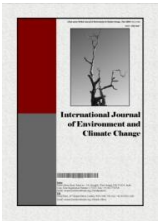

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# Interactive Effect of Elevated $CO_2$ and Drought Stress on Leaf Anatomy in *Brassica Species*

**Ranjan Das[1*#]**

*[1]Department of Crop Physiology, AAU, Jorhat Assam, India.*

***Author's contribution***

*The sole author designed, analyzed, interpreted and prepared the manuscript.*

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## ABSTRACT

The aim of present study was to understand how the ultrastructure of the leaf mesophyll cells in *Brassica* leaf can be altered under elevated $CO_2$ by interactive effect of elevated $CO_2$ on leaf anatomy and ultra structure of Brassica species under moisture stress conditions. Results of the experiment revealed that the crop genotypes differ greatly in response to elevated $CO_2$ and moisture stress conditions. Elevated $CO_2$ brought about an increase in cell and chloroplast expansion in *Brassica* genotypes. Elevated $CO_2$ also increased the thickness of epidermis, size ofmesophyll cells, accumulation of starch and size and number of starch granules per chloroplast in *Brassica juncea* and *Brassica juncea* cultivars. These alterations in the ultra structure of cells in plants might help to plant adjustment to changing climate in the future.

*Keywords: Elevated $CO_2$; moisture stress; chloroplast; starch granule and leaf thickness.*

## 1. INTRODUCTION

The $CO_2$ concentration in the atmosphere was in a steady state at 280 mol $mol^{-1}$ till the pre-industrial period (1850). If it continues at the present rate i.e.1.5 ppm (exponential rise of $CO_2$ in the atmosphere); it may be doubled by the end of 21st century (Luo and Moony 1996). Atmospheric $CO_2$ increased to 403.3 ± 0.1 µmol $mol^{-1}$ in 2016, approximately 145% of pre-industrial level [1]. This increase will inevitably continue [2]. $CO_2$ has biological effect as well,

**Corresponding author: E-mail: rdassam1966@gmail.com;*
*[#]Present address*

because it serves as the main substrate for photosynthetic carbonassimilation. However, there is no consensus on the quantitative effects of increased C $CO_2$ on plant processes and growth, due to differences in response with stages of growth, type of species and crops and because of growth limiting environmental factors. Hence, the various researches are being carried on to study the crop response to elevated $CO_2$.The current increase in atmospheric $CO_2$ at the rate of 1.5-1.8 μmol $mol^{-1}$ per annum is much too small a signal for field experiment to detect a response. Therefore, various models were used for $CO_2$ experiments using Open Top Chambers (OTCs) and Free Air $CO_2$ Enrichment (FACE) technologies that provide alarge input signal or pulse which allows tracing of crop responses for a short time frame. Crops responds directly to rising $CO_2$ through photo-synthesis and stomatal conductance [3,4], which consequently promotes crop yield [5,6]. The direct fertilization effect of rising atmospheric $CO_2$ is expected to offset the reduction of crop yields induced by climate change [7]. Besides the physiological and biochemical process elevated $CO_2$ has also effect on anatomical processes depending on species. Roger et al. [8] reported in increasing in thickness of all layers of leaf of *Pinustaeda* and *Liquidambar styraciflu a* grown in high $CO_2$. The difference in leaf thicknesswas reflected in difference in the ratio of leaf weight to leaf area [9]. Leadley [10], Wu et al. [11] reported that soybean plants grown in the high $CO_2$ treatment had thickerleaves but less palisade cell surface area per unit leaf area. Surface area of the mesophyll per unit leaf area was unaffected by $CO_2$. Overdieck and Ungemach [12] recorded that elevated $CO_2$ induced increase in density of palisade and spongy mesophyll at the expense of intercellularspaces in *T. repens* and *Lolium perenne*. Chloroplasts of $CO_2$ enriched leaf tissues of plants were more deeply stained by crystal violate and appear eddenser. Elevated $CO_2$ stimulated cell production in roots cells [13]. Elevated $CO_2$ brought about an increase in cell and chloroplast expansion of wheat (*Triticum aestivum*) leaves 10 and 25%,respectively and observed that older leaves chloroplast contained larger starch deposits [14]. Similarly, Hao et al. [15], reported that elevated $CO_2$ increased the number and size of starch grains in chloroplasts of two cultivars of soybean. In general, simultaneous exposure to increased O3 reduced the impact of increased $CO_2$ [16]. The Transmission Electron Microscopy (TEM) study of leaf tissues showed a significant increase in the thickness of epidermis, size of mesophyll cells, accumulation of starch and size and number of starch granules per chloroplast in *Brassica juncea* [17].

Changes in chloroplast density or volume which occur in plants grown in $CO_2$ enriched environment have been generally attributed to increased starch accumulation in the chloroplasts of tomato and soybean [18]. In view of the wide ranges of results that have been obtained through previous research further studies are in the need to understand its ramifications and connection with ongoing rise in the ambient $CO_2$ content particularly in timing and mechanism of plant adjustment to elevated $CO_2$ through anatomical modification which will significantly influence in the biomass production potential or productivity of crop *Brassica species* viz. *Brassica juncea* cv.**'RH-30'** and *Brassica campestris* cv.'**Pusa Gold'** under elevated $CO_2$ and moisture stress condition.

## 2. MATERIALS AND METHODS

### 2.1 Plant Material

*Brassica* cultivars *viz. Brassica juncea* cv. RH-30 and *Brassica campestris* cv. Pusa Gold were collected and grown for the present investigation.

### 2.2 Experimental Site and Growth Conditions

The response of both the species to elevated $CO_2$ was studied using Free Air $CO_2$ Enrichment Technology (FACE) to simulate the doubling $CO_2$ concentration at, IARI, New Delhi-12. The crops were grown in the field and inside the Mid Free Air $CO_2$ enrichment (FACE) facility in 8 m diameter circles. An elevated $CO_2$ concentration of 550 μmol$mol^{-1}$ was maintained throughout the crop growth period with the help of computer-based PID valves. There was no exogenous supply of $CO_2$ to the normal air under ambient field condition. Field was prepared by recommended agronomic practices.

### 2.3 Cultural Practice

Farm yard manure was applied at the rate of 5 tons per hectare at the time of field preparation. The plant spacing, fertilizer application at the rate of 30+30:60:40 kg per hectare of nitrogen, phosphorus and potassium and other cultural practices were followed as reported by Uprety et al. [17].

## 2.4 Moisture Stress Treatment

Moisture stress treatment was given by restricting irrigation and bringing the soil moisture level between 7 and 10% compared to 22-25% under irrigated condition. All the observations were taken in triplicate for each treatment at Stage-1: vegetative (25 days after sowing), Stage-2: Flower bud initiation (45 DAS), Stage- 3: 50% flowering (60 DAS) and Stage-4: post flowering (75DAS).

## 2.5 Leafanatomy

For the measurement of leaf thickness, leaf segment of each treatment was initially placed in fixative FFA, comprising of formalin: acetic acid: ethyl alcohol: Water (10: 5: 50: 35V/V). It was then dehydrated in ethyl-butyl alcohol series and embedded in paraffin wax. Cross section was made on ultra-microtom and stained with safranine (0.5%. w/v) in 50 %,(v/v)alcohol. The thickness of palisade, mesophyll and combined epidermal layer were measured in terms of micrometer (μm) [19].

## 2.6 Leaf Ultra structure Study

Ultra structural measurements were done on fully expanded leaf ($6^{th}$ node from the top) of mainstream using Transverse Electron Microscope (TEM) technology as reported by Robertson and Leech [20]. From the base of the leaf, slices of 2-3 mm thickness were cut and fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH7.2) at room temperature for 1 hr.

The leaf tissues were washed three times in 0.1 M phosphate buffer, pH 7.2, before post-fixation in 1 percent (v/v) osmium tetra oxide in 0.1 M phosphate buffer for two hours at room temperature. After three more washes in 0.1 M phosphate buffer, the tissue was dehydrated through an acetone series and embedded in Spurr' epoxy resin (TABB Laboratories Equipment Ltd., UK,) [21]. Ultra-thin sections were cut for TEM using a diamond knife. Double staining in uranyl acetate and lead citrate was done for taking observation in electron microscope.

# 3. RESULTS

## 3.1 Anatomical Characters Leaf Thickness

Elevated level of $CO_2$ brought about significant increase in leaf thickness (Plate 1, Table 1). The increased over the ambient plant was 39% (palisade), 34%(spongy), 36%(mesophyll), 29% (upper epidermis), 28 %( lower epidermis) and 34% (total leaf thickness).Moisture stress treatment significantly reduced the leaf thickness. The reduction was 22% (palisade), 19% (spongy), 18% (mesophyll), 16% (upper epidermis), 25%( lower epidermis) and 18 (total leaf thickness) compared to control. The stress induced reduction in Pusa gold under ambient condition 39% (palisade),37%(spongy),38% (mesophyll), 31% (upper epidermis),35% (lower epidermis), 37% (total thickness), where as reduction under elevated $CO_2$ condition was 24% (palisade),21% (spongy), 23% (mesophyll), 22% (upper epidermis),21% (lower epidermis), 22% (total leaf thickness). The stress induced reduction in RH-30 under ambient condition 35% (palisade), 33% (spongy), 34% (mesophyll), 25% (upper epidermis), 25% (lowerepidermis) and 35% total leaf thickness where as reduction in elevated $CO_2$ condition 21% (palisade), 17% (spongy), 19% (mesophyll), 17% (upper epidermis), 16% (lower epidermis) and 19% (total leaf thickness).

## 3.2 Leaf Cell Size Palisade Cells Size

The increased concentration of $CO_2$ significantly enhanced the palisade cell size in *Brassica* cultivars Table 2, Plate 2 The increase was 36% (length), 23% (breath) and 65%(area). The larger palisade cell was observed in leaves of RH-30 compared to Pusa gold. Moisture stress treatment significantly reduced cell size. The reduction was 38% (length) and 28% (breath) and 74%( area). The stress-induced reduction in Pusa gold under ambient condition was 35% length, 28% breath, and 53% area whereas reduction under elevated $CO_2$ was 22% (length), 16% (breath), and 35% (area). The stress-induced reduction in RH-30 under ambient conditionwas 28% length, 23% breath and 53% (area) where as in elevated $CO_2$ reduction was 19% (length),12%(breath) and 30% (area).

## 3.3 Spongy Cell Size

There was no any significant effect on spongy cell size in leaves of *Brassica* cultivars due to $CO_2$ enrichment (Table 3). The larger spongy cell was observed in leaves of Pusa gold compared toRH-30.Moisture stress treatment significantly reduced spongy cell size. The reduction was 12%( length), 12%( breath) and 21% (area). The stress-induced reduction in Pusa gold under ambient condition was 12% (length), 16% (breath) and 26% (area) however

in RH-30 was 17% (length), 17% (breath) and 19% (area).

## 3.4 Palisade Number

The increased concentration of $CO_2$ significantly enhanced (12.33%) the palisade cellnumber in *Brassica* cultivars. (Table 2).The more number of palisade cell was observed in leaves of RH-30 compared to Pusa gold. Moisture stress treatment significantly reduced (31%) number of palisade cell. The stress induced reduction in Pusa gold under ambient was 31% where it was 21% in elevated $CO_2$ condition. The reduction in RH-30 under ambient was 24% where as 17% in under elevated $CO_2$ condition.

## 3.5 Spongy Cell Number

There was no any effect on spongy cell number due to $CO_2$ enrichment (Table 3). The more number of spongy cells was observed in leaves of Pusa gold compared to RH-30. Moisture stress treatment significantly reduced (14%) number of spongy. The stress reduction in Pusa gold under ambient was 20.00% where as in RH-30 it was15%.

## 3.6 Chloroplast Ultra-structure

### 3.6.1 Mesophyll chloroplast number

The $CO_2$ enrichment brought about significant increase (72 %) in the chloroplast numberin the mesophyll. (Table 4) The more number of chloroplast was recorded in RH-30 cultivar compared to Pusa gold. Moisture stress significantly decreased (25%) the chloroplast number in mesophyll cell. The stress induced reduction in chloroplast number under ambient was 34% whereas under elevated 22% in Pusa gold. The reduction in RH-30 under ambient was 32%, where as in elevated 19%.

### 3.6.2 Vascular sheath chloroplast number

The $CO_2$ enrichment significantly enhanced (36%) the chloroplast number in vasculars heath cell. There was no significant difference between cultivars. Moisture stress significantly decreased (25%) in the chloroplast number in vascular bundle cell. The stress induced reduction in chloroplast number under ambient was 34% whereas in elevated condition, 21% in Pusa gold.The reduction in RH-30 under ambient was 33%, where elevated $CO_2$ condition only 17%.

### 3.6.3 Number of starch granule in mesophyll chloroplast

Elevated $CO_2$ brought about significant increase (35%) in the number of starch granule inthe mesophyll chloroplast. Moisture stress significantly decreased (33%) the number of starch granule in the mesophyll chloroplast. The stress induced reduction in number of starch granule in chloroplast under ambient was 48% where in elevated 24% in Pusa gold. The reduction inRH-30underambientwas 46%, where elevated 21%.

### 3.6.4 Number of starch granule in vascular sheath chloroplast

The $CO_2$ enrichment significantly increased (41%) the starch granule in the vascular sheath chloroplast (Table 4). Moisture stress significantly decreased (19%) the number of starch granule in the vascular sheath chloroplast. The stress induced reduction in number of starch granule in chloroplast under ambient was 42% where as elevated condition 20% in Pusa gold. The reduction in RH-30 under ambient was 33%, where elevated condition 15%.

## 4. DISCUSSION

Changes in rate of cell division, cell expansion and cell cycling was reported by various scientist due to exposure to elevated $CO_2$ and that might alter plant structure for example an alteration in plant structure under elevated $CO_2$ at organ level is possibly a result of metabolic changes induced at cellular level [22]. Elevated $CO_2$ brought about an increasein leaf thickness, which was also more prevalent in 'RH-30', whereas stress-induced reduction in leaf thickness greatly ameliorated Plate 1. The increase of the palisade and spongy layers alongwith that of epidermal cell layers contributed to the total leaf thickness under elevated $CO_2$ though increased $CO_2$ concentration and has no significant effect on cell size and number inspongy cells but a significant increase in palisade cell size was recorded and this results were in accordance with the results of Zheng et al. [23] reported in soybean which showed spongy tissue area of 15 and 28% as $CO_2$ concentration increased from 400 ppm to 600 ppm. Moisturestress significantly decreased the cell size and number in spongy layer. The increase in epidermal layer was one of most important strategy of plant for

**Table 1. Interactive effect of elevated $CO_2$ and drought stress on leaf anatomy *Brassica Species***

| Treatments | | | Pusa gold | | | | RH-30 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Palisade (µm) | Spongy (µm) | Mesophyll (µm) | Upper epidermis(µm) | Lower epidermis (µm) | Total (µm) | Palisade (µm) | Spongy (µm) | Mesophyll(µm) | Upper epidermis (µm) | Lower Epidermis (µm) | Total(µm) |
| FACEIRR | 88.19 | 99.90 | 188.10 | 27.21 | 19.40 | 230.71 | 73.89 | 84.21 | 158.10 | 23.20 | 15.30 | 196.60 |
| FACEMS | 66.34 | 78.40 | 144.80 | 21.20 | 15.20 | 181.20 | 57.89 | 69.12 | 127.01 | 19.10 | 12.70 | 158.81 |
| AMBIRR | 70.23 | 80.40 | 150.60 | 22.200 | 16.10 | 188.90 | 57.94 | 70.14 | 128.08 | 19.20 | 12.80 | 160.08 |
| AMBMS | 42.21 | 50.21 | 92.40 | 15.10 | 10.40 | 117.90 | 37.21 | 46.90 | 84.11 | 14.30 | 9.6 | 108.01 |
| CDat0.05 | | | | | | | | | | | | |
| CV. | 2.11 | 6.21 | 8.89 | 1.86 | 0.62 | 9.14 | 2.11 | 6.21 | 8.89 | 1.86 | 0.62 | 9.14 |
| CO2 | 4.32 | 3.14 | 12.58 | 0.70 | 0.31 | 12.98 | 4.32 | 3.14 | 12.58 | 0.70 | 0.31 | 12.98 |
| CVxCO2 | 6.99 | 4.45 | 14.97 | 0.99 | 0.44 | 15.19 | 6.99 | 4.45 | 14.97 | 0.99 | 0.44 | 15.19 |
| MS | 2.56 | 5.30 | 6.67 | 1.01 | 0.53 | 7.01 | 2.56 | 5.30 | 6.67 | 1.01 | 0.53 | 7.01 |
| CV.xMS | 7.34 | 7.50 | 13.89 | 1.43 | 0.75 | 14.67 | 7.34 | 7.50 | 13.89 | 1.43 | 0.75 | 14.67 |
| CO2xMS | 9.77 | 7.49 | 16.76 | 1.53 | 0.85 | 17.45 | 9.77 | 7.49 | 16.76 | 1.53 | 0.85 | 17.45 |
| CV.xCO2xMS | 12.56 | 10.60 | 20.55 | 2.03 | 1.36 | 21.55 | 12.56 | 10.60 | 20.55 | 2.03 | 1.36 | 21.55 |

*FACE = Free air $CO_2$ enrichment, IRR= Irrigated, MS= moisture stress, AMB= ambient*

**Table 2. Interactive effect of elevated CO2 and moisture stress in leaf palisade cell number and cellsize**

| Treatment | | Pusa gold | | | | RH-30 | | |
|---|---|---|---|---|---|---|---|---|
| | Number | Length(µm) | Breath(µm) | Area(µm$^2$) | Number | Length(µm) | Breath(µm) | Area(µm$^2$) |
| FACEIRR | 23.00 | 37.40 | 22.60 | 845.24 | 27.00 | 48.50 | 25.90 | 1256.15 |
| FACEMS | 18.00 | 28.90 | 18.81 | 543.61 | 22.30 | 38.90 | 22.60 | 879.14 |
| AMBIRR | 19.00 | 29.20 | 19.70 | 575.24 | 21.00 | 39.12 | 22.80 | 891.94 |
| AMB MS | 13.00 | 18.90 | 14.10 | 266.49 | 15.90 | 28.0 | 17.40 | 487.20 |
| CV. | 1.01 | 1.67 | 1.93 | 37.96 | 1.01 | 1.67 | 1.93 | 37.96 |
| CO2 | 1.58 | 2.22 | 2.45 | 81.35 | 1.58 | 2.22 | 2.45 | 81.35 |
| CV.xCO2 | 2.56 | 3.78 | 3.47 | 115.04 | 2.56 | 3.78 | 3.47 | 115.04 |
| MS | 1.67 | 2.92 | 2.43 | 76.90 | 1.67 | 2.92 | 2.43 | 76.90 |
| CV.xMS | 2.87 | 4.01 | 3.44 | 108.75 | 2.87 | 4.01 | 3.44 | 108.75 |
| CO2xMS | 3.54 | 5.85 | 3.69 | 118.75 | 3.54 | 5.85 | 3.69 | 118.75 |
| CV.xCO2xMS | 4.09 | 7.44 | 4.86 | 153.80 | 4.09 | 7.44 | 4.86 | 153.80 |

**Table 3. Interactive effect of elevated $CO_2$ and moisture stress leaf spongy cell number and cellsize**

| Treatment | Pusagold | | | | RH-30 | | | |
|---|---|---|---|---|---|---|---|---|
| | Number | Length(µm) | Breath(µm) | Area(µm$^2$) | Number | Length(µm) | Breath(µm) | Area(µm$^2$) |
| FACEIRR | 17.00 | 31.20 | 22.90 | 714.48 | 14.00 | 26.60 | 19.44 | 517.1 |
| FACE MS | 16.00 | 30.64 | 21.42 | 650.18 | 13.00 | 24.97 | 18.91 | 472.18 |
| AMBIRR | 18.00 | 32.62 | 24.30 | 792.67 | 15.00 | 28.22 | 22.46 | 533.64 |
| AMBMS | 15.00 | 28.62 | 20.30 | 580.98 | 13.00 | 23.42 | 18.44 | 431.86 |
| CV. | 1.5 | 1.56 | 0.62 | 50.44 | 1.5 | 1.56 | 0.62 | 50.44 |
| $CO_2$ | NS | NS | 0.87 | NS | NS | NS | 0.87 | NS |
| CV.x$CO_2$ | 0.22 | NS | 1.28 | NS | 0.22 | NS | 1.28 | NS |
| MS | 0.44 | 0.83 | 0.61 | 18.55 | 0.44 | 0.83 | 0.61 | 18.55 |
| CV.xMS | 0.63 | 1.13 | 1.02 | 30.87 | 0.63 | 1.13 | 1.02 | 30.87 |
| $CO_2$xMS | 0.83 | 2.44 | 1.99 | 42.43 | 0.83 | 2.44 | 1.99 | 42.43 |
| CV. x $CO_2$ xMS | 1.21 | 3.10 | 2.84 | 67.54 | 1.21 | 3.10 | 2.84 | 67.54 |

**Table 4. Interactive effect of elevated $CO_2$ and drought stress ultra structure of chloroplast**

| Treatments | Number of chloroplastin mesophyll | | Number of starch granule in mesophyll chloroplast | | Number of chloroplastin Vascular sheath | | Number of starch granule in vascular sheath chloroplast | |
|---|---|---|---|---|---|---|---|---|
| | Pusa gold | RH-30 | Pusa gold | RH-30 | Pusa gold | RH-30 | Pusagold | RH-30 |
| FACEIRR | 7.50 | 8.90 | 3.70 | 3.90 | 3.20 | 3.50 | 3.00 | 3.20 |
| FACEMS | 5.80 | 7.20 | 2.80 | 3.10 | 2.50 | 2.90 | 2.40 | 2.70 |
| AMBIRR | 4.70 | 5.50 | 2.90 | 2.90 | 2.60 | 2.70 | 1.90 | 1.80 |
| AMBMS | 3.10 | 3.70 | 1.50 | 1.50 | 1.70 | 1.80 | 1.10 | 1.20 |
| Cv. | 84 | | | NS | | NS | | NS |
| $CO_2$ | 11 | | | 0.08 | | 0.09 | | 0.07 |
| Var.x $CO_2$ | 18 | | | 0.12 | | 0.12 | | 0.10 |
| MS | 09 | | | 0.11 | | 0.06 | | 0.05 |
| Var. xMS | 43 | | | 0.22 | | 0.12 | | 0.11 |
| $CO_2$xMS | 67 | | | 0.43 | | 0.30 | | 0.21 |
| Var. x $CO_2$ xMS | 42 | | | 0.61 | | 0.42 | | 0.30 |

amelioration of moisture stress effect because the thicker epidermis may reduce the rate of transpiration through the accumulation of cuticular wax. These results were in conformity with that of Thomas and Harvey (1983) in *Glycin max*) and Lee *et al.* (1993) in *Betula pendula*. Uprety et al. [17] also reported at elevated $CO_2$ concentration has significant effect on leaf structure of *Brassica juncea* L.cv.

Line Bio-141(95) under moisture stress which revealed that $CO_2$ elevated to 600 $\mu molmol^{-1}$ increased the length of epidermal cell and length of palisade parenchyma cells Ferris and Taylor (1999) in *Populus* demonstrated that carbon induced growth effect occurred because of the enhanced wall extensibility and the effect of wall loosening enzyme, xyloglucan endo transglycosylase (XET). Masale [24] and Zhuo et al. [13] observed increased supply of soluble carbohydrates stimulated both cell wall expansion and cell production but the mechanism remained elusive. Ferris et al. [25] reported that leaf expansion was due to the production oflarger cells in their in *P. nigra* when they raise the atmospheric $CO_2$ under FACE experiment. According to Thomas and Harvey [26] differences in thickness were due to the result of increased palisade at initiation and development. According to them in soybean and sweet gum at elevated $CO_2$ due to higher carbon accessibility increased osmotic potential in the leaves that in turn causes expansion of cells of palisade area. Elevated $CO_2$ induced one more layer ofpalisade cells in the mesophyll and greater cell expansion in *Brassica* leaves in the present investigation.

Vaz et al. [27] reported that when leaves of *Quer cussube*r was exposed to elevated $CO_2$ it resulted in increased leaf thickness and an additional layer of palisade cells was also observed.

Uprety 2001 opined that increase in mesophyll layer served as an important approach for *Brassica juncea* to provide room for higher starch accumulation under high $CO_2$. In the present study, with *Brassica,* the length and width of epidermal cells were increased at high $CO_2$ condition, indicating greater effect on cell expansion, which might be associated with an increase in osmotic potential due to accumulation of saccharides. These changes along with those in stomatal characters might help in regulating and reducing transpiration, gas exchange and also regulating osmotic movement of cell sap to maintain turgor under moisture stress condition. .Increased chloroplast size has been a reliable observation under elevated $CO_2$ enrichment asreported by some workers [28,17]. According to them this has been mostly attributed to increased cross-sectional width of chloroplast rather than length.

The $CO_2$ enrichment significantly increased the chloroplast number in both mesophyll and vascular bundle sheath of the cell in *Brassica* leaves but it markedly decreased under moisture stress condition. The stress induced reduction in chloroplast number significantly ameliorated in elevated $CO_2$ condition. Mesophyll cell had more number of chloroplast compared to the vascular bundle sheath cell and laying near the cell wall under elevated $CO_2$ (Plate 2). The increased chloroplast number could be related with increase in the cell size. These findings indicated that cells have got more space to develop number of chloroplast. Similarly $CO_2$ enrichment brought about significant increase in the starch granule number within thechloroplast both mesophyll and vascular bundle sheath cell. There was no significant difference in terms of cultivars. Moister stress decreased the starch granule number within chloroplast in both mesophyll and vascular bundle sheath cells (Plates 3 and 6).

Several workers have studied the response of higher level of atmospheric $CO_2$ on starchaccumulation in the leaves of various plants for example; number and size of starch increased in cucumber [29]; in *Gmelina arborea* [30] and in *Isatis indigotica* [15] grown under Free air atmospheric $CO_2$ enrichment facility. And same trend was also recorded [31] in the leaves of *Impatiens hawker* when treated with higher level of $CO_2$ in growth chamber.

The Transmission Electron Microscope (TEM) study of leaf tissues showed a significant increase in the thickness of epidermis, size of mesophyll cells, accumulation of starch and size and number of starch granules per chloroplast in *Brassica juncea* [17]. According to them the production of photo-assimilates under elevated $CO_2$ might have increased the sink capacity to adjust the starch granules in the chloroplast without disturbing the thylakoids membrane. The important phenomenon observed in the present study showed that $CO_2$ enrichment not only increased chloroplast size but also strengthened the chloroplast membrane.The elevated $CO_2$ increased the starch granule size and it

deposited them to near chloroplastmembrane stretching without any damage [Plate 4.(a) & (b)]. Due to deposition of starch granule near the membrane the thylakoid and grana were protected from mechanical damages in the middle portion of chloroplast and ultimately avoid the feedback inhibition by metabolite adjustment Plate 5. The greater size and number of starch granules additionally helped the osmotic phenomenon [32,33].

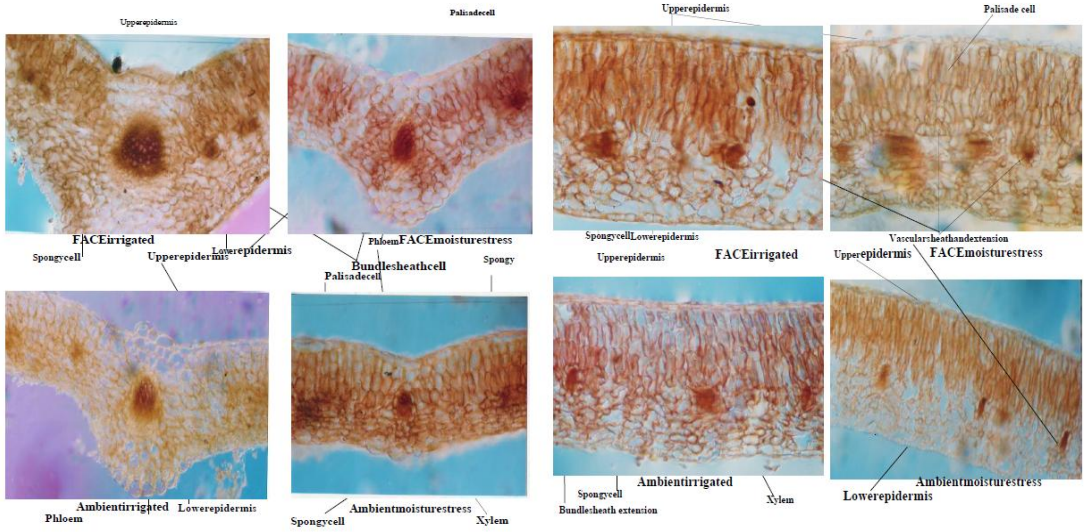


**Plate 1. Effect of elevated CO2 and moisture stress on leaf anatomy of *B. Campestris* cv Pusa gold and *B. juncea*, cv. RH-30 (LM100x)**

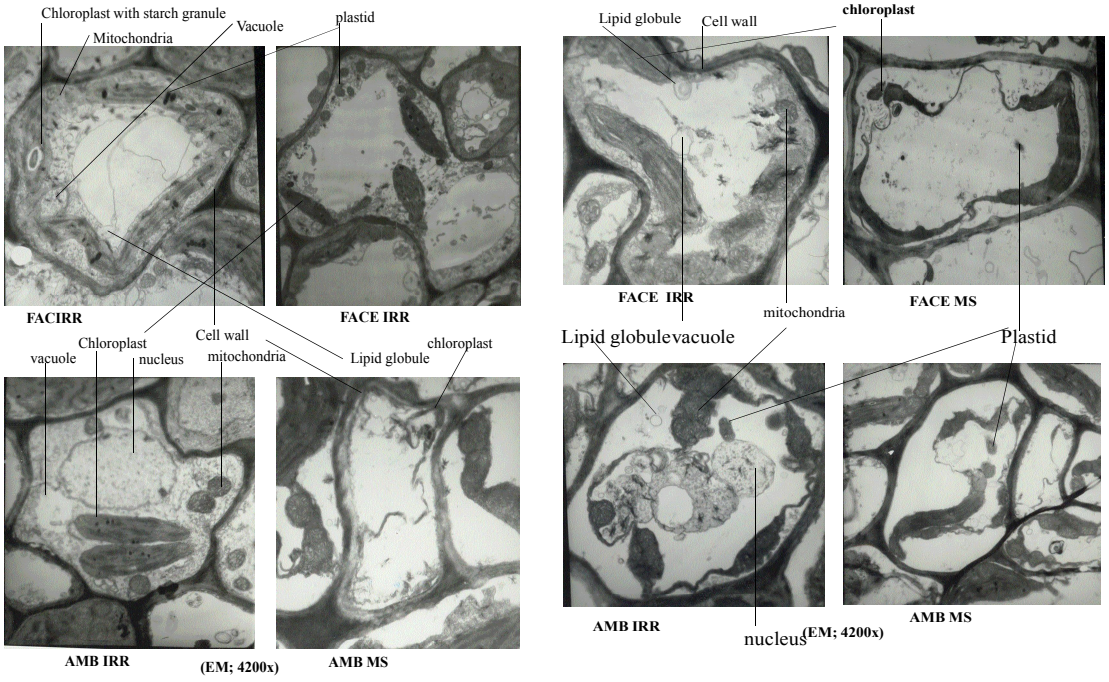


**Plate 2. Effect of elevated $CO_2$ and moisture stress on vascular bundle cell size and number of chloroplast in the leaves *Brassica campestris cv.Pusa gold Brassica juncea* cv. RH-30**

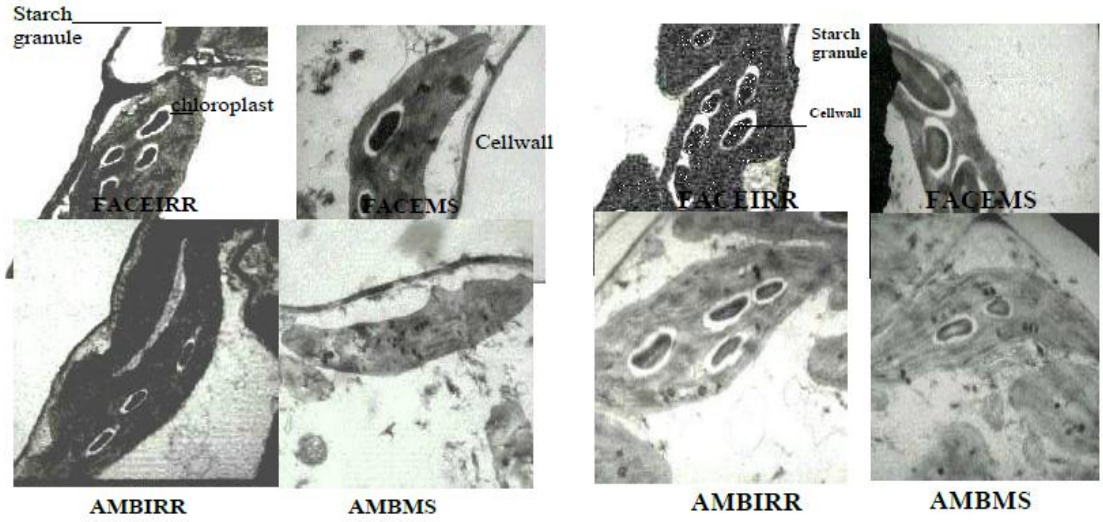


**Plate 3. Effect of elevated CO2 and moisture stress on chloroplast structure in the leaves of *Brassica juncea*cv. RH-30. (EM;8200x)**

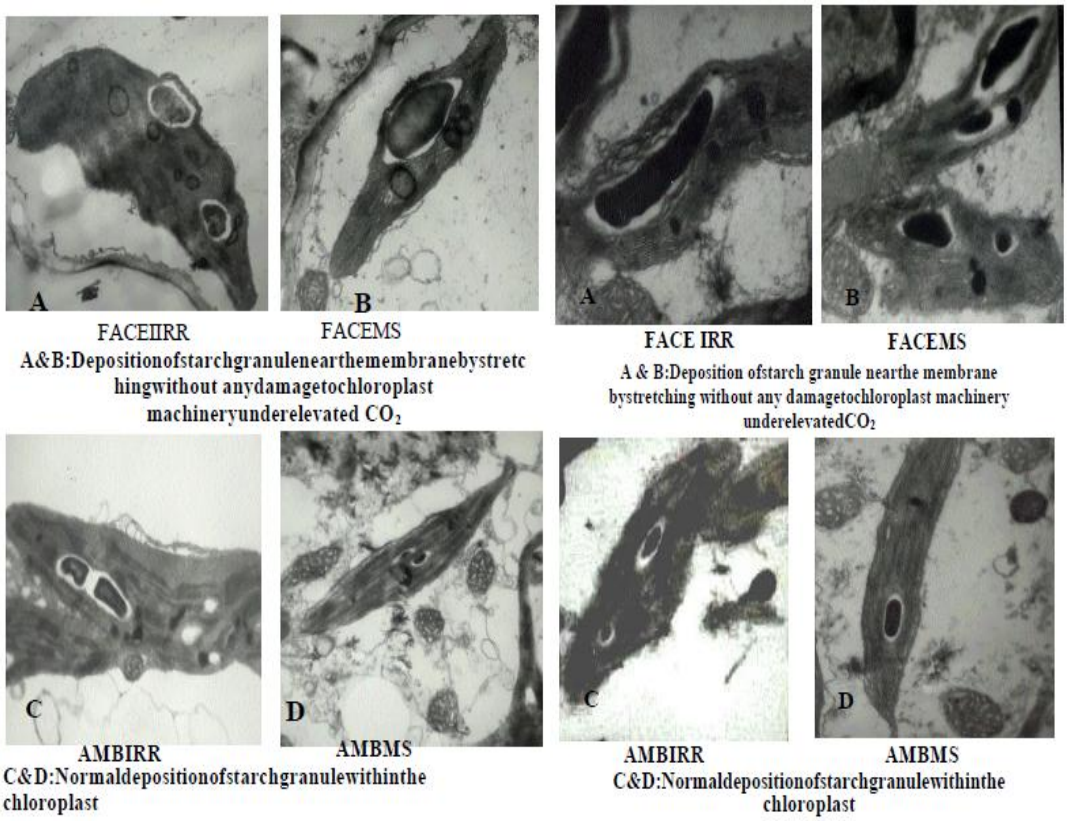


**Plate 4(a). Effect of elevated CO2 and moisture stress on starch granules in the leaves of *Brassica juncea* cv.RH-30(EM 8400x.)**




**Plate 4(b). Effect of elevated CO2 and moisture stress on starch granule in the leaves of *Brassica campestris* cv. Pusa Gold (EM 8400x)**

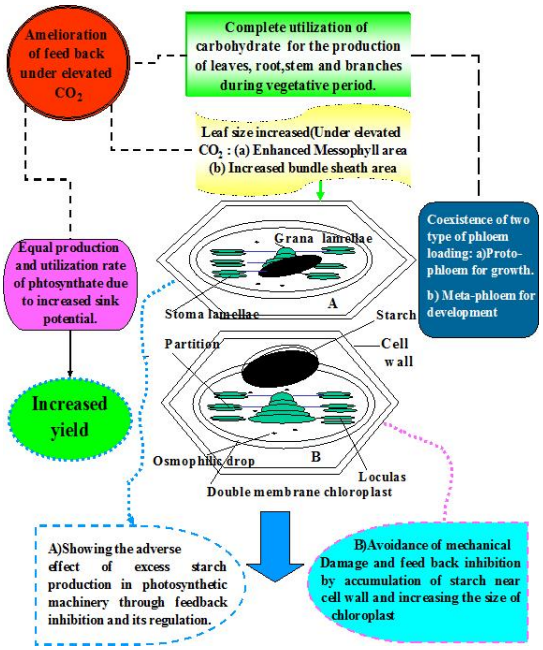


**Plate 5. Avoidance of feedback inhibition in *Brassica* cultivar under elevated $CO_2$**

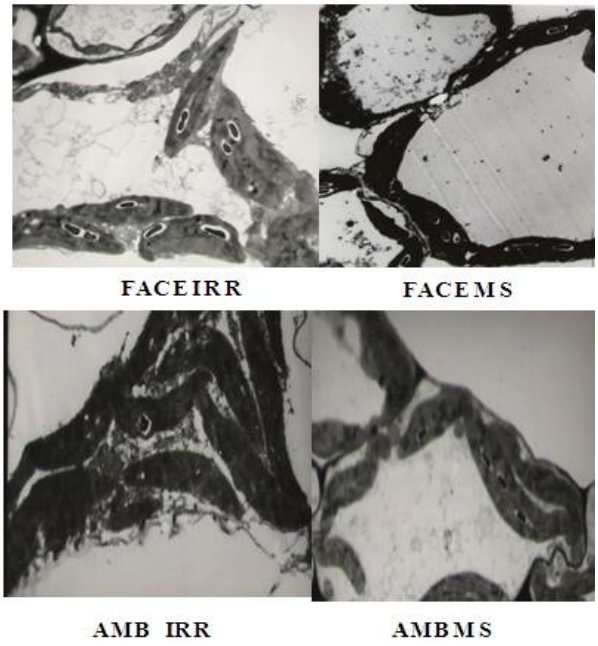


**Plate 6. Effect of elevated $CO_2$ and moisture stress on number of mesophyll cell chloroplast in leaves of *Brassica Juncea* cV. RH-30 (EM; 2050x)**

## 5. CONCLUSION

To understand the mechanism of responses to elevated $CO_2$, an alteration in anatomical and leaf ultra-structure is very much pertinent which provides the pave for an idea about adaptation strategies of plant under future environment. Thus, in the present investigation the response of *Brassica* species to the interaction of elevated $CO_2$ and moisture stress was characterized using Free Air $CO_2$ Enrichment technology.

Among the two cultivar cv. RH-30 showed a better adaptation by optimizing various ultra-structure such as number starch granule in chloroplast, leaf thickness, cell size etc. compared to cv. Pusa gold This data base on *Brassica* species might help in developing model,

Identification of cultivars and modification of cultivation and nutrient application technologies for future environments.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## COMPETING INTERESTS

Author has declared that no competing interests exist.

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