RESEARCH ARTICLE

Antioxidant Defence Response and Micronutrient Content Availability in *Cyamopsis* Varieties in eCO_2 Concentration

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Abstract

There is need for research that explores the impact of elevated carbon dioxide on the antioxidant defense system, crop nutrition and soil micro-nutrients availability which was not investigated much in past studies.

A pot experiment was performed to analyse antioxidant defence response including Superoxide dismutase (SOD) enzyme assay, total Ascorbate, Ascorbate peroxidase (APX) leaf data assay, Flavanoid and Total Phenolic content (TPC) in leaves samples. Micronutrients analysis and nutritional quality were estimated including Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo and Pb elements. Micronutrients analysis were determined in soil, leaves, pods and seeds of RGC 1002 and RGC 1066 *Cyamopsis* varieties fumigated under e[CO_2]=550±20ppm and a[CO_2]=420±20ppm maintained at FACE setup at CSIR- NBRI, Lucknow.

Superoxide dismutase activity was found to decline in RGC 1002 [-16.63%] and RGC 1066 [-17.90%] while total ascorbate, ascorbate peroxidise activity, total phenol and flavonoid content increased in RGC 1002 [+9.37%, +6.30%, +11.53%, +10.46%] and RGC 1066 [+66.32%, +12.17%, +76.50%, +19.82%] under elevated carbon dioxide e[CO_2] concentration in both the cultivars. Micronutrient content declined in leaves but it was enhanced in pods and seeds of both the cultivars under e[CO_2] concentration. In leaves, pods and seeds of RGC 1002 micro-nutrient contents were Fe [-56.00%, +6.00%, +9.75%], Cu[-23.16%, +7.45%, +10.46%], Zn[-30.61%,+28.30%,+7.41], Mn[-32.29%, +23.05., +7.52] content. However in RGC 1066, there was a differential response regarding some of the metals Cu[-52.81%,+5.58%, +6.42%], Zn[-20.29%.+9.50%.+6.50%], Mn[+31.54%, +11.18%, +9.96%], Fe[+32.99%, +4.00%, +8.71%] content was found to increased under e[CO_2] concentration.

Antioxidant response in both the cultivars was enhanced under e[CO_2] concentration that leads to the scavenging of ROS particles thus leading to declining of ROS and mitigating the plant against abiotic stress condition. This conditions leads to altogether improvement in plant antioxidant defences system. It was observed that the interaction between e[CO_2] and both plant varieties increased uptake of micro-nutrients in pods and seeds in both the cultivars. Apart from these RGC 1066 varieties showed better uptake and translocation of micro-nutrient content (Fe, Cu, Zn) than RGC 1002 plant variety under e[CO_2] concentration. Thus, it can be concluded that RGC 1066 is better than RGC 1002 plant variety which is adapting and performing in better way under e[CO_2] concentration.

Keywords: e[CO_2], a[CO_2], FACE, ROS, SOD, APX

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Introduction

Globally atmospheric carbon dioxide (CO_2) concentration dramatically increased up to 550 ppm due to ongoing anthropogenic activities involving greenhouse gas emissions and fossil fuel combustion leading to global surface temperature rising by 2°C at the end of this century (IPCC 2018). While elevated CO_2 positively effect the crop yield (Lam *et al.,* 2012), and biomass but negative (Hogy *et al.,* 2013, Myers *et al.,* 2014), positive (Carvalho *et al.,* 2020) or neutral effects on nutrients dynamics. Much research had been conducted on plant translocation and accessibility of macronutrients specially nitrogen, phosphorus and potassium but limited papers deals with both macro and micronutrient studies. However it remains still unclear how the changes in plant growth under CO_2 enrichment affect the availability of soil micronutrients and their accessibility to plant uptake. Not only the CO_2 enrichment concentration affects the uptake of micronutrients to plants but their mobility in soil is mediated by plant mediated soil processes.

Micronutrients also known as "magic wands" plays an important role in plant development, enzyme production and reproduction. The deficiency of micronutrients often results in specific diseases that negatively affect plant growth, as they are involved in a series of enzyme formations and metabolic processes (Khoshgoftarmanesh *et al.,* 2010). The availability of these micronutrients is not only regulated by their total amount in soil but also controlled by the climatic conditions, especially under the current enrichment of atmospheric CO_2 (Jhonson, 2013). Loladze (2002) suggested that terrestrial plants were experiencing a global imbalance of essential elements with carbon assimilation under CO_2 enrichment.

Recent studies provides convincing evidences that staple cereals like wheat, rice as well as legumes have lower

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concentration of iron and zinc (Myers *et al.,* 2014) and this has raised the spectular cause of malnutrition world wide. For example countries like Europe, East India, Malta and many reported Fe deficiency in staple crops like wheat and rice. Similarly Zn deficient countries like Meditteranian, Australia, Asia, Africa, America reported Zn deficiency in maize and rice. More over similar scenario of Mn deficiency is reported in soils of Europe, China, India, Australia, American and African countries.

Previous studies have reported a huge reduction in micronutrients that leads to significantly reduction in plant growth and yield parameters of plants varieties in soil deficient in Fe, Cu, Zn, Mn and other micronutrients (Khoshgoftarmanesh *et al.,* 2007)

Recently World Health Organisation (WHO) has repoted 30% of the world population under the threat of Fe deficiency, one of the most universal nutritional disorder in the world. Two billion of world population are anemic due to iron deficiency. More over in many developing countries iron deficiency aggravated worm infection leading to malaria and infectious diseases. Similarly Zn, Mn, Se deficiencies are also widely spreading among the population in developing and developed countries. Zn is the trace metal essential for gene expression, cell development and replication. Manganese is an essential trace element for living organisms, including human beings because some enzymes require manganese (e.g. manganese superoxide dismutase), and some are activated by this element (e.g. kinases, decarboxylases). Adverse health effects can be caused by inadequate dietary intake or overexposure (Ref).

A part from micronutrient availability and accessibility stress responses created due to the environmental conditions are very less documented in plants under elevated carbon dioxide concentration. However, the importance of these experimental studies are required and needed to be increasing in future scenarios (Xu *et al.,* 2015; Feng *et al.,* 2014) under elevated carbon dioxide concentration. Stress response in plants resulted due to the formation of reactive oxygen species (ROS) radicals. The phenomenon comprising of O_2 molecule (free radical) in ROS with unpaired electrons (two) that have the parallel spin quantum number. This spin restriction allows O_2 to accept its electrons one at a time, leading to the creation of the so called ROS, which are highly reactive, toxic in effect and damage lipids, protein, carbohydrates and DNA machinery leading to oxidative stress in the cells and tissue biochemistry. The ROS in plants, comprises of various forms of free radicals like superoxide radicals ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$), and the perhydroxy radical ($HO_2\cdot$) and molecular forms (non-radical) hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$).When plants are exposed to abiotic stress these reactive radicals gets accumulate under stressful condition, while the antioxidant defense system with enzymatic (Superoxide dismutase, SOD; Catalase, CAT; Ascorbate peroxidase, APX etc.) and non-enzymatic (Phenolic Compounds, Flavanoids, Ascorbic acid, ASH; Alkaloids etc.) machinery scavenge the free radicals and helps to protect against damage due to oxidative stress in plants. ROS generation occurs particularly in the stressful environmental changes and are produced continuously as by products of various metabolic pathways contained in different cellular organelles such as chloroplast, mitochondria and peroxisomes (Navrot *et al.,* 2010). Phenolics being the foremost group of phytochemicals that causes the majority of the antioxidant activity in plants or plant products. Phenolics acquire a wide range of biochemical activities like antioxidant, antimutagenic, anti carcinogenic, as well as capacity to transform the gene expression. It is already projected that eight thousand plant phenolics and about fifty percent of them are flavonoids (Harborne *et al.,* 1993) occurs naturally. Flavonoids are the part of phenolic compounds found in different parts of plants, occurring naturally both in free state and as glycosides. These compounds possess many biological activities including antimicrobial, antiarthritic, antiulcer, anticancer, antiangiogenic etc. The most extensively distributed of all the Phenolic are flavones and flavonols (Peter *et al.,* 1999). Flavonoids are valuable as an antioxidants and protects against cardiovascular disease, certain forms of cancer and age related degeneration of cell components. They have polyphenolic structure moiety that enable them to scavenge injurious free radicals like super oxide and hydroxyl radicals.

The present experimental study predicts the impact of elevated carbondioxide concentration on micronutrients uptake in soil, leaves, pods and seed and antioxidant defence mechanism in *Cyamopsis tetragonoloba*, a legume.

Materials And Methods

Site Description

The experimental plan was performed in National Botanical Research Institute (N.B.R.I) an urban site (26° 55' N, 80° 59' E) located at main city of Lucknow, India with whole Free Air Carbon dioxide Enrichment (FACE) setup, situated 135 m above sea level, with loamy sandy soil (sand 55%, silt 31%, clay 14%), pH in range of 8.3-8.7 and the electrical conductivity in range of 229-234 µS cm^{-1}. Climate condition in this area is basically tropical hot and humid with average mean temperature of 34°C and heat index 40°C. Precipitation was estimated was 0.04.

Experimental Setup

Two varieties of *Cyamopsis tetragonoloba* RGC 1002 and RGC 1066 seeds were collected from semi arid areas of Jodhpur, Jaiselmer and Barmer were selected on screening *Cyamopsis* varieties (8 in no.) on basis of better adaptation potential and physiological performances under elevated carbon dioxide concentration (e[CO_2]) . Seeds were surface-sterilised in 1 % (V/V) mercuric chloride solution for 15-20 min. After rinsing in distilled water, were kept in glass beakers, imbibed for 12 hrs and then were sown in 14 inches of pots filled with 10 kg of sandy and loamy garden soil and proper irrigation was given during experiment.

Pots were ploughed to maintain proper aeration and recommended doses of NPK fertilisation (120:60:60) was applied prior to seed sowing. Pots were kept in open field at photosynthetic active radiation (PAR) in range of 678-768µmol/m^2/s, at a temperature of 32.1°-35.9°C, at relative humidity between 63-74RH and wind speed in range of 0-0.7m/s. Seeds were germinated, seedlings grew and after 40 days plant were transferred in rings under elevated and ambient carbon dioxide enrichment concentrations. RGC 1002 and RGC 1066 pots were setup and kept under aluminium framework of circular rings

in three replicates maintained under e[CO_2] (triplicate rings) (550 ± 20 ppm) and a[CO_2] (triplicate rings) (420 ± 20ppm). Pure carbon dioxide gas mixed with pure air is used in enrichment. A circulating pump and regulator were used to infuse carbon dioxide into the ring chambers. Carbon dioxide concentration was adjusted by flow meter to the target level. Carbon dioxide was fumigated for 125 days from 9:30am to 5:30pm. FACE setup is computer automated with eight data scanner and wind monitor logger which monitor and capture per day data. Plants grown in the above condition for 160 days (5 months) including seed sowing to seedling stage of 40 days from 20 April 2015 to 12 Nov 2015 and plant stage with routine and proper irrigation were maintained.

Plant and Soil Sampling

The samples of plants including root,stem, leaves, pods and seeds were collected from each rings of pots by harvesting the plant parts, dried and crucified in motor and pestle and ashed. Randomised plants sample were selected in 2015 and roots and leaves were separated. Samples were washed to remove soil particles, soaked with tissue paper. All samples were dried in oven at 105°C and then over dried at 70°C. These samples were grounded with motor and pestle and followed by 0.25 mm sieve prior to analysis.

Soil samples from the roots legume-soil system were collected from each ring of pots in polybags.The root legume-soil system were excavated at the depth of 3 cm to carefully separate roots-legume from soil system. The soil was mixed evenly to form composite sample of rhizoshpheric soil. All the plant residues including plant parts, roots residue were separated and removed. The soil were sieved through 2 mm mesh sieve and transferred to ziplog plastic bags. These soil samples were kept and stored in 4°C prior to further analysis.

Soil MBC

Soil MBC was determined (three replicates) by chloroform-fumigation extraction method (Vance *et al.,* 1987) followed by 12.5 gm of moist sample of non fumigation and fumigation were extracted with 0.5M potassium sulphate (K_2SO_4) and oxidized with 0.4 N Potassium di chromate ($K_2C_rO_7$), after oxidation process titrated with 0.4N ammonium ferrous sulphate. The MBC sample was calculated by a formula: microbial biomass C=fumigated – non fumigated and the kinetic value of C is 0.33.

Biochemical Assays

Superoxide Dismutase (SOD) Enzyme Assay

Assay for superoxide dismutase was performed on leaves sample (three replicates) in terms of SOD's ability to inhibit reduction of nitroblue tetrazolium (NBT) to form formazan by superoxide (Beyers *et al.,* 2012). Plant tissue (0.5 g) was homogenized in phosphate buffer (pH 7.5), 1mM ethylenediaminetetraaceticacid and 2% (w/v) polyvinylpyrrolidone (PVP) in a chilled motor and pestle. The homogenate was centrifuged at 14,000 × g for 10 minutes at 4°C and supernatant separated and applied for the assay. The assay mixture consisted of phosphate buffer (pH 7.8), Lmethionine, NBT, Triton X-100 and riboflavin. To the assay mixture sample and few microliters of riboflavin was added in glass vials and glass vials were kept in light box for 7–10 min. After 7 min. glass vials are taken out wrapped in black cloth. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm (Unit: mg^{-1} protein). One unit of SOD activity is defined as the amount of enzyme that causes 50% inhibition of NBT reduction in one minute.

Total Ascorbate (ASH)

Total ascorbate was determined by the method of Hodges *et al.,* 2003. Plant tissue was homogenized in 10% TCA and 50 mM phosphate buffer (pH 7.4) which contained 3mM EDTA and 1 mM DTT. For assay of total ascorbate, DTT was used and the extract was centrifuged at 14,000×g for 15 transactions. Now the aliquot was incubated at 25°C for 10 minutes. To the aliquot was added N-ethylmaleimide (0.5% w/v), 0.61M TCA, 0.8M H_3PO_4, 65 mM α-α-bipyridil and 110 mM ferric chloride. The mixture was then incubated in a water bath at 55°C for 10 minutes and absorbance was recorded at 525nm (Unit: micro mol mg^{-1} protein).

Ascorbate Peroxidise (APX)

Ascorbate peroxidase (APX) is one of the key in ascorbate-glutathione cycle. APX assay was performed by H_2O_2 dependent oxidation of ascorbate (Chen *et al.,* 1989). Reaction mixture consisted of 50mM phosphate buffer (pH 7.0), 0.6mM ascorbate, plant tissue extract and 10% H_2O_2. Reaction was initiated by using H_2O_2 and decrease in absorbance was noted at 290 nm. Extinction coefficient 2.8 $mM^{-1}cm^{-1}$ was used to calculate the activity.

Total Phenolic Content

The total phenolic content were determined by the method Folin-Ciocalteu assay. An aliquot (1-mL) of extracts or standard solution of Gallic acid (100, 200, 300, 400, and 500 µg/mL) was prepared and was added to 25 mL of volumetric flask, containing 9 mL of distilled water. Total phenoic content was performed by mixing sample with 50% CH_3OH : H_2O. The solution is vortex in centrifuge at 1000 rpm, 4°C for 10 min. and left for over night. The sample in different volumes were taken in glass voils and volume makeup was made by milliq water (0.5 mL) to which folin reagent (0.5 mL) and 20% Na_2CO_3 (1-mL) were added. Finally solution was make to the volume (12.5 mL) by adding milliq water, vortex, incubate for half an hour and absorbance was noted at 720 nm.

Total Flavanoid Content

Total flavanoid content was measured by the aluminum chloride colorimetric assay. An aliquot comprising extracts (1-mL) or standard solutions prepared of quercetin (20, 40, 60, 80 and 100 µg/mL) was added to volumetric flask (10 mL) containing distilled water (4 mL). To the above volumetric flask was added 5% $NaNO_2$ (0.30 mL), after five minutes 10 % $AlCl_3$ (0.3 mL) was added. After five minutes, 1M NaOH (2 mL) was added and the volume was made up to 10 mL with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm. The total flavanoid content was expressed as mg quercetin equivalents (QE).

Sample Analysis for Micro-nutrient Assay

Plant samples (0.25 g) including root, leaves, pods and seed are weigh and was digested in 100 mL digestion tube with 5 mL

HNO_3 and 1ml perchloric acid (5:1,V:V) at 170°C .The samples were kept in digestion block was digested in hot block digester (Kjeldhal-Digestion unit) for 7–8 hours till white residue is obtained. The filterate is filtered and the volume was make to 50 mL with distilled water. The concentration of micronutrients were determined with Integrated Coupled Plasma Mass Spectrometry (ICP-MS Agilent technologies 7500cx).

Soil basic measurements like soil pH, EC was conducted and measured using the glass electrode (1/3 soil/water ratio) with Metteler-Toledo pH meter.

Soil samples (0.1g) are weigh and was pre-digested in 100ml digestion tube with 5 mL HNO_3 and 1-mL perchloric acid (5:1,V:V) at 170°C. The samples were digested in hot block digester (Kjeldhal-Digestion unit) for 7-8 hours till white residue is obtained. The filterate is filtered and the volume was make to 50 mL with distilled water. The concentration of micronutrients were determined with Integrated Coupled Plasma Mass Spectrometry (ICP-MS Agilent technologies 7500cx).

Statistical Analysis

The experimental plan was performed with three replicates of each treatment dose from triplicate rings maintained at e[CO_2] and a[CO_2] concentrations in a randomized plan. Statistical analysis of the data was performed by by SPSS 16.0 software version (One way Anova). The standard deviation (SD) values between the treatments were calculated at 5% probability levels and significance of data is analyzed $p \leq 0.05$.

Results

Plant and Soil Analysis for Micro-nutrients

Micro-nutrient analysis were done and estimated in both the *Cyamopsis* varieties under e[CO_2] concentration. Micro-nutrient included were Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo and Pb. These micro-nutrients analysis were done in both leaf and soil samples. Cr content declines significantly ($p \leq 0.05$) in 1002 (62.14%) contrary, it was increased in 1066 (92.0%) under e[CO_2] concentration. Mn content increased in both the plant varieties significantly however, it was more (17.25%) in 1002 and (4.12%) in 1066. Fe content declined significantly in (56%) in 1002 and it increased (32.99%) in 1066 under e[CO_2] concentration. Co content was found to decline (9.11%) in 1002 but it was increased and enhanced (15.30%) in 1066 under e[CO_2] concentration. Cu content declined in both the varieties significantly, was reported less in (23.16%) in 1002 and (52.81%) in 1066 under e[CO_2] concentration. Zn content also declined significantly ($p \leq 0.05$) in both plant varieties to (30.61%) in 1002 and (20.29%) in 1066 under e[CO_2] concentration. As content declined to (43.52%) in 1002 however, no significant changes were reported in 1066 under e[CO_2] concentration. Se content declined in both and was (13.57%) in 1002 and (19.00%) in 1066 reported under e[CO_2] concentration. Mo content declines in both the varieties and it was more (38.42%) in 1002 than (31.54%) in 1066 under e[CO_2] concentration. Lastly Pb content reported a decline significantly (49.12%) in 1002 and (40.21%) in 1066 under e[CO_2] concentration. Similar analysis of micro-nutrients were done in soil under e[CO_2] concentration. Cr content in soil was found to increased in both was (35.42%) in 1002 and (16.73%) in 1066 but no significant differences were reported in both varieties under e[CO_2] concentration. Mn content was also found to increase and was reported more (32.29%) in 1002 soil than (14.40%) in 1066 soil under e[CO_2] concentration however, both varieties showed non significant differences. Fe content was found more in (71.86%) in 1002 soil contradictory, it decreased non significantly in (3.91%) in 1066 soil under e[CO_2] concentration. Co and Ni content donot reported any significant changes in both varieties soil under e[CO_2] concentration. Cu content was reported more in (58.68%) 1002 soil than (18.56%) in 1066 soil under e[CO_2] concentration. Zn content increased significantly in (29.63%) 1002 soil however, it was found to decline in (24.82%) 1066 soil under e[CO_2] concentration. As content was more significant ($p \leq 0.05$) in (101.5%) 1002 soil than in (26.77%) 1066 soil under e[CO_2] concentration. Se content donot showed significant differences in both the varieties. Mo content was more reported in both the varieties and was (34.13%) in 1002 soil than (69.73%) in 1066 soil under e[CO_2] concentration. Pb content was reported to increase in (21.45%) 1002 soil however, it was decline in (12.49%) 1066 soil under e[CO_2] concentration.

Micronutrient in Pods and Seeds

Micro nutrient analysis were done in pods and seeds of *Cyamposis tetragonoloba* which includes Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Pb. Cr content increased significantly ($p <= 0.05$) in pods of both RGC 1002 (16.68%) and RGC 1066 (21.96%). Similarly increase trend was found in seeds with RGC 1002 (5.08%) and RGC 1066 (21.86%). Mn content increased significantly in pods of both the varieties with RGC 1002 (23.05%) showing more increment than RGC 1066 (11.81%) (Table 3). However in seeds

Table 1: Micro-nutrient content concentration in RGC 1002 and RGC 1066 under (e[CO_2]=550 ± 20ppm) and (a[CO_2]=420 ± 20 ppm) concentration in leaves. Different lower case letters depicts significant differences in mean value at $p \leq 0.05$.

Micronutrients→ (leaves) varieties↓	*Cr (mg/kg)*	*Mn (mg/kg)*	*Fe (mg/kg)*	*Co (mg/kg)*	*Cu (mg/kg)*	*Zn (mg/kg)*	*As (mg/kg)*	*Se (mg/kg)*	*Mo (mg/kg)*	*Pb (mg/kg)*
e[CO_2] 1002	5.18 ± 0.97^{bc}	50.53 ± 2.80^{ab}	656.46 ± 55.00^{c}	1.517 ± 0.265^{a}	10.03 ± 2.24^{bc}	76.23 ± 2.19^{bc}	1.08 ± 0.12^{b}	1.25 ± 0.13^{b}	2.91 ± 1.28^{b}	1.95 ± 0.16^{b}
a[CO_2] 1002	13.68 ± 2.27^{b}	43.10 ± 2.69^{a}	1491.94 ± 46.33^{b}	1.656 ± 0.184^{b}	13.06 ± 2.46^{c}	109.87 ± 5.35^{c}	1.91 ± 0.68^{ab}	1.44 ± 0.06^{b}	4.73 ± 1.21^{ab}	3.83 ± 0.49^{b}
e[CO_2] 1066	7.67 ± 1.29^{a}	62.86 ± 7.63^{b}	920.11 ± 84.58^{a}	0.921 ± 0.073^{a}	7.71 ± 1.18^{ab}	67.08 ± 5.90^{a}	1.58 ± 0.15^{a}	1.39 ± 0.16^{ab}	5.24 ± 1.42^{b}	2.48 ± 0.41^{a}
a[CO_2] 1066	3.99 ± 0.74^{ac}	60.37 ± 11.71^{a}	691.83 ± 124.30^{c}	0.798 ± 0.060^{b}	16.35 ± 3.85^{a}	84.16 ± 9.54^{b}	1.58 ± 0.18^{ab}	1.71 ± 0.21^{a}	7.65 ± 1.23^{a}	4.15 ± 0.24^{a}

Table 2: Micro-nutrient content concentration in RGC 1002 and RGC 1066 under (e[CO_2]=550 ± 20 ppm) and (a[CO_2]=420 ± 20 ppm) concentration in soil. Different lower case letters depicts significant differences in mean values at p ≤ 0.05

Micronutrients→ (Soil) varieties↓	*Cr (mg/kg)*	*Mn (mg/kg)*	*Fe (mg/kg)*	*Co (mg/kg)*	*Cu (mg/kg)*	*Zn (mg/kg)*	*As (mg/kg)*	*Se (mg/kg)*	*Mo (mg/kg)*	*Pb (mg/kg)*
e[CO_2] 1002	15.93 ± 3.45^{a}	431.78 ± 72.05^{a}	22864.11 ± 917.90^{a}	8.25 ± 1.31^{a}	37.33 ± 3.12^{a}	132.24 ± 8.05^{b}	7.09 ± 1.53^{a}	2.13 ± 0.18^{a}	4.81 ± 1.04^{b}	25.12 ± 3.37^{bc}
a[CO_2] 1002	11.76 ± 1.76^{a}	326.38 ± 75.60^{a}	13303.33 ± 1184.12^{a}	6.28 ± 1.49^{a}	23.53 ± 3.74^{a}	102.01 ± 25.88^{b}	3.52 ± 0.92^{ab}	2.05 ± 0.21^{a}	3.58 ± 0.35^{a}	20.68 ± 2.94^{ab}
e[CO_2] 1066	16.29 ± 0.66^{b}	424.88 ± 72.48^{a}	20952.94 ± 3410.13^{b}	8.31 ± 1.54^{a}	40.92 ± 5.31^{b}	129.98 ± 16.58^{b}	5.17 ± 0.41^{b}	1.80 ± 0.10^{a}	5.01 ± 0.25^{b}	26.63 ± 1.75^{c}
a[CO_2] 1066	13.96 ± 0.80^{ab}	371.39 ± 39.60^{a}	21806.17 ± 575.75^{a}	8.14 ± 0.35^{a}	34.51 ± 1.41^{a}	172.91 ± 22.25^{a}	7.06 ± 1.32^{a}	1.95 ± 0.18^{a}	2.95 ± 0.13^{c}	30.43 ± 1.36^{a}

Table 3: Micro-nutrient content concentration in RGC 1002 and RGC 1066 under (e[CO_2]=550 ± 20ppm) and (a[CO_2]=420 ± 20ppm) concentration in pods. Different lower case letters depicts significant differences in mean values at p≤0.05.

Micronutrients→ (pod) varieties↓	*Cr (mg/kg)*	*Mn (mg/kg)*	*Fe (mg/kg)*	*Co (mg/kg)*	*Cu (mg/kg)*	*Zn (mg/kg)*	*As (mg/kg)*	*Se (mg/kg)*	*Mo (mg/kg)*	*Pb (mg/kg)*
e[CO_2] 1002	18.53± 0.42^{c}	20.55± 1.08^{c}	149.35± 4.49^{a}	2.62± 0.18^{c}	38.43± 0.78^{c}	6.95± 0.72^{c}	3.81± 0.08^{c}	192.17± 1.53^{c}	2.38± 0.08^{c}	2.11± 0.10^{c}
a[CO_2] 1002	15.88± 0.43^{d}	16.70± 0.38^{d}	140.80± 1.69^{c}	2.13± 0.16^{d}	35.77± 0.43^{d}	5.42± 0.44^{d}	3.31± 0.10^{d}	161.17± 1.61^{d}	2.13± 0.10^{d}	1.44± 0.13^{d}
e[CO_2] 1066	24.71± 1.01^{a}	24.35± 0.41^{a}	141.27± 1.37^{b}	4.00± 0.35^{a}	46.62± 1.41^{a}	8.77± 0.35^{a}	5.14± 1.19^{a}	405.83± 14.09^{a}	6.85± 0.43^{a}	6.53± 0.63^{a}
a[CO_2] 1066	20.26± 0.61^{b}	21.78± 0.63^{b}	135.25± 0.85^{d}	3.12± 0.18^{b}	44.15± 0.48^{b}	8.00± 0.13^{b}	4.41± 0.10^{b}	349.17± 4.01^{b}	3.95± 0.28^{b}	5.29± 0.26^{b}

it was enhanced and was found to be less in RGC 1002 (7.52%) than RGC 1066 (9.96%). Fe content increased significantly in pods with RGC 1002 (6.00%) than RGC 1066(4.00%), but in seeds it was more in RGC 1002 (9.75%) than RGC 1066 (8.71%). Co content increased significantly and was more in pods of RGC 1066 (28.00%) than RGC 1002 (262.65%) and in seeds also it was reported more in RGC 1066 (35.23%) than RGC 1002 (22.05%). Cu content was more in RGC 1002 (7.45%) than in RGC 1066 (5.58%) but in seeds it follows opposite trend was increased in RGC 1066 (10.16%) than in RGC 1002 (6.42%). Similarly Zn content was more in pods of RGC 1002 (28.30%) than RGC 1066 (9.58%) (Table 3) but in seed it follws opposite trend was more in RGC 1066 (7.14%) than RGC 1002 (6.10%). As content was more in RGC 1066 (16.64%) than in RGC 1002 (15.12%) however in seeds was reported more in RGC 1002 (13.76%) than in RGC 1066 (10.49%). Se content increased and was more in pods of RGC 1002 (19.00%) than in RGC 1066 (16.00%) and similar increased trend was reported in seed of RGC 1002 (16.87%) than RGC 1066 (10.87%). Mo content increased most significantly was reported more in pods of RGC 1066 (73.41%) than in RGC 1002 (11.71%) (Table 3) and similar trend was reported in seeds of RGC 1066 (9.48%) than in RGC 1002 (5.00%). Pb content increased significantly was more in pods of RGC 1002 (46.24%) than RGC 1066 (23.30%) (Table 4) and similar trend was reported in seeds with RGC 1002 (40.65%) than in RGC 1066 (10.05%).

Soil MBC

Soil microbial biomass carbon shows soil fertility. The MBC was significantly higher (p value< 0.05) in elevated condition in (Table 5). Rhizospheric soil of both RGC 1002 and RGC 1066 varieties in both pre and post flowering stages. There are significant changes found in RGC 1002 and RGC 1066 in both e[CO_2] and a[CO_2] concentration grown plants which shows higher microbial activity and it may possible due to the fast growing nature having high biomass and biological N_2-fixation capacity, compared to RGC 1002 legume.

Effect of Elevated CO_2 on SOD, ASH and APX

Elevated carbon dioxide significantly affects the antioxidant such as SOD, ASH, and APX. SOD activity declined in a significant way ($p \leq 0.05$) under elevated carbon dioxide concentration in both the plant varieties however it was more pronounced in RGC 1066 (17.90%) than in RGC 1002 (16.63%) when compared with ambient carbon dioxide concentration grown plants. However between the varieties insignificant differences were found. Total ascorbate content increased in both varieties however was more pronounced and almost many fold times in RGC 1066 (66.32%) than RGC 1002 (9.37%) under elevated carbon dioxide concentration. Thus a significant difference in ascorbate content was observed in elevated and ambient grown plant varieties. APX activity was significantly high ($p \leq 0.05$) and almost double in RGC 1066 (12.17%) than in RGC 1002 (6.3%). Thus statistical analysis shows that antioxidant activity were affected in elevated carbon dioxide grown plant varieties when compared with their ambient counterparts.

Effect of Elevated CO_2 on Total Phenolics and Flavanoids

Elevated carbon dioxide enhanced the synthesis of total phenol and flavanoid content in both the plant varieties. Thus it significantly affected the phenolic and flavanoid content both in leaves and roots. The phenolic content increased more significantly ($p \leq 0.05$) in leaves of RGC 1066 (76.50%) than RGC 1002 (11.53%) however in roots it followed the same trend, was

Table 4: Micro-nutrient content concentration in RGC 1002 and RGC 1066 under ([CO_2]=550 ± 20ppm) and (a[CO_2]=420 ± 20ppm) concentration in seeds. Different lower case letters depicts significant differences in mean values at p ≤ 0.05.

Micronutrients→ (seeds) varieties↓	*Cr (mg/kg)*	*Mn (mg/kg)*	*Fe (mg/kg)*	*Co (mg/kg)*	*Cu (mg/kg)*	*Zn (mg/kg)*	*As (mg/kg)*	*Se (mg/kg)*	*Mo (mg/kg)*	*Pb (mg/kg)*
e[CO_2] 1002	8.68± 0.13^{c}	13.15± 1.00^{c}	135.72± 4.48^{a}	1.38± 0.08^{c}	17.38± 1.16^{c}	4.63± 0.10^{c}	4.00± 0.08^{c}	188.16± 2.3^{c}	4.86± 0.08^{a}	2.13± 0.10^{c}
a[CO_2] 1002	8.26± 0.10^{d}	12.23± 0.40^{d}	123.65± 1.59^{c}	1.13± 0.08^{d}	16.33± 0.20^{d}	4.37± 0.08^{d}	3.51± 0.08^{d}	161± 1.3^{d}	4.64± 0.08^{c}	1.52± 0.08^{d}
e[CO_2] 1066	13.08± 0.53^{a}	17.37± 0.43^{a}	129.98± 3.80^{b}	1.75± 0.10^{b}	23.83± 0.28^{a}	5.00± 0.13^{a}	4.56± 0.08^{a}	234.5± 8.7^{a}	4.81± 0.13^{b}	3.10± 0.15^{a}
a[CO_2] 1066	10.31± 0.18^{b}	15.80± 0.33^{b}	119.57± 1.44^{d}	2.37± 0.10^{a}	21.63± 0.31^{b}	4.67± 0.08^{b}	4.13± 0.05^{b}	211.5± 2^{b}	4.39± 0.10^{d}	2.82± 0.08^{b}

Table 5: The data of soil Microbial Biomass Carbon (MBC) in RGC 1002 and RGC 1066 in rhizospheric soil sample under e[CO_2] and a[CO_2] concentration in pre-flowering and post-flowering stages. Different lower case letters signifies significant differences in mean values at p ≤ 0.05.

Stages	*Varieties*	*MBC (µg/g)[Rizhospheric]*
PRE FLOWERING	e[CO_2] 1002	277.90±0.34^{b}
	a[CO_2] 1002	198.95±0.48^{d}
	e[CO_2] 1066	283.00±0.88^{a}
	a[CO_2] 1066	201.07±0.92^{c}
POST FLOWERING	e[CO_2] 1002	285.91±0.42^{b}
	a[CO_2] 1002	218.75±0.42^{d}
	e[CO_2] 1066	293.47±0.13^{a}
	a[CO_2] 1066	227.99±0.32^{c}

more in RGC 1066 (25.63%) than RGC 1002 (9.33%). Flavanoid content also increased significantly but was found more in leaves of RGC 1066 (19.82%) than RGC 1002 (10.46%) ,in roots it was increased and was found more in RGC 1066 (23.26%) than RGC 1002 (18.93%).

Discussion

Micronutrient availability is the capacity of a soil to provide sufficient micronutrients to a plant, which is specific to soil and plant (White *et al.,* 1999). Our results depict that the micro-nutrient Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, and Pb content increased availability in 1002 soil exposed to e[CO_2] concentration. However in 1066 soil Cr, Mn, Co, Cu, and Mo content availability was increased while Fe, Zn, As, Se, and Pb availability declined under e[CO_2] concentration. The changes of soil micronutrient availability can be partly explained by the root exudate-induced changes in soil pH and partly by increase in soil moisture under e[CO_2] concentration as observed by Wang *et al.,* 2016a in wheat. As observed in our previous experimental study, soil pH decreased significantly under e[CO_2] concentration (Mehrotra *et al.,* 2017). The outcome of reduction in soil pH leads to soil acidosis which resulted in translocation of reduced amount of micro-nutrients in plant. It was well known that reduction in soil pH effects on mineral dissolution and acidity. Previous experimental studies reported that the chief source of soil acidity is the result of carbon dioxide contributed by the plant roots respiration, microbial respiration, and atmospheric diffusion. The mechanism behind soil acidity is carbon dioxide reacts with soil moisture and forms carbonic acid (Oh Hwan *et al.,* 2004). Thus the H^+ and HCO_3 ion generated resulted in lowering of soil pH by proton to soil pool under carbon dioxide elevation. Thus it leads to reduction in overall soil because of elevated carbon dioxide concentration (Oh Hwan *et al.,* 2004). However this proton pool outpaces mineral dissolution because of rapid kinetics of exchangeable surface cations generated from carbonic acid, so mineral dissolution is buffered.

Our results showed that availability of Fe, Mn, Co and Cr was negatively correlated with soil pH across different varieties ($p \leq 0.05$) (Table 2). A lot of studies have reported that soil pH was one of the main factors influencing the solubility and availability of trace elements in arable soils (Öborn *et al.,* 1995). Although we donot have estimated plant root exudates in our study but, other studies have shown that plants exposed to elevated CO_2 exudate more carbohydrates into the rhizosphere than those under ambient CO_2 (Bhattacharyya *et al.,* 2013; Yin *et al.,* 2013). The root exudates are composed of organic acid, polymeric carbohydrates, polysaccharides, polymer degrading enzymes, amino acids, and fatty acids (Franson *et al.,* 2010; Doornbos *et al.,* 2012), which acidifies rhizosphere soil. Root exudates also resulted in enhance availability (Table 1.) of some micronutrients (e.g., Mn) by changing the oxidation-reduction potential (Hinsinger *et al.,* 2001). The increase in soil moisture under CO_2 enrichment was investigated by many researchers (Wang *et al.,* 2016b) that resulted in increased reducing condition near rhizospheric soil. Under reducing condition, Mn in soils is present in the form of Mn^{2+}, which is more soluble than Mn^{4+}.

Additionally, CO_2 enrichment could increase root exudates and organic debris sloughing off belowground, which enhanced soil microbial turnover in rhizosphere (Jin *et al.,* 2014). Our results depicts that e[CO_2] increased MBC significantly in both the varieties and soil MBC can be positively co-related with the availability of Cr, Mn, Co, Cu and Mo micro-nutrient in rhizospheric region which ultimately affect nutrient avaliability by modulating key elemental cycling (Allard *et al.,* 2005).

Under e[CO_2] concentration scenarios, seed nutrient status is becoming a global concern for future requirement of human nutrition (Pleijel *et al.,* 2018; Jin *et al.,* 2019). Micro nutrient content was however not restricted in pods and seeds of both the RGC 1002 and RGC 1066 plant varieties under e[CO_2] concentration. Majority of the nutrients contents like Mn, Fe, Co, Cu, Zn, As, Se, Mo, Pb were found to be significantly higher ($p <= 0.05$) in both RGC 1002 and RGC 1066 vartieties under e[CO_2] concentration. RGC 1002 follows the decreasing trend

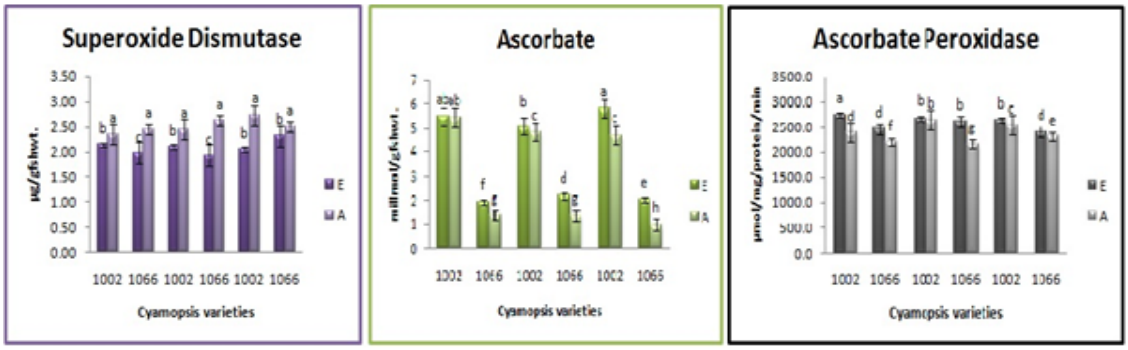

Fig. 1: (a-c) Antioxidant assay in leaves of *Cyamopsis tetragonoloba* under elevated and ambient carbon dioxide concentration. (a) Superoxide enzymatic assay (SOD) (b) Total ascorbate content (ASH) and (c) Ascorbate peroxidise (APX) enzymatic assay. Significant difference is calculated at p≤0.05 probability level. Different lower case letter signifies significant differences and same letters signifies non significant differences between the mean values.

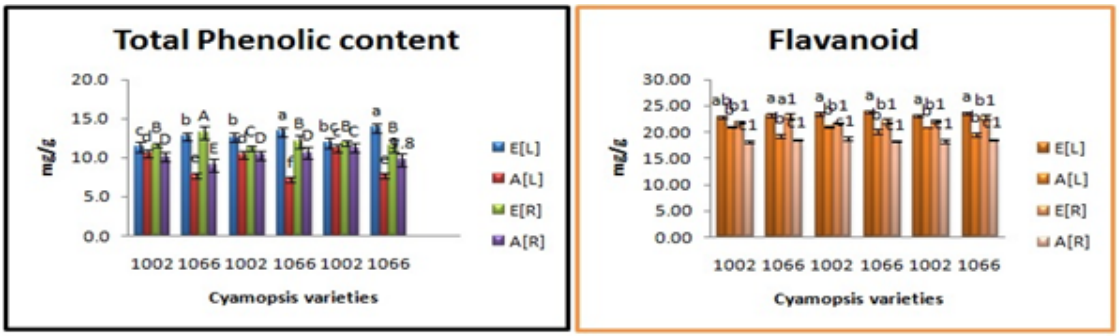

Fig. 2: Total Phenolic content (a) and Flavanoids (b) in leaves[L] and roots[R] of *Cyamopsis tetragonoloba* plant varieties RGC 1002 and RGC 1066 under elevated and ambient carbon dioxide concentration. Different symbols are used to define the mean values in leaves and roots under elevated (E) and ambient (A) carbon dioxide concentration. Different upper and lower case letter shows significant differences (p≤0.05) of mean values.

of micro-ntrient in pods Se>Fe> Cu>Mn>Cr>Zn>As>Mo>Co>Pb under e[CO_2] concentration, however in seeds it follows slight different trend Se>Fe>Cu> Mn>Cr>Mo>As>Zn>Pb>Co. Thus in seed Mo and Pb translocation and concentration was more than pod. RGC 1066 follows the decreasing trend of micro-nutrient in pods Se>Fe>Cu>Cr>Mn>Zn>Mo>Pb>As>Co, however in seeds it follows slightly different trend Se>Fe>Cu>Mn>Cr>Zn>Mo>As>Pb>Co. Similarly in seeds Mn and As translocation was more but concentration was less than pods. Baliger *et al.* 2017 also reported micro-nutrient uptakes were significantly influenced by leguminous cover crop species. According to his findings significant variability in nutrient uptake among various cover crop species was associated with different growth habits, the amount of dry matter accumulated in the shoot and the specific requirement of the plant for any particular nutrient.

In all the legumes the mean P, Mg, Cu, Fe and Zn content increased significantly with increasing PPFD. Accumulation of nutrients was in the order of Mn > Fe > Zn >Cu for micronutrients. Similar trends in higher Mn and Fe uptake in other perennial legume cover crops have been reported Baliger *et al.* 2006. Although our studies did not have estimated nutrient influx, transport and nutrient efficiency but these parameters were influenced by various leguminous species and crop species have significant effect on transport of micro-nutrients (Baliger *et al.,* 2006).

Antioxidant enzyme assay includes Superoxide dismutase enzyme, ascorbic peroxidase and total ascorbate estimation while non enzymatic assay includes total phenol and flavanoides. Superoxide dismutase enzyme activity slightly declines however, elevated carbon dioxide concentration increased the level of ascorbate peroxidation, total ascorbate, total phenol, and flavanoids (Fig.1 and 2) with significant enhancement in the antioxidant capacity and leading to decline in ROS level. This changes in ROS level occurs particularly in the pace of stressful environmental changes, such as adverse climatic changes like droughts and heatwave (Sekmen *et al.,* 2014; Zinta *et al.,* 2014). It is reported that when plant is going through senescence stage some antioxidants compounds starts increasing and others decreasing as ROS accumulates in a large amount, and the antioxidant machinery disturbs so it doesnot work well. This is often indicated by enhanced lipid peroxidation and decreased levels of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) particularly under severe abiotic stress (Kumari *et al.,* 2013).

Previous findings justifies our results (Fig.1) and interpret the same finding in the plants exposed to elevated CO_2 caused enhanced level of ASC and phenol as were obtained in *Betavulgaris* by Kumari *et al.,* 2013 and a similar increase in the ASC, as well as in the redox status, were found in *L. perenne* and *M.lupulina* by Farfan-Vignolo and Asard, 2012. Ascorbate synthesis was activated and may be enhanced by too much

carbohydrate production due to elevated CO_2 (Farfan-Vignolo and Asard, 2012; Smirnoff *et al.,* 2000), which can be closely correlated to carbon metabolism (Smirnoff *et al.,* 2000), and alltogether resulted in improvement in the plant-antioxidant defense system. Moreover, elevated CO_2 not only causes delay in the onset of senescence and it is commonly accepted that the antioxidant enzyme activity and accumulation of antioxidant compounds show better performance in dealing with the biological process of senescence (Hodges *et al.,* 2003). However, oxidative stress was found to reduced in *Catharanthus roseus* (Singh *et al.,* 2015), a bean, *A. thaliana* plants (*Baliger et al.,* 2006) and *Vigna radiate* (Mishra *et al.,* 2014) under elevated CO_2. However, under elevated CO_2-alleviated oxidation stress indication coming from a various reports (Xu *et al.,* 2014), but these results havenot been confirmed in some species, such as in *Spinacia oleracea* leaves (Hodges *et al.,* 2003). Farfan-Vignolo and Asard (2012) reported that CO_2 enrichment could aggravate lipid peroxidation in *M.lupulina*, but not in *L. perenne* plants, with no rising-CO_2 responses in the ascorbate peroxidase (APX) and peroxidase (POX) in *M.lupulina*. According to the previous findings the activities of SOD, and APX, as well as the sum of dehydroascorbate and ASC, were reduced in CO_2-elevated environments (Schwanz *et al.,* 1998) however, in C_3 grasses (*L. perenne, Poapratensis*) and C_3 legumes (*M.lupulina*, *Lotus corniculatus*) elevated CO_2 reduce the H_2O_2 level, while it decreased the SOD, but did not affect the ASC-GSH cycle (AbdElgawad *et al.,* 2014).Thus, the predominant form of the enzymatic antioxidant defense may strongly depend on the species and the abiotic stress (Zinta *et al.,* 2014; Duarte *et al.,* 2013). Many papers reported that elevated carbon dioxide helps in mitigating the stress effects but the abiotic stress impact vary considerably. The underline mechanism apart from providing extra C, it also induces stomatal closing. However, reduced oxidative stress, damage and ROS level involves metabolic changes, more specifically extra C assimilates synthesis and availability that results in increased supply of defence molecules (antioxidants) that are responsible for protection against oxidative damage under elevated carbon dioxide concentration.

Conclusions

It can be concluded from the above experimental study that soil micro-nutrient availability was improved frequently as observed under e[CO_2] concentration. Soil pH and Microbial Biomass Carbon (MBC) were key factors influencing the availability of soil micro-nutrients under e[CO_2] concentration. However, it was observed that the interaction between e[CO_2] and varieties growth stages (pre-flowering and post-flowering) increased uptake of micro-nutrients. Apart from these RGC 1066 variety showed better uptake and translocation of micro-nutrient efficiency (Cr, Mn, Co, Cu, Mo) than RGC 1002 plant variety (Mo) under e[CO_2] concentration.Apart from above, antioxidant response in both the cultivars was enhanced under elevated carbon dioxide concentration that leads to the scavenging of ROS thus leading to declining of ROS against abiotic stress condition. This condition leads to altogether improvement in plant antioxidant defences system. However, under e[CO_2] concentration, overall RGC 1066 plant variety was found to be showing higher antioxidant activity than including enzymatic SOD, ASH, APX and non- enzymatic including total phenolics and flavonoids RGC 1002. Thus, it can be concluded that RGC 1066 is better than RGC 1002 plant variety which is adapting and performing in better way under e[CO_2] concentration.

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Authors Contribution

The following author's individual contributions are Sonali Mehrotra: Conception and Experiment designed, Data collection, Analysis and Data Processing, Article Processing. K.P. Tripathi: Article editing, final approval of the article.

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