

Physiological and Biochemical responses of two legume species to elevated levels of carbon dioxide

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ABSTRACT

Effects on some selected physiological and biochemical parameters of *Glycine max* and *Phaseolus vulgaris* were inspected in present investigation using exposure to enhanced CO₂ levels. Enriched CO₂ levels (2 and 3%) were used to expose two weeks old *Glycine max* and *Phaseolus vulgaris* seedlings for 15 minutes per day, for 40 days duration. Aeration with enriched CO₂ air resulted in higher carbohydrate content as compared to plants grown in ambient CO₂ condition. However, reduction in protein was found under elevated levels of CO₂, the effect was more pronounced at 3% CO₂ level as compared to 2%. The amount of various amino acids was increased in both CO₂ treatments, while decrease was observed in the quantity of glycine and serine. In present study, we furthermore investigate the changes in the activity of peroxidase enzyme, which involved in defense mechanisms of plants under oxidative stress. The activity of peroxidase enzyme was found to be increased, this increase continued till the end of experimental period and higher activity of peroxidase was found in the treated plants than control.

Key words: Amino acid; Carbohydrate; Peroxidase; protein; *Glycine max*; *Phaseolus vulgaris*

1. INTRODUCTION

CO₂ is a natural component of the earth's atmosphere and it is considered as important gaseous nutrient for green plants in order to carry out photosynthesis¹. The concentration of CO₂ in the air has enhanced slowly since about 1850 from about 280 µl/l to slightly over 340 µl/l at present². The amount of CO₂ that requires by plants to grow vary from plant to plant, it was observed that most of plants will stop growing when the CO₂ level decreases below 150 ppm, 370 ppm was found to be averaged level for normal plant growth, according to another report 1000 to 1400 ppm CO₂ level was found to ideal level for plant growth and it dramatically increase photosynthetic rates and hence the growth³. Elevated CO₂ is the primary variable that influences growth, yield and increases aboveground biomass⁴. In C₃ crops elevated CO₂ stimulates photosynthesis because ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is not CO₂ saturated at current CO₂ level and because elevated level of CO₂ inhibits photorespiration⁵. There has been no significant stimulation of yield was established in C₄ crops because C₄ photosynthesis is saturated under ambient CO₂⁶⁻⁹.

On other hand CO₂ is considered as major air pollutant¹⁰, because it is an important green house gas and absorbing infrared radiation from the earth and predicated to cause global warming through a process called the green house effect¹¹. CO₂ enters the atmosphere through burning of fossil fuels, deforestation and also chemical reactions undergoing in different factories¹². Many studies concluded that natural phenomena like volcanoes also produced the warming of earth climate¹³. Global warming and increased atmospheric CO₂ is having a major impact on plant distributions; in general, plants benefit from slightly warmer temperatures and higher CO₂, but not all plants will benefit equally from these conditions, and some may even be harmed¹⁴.

The main intention of this study was to determine the effect of elevated level of CO₂ on total carbohydrate, total protein and individual amino acid content of *Glycine max* and *Phaseolus vulgaris* and to determine the effect of elevated levels of CO₂ exposure on peroxidase activity, which play important role in protecting plants against injury of oxidative stressors. In this study we tested hypothesis that magnitude of responses of both legume species differ with exposure to treatments of CO₂(2 and 3%) and physiological parameters were used to recognize the difference.

2. EXPERIMENTAL

Experiments were conducted on *Glycine max* and *Phaseolus vulgaris*. The seeds were obtained from local Market. Healthy seeds of both species were selected and sterilized with 0.1% Mercuric chloride solution for 5 minutes followed by rinsing with tap and distilled water. Seeds were sown in 8cm diameter plastic pots containing 300gm of sterilized soil. Half-strength Hoagland's solution was used to irrigate the plants throughout experimental period¹⁵. Two weeks old seedlings were exposed to two different 2 and 3% levels of CO₂ in controlled environment chamber for 40 days. Air is drawn into chamber by cylinder which were obtained from "The National Gas Limited Pakistan", enriched with CO₂, and blown through the chamber. All the chambers were maintained at 2 and 3% CO₂ levels at 30°C temperature. Plants were fumigated with elevated CO₂ for 15 minutes per day for 40 days duration, inside chamber light was delivered by fluorescent

and luminescent lamps, which adjusted at top of the canopy. After CO₂ exposure the pots were returned to natural environmental condition in the net house for growth. The non treated plants serve as control. There were there replicates for each treatment.

2.1 Plants responses measurements

Control and treated leaf samples were collected in early hours of the morning and were kept in labeled sample bags. The plants samples were analyzed for following biochemical parameters.

2.2 Estimation of carbohydrate content

Carbohydrate content was measured according to the method of Yemm & Willis¹⁶ method using anthrone reagent. 1.0 gm of leaves was homogenized in 10 ml of distilled water and centrifuged at 500 rpm for 5 minutes. The supernatant was used for estimation of total carbohydrate content. The reaction mixture contained 0.5 ml of supernatant and 5 ml of anthrone reagent which was boiled at 100°C for 30 minutes. Absorbance was determined at 620nm. The carbohydrate content expressed as $\mu\text{g}\cdot\text{mg}^{-1}$ fresh weight.

2.3 Estimation of peroxidase activity

The activity of Enzyme peroxidase was assayed according to the method of Alvarez¹⁷. 0.5g leaf were ground ice chilled 3ml of 0.15M phosphate buffer of pH 5.6 then drop of 3mM Sodium EDTA was added after that the extract was centrifuged at 4000 rpm for 10 minutes at 0°C in a refrigerated centrifuge, the supernatant was used for the estimation of peroxidase activity and soluble protein. Soluble protein was estimated using Folin reagent and Bovine serum albumin using as standard reference (Lowry *et al.*, 1951)¹⁸. For determination of peroxidase activity 0.5ml of crude enzyme extract was mixed with 2.5ml of 0.15M phosphate buffer (pH 5.6) and then 0.25ml of 0.1M H₂O₂ and 0.01M pyrogallol was added and after incubation at 28°C absorbance were recorded at 410nm. Activity of peroxidase expressed as $\mu\text{g}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$ of leaf fresh weight.

2.4 Extraction and Estimation of protein

Estimation of Protein was done in plant extracts by the method of Lowry *et al*¹⁸. For protein assays, 0.5g leaves were ground in 10ml of distilled water. The homogenates were centrifuged for 10 minutes at 3000rpm and the resulting supernatants were used for determination of protein content assays. To 0.2ml of plant sample, add 3ml alkaline copper sulfate in the presence of tartrate, let the mixture stand at room temperature for 30minutes. This incubation is then followed by the addition of Folin reagent furthermore read the absorbance at 750nm and Bovine serum albumin is used to make the standard curve. The amount of total protein content was calculated from a standard curve of Bovine serum albumin and expressed as $\mu\text{g}\cdot\text{mg}^{-1}$ of leaf fresh weight.

2.5 Amino acid Estimation

Amino acid were extracted and estimated by the method given by Harbon¹⁹, 1.0g of plant material were heat at 60°C for 10 minutes in 10ml of 80% ethanol then material kept over night. Next day it was centrifuged twice at 4000rpm and supernatant was taken in vial and their volume is reduced up to 1ml at 40°C temperature then in reduce extract 1ml of 50% ethanol was added. Separation of amino acids were done by using thin layer chromatography (TLC) technique, on thin layer of silica gel amino acid extract were loaded and run in n-butanol - acetic acid -water (4:1:1) as solvent. After chromatogram dried at room temperature spraying was done with 2% ninhydrine in acetone then spraying the plate and heated for 10 minutes at 105°C when most amino acids give purple or grey-blue color their R_f values were calculated. Standard of amino acids were prepared by dissolving 250 μg amino acid in 1 ml absolute ethanol.

3. STATISTICAL ANALYSIS

The data for protein, carbohydrate and peroxidase activity of *Glycine max* and *Phaseolus vulgaris* under different treatments were analyzed using the "COSTAT" statistical program by two-way analysis of variance (ANOVA) to compare the means of different treatment and "SIGMA PLOT" program was used for graphic presentation of the data.

4. RESULTS

The results achieved for the effect of different concentration of CO₂ on total carbohydrate content of *Glycine max* and *Phaseolus vulgaris* leaves are shown in Fig. 1. Significant (**P<0.01) increase in total carbohydrate content was observed in treated samples as compared to control samples throughout experimental period. The result for the effect of elevated level of CO₂ treatment on enzymatic activity of peroxidase of *Glycine max* and *Phaseolus vulgaris* are showing in Fig. 2,

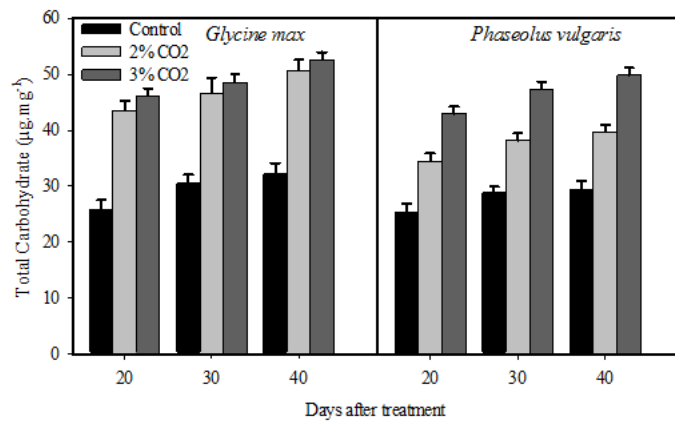


Fig-1: Total carbohydrate content in *Glycine max* and *Phaseolus vulgaris* leaves grown under different Carbon dioxide concentration.

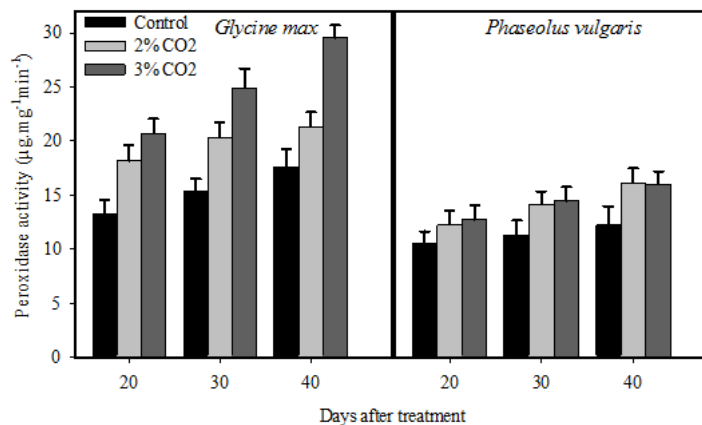


Fig-2: Peroxidase activity in *Glycine max* and *Phaseolus vulgaris* leaves grown under different Carbon dioxide concentration

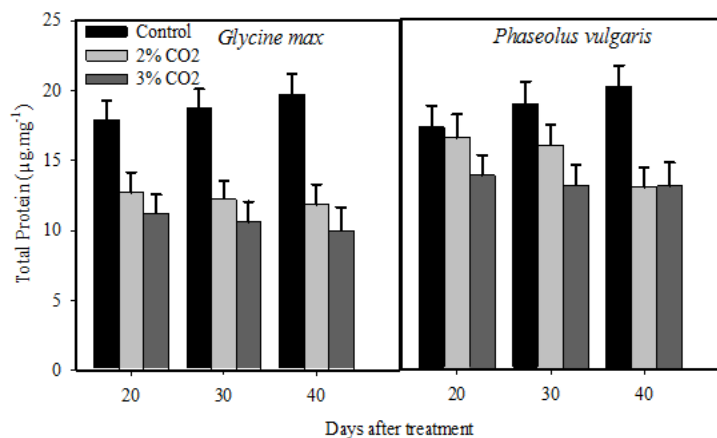


Fig-3: Total protein content in *Glycine max* and *Phaseolus vulgaris* leaves grown under different Carbon dioxide concentration

the result obtained were statistically significant ($P < 0.01$). Both CO₂ treatment of *Glycine max* and *Phaseolus vulgaris* demonstrate an increase in activity of peroxidase as compare to control.

The result get for the effect of different concentration of CO₂ on total protein content of *Glycine max* and *Phaseolus vulgaris* are shown in Fig. 3. From Fig. 3 it is clear that Significant decrease in amount of total protein in leave of *Phaseolus vulgaris* ($P < 0.05$) and *Glycine max* ($P < 0.01$) was observed throughout the experimental period, whereas, increase was observed in control of both species. Individual amino acids content of *Glycine max* were showing in Table 1, at 20th day tyrosine and tryptophan were absent in *Glycine max*. Lysine, proline, valin and phenylalanine were absent only

in control while their traces were present in CO₂ treatment. Traces of threonine and asparagines present in control and 3% CO₂ however they absent in 2% CO₂ treatment. Arginine, alanine, cystine and histidine traces, but low quantity of aspartate, glutamate and glutamine were found in both CO₂ treatments as well as in control of *Glycine max*. In control of *Glycine max* glycine and serine was found in low amount whereas their trace were present in both CO₂ treatment. Moderate amount of methionine, leucine and isoleucine was found in 2 and 3% CO₂ treatment furthermore their traces were found in control of *Glycine max*.

At 30th and 40th day arginine and tyrosine were absent whereas traces of cystine, threonine, glycine, alanine, valine, methionine and phenylalanine and low amount of glutamate and glutamine and aspartate were present in control and both CO₂ treatment of *Glycine max*, rest of the amino acids illustrate the uneven trend.

Individual amino acids content of *Phaseolus vulgaris* were presented in Table 2, at 20th day *Phaseolus vulgaris* illustrate bit lower amount i.e. traces of Cystine, asparagines, alanine, valine, methionine, leucine and glutamine were present while isoleucine and tyrosine were absent in *Phaseolus vulgaris*, in addition histidine and serine were present in traces in control, but lacking in both CO₂ treatment of *Phaseolus vulgaris*. Traces of lycine, arginine, prolein, phenylalanine, tryptophan and aspartate were found equally in 2% and 3% CO₂ treatments while missing in control of *Phaseolus vulgaris*. In both CO₂ treatment and control of *Phaseolus vulgaris* glutamate was found in low amount whereas threonine and glycine were present in control but lacking in CO₂ treatments.

At 30th day cystine, histidine, aspartate, threonine, asparagines, methionine, serine and glutamine traces but moderated amount of glycine, glutamate and arginine were present while lycine, tyrosine, proline, tryptophan, alanine, valine, phenylalanine, leucine and isoleucine were missing in control of *Phaseolus vulgaris*. In 2% CO₂ treatment cystine, tyrosine and serine were absent while the traces of all other amino acid were present in *Phaseolus vulgaris*. Cystine, histidine, arginine, methionine, glutamine, phenylalanine, leucine, alanine and valine were present in traces, while low quantity of aspartate, glutamate, proline and asparagines were present in 3% CO₂ treatment of *Phaseolus vulgaris*.

At 40th day amount of various amino acids were found in moderate amount in 2% and 3% CO₂ treatments of *Phaseolus vulgaris* while in control of *Phaseolus vulgaris* traces of various amino acids were observed.

Table-1: Changes in the free amino acids quality in leaves of *Glycine max* under different concentration of CO₂

Amino Acids	Control			2% CO ₂			3% CO ₂		
	Days after the treatment application								
	20	30	40	20	30	40	20	30	40
Cystine	+	+	+	+	+	+	+	+	+
Histidine	+	+	+	+	+	+	+	-	-
Lycine	-	+	-	+	+	+	+	+	+
Aspartate	++	+	++	++	++	++	++	++	++
Threonine	+	+	+	-	+	+	+	+	+
Glycine	++	+	+	+	+	+	+	+	+
Glutamate	++	++	++	++	++	++	++	++	++
Arginine	+	-	-	+	-	-	+	-	-
Tyrosine	-	-	-	-	-	-	-	-	-
Proline	-	-	-	+	+	++	+	+	++
Asparagine	+	+	+	-	-	-	+	+	+
Tryptophan	-	-	-	-	-	-	-	+	+
Alanine	+	+	+	+	+	+	+	+	+
Valine	-	+	+	+	+	+	+	+	+
Methionine	+	+	+	+++	+	+	+++	+	+
Phenylalanine	-	+	+	+	++	++	+	+	+
Leucine	+	-	-	+++	+	-	+++	+	+
Serine	++	++	++	+	+	+	+	-	-
Isoleucine	+	-	-	+++	+	+	+++	-	++
Glutamine	++	++	++	++	+	++	++	++	++

+ Traces, ++ low, +++ moderate and ++++ high.

Table-2: Changes in the free amino acids quality in leaves of *Phaseolus vulgaris* under different concentration of CO₂

Amino Acids	Control			2% CO ₂			3% CO ₂		
	Days after the treatment application								
	20	30	40	20	30	40	20	30	40
Cystine	+	+	+	+	-	+++	+	+	-
Histidine	+	+	+	-	+	+++	-	+	+++
Lycine	-	-	+	+	+	++	+	-	-
Aspartate	-	+	+	+	+	+++	+	++	+++
Threonine	+	+	+	+	+	+++	-	-	+++
Glycine	++	++	+	+	+	+	-	-	-
Glutamate	++	++	+	++	+	++	++	++	-
Arginine	-	++	+	+	+	+++	+	+	+++
Tyrosine	-	-	-	-	-	-	-	-	++
Proline	-	-	-	+	+	++	+	++	+
Asparagine	+	+	+	+	+	+	+	++	++
Tryptophan	-	-	+	+	+	+++	+	-	+++
Alanine	+	-	+	+	+	++	+	+	-
Valine	+	-	+	+	+	-	+	+	++
Methionine	+	+	+	+	+	++	+	+	+++
Phenylalanine	-	-	-	+	+	+++	+	+	++
Leucine	+	-	-	+	+	+	+	+	++
Serine	+	+	+	-	-	-	-	-	-
Isoleucine	-	-	-	-	+	++	-	-	-
Glutamine	+	+	-	++	+	+++	+	+	+++

+ Traces, ++ low, +++ moderate and ++++ high

5. DISCUSSION

In present investigation increased in total carbohydrate content was observed. Elevated CO₂ conditions produced an average increase in total carbohydrate contents of 28% for clover and 16% for phalaris²⁰. Short-term exposure of elevated CO₂ for plants generally leads to increased rates of leaf-level photosynthesis due to better activity of ribulose-1.5-bisphosphate carboxylase/oxygenase²¹. Many studies reported that during growth under twice-ambient CO₂, leaf soluble carbohydrate content increased on average by 52% and starch content by 160%²¹. In the short term (hours to days), elevated CO₂ increases the rate of photosynthesis in plants. However, over the longer term (days to weeks), growth in elevated CO₂ often decreases photosynthetic capacity because of decreased in the content of photosynthetic enzymes²². According to an other report an increased in total carbohydrate content of two *Vigna* species was observed, which exposed to enriched CO₂ air for short duration²³.

Increase in peroxidase activity of *Glycine max* and *Phaseolus vulgaris* revealed in present investigation. Short term exposure of enriched CO₂ is reported to enhance the activity of peroxidase²⁴. Peroxidase is involved in numerous physiological mechanisms and it is commonly reported that frequently oxidizing agents are responsible for the stimulation of peroxidase activity in plants²⁵. Peroxidase is believed to assist the production and breakdown of hydrogen peroxide and other reactive oxygen species²⁶⁻²⁸. Elevated level of CO₂ and ozone, alone and in combination with one another, found to increase the activity of peroxidase, which is the first line of enzymatic defense against oxidative stress, Thus, atmospheric CO₂ enrichment increased the activities of peroxidase enzymes that function to keep cells from experiencing oxidative damage, particularly to their membranes²⁹.

In the present investigation, decrease in the total protein content is evident with fumigation of the elevated levels of CO₂, however increased in various amino acid content was observed on one hand, on the other lower glycine and serine was observed. Elevated levels of CO₂ caused significant reduction in protein content in wheat³⁰. Barbehenn suggested that plants protein concentration decreased much more in the C₃ species (22%) than in the C₄ species (7%) when the plants were grown in CO₂ enriched air³¹. Likewise, Agrawal and Deepak³² determined that CO₂ enrichment results in diminish leaf protein levels by 3-4% in wheat. Zeigler suggested that such decrease could be attributed to break down of existing protein and reduced in synthesis³³. Rise in the atmospheric CO₂ content typically lead to greater decreases in the concentrations of nitrogen and, therefore, protein in the foliage of C₃ as compared to C₄ grasses³⁴. Another investigation supported that the CO₂-induced decrease in leaf protein concentration but it did not reduces the growth rate of plants³⁵. Rogers et al. found that total amino acid and Nitrogen content increased markedly at elevated level of CO₂³⁶. Although Amino acids involved in photorespiration (glycine, serine) make a large contribution to the total amino acid pool; as a consequence of the lower rates of photorespiration these amino acids typically decrease at elevated level of CO₂³⁷.

Ferrario-Mery et al suggested that, reducing photorespiration at elevated CO₂ level may result in large decreases in leaf concentration of glycine and serine and ammonium³⁸. Stitt & Krapp recommended that decreased in pool sizes of glycine and serine furthermore associated with lower protein content in leaves at elevated CO₂ (37). Reduced photorespiration decreases the rate of nitrate photo reduction and this may contribute to lower protein content in leaves that grow at enriched CO₂ circumstance³⁹.

6. CONCLUSION

Exposure of elevated level of CO₂ led to increased carbohydrate content of both legume species. The decrease in protein content and free amino acids (glycine and serine) of *Glycine max* and *Phaseolus vulgaris* was observed. While activity of Peroxidase enzyme, which participate in defense mechanisms of plants under oxidative stress, increases at enriched CO₂ condition.

7. REFERENCE

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