton *et al.*, 2002; Plaxton and Podesta, 2006). In addition, PK activity

may be a good physiological indicator of cation levels (K⁺, Mg²⁺, and Ca²⁺) in plant tissues (Ruiz *et al.*, 2002).

ATPase activity in roots, mainly in the plasma membrane, partly reflects ion transport. A direct relationship has been found between ATPase activity and the root concentration of cations. In addition, the activity of ATPase has reportedly been stimulated by cations, K⁺ being regarded as the most effective (Morsomme and Boutry, 2000).

Cations such as K⁺, Mg²⁺, and Ca²⁺ are important major nutrients for higher plants. Like other nutrients, these cations display individual functions as well as mutual interactions. The absorption of a given ion may be influenced by the presence in the medium of another ion or ions. In high concentrations, an ion or ions such as K⁺ in the external solution are taken up at high rates, and this may lead to excessive accumulation in the tissue. Excessive uptake of K⁺ or other ions may inhibit the uptake of the other mineral nutrients into the root and their transport to the shoot, thereby leading eventually to a deficiency in the tissue (Mäser *et al.*, 2002).

High levels of K⁺ decreased Mg²⁺ and Ca²⁺ uptake and translocation from root to shoot. Magnesium fertilization decreased leaf Ca²⁺ and K⁺ concentrations, and Ca²⁺ fertilization decreased leaf Mg²⁺ concentration (Kronzucker *et al.*, 2003; Tobe *et al.*, 2003).

In current agriculture, the main types of stress commonly generated as a consequence of the heavy use of inorganic fertilizers are related to the nutritional status of certain nutrients, chiefly N, given its excessive use (Sánchez *et al.*, 2004). An adequate supply of N is a key factor for the growth and productivity of many crops. In general, plant growth is delayed when N supply exceeds 20 mM, a value considered above the toxicity threshold for many species. Plants can tolerate nitrate excess better than ammonium excess. Ammonium

levels can be toxic to plants if they are not incorporated into C-N compounds after uptake. Ammonium can also restrict the uptake of K^+ , Ca^{2+} and Mg^{2+} through competition for uptake sites on the root (Ruan *et al.*, 2007).

Most higher plants develop severe toxicity symptoms when grown on ammonium (NH_4^+) as the sole N source (Gerendás and Ratclife, 2000; Brito and Kronzucker, 2001). Several hypotheses have been advanced to explain why NH_4^+ is toxic to plants, but none is considered satisfactory (Bruto *et al.*, 2002; Szczerba *et al.*, 2009). As NH_4^+ uptake mechanisms are coupled to H^+ extrusion into the rooting medium and H^+ release is also associated with NH_4^+ incorporation into protein, it has been repeatedly suggested that root medium acidification and/or intracellular pH disturbance may explain the observed symptoms.

The aim of the present study was to analyze the effect of different application rates of N on enzymatic activities of ATPase and PK as possible bioindicators of the levels of K^+ , Mg^{2+} , and Ca^{2+} in the roots and leaves of green bean plants (*Phaseolus vulgaris* L.).

Material and methods

Crop design and plant sampling

Seeds of *Phaseolus vulgaris* cv. Strike were sown and grown in a growth chamber under controlled environmental conditions, with relative humidity of 60–80%, temperature 30/20°C (day/night), and a photoperiod of 16/8 h under a photosynthetic photon flux density of $350 \mu\text{mol m}^{-2} \text{s}^{-1}$ (measured at the top of the plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE). Four plants were grown in 8 L pots (25 cm upper diameter, 17 cm lower diameter, 25 cm height), filled with vermiculite. For 30 days (including 8 days for germination), before the experimental treatments, the plants received a nutrient solution consisting of 6 mM NH_4NO_3 , 1.6 mM H_3PO_4 , 4.0 mM K_2SO_4 , 4 mM $CaCl_2 \cdot 2H_2O$, 1.4 mM $MgSO_4 \cdot H_2O$, 5 μM Fe-EDDHA, 2 μM $MnSO_4 \cdot H_2O$, 1 μM $ZnSO_4 \cdot 7H_2O$, 0.25 μM $CuSO_4 \cdot 5H_2O$, 0.3 μM $Na_2MoO_4 \cdot 2H_2O$, and 25 μM H_3BO_3 . The nutrient solution (pH 6.0 ± 0.1) was renewed every 3 days.

At 30 days after sowing, the different N treatments in the form of NH_4NO_3 (N1 = 1.5 mM, N2 = 3.0 mM, N3 = 6.0 mM, N4 = 12.0 mM, N5 = 18.0 mM, and N6 = 24.0 mM) were applied for 30 days (until harvest). The concentration 6.0 mM of N was considered optimal, according to Sánchez *et al.* (2004). This experimental

design was a complete randomized block with six replicates (individual pots) with 24 plants per treatment.

The plants were sampled at 60 days after sowing, at full pod development. All the root and leaf samples were taken in the mature state. The material was rinsed three times in distilled water after disinfection with non-ionic detergent at 1%, then blotted on filter paper. At each sampling, fresh root and leaf matter were used fresh for the analysis of ATPase and pyruvate kinase (PK). A subsample of leaf and root was dried in a forced air oven at 70°C for 24 h, ground in a Wiley mill and then placed in plastic bags for further analyses of the total and soluble concentrations of K^+ , Mg^{2+} , and Ca^{2+} . Dry weight (DW) was recorded and expressed as mg DW per root or per leaf. All determinations were performed in triplicate.

Plant analysis

Pyruvate kinase assay

The activity of basal PK was determined by the methods of Pulgar *et al.* (1996) and Ruiz *et al.* (1999). A total of 0.5 g of fresh samples (roots and leaves) was ground with a mortar and pestle (at 0–4°C) in 50 mM Tris-HCl buffer (pH 7.5), 50% glycerol (v/v), and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 3,000 g for 5 min at 0°C and then the supernatant was centrifuged again at 24,000 g for 15 min resulting in the enzymatic extract. To prepare the reaction mixture, 0.1 mL of the desalted extract was combined with 0.5 mL of 50 mM Tris-HCl buffer (pH 7.4), together with 0.25 mM sodium molybdate, 25 mM PEP, 5 mM ADP, 5 mM $MgCl_2$ and 0.2 mL of H_2O . The mixture was incubated at 37°C for 10 min, and the reaction was stopped by adding 0.5 mL of 2,4-dinitrophenyl-hydrazine 0.0125% (w/v) to 2 M HCl and 0.5 mL 2 M NaOH to avoid possible absorbance changes from altering the pH of the reaction mixture. After centrifugation for 5 min at 3,000 g, the absorbance at 510 nm was measured and compared against a standard curve of pyruvate. Triplicate assays were performed for each extract. To ascertain whether pyruvate was formed exclusively by PK, the activity of acid phosphate was determined by the method of Besford (1979). Enzymatic activity was expressed as μM of pyruvate formed per gram of fresh weight (FW) per hour.

Assay of PK in the presence of K^+ , Ca^{2+} , and Mg^{2+} followed the same method as for basal PK, with the

difference of adding to the reaction mixture either 0.1 mL of K^+ in the form of KCl (50 mM), 0.1 mL of Ca^{2+} in the form of $CaCl_2$ (50 mM), or 0.1 mL of Mg^{2+} in the form of $MgSO_4$ (50 mM). The processes of incubation, centrifugation, and activity measurement were the same as described for basal PK. The soluble proteins from the supernatants or crude enzyme extracts were determined by Bradford's method (1976), with bovine serum albumin (BSA) as the standard.

Preparation of microsomal fraction

Microsomal fractions were prepared according to the method of Ferrol *et al.* (1993). Briefly, 2 g of roots were homogenized with mortar and pestle and then with a glass homogenizer in 5 mL g^{-1} FW of grinding medium containing 25 mM 1,3-bis-tris(hydroxymethyl) methyl-amino-propane-2-(N-morpholino) ethane-sulfonic acid (BTP-MES) (pH 7.6), 250 mM sucrose, 2 mM dithiothreitol (DTT), 2 mM $MgSO_4$, 2 mM ATP, 10% glycerol (v/v), 2 mM ethyleneglycol-bis(β -aminoethyl ether)-N, N,N',N'-tetraacetic acid (EGTA), 1 mM phenylmethane-sulfonyl fluoride (PMSF) and 0.5% albumin from bovine serum (BSA) (w/v). The homogenate was filtered and centrifuged for 10 min at 13,000 g. The supernatant was centrifuged for 35 min at 80,000 g and then the pellet (crude microsomes) was resuspended in a medium containing 2 mM BTP-MES (pH 7), 250 mM sucrose, 1 mM DTT, 10% glycerol (v/v) and 0.2% BSA (w/v) to obtain the microsomal fraction. All these procedures were carried out at 0 to 4°C.

Plasma membrane ATPase assays

The basal plasma membrane ATPase defined as vanadate-sensitive, molybdate-insensitive, nitrate-insensitive and azide-insensitive Mg-ATP hydrolysis was performed as described by Gibrat *et al.* (1989). Enzymatic activity was assayed for 30 min at 30°C in a basal reaction medium containing 3 mM ATP-BTP (pH 6.5), 2 mM BTP-HCl (pH 6.5), 0.1 mM sodium molybdate, 0.25 mM sodium orthovanadate, 100 mM KNO_3 and 1 mM $NaNO_3$ in a final volume of 1 mL. The amount of membrane protein routinely used was 10-15 μ g per assay.

The corresponding inhibitor-sensitive ATPase activities were calculated as differences of activities mea-

sured in the presence and in the absence of the corresponding inhibitor. The ATPase activity was determined as the release of inorganic phosphate (Pi), this being quantified by the formation of the phosphomolybdic colored complex. Protein was determined by the method of Bradford (1976). Triplicate assays were performed for each extract.

Assay of plasma membrane ATPase in the presence of K^+ , Mg^{2+} , and Ca^{2+} followed the same method as for basal ATPase, with the difference of adding to the reaction mixture either 0.1 mL of K^+ in the form of KCl (50 mM), 0.1 mL of Mg^{2+} in the form of $MgSO_4$ (50 mM), and 0.1 mL of Ca^{2+} in the form of $CaCl_2$ (50 mM). The processes of measuring, incubation, centrifugation, and activity were the same as described for basal ATPase.

Cations determination

Dry matter (roots and leaves) were digested with 96% H_2SO_4 in the presence of hydrogen peroxide (H_2O_2). Total and soluble potassium (K^+) were determined according to the flame photometer method (Lachica *et al.*, 1973), whereas total and soluble calcium (Ca^{2+}) and magnesium (Mg^{2+}) were analyzed by atomic absorption spectrophotometry (Ruiz *et al.*, 2002). All cations were expressed as mg g^{-1} of DW.

Statistical analysis

Data were analyzed using ANOVA (SAS, 1987). When F tests were significant, differences between treatment means were compared using LSD at the 0.05 probability level. Also, correlation analyses were made between the different variables. Levels of significance were represented by * at $p < 0.05$, ** at $p < 0.01$, *** $p < 0.001$ and NS: not significant. Data shown are mean values.

Results and discussion

Like N, K^+ is an essential nutrient for plant growth and development (Mäser *et al.*, 2002). In our experiment, the application of different doses of N significantly favored the concentration of both total and soluble forms of K in roots and leaves ($p \leq 0.05$; Table 1), presenting the higher levels of total and soluble K in

Table 1. Effect of N treatments (N1: 1.5 mM, N2: 3.0 mM, N3: 6.0 mM, N4: 12.0 mM, N5: 18.0 mM, and N6: 24.0 mM of N) on the total and soluble of K^+ , Mg^{2+} , and Ca^{2+} concentrations ($mg\ g^{-1}$ DW) in the roots and leaves of green bean plants. Data are means ($n = 6$). Treatments with different letters are significantly different, ANOVA ($p < 0.05$) LSD_{0.05}

Treatment	Total			Soluble		
	K^+	Ca^{2+}	Mg^{2+}	K^+	Ca^{2+}	Mg^{2+}
<i>Roots</i>						
N1	18.58 ^d	15.05 ^{cd}	5.56 ^{cd}	2.03 ^d	5.12 ^{cd}	1.15 ^{cd}
N2	20.63 ^{cd}	16.75 ^c	6.56 ^c	3.92 ^{cd}	5.76 ^c	1.96 ^c
N3	31.19 ^a	20.44 ^{ab}	8.72 ^{bc}	11.13 ^a	9.25 ^{ab}	2.13 ^{bc}
N4	27.64 ^b	21.36 ^a	11.98 ^{ab}	8.03 ^b	10.43 ^a	5.24 ^{ab}
N5	25.80 ^{bc}	19.58 ^b	12.74 ^a	6.68 ^{bc}	8.50 ^b	6.31 ^a
N6	22.88 ^c	18.30 ^{bc}	10.25 ^b	5.71 ^c	7.49 ^{bc}	3.67 ^b
<i>Leaves</i>						
N1	20.41 ^d	18.46 ^{bc}	6.72 ^{cd}	5.43 ^d	11.46 ^{bc}	2.70 ^{cd}
N2	23.02 ^{cd}	19.51 ^b	7.02 ^c	7.27 ^{cd}	12.06 ^b	3.17 ^c
N3	33.67 ^a	21.29 ^{ab}	9.29 ^{bc}	15.09 ^a	14.98 ^{ab}	4.71 ^{bc}
N4	29.32 ^b	22.46 ^a	14.50 ^{ab}	13.40 ^b	15.47 ^a	6.37 ^{ab}
N5	27.50 ^{bc}	20.74 ^{ab}	16.04 ^a	11.40 ^{bc}	13.62 ^{ab}	8.10 ^a
N6	25.88 ^c	20.35 ^{ab}	11.33 ^b	9.79 ^c	13.29 ^{ab}	5.82 ^b

treatment N3, with increments of 40% and 82% in roots, and 39% y 64% in leaves, respectively, in relation to the lower levels found in treatment N1.

Ammonium levels can be toxic to plants if ammonium is not incorporated into C-N compounds after uptake. Ammonium can also restrict K^+ uptake because of competition for uptake sites in the root (Siddiqi *et al.*, 2002). In our study, it was observed that as N was increased to level N3, the uptake, transport and accumulation of both total and soluble K in roots and leaves also increased (Table 1). However, at high N doses (N4, N5 and N6), the accumulation of total and soluble K in both organs diminished, due probably to the existence of elevated levels of NH_4^+ in the nutrient solution applied in treatments N4, N5 and N6; this cation competes with K^+ for sites of absorption, causing a decrease in the K^+ levels in the two organs. Our results are in agreement with those found by Mäser *et al.* (2002), who mentioned that elevated application of NH_4^+ tends to depress K^+ concentration due to: (1) interference with K^+ diffusion from clay layers in soil, and (2) competition with K^+ uptake.

Several hypotheses have been advanced to explain why NH_4^+ is toxic to plants, but none is considered satisfactory (Brito *et al.*, 2002; Ruan *et al.*, 2007; Szczerba *et al.*, 2009). As NH_4^+ uptake mechanisms are coupled to H^+ extrusion into the rooting medium and H^+ release is also associated with NH_4^+ incorporation into protein, it has been repeatedly suggested that root medium

acidification and/or intracellular pH disturbance may explain the observed symptoms (Gerendás and Ratcliffe, 2000).

On other aspect, the levels of total and soluble K were higher in leaves than in roots, because this cation shows a high mobility, which allows K^+ efficient transport from roots to leaves.

With regard to the Ca^{2+} cation, in this study the application of different N doses significantly enhanced the concentration of total and soluble Ca^{2+} ($p \leq 0.05$; Table 1), showing maximum (Ca^{2+}) levels in treatment N4, with 30% and 50% increments in roots, as well as 18% and 26% in leaves, respectively, when compared to the minimum levels attained in treatment N1.

The uptake of Ca^{2+} is influenced by the source of N; for example the application of N- NO_3^- is generally associated to an increment in the absorption and transport of several ions, especially Ca^{2+} and K^+ . However, when the N source is NH_4^+ , Ca^{2+} uptake is affected (Mengel and Kirkby, 2001).

In this research, it was observed that as N dose increased, levels of total and soluble Ca^{2+} in roots and leaves raised up to level N4 (Table 1) and then drastically diminished; this probably was due to the antagonistic effects that high levels of NH_4^+ have on the uptake and transport of Ca^{2+} . It is also noted that levels of total and soluble Ca^{2+} are very similar in roots and leaves, probably due to the low mobility of this cation in the plant.

For magnesium, in our experiment it was observed that the application of different N levels followed a trend alike that of K^+ ($p \leq 0.05$; Table 1). Magnesium had its higher concentrations under treatment N5, with increments of 56% and 82% in roots, and 58% and 66% in leaves, respectively, with regard to the lower concentrations registered in N1 treatment.

Similarly to both K^+ and Ca^{2+} , Mg^{2+} concentration is also affected by N source, especially by NH_4^+ (Mengel and Kirkby, 2001; Mäser *et al.*, 2002). In our work, we observed that elevated doses of N (N5 and N6) drastically diminished total and soluble Mg in roots and leaves (Table 1), which indicates that probably the elevated levels of NH_4^+ in the nutrient solution compete for uptake sites with Mg^{2+} during absorption. Noteworthy, Mg^{2+} levels in leaves were higher than those in roots, a fact probably explained by magnesium mobility which facilitates its transport from roots to leaves (Shaul, 2002).

In general, N-deficient treatments (N1 and N2) were characterized by the smallest accumulation of K^+ , Ca^{2+} and Mg^{2+} in roots and leaves, which was reflected in lower biomass production by green bean plants. Meanwhile, the N dose which was considered optimal (N3, along with treatment N4), showed the highest accumulation of these cations, as well as the best biomass production ($p \leq 0.05$; Fig. 1). On the other side, elevated N doses (N5 and N6) were characterized by a drastic decline in K^+ , Ca^{2+} and Mg^{2+} accumulation, and a lower green bean biomass (Fig. 1). The latter is probably explained by the high levels of NH_4^+ in the nutrient solution in treatments N5 and N6; this toxicity by NH_4^+

can affect, in decreasing order, the uptake and translocation of the cations K^+ , Ca^{2+} and Mg^{2+} , which is in agreement with Gerendás and Ratclife (2000) who mentions that NH_4^+ toxicity can induce deficiencies by antagonistic action with other nutrients, especially cations like K^+ , Ca^{2+} and Mg^{2+} .

One of the enzymes utilized as a bioindicator of the nutritional status of the cations K^+ , Ca^{2+} and Mg^{2+} is the PK activity (Ruiz *et al.*, 2002). This enzyme catalyzes the synthesis of pyruvate and ATP from PEP and ADP. The role of this enzyme is essential, since it is the first control step of glycolysis, a determinant process in plant growth and development (Muñoz and Ponce, 2003). Studies on the kinetics of this enzyme reveal that its activity depends on the levels of PEP, ADP, and the presence of cations like K^+ and Mg^{2+} (Baud *et al.*, 2007).

As it was earlier mentioned, one of the objectives of this study was to determine whether PK activity reflects the concentration of cations in different analyzed organs, and it is influenced by N treatments. In our experiment, the activities of PK basal and those induced with K^+ (PK + K), Ca^{2+} (PK + Ca) and Mg^{2+} (PK + Mg) augmented as N increased; maximum enzyme activities were registered on treatment N6, in roots as well leaves, with increments greater than 16% for the activity of basal PK, 29% for PK + K, 30% in PK + Ca and 33% for the activity of PK + Mg, in relation to the minimum activities found in treatment N1 ($p \leq 0.01$; Table 2). Therefore, and taking into account the dependence of PK activity on cations, this enzyme has been utilized as a bioindicator of the levels of these cations (K^+ , Mg^{2+}

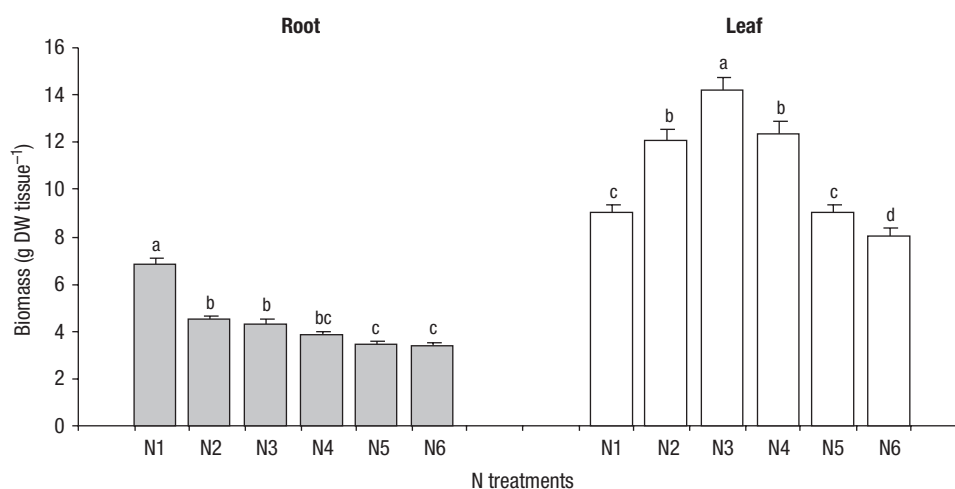


Figure 1. Root and foliar biomass in green bean plants in response to N treatment. Data are means ($n = 6$). Treatments with different letters are significantly different, ANOVA ($p < 0.05$) LSD_{0.05}.

Table 2. Effect of N treatments on basal PK activity and on the PK activity in the presence of 50 mM K⁺ (PK + K⁺), 50 mM Mg²⁺ (PK + Mg²⁺) and 50 mM Ca²⁺ (PK + Ca²⁺) in the roots and leaves of green bean plants. Data are means (n = 6). Treatments with different letters are significantly different, ANOVA ($p < 0.05$) LSD_{0.05}

Treatment	PK activity (nmol piruvate mg ⁻¹ protein s ⁻¹)			
	Basal PK	PK + K ⁺	PK + Ca ²⁺	PK + Mg ²⁺
<i>Roots</i>				
N1	19.51 ^c	18.04 (−7.53%) ^{cd}	18.50 (−5.17%) ^c	19.08 (−2.20%) ^{bc}
N2	20.15 ^b	19.25 (−4.46%) ^c	19.56 (−2.92%) ^{bc}	19.90 (−1.24%) ^{bc}
N3	21.50 ^b	21.75 (+1.16%) ^{bc}	22.16 (+3.06%) ^b	22.45 (+4.41%) ^b
N4	23.06 ^{ab}	26.35 (+14.26%) ^b	24.11 (+4.55%) ^b	24.25 (+5.16%) ^{ab}
N5	24.91 ^{ab}	28.68 (+15.13%) ^{ab}	26.35 (+5.78%) ^{ab}	26.44 (+6.14%) ^a
N6	25.73 ^a	30.70 (+19.31%) ^a	28.77 (+11.81%) ^a	27.76 (+7.88%) ^a
<i>Leaves</i>				
N1	16.16 ^b	15.25 (−5.63%) ^c	15.54 (−3.83%) ^c	15.77 (−2.41%) ^c
N2	16.76 ^b	15.96 (−4.77%) ^c	16.30 (−2.74%) ^c	16.45 (−1.84%) ^c
N3	17.82 ^{ab}	18.04 (+1.23%) ^b	18.35 (+2.97%) ^b	18.62 (+4.48%) ^b
N4	18.25 ^{ab}	20.32 (+11.34%) ^{ab}	19.46 (+6.63%) ^{ab}	19.87 (+8.87%) ^{ab}
N5	18.86 ^a	21.96 (+16.43%) ^{ab}	20.36 (+7.95%) ^{ab}	20.88 (+10.71%) ^{ab}
N6	19.20 ^a	23.45 (+22.13%) ^a	22.15 (+15.36%) ^a	22.76 (+13.33%) ^a

and Ca²⁺) in different organs studied (Ruiz *et al.*, 2002). In this research, a positive and significant relationship was found between the content of K⁺, Ca²⁺ and Mg²⁺, and the enzymatic activities of PK basal in both organs (*roots*: K total-PK basal, $r = 0.90^{**}$; Ca total-PK basal, $r = 0.81^{**}$; Mg total-PK basal, $r = 0.80^{**}$; K soluble-PK basal, $r = 0.92^{**}$; Ca soluble-PK basal, $r = 0.85^{**}$; Mg soluble-PK basal, $r = 0.84^{**}$; *leaves*: K total-PK basal, $r = 0.92^{**}$; Ca total-PK basal, $r = 0.86^{**}$; Mg total-PK basal, $r = 0.84^{**}$; K soluble-PK basal, $r = 0.94^{***}$; Ca soluble-PK basal, $r = 0.90^{**}$; Mg soluble-PK basal, $r = 0.87^{**}$). Similar results have been reported by other authors that have worked with different vegetal species and organs (Ruiz *et al.*, 1999, 2000).

Several studies suggest that PK activity could be a good physiological indicator of K⁺, Mg²⁺ and Ca²⁺ content in vegetal tissue (Ruiz *et al.*, 2000, 2002). In this study, Table 2 shows the PK activities induced with K⁺, Ca²⁺ and Mg²⁺, and also the increase and decrease of these activities with regard to the activity of basal PK. The PK activity was induced with K⁺ in treatment N6 in both organs, reaching its maximum activity and the largest increment (19% in roots and 22% in leaves), with regard to the basal PK activity in the same treatment, possibly reflecting K⁺ deficiency levels in N6 due to the elevated concentrations of NH₄⁺ in the nutrient solution and the plant (Ruiz *et al.*, 2002). The opposite trend occurred in roots and leaves of treatment

N1, possibly due to the high levels of K⁺, a condition that could inhibit PK activity due to the direct action of K⁺ on this enzyme or due to the antagonism between NH₄⁺ and K⁺. On the other side, on both organs under treatment N3, the activity of PK induced with K⁺ showed minimal change (about 1% in roots and leaves) with respect to the basal activity, indicating appropriate conditions of this cation (Ruiz *et al.*, 2002).

The behavior of PK stimulated by Ca²⁺ (Table 2) was similar to the PK activity induced by K⁺ in both organs ($p \leq 0.05$; Table 2), being treatment N6 which achieved the highest activity, and also the greatest increase (12% in roots and 15% in leaves) when compared to the basal PK activity in the same treatment; this could reflect levels of Ca²⁺ deficiency in N6 due mainly to: (1) the elevated levels of NH₄⁺ that provoked a diminution in Ca²⁺ uptake, (2) strong competition between Ca²⁺ and the cations K⁺ and Mg²⁺, being the presence of any of them essential for PK activity; (3) a rise in the formation of complexes among Ca²⁺ and the substrates of PK (Ca-PEP y Ca-ADP), which could diminish the affinity of Ca with the enzyme and therefore its activity (Plaxton *et al.*, 2002; Plaxton and Podesta, 2006); and (4) finally, a decrease in the N metabolism in this treatment (Ruiz *et al.*, 1999). On the contrary, in treatment N1, there was a decrease on the activity of the PK activity induced by Ca²⁺ (−5% in roots and −4% in leaves) with respect to the values of basal PK, suggesting that the foliar

concentration of Ca^{2+} in the two organs was adequate. Ruiz *et al.* (2000), working with Ca^{2+} applications in tobacco plants, found that an increase on the application of Ca^{2+} decreased K^+ and Mg^{2+} content in leaves, and diminished the activity of PK, hence suggesting the activity of this enzyme as a bioindicator of the content of Ca^{2+} , Mg^{2+} and K^+ .

With regard to PK activity induced by Mg^{2+} , in this study, this enzyme presented a behavior similar to that of PK induced by K^+ and Ca^{2+} ($p \leq 0.05$; Table 2). The only notorious characteristic was that the increases or decreases of the Mg-induced activity of PK in roots and leaves were less intense than those registered for K^+ and Ca^{2+} . This effect could be attributed firstly to the more negative effect of NH_4^+ over K^+ and Ca^{2+} and on minor degree on Mg^{2+} ; and secondly, to the fact that the kinetics of PK activation depends on the presence of cations, especially Mg^{2+} (Plaxton *et al.*, 2002; Plaxton and Podesta, 2006).

From the above, according to the results of the activity of PK induced with K^+ , Ca^{2+} and Mg^{2+} , there is a physiological need for these cations, especially under the treatments of high-N (N4, N5 and N6), due to the antagonistic effects that the high levels of NH_4^+ exert over the uptake of K^+ , Ca^{2+} and Mg^{2+} .

In relation to the root plasmatic ATPase, it plays a central role in plant physiology. The ATPase activity in the roots acts principally in the plasma membrane, favouring ion transport. A direct relationship has been found between ATPase activity and the concentration of cations like K^+ , Ca^{2+} and Mg^{2+} in the root. Besides, ATPase activity is induced by the presence of cations, being K^+ the most effective one (Gévaudant *et al.*, 2007). In our experience, the application of the different N doses was accompanied by a statistically significant increase in the activities of basal ATPase (ATPase basal) and those induced with K^+ (ATPase+K), Ca^{2+}

(ATPase + Ca) and Mg^{2+} (ATPase + Mg) in roots ($p \leq 0.05$; Table 3), attaining maximum activity in treatment N6, with 12%, 27%, 24% and 21% respectively, in relation to N1 which yielded the minimum activity ($p \leq 0.05$; Table 3). The ATPase activity followed a similar trend to that of root PK, which indicates the level of cations in roots; therefore, these enzymes can be considered good physiological indicators of these cations, and this is in agreement with studies by Ruiz *et al.* (1999, 2002).

Ruiz *et al.* (2002) suggest that the activity of plasma ATPase could be a good physiological indicator of the contents of K^+ , Mg^{2+} , and Ca^{2+} in plant tissue. In our study, the application of different N doses favored ATPase activity in presence of K^+ , presenting the lowest activities in treatment N1 ($p \leq 0.05$; Table 3), coinciding with the low concentrations of total and soluble K in roots (Table 3). Furthermore, independently of the N treatment applied, the activity of ATPase in the presence of K^+ was greater than the activities of basal ATPase (Table 3). The ATPase activity is induced mainly by monovalent cations, being K^+ the most effective, mainly because this cation increases the affinity of ATPase for ATP (Morsomme and Boutry, 2000). Nonetheless, Ruiz *et al.* (2002) found that K^+ stimulates the ATPase activity under limiting or inadequate nutrient conditions, particularly in the case of K^+ . These results could explain the stimulation of the activity of ATPase in the presence of K^+ with respect to the basal activity on all N treatments and especially at high-N doses (N4, N5 and N6). Likewise, our results could indicate an inadequate nutrient status of K^+ in the roots of green bean plants grown under the nutrient conditions of the present study.

With regard to ATPase activity in the presence of calcium, the maximum activity of ATPase was noticed in plants were elevated N doses (N6) were applied,

Table 3. Effect of N treatments on basal ATPase activity and on the ATPase activity in the presence of 50 mM K^+ (ATPase + K^+), 50 mM Ca^{2+} (ATPase + Ca^{2+}) and 50 mM Mg^{2+} (ATPase + Mg^{2+}) in the roots of green bean plants. Data are means ($n = 6$). Treatments with different letters are significantly different, ANOVA ($p < 0.05$) LSD_{0.05}

Treatment	ATPase activity ($\mu\text{mol Pi mg}^{-1} \text{protein h}^{-1}$)			
	Basal ATPase	ATPase + K^+	ATPase + Ca^{2+}	ATPase + Mg^{2+}
N1	32.68 ^b	31.04 (−5.01%) ^b	31.55 (−3.45%) ^b	31.87 (−2.47%) ^c
N2	33.61 ^b	32.69 (−2.73%) ^b	32.89 (−2.14%) ^b	33.25 (−1.07%) ^b
N3	34.83 ^{ab}	35.25 (+1.20%) ^{ab}	35.77 (+2.69%) ^{ab}	36.11 (+3.67%) ^{ab}
N4	35.15 ^{ab}	37.72 (+7.31%) ^{ab}	36.35 (+3.41%) ^{ab}	36.86 (+4.86%) ^{ab}
N5	36.45 ^a	39.80 (+9.19%) ^a	38.45 (+5.48%) ^a	38.78 (+6.39%) ^a
N6	37.16 ^a	42.78 (+15.12%) ^a	41.36 (+11.30%) ^a	40.54 (+9.09%) ^a

showing increments of 24% respect to N1. Another aspect is that ATPase activity was significantly increased in the presence of Ca^{2+} in relation to basal ATPase ($p \leq 0.05$; Table 3). These results are in accordance with Ruiz *et al.* (2002), who registered that ATPase activity is induced in minor degree by Ca^{2+} than for K^+ , with some cases registering an inhibition of enzymatic activity. However, our results could be explained by the inadequate nutrient status of Ca^{2+} in the roots of the green bean plants of our experiment.

In relation to the ATPase activity induced by Mg^{2+} , it presented a tendency similar to that of ATPase induced by K^+ and Ca^{2+} ($p \leq 0.05$; Table 3). The only remarkable aspect was that the increases or decreases of ATPase induced with Mg^{2+} in roots were less intense than those registered for K^+ and Ca^{2+} . This effect could be caused in first place, by the fact that high NH_4^+ levels have a more negative effect on the concentration of K^+ and Ca^{2+} , and with less degree on Mg^{2+} ; and in second place, to the activation kinetics of ATPase, which depends on the presence of cations, in special Mg^{2+} (Plaxton *et al.*, 2002; Plaxton and Podesta, 2006).

The cation content in the root is predetermined by ion transport through the plasma membrane and this requires the participation of ATPase (Gévaudant *et al.*, 2007). It has been demonstrated that basal ATPase in the roots indicates ion transport, and in some cases shows a direct relationship between the activity of this enzyme and the concentration of cations in roots (Ruiz *et al.*, 2002). In our experience, a significant and positive correlation was found between the basal ATPase activity and the contents of K^+ , Ca^{2+} and Mg^{2+} in roots (K total-ATPase basal, $r = 0.92^{**}$; Ca total-ATPase basal, $r = 0.90^{**}$; Mg total-ATPase basal, $r = 0.84^{**}$; K soluble-ATPase basal, $r = 0.94^{**}$; Ca soluble-ATPase basal, $r = 0.92^{**}$; Mg soluble-ATPase basal, $r = 0.87^{**}$).

Conclusions

Results proved that green bean plants under N-deficient conditions (N1 and N2) were characterized by the lowest accumulation of total and soluble K^+ , Mg^{2+} and Ca^{2+} , and also the minimum activities of PK and ATPase induced by K^+ , Mg^{2+} and Ca^{2+} with respect to the activity of PK and basal ATPase, which indicates near-optimum conditions of these cations. On the opposite, high-N (N4, N5 and N6) treated green bean plants had decreasing concentrations of total and soluble K^+ , Mg^{2+} and Ca^{2+} , both in roots and leaves; however,

the activities of PK and ATPase induced with K^+ , Mg^{2+} and Ca^{2+} were increased reaching their maximum activities with regard to PK and basal ATPase. This indicates a physiological need for these cations in the high-N (N4, N5 and N6) treated plants. Finally, the activity of basal ATPase and induced with K^+ , Mg^{2+} and Ca^{2+} followed a trend similar to that of the activity of PK, both enzymes mirroring the concentration of cations in roots and leaves, therefore being considered good physiological indicators of these cations.

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