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Biosynthesis of proline in fruits of green bean plants: deficiency *versus* toxicity of nitrogen

(With 2 Tables & 1 Figure)

Biosíntesis de prolina en frutos de plantas de frijol: deficiencia versus toxicidad de nitrógeno

(Con 2 Tablas y 1 Figura)

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Abstract. The objective of this work was to determine the effect of deficiency *versus* toxicity of N on biosynthesis of proline in fruits of green bean plants (*Phaseolus vulgaris* L. cv. Strike). Nitrogen was applied to the nutritive solution in the form of NH_4NO_3 at 1.5 mM (N1), 3.0 mM (N2), 6.0 mM (N3, optimal level), 12.0 mM (N4), 18.0 mM (N5), and 24.0 mM (N6). Nitrogen deficiency (N1 and N2) was characterized by having lower proline accumulation in pods and seeds, mainly because proline degradation was stimulated by the enzyme proline dehydrogenase. On the other hand, N toxicity (N4, N5, and N6) was characterized for accumulation of greater amounts of proline in pods and seeds due primarily to the greater activity of the enzyme ornitine-δ-aminotransferase. These results suggest a predominance of the ornithine over the glutamine pathway. Under our experimental conditions, proline can be defined as a good bioindicator of N excess in green bean

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plants. The accumulation of proline in both organs (pods and seeds) is also considered a good bioindicator of N toxicity in the study plant species.

Key words: *Phaseolus vulgaris* L., proline metabolism, green bean, nitrogen, deficiency, toxicity.

Resumen. El objetivo de este trabajo fue determinar el efecto de la deficiencia *versus* la toxicidad de N sobre la biosíntesis de prolina en frutos de plantas de frijol (*Phaseolus vulgaris* L. cv. Strike). El N se aplicó a la solución nutritiva como NO_3NH_4 a 1,5 mM (N1); 3,0 mM (N2); 6,0 mM (N3, nivel óptimo); 12,0 mM (N4); 18,0 mM (N5), y 24,0 mM (N6). La deficiencia de N (N1 y N2) se caracterizó por tener una menor acumulación de prolina en vainas y semillas, principalmente debido a que la degradación de la prolina fue estimulada por la enzima prolina deshidrogenasa. Por otro lado, la toxicidad de N (N4, N5, y N6) manifestada por la acumulación de mayores cantidades de prolina en vainas y semillas, fue debida principalmente a la mayor actividad de la enzima ornitina-δ-aminotransferasa. Estos resultados sugieren una predominancia de la vía de la ornitina más que la vía de la glutamina. Bajo nuestras condiciones experimentales, la prolina puede ser definida como un buen indicador biológico del exceso de N en plantas de frijol. La acumulación de prolina en ambos órganos (vainas y semillas) se considera como un indicador biológico de la toxicidad de N en plantas de la especie estudiada.

Palabras clave: *Phaseolus vulgaris* L., metabolismo de la prolina, frijol, nitrógeno, deficiencia, toxicidad.

INTRODUCTION

Beans 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 an integral part of dietary protein for 50 % of the world's population (Deshpande et al., 1984). Beans are produced in large quantities in the American Continent and East Africa (Singh, 1999).

Different roles have been proposed for proline accumulation as an adaptive response; it has been suggested that proline may function as an osmoticum, a sink of energy and reducing power, a nitrogen-storage compound, a hydroxy-radical scavenger, and a compatible solute that protects enzymes. It may also play a role in the regulation of cellular redox potentials (Saradhi & Saradhi, 1991).

In plants, proline is synthesized from either glutamate or ornithine. It has been demonstrated that the glutamate pathway is predominant under conditions of osmotic stress (Delauney et al., 1993). The main step of proline biosynthesis from glutamate is catalysed by a single bifunctional enzyme, Δ^1-pyrroline-5-carboxylate synthetase (P5CS), which produces γ-glutamyl kinase (γ-GK) and glutamic acid-5 semialdehyde (GSA) dehydrogenase (or γ-glutamyl phosphate reductase). The GSA produced by these reactions is spontaneously converted into pyrroline-5-carboxylate (P5C), which is then reduced by P5C reductase (P5CR) to proline (Zhang et al., 1995).

Plants also synthesize proline from ornithine, through ornitine-δ-aminotransferase (OAT). If the α-amino group of ornithine is transaminated, the product is α–keto-δ-amino-valerate, which cyclizes to Δ^1-pyrroline-2-carboxylate (P2C) and is then reduced to proline. Alternatively, trans-amination of the δ-amino group yields GSA, which is converted to proline via P5C (Delauney & Verma, 1993).

On the other hand, the metabolism and accumulation of proline also depends on its degradation, which is catalysed primarily by the action of the mitochondrial enzyme, proline dehydrogenase (PDH) (Hare et al., 1999).

In present-day agriculture, the main types of stress commonly resulting from the heavy use of inorganic fertilizers are related to the nutritional status of certain nutrients, primarily nitrogen (N), given its extensive use (Ruiz & Romero, 1998, 1999). Rabe (1990) reviewed the influence of numerous kinds of abiotic and biotic stresses on the composition of N-containing compounds in plants. The amino compounds most often accumulated in different organs of the plant as a function of stress include glutamine, asparagine, arginine, citruline, ornithine and mainly proline. In general, although only scant literature is available on the subject, it appears that the relationship between N availability and proline accumulation is usually positive (Andersen et al., 1995). Nevertheless, little information is available on proline metabolism and accumulation in fruits, or on the possible physiological functions of this compound. Therefore, the objective of the present work was to determine the effects of N deficiency and toxicity on proline biosynthesis in fruits of green bean plants (*Phaseolus vulgaris* L. cv. Strike).

MATERIALS 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. Relative humidity, temperature and photoperiod were 60-80 %, 30/20 ºC (day/night), and 16/8 h (day/night) respectively. Photosynthetic photon flux density was 350 µmol/m^2/s , measured at the plant tops with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE. Four plants were grown in 8-liter pots (25 cm uppest diameter, 17 cm lowest diameter, 25 cm height) filled with vermiculite. During 30 days, before the experimental treatments, plants received a nutritive solution of 5.4 mM NH_4NO_3; 1.6 mM K_2HPO_4; 0.3 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 $CuS0_4 \cdot 5H_2O$; 0.3 µM $Na_2MoO_4 \cdot 2H_2O$, and 0.5 µM H_3BO_3. The nutritive solution (pH 6.0-6.1) was renewed every 3 days.

Thirty days after sowing, the different N treatments in the form of NH_4NO_3 were applied for 30 days (until harvest): 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. The optimal N dose for *Phaseolus vulgaris* under the cultivation conditions of our experiment was the N3 treatment (Carbonell-Barrachina et al., 1997). A complete randomized block experimental design was used with 6 replicates (individual pots) of 24 plants per treatment.

Sampling and plant analysis. Plants were sampled at 60 days after sowing, at full pod development and maturity. Seeds and pods were sorted out for analysis. Plant material was rinsed three times in distilled water after disinfecting with non-ionic detergent at 1% (Wolf, 1982) and then blotted on filter paper. A subsample of pods and seeds were used fresh for the analysis of P5CS, OAT, PDH, NO_3^-, NH_4^+, and proline. Triplicate assays were made for each extraction.

Statistical analysis. Data were analyzed using ANOVA. 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 $p < 0.01$, *** at $p < 0.001$, and NS: not significant. Data shown are mean values ± SE.

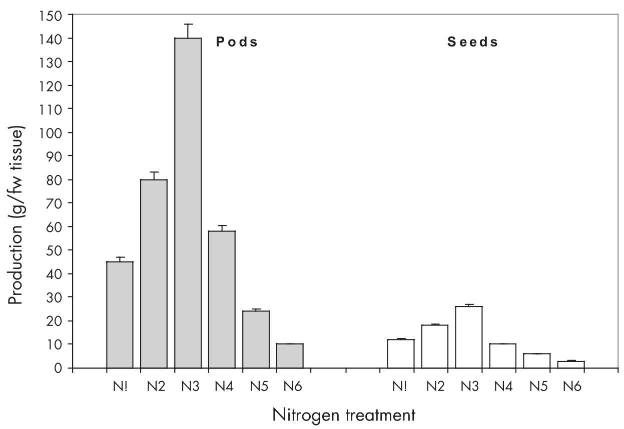
RESULTS AND DISCUSSION

Adequate N levels are essential for growth and productivity in most crops. Effectiveness of the N treatments in our experiment is reflected in the production of pods ($p < 0.001$) and seeds ($p < 0.001$), which diminished sharply as the N dose increased (Fig. 1). That is, N6 presented markedly less pod and seed production in relation to N3. These results indicate that N3 stimulated growth (optimal) in our experiment, in agreement with the results of Carbonell-Barrachina et al. (1997). On the other hand, application of the N6 treatment resulted in N toxicity in our experimental plants.

Proline accumulates in plants under drought and salinity stresses in a number of species, and it is thought to play an important role in plant cells for adaptation to water stress (Delauney & Verma, 1993). Two metabolic pathways, glutamate and ornithine metabolism, are key to proline formation. In plants, proline is synthetisized from glutamate via Δ^1-pyrroline-5-carboxilate (P5C) by two successive reductions, with are catalysed by P5C synthetase

Fig. 1. Pod and seed production in green bean plants in response to N application (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). Data are means ± s.e. (n = 6).

Fig. 1. *Producción de vainas y semillas en plantas de frijol en respuesta a la aplicación de N (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 de N). Los datos son promedio ± e.e. (n = 6).*



(P5CS) and P5C reductase (P5CR); nevertheless, the P5CS enzyme constitutes the limiting step under this proline-synthesis pathway in plants (Kavi Kishor et al., 1995). Regarding the metabolic pathway of glutamate for proline biosynthesis, the N dose significantly affected the behaviour of the pod and seed activity of P5CS ($p < 0.01$; Table 1), with the lowest and highest activities shown in the N6 and N1 treatments, respectively.

With reference to the metabolic pathway of ornithine, the activity of OAT (which transforms ornithine and α-ketoglutarate to GSA and glutamate, the latter transforming into proline) was also significantly influenced by the N treatments. In our experiment, the application of the highest N dose boosted the activities of pod and seed OAT ($p < 0.001$; Table 1), presenting the highest and lowest activities at N6 and N1, respectively.

The highest concentrations of proline on pods and seeds appeared under treatment N6 ($p < 0.001$; Table 1). Our results reveal an inverse relationship between enzymatic activity of P5CS and proline concentration, both in the pods ($r = -0.80^{**}$) and the seeds ($r = -0.75^{**}$). Both organs showed a directly proportional relationship between OAT activity and the proline concentration (pods, $r = 0.81^{**}$; seeds, $r = 0.97^{***}$).

The other important factor that controls proline levels in plants is degradation. L-proline is oxidized to P5C in plant mitochondria by PDH. This oxidation is inhibited during proline accumulation under water stress, and is activated in rewater-stressed plants (Rayapati & Stewart, 1991). Proline degradation produces glutamate, which is utilized as a N source for the synthesis of other amino acids. In our experiment, the PDH pod and seed activities ($p < 0.01$; Table 1) diminished as N dose increased, presenting minimum activities at N6, with respect to the highest activity at N1. As indicated above, concentrations of pod and seed proline (Table 1) were highest at N6; this can be explained by the inverse relationship between proline and PDH activity both in the pods ($r = - 0.75^{**}$) and in the seeds ($r = - 0.81^{**}$).

Our results for proline metabolism in pods and seeds of *Phaseolus vulgaris* at the highest N rate could be explained by the toxicity effects of this nutrient. As indicated in several works, N toxicity reduces root growth,

Table 1. Response of proline metabolism in pods and seeds of green bean plants subjected to different 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). Data are means ± s.e. (n=6).

Tabla 1. *Respuesta del metabolismo de la prolina en vainas y semillas de plantas de frijol expuestas a diferentes tratamientos de N (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 de N). Los datos son el promedio ± e.e. (n=6).*

Treatment	P5CS	OAT	Proline	PDH
		Pods		
N1	0.282 ± 0.03	2145.4 ± 75.3	167.4 ± 9.7	184.2 ± 9.8
N2	0.246 ± 0.02	2321.6 ± 91.2	189.6 ± 10.2	155.4 ± 9.2
N3	0.230 ± 0.01	2610.9 ± 113.2	238.8 ± 12.8	136.3 ± 8.1
N4	0.214 ± 0.01	2820.8 ± 108.3	328.6 ± 10.7	124.2 ± 7.9
N5	0.194 ± 0.01	3627.7 ± 183.6	422.7 ± 13.1	117.2 ± 3.1
N6	0.188 ± 0.01	3824.8 ± 128.5	485.9 ± 15.4	105.9 ± 3.8
		Seeds		
N1	0.240 ± 0.03	1243.2 ± 38.7	065.3 ± 4.3	138.1 ± 8.2
N2	0.225 ± 0.02	1361.3 ± 46.1	091.2 ± 6.4	125.7 ± 6.5
N3	0.216 ± 0.01	1430.1 ± 75.3	102.0 ± 8.5	94.40 ± 4.9
N4	0.203 ± 0.02	2158.4 ± 56.4	108.0 ± 3.1	73.20 ± 3.6
N5	0.191 ± 0.01	2944.1 ± 45.5	192.1 ± 4.1	68.80 ± 5.7
N6	0.178 ± 0.01	3040.4 ± 61.1	290.5 ± 7.3	69.10 ± 7.1

Pyrroline-5-carboxylate synthetase (P5CS) expressed in μM Pi/mg protein/min;
Ornitine-δ-aminotransferase (OAT) expressed in nmol NADH oxidade/mg protein/min;
Proline dehydrogenase (PDH) expressed in nmol NAD reduced/mg protein/min;
Proline expressed in mg/g fresh weight.

Pyrrolina-5-carboxylate sintetasa (P5CS) expresada en μM Pi/mg proteina/min;
Ornitine-δ-aminotransferasa (OAT) expresada en nmol NADH oxidasa/mg proteina/min;
Prolina dehydrogenasa (PDH) expresada en nmol NAD reducido/mg proteina/min;
Prolina expresada en mg/g de peso fresco.

disrupts vascular tissues and depresses water uptake (Benton Jones, 1997); this latter symptom is similar to that caused by drought and salinity (Delauney & Verma, 1993). Normally, plants respond to water stress by activating proline biosynthesis; this result is similar to that found in our experiment.

Metabolic responses of proline under N toxicity can be seen primarily in the seeds, where proline accumulation was higher than that found in the pods. This was because PDH activity proved to be strongly inhibited by N toxicity in the seeds. These results define proline accumulation as a bioindicator of N toxicity in the seeds of green bean plants.

Other factors which can determine the regulation processes of proline synthesis in plants are the availability and concentration of inorganic N forms (NO_3^- and NH_4^+) (Delauney et al., 1993). In higher plants, the purpose of proline accumulation is yet to be completely elucidated. In addition of acting as an osmolite, proline accumulation has other important cell functions. Proline can act as a N source in the cell under stress conditions; the accumulation of this nitrogenous compound could be utilized as a form of stored N (Dandekar & Uratsu, 1988). In our experiment, the application of high quantities of N drastically increased NO_3^- and NH_4^+ contents in pods and seeds ($p < 0.001$; Table 2). Our results indicate a positive and significant relationship in pods and seeds between NO_3^- and NH_4^+ levels and proline contents (pods: NO_3^- proline, r = 0.83** and NH_4^+-proline, r = 0.70**; seeds: NO_3^--proline, r = 0.94*** and NH_4^+-proline, r = 0.90***, respectively).

Table 2. Accumulation of NO_3^- and NH_4^+ in pods and seeds of green bean plants subjected to different 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). Data are means ± s.e. (n=6).

Tabla 2. *Acumulación de NO_3^- y NH_4^+ en vainas y semillas de plantas de frijol expuestas a diferentes tratamientos de N (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). Los datos son el promedio ± e.e.(n=6).*

	Pods		**Seeds**	
Treatment	**NO_3^-**	**NH_4^+**	**NO_3^-**	**NH_4^+**
N1	24.1 ± 2.1	12.4 ± 1.1	33.1 ± 2.8	22.6 ± 2.2
N2	32.2 ± 3.4	18.3 ± 1.6	44.2 ± 3.7	30.1 ± 2.6
N3	48.5 ± 3.7	24.2 ± 2.2	64.3 ± 4.5	38.5 ± 2.9
N4	74.4 ± 4.9	34.6 ± 2.3	96.8 ± 7.8	46.9 ± 3.6
N5	81.5 ± 5.6	38.3 ± 3.2	108.4 ± 8.5	50.2 ± 4.2
N6	92.6 ± 6.7	44.5 ± 4.1	114.5 ± 9.6	55.3 ± 4.8

Nitrates (NO_3^-) and Ammonium (NH_4^+) expressed in mg/g fresh weight.

Nitratos (NO_3^-) y amonio (NH_4^+) expresados en mg/g de peso fresco.

Nitrogen deficiency (N1 and N2) was characterized by having lower proline accumulation in pods and seeds, mainly because proline degradation was stimulated by the enzyme proline dehydrogenase. On the other hand, N toxicity (N4, N5, and N6) was characterized for accumulation of greater amounts of proline in pods and seeds due primarily to the greater activity of the enzyme ornitine-δ-aminotransferase. These results suggest a predominance of ornithine over the glutamine pathway. Finally, under our experimental conditions, proline accumulation can be defined as a good bioindicator of N excess and toxicity in green bean plants.

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