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Research Article

Study of the Responses Stomatal Configuration in *Brassica* to Elevated CO_2 Under Moisture Stress condition

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Introduction

Increasing atmospheric carbon di oxide (CO_2) concentration in atmosphere has major impact on global climate change. carbon di oxide has increased from level of 280 ppm (pre-industrial 1750), to level of 400 ppm (present) it and in the end of the 21st century it is likely to be increase to 900 ppm (Xu et al,. 2016). Stomata are considered the first molecular link between the biosphere and the atmosphere. Stomata are important access point in the crop and the atmosphere may play a significant role in crop responses to environments (Nilson et al., 2007 & Xu, and Zhou 2008). Variables of environment viz. extreme temperature (Beerling et al., 1993) elevated CO_2 (Woodward 1987) moisture stress, (Zhao 2001 et al., Galmes et al., 2007) controlled by stomatal density and its related parameters ultimately influence the photosynthesis process and yield. Various reports have indicated that the stomatal configuration is altered by elevated CO_2 concentration. There is close correlation between photosynthetic and stomatal response to CO_2, the photosynthetic C reduction pathway in the subsidiary cells of the stomatal complex may have an intricate role (Jarvis and Mansfield 1981), in which case, stomatal aperture would be expected to acclimate to elevated CO_2 as a results of photosynthetic acclimation. The role of CO_2 as a chief input to plant life has greater significance when the plant is to face various stressful environments. The water status of plant also significantly improved under elevated CO_2 concentration possibly by increasing stomatal resistance and/or increased root growth. Uprety et al. (2002) reported that elevated CO_2 brought about significant increase in size of stomatal guard cells, stoma and epidermal cell, and such acclimation involve in the regulation of photosynthesis. Water deficit overrides both light and CO_2 concentration, triggering stomatal closure (Murray, 1995). The main aim of the present study is to see whether elevated CO_2 regulate / influences the stomatal function under moisture stress condition. To test the hypothesis two different species of Brassica were grown under two CO_2 and moisture regimes and compared with a control.

Materials and Methods

Plant Material

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

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 µmolmol-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.

Cultural Practice

Farmyard 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. 2001.

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).

Stomatal Study

Leaf Stomata

Fully expanded leaf samples at flowering stage were collected in polythene bag. Clear nail polish was applied to both leaf surfaces and allowed to dry for approximately five minutes: Double sided cellophane tape was used to peel dried impression from the leaves. 5 cm long section of the impression at the tip, centre and base positions of each leaf surface were mounted on microscope slides and cover slip was placed. Stomata of each surface were counted in 1.85-mm2 microscopic field (100 x magnifications) on both adaxial and abaxial surface (Cohen et al., 1982)

Siliquae Stomata

Siliquae at 36 days after flowering (DAF) from middle position (13th, 14th and 16th number) of main inflorescence collected from each treatment. The tip and base portion of siliquae was removed and placed in a 100ml beaker containing 5 per cent CuSO4 and boiled on hot plate. The sample material was cooled under running tap water and 1 per cent HCl was added and kept for 20 min. Samples were re-washed in distilled water and impression was removed by hand peeling. The impression was mounted on slide for stomatal studies as method described by Cohen et al. (1982).

Stem Stomata

The stem at 7th internodes from base of main stem (during flowering) was collected from each treatment and peeling of impression was done as followed for siliquae.

Stomata Size

Stomatal size was estimated by measuring exterior guard cell length and breadth of five stomata per sample field using 100 x magnifications (Cohen et al. 1982). Calibration was done with the help of stage and ocular micrometer.

Stomatal Index (SI)

The stomatal index was calculated as follows:

SI = (No.of stomata)/(No.of guard cell + epidermal cell) × 100

Results

Stomatal Characters in Leaves

Stomatal density

The elevated CO_2 brought about significant reduction in the stomatal density at adaxial and abaxial side of Brassica leaves (Table 1). The reduction was 22% (adaxial side) and 25% (abaxial side). The stomatal density was lower in 'RH-30' compared to Pusa Gold. Moisture stress reduced the stomatal density 28% at adaxial side) and 27% at abaxial side. The stress-induced reduction in 'Pusa Gold' under ambient and elevated conditions was 15% (adaxial side) and 19% (abaxial side) and 27% (adaxial side) and 28% (abaxial side) respectively, whereas, reduction was 13% (adaxial side) and 15% (abaxial side) and 23% (adaxial side) and 27% (abaxial side) respectively in 'RH-30'.

Stomatal Index

Elevated CO_2 significantly increased the stomatal index at adaxial (25%) and abaxial (28%) side of leaves (Table 1). The higher stomatal index was observed in 'RH-30'. Moisture stress brought about reduction in stomatal index at the adaxial side (30%) and abaxial side (35%) of the leaves. This reduction in 'Pusa Gold' under ambient and elevated CO_2 condition was 26% (adaxial side) and 28% (abaxial side) and 20% (adaxial side) and 22% (abaxial side) respectively. The stress-induced reduction in 'RH-30' under ambient and elevated CO_2 was 22% (adaxial side) and 23% (abaxial side) 16% (adaxial side) and 18% (abaxial side) respectively.

Leaf Stomatal Size

Adaxial Stomata Size

The increased concentration of CO_2 significantly increased length (28%), breadth (27%) and area (61%) of stomata at adaxial side (Table 2). The large size of stomata in adaxial side observed in the leaves of 'RH-30'. Moisture stress significantly reduced the stomata size of adaxial side. The reduction was 31% in length, 32% in breadth and 61% in area. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 was 29% in length, 29% in breadth and 50% in area and 16% in length, 16% in breadth and 30% in area respectively, whereas, it was 22% in length, 23% in breadth and 39% in area and 14% in length, 15% in breadth and 27% in area in 'RH-30', respectively.

Abaxial Stomata Size

The higher level of CO_2 increased length (24%), breadth (27%) and area (70%) of the stomata at abaxial

side (Table 2). It was larger in 'RH-30'. Moisture stress significantly reduced the stomatal length (33%), breadth (35%) and area (77%) in abaxial side. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 condition was 30% (length), 31% (breadth), and 52% (area) and 19% (length), 18% (breadth) and 34% (and area) respectively, whereas, it was 26% for length, 29% for breadth, and 47% for area, 17% for length, and 15% for breadth and 30% for area in 'RH-30' respectively.

Stomatal Pore Length

A significant increase in adaxial (26%) and abaxial (23%) stomatal pore length in Brassica was observed with increased concentration of CO_2 (Table 4). The large pore length of stomata was observed for both adaxial and abaxial side of leaves of 'RH-30' compared to 'Pusa gold'. Moisture stress significantly reduced stomatal pore length from adaxial side (32%) and abaxial side (34%). The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 conditions was 30% (adaxial side) and 31% (abaxial side) and 18% respectively, whereas, it was 25% (adaxial) and 23% (abaxial) and 15% (adaxial) and 12% (abaxial) respectively in 'RH-30'.

Stem Stomatal Characters

Stomatal Density

The increased concentration of CO_2 brought about significant reduction (28%.) in the stomatal density in Brassica stem (Table 1). The stomatal density was lower in 'RH-30' compared to 'Pusa gold' in the stem. Moisture stress treatment significantly reduced (37%) the stomatal density in Brassica stem. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 condition was and 20% respectively, whereas, 33% and 20% respectively in 'RH-30'.

Stomatal Index

The elevated CO_2 significantly increase in the stomatal index Brassica stem (31%) (Table 1). The stomatal index was higher in 'RH-30' compared to 'Pusa Gold'. Moisture stress treatment significantly reduced the (35%) stomatal index of Brassica stems. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 was 32% and 17% respectively, whereas this reduction in 'RH-30' was 32% and 14% respectively.

Stomata Size

Elevated CO_2 significantly increased the length (36%), breadth (34%) and area (79%) of stomata (Table 3). The large size of stomata was observed in the stem of 'RH-30' compared to 'Pusa Gold'. Moisture stress reduced the length (41%), breadth (40%) and area (93%) of stomata. The stress-induced reduction in 'Pusa gold' under ambient and elevated CO_2 condition was 35% for length, 35% for breath, and 58% for area and 24% for length, 21% for breadth and 40% for area respectively in 'Pusa Gold', whereas, it was 28% for length, 30% for breadth, and 50% for area and 16% for length, 15% for breadth and 29% for area in 'RH-30' respectively.

Stomatal Pore Length

Pore length of stem stomata was increased by 28% due to elevated CO_2. It was larger in 'RH-30' (Table 4). Moisture stress treatment significantly reduced (30%) the stomatal pore length of stem. This reduction under ambient and elevated CO_2 condition was 23% and 15% respectively in 'Pusa Gold' and 21% and 12% respectively in 'RH-30'.

Siliquae Stomatal Parameter

Stomatal Density

The elevated level of CO_2 brought about significant reduction in stomatal density (32%) of siliquae (Table 1). The stomatal density was lower in 'RH-30'. Moisture stress treatment significantly reduced (37%) the stomatal density of siliquae of Brassica. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 conditions was 19% and 21% respectively and 15% and 25% in 'RH-30' respectively.

Stomatal Index

The increased concentration of CO_2 significantly increased (32%) the stomatal index in siliquae of Brassica (Table 1). The stomatal index was higher in the siliquae of 'RH-30'. Moisture stress reduced (35%) the stomatal index. The stress-induced reduction in 'Pusa Gold' under ambient and elevated CO_2 was 33% and 19% respectively, whereas, it was 27% and 15% respectively in 'RH-30'.

Siliquae Stomata Size

The elevated CO_2 significantly increased the length (23%), breadth (24%) and area (54%) of sliquae stomata (Table 3). The large size of stomata was recorded in the siliquae of 'RH-30' compared to 'Pusa Gold'. Moisture stress significantly reduced its length (40%), breath (41%) and area (92%). This reduction in 'Pusa Gold' under ambient and elevated condition was 37% for length, 38% for breadth and 61% for area and 22% for length, 20% for breath and 38% for area respectively, similarly, it was 30% for length, 28% for breadth and 50% for area and 17% for length, 16% for breadth and 31% for area respectively in 'RH-30'.

Stomatal Pore Length

CO_2 enrichment significantly increased the (27%) length of the pore of siliquae (Table 3). The large size of pore length of sliquae in stomata was observed in 'RH-30'. Moisture stress significantly reduced (32%) the stomatal pore length of siliquae. The stress induced reduction in 'Pusa Gold' under ambient and elevated CO_2 condition was 23% and 14% respectively, whereas, it was 20 and 12% respectively in 'RH-30'.

Discussion

Stomata play a crucial role in the exchange of gases between vegetation and the atmosphere (Xu et al., 2016). Present results indicated that the CO_2 enrichment brought about significant decrease of stomatal density (SD) in the leaves (abaxial and adaxial), stem and siliquae of Brassica cultivars. Similarly, moisture stress markedly reduced the stomatal density in these organs. The reduction in stomatal density was significantly more in elevated CO_2 under drought in leaves and siliquae although higher reduction was observed in stem in both the cultivars. Net reduction was more in Pusa gold compared to RH-30. A number of previous studies (Teng et al., 2009, Beerling & Chaloner, 1993; Madsen, 1973; Lin et al., 2001;) have also indicated that the decrease in SD when crops were grown in higher level of CO_2. A decrease of 5% in SD due to elevated CO_2 from a meta-analysis on stomatal responses was observed by Ainsworth and Rogers (2007). The complete opposite trend was observed in case of stomatal index (abaxial and adaxial) in present study. The CO_2 enrichment significantly increased stomatal index in the leaves, stem and siliquae and the moisture stress decreased it. But, the stress-induced reduction was ameliorated in elevated CO_2 condition in both the cultivars. However, this reduction was less in RH-30 cultivar compared to Pusa Gold, indicating genotypic variation within the species. This behaviour of stomata may be related to adaptive characters of particular plant. Crops must involved in alternation of stomatal behaviour and characters for balance intake of CO_2 under changing environment in maintaining of photosynthesis as well as transpiration (Lawson et al., 2014, Haworth et al., 2013 and Gray et al., 2000;). Depending on the species and genotypes stomatal short-term behaviour (e.g., stomatal closure) and a long-term developmental (e.g., stomatal size and its density) responses to environmental changes might occur together. (DaMatta et al., 2016, Gray et al., 2000; Haworth et al., 2013; Ainsworth and Rogers, 2007).

The CO_2 enrichment markedly increased the stomatal size in terms of length, breath and area and pore length in leaf (abaxial and adaxial side) and stem and siliquae in both the Brassica species. But the stomatal size and pore length in leaves (abaxial and adaxial), stem, and siliquae were also significantly reduced by moisture stress. Adverse stress effect on stomatal characters was markedly lesser under elevated CO_2 condition. This results was consistent with the results of Thinh et al. (2018), who reported that under elevated CO_2 condition SD on the leaf blade (abaxial side) was greater compared to ambient CO_2 and as well as pore length of stomata was greater under elevated level of CO_2 than ambient CO_2 condition. This alternation of stomatal configuration may be related to stomatal behaviour of the plant which could ultimately influence the process of photosynthesis. Chen et al., 2015 reported from his studies that when crop treated with elevated CO_2, it enhance the net assimilation rate with a lower amount of stomatal conductance but high RuBP carboxylase activity throughout the drought as well as re-watering. According to him this may point out the mitigation of metabolic constraint caused by moisture stress damages than the stomatal limitations imposed by elevated CO_2 in tall fescue (Festuca arundinacea). Similarly Xu et al., 2014 also were in agreement that higher level of CO_2 may reduce non-stomatal restrictions by defending the apparatus of photosynthesis process throughout the drought period. But recent report of Raki´c et al., 2015 opined that plants with smaller stomata have greater resistance to drought than plant which have bigger stomata indicating heritable difference. Xu et al. 2016 also reported that the decline in stomatal density (SD) may be the effect of a long-term heritable difference or short-term structural plasticity under elevated CO2. Stomatal size has greater role in water loss and exchange of gas in the plant. Elevated CO2 may enhanced the plant water status by reducing stomatal conductance and by this means raising water use efficiency, ameliorating the adverse effects of stress on growth and development (Ainsworth and Rogers, 2007; Xu et al., 2013, 2014). Elevated CO_2 significantly decreased stomatal density, stomatal widths and stomatal aperture on the abaxial surface of leaves under moderate drought stress. Liu et al. 2018 reported that elevated CO_2 can alleviate the negative effects of drought stress by improving the drought resistance of cucumber seedlings through stomatal modifications and leaf structure.

Conclusion

An increase in stomatal index and consequent decrease of stomatal density due to the elevated CO_2 in present investigation may be attributed to increase of epidermal cell and subsequent decrease of stomata in leaves and siliquae. However, in stem the CO_2 has no effect on epidermal cell differentiation thus stomatal density was not significantly altered by the interaction of elevated CO_2 and moisture stress. These findings also indicated the site-specific response of CO_2 under different climate and give further research opportunity for in-depth study. The reduction in stomatal density ultimately decreased the transpiration and may increase the WUE in Brassica.

Table: 1. Interactive effect of of elevated CO_2 and moisture stress on stomatal density and intensity

Treatment	Leaf adaxial				Leaf abaxial				Stem				Siliquae			
	Pusa gold		RH- 30		Pusa gold		RH- 30		Pusa gold		RH- 30		Pusa gold		RH- 30	
	Density (No. 100 µm-2)	Intensity (%)	Density No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)	Density (No. 100 µm-2)	Intensity (%)
FACE IRR	179.80	15.20	126.50	19.90	285.20	18.40	247.50	22.40	86.70	8.40	43.20	10.80	74.40	7.80	50.10	9.80
FACE MS	131.20	12.10	90.20	16.70	202.80	14.30	168.30	18.30	69.20	6.90	34.10	9.20	59.10	6.30	37.30	8.30
AMB IRR	202.70	13.20	159.40	16.90	335.40	14.90	287.60	18.100	123.30	7.10	57.400	9.60	97.30	6.50	72.20	8.50
AMB MS	171.60	9.70	130.30	13.10	270.70	10.60	230.20	13.90	82.40	4.80	38.20	6.90	68.10	4.30	48.10	6.20
Var.	4.35	0.61	4.35	0.61	2.89	0.62	2.89	0.62	1.97	0.43	1.97	0.43	1.86	0.39	1.86	0.39
CO2	2.20	0.86	2.20	0.86	4.12	0.44	4.12	0.44	4.38	0.61	4.38	0.61	1.57	0.74	1.57	0.74
Var. x CO2	3.11	1.22	3.11	1.22	6.11	0.63	6.11	0.63	6.19	0.86	6.19	0.86	2.22	0.82	2.22	0.82
MS	5.37	0.76	5.37	0.76	4.33	0.52	4.33	0.52	2.53	0.39	2.53	0.39	3.71	0.51	3.71	0.51
Var. x MS	7.61	1.67	7.61	1.67	7.99	0.74	7.99	0.74	5.08	0.63	5.08	0.63	5.25	0.66	5.25	0.66
CO2 x MS	8.32	2.78	8.32	2.78	10.01	0.86	10.01	0.86	6.14	0.90	6.14	0.90	6.01	0.86	6.01	0.86
Var. x CO2 x MS	10.76	3.34	10.76	3.34	12.24	1.45	12.24	1.45	8.34	1.21	8.34	1.21	7.42	1.32	7.42	1.32

Table: 2. Interactive effect of elevated CO_2 and moisture stress on leaf Adaxial and Abaxial stomatal size

Treatment	Adaxial						Abaxial					
	Pusa gold			RH-30			Pusa gold			RH-30		
	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)
FACE IRR	15.20	12.40	188.48	18.10	16.40	296.84	16.70	13.70	228.79	19.60	17.70	346.92
FACE MS	12.70	10.30	130.81	15.50	13.90	215.45	13.40	11.20	150.08	16.20	14.90	241.92
AMB IRR	12.90	10.60	136.74	15.70	14.10	221.37	14.60	11.90	173.74	16.90	15.40	260.26
AMB MS	9.100	7.50	68.250	12.20	10.90	132.98	10.10	8.20	82.82	12.40	10.90	135.58
Var.	0.62	0.58	6.93	0.62	0.58	6.93	1.86	1.43	4.97	1.86	1.43	4.97
CO2	0.44	0.39	8.77	0.44	0.39	8.77	0.70	0.61	7.67	0.70	0.61	7.67
Var. x CO2	0.63	0.57	9.98	0.63	0.57	9.98	0.99	0.93	8.84	0.99	0.93	8.84
MS	0.53	0.49	4.49	0.53	0.49	4.49	1.01	0.56	4.32	1.01	0.56	4.32
Var. x MS	0.74	0.70	8.91	0.74	0.70	8.91	1.43	1.11	7.98	1.43	1.11	7.98
CO2 x MS	0.85	0.81	10.47	0.85	0.81	10.47	1.73	1.54	9.97	1.73	1.54	9.97
Var. x CO2 x MS	1.43	1.37	14.72	1.43	1.37	14.72	2.02	1.89	13.44	2.02	1.89	13.44

Table: 3. Interactive effect of elevated CO_2 and moisture stem and siliqua stomatal size

Treatment	Stem						Siliquae					
	Pusa gold			RH-30			Pusa gold			RH-30		
	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)	Length (µm)	Breath (µm)	Area (µm2)
FACE IRR	10.40	9.70	100.80	12.80	12.10	154.88	9.90	9.30	92.07	12.20	11.90	145.18
FACE MS	7.90	7.60	60.04	10.7	10.20	109.14	7.70	7.40	56.98	10.10	9.90	99.90
AMB IRR	8.100	7.90	63.99	10.80	10.50	113.40	7.80	7.60	59.28	10.40	10.10	105.04
AMB MS	5.20	5.10	26.52	7.70	7.30	56.21	4.90	4.70	23.03	7.20	7.20	51.84
Var.	1.67	1.47	7.63	1.67	1.47	7.63	0.48	0.58	4.95	0.48	0.58	4.95
CO2	0.44	0.56	3.25	0.44	0.56	3.25	0.67	0.73	5.72	0.67	0.73	5.72
Var. x CO2	0.63	0.89	4.60	0.63	0.89	4.60	0.97	1.03	6.89	0.97	1.03	6.89
MS	0.75	0.65	5.27	0.75	0.65	5.27	0.44	0.42	3.50	0.44	0.42	3.50
Var. x MS	1.05	1.12	7.46	1.05	1.12	7.46	0.62	0.69	5.21	0.62	0.69	5.21
CO2 x MS	1.56	1.47	8.19	1.56	1.47	8.19	0.86	0.92	7.35	0.86	0.92	7.35
Var.xCO2 x MS	1.98	1.89	10.54	1.98	1.89	10.54	1.26	1.34	11.24	1.26	1.34	11.24

Table: 4. Interactive effect of elevated CO_2 and moisture stomatal pore length ((µm) of leaf, stem and siliqua

Treatment	Leaf adaxial		Leaf abaxial		Stem		Siliquae	
	Pusa gold	RH-30	Pusa gold	RH-30	Pusa gold	RH-30	Pusa gold	RH-30
FACE IRR	8.80	11.10	10.10	13.30	4.60	6.30	4.20	6.10
FACE MS	7.20	9.40	8.20	11.70	3.90	5.50	3.60	5.39
AMB IRR	7.50	9.90	9.30	11.10	4.00	5.20	3.80	5.40
AMB MS	5.20	7.40	6.40	8.50	3.10	4.10	2.90	4.10
Var.	0.67		0.62		0.23		0.19	
CO2	0.78		0.83		0.44		0.35	
Var. × CO2	1.01		1.17		0.62		0.58	
MS	0.33		0.31		0.43		0.31	
Var. × MS	0.88		0.43		0.61		0.52	
CO2 × MS	0.93		0.97		0.71		0.69	
Var. × CO2 × MS	1.67		1.56		0.86		0.81	

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