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Role of Calcium in Ameliorating Photosynthetic Capacity, Nitrogen Fixation, Enzyme Activities, Nutraceuticals and Crop Productivity of Hyacinth Bean (*Lablab purpureus* L.) under Calcium-Deficient Soil

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ABSTRACT

Plant biological yield appears to be comparatively low in calcium (Ca)-deficient soils of Aligarh, Western Uttar Pradesh, India. Here, Ca deficiency poses a serious yield and quality limitation for several crops, including various legumes. Hyacinth bean (*Lablab purpureus* L.) is a good source of vegetable protein in the human diet. Its seeds and pods contain as much as 20-28% protein. It contains tyrosinase enzyme, which has potential use in the treatment of hypertension. Because of the importance for hyacinth bean as a bio-functional medicinal legume, an experiment was designed to determine whether Ca application through soil could enhance hyacinth bean production, nitrogen fixation, photosynthesis, enzymatic activities, nutraceuticals and quality attributes for this legume. The plants were grown in pots containing soil and supplied with five levels of calcium, viz. 0, 40, 80, 120 and 160 mg Ca kg^{-1} soil applied as calcium chloride ($CaCl_2$). The performance of the crop was assessed in terms of various growth, physiological, biochemical, yield and quality attributes at 60, 90, 120 and 150 days after sowing (DAS). Ca application proved to be significantly effective on most of the parameters studied. Of the five levels, Ca at 120 mg kg^{-1} soil showed the best results, significantly stimulating most of the attributes studied at 60, 90, 120 and 150 DAS. In fact, this level of Ca increased seed yield, seed protein content and tyrosinase activity by 30.3, 16.6 and 20.3%, respectively, compared to control plants. This need for Ca by hyacinth bean should be included as a fertilizer recommendation for this region.

Keywords: calcium, carbonic anhydrase, nitrate reductase, nutraceuticals, tyrosinase

INTRODUCTION

Hyacinth bean (*Lablab purpureus* L.) has great potential as a medicinal legume. It constitutes an important source of therapeutic agents used in the modern as well as traditional systems of medicine (Morris 2003). The plant is reported to be a multipurpose crop used for food, forage, soil improvement, soil protection, and weed control (Pengelly and Maass 2001; Morris 2003). The beans are naturally rich in carbohydrates, proteins, fats, and fibers as well as minerals, including calcium, phosphorus and iron. Furthermore, several legumes have tremendous potential as nutraceuticals because of their healing properties (Morris 2003). The young pods and tender beans of hyacinth bean are used as vegetables in India and tropical and warm temperate Asia. The seeds are used as a laxative, diuretic, anthelmintic, antispasmodic, aphrodisiac, anaphrodisiac, digestive, carminative, febrifuge and stomachic (Kirtikar and Basu 1995). It also contains fiber, which is known to prevent cancer, diabetes, heart disease, obesity, and is also used as a laxative (Beckstrom-Sternberg and Duke 1994). Hyacinth bean also contains a flavonoid known as kievitone that is a potential breast cancer-fighting agent (Hoffman 1995). The flavonoid, genistein found in hyacinth bean may play a role in the prevention of cancer (Kobayashi *et al*. 2002) and as a chemotherapeutic and/or chemopreventive agent for head and neck cancer (Alhasan *et al.* 2001).

In fact, legumes require a higher amount of calcium (Ca), especially when they depend upon symbiotic nitrogen (N) fixation alone. Usually, a higher amount of Ca is needed for the formation of nodules for N fixation and plant growth in legumes (Lowther and Loneragan 1968; Munns 1978; Brauer *et al*. 2002). However, hyacinth bean requires higher amounts of Ca both for nodule formation and N fixation. The soil analysis of this region reveals that it is Ca-deficient and it appears that soil-Ca-deficiency could be a serious limitation for hyacinth bean production. The low Ca level of the soil in this region of India could be a reason for its poor bean yield. Hence, the present study was conducted to investigate whether the basal application of Ca could ameliorate crop productivity, photosynthesis, N fixation and enzyme activities as well as quality attributes of hyacinth bean in Aligarh, Western Uttar Pradesh, India.

MATERIALS AND METHODS

Plant material and growth conditions

Healthy seeds of hyacinth bean were received from the USDA, ARS, Plant Genetic Resources Conservation Unit, Griffin, GA, USA. Seeds of uniform size were selected and their viability was tested using 1% tetrazolium salt. The seeds were surface sterilized with 95% ethyl alcohol for 5 min and then washed thoroughly with distilled water before sowing. Since hyacinth bean is a leguminous crop, no N fertilizer was applied. The requirement of N was expected to be fulfilled by the crop itself through biological N fixation because hyacinth bean seeds were inoculated with *Rhizobium leguminosarum* (QA 08). A healthy and viable *Rhizobium* culture, compatible for hyacinth bean, was obtained from the Culture Laboratory, Government Agriculture Farm, Quarsi, Aligarh. This *Rhizobium* culture was prepared using similar methods by Subba Rao (1972). Colourless Gum Arabic (coating material; 200 g) and 50 g of sugar were dissolved in 500 mL warm water. After cooling, 100 g of the *Rhizobium* culture was mixed with the coating material. The required amount of seeds (25) was mixed vigorously with the inoculum (100 mL) until the seeds were evenly

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coated by the inoculum mixture. The inoculated seeds were spread in a clean tray and dried for 1 h in the shade prior to sowing.

Prior to seed sowing, 5.0 kg of a homogenous soil mixture containing farmyard manure (4: 1) was used in each pot. Physico-chemical characteristics of the soil were: texture-sandy loam, pH (1:2) 8.0, E.C. (1:2) 0.50 m mhos/cm, available N, P and K (96.87, 7.68 and 149.8 mg kg^{-1} soil, respectively) and calcium carbonate was low. The soil samples were tested at the Government Soil Testing Laboratory, Quarsi Farm, Aligarh. A uniform basal level of P (10 mg kg^{-1} soil) was basally applied. Then seeds were sown at a depth of 2 cm in an earthen pot (25 cm diameter × 25 cm height) containing soil. One healthy plant was maintained per pot. Dimecron 100 SCW (Syngenta India Ltd., Mumbai, India) was sprayed to protect plants from aphid infestation.

Experimental design

A pot culture experiment was conducted in the net house at the Botany Department, A.M.U., Aligarh (27° 52' N latitude, 78° 51' E longitude, and 187.45 m altitude). Growth and biochemical attributes of hyacinth bean were determined at 60, 90 and 120 days after sowing (DAS). At 60, 90 and 120 DAS, three plants from each treatment were uprooted. The root nodules of each plant were washed under tap water, and nodule number $plant^{-1}$ was counted. The plants were dried at 80°C for 24 h, and the dry weight of the nodules and plants were recorded. The experiment was conducted according to a simple randomized complete block design using five levels of Ca, viz. 0, 40, 80, 120 and 160 mg Ca kg^{-1} soil (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) applied as calcium chloride ($CaCl_2$) at seed sowing. Each treatment was replicated three times and each replicate had three plants. The pots were watered thoroughly. Plants were grown under naturally illuminated environmental conditions.

Growth analysis

A simple pot experiment was designed to evaluate crop productivity, photosynthesis, N-fixation, enzyme activities, nutraceuticals, and the complete life cycle of hyacinth bean. The plants were sampled at the vegetative stage (60 DAS), flowering stage (90 DAS) and pod-filling stage (120 DAS), respectively. During the three growth stages, three plants from each treatment were harvested by carefully removing the roots and washing with tap water to remove adhering foreign particles. Water adhering to the roots was removed with blotting paper and fresh weights of plants were recorded. The root nodules of each plant were washed under tap water, and number of nodules $plant^{-1}$ was counted. The plants were dried at 80°C for 24 h, and the dry weight of the nodules and plants were recorded.

Yield analysis

At harvest (150 DAS), nine plants from each treatment were uprooted randomly and used for computing yield attributes, including number of pods $plant^{-1}$, number of seeds pod^{-1}, 100-seed weight and seed-yield $plant^{-1}$. The pods were then threshed and seeds were cleaned and counted. Afterward, the number of seeds pod^{-1} and 100-seed weight were recorded. Seed-yield was calculated accordingly.

Physiological and biochemical analysis

The youngest fully expanded fresh leaves were used for the analysis of various physiological and biochemical attributes including total Chlorophyll and carotenoid content, nitrate reductase activity and carbonic anhydrase activity except leaf-N, -P, -K and -Ca contents.

Net photosynthetic rate, stomatal conductance and transpiration rate

Net photosynthetic rate, stomatal conductance and transpiration rate were measured on cloudless clear days at 11:00 a.m. on fully expanded hyacinth bean leaves using an IRGA (Infra Red Gas Analyzer), LiCor 6200 Portable Photosynthesis System (Lincoln, Nebraska, USA). The IRGA was calibrated and zero was adjusted approximately every half an hour during the measurement period. The youngest fully expanded leaves of hyacinth bean were enclosed in a 1 L gas exchange chamber for 60 sec. These measurements were recorded three times in each treatment. Photosynthesis was measured only at 90 DAS (flowering stage) using youngest fully expanded leaves since photosynthetic pigments (Chlorophyll and carotenoids contents) were maximum at 90 DAS. Moreover, we noticed that all biochemical parameters (Chlorophyll and carotenoids contents, enzyme activities and nutrient elements) decreased sharply at the fruiting stage (120 DAS) hence the selection of 90 DAS only.

Total Chlorophyll and carotenoid content

Total Chlorophyll (Chl) and carotenoid content from fresh leaves were estimated using the method of MacKinney (1941) and MacLachlan and Zalik (1963), respectively. Fresh tissue from interveinal leaf areas was ground in a mortar and pestle containing 80% acetone. The optical density (OD) of the solution was recorded at 645 and 663 nm for Chlorophyll content and at 480 and 510 nm for carotenoid content estimation using a spectrophotometer (Spectronic 20D, Milton Roy, USA). These contents were expressed as mg g^{-1} FW.

Nitrate reductase (NR) activity

The NR (E.C. 1.6.6.1) activity was estimated by the intact tissue method developed by Jaworski (1971), which is based on the reduction of nitrate to nitrite based on the following biochemical reaction:

$$NO_3^- + NADH + H^+ \xrightarrow{NR} NO_2^- + NAD^+ + H_2O$$

The nitrite formed was determined spectrophotometrically. Freshly chopped leaves (200 mg) were weighed and transferred to a plastic vial. Each vial contained 2.5 mL phosphate buffer (pH 7.5), 0.5 mL potassium nitrate solution and 5% isopropanol. After incubation, 1% sulphanilamide and 0.02% *N*-(1-naphthyl)ethylenediamine dihydroChloride (NED-HCL) were added. The test tubes were stored for 20 min at room temperature for colour development. The OD of leaf colour was recorded at 540 nm using a spectrophotometer. Nitrate reductase activity was expressed as n M NO_2^- g^{-1} FW h^{-1}.

Carbonic anhydrase (CA) activity

The CA (E.C. 4.2.1.1) activity in fresh leaves was analyzed using the method described by Dwivedi and Randhawa (1974). Fresh leaf pieces (200 mg) were weighed and transferred to Petri dishes. The leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride for 20 min at 4°C. Four mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue was added to the homogenate. The reaction mixture was titrated against 0.05 N HCl using methyl red as an indicator. Carbonic anhydrase activity was expressed as μM CO_2 kg^{-1} leaf FW s^{-1}.

Nutrient analysis

Leaf samples from each treatment were used for the estimation of leaf -N, -P, -K and -Ca content. The leaves were dried in a hot air oven at 80°C for 24 h. Dried leaves were powdered using a mortar and pestle and passed through a 72 mesh. The sieved powder was used to evaluate N, P, K and Ca content. Oven-dried leaf powder (100 mg) was carefully transferred to a digestion tube and 2 mL of analytical reagent (AR) grade concentrated sulphuric acid was added to it followed by heating on a temperature-controlled assembly for 2 h. After heating, the contents of the tube became black. The homogenate (solution) of digested leaves was cooled for about 15 min at room temperature and then 0.5 mL of 30% hydrogen peroxide (H_2O_2) was added. The addition of H_2O_2 followed by heating was repeated until the contents of the tube became colourless. The prepared aliquot (H_2O_2-digested material) was used to estimate N, P, K and Ca content.

Nitrogen content

Leaf-N content was estimated using the method of Lindner (1944) with a slight modification by Novozamsky *et al*. (1983). A 10 mL aliquot (H_2O_2-digested material) was poured into a 50 mL volumetric flask. Two mL of 2.5 N NaOH and 1 mL of 10% sodium silicate solutions were added to neutralize the excess acid and to prevent turbidity, respectively. Five mL aliquot of this solution containing 0.5 mL Nessler's reagent (E. Merck India Ltd., Mumbai, India) were poured into a 10 mL graduated test tube. The contents of the test tubes were stored for 5 min to allow for maximum colour development. The OD of the solution was recorded at 525 nm using a spectrophotometer. The reading from each sample was compared with the standard calibration curve of ammonium sulphate to estimate the percent N content.

Phosphorus content

The method of Fiske and Subba Row (1925) with a slight modification by Rorison *et al*. (1993) was used to estimate the leaf-P content in the digested material. The same aliquot was used to determine the leaf-P content. A 5 mL aliquot was taken in a 10 mL graduated test tube. Afterwards, 1 mL molybdic acid (2.5%) was added carefully, followed by addition of 0.4 ml 1-amino-2-naphthol-4-sulphonic acid. When the colour became blue, the volume was increased to 10 mL with the addition of double distilled water. The solution was shaken for 5 min and the OD of the solution was recorded at 620 nm using a spectrophotometer.

Potassium and calcium contents

K and Ca contents were analyzed using flame-photometry. In the flame-photometer, the solution (peroxide-digested material) is discharged through an atomizer in the form of a fine mist into a chamber, where it is drawn into a flame. Combustion of the elements produces light of a particular wavelength (λ_{max} for K = 767 nm (violet)). The light produced was conducted through the appropriate filters to impinge upon a photoelectric cell that activates a galvanometer. Both leaf-K and -Ca content in the same aliquot were estimated and recorded with the aid of emission spectra using specific filters in a flame-photometer (Model, C150, AIMIL, India). Leaf -N, -P, -K and -Ca content were expressed in terms of percent dry weight.

Nodule-nitrogen and leghemoglobin content

Nodule-N content was estimated similarly to Lindner (1944).

Leghemoglobin (Lb) content in fresh nodules was determined as described by Sadasivam and Manickam (2008). The solution's OD was recorded at 556 and 539 nm. The Lb content was calculated using the following formula:

$$\text{Lb concentration (mM)} = \frac{\text{OD } 556 - \text{OD } 539 \times 2D}{23.4}$$

where OD_{556} and OD_{539} represented absorbance (OD) and D is the initial dilution.

Seed-protein content

The seed-protein content was estimated using the method of Lowry *et al.* (1951). Hyacinth bean seed was obtained at 150 DAS and ground to a powder using a mortar and pestle. The seed powder was transferred to a mortar containing 5% cold trichloroacetic acid (TCA, E. Merck India Ltd., Mumbai, India). Finally, extracted protein was measured at 660 nm using a spectrophotometer. The reading was compared with a calibration curve obtained by using a known dilution of standard egg albumin solution and the percent seed protein content was calculated on a dry weight basis.

Seed-carbohydrate content

The carbohydrate content in seeds was analyzed as described by Sadasivam and Manickam (2008). Hyacinth bean powder (100 mg) was poured into a tube containing boiling sulphuric acid and centrifuged at 4,000 rpm. Four mL of anthrone reagent was added and the resulting dark green colour was recorded at 630 nm. The reading was compared with the calibration curve obtained using a known dilution of a glucose standard and the per cent carbohydrate content was calculated on a dry weight basis.

Tyrosinase (polyphenol oxidase) activity

Tyrosinase (EC 1.14.18.1) was extracted according to the method used by Paul and Gowda (2000). The enzyme activity was assayed spectrophotometerically using the procedure of Cosetang and Lee (1987). The enzyme assay mixture contained 0.9 mL of 0.05 M sodium acetate buffer (pH 4.5), 0.1 mL of substrate (L-3,4-dihydroxy phenylalanine) (L-DOPA) and 10-100 µg of the enzyme extract. The optical density of the coloured solution developed due to formation of the compound dopachrome and was recorded at 480 nm. One unit of the enzyme activity corresponded to an amount of enzyme that caused an increase in the absorbance of 0.001 min^{-1} at 25°C. The reference cuvette contained all the ingredients except the enzyme in a final volume of 1 mL. The activity of tyrosinase was expressed as U mg^{-1} protein.

Statistical analysis

The data were statistically analysed using analysis of variance (ANOVA) by SPSS (ver. 12; SPSS Inc., Chicago, IL, USA). The treatment means were separated by Duncan's multiple range test ($p< 0.05$) using different letters in tables (means of three replicates ± SE). LSD ($p\leq0.05$) was employed above bars of each figure to separate the means.

RESULTS

According to plant growth stages, growth, physiological, biochemical, yield and quality attributes were recorded at 60, 90, 120 and 150 DAS. The optimum treatment (Ca_3) gave the highest values for most of the attributes at 90 DAS.

Growth attributes

At 60, 90 and 120 DAS, the effect of calcium application was significant on all growth attributes studied (fresh and dry weights $plant^{-1}$, number of nodules and dry weight of nodules $plant^{-1}$) (**Table 1**). Ca_3 proved superior to the rest of the Ca levels, giving maximum fresh weight at 60, 90 and 120 DAS. Ca_3 produced 25.3, 31.6 and 29.2% higher values than the control for fresh weight at 60, 90 and 120 DAS, respectively. Ca_0 resulted in the poorest performance (**Table 1**). Ca_3 also enhanced the dry weight by 40.6, 54.2 and 35.3% over the control plants at 60, 90 and 120 DAS, respectively (**Table 1**). However, the effect of the Ca_4 treatment was statistically at par to Ca_3 at all three stages for the attributes studied (**Table 1**).

Treatment Ca_3 produced the maximum number of nodules $plant^{-1}$. Ca_3 increased the nodule number by 41.3, 43.8 and 38.3% at 60, 90 and 120 DAS, respectively, when compared to the control (Ca_0) (**Table 1**). The dry weight of nodules was also enhanced significantly by Ca application at all crop stages when compared to the control. Ca_3 gave the highest dry weight of nodules, exceeding the control by 27.6, 35.3 and 32.2%, at 60, 90 and 120 DAS, respectively. For all growth stages, Ca_4 was statistically similar to Ca_3 (**Table 1**).

Yield attributes

Ca application affected all the yield attributes significantly except number of seeds pod^{-1} and 100-seed weight (**Table 2**). Of the five Ca treatments, Ca_3 produced maximum number of pods $plant^{-1}$ (28.7% higher than Ca_0). However, the effect of Ca_4 was similar to that of Ca_3 (**Table 2**). Seed yield was significantly affected by Ca levels and a progressive increase was observed up to Ca_3 (**Table 2**). The maximum seed yield was recorded by Ca_3 (30.3% higher than the control).

Table 1 Effect of five levels of calcium (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on plant fresh and dry weights (g), number and dry weight (g) of nodules plant^{-1} of hyacinth bean (*Lablab purpureus* L.) at 60, 90 and 120 DAS (Means of three replicates). The data shown are means of three replicates ± SE. Means within a column followed by the same letters are not significantly different ($p \leq 0.05$)

Attributes	DAS	Calcium (mg Ca kg^{-1} soil)				
		Ca_0	Ca_1	Ca_2	Ca_3	Ca_4
Fresh weight plant^{-1}	60	23.42 ± 0.62 c	25.44 ± 0.64 b	27.25 ± 0.63 b	29.35 ± 0.50 a	29.12 ± 0.96 a
	90	50.62 ± 1.14 d	55.84 ± 1.19 c	60.36 ± 1.21 b	66.60 ± 1.18 a	66.35 ± 1.46 a
	120	68.21 ± 1.18 d	75.46 ± 1.24 c	79.64 ± 1.16 b	88.12 ± 1.29 a	88.76 ± 0.96 a
Dry weight plant^{-1}	60	4.70 ± 0.16 c	5.21 ± 0.14 c	6.00 ± 0.28 b	6.61 ± 0.16 a	6.56 ± 0.09 a
	90	9.96 ± 0.15 d	12.20 ± 0.15 c	13.47 ± 0.20 b	15.36 ± 0.31 a	14.57 ± 0.36 a
	120	14.48 ± 0.24 d	16.30 ± 0.23 c	17.44 ± 0.22 b	19.59 ± 0.17 a	19.34 ± 0.24 a
Number of nodules plant^{-1}	60	28.3 ± 0.87 d	32.7 ± 0.35 c	35.3 ± 0.98 b	40.0 ± 0.58 a	40.0 ± 0.58 a
	90	45.7 ± 1.08 d	54.3 ± 1.15 c	60.0 ± 1.15 b	65.7 ± 1.16 a	64.0 ± 1.15 a
	120	31.3 ± 0.81 d	35.3 ± 0.98 c	38.7 ± 0.75 b	43.3 ± 0.98 a	42.0 ± 1.03 a
Dry weight of nodules plant^{-1}	60	0.246 ± 0.007 c	0.277 ± 0.005 b	0.291 ± 0.004 b	0.314 ± 0.006 a	0.315 ± 0.005 a
	90	0.434 ± 0.009 c	0.515 ± 0.003 b	0.525 ± 0.008 b	0.587 ± 0.009 a	0.564 ± 0.006 a
	120	0.276 ± 0.003 d	0.308 ± 0.006 c	0.341 ± 0.005 b	0.365 ± 0.007 a	0.346 ± 0.002 ab

Table 2 Effect of five levels of calcium (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on number of pods plant^{-1}, number of seeds pod^{-1}, 100-seed weight (g) and seed-yield (g) plant^{-1} of hyacinth bean (*Lablab purpureus* L.) at 150 DAS (Means of three replicates). The data shown are means of three replicates ± SE. Means within a column followed by the same letters are not significantly different ($p \leq 0.05$)

Attributes	Calcium (mg Ca kg^{-1} soil)				
	Ca_0	Ca_1	Ca_2	Ca_3	Ca_4
Number of pods plant^{-1}	41.5 ± 0.69 c	43.7 ± 0.81 bc	45.6 ± 0.81 b	53.4 ± 0.75 a	53.0 ± 0.40 a
Number of seeds pod^{-1}	4.4 ± 0.35 a	4.3 ± 0.32 a	5.0 ± 0.0.06 a	5.0 ± 0.0.06 a	4.3 ± 0.32 a
100-seed weight	33.10 ± 0.06 a	33.15 ± 0.05 a	33.21 ± 0.08 a	33.30 ± 0.07 a	33.21 ± 0.08 a
Seed-yield plant^{-1}	44.82 ± 0.17 d	47.35 ± 0.19 c	50.59 ± 0.16 b	58.42 ± 0.17 a	57.86 ± 0.25 a

Physiological and biochemical attributes

Ca at the Ca_3 level accelerated the net photosynthetic rate, transpiration rate and stomatal conductance by 24.8, 13.7 and 14.1%, respectively when compared to the control plants (**Fig. 1**). Total Chl and carotenoid content, nitrate reductase activity, carbonic anhydrase activity, leaf-N, -P, -K and -Ca contents, nodule-N and Lb contents, studied at 60, 90 and 120 DAS, were significantly increased by Ca application (**Fig. 1-3**). The application of Ca to hyacinth bean plants favourably influenced the concentrations of the photosynthetic pigments (Chl and carotenoid) at 60, 90 and 120 DAS (**Fig. 1**). The Chlorophyll and carotenoid contents increased with an increase in Ca level. Compared to the control, treatment Ca_3 increased the total Chl content by 14.3, 19.4 and 17.6% at 60, 90 and 120 DAS, respectively (**Fig. 1**). Total carotenoid content was maximally increased by Ca treatment at 60, 90 and 120 DAS. Of the five levels, Ca_3 and Ca_4 resulted in higher carotenoid content when compared to the control. The maximum increase in carotenoid content shown by Ca_3-treated plants was 9.5, 15.6 and 14.3% at 60, 90 and 120 DAS, respectively, compared to the non-treated plants (**Fig. 1**).

All the Ca levels enhanced NR activity over the control at 60, 90 and 120 DAS. The maximum NR activity was recorded by the treatment Ca_3 being 41.1, 36.7 and 36.1% higher than the control (Ca_0) at 60, 90 and 120 DAS, respectively (**Fig. 2**). CA activity was significantly affected by Ca application at all three growth stages (**Fig. 2**). CA activity was highest in Ca_3-treated plants at all three growth stages. Compared to the control (Ca_0) Ca_3 treatment increased CA activity by 16.7, 14.6 and 12.2% at 60, 90 and 120 DAS, respectively (**Fig. 2**).

A progressive increase in nodule-N content at 60 DAS was observed due to the application of Ca. The maximum value (in Ca_3-treated plants) was 6.7, 4.3 and 4.8% at 60, 90 and 120 DAS, respectively, when compared to the control plants (**Fig. 2**). The effect of Ca application on Lb content was also positive at 60, 90 and 120 DAS. The best results were provided by the Ca_3 level increasing the Lb content by 22.4, 20.4 and 16.9% at 60, 90 and 120 DAS, respectively over the non-treated plants (**Fig. 2**). Ca_4 was similar to Ca_3 at all growth stages (**Fig. 2**).

Ca treatments also showed a positive effect on leaf-N content at 60, 90 and 120 DAS (**Fig. 3**). Of the five levels, Ca_3 increased the leaf-N content significantly by 24.3, 19.2 and 17.3% at 60, 90 and 120 DAS, respectively. The maximum increase in P content was given by Ca_3 (17.2, 24.2 and 22.6%, higher than the control at 60, 90 and 120 DAS, respectively). The control (Ca_0) gave the lowest values at all stages (**Fig. 3**). Maximum leaf-K content was noted in the Ca_3 treatment at all growth stages. Ca_3-treated plants showed an increase of K content by 23.5, 20.1 and 19.2% at 60, 90 and 120 DAS, respectively over Ca_0 (**Fig. 3**). Ca application also contributed to an increase in total Ca content at 60, 90 and 120 DAS. Ca_3 maximally enhanced the Ca content by 29.4, 16.8 and 26.7% at 60, 90 and 120 DAS, respectively compared to Ca_0, which registered the minimum values at all three stages (**Fig. 3**).

The data presented in **Fig. 4** reveals that protein content was maximally accumulated in the seeds as a result of basal Ca application. Among all Ca levels, Ca_3 proved to be best and gave 11.6% higher seed-protein content than the control (**Fig. 4**). The effect of Ca_4 was statistically equal to that of Ca_3. Similarly, the carbohydrate content increased progressively according to the increment in Ca level. Compared to the control, Ca_3 treatment showed a 8.73% increase in carbohydrate content at 150 DAS (**Fig. 4**). There was enhanced tyrosinase activity in Ca-treated plants compared to the control plants. Treatment Ca_3 increased tyrosinase activity by 20.3% over the control at 150 DAS (**Fig. 4**).

DISCUSSION

Effect of calcium on growth and yield attributes

Ca_3 level of Ca (120 mg Ca kg^{-1} soil) proved to be best in hyacinth bean. Ca_3 treatment increased plant fresh and dry weights significantly compared to the controls at all plant growth stages (**Table 1**). Among the growth attributes, dry weight is considered the most important parameter because all physiological and biochemical activities lead to the production of dry matter. Utilization of internal Ca for different physiological and metabolic processes contributes to the biomass production (Dieter *et al*. 1984). In fact, Ca controls a wide variety of physiological and cellular processes (Nayyar 2003) in the plant and hence plant growth depends on the availability of Ca in the soil. Furthermore, Ca regulates many physiological functions such as, cell elongation, cell division and differentiation, stabilization of cell wall and

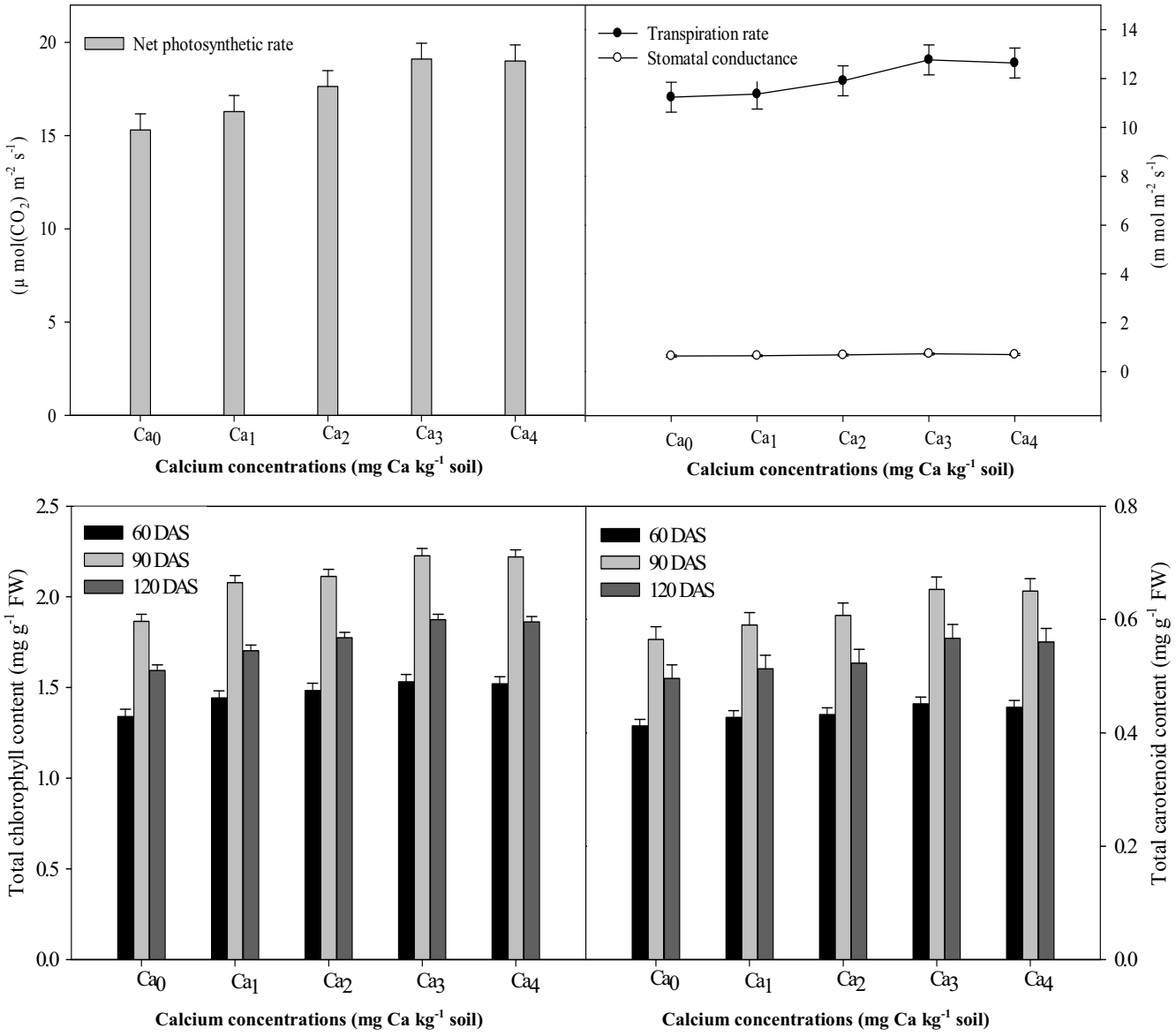

Fig. 1 Effect of five calcium levels (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on net photosynthetic rate, transpiration rate and stomatal conductance (90 DAS), total chlorophyll and carotenoid content of hyacinth bean (*Lablab purpureus* L.) studied at 60, 90 and 120 DAS (Means of three replicates). LSD ($p \leq 0.05$) was employed at the top of bars to separate the means.

plasma membrane, membrane stability and cell integrity, polymerization of proteins, regulation of enzymes and fruit development (Kirkby and Pilbeam 1984; Marschner 2002; Nayyar 2003; White and Broadley 2003; Hirschi 2004). The most important function of cytoplasmic Ca is related with the Ca-binding protein (CaM). Ca is sequestered by CaM (White and Broadley 2003) and is crucial in plant growth and development by regulating cell division and modulating the enzyme activities of the cells (Rasmussen and Means 1989). The ameliorating effect of Ca on fresh and dry weights of this medicinal plant is similar to the results of Savithramma (2004) for *Boswellia ovalifoliolata* Bal. & Henry, *Pterocarpus santalinus* L. and *Syzygium alternifolium*, Naeem *et al*. (2005) for mungbean (*Vigna radiata* L.), Arshi *et al*. (2005, 2006) for senna (*Cassia angustifolia* Vahl.) and chicory (*Cichorium intybus* L.), Khan and Naeem (2006) for mungbean (*Vigna radiata* L.), Naeem and Khan (2006) for *Cassia tora* L., Murillo-Amador *et al*. (2007) for cowpea (*Vigna unguiculata* L.), kidney bean (*Phaseolus vulgaris* L.), Dordas (2009) for oregano (*Origanum vulgare* sp. *Hirtum*) and Naeem *et al*. (2009a) for senna sophera (*Cassia sophera* L.). All these authors observed a positive effect of calcium application on different growth characteristics in their study.

The effect of soil-applied Ca on the number and dry weight of nodules was significant at 60, 90 and 120 DAS, as shown in **Table 1**. Of all the Ca treatments, Ca_3 proved most effective compared to other treatments and exhibited a considerable improvement in the number and dry weight of nodules at all growth stages. The number and dry weight of nodules were highest at 90 DAS and lowest at 120 DAS as observed in the present study (**Table 1**). The favourable response of plants to applied Ca regarding number and dry weight of nodules was most probably due to sufficient production and supply of photosynthates from the source to sink sites. Generally, legumes require a higher amount of Ca, especially since they depend upon symbiotic N fixation. Soil-Ca increases nodulation on the basis of the current understanding of the role of Ca in the nodulation process (Purcino and Lynd 1986; Brauer *et al*. 2002). In fact, a higher amount of Ca is needed for the formation of nodules than for N fixation and plant growth in legumes (Brauer *et al*. 2002). It is well documented that Ca increases the number of nodules and is required at the early stages of nodule initiation (Balatti *et al.* 1993; Brauer *et al*. 2002; El-Hamdaoui *et al*. 2003). Favourable effects of Ca application on the number and dry weight of nodules has also been reported by Bell *et al*. (1989a, 1989b) for tropical food legumes, Pijnenborg and Lei (1990), Brauer *et al*. (2002) and Grewal and Williams (2003) for alfalfa (*Medicago sativa* L.),

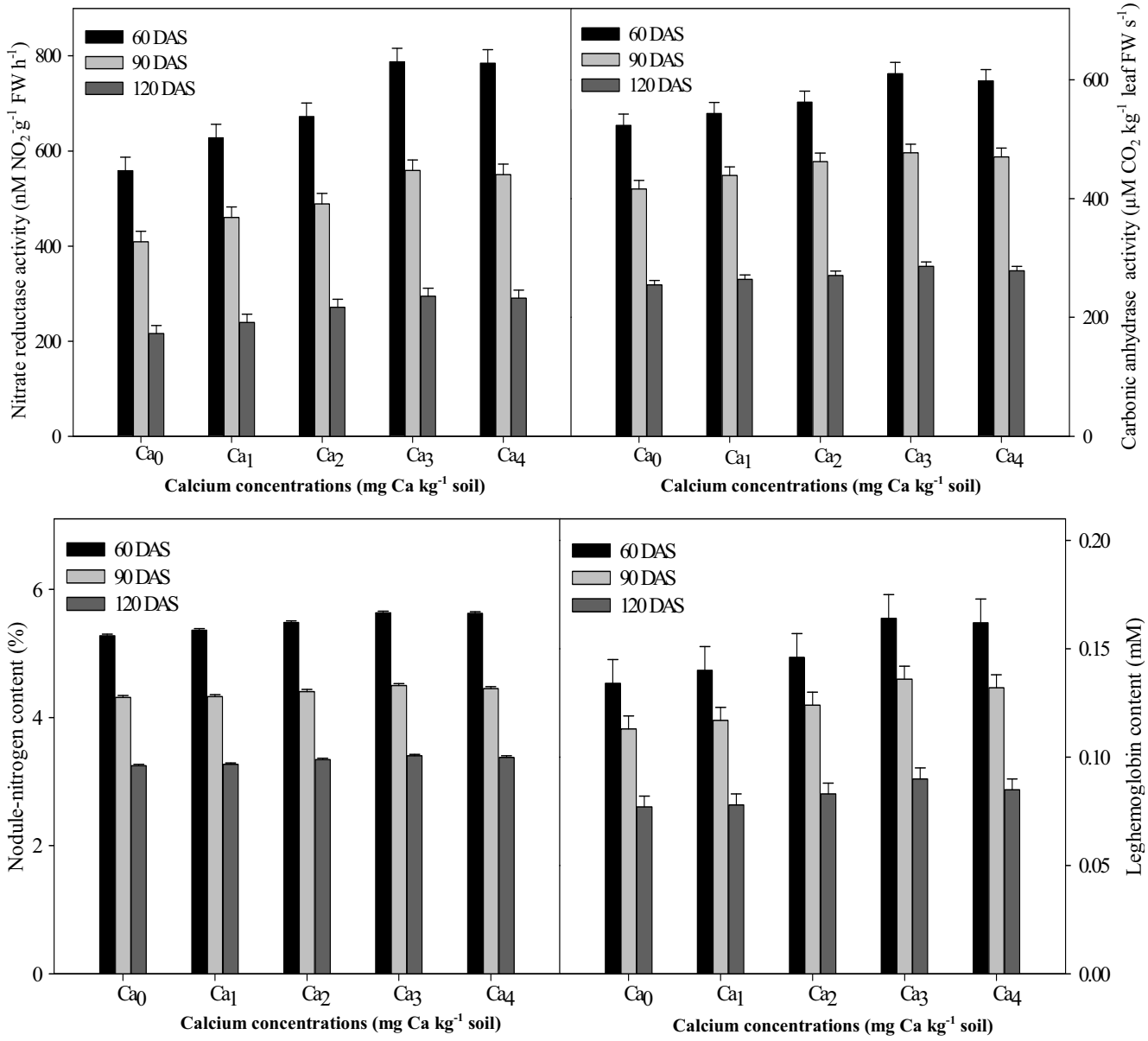

Fig. 2 Effect of five calcium levels (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on nitrate reductase activity and carbonic anhydrase activity, nodule-nitrogen content and leghemoglobin content of hyacinth bean (*Lablab purpureus* L.) studied at 60, 90 and 120 DAS (Means of three replicates). LSD ($p \leq 0.05$) was employed at the top of bars to separate the means.

Naeem *et al*. (2005) and Khan and Naeem (2006) for mungbean (*Vigna radiata* L), respectively.

All Ca levels had a significant effect on yield attributes including number of pods and seed yield plant^{-1}. A greater seed yield of Ca-treated plants (Ca_3) was mainly due to increased number of pods (**Table 2**). The role of Ca in increasing seed-yield can possibly be ascribed to its involvement in the processes of photosynthesis and translocation of carbohydrates to younger pods (Sawan *et al*. 2001). This indicates that availability of Ca at an early stage of growth helps in plant active growth and metabolism, which ultimately leads to an increase in yield. Our results are similar to results of other researchers in this regard (Khan *et al*. 2001; Sarkar and Malik 2001; Arshi *et al*. 2006; Khan and Naeem 2006; Murillo-Amador *et al*. 2007; Dordas 2009; Naeem *et al*. 2009a). The above researchers found a significant effect of Ca application on yield attributes in case of mustard (*Brassica juncea* L. Czern & Coss), wheat (*Triticum aestivum* L.), grasspea (*Lathyrus sativus* L.), senna (*Senna aungustifolia* L.), mungbean (*Vigna radiata* L.), cowpea (*Vigna unguiculata* (L.) and kidney bean (*Phaseolus vulgaris* L.) and senna sophera (*Cassia sophera* L.), respectively.

Effect of calcium on physiological and biochemical attributes

Total Chl and carotenoid content were maximally exhibited in response to Ca application at all growth stages (**Fig. 1**). Treatment Ca_3 also enhanced the photosynthetic pigments (Chl and carotenoid content) at all growth stages of hyacinth bean (**Fig. 1**). In the present study, the minimum values for photosynthetic pigments were recorded in non Ca-treated plants (**Fig. 1**). Our results are supported by the findings of Savithramma (2004) for *ovalifoliolata* Bal. & Henry., *Pterocarpus santalinus* L. and *Syzygium alternifolium*, Naeem *et al*. (2005) for mungbean (*Vigna radiata* L.), Arshi *et al*. (2005, 2006) for senna (*Cassia angustifolia* Vahl.) and chicory (*Cichorium intybus* L.), Khan and Naeem (2006) for mungbean (*Vigna radiata* L.), Naeem and Khan (2006) for *Cassia tora* L., Murillo-Amador *et al*. (2007) for cowpea (*Vigna unguiculata* L.) and kidney bean (*Phaseolus vulgaris* L.) and Naeem *et al*. (2009a) for senna sophera (*Cassia sophera* L.). They observed a positive effect of calcium treatment in their study for the above mentioned plants.

The contents of total Chl and carotenoid in hyacinth

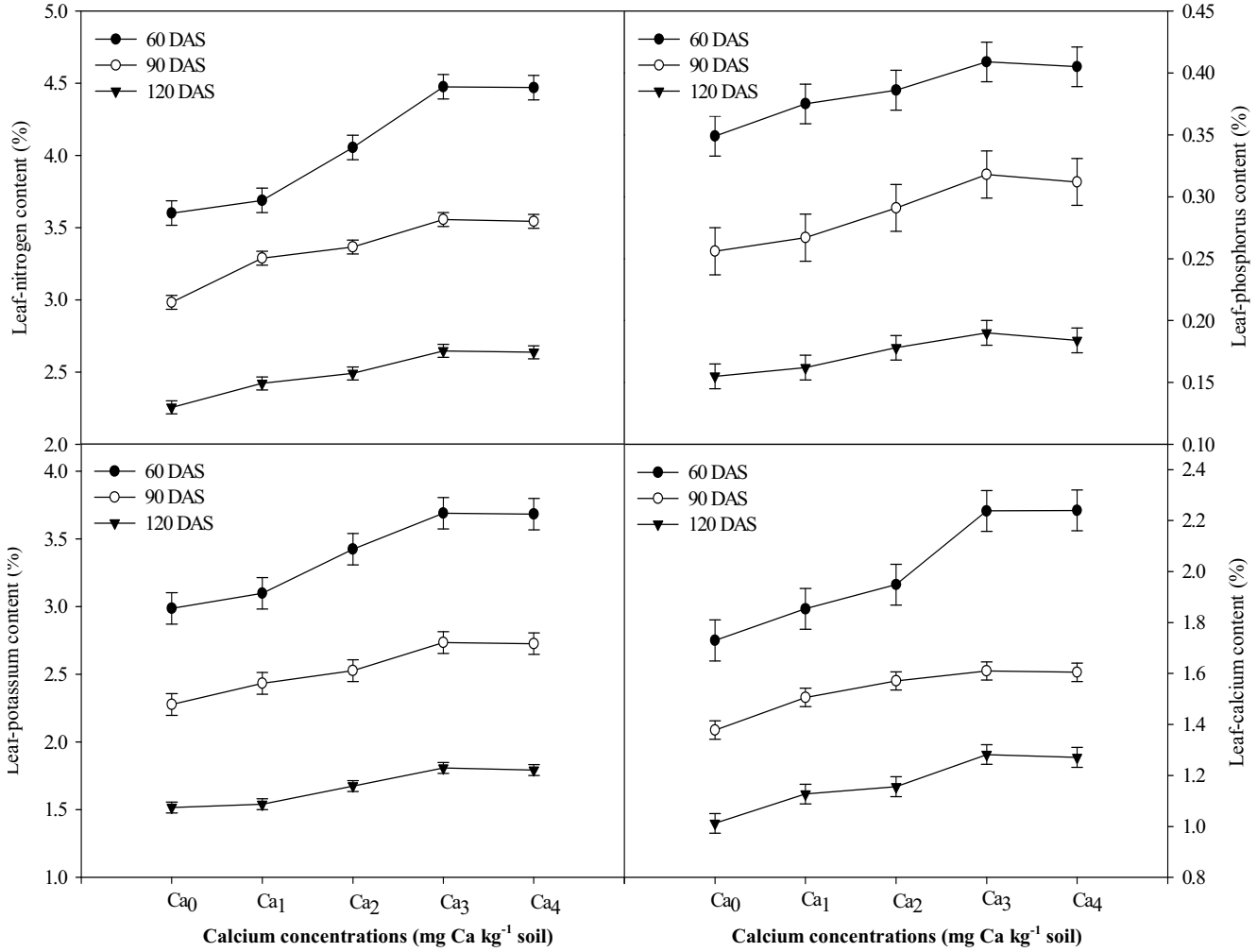

Fig. 3 Effect of five calcium levels (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on leaf -nitrogen, phosphorus, potassium and calcium content of hyacinth bean (*Lablab purpureus* L.) studied at 60, 90 and 120 DAS (Means of three replicates). LSD ($p \leq 0.05$) was employed at the top of bars to separate the means.

bean leaves was maximum at the flowering stage (90 DAS) and minimum levels were observed at the pod-filling stage (120 DAS), respectively (**Fig. 1**). Generally, degradation of the Chl and carotenoid content presumably begin with leaf senescence as a result of the aging effect, however the highest concentrations of photosynthetic pigments occur when the leaf blade is fully mature for photosynthesis (Lopez-Cantarero *et al*. 1994).

It has been observed that the leaves of Ca-treated plants trap more sunlight to increase the rate of photosynthesis compared to control plants (Khan *et al*. 2001). Furthermore, Ca is very important for plant metabolism in general and photosynthetic processes in particular (Ramalho *et al*. 1995). Ca serves a dual function in photosynthesis including Ca^{2+} ions, binding to photosystem II (PS II) involved in oxygen evolution and provide a structural role in the peripheral antenna assembly (Ramalho *et al*. 1995). Moreover, Ca is effective in protection of Chlorophylls and proteins, as well as in the functional ability of PS-II (Swamy *et al*. 1995; Savithramma 2004).

Nitrate reductase activity was maximum in the soil-applied Ca_3-treated plants when compared to the control plants at all growth stages (**Fig. 2**). The reduction of nitrate in hyacinth bean leaves depends upon the presence of Ca ions and a metabolic connection exists between nitrate assimilation as well as Ca content of leaves (Dekock *et al*. 1979). However, the role of calcium in N metabolism depends upon the N source. If, the N source is NH_4^+, the application of Ca increases NH_4^+ uptake and improves N utilization in the plant, resulting in increased production of dry matter (Fenn *et al*. 1994). The role of Ca as an activator for nitrate reductase activity in leaves has been reported by Sane *et al*. (1987), Fenn *et al*. (1994), Ruiz *et al*. (1999), Naeem and Khan (2006) and Naeem *et al*. (2009a). In addition, NR activity decreased with increasing age of the plants, comparatively slowly from the vegetative to the flowering stage and it decreases rapidly from the flowering to the fruiting stage (Naeem and Khan 2009).

CA enzyme plays an active role in photosynthesis, which is established by its presence in all photosynthesizing tissues. This enzyme catalyzes the reversible hydration of CO_2, thereby increasing its availability for RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase (Badger and Price 1994; Khan *et al*. 2004). The enhancement in CA activity due to external Ca application could be the result of readily availability of this nutrient at the site of its metabolism. Furthermore, the increase in CA activity would enhance the rate of CO_2 assimilation. Consequently this was reflected in production of enhanced dry matter (**Table 1**) presumably owing to the improvement in the net photosynthetic rate. A probable cause for the enhancement of CA activity might be the influence of Ca on the *de novo* synthesis of CA, which involves translation/transcription of the plant cells (Okabe *et al*. 1980).

The effect of Ca on leaf-N, -P, -K and -Ca content was positively significant at all three growth stages. The control plants, which were not supplied with these nutrients, had to totally depend on soil nutrients present in low concentrations. On the other hand, Ca-supplied plants received an adequate supply, ensuring continuous absorption by roots followed by smooth translocation to the foliage. The enhanced content of N, P, K and Ca due to Ca supply might be because of enhanced dry matter accumulation, effective nodulation, synthesis of higher amounts of Chlorophyll and

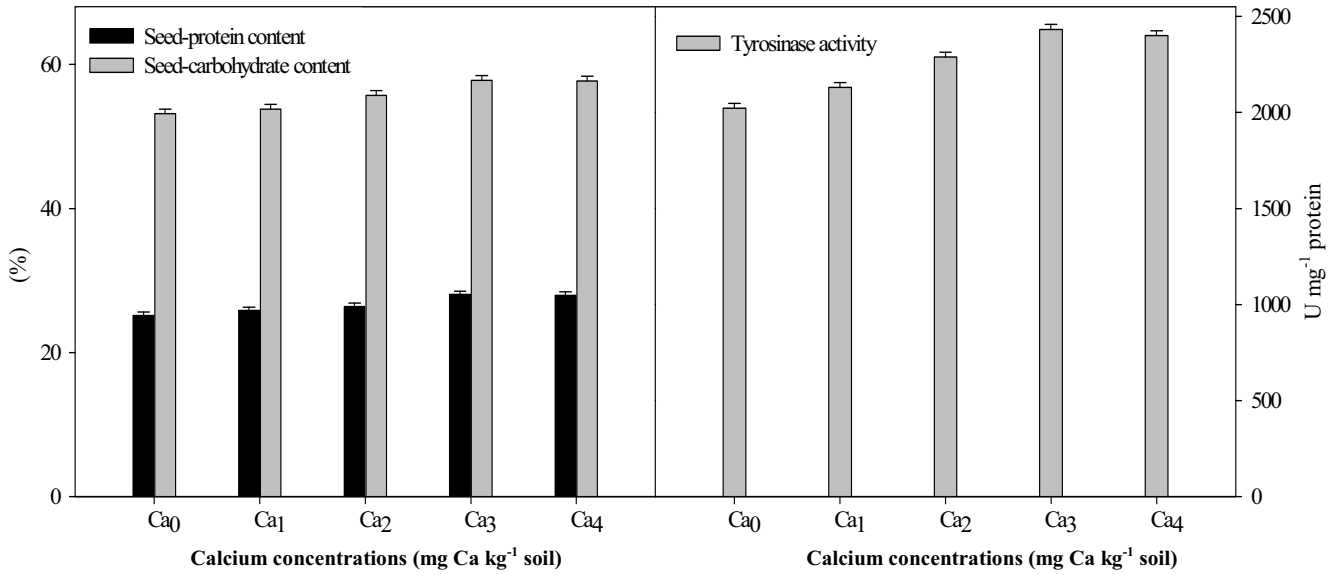

Fig. 4 Effect of five calcium levels (Ca_0, Ca_1, Ca_2, Ca_3 and Ca_4, respectively) on seed-protein content, -carbohydrate content and tyrosinase activity of hyacinth bean (*Lablab purpureus* L.) studied at 150 DAS (Means of three replicates). LSD ($p \leq 0.05$) was employed at the top of bars to separate the means.

carotenoids and increased activity of NR (**Table 1, Fig. 1**). In fact, it has been reported that the effect of soil Ca concentration on nutrient absorption rates plays an important role in nutrient uptake from soil by legume crops (Andrew and Johnson 1976; Bell *et al*. 1989a). In fact, an increasing supply of calcium has been reported to markedly augment the absorption of N, P, K and Ca contents in legumes (Bell *et al*. 1989a). Leaf-N, -P, -K and -Ca content decreased with increasing age of the plants, as presented in **Fig. 3**. Such a decrease in N, P, K and Ca contents of leaves may be due to continuous utilization of these nutrients by the developing pods and their translocation from vegetative (sink) to reproductive parts (source). These results are similar to the results of Naeem and Khan (2006) for *Cassia tora* L. and Naeem *et al.* (2009a) for *Cassia sophera* L.

In comparison to control plants, we observed a positive effect of Ca application on nodule-N and Lb contents in hyacinth bean (**Fig. 2**) as noted at 60, 90 and 120 DAS. Our study reveals that Ca-treated plants produce maximum nodule-N and Lb contents compared to untreated plants. Further, Ca might have increased soil pH, resulting in increased nodulation in legumes through calcium binding protein Rhicadhesin (Smit *et al*. 1989; Kiranmayee *et al.* 1995). The results of Suttan *et al*. (1994) indicated the presence of a Ca-binding nodulation protein (*NodO*) from *Rhizobium leguminosarum* biovar *viciae*. This suggests that Ca could be a regulator agent in N-fixation in nodules. The results of the present study are also supported by the results of Kiranmayee *et al*. (1995), Carpena *et al*. (2000), Brauer *et al*. (2002), El-Hamdaoui *et al*. (2003) and Khan and Naeem (2006) for cowpea (*Vigna unguculata* (L.), pea (*Pisum sativum* L.), clover (*Trifolium repens* L.) and mungbean (*Vigna radiata* L.), respectively in which nodulation was increased by applying the following levels of calcium: (2 and 3.6 mM Ca), (0.68, 1.36, 2.72 and 5.44 mM Ca) and (0 and 35 kg Ca ha^{-1}). Generally, leguminous crop require a higher amount of Ca, especially since they depend upon symbiotic N fixation. As we have observed that external Ca supplied to the crop enhanced the capacity of N fixation (nodulation, nodule-N and Lb content) in hyacinth bean plants.

Additionally, this study reveals that nodule-N and Lb contents decreased slowly from 60 to 90 DAS and sharply from 90 to 120 DAS (**Fig. 2**). In this study, nodule-N and Lb contents declined with the age of the crop. It is logical to state that bacteria in the nodules depend upon the plants for their energy source. Therefore, prior to flowering, nodules can compete as a carbohydrate sink. However, once the plant enters the reproductive phase, the seeds act as a stronger sink for carbohydrates than nodules. Consequently the latter showed comparatively a decreased dry weight of nodules and lower N-fixing capacity (Samiullah and Khan 2003; Naeem *et al*. 2009b).

Effect of calcium on quality attributes

Like leaf-nutrients and yield attributes, seed-protein content was also significantly enhanced by the application of Ca. Treatment Ca_3 enhanced the seed-protein content over no-Ca treatments considerably more than the other treatments (**Fig. 4**). It is an established fact that Ca binds certain proteins known as CaMs that are directly stimulated by calcium. These proteins activate several enzymes through phosphorylation (Marschner 2002). Moreover, Ca increased accumulation of N, P, K and Ca contents that might have also been responsible for enhanced proteins synthesis during seed development. A favourable effect of Ca application on seed-protein content has also been reported by Savithramma (2004), Naeem and Khan (2006) and Naeem *et al*. (2009a) in case of *Boswellia ovalifoliolata* Bal. & Henry, *Pterocarpus santalinus* L. and *Syzygium alternifoliu*, *Cassia tora* L. and senna sophera (*Cassia sophera* L.), respectively. The above researchers applied different levels of calcium (0, 10, 20, 30, 40 and 50 mM Ca) and (0, 40, 80, 120 and 160 mg Ca kg^{-1} soil) in their study, and found that seed protein content was increased by 19.6, 33.3 and 12.8 %, respectively in comparison to control. Furthermore, we observed a beneficial effect of Ca application on carbohydrate content (**Fig. 4**).

The tyrosinase/polyphenol oxidase (PPO) activity was higher in Ca-treated plants than in control plants (**Fig. 4**). In this study, the enzyme responsible for phenolic oxidation was positively affected by Ca. In fact, it has been reported that Ca acts on PPO, which normally is found in its latent form, modifying the conformational state of this enzyme and thus consequently boosting the PPO activity. Soderhall (1995) and Ruiz *et al*. (2003) also found a positive effect of Ca on the activity of this enzyme in carrot (*Daucus carota* L) and tobacco (*Nicotiana tobacum* L.) plants.

CONCLUSION

On the basis of our findings it may be postulated that a basal dose of Ca (120 mg kg^{-1} soil) improved the overall performance of the crop. The low level of Ca in this region's soil may be one of the main causes of poor hyacinth bean yield. Thus, the economic dose of Ca can be recom-

mended for maximizing the productivity and quality of hyacinth bean used as a drug in the modern as well as traditional systems of medicine.

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