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Changes in nitrogen metabolism of *Vigna radiata* in response to elevated CO_2

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Abstract

With the aim to determine the effects of CO_2 on nitrogen metabolism mungbean (*Vigna radiata*) plants were grown from seedling emergence to maturity inside open top chambers under ambient CO_2 (CA, 350 ± 25 μmol mol^{-1}) and elevated CO_2 (CE, 600 ± 50 μmol mol^{-1}) concentrations at the Indian Agricultural Research Institute, New Delhi. Leaflet blades of the same physiological age were sampled at 20, 35 and 50 d after germination. Total nitrogen concentration in dry mass was consistently lower under CE than in CA. Non-protein nitrogen and protein nitrogen were also decreased under CE. Total soluble protein content also decreased up to 35 d after germination under CE. However, a 27 % increase in protein content at 50 d after germination due to CE was observed. A significant decrease in total free amino acid under CE at 20 d after germination was observed. CE also brought about a remarkable decrease in the activity of nitrate reductase in leaves at 20 d after germination but increase at 35 d and 50 d after germination. Nitrogenase activity increased at all growth stages due to CE. Although total harvested leaves of CE plants accumulated more nitrogen, the relative amount of nitrogen on a percentage basis was low, probably due to a comparatively greater accumulation of sugars in the leaves of CE plants.

Additional key words: mungbean, nitrate reductase, nitrogen fixation, protein content.

Introduction

Earlier studies indicated that CO_2 enrichment of C_3 plants can result in accumulation of starch and other non-structural polysaccharides in the leaves and increase in total biomass of the plants (Warrick and Gifford 1986, Sharma and Sengupta 1990, Ulman *et al.* 2000). Enhanced growth and photosynthetic rate under elevated CO_2 is also reported in mungbean (Gifford *et al.* 1985) and soybean (Griffin and Luo 1999).

Carbon and nitrogen metabolism are linked in several ways. At the cellular level, uptake of nitrate, reduction of NO_3^- and incorporation of ammonium into amino acids and protein are dependent on carbon metabolism for the production of energy, reductant and carbon skeletons. Photosynthetic rate of a plant, on the other hand, is influenced by the nitrogen status (Evans 1989).

Some of the earlier experiments indicated that CO_2 enrichment lowered the concentration of nitrogen in leaves and altered the distribution pattern of nitrogen within the plant (Larigauderie *et al.* 1988, Hocking and Meyer 1985, Uprety and Rabha 1999). It has been shown that less enzyme (Rubisco) and leaf protein nitrogen are required to produce a given amount of dry matter (Warrick and Gifford 1986, Allen *et al.* 1988). Consequently, CO_2 enrichment may improve the nitrogen use efficiency of C_3 crop species (Schmitt and Edwards 1981, Goudriaan and de Ruiter 1983), weeds (Hocking and Meyer 1985) and grasses (Larigauderie *et al.* 1988). In contrast to the above findings, plants accumulated more nitrogen in wheat under elevated CO_2 conditions but the proportional increase in the nitrogen content was not as great as dry matter (Hocking and Meyer 1991). Nitrogen metabolism in legumes under high CO_2 will be

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Abbreviations: CA - ambient CO_2 (350 ± 25 μmol mol^{-1}); CE - elevated CO_2 (600 ± 50 μmol mol^{-1}); DM - dry mass; FM - fresh mass; LA - leaf area; NR - nitrate reductase, TNA - nitrogenase.
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different, because symbiotic nitrogen fixers receive all of their carbon from photosynthates and provide a source of available nitrogen to their host plant. Earlier experiments have shown that symbiotic nitrogen fixation in many agriculturally important legumes is stimulated greatly when the plants were grown in elevated level of atmospheric CO_2 (Masterson and Sherwood 1978, Finn and Brun 1982) but the response of mungbean to elevated CO_2 has not been sufficiently described.

This experimental study was designed to determine whether nodulation and symbiotic nitrogen fixation in mungbean is stimulated by elevated CO_2 analogously to the responses in other agronomic legumes. The emphasis of this study is to understand changes in nitrogen metabolism due to elevated CO_2 in summer grown mungbean.

Materials and methods

Plants and treatments: Mungbean (*Vigna radiata* (L.) Wilczek cv. PS16) was grown in pots at the Plant Physiology Division, Indian Agricultural Research Institute, New Delhi (India) from 10 April, 1999 to 18 June, 1999. In the experiment, a modified open top chamber (OTC), as described by Rogers *et al.* (1983) was developed to study crop responses to elevated CO_2. The height and diameter of the OTC was 1.8 m and 1.6 m, respectively. One set of plants was grown under ambient CO_2 (CA, 350 ± 25 μmol mol^{-1}) and other group of plants was grown under elevated CO_2 (CE, 600 ± 50 μmol mol^{-1}) in a naturally lit OTC. A total of 16 pots were used in each chamber for the treatment. The concentration of CO_2 in the chamber was monitored by an infra red gas analyser (*LiCOR 6200*, Lincoln, USA).

Leaflet blades of the same physiological age were sampled in May - June, 1999 at 20, 35 and 50 d after germination for analyzing amino acids, protein, nitrate reductase and total nitrogen. Dry matter of leaves was determined after drying in an oven at 80 °C for 4 h and then at 60 °C till a constant mass using an electronic balance (*Sartorius 1212 MP*, Göttingen, Germany)

Assay of nitrate reductase activity (EC 1.6.6.1): Three samples of 5 leaves of the same physiological age were collected at midday from both treatments and carried to the laboratory in an ice bucket. An *in vivo* procedure was used (Klepper *et al.* 1971), in which fresh leaves were finely chopped in a cold room at 3 °C and 0.3 g subsamples transferred to flasks containing 2.5 cm^3 of 60 mM potassium nitrate, 2.5 cm^3 of 0.1 M phosphate buffer (pH 7.5) and 0.1M *n*-propanol. The solution was infiltrated into the leaf tissue by creating a vacuum in the tubes using a vacuum pump. The infiltration was done for 5 min and then the infiltrated material was kept for incubation at 35 °C for 20 min; boiling the reaction mixture on a hot plate for 2 min stopped the reaction. A volume of 0.3 cm^3 was taken in test tubes with 3 replicates to assay NO_2^-. The absorbance was recorded at 535 nm by using spectrophotometer *Spectronic-20* (*Bausch and Lamb*, Rochester, USA)

Total nitrogen concentration: Non-protein and protein nitrogen portions in the form of reduced nitrogen in the trichloro acetic acid (TCA) preserved leaf material were determined by using *N-Kjeltech Auto 1030* analyzer, following the procedure detailed in *Tecator* manual, 1987 (*Tecator Company*, Hoganas, Sweden).

Leaf samples were collected randomly between plants in polyethylene bags and 0.5 g of leaf from each sample was preserved in 10 cm^3 of 10 % TCA. Homogenized leaves were centrifuged twice at 20 124 *g* for 10 min. Supernatant (non-protein) and residues (protein nitrogen) were subjected to digestion and distillation.

Total soluble protein and amino acids: Leaflets blades were chopped into small pieces and 2 g of leaf material was homogenized in an extraction buffer of approximately 0.5 M Tris-HCl (hydroxymethyl) amino ethane HCl buffer (pH 6.8). The homogenate was centrifuged at 20 124 *g* for 10 min at 4 °C. Soluble protein in the supernatant was measured by the method of Lowry *et al.* (1951).

For the estimation of free amino acids, 0.5 g of leaf material from each treatment was homogenized with 10 cm^3 distilled water. The supernatant was used as a crude extract for amino acid assay. Free amino acid amounts were estimated following the method described by Lee and Takahashi (1966).

Nitrogenase activity in root nodules: Total nitrogenase activity (TNA) of the plants was assayed by the acetylene reduction technique (Hardy *et al.* 1968). Nodulated root systems were carefully uprooted, washed clean of soil, separated from the shoot, and incubated in the dark for 60 min in plastic bottles with 10 cm^3 acetylene. The manipulation of the root system and detachment from the shoot probably resulted in lower values of TNA than would have been obtained from the intact plant (Wheeler *et al.* 1978), but errors due to differential gas diffusion through the soil were thereby avoided. Ethylene production was measured on a *Perkin Elmer Model 2000,* (Boston, USA) gas chromatograph. An external ethylene standard was used. The nodules were removed from the roots, dried at 70 °C for 4 h and then at 60 °C till a constant mass and the dry mass was recorded.

Statistical analysis of the data was done by analysis of variance (*ANOVA*) given by Panse and Sukhatme (1967). The critical difference (CD) values were calculated at 5 % probability level.

Results

A significant increase in leaf mass and total biomass in mungbean was observed under CE. Major difference in leaf dry mass had occurred at 20 d after germination where average dry mass was 0.57 g and 1.33 g for CA and CE grown plants, respectively (Table 1).

Non-protein nitrogen concentrations in leaves of CE grown plants was lower than in CA grown plants at 20 and 35 d after germination but at 50 d after germination, no significant difference was found (Table 1). Protein nitrogen concentration in leaves harvested at 20 d after germination was significantly lower in CE than in CA but no significant difference at 35 and 50 d after germination was found (Table 1). Similarly, total nitrogen concentrations was consistently lower under CE than under CA (Table 1). Although the nitrogen percentage decreased under CE, accumulation of nitrogen in total harvested leaves increased significantly in terms of non-protein, protein and total nitrogen content at early growth stages (Table 2).

Table 1. Growth characteristics, nitrogen, total soluble protein and total free amino acids of mungbean at different growth stages under ambient and elevated CO_2 conditions. Means ± SD of five replications (* - differences between CA and CE significant at $P < 0.05$). SLM - specific leaf mass; LFM - leaf fresh mass; LDM - leaf dry mass; TB - total biomass; NPN - non-protein nitrogen; PN - protein nitrogen; TN - total nitrogen; TSP - total soluble protein; TFAA - total free amino acid.

		SLM	LFM [g plant^{-1}]	LDM [g plant^{-1}]	TB [g plant^{-1}]	NPN [g g^{-1}(d.m.)]	PN [g g^{-1}(d.m.)]	TN [g g^{-1}(d.m.)]	TSP [g g^{-1}(d.m.)]	TFAA [μmol g^{-1}(d.m.)]
20 d	CA	0.51±0.04	3.98±0.39	0.57±0.10	1.03±0.10	13.39±0.91	18.36±0.48	31.75±1.29	32.63±0.78	118.80±5.38
	CE	0.91±0.12*	6.13±0.85*	1.33±0.09*	2.22±0.13*	12.18±0.46	12.30±0.37*	24.46±0.74*	29.43±0.22	85.50±2.91*
35 d	CA	0.59±0.07	6.38±0.36	0.90±0.09	2.21±0.16	14.85±0.55	13.43±0.62	28.28±0.48	52.69±2.36	60.30±4.79
	CE	0.58±0.06	8.93±0.28*	1.53±0.05*	3.90±0.51*	10.61±0.28*	10.97±0.41	21.58±0.58*	50.36±3.69	69.62±6.88
50 d	CA	0.83±0.09	7.81±0.77	1.45±0.09	3.73±0.17	8.98±0.38	11.03±0.56	20.01±0.72	31.50±1.44	74.12±7.08
	CE	0.59±0.05*	9.92±0.45	1.63±0.11	4.30±0.14*	9.14±0.43	10.69±0.35	19.83±0.65	40.14±2.02*	84.02±6.97

Table 2. Nitrate reductase, nitrogenase activity and accumulation of nitrogen, total soluble protein and total free amino acids in total harvested leaves of mungbean at different growth stages under ambient and elevated CO_2 conditions. Means ± SD of five replications (* - differences between CA and CE significant at $P < 0.05$). NPN - non-protein nitrogen; PN - protein nitrogen; TN - total nitrogen; TSP - total soluble protein; TFAA - total free amino acid; NR - nitrate reductase; TNA - nitrogenase.

		NPN [g g^{-1}(d.m.)]	PN [g g^{-1}(d.m.)]	TN [g g^{-1}(d.m.)]	TSP [g g^{-1}(d.m.)]	TFAA [μmol g^{-1}(d.m.)]	NR [μmol(NO_2) g^{-1}(d.m.)]	TNA [nmol(C_2H_4) g^{-1}(d.m.)]
20 d	CA	7.63±1.64	10.47±1.78	18.10±3.40	18.59±3.26	67.22±11.88	3.80±0.64	0.179±0.014
	CE	16.19±0.62*	16.36±1.45*	32.53±2.07*	39.14±2.78*	113.72± 8.08	2.06±0.69*	0.236±0.017
35 d	CA	13.37±1.41	12.09±0.86	25.46±2.07	47.42±4.59	54.27± 5.26	6.29±0.25	0.261±0.004
	CE	16.23±0.91	16.78±0.81*	33.01±1.63*	77.05±2.33*	106.52± 3.21*	7.59±0.62	0.490±0.012*
50 d	CA	13.02±0.51	15.99±0.83	29.01±1.05	45.68±2.98	107.47± 7.01	3.15±0.42	0.176±0.003
	CE	14.89±0.25	17.42±1.02	32.31±1.23	65.43±4.29*	136.95± 8.99*	6.85±1.15*	0.185±0.003

Total soluble protein content also exhibited a continuous decrease up to 35 d after germination under CE. However, a 27.4 % increase in protein content at 50 d after germination under CE was recorded (Table 1). Similar to nitrogen content, accumulation of total soluble protein in leaves also increased substantially under CE, and the increase was more at 50 d after germination (Table 2).

Total free amino acid content was significantly reduced under CE at 20 d after germination. (Table 1). However, at 35 and 50 d a marginal increase in amino acid content due to CE was observed. Accumulation of total free amino acid in leaves was also higher under CE at all growth stages (Table 2).

CE brought about a remarkable decrease in the activity of nitrate reductase in leaves of mungbean at 20 d after germination (Table 2). However, a significant increase in the activity of nitrate reductase due to CE was

obtained at 35 and 50 d after germination.

Total nitrogenase activity was increased by CE at all growth stages but the major increase was observed at 35 d after germination (Table 2).

Discussion

In the present study, increase in dry and fresh masses of leaves and total biomass was recorded due to CE. This phytomass accumulation in response to CO_2 confirms the lack of significant inhibition of photosynthetic rate that was shown by Jones *et al.* (1985) in soybean. Earlier studies on mungbean have also shown a substantial carbon gain at CE (Sharma and Sengupta 1990). The present study showed that leaf nitrogen concentration (protein and non-protein) decreased when the plants were grown under CE. Thus, it appears that the nitrogen taken up by the plants cannot proportionately keep up with the carbon gain by the plants under CE. Lowering in nitrogen concentration has been reported in wheat (Manderscheid *et al.* 1995, Hocking and Meyer 1991, McKee and Woodward 1994), soybean (Reeves *et al.* 1994) and common bean (Mjwara *et al.* 1996). The reason for low nitrogen concentration under CE could be the suppression of photorespiration which will reduce the flux of nitrogen through the glycolate pathway or greater photosynthetic efficiency will result in a reduced nitrogen allocation to Rubisco (Sage 1994).

Similar to reduced nitrogen concentration, low soluble protein and free amino acids were recorded under CE at early growth stages in the present study but relatively higher amino acid and total soluble protein were obtained at later stages. Earlier findings for amino acids and soluble protein are variable. In most of the studies, lower soluble protein is reported under CE (Wilkins *et al.* 1994, Vu *et al.* 1997). In contrast to the above findings, Allen *et al.* (1988) reported no difference in leaf blade protein for soybean under CE. In another study, Vu *et al.* (1989) reported a continuous rise in soluble protein levels with increasing CO_2 concentration in soybean. The reason for the reduction in total soluble protein could be the reduced allocation of nitrogen to protein under CE because we have recorded similar reduction in protein nitrogen under CE.

We recorded less amino acids at 20 d after germination in CE grown plants. The reason for the reduction in amino acid content could be inhibition of photorespiration (Madore and Grodzinski 1985).

The nitrate reductase activity also showed decrease at 20 d after germination; but a significant increase at later growth stages. Low NR activity has been reported in wheat due to CE (Hocking and Meyer 1991) while increased activity has been reported in barley (Cramer *et al.* 1996).

CE resulted in a significant increase in total nitrogenase activity (TNA) in mungbean. CE presumably increased the nitrogenase activity either directly or indirectly by increasing the rate of photosynthesis. Symbiotic nitrogen fixation has been closely linked to photosynthetic capacity which supports a link between a CO_2 induced stimulation of carbon and nitrogen fixation in legumes. The link between carbon and nitrogen fixation in these experiments was manifested primarily through nodulation or nodule development; there was no evidence for stimulation of specific nitrogenase activity however, total nitrogenase activity increased in our case. Finn and Brun (1982) also found that CE increased TNA of peas and soybeans, because of increased nodule mass and not because of an increase in SNA.

The higher levels of TNA in mungbean grown in CE did not result in a significant increase in nitrogen percentage relative to that of control plants similar to earlier findings (Manderscheid *et al.* 1995). However, CE plants accumulated more nitrogen, protein and free amino acids in leaves than did control plants at all growth stages. The accumulation of nitrogen, protein and amino acids was probably due to the increase in total nitrogenase activity. However, nitrogen fixation apparently comprised a relatively small proportion of the total nitrogen assimilation in these plants, so that we did not observe higher nitrogen percentages in CE grown plants.

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