Assimilatory function and biochemical changes in *Stylosanthes hamata* grown under elevated CO_2

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ABSTRACT

We studied the impact of 360 ± 50 µL/L (ambient) and 600 ± 50 µL/L (elevated) CO_2 on growth performance, biomass production, photosynthetic efficiency, carbon isotope discrimination, protein profile and some antioxidant enzymes on *Stylosanthes hamata*. This crop responded significantly to photosynthetic rate, stomatal conductance and transpiration rate under elevated CO_2. The biomass production in terms of fresh and dry was increased in elevated CO_2 by 126.81% (fresh) and 114.55% (dry) over ambient CO_2. Long term exposure to elevated CO_2 enhanced photosynthetic water use efficiency by 127.77%. The photosynthetic pigment, total chlorophyll and chlorophyll *a*/*b* ratio also increased by 220.56 and 132.86%, respectively in elevated over ambient CO_2. Around 149% increase in the soluble protein accumulation (mg/g FW) was recorded under elevated over ambient CO_2, which was also reflected in the polyacrylamide gel profile. The isoforms of superoxide dismutase and esterase isozymes showed remarkable difference under elevated as compared to ambient. Measurement of $^{13}\delta$ in different plant parts indicated a significant increase in discrimination against ^{13}C when plants were grown at elevated relative to ambient CO_2. Maximum increase was recorded in roots (439.72%) followed by leaf and the stem recorded least increase in $^{13}\delta$ (119.94%) in elevated over ambient CO_2.

Keywords: photosynthesis; antioxidant enzymes; biomass production; $^{13}\delta$ discrimination

Atmospheric CO_2 concentrations increased significantly in the past two centuries, rising from about 270 µL/L in 1750 to current concentrations larger than 385 µL/L (Le Quéré et al. 2009). The expected continued rise in atmospheric CO_2 concentration, apart from possible influences of increasing temperature and other changes in environmental factors, is anticipated to stimulate biomass of many C_3 crops (Ainsworth and Long 2005, Prasad et al. 2005). Changes in atmospheric CO_2 are known to affect the fundamental plant processes of photosynthesis (P_n), respiration and stomatal conductance (g_s). Elevated CO_2 typically improves net photosynthetic rate under saturated irradiance, however the photosynthesis is not stimulated by CO_2 concentration under low light intensities, when photons are limiting substrate of the photosynthesis (Spunda et al. 2005), plant-water relations and photosynthetic water use efficiency (Ainsworth and Long 2005, Prasad et al. 2005). Antioxidant enzymes like superoxide dismutase (SOD), esterase (EST) etc. are produced by the plants to minimize the cellular oxidative damage. These molecules catalyze reaction that directly or indirectly detoxify reactive oxygen species (ROS) (Schwanz et al. 1996). *Stylosanthes hamata* is regarded as the most important C_3 range legume for the humid to semi-arid tropics. It is extensively utilized in pastoral, agro-pastoral and silvi-pastoral systems for animal production. Due to its ability to restore soil fertility, improve soil physical properties and provide permanent vegetation cover, it plays a vital role in the development of west lands. It is also considered a nurse crop in plantation on degraded lands. In light of rising global atmospheric CO_2 concentrations it is an attempt to understand the effects of atmospheric CO_2 enrichment on assimilatory function and biochemical changes in *Stylosanthes hamata* under elevated CO_2 environment.

MATERIAL AND METHODS

Plant material, experimental environment and treatment. The experiment was conducted at the

Indian Grassland and Fodder Research Institute, Jhansi, India (25°27'N, 78°35'E, 271 m a.s.l.). As per weather data concerned the maximum temperature ranges from 24–42°C, minimum temperature ranges from 4–26°C, the precipitation varies from 550–600 mm, the relative humidity ranges from 30–90% and the total sunshine hours varies from 3–9 h per day. The soil was clay loam in texture, neutral in reaction (pH 6.54), and non saline (EC 0.29 mS/m). The contents of organic carbon (0.48%) and available nitrogen (23.52 g/m^2) in the surface soil were low. The available phosphorus and potassium contents were in the medium range [1.27 g/m^2 (P) and 23.14 g/m^2 (K)]. Stylosanthes seeds were sown in side the open top chambers (OTCs) at 50 cm row to row and 25 cm plant to plant spacing. The OTCs are circular in nature with diameter of 3 m. Nitrogen and phosphorus were applied as basal at the rate of 60 kg N and 17.6 kg P per hectar before sowing on the onset of monsoon i.e. in the month of July. The plants were maintained as per recommended agronomical package of practices. Pure CO_2 gas (discrimination value ranges from 7.1–7.8%) was used for enrichment of CO_2 inside the open top chambers. Two chambers were maintained as elevated CO_2 (600 ± 50 µL/L) and the other two chambers were maintained without elevated CO_2, i.e. ambient CO_2 (360 ± 50 µL/L) (C_{360}). The flow of CO_2 was adjusted with the help of a flow meter to get the target concentration of CO_2 inside the chambers and the CO_2 concentration was monitored by an IRGA (LICOR, Nebraska, USA). The period of CO_2 enrichment was from 8.0 a.m. to 5.0 p.m. round the cropping season from 2 week seedling stage to harvest (flowering). The soil water condition was never a limited resource for the whole cropping season, however, the irrigation was given when the soil moisture reached to 20–25%. Measurements of each parameter were done in three replicates from each chamber and the data presented is the mean of six data recorded from two chambers of each elevated and ambient CO_2. The data was statistically analyzed by using statistical package MSTAT C and the *LSD* value is given in the table.

Photosynthesis and related parameters. The assimilatory characters such as P_n, transpiration (E), g_s and intercellular CO_2 concentration (C_i) were recorded in the second fully expanded leaf from the top of the plant by using the portable photosynthesis system (LI-6200, LICOR, Nebraska, USA). The micro environmental parameters like, air temperature, photosynthetically active radiation (PAR), relative humidity (RH) and leaf temperature (LT) were also recorded by the attached sensors of the photosynthesis system. All the measurements were made between 11.00 and 12.00 h under saturated light condition and the PAR in the OTCs ranges from 1400–1600 µmol/m^2/s during the measurements. The leaf temperature ranges from 1.0–1.5°C among the observations. The ratio P_n/E (photosynthetic water use efficiency) and P_n/C_i (carboxylation efficiency) were also calculated.

Biomass production. The above ground fresh and dry biomass of the whole plant was taken just after harvesting at 50% flowering stage. For dry matter yield, the plant samples were dried in a hot air oven at 80°C for 48 h. The leaf area per unit land area, leaf area index (LAI) was estimated by taking twenty representative leaf blades from plants in each sample at random and their total area was measured by an automatic leaf area meter (LICOR-3000, Nebraska, USA). The LAI was calculated following Tanaka and Kuwano (1966).

Photosynthetic pigments. Chlorophyll *a* and *b* contents were determined by extraction in dimethyl sulphoxide (DMSO) using a non-maceration technique of Hiscox and Israelstam (1979).

Enzyme and soluble proteins. Fresh leaves were ground in a pre-chilled pestle and mortar in grinding medium (1 mL/L g tissue) containing 50 mmol Tris HCl (pH 8.0), 50 mmol $MgCl_2$, 5 mmol 2-mercaptoethanol and 1 mmol. Homogenate was centrifuged at 4°C for 20 min at 15 000 × g. This extract was used for estimating soluble protein following the procedure of (Lowry et al. 1951). The same extract was used for estimating PEPC activity by a decrease in absorbance at 340 nm per s in a mixture of 925 µL 0.1 mol Tris HCl (pH 7.8), 500 µL $MgCl_2$ (20 mmol), 500 µL $NaHCO_3$ (10 mmol), 500 µL PEP (5 mmol), 50 µL NADH and 50 µL enzyme extract. The activity was expressed in unit per mg protein, whereas one enzyme unit was defined as a change of 0.1 absorbance per min caused by the enzyme aliquot.

SDS-polycarylamide gel electrophoresis (PAGE). SDS-PAGE was performed using 12% gel according to (Laemmli 1970) with minor modification. An aliquot (15 cm^3) of the above extract was mixed with 2X sample buffer (0.25 mol/L Tris-Cl, pH 6.8; 0.2% sodium dodecyl sulphate (SDS), 10% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue. The electrophoresis was performed at 30 mA on a 1.0 × 1.5 mm gel.

Isozymes. Fresh leaf samples were extracted with 1:2 (m/v) volume of Tris-Cl buffer (pH 7.6) containing 5 mmol β-mercaptoethanol. For esterase (EST, E.C.3.1.1.2) isozymes (EST), anionic PAGE

was used by loading approximately 150 µg protein. For staining EST was stained with 50 mmol phosphate buffer (pH 6.0) containing 0.02% α-naphthyl acetate (dissolved in 2 cm^3 of 60% acetone) and 0.05% Fast Blue RR salt. For Super oxide dismutase (SOD, E.C.1.15.1.1), when the run was over gel was removed from the gel assembly and incubated in dark for 30 min in 100 mL of 50 mmol Tris buffer (pH 8.0) containing 2 mg riboflavin, 1 mg EDTA and 10 mg NBT (Nitroblue tetrazolium). After the incubation gel was shifted to bright and intense light for 30 min and then gel was washed with distilled water. The bands appeared in form of negative bands against the blue background.

Carbon isotope discrimination. Carbon isotope ratios were determined with an Isotope Ratio Mass Spectrometer (Delta-plus, Thermo Finnigan, Bremen, Germany) interfaced with an Elemental Analyzer (NA 1112, CarloErba, Milano, Italy) via a continuous flow device (Conflo-III, Thermo Finnigan, Bremen, Germany). A composite leaf sample comprising of 10 mature leaves representing all positions of the plant canopy were harvested from both the CO_2 levels and oven dried for 3 days at 70°C and homogenized to fine powder with a ball mill. Three replications for both the grasses were analyzed for $\delta^{13}C_{lb}$ with an analytical uncertainty of less than 0.1‰. Carbon isotope discrimination ($\delta^{13}C$) expressed in ‰ notation) was computed as follows, assuming the isotopic composition of atmospheric air ($\delta^{13}C_a$) to be –‰ (Farquhar et al. 1989): $\delta^{13}C = [\delta^{13}C_a - \delta^{13}C_{lb]}/[1 + (\delta^{13}C_{lb}/1000)]$

RESULTS AND DISCUSSION

Photosynthesis and related parameters. P_n and related parameters like internal CO_2 concentration (C_i) and transpiration (E) were increased significantly in *Stylosanthes hamata* grown under elevated CO_2 as compared to the crop grown under $C_{360.}$ However there was a significant decrease in stomatal conductance (g_s) observed under the elevated CO_2 environment. The LAI increased under elevated CO_2. Elevated CO_2 could increase the LAI through at least two mechanisms, such as by increasing photosynthetic efficiency, elevated CO_2 would lower the light compensation point (LCP) of photosynthesis, allowing leaves to maintain a positive carbon balance in elevated CO_2 than at present atmospheric CO_2. Alternatively, greater carbohydrates supply and improved water use ef-

Table 1. Effect of elevated CO_2 (600 ± 50 µL/L) on photosynthesis, transpiration, chlorophyll accumulation and carbon isotope discrimination ($^{13}\delta$) in *Stylosanthes hamata*

S. No.	Parameters	OTC with		$LSD_{0.05}$
		360 ± 50 µL/L CO_2	600 ± 50 µl/L CO_2	
1	P_n (µmol/m^2/s)	15.13	20.49 (135.43)	4.490
2	C_i (µmol CO_2/mol air)	338.96	538.26 (158.79)	71.93
3	g_s (mol H_2O/m^2/s)	0.215	0.158 (73.49)	0.179
4	E (mmol H_2O/m^2/s)	8.979	9.159 (102.00)	1.439
5	LAI	5.01	5.61 (111.98)	0.812
6	P_n/C_i (µmol/mol)	0.045	0.047 (104.44)	0.023
7	P_n/E (µmol/m^2/s)	1.685	2.153 (127.77)	0.963
8	chl a + b (mg/g/FW)	1.07	2.36 (220.56)	0.109
9	chl a:b	5.69	7.56 (132.86)	1.235
10	soluble protein (mg/g/FW)	12.36	18.42 (149.03)	4.68
11	fresh biomass (g/m^2)	2361	2994 (126.81)	138.83
12	dry biomass (g/m^2)	880	1008 (114.55)	101.42
13	$^{13}\delta$ (leaf)	18.17	24.43 (134.45)	5.42
14	$^{13}\delta$ (stem)	20.76	24.90 (119.94)	3.63
15	$^{13}\delta$ (root)	4.28	18.82 (439.72)	12.41

Data in parentheses indicates the percentage increase/decrease over control-calculated keeping the ambient CO_2 value as 100

ficiency may lead to larger individual leaves and more rapid canopy development, thereby increasing the LAI (Ferris et al. 2001). A negligible change in the carboxylation efficiency (P_n/C_i) was recorded in C_{600} but there was a significant increase in the photosynthetic water use efficiency (P_n/E) (Table 1). Changes in P_n are the result of changes in both g_s and mesophyll capacity for photosynthesis. The impact of elevated CO_2 on mesophyll capacity in turn depends on the carboxylation efficiency (activity, amount and kinetic properties) of Ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) and the capacity for photosynthetic electron transport and ribulose 1,5-bis-phosphate regeneration (Bunce 2001). Elevated CO_2 resulted in an increase in photosynthesis of the leaves exposed to C_{600}. This is in agreement with the majority of reports in the literature where such an increase in CO_2 has resulted in enhanced P_n in C_3 plants (Ainsworth and Long 2005, Ainsworth and Rogers 2007). Fleisher et al. (2008) also reported in potato that plants grown under elevated CO_2 had consistently larger photosynthetic rates through most of the growth season, with the maximum canopy photosynthesis at 1600 μmol photons/m^2/s. In the contrary, Spunda et al. (2005) reported that Norway spruce trees cultivated under elevated CO_2 coupled with low irradiance led to the diminution of the midday photosynthesis depression that was predominantly caused by stomatal closure and subsequent decrease in the intercellular CO_2 (C_i). Stomatal closure in response to elevated CO_2 is a common phenomenon. In our study, the decline in g_s probably resulted from direct effects of CO_2 on the stomata guard cells, because plants were watered regularly. CO_2 sensing is an intrinsic property of guard cells, which are thought to respond to C_i rather than CO_2 at the leaf surface. Bernacchi et al. (2003) suggested that photosynthetic CO_2 uptake within a leaf is either limited by the rate of ribulose-1,5-biphosphate (RuBP) regeneration or the activity of RuBP carboxylase oxygenase (RuBisCO). The same author reported a re-parameterization at the temperature responses of Rubisco activity that proved robust when applied to a range of species. At the same time stomatal conductance is reduced, leading to lower transpiration and less evaporative cooling (Leakey et al. 2009). One of the major limitations of carbon uptake is stomatal conductance (g_s). Linear and non linear decreases in g_s were observed in C_3 annual grass grown across a sub-ambient to super-ambient CO_2 gradient and these decreases were quite consistent when plants were grown in elevated CO_2 (Ainsworth and Rogers 2007). However, the response of A to C_i is non-linear, so g_s decreases significantly with increasing CO_2 concentration, stomatal limitation to photosynthesis as CO_2 increased and that the shift in photosynthetic control from Rubisco to RuBP regeneration at elevated CO_2 was sufficient to offset the decrease in g_s (Bernacchi et al. 2005). Decrease in stomatal conductance in response to increased CO_2 is more than compensated by the larger substrate for carboxylation (Del Pozo et al. 2005). Zhou et al. (2005) who found g_s of *Pinus sylvestriformis* was 22% lower in plants grown under elevated CO_2 compared with ambient CO_2 (350 μmol/mol) grown plants. Following a decrease in g_s with concomitant rise in the atmospheric CO_2, many C_3 species use less water and thus become more water use efficient.

Carbon isotope discrimination function ($^{13}\delta$). Measurements of $^{13}\delta$ made on dried plant parts indicated a significant increase in discrimination against $^{13}\delta$ when plants were grown at elevated relative to ambient CO_2 (Table 1).$^{13}\delta$ showed an increase in the $^{13}\delta$ in all the plant parts grown under elevated CO_2 environment. The maximum increase was recorded in root followed by leaf and stem recorded the least increase in $^{13}\delta$ under C_{600} over C_{360} (Table.1). The $^{13}\delta$ values (indicating their ability to discriminate CO_2 on the basis of the ^{12}C or ^{13}C isotope) in the different plant parts differed significantly ($P < 0.05$) with higher CO_2. The discrimination was lower in stem followed by leaf and root. The increase in $^{13}\delta$ possibly due to increased capture of CO_2 in different plant parts under elevated CO_2 and also reduced leakiness. The switching of air isotopic composition from $^{12}CO_2$ to pure $^{13}CO_2$ when the [CO_2] was changed, showed that the efflux of $^{12}CO_2$ (which represents net respiratory CO_2 evolution originating from respiratory substrates minus the CO_2 being re-fixed by carboxylases) was reduced in leaves of C_3 plants, but not C_4, when the [CO_2] increased (Pinelli and Loreto 2003). We therefore, hypothesized that for any given species the length of exposure of the plant to elevated CO_2 account for the majority of $^{13}\delta$ variation.

Biomass production. Long term exposure of *Stylosanthes hamata* to C_{600} in open top chambers resulted in a significant enhancement of the biomass production. Fresh and dry biomass production (g/m^2) was recorded and a significant increase in both fresh and dry biomass was observed in the crop grown under C_{600} over C_{360} (Table 1). The significant increase in biomass production was due to the production of more photosynthates and their partitioning to different plant parts which

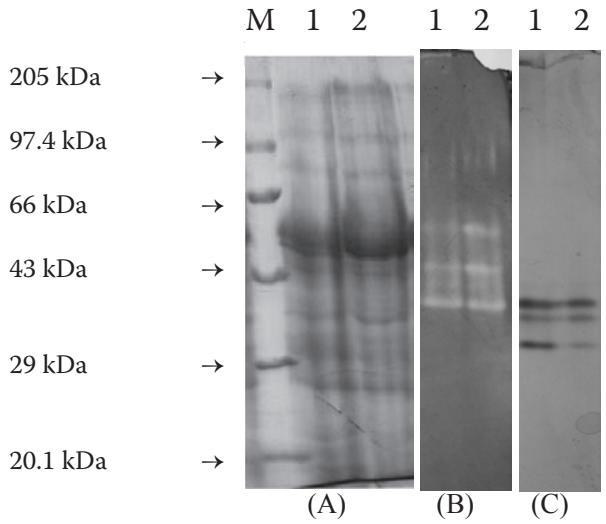

Figure 1. SDS-PAGE protein profile (A), super oxide dismutase (B) and esterase (C) of *Stylosanthes hamata* grown under ambient (C_{360}) and elevated (C_{600}) CO_2 environment. M – molecular weight marker; 1 – *S. hamata* (C_{360}); 2 – *S. hamata* (C_{600})

ultimately increased the total biomass production. Sharma and Sengupta (1990) also observed that the extra carbon fixed by the plants due to CO_2 enrichment was translocated to the growing axis. Increased fodder production of white clover and increased yield in rice grown under elevated CO_2 was reported by Saebo and Mortensen (1995) and Uprety et al. (2002), respectively. The increase in P_n and LAI (data not shown) under elevated CO_2 might have resulted in greater accumulation of assimilates which resulted in the production of more biomass. The *Stylosanthes hamata* grown in elevated CO_2 produced greater total biomass than those grown in ambient CO_2. Growth in CO_2 enriched environments typically enhances growth and photosynthesis by directly increasing the amount of carbon available for fixation, decreasing CO_2 loss to photorespiration and reducing oxygenase activity of rubisco (Lawlor and Mitchell 1991). Increased leaf biomass, stem biomass and total biomass due to long-term exposure to CO_2 enrichment in 90 day old seedlings of white birch (*Betula papyrifera*) was reported by (Cao et al. 2008) also summarized the increase in the above ground biomass of cotton (*Gossypium hirsutum* L.) in response to elevated CO_2 of 600 µL/L.

Biochemical changes. The accumulation of photosynthetic pigments was also influenced by the elevated CO_2. Significant increase in total chlorophyll and chlorophyll *a*/*b* ratio was recorded in the leaves of *S. hamata* grown under C_{600}. The increase in the soluble protein was recorded in the leaves of *S. hamata* grown under C_{600}, which was reflected in the SDS-PAGE pattern with the more intensified band in the range of 55 kDa in the plants grown under C_{600}. The isozyme pattern of EST and SOD differ under the C_{600}. The isoforms of the SOD are less intensified in C_{600} than the C_{360}. The isoforms of the EST also differed with less intensification of the 2nd and 3rd isoforms under the elevated CO_2 environment (Figure 1). The accumulation of photosynthetic pigments was also influenced by the elevated CO_2. Significant increase in total chlorophyll and chlorophyll *a*/*b* ratio was recorded in the leaves of *S. hamata* grown under C_{600}. The increase was recorded to the tune of 54.66% in total chlorophyll (chl *a* + *b*) and 24.73% in chlorophyll *a*/*b* ratio. In agreement with this, the chl *a*/*b* ratio also significantly increased. Moreover, Wang et al. (2004) reported that elevated CO_2 increased chloroplast number per unit cell area. In general it is thought that the status (up or down regulated) of plant oxidant systems is controlled by the extent of oxidative stress (Polle et al. 1997). Therefore it follows that the reduction of oxidative stress resulting from growth in CO_2 enriched atmosphere. Further, because antioxidant activities are stimulated by water stress (Schwanz et al. 1996) and growth in elevated CO_2 is often reported to alleviate water stress (Rogers et al. 1983), plants growing in CO_2 enriched atmospheres may exhibit lower antioxidant activities. Clearly, the relative contributions of these mechanisms to the observed reductions in antioxidative enzymes remain to be elucidated. Data obtained in the present study lend support to the hypothesis that plants growing in CO_2 enriched atmospheres will have lower antioxidative enzymes activity because of a decrease in cellular ROS production. In our study less intensified isoforms were observed for SOD and EST.

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