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Asparagine and Glutamine affect the Growth and Cause Metabolic Changes in *Phaseolus vulgaris in Vivo*

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ABSTRACT

The objective of this study was to investigate the effects of amide nitrogen compounds, mainly L-asparagine or L-glutamine (0-5 mM) on growth, pigment content and metabolism of intact *Phaseolus vulgaris* plants *in vivo*, at two stages (seedling and vegetative) of plant growth and development. All growth parameters – specifically chl *a* and *b* and carotenoid contents, total carbohydrates and its fractions – were increased by 1 and 2 mM asparagine or glutamine but decreased in response to other concentrations (3, 4 and 5 mM) and to the control (i.e. untreated plants), during both stages of development. Treatment with any concentration of asparagine or glutamine to plants grown *in vivo* generally induced a marked increase in amide nitrogen, total nitrogen and protein content and a decrease in ammonia, peptide and total soluble nitrogen during both stages. Ion content (K^+, Na^+, Ca^{2+} and Mg^{2+}) increased significantly when treated with 1 mM asparagine or glutamine and decreased markedly when other concentrations were used. Both asparagine and glutamine treatments increased and decreased growth promoter (auxins, gibberellins and cytokinins) level at low and high concentrations, respectively, but a reverse trend was observed for abscisic acid. The activity of several enzymes (asparagine synthetase, glutamine synthetase, nitrate reductase and protease) decreased when asparagine or glutamine concentrations were increased during both stages of *P. vulgaris* development.

Keywords: amide, enzyme, French bean, hormone, ion

INTRODUCTION

Nitrogen is an essential element for growth and development and it is an indispensable element incorporated in most important structural and functional macromolecules, such as proteins and for biological molecules such as amino acids, nucleotides, proteins and DNA (Redinbaugh and Campbell 1991; Crawford 1995; Suarez *et al*. 2003). Kingston-Smith *et al*. (2006) found that plants of white clover (*Trifolium repens*) could be grown from seed when supplied with a nutrient solution containing 2.5, 5.0, 7.5 or 10 mM nitrate. Protein, free amino acid and protease activity were determined in leaves. They found that, regardless of nitrate supply, 50% of the protein was degraded in 6 h and 80% after 24 h. As the extent of protein decrease was determined by initial protein content, more protein degradation occurred in those plants grown with the highest nitrate supply.

The importance of nitrogen in plant biology extends far beyond its role as a nutrient. It is now clear that several different nitrogen compounds and some products of their assimilation exert strong regulatory effects on both plant metabolic and developmental pathways (Forde and Clarkson 1999; Stitt 1999; Zhang and Forde 2000; Coruzzi and Bush 2001; Coruzzi and Zhou 2001; Tabatabaei *et al*. 2008). Nitrogen assimilation is a vital process controlling plant growth and development. Inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, asparagine and aspartate, which serve as important nitrogen carriers in plants (Lam *et al*. 1996). Nitrogen-fixing plants can be classified as amide exporters or ureide exporters based on the xylem fluid collected from excised nodules or nodulated root systems (Green *et al.* 1990; Ireland 1990). The amide exporters transport asparagine, glutamine or 4-methylene glutamine while ureide exporters transport either allantoin or citrulline. Asparagine and glutamine appear to be the most common nitrogen-transport compounds and are particularly prevalent in the xylem sap of root NO_3 assimilators, plants assimilating soil NH_4^{2+} and most temperate nitrogen-fixing species (Bollard 1960; Lea and Miflin 1980; Pate 1980).

There is now clear evidence that soluble asparagine accumulates in most if not all plant organs during periods of low rates of protein synthesis and a plentiful supply of reduced nitrogen. The accumulation of asparagine occurs during normal physiological processes such as seed germination and nitrogen transport. However, in addition, stress-induced asparagine accumulation can be caused by mineral deficiencies, drought, salt, toxic metals and pathogen attack. The properties and gene regulation of the enzymes involved in asparagine synthesis (AS) and breakdown in plants are discussed in detail by Lea *et al*. (2007).

Sivasankar and Oaks (1996) reported that the amide asparagine is important both as a protein amino acid and as a major nitrogenous transport and storage compound. They also discovered that in legumes it is known to be synthesized in high levels in cotyledons of germinating seedlings, roots and nitrogen-fixing nodules. Asparagine is also synthesized by leaves, and its synthesis is more active in mature leaves than in growing leaves; in contrast the utilization of asparagine is more active in the growing leaves than in mature leaves (Yoneyama 1984). El-Saht (1994) found an increase in all growth parameters (root length, shoot length, fresh weight, dry weight and water content) of both *Phaseolus vulgaris* and *Vicia faba* seedlings grown on 1 and 3 mM asparagine. On the other hand, Geisler (1985) noted that the leaf area, root surface area and total dry matter of two-weeks-old seedlings of spring barley, field bean and maize were reduced by increasing nitrogen concentration in the soil. He also found that the shoot and root dry matter were related to the root surface. Zhang *et al*. (1999) stated that ammonia and glutamine can serve as alternative nitrogen sources for *Arabidopsis*, although, at high concentrations (≥ 1 mM), they can inhibit growth. Moreover, Schubert (1983) showed that asparagine could act as an amide group donor similar in role to glutamine. de Pinheiro Henriques and

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Original Research Paper

Marcelis (2000) found that there was an increase in leaf dry matter percentage of lettuce at low nitrogen supply, and at high nitrogen supply dry matter was closely related to plant nitrogen concentration. Menéndez *et al*. (2002) enriched water with distinct forms of nitrogen to study the effect of dissolved nutrients on growth, nutrient content and uptake rates in *Chaetomorpha linum*. They observed that nitrogen enrichment was followed by an increase in chlorophyll (chl) content after 4 days of treatment and that this was followed by an increase in biomass after 10 days. Furthermore, Nakano *et al*. (1998) studied the effect of different nitrogen concentrations on chl content of rice and *P. vulgaris* and observed that nitrogen treatment – particularly at lower concentrations – led to a decrease in chl content in rice but not in *P. vulgaris*. Martin *et al*. (2002) detected a significant reduction in *Arabidopsis* seedling chl when treated with low levels of nitrogen (0.1 mM). On the other hand, Tremblay *et al*. (1999) stated that when *P. vulgaris* and sweet corn were grown at different nitrogen concentrations, the chl contents of both were not correlated to nitrogen application. Wheat and maize, when grown for 21 days in nutrient solution containing 10 mM asparagine, plastid pigment (chl *a* and *b* and carotenoids) content was higher than untreated plants (Stancheva and Dinev 1995). Sagi *et al*. (1998) reported an increase in cations in *Lolium multiflorum* following the addition of nitrogen at 0.5, 4.5 or 9.0 mM to the growth medium. Moreover, Kubik-Dobosz and Buczek (1999) observed that *Pisum sativum* plants supplied with 1 mM glutamine or asparagine took up ammonium and potassium at a rate lower than that of control plants. They also found that the efflux of NH_4^+ and K^+ from root to ambient solution was enhanced under these treatments. Mercier and Kerbauy (1998) studied the effect of two nitrogen sources, glutamine and ammonium nitrate, on the auxins and cytokinin levels of 3 bromeliad species with different growth habits and found, in general, that the highest IAA level was obtained with NH_4NO_3. However, in *Tillandsia pohliana* the highest IAA content and the largest amounts of total cytokinin were obtained in plants cultivated with glutamine. For *Pitcairnia flammea* and *Vriesea philippocoburgii*, the largest amounts of total cytokinins were found when the bromeliads were cultivated with glutamine.

AS is the primary enzyme involved in the production of asparagine in plants. It catalyzes a reaction where asparagine is biosynthesized from aspartic acid using an ATP-dependent amide group with either glutamine or ammonia as the nitrogen source (Romagni *et al*. 2000). Glutamine synthetase (GS) is necessary for the biosynthesis of nucleic acids, proteins, complex polysaccharides, and various coenzymes. In this respect, Zhang *et al*. (1998) noted that in rice cv. 'IR72' leaf GS activity was greater than root GS activity, regardless of nitrogen application. Root and leaf GS generally declined as plants aged, and the decline was greater in root than leaves. Ogawa *et al*. (1999) proved that after the addition of glutamine to nitrate-containing medium, nitrate reductase (NR) activity in cultured spinach cv. 'Hoyo' cells was repressed. Lu and Peng (2001) showed that in forage rice NR and GS activities decreased as the grain matured. However, proteinase activity increased during the late stage of grain maturity.

Here we study the effects of some different amide compounds (L-asparagine and L-glutamine) at five levels (1, 2, 3, 4 and 5 mM) on the growth of French bean (*Phaseolus vulgaris* cv. 'Contendor') during two developmental stages. The effect on certain metabolic activities such as pigments, carbohydrates, and nitrogen, protein, ion as well as plant growth regulators (PGRs) (auxins (IAA), gibberellic acid (GA_3), cytokinin (cyt) and abscisic acid (ABA)) contents were assessed. In addition, the activities of certain related enzymes (AS, GS, NR) and protease) were determined during these phases of development.

MATERIALS AND METHODS

Time course experiment

A homogenously-sized lot of *Phaseolus vulgaris* (French or common bean) seeds were selected. The seeds were surface sterilized by soaking in 0.01% $HgCl_2$ solution for about 3 min, then washed thoroughly with continuously flowing tap water for about 1 h. After this, 25 seeds were allowed to germinate in plastic dishes (length: 30 cm; width: 20 cm; height: 12 cm), covered with Whatman filter paper No. 1 and watered with equal amounts of Hoagland's nutrient solution (Arnon and Hoagland 1940). The nutrient solution used was ¼-strength of Pfeffer (1900) nutrient mixture of macroelements. Micronutrients were supplied to the nutrient solution at concentrations used by Arnon and Hoagland (1940). All chemicals used were of the purest grade available commercially (El-Gomhouria company for chemicals, Cairo, Egypt). The pH value of this nutrient solution was 5.7 ± 0.3.

The dishes were incubated in the dark at 25 ± 1°C to allow seeds to germinate. After 48 h six uniform seedlings (the length of the radical was about 2 cm; leaves had not yet differentiated) were placed in black-painted beakers (600 ml) containing ¼-strength Hoagland's nutrient solution either alone or supplemented with asparagine or glutamine at 1, 2, 3, 4 or 5 mM; **Plate 1A-D**). The beakers were placed in a growth chamber adjusted at optimum growth conditions: temperature: 28 ± 2°C; light intensity: 3000-5000 lux; relative humidity: 60-70%; continuous aeration from an air pump at a rate of 2 L/h/beaker according to Steing Rover (1983).

Throughout the experimental period, various growth parameters and metabolic activities (pigments content, carbohydrate fractions, nitrogen fractions, protein and ion content), in addition to levels of PGRs (total auxins, GA_3, total cytokinin and ABA) and activities of certain enzymes (asparagine synthetase, glutamine synthetase, nitrate reductase and protease), were measured after 6 and 15 days from sowing, which represent the seedling and vegetative stages, respectively.

Data from the different groups of seedlings were statistically analyzed and comparison among means was carried out using Statgraphic Ver. 4.2, Display (one-tailed ANOVA), as described by Snedecor and Cochran 1980).

Estimation of photosynthetic pigments

The protocol to measure the plant photosynthetic pigments (chls *a* and *b* and carotenoids), which were determined at both stages of plant development, is based on the method of Arnon (1949) for chls and that of Horvath *et al*. (1972) for carotenoids as adopted by Kissimon (1999).

Estimation of carbohydrates

Total soluble sugars and sucrose were extracted and determined using modifications of the procedures of Yemm and Willis (1954) and Handel (1968), respectively. The method used for estimation of polysaccharides was that of Thayermanavan and Sadasivam (1984).

Estimation of nitrogenous constituents

The method used in this study was essentially that adopted by Yemm and Willis (1956). Ammonia-N was estimated spectrophotometrically by the method adopted by Delory (1949) using Nessler's reagent as modified by Naguib (1964). The method used for estimation of amide-N was that recommended by Naguib (1964). Estimation of peptide nitrogen was according to Kwon *et al*. (2000). Total soluble nitrogen was determined by the conventional semi-micromodification of Kjeldahl method (Pirie 1955). Total nitrogen was determined by the conventional semi-micromodification of the Kjeldahl method of Chinbal *et al*. (1943).

Estimation of protein

The method of protein extraction adopted was that of Scarponi and Perucci (1986). Protein content was determined spectrophotomet-

Plate 1 Effect of different asparagine (**A, B**) or glutamine (**C, D**) concentrations on growth of *Phaseolus vulgaris* plant at the seedling (**A, C**) or vegetative (**B, D**) stage.

rically by using a double beam recording spectrophotometer according to the Bradford (1976) method.

Determination of K^+, Na^+, Mg^{++} and Ca^{++} ions

Flame spectrophotometry was used to determine K and Na, while Ca and Mg were measured by atomic absorption spectrophotometry according to the method described by Chapman and Pratt (1978).

Determination of enzyme activities

Determination of AS activity

The method used in the present study was essentially that of Ravel (1970) where the enzyme is most easily measured by substituting hydroxylamine for ammonia then the amount of aspartyl hydroxamic acid formed is determined colorimetrically with ferric chloride reagent.

Determination of GS activity

The method used in this investigation is as described by Sadasivam and Manicham (1992). Glutamine synthetase catalyses the γ-glutamyl transfer reaction. Hence, it can be assayed by measuring the production of γ-glutamyl hydroxamate. γ-Glutamyl hydroxamate reacts with ferric chloride to produce a brown colour in acidic medium. When the activity is measured in the presence of Mn^{++}, it represents total glutamine synthetase activity (adenylated and unadenylated forms). The biologically active unadenylated form may be measured by inhibiting the adenylated form by the addition of 60 mM Mg^{++}.

Determination of NR activity

The method of Hageman and Reed (1980) was employed.

Determination of protease activity

The method of Colowick *et al*. (1951) was used. The assay of protease activity is essentially that of Basha and Beevers (1975) and Salmia *et al*. (1978).

Extraction, separation and bioassay of growth bioregulators

Extraction and separation were performed according to Shindy and Smith (1975) and as adopted by Haroun (1985).

Auxins (IAA) were bioassayed by using the straight-growth test of *Hordeum vulgare* cv. 'Giza 118' coleoptile sections (Foda and Radwan 1962).

Gibberellins (GAs) were bioassayed by the growth of *Lactuca sativa* (cv. 'Roumine') hypocotyls, which can be used to bioassay a number of GAs and GA-like substances (Frankland and Wareing 1960; Crozier *et al*. 1970).

Cytokinin was bioassayed by assessing the growth of the cotyledon tissue of *Xanthium brasilicum* seeds, which expresses a rapid cytokinin response which can be obtained in solutions of very small volumes (Esashi and Leopold 1969).

ABA was bioassayed by using *Triticum aestivum* L. grains, which were germinated in the dark for 70 h at 25°C, according to the procedure used by Wright (1956).

RESULTS AND DISCUSSION

Plant organs contain numerous compounds, which generally correspond to minor quantities. Therefore, they often classified among "secondary plant products" (Mothes 1980; Ali 2000). Arora (1982) stated that a significant fraction of these secondary products consists of non-protein nitrogenous compounds. Many of them have been reviewed such as the case of purine and pyrimidine derivatives, non-protein or uncommon amino acids, amines, amides and alkaloids (Finar 1986; Lea 1993).

Schröder *et al*. (2005) stated that pyrimidines are particularly important in dividing tissues as building blocks for nucleic acids, but they are equally important for many biochemical processes, including sucrose and cell wall polysaccharide metabolism.

N availability in the root zone is crucial in determining productivity under intensive plantation culture, given ade-

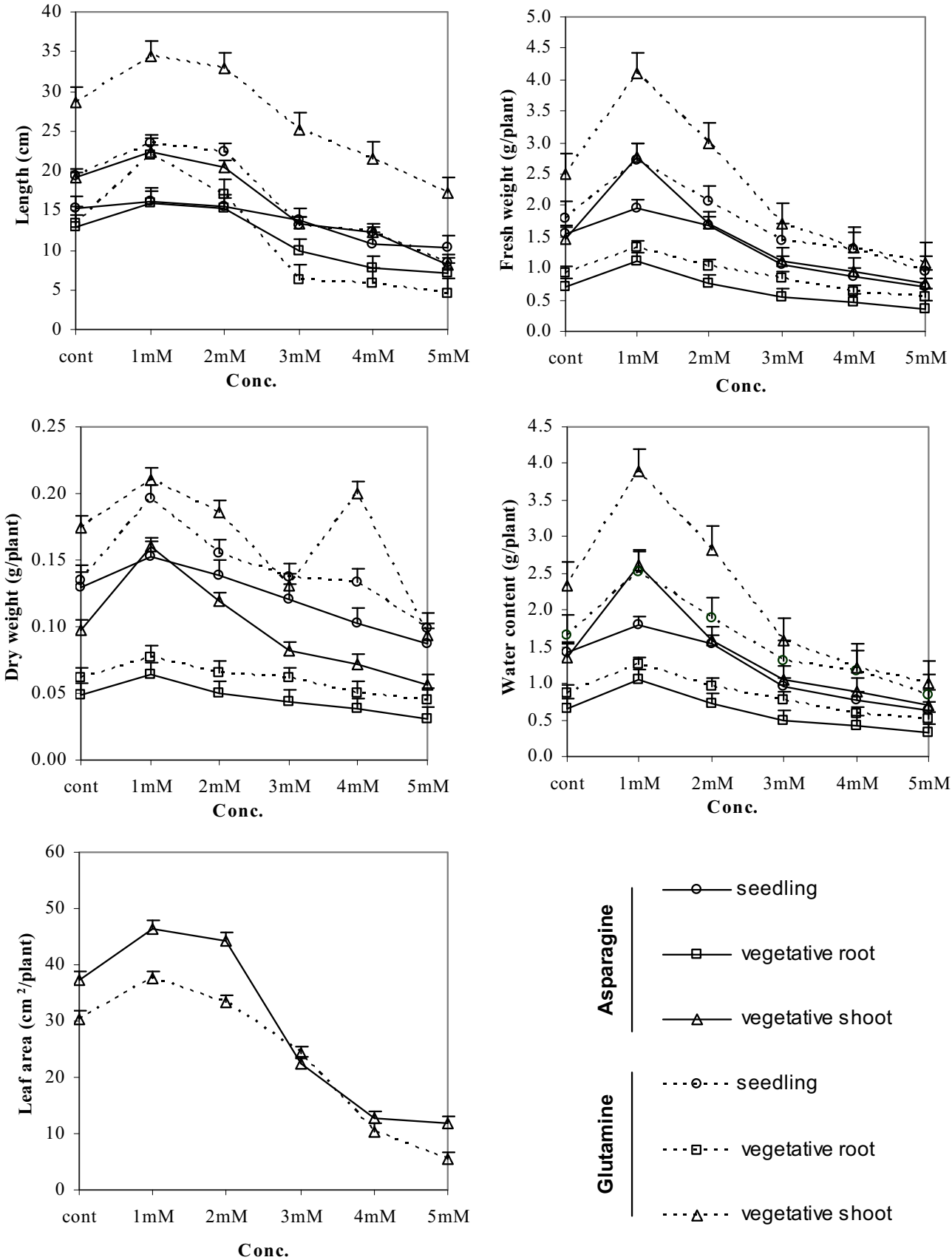


Fig. 1 Effect of increasing concentrations of asparagine or glutamine on five (plant/seedling length, fresh weight) growth parameters of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = the value of LSD at 0.05.

quate soil moisture. Responses of growing plants to N fertilization indicate increases in leaf area and plant biomass (Heilman and Eu-Guvang 1993), shoot/root ratios (Pregitzer *et al*. 1990), light-dependent photosynthetic capacity (van Hove *et al*. 1989), leaf nitrogen and Chl content (Mulligan 1989). Whereas biomass accumulation and photosynthetic capacity can be promoted under high N concentrations, high N plants may also be more susceptible to environmental conditions (Mazzoleni and Dickmann 1988). Physiological responses to N availability may also be modified by environmental conditions (Liu and Dickmann 1993) since high plant N levels enhance the rate of physiological processes but the promotive effect of N is dependent on soil-water status, plant species, culture conditions and duration of the ex-

periment (Liu and Dickmann 1992). Controlled environmental studies can be used to define physiological interactions among soil resources and ultimately provide initial guidelines for management trails in test plantations (Liu and Dickmann 1996; Ali 2000).

As stated in the introduction, the main goal of the present investigation was to assess the possible effects of the two different amide-N compounds (asparagine and glutamine) on growth, the content of pigment, carbohydrate, N, protein, ion, and PGRs and the activity of some related en-

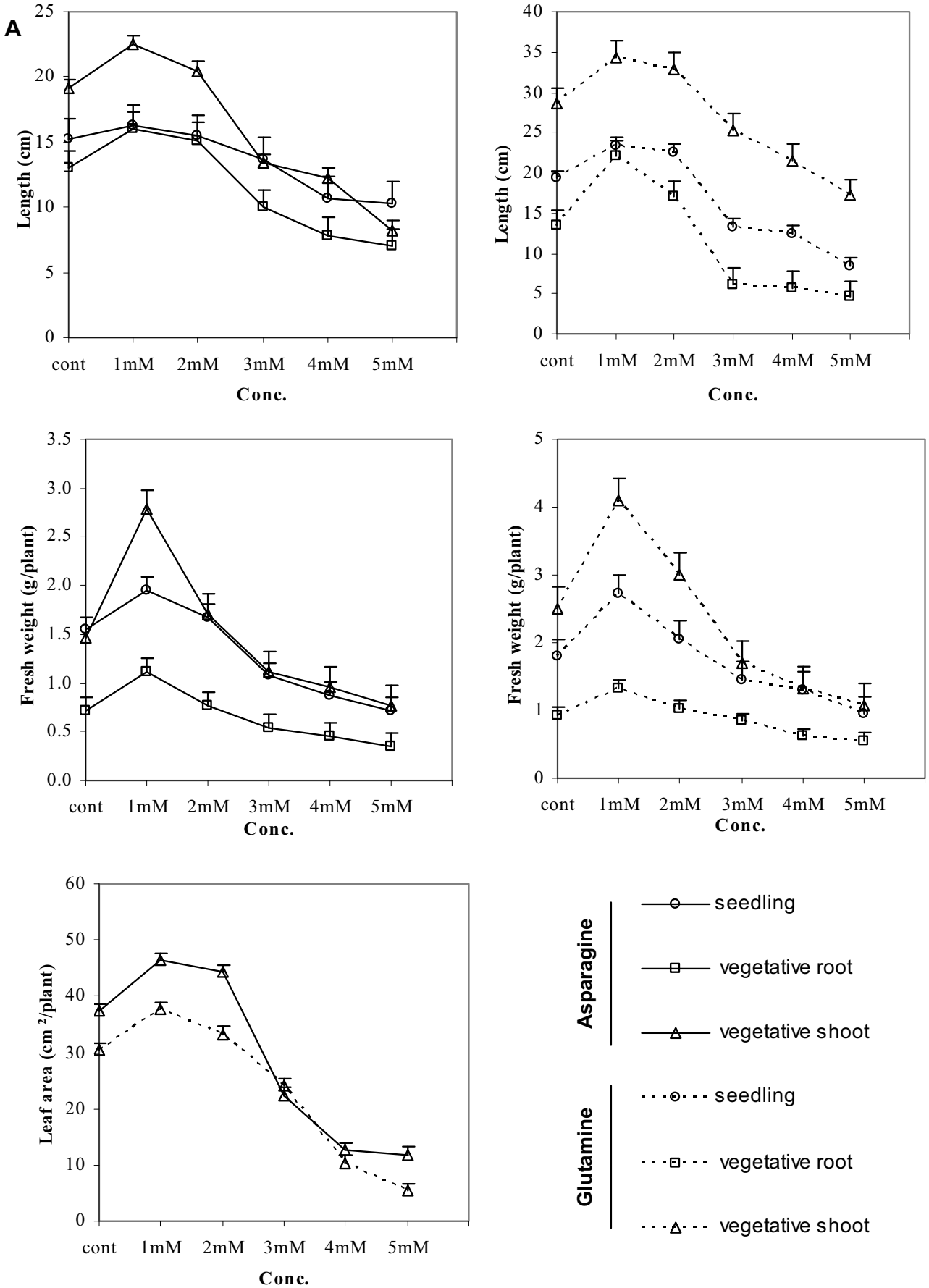
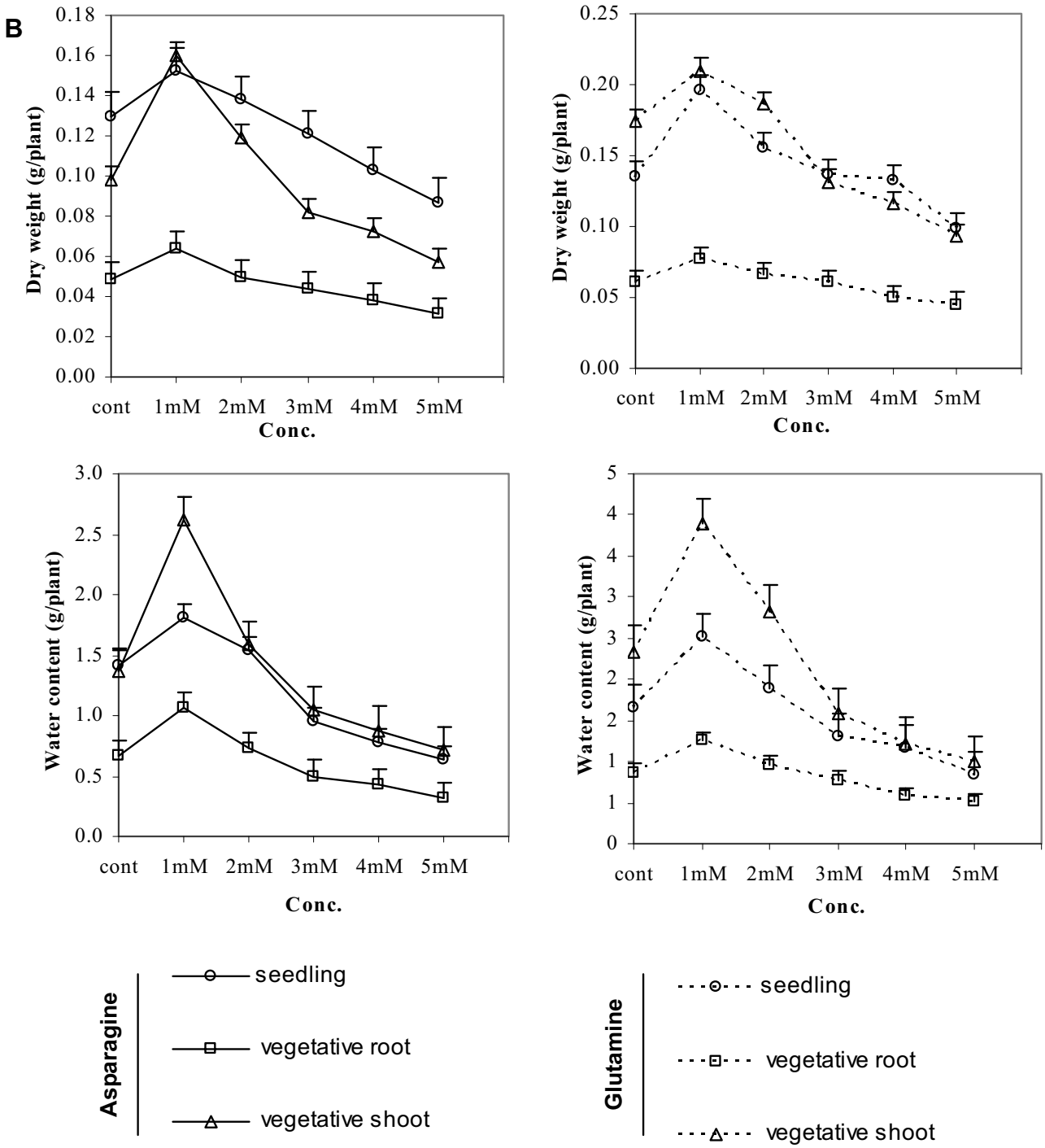


Fig. 2 Effect of increasing concentrations of asparagine or glutamine on five growth parameters (A = plant/seedling length, fresh weight, leaf area; B = dry weight, water content) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.



zymes to amide metabolism such as AS, GS, NR and protease in the intact French bean seedlings (*in vivo*).

The analysis reported in this study is indicative of the extent to which growth parameters and different metabolite contents as well as enzyme activities were perturbed by the different amide treatments.

Changes in growth parameters

Different growth parameters of intact *Phaseolus* plant (shoot length, root length, fresh and dry weights, water content and leaf area) and bean (vegetative length, fresh and dry weights and water content) showed a general significant increase by 1 mM asparagine or glutamine (**Figs. 1, 2A, 2B**). This increase may thus be directly related to the increased flux of amide to the leaf and/or to the subsequent reduction processes involved, which were described by Sprent and Thomas (1984) and Sutherland *et al.* (1985).

Proietti and Tombesi (1996) indicated that asparagine and glutamine stimulated growth of olive (*Olea europaea* L.) and they suggested that asparagine and glutamine may be involved in the inductive process as messengers in relation to the assimilate reserves in the plant. Lee and Rudge (1986) indicated that after about 6 days, young barely (*Hordeum vulgare* L.) plants depend on an external supply of N sustaining their maximum rate of growth. Moreover Keller *et al*. (2001) found that vine (*Vitis vinifera* L.) leaf area increased in response to N application at 100 kg/ha.

On the other hand, treatment with 3, 4 and 5 mM of asparagine or glutamine significantly decreased the above mentioned parameters (**Fig. 2A, 2B**). Geisler (1985) also stated that two weeks-old seedlings of maize (*Zea mays* L.), spring barely (*Hordeum vulgare* L.) and field beans (*Vicia faba* L.) which were grown under different N concentrations (10, 20, 30 µM) ammonia nitrogen showed a general reduction in leaf area, and root surface area with increasing N concentration (30 µM). de Pinheiro Henriques and Marcelis (2000) stated that the effect of N (10 µM NO_3^-) on growth was mediated by its effect on leaf area development and hence on light interception.

The negative effect of amide on root and shoot fresh weight might be due to the fact that amide decreases water

uptake and relative water content as suggested by Vassilev *et al*. (1997) using young barely plants.

The importance of leaf area in controlling plant dry matter and growth rates has long been appreciated (Aldesuquy 2000).

The increase and decrease of different growth parameters (**Fig. 2A, 2B**) in response to asparagine or glutamine treatments may be mediated by a change in the level of naturally synthesized hormones (**Fig. 8A, 8B**). Groot *et al*. (2003) found that relative growth rate increased sharply

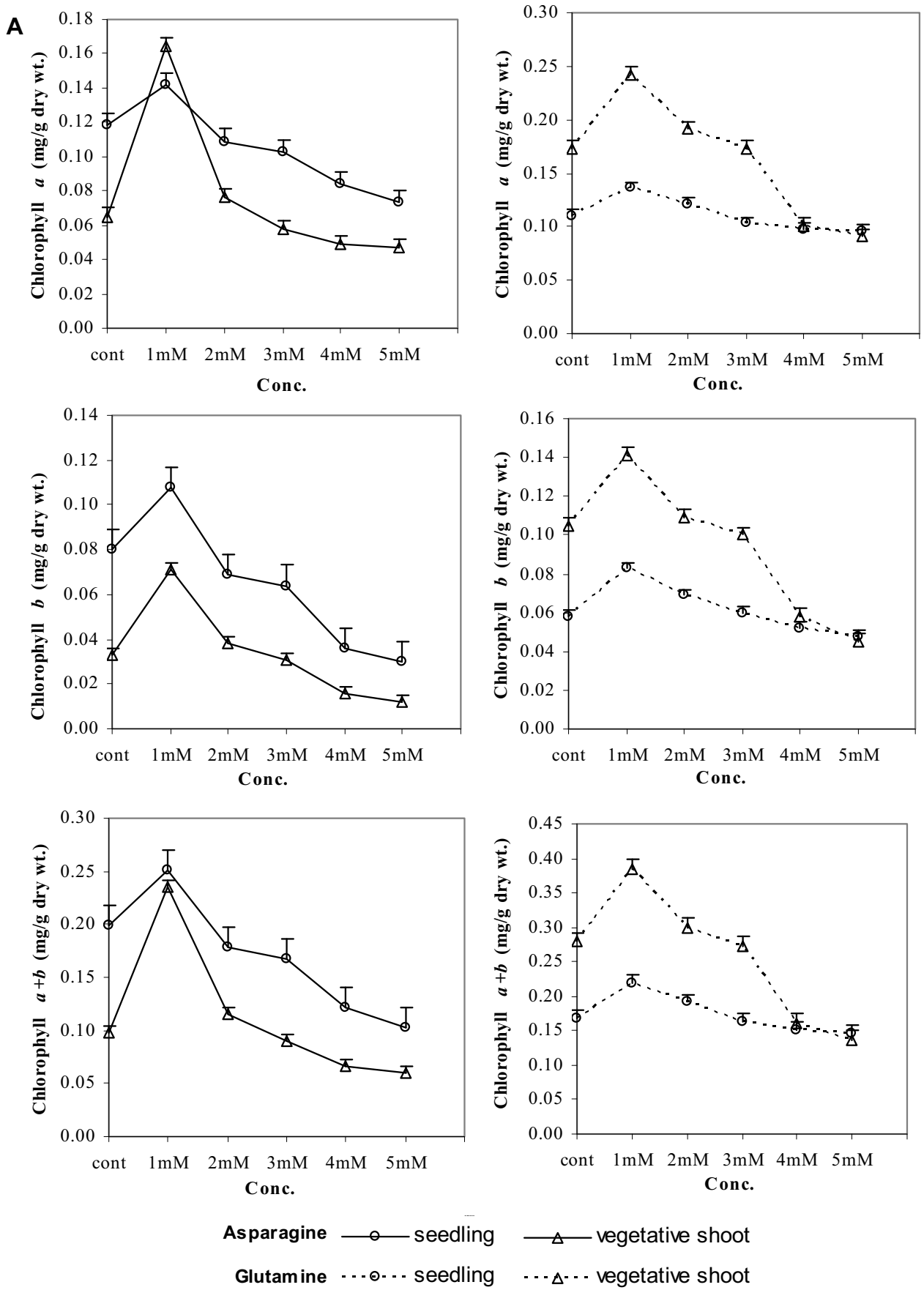
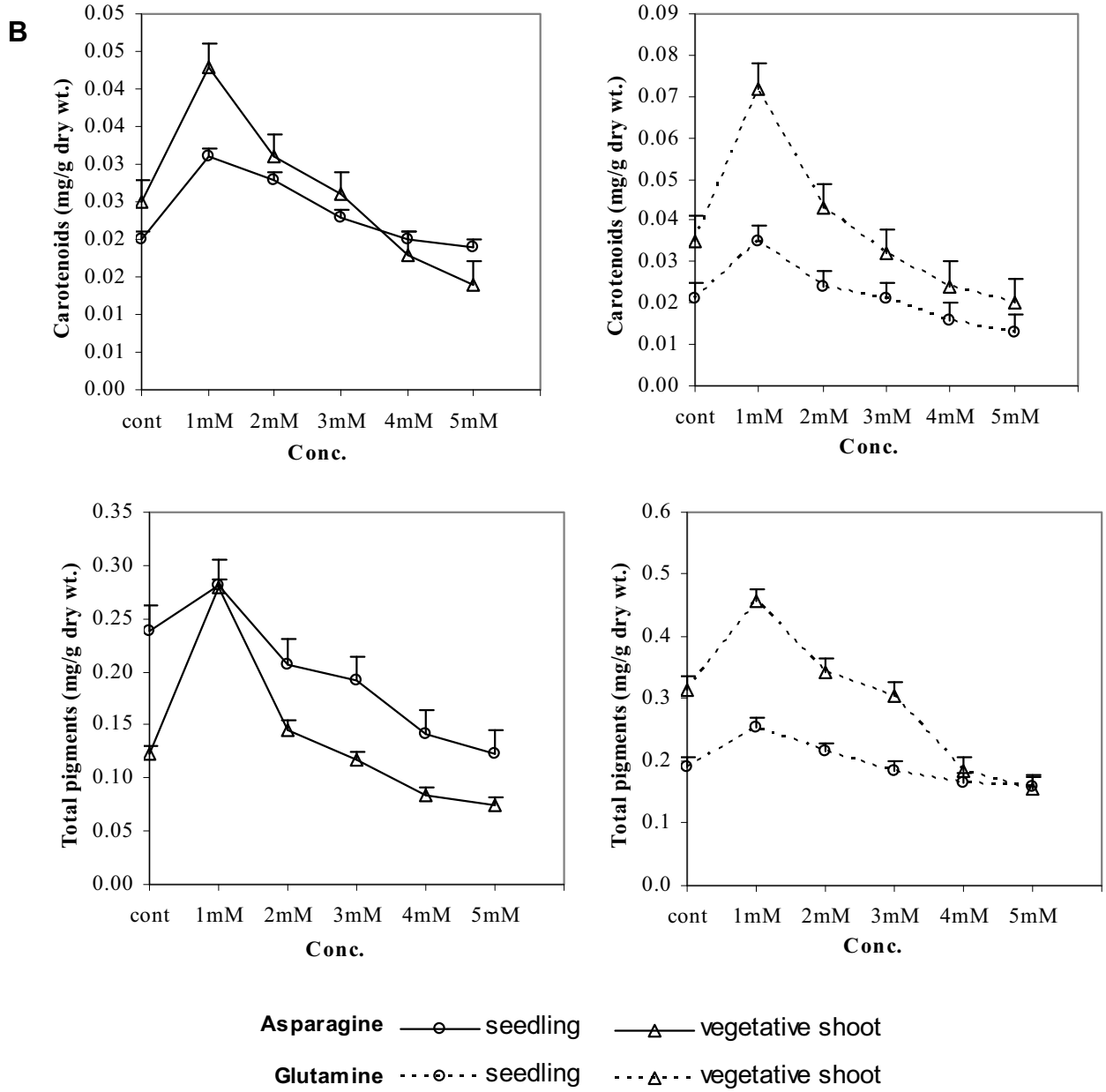


Fig. 3 Effect of increasing concentrations of asparagine or glutamine on photosynthetic parameters (A = Chl *a*, Chl *b*, Chl *a*+*b*; B = carotenoids, total pigments) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.



with increasing plant P and N concentrations of young tomato plants (*Lycopersicon esculentum* Mill. cv. 'Capita'). Thus in the present study, it can be concluded that an adequate amount of amides (asparagine or glutamine), after being applied to the media, were taken up in the root tissues as such and consequently translocated and assimilated within French bean and hence caused the observed changes (**Fig. 2A, 2B**) in the various growth parameters.

Changes in pigment contents

Further evidence of the role played by asparagine and glutamine modifying plant metabolism can be obtained from the data of changes in pigments (**Fig. 3A, 3B**) which clearly demonstrated that the biosynthesis of chls in treated French bean plant was markedly activated by low level (1 mM) and inhibited by the higher levels (3, 4, 5 mM) of intact seedlings and vegetative stages of french bean plants. In accord with those results, Keller *et al.* (2001) demonstrated an increase in chl content and photosynthesis of vine plant by high soil nitrogen. On the other hand, Martin *et al.* (2002) observed a significant reduction in chl content in *Arabidopsis* seedlings grown in low nitrogen concentration.

The observed progressive increases as well as the progressive decreases (**Fig. 3A, 3B**) in pigment contents (chls *a* and *b*, carotenoids and total chl) of French bean throughout the entire periods of experiments treated with different concentrations of either asparagine or glutamine are in good support to the growth rate (**Fig. 2A, 2B**) as well as to the change in carbohydrate content (**Fig. 4A, 4B**) of the same tissues. In this sense, Wettlaufer and Obendorf (1991) stated that, treatment of soybean with glutamine or asparagine (at 62 mM) resulted in increasing fresh weight and retention of green colour.

The non-significant responses in pigments content by 2 mM asparagine or glutamine (**Fig. 3A, 3B**) are in harmony with those obtained by Tremblay *et al.* (1999) who applied nitrogen fertilizers at 5 rates (0, 15, 30, 60 and 120 kg N/ha) and stated that chl content was not correlated with nitrogen application.

Changes in carbohydrate content

The experimental data in **Fig. 4A, 4B** show that there is a significant increase in glucose, sucrose, total soluble sugars and total carbohydrate with 1 and 2 mM asparagine or glutamine; the magnitude of increase was most pronounced at a lower concentration (1 mM asparagine or glutamine). In accord with these results Nakano *et al.* (1998) determined starch and sucrose content in rice and *P. vulgaris* when both species were grown at different N concentrations and stated that starch and sucrose contents in both species were increased at lower nitrogen rates, but the amount of accumulated starch in *P. vulgaris* was much greater than in rice

(*Oryza sativa* L.). Also, glutamine was utilized as a major carbon source by the pea embryo (Murray and Cordova-Edwards 1984).

An opposite pattern of changes was shown (**Fig. 4A, 4B**) for the different carbohydrate compounds in French bean seedlings treated with 3, 4 and 5 mM asparagine or glutamine. Accordingly, an enhancement of protein content accompanied by parallel decrease in carbohydrates was observed with increasing nitrogen concentrations in the medium of *Anabaena variabilis* cells (Sanz *et al*. 1995). The obtained results are in agreement with those of Druege *et al*. (2000) who found that increasing N supply (at 0.6,1.5 and 6 g N) resulted in substantial lower starch level and higher sucrose concentration in leaves of two *Chrysanthemum* cultivars ('Puma' and 'Cassa'). Also they stated that fructan concentration was low and decreased with increasing N

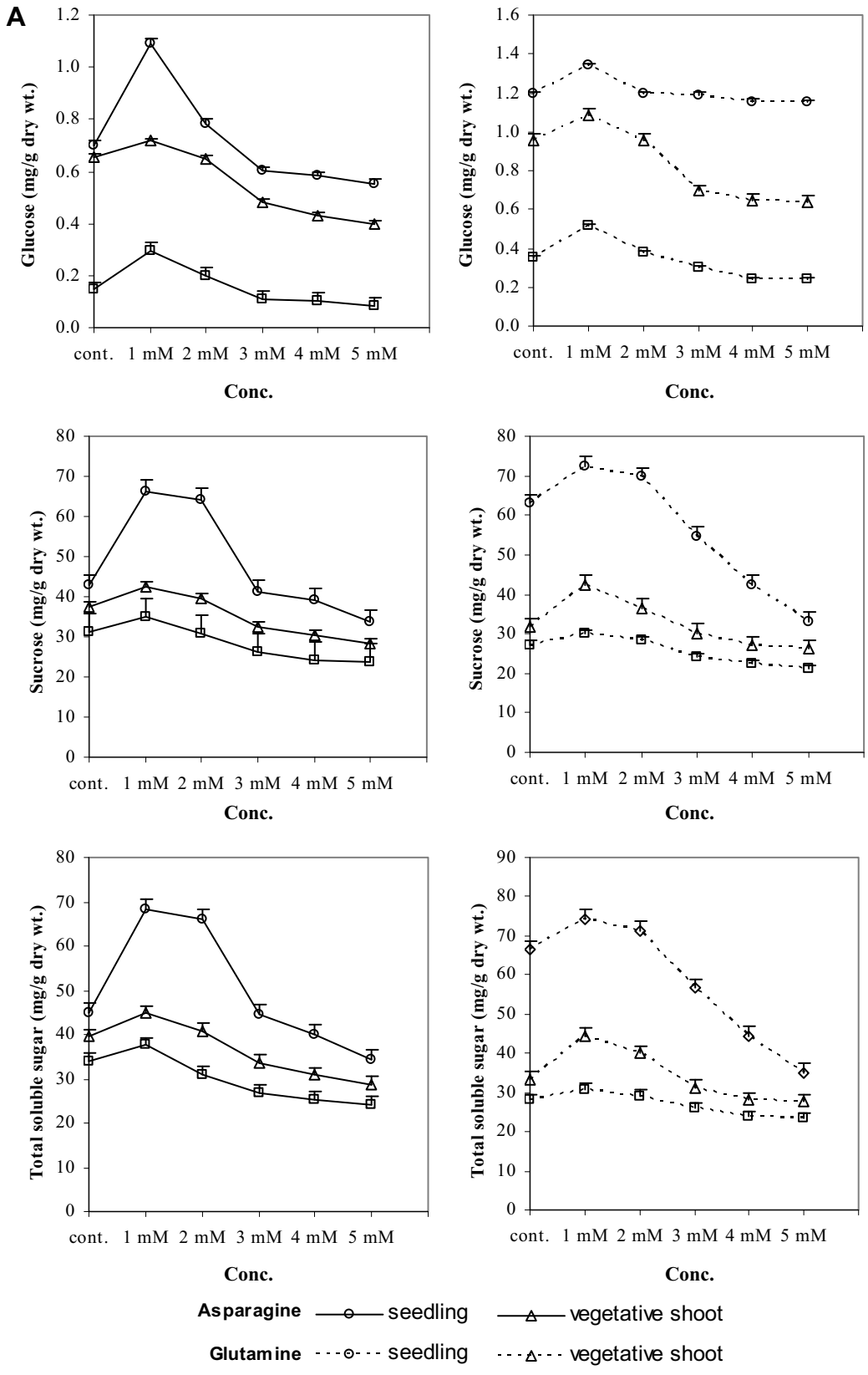
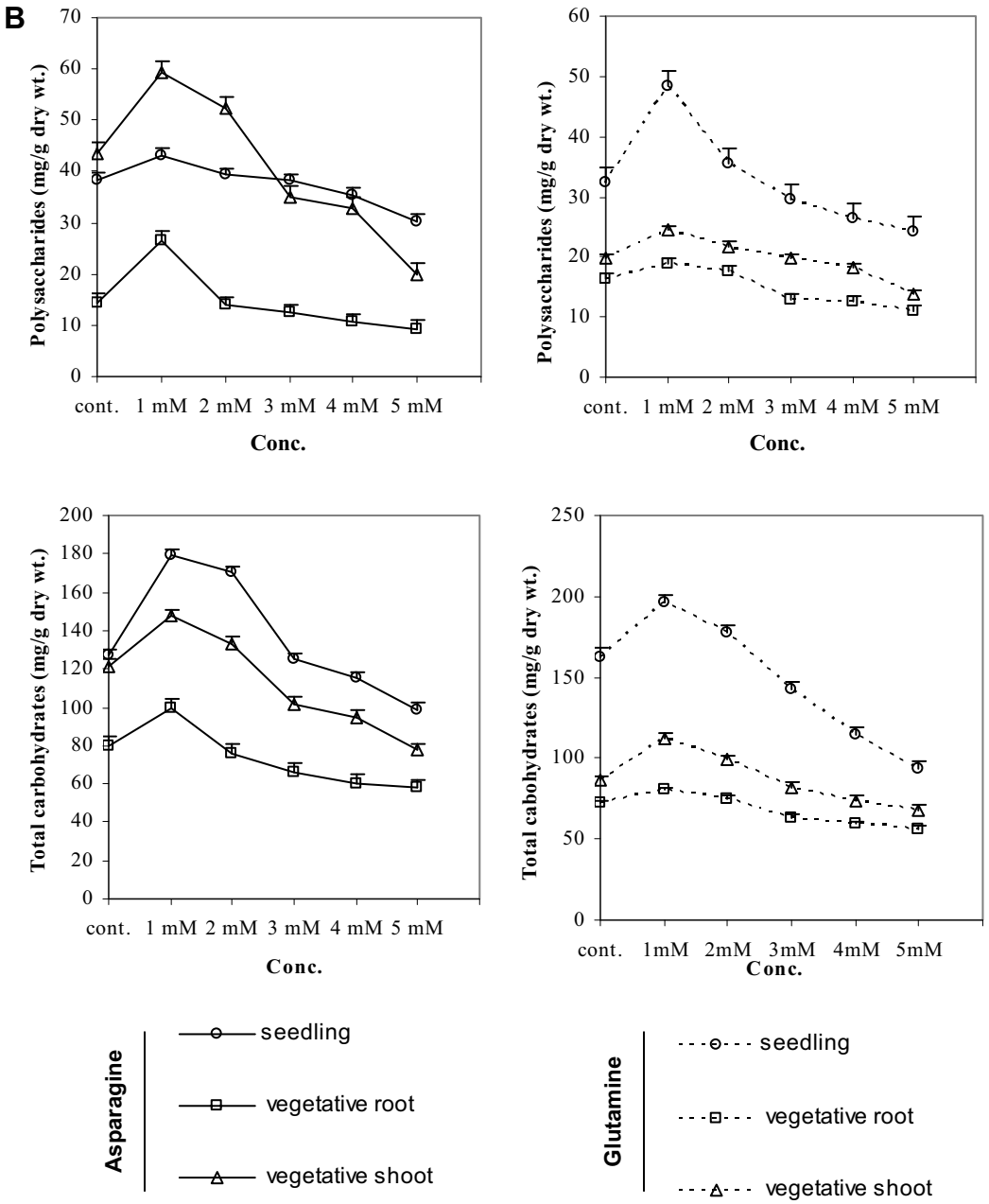


Fig. 4 Effect of increasing concentrations of asparagine or glutamine on carbohydrate parameters (A = glucose, sucrose, total soluble sugars; B = polysaccharides, total carbohydrates) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.



levels.

The observed changes in glucose, sucrose, polysaccharides and total carbohydrates content in intact French bean seedlings treated with different levels of asparagine or glutamine, namely 1 and 2 mM, throughout the entire period of the two experiments can be explained by (1) rapid decomposition of polysaccharides (starch) by hydrolytic enzymes, (2) changes in net photosynthetic rates and (3) increased oxidative phosphorylation. In support of these results, Aslam and Oaks (1975) in corn root, Aslam and Huffer (1984) in primary leaves of barley, and Oaks and Hirel (1985) in roots examined the relationship between enzyme carbohydrate level and the component of leaf system to reduced N. They found that when carbohydrate levels were high, the nitrate reduction and the reduction of ammonia were also relatively high. A similar situation was apparent in roots. For example, when wheat seedlings were subjected to N starvation there was a relative increase in carbohydrates and a corresponding enhanced capacity to reduce transport N (Talouise *et al*. 1984).

Changes in N content

Glutamine and asparagine are the key intermediates in the pathway of N assimilation (Pal'ove-Balang 2002). Thus, in this investigation, treatment of French bean plants with increasing concentrations of either asparagine or glutamine induced a progressive significant increase in amide, protein and total N contents during seedling and vegetative stages, as compared with control values. On the other hand, ammonia, peptide and total soluble N were found to decrease progressively with an increasing in concentration of either asparagine or glutamine through both stages of plant growth and development (**Fig. 5A, 5B**).

In this respect, Youssefi *et al*. (2000) stated that leaf-N concentrations were related positively to concentrations of applied amino acids (especially asparagine and glutamine). At the same time the amino acid concentrations in phloem exudate and xylem sap were highest in trees of cv. 'Mission' almond (*Prunus dulics* L.), when grown under the highest N fertilizer regime. It is suggested that the high amino acid concentrations in phloem and xylem saps are indicative of a large pool of amino N cycling throughout the vasculature of high N status trees. On the other hand, increasing the N supply to *Z. mays* led to an increase in the activity of certain enzymes, starch and the levels of N compounds (total N, soluble protein and free amino acids) and decreased the levels of carbon metabolites (sucrose and reducing sugars) in the tested plant (Cazetta *et al*. 1999).

Sawaguchi (1987) found that increasing fertilizer N

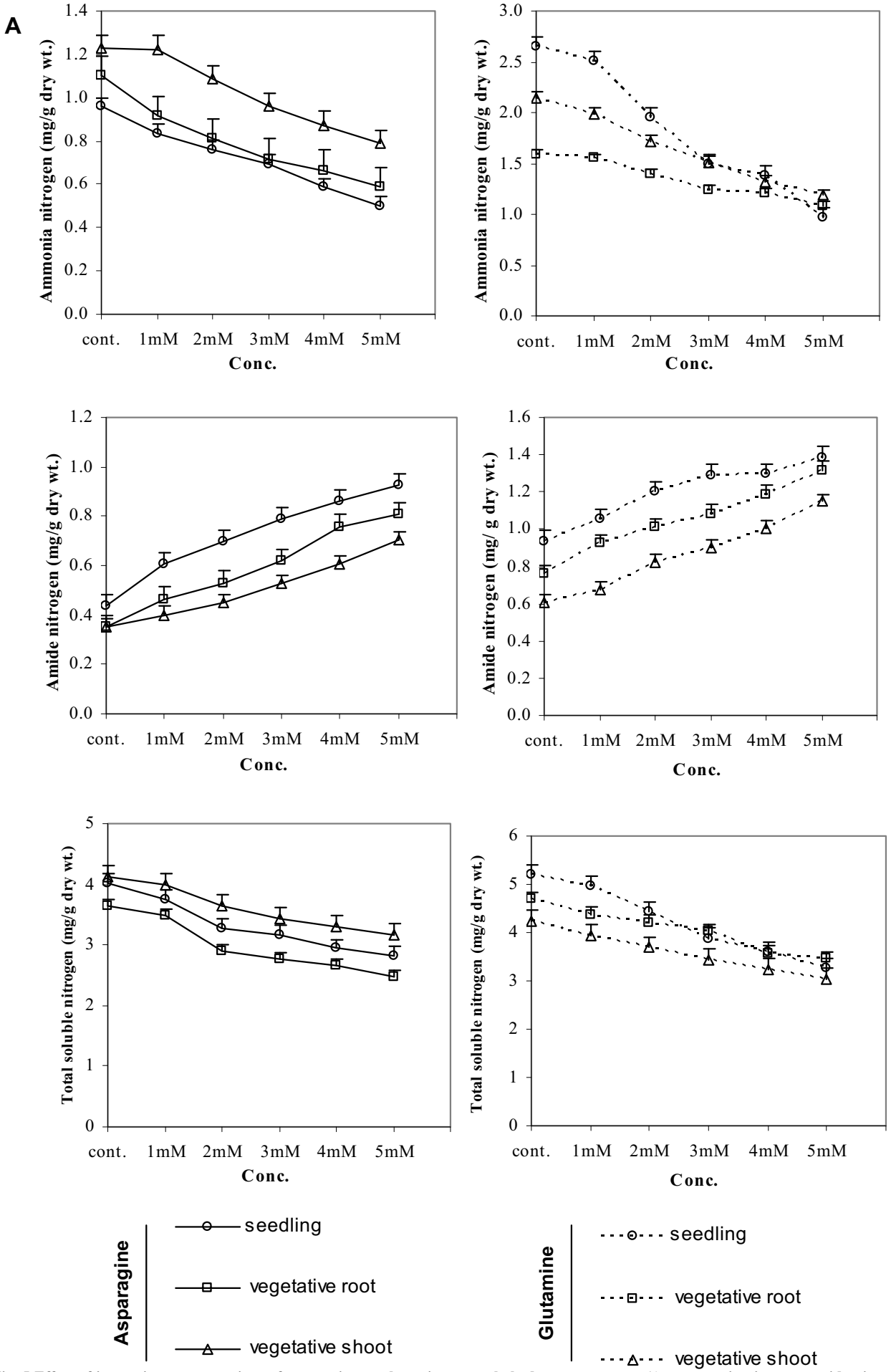
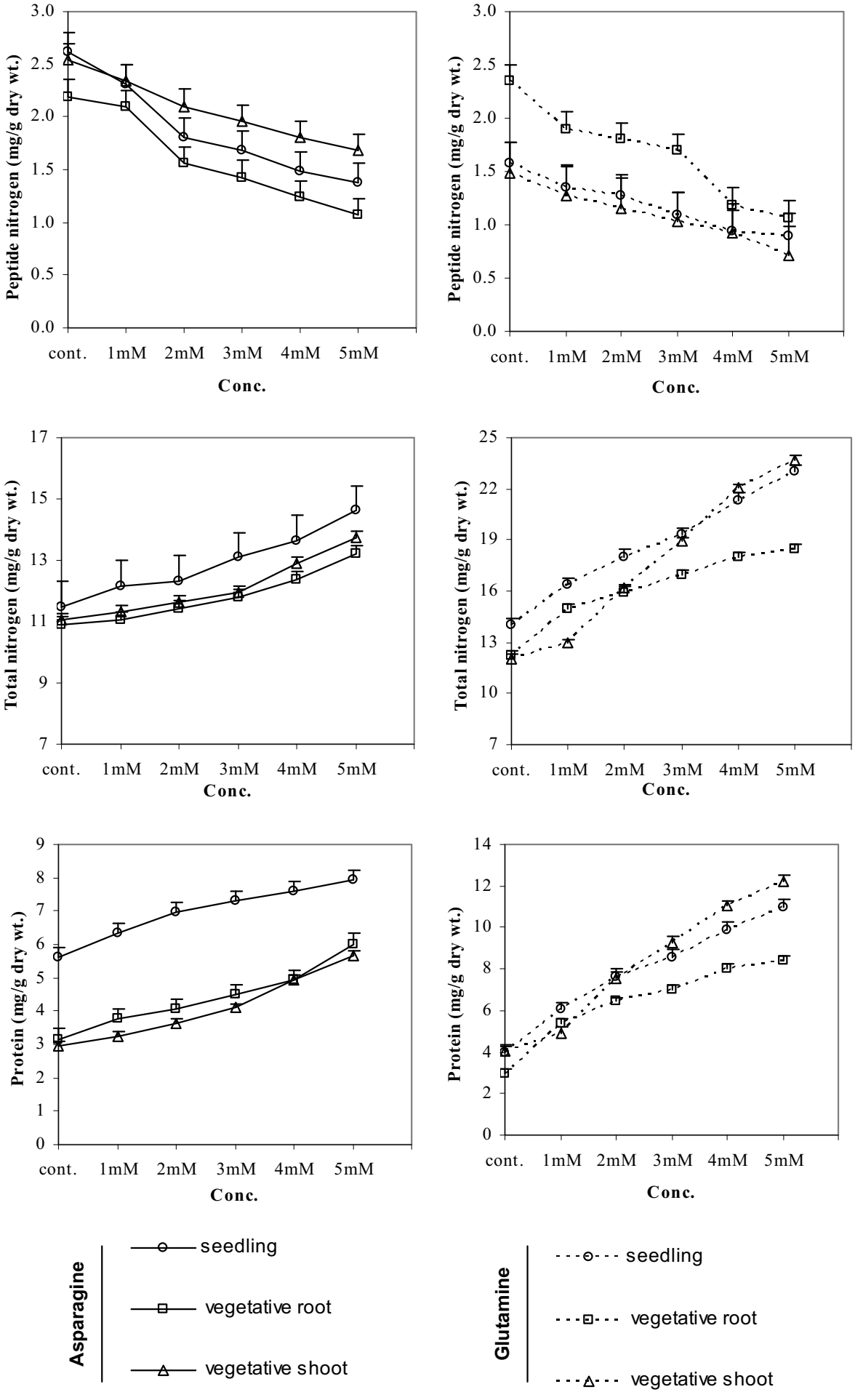


Fig. 5 Effect of increasing concentrations of asparagine or glutamine on carbohydrate parameters (A = ammonia nitrogen, amide nitrogen and total soluble nitrogen content; B = peptide nitrogen, total nitrogen and protein) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.

B


Peptide nitrogen (mg/g dry wt.)
Conc.
cont. 1mM 2mM 3mM 4mM 5mM
Total nitrogen (mg/g dry wt.)
Protein (mg/g dry wt.)
Asparagine
Glutamine
seedling
vegetative root
vegetative shoot

application increased N content in *Vigna angularis*. Also, the increase in ammonia and amino acids was the result of protein degradation (Vincent *et al*. 1997). In addition, Stoermer *et al*. (1997) found that total N content of *Picea abies* was increased by N fertilizers.

In conclusion, N metabolism is expected to be influenced by the two applied amide-N compounds (asparagine and glutamine) used in the present study (**Fig. 5A, 5B**), because the treatments involve the biosynthesis of proteins and/or amino acids and ammonia. Ammonia is considered to be the unit of N metabolism from which different amino acids are produced, these being further incorporated in the protein synthesis. For its role in N metabolism and protein synthesis, ammonia liberated from hydrolysis of amides has been found to be very important for plant survival if could be utilized by the plant cell. Instead, its accumulation without being utilized would be harmful to the plant tissues (**Fig. 5A, 5B**).

Changes in protein content

The results presented in **Fig. 5B** showed that a general significant increase in protein content, in *Phaseolus* plant, at seedling and vegetative stages, when asparagine or glutamine concentrations were increased. In agreement with this, Awonaike *et al*. (1978) stated that asparagine stimulated protein synthesis in pea (*Pisum sativum* L.), up to three-fold.

On the other hand, Ogawa *et al*. (1999) detected low levels of protein when glutamine was added to the medium of cultured *Spinach* cv. 'Hoyo' cells. Izmailov *et al*. (1981) reported that in maize seedlings grown in sterilized sand and given ^{14}C-asparagine and ^{14}C-glutamine through the roots, asparagine was slowly transformed into amino acids, mainly transport and storage nature, whereas glutamine served as an active metabolic source. Moreover, the effect of different N supply on amino acid export to the phloem was studied in young wheat (*Triticum aestivum* L.) plants (Caputo and Barneix 1997). They stated that this N is used for the synthesis of leaf protein when the supply is low, ex-

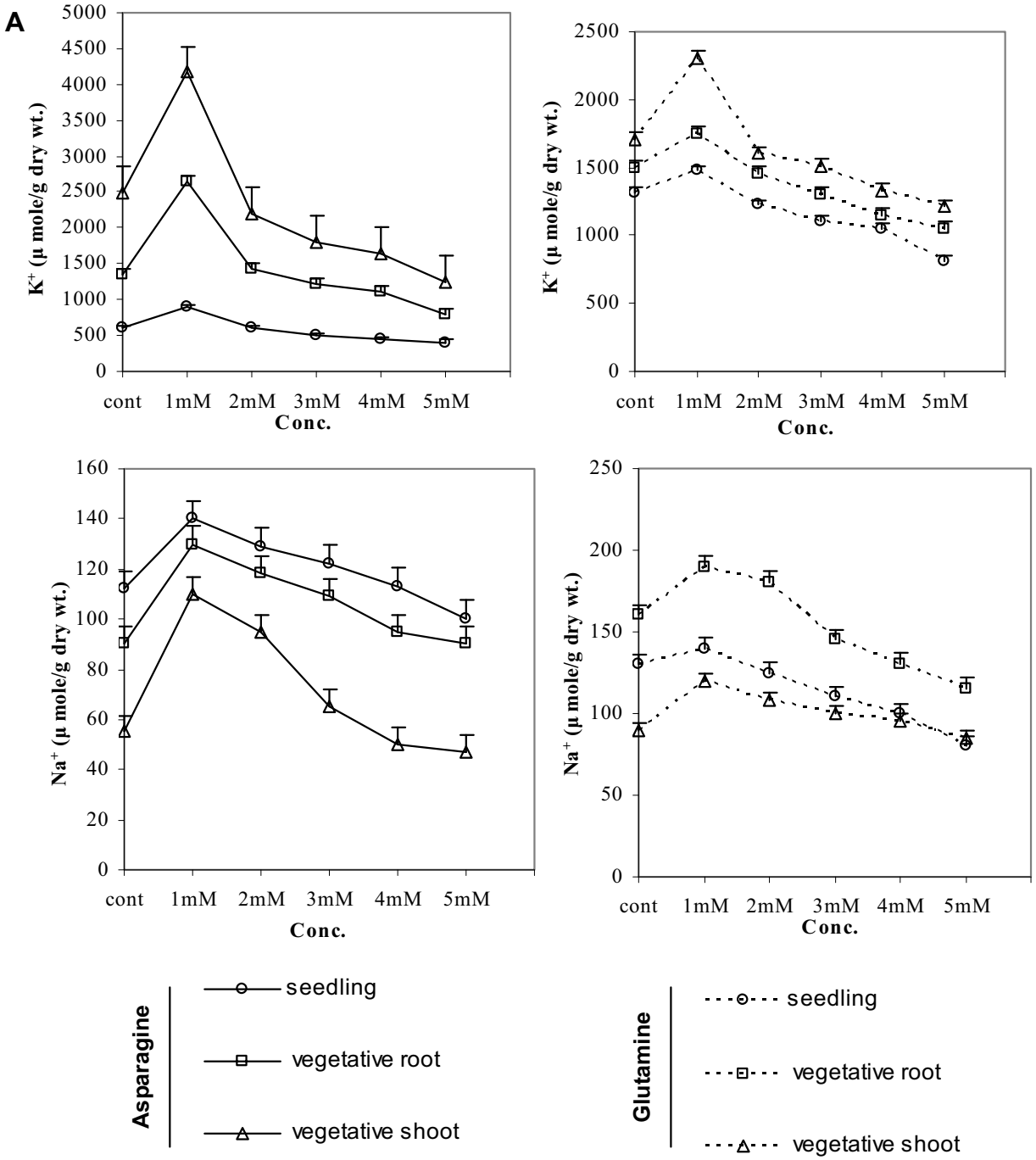
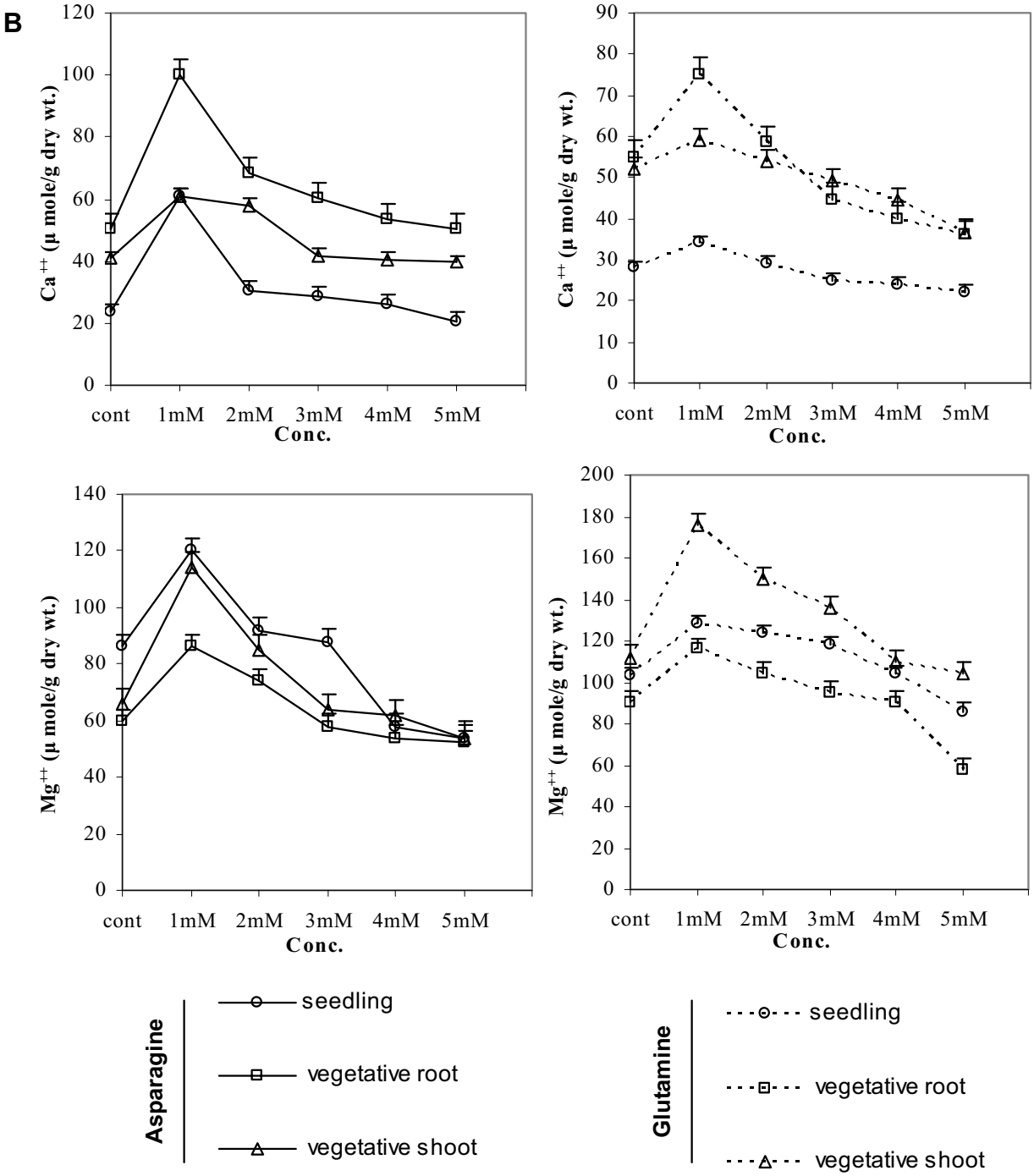


Fig. 6 Effect of increasing concentrations of asparagine or glutamine on carbohydrate parameters (A = K^+ and Na^+ contents; B = Ca^{++} and Mg^{++} contents) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.



ported to phloem when supply is adequate, and accumulated in the storage pool when supply is above plant demand.

Recently, Lea and Azevedo (2006) highlighted the latest developments in the isolation and characterisation of the genes involved in the uptake of N from the soil, normally present as nitrate or ammonium ions, and the utilisation efficiency, the ability of the plant to transfer N to grain, predominantly present as protein.

The nitrogen use efficiency (NUE) of crop plants can be expressed very simply as the yield of N per unit of available N in the soil. This NUE can be divided into two processes: uptake efficiency, the ability of the plant to remove N from the soil.

Changes in ion content

In general, all determined elements (K^+, Na^+, Mg^{++} and Ca^{++} ions) of French bean increased significantly with 1 and 2 mM asparagine or glutamine (**Fig. 6A, 6B**), while there is a general significant decrease at higher concentrations (3, 4 and 5 mM).

The magnitude of decrease in mineral contents was most pronounced with the increase in asparagine or glutamine concentrations. Kubik-Dobosz and Buczek (1999) supplemented *Pisum sativum* plants with 1 mM glutamine or asparagine and found that plants took up ammonium and K at a rate lower than control plants. On the other hand, Keller *et al*. (2001) stated that N supply (100 kg/ha) increased the translocation of K^+ and Ca^{++} in grape vines.

As regards **Fig. 6A, 6B**, the magnitude of increase and decrease in inorganic ion contents (K^+, Na^+, Mg^{++} and Ca^{++}) at low and high concentrations, respectively by asparagine or glutamine is expected to be influenced by amide-N compounds on protein synthesis as shown in **Fig. 5B**, in French bean.

Proteins are required to transport protons, inorganic ions and organic solutes across the plasma membrane and tonoplast at rates sufficient to meet the needs of the cells (Schroeder *et al*. 1999). Membranes contain different types of transport proteins, ATPases or ATP-powered pumps, channel proteins and cotransporters (Lalonde *et al*. 1999; Sze *et al*. 1999). ATPase utilizes the energy released upon

hydrolysis of ATP to move ions across the plasma membrane against chemical and electrical gradients. Channel proteins facilitate the diffusion of water and ions down energetically favorable gradients. Cotransporters, the third class of membrane-transport proteins, can move solutes either up or down gradients (Sze *et al*. 1999).

Changes in growth regulators

The relation between N metabolism and endogenous hormones is reciprocal. In this study, for French bean, there was a significant increase in PGRs (IAA, GA_3 and Cyt) with 1 and 2 mM asparagine or glutamine while a significant decrease occurred with higher concentrations (3, 4 and 5 mM; **Fig. 7A,7B**).

On the other hand a reverse situation occurred in the ABA level where 1 mM of asparagine or glutamine caused a significant decrease while 2, 3, 4 and 5 mM caused an increase in ABA content in French bean shoots. The magnitude of decrease in promoters and increase of inhibitor is most pronounced by increasing the concentrations of asparagine or glutamine. Zhang *et al*. (1999) suggested an overlap between the N and auxin response pathways. They found that high rates of nitrate supply to the roots had a systemic inhibitory effect on lateral root development that acted specifically at the stage when the laterals had just emerged from the primary root, apparently delaying final activation of the lateral root meristem.

Mercier and Kerbawy (1998) studied the effect of glutamine on auxin and cytokinin levels of three bromeliad species and detected significant increases in these PGRs in response to glutamine treatment. They also found a positive correlation between these increases and the promotion of shoot growth. This correlation supports our results: the promotion of growth (in response to low amide levels) occurred concurrently with increasing levels of IAA, GA and Cyt and decreased ABA content, whereas opposite changes in these PGRs were found to also inhibit growth (**Fig. 2A**).

In this study, the application of amides obviously caused an increase in ABA levels in two stages of plant growth and development. This increase in ABA content may probably be due to its biosynthesis within seedlings or it may possibly be translocated from the leaves. From another point of view, amides may act by interference with hormone metabolism by preventing ABA catabolism in French bean plant (Walton 1980). On the other hand, the decrease in IAA as a result of amide, particularly the 5 mM treatment, might be due to amide stimulating the formation

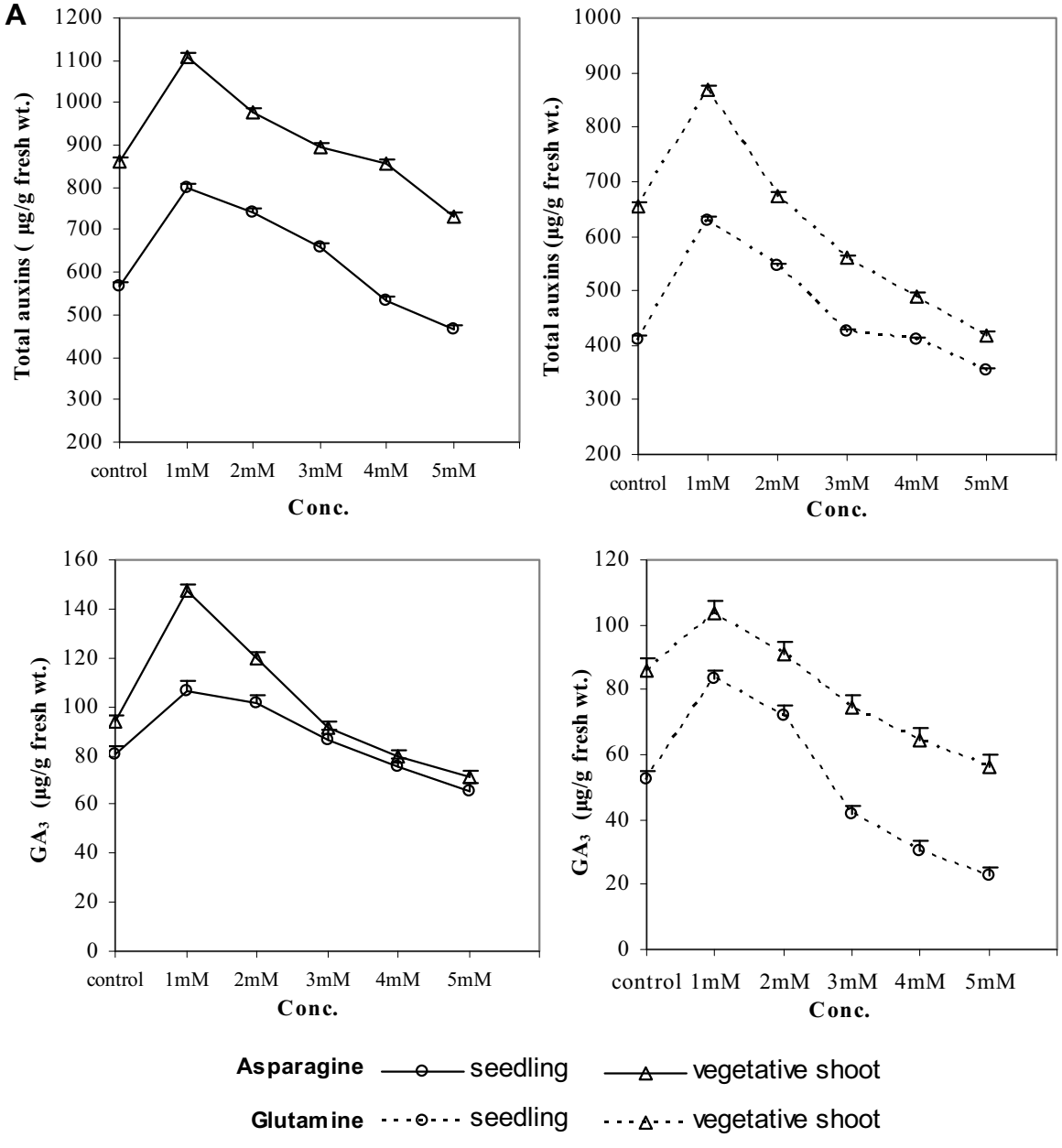
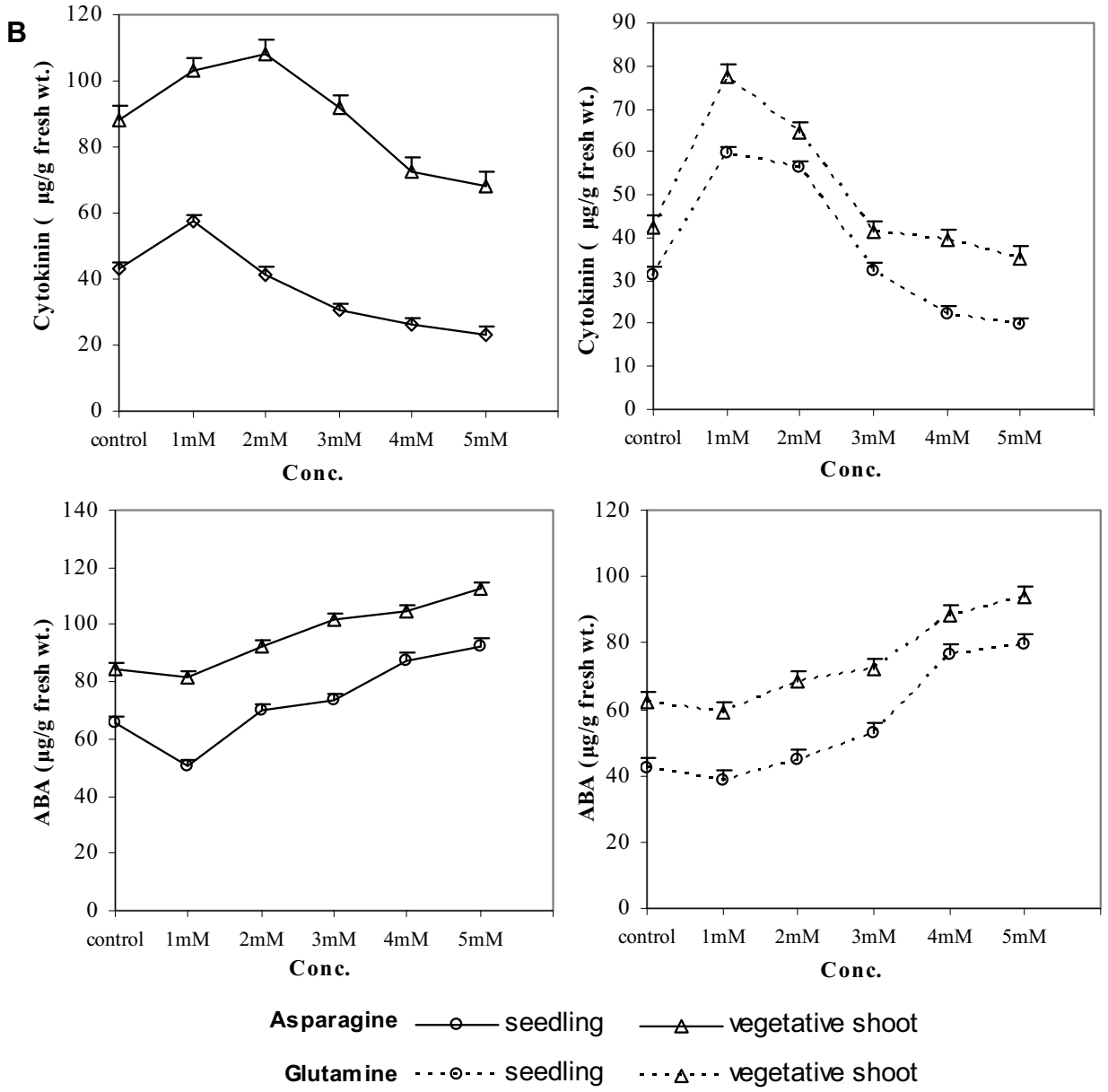


Fig. 7 Effect of increasing concentrations of asparagine or glutamine on carbohydrate parameters (A = total auxins and GA_3 contents; B = cytokinin and ABA contents) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.



of IAA-oxidase and peroxidase leading to destruction of IAA in French bean plant and/or due to a decrease in IAA biosynthesis in plant tissues (Torrey 1976).

The noticeable decline in GAs of French bean plant caused by the application of amide may result from the conversion of free active GAs into bound inactive GA (Ungarl and Binet 1975).

Changes in enzyme activities

AS and GS are key enzymes of N metabolism in higher plants (Muhitch and Felker 1994; Romagni *et al*. 2000). The activities of AS, GS, NR and protease, in general, decreased significantly with increasing asparagine or glutamine concentrations in intact French bean during both stages of plant growth (**Fig. 8A, 8B**).

Sivasankar and Oaks (1995) and Sivasankar *et al*. (1997) also found that the application of exogenous amides (asparagine or glutamine) to maize seedlings led to a decrease in NR activity. Also, after the addition of glutamine to nitrate-containing medium (*in vitro*), NR activity in cultured spinach cv. 'Hoyo' cells was repressed (Ogawa *et al*. 1999).

Pal'ove-Balang and Mistrik (2001) examined NR activity in maize, and after treatment with two different concentrations (1 and 10 mM) of glutamine or asparagine they found that the low concentration used (1 mM) showed no effect on NR activity, while the higher concentration (10 mM) stimulate this enzyme. The same result was obtained by Stancheva and Dinev (1995) on the same plant. In addition, Aslam *et al*. (2001) noted that pretreatment of barley with glutamine or asparagine had no effect on the subsequent induction of NR. They suggest that this inhibition is a result of the lack of substrate availability due to inhibition of the nitrate uptake system.

GS is the enzyme responsible for the primary assimilation of ammonium produced by nitrate reduction or fixation of dinitrogen as well as the reassimilation of ammonium released by photorespiration and other metabolic processes (Gomez-Maldonado *et al*. 2004).

Furthermore, glutamine supplementation reduced GS activity of *Asparagus officinalis* L. (Seelye *et al*. 1995). Meanwhile, Ma and Li (2002) found that in root and leaves of sugar beet, GS and NR gradually increased with increasing N treatment.

It is interesting to mention that the decrease in enzyme activities are more pronounced in roots than in shoots of intact French bean (**Fig. 8A, 8B**). Zhang *et al*. (1998) found that in rice cv. 'IR72' plants treated with fertilizer N, GS activity in leaves was greater than in roots, regardless of N application.

GS functions as the major assimilatory enzyme for ammonia produced from N fixation, and nitrate or ammonia nutrition. It also reassimilates ammonia released as a result of photorespiration and the breakdown of proteins and N transport compounds. GS is distributed in different subcel-

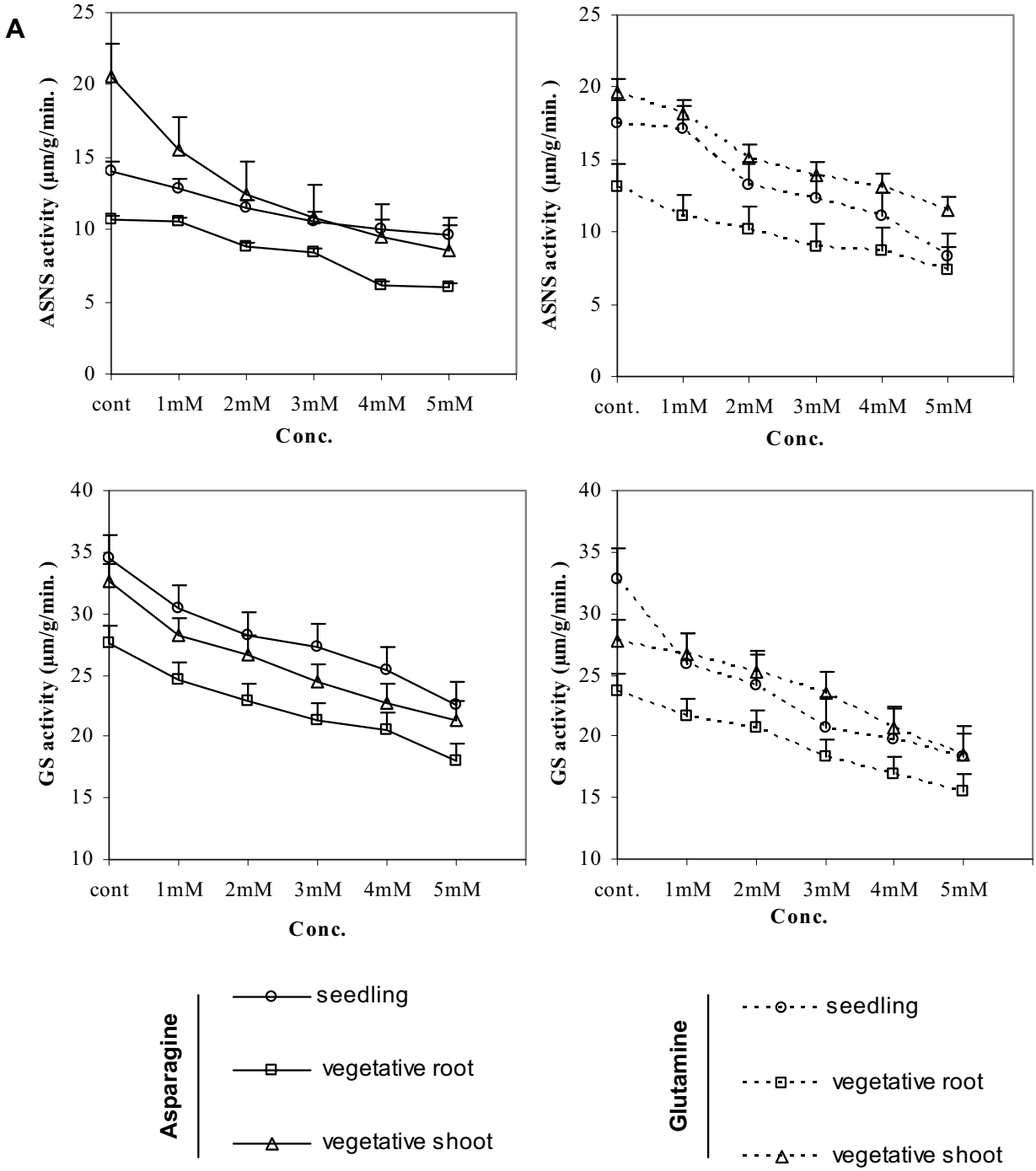


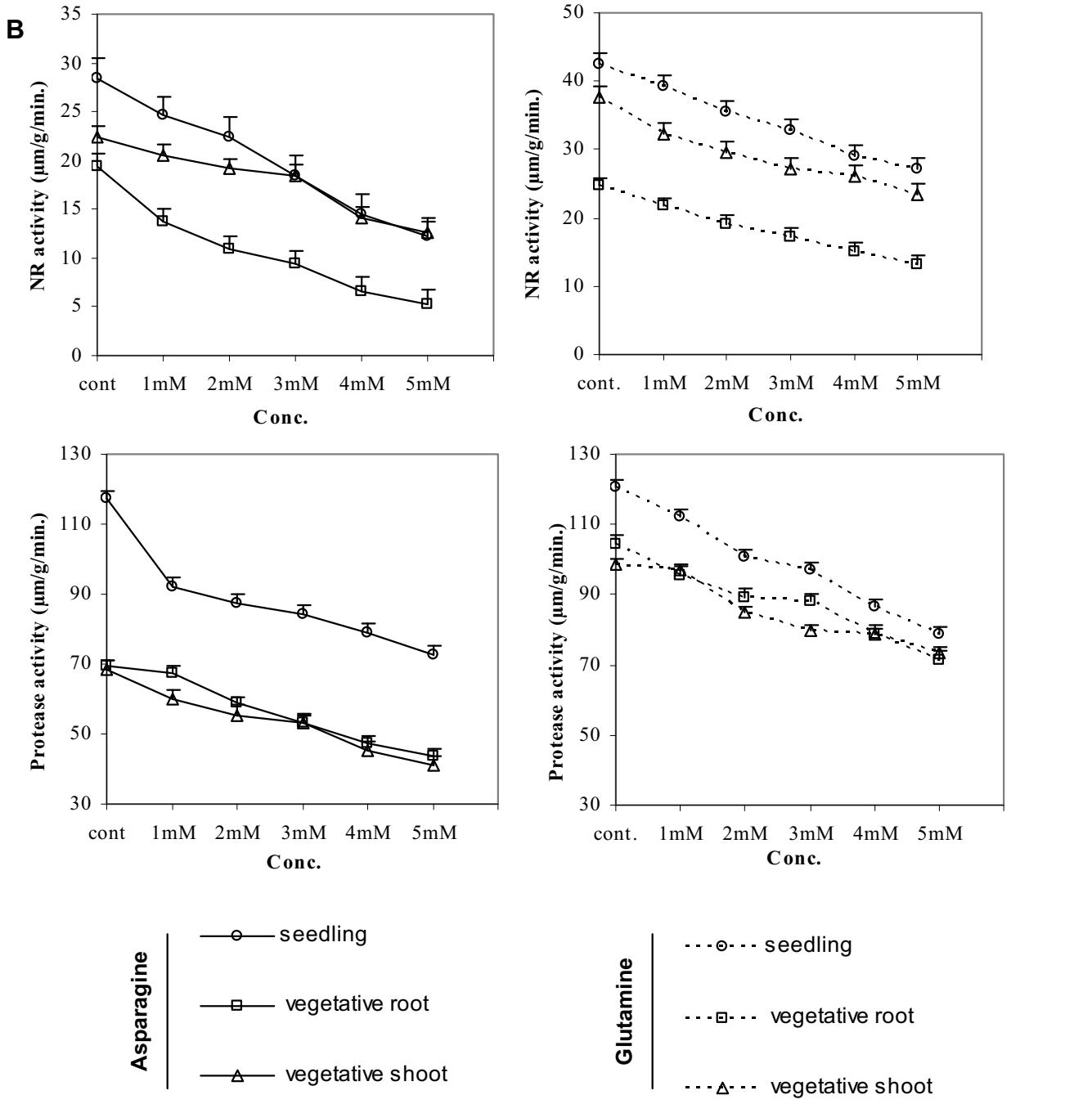
Fig. 8 Effect of increasing concentrations of asparagine or glutamine on carbohydrate parameters (A = asparagine synthetase (ASNS) and glutamine synthetase (GS) activities; B = nitrate reductase (NR) and protease activities) of *Phaseolus vulgaris* at seedling and vegetative stages. Vertical bar = value of LSD at 0.05.

lular locations (chloroplast and cytoplasm) and in different tissues and organs. This distribution probably changes as a function of the development of the tissues (Miflin and Habash 2002). Ke *et al*. (2001) stated that in forage rice cvs. 'Xiang zaoxian 24' and 'Zhong youzao 81', NR, glutamine transferase (GT) and GS activities decreased but proteinase activity increased, as N degradation and transmission increased. This is in agreement with the data of N fractions in this study (**Fig. 5A, 5B**).

Lea and Azevedo (2007) stated that the genes controlling the enzymes of amino acid metabolism that may be involved in transferring N to the protein in the grain. Evidence is now accumulating from the use of knockout mutants, of the role of individual isoenzymes involved in amino acid metabolism, which are encoded by specific genes that are often members of a multigene family. In addition, a significant number of overexpressing plant lines have been obtained, which have increased activities of cytosol located, GS, asparagine synthetase and alanine aminotransferase that appear to have improved NUE.

Recently, Majerowicz and Kerbauy (2002), using *Catasetum fimbriatum*, demonstrated that glutamine treatment *in vivo* resulted in the accumulation of dry matter in shoots due to higher values of N assimilatory enzyme activities (NR, GS and glutamate dehydrogenase (GDH)) as well as free ammonia content. This was in harmony with our results (**Fig. 2A, 2B**; **5A, 5B**).

In conclusion, the changes in the different enzyme activities (AS, GS, NR and protease) by application of either asparagine or glutamine in French bean plants may be attributed to the amide action on the biosynthesis of enzyme protein, enzyme activation and/or membrane permeability.



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