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EFFECT OF ELEVATED CARBON DIOXIDE ON KINETICS OF NITRATE UPTAKE IN WHEAT ROOTS

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SUMMARY

Wheat (*Triticum aestivum* L.) *cv* PBW 343 was grown in Hoagland solution devoid of nitrogen (-N) and with 1 mM KNO_3 (+N) under two carbon dioxide levels, viz. ambient (370 µl/l, AC) and elevated (600±50 µl/l, EC) for twenty days in growth chambers. The pattern of nitrate uptake was similar under both AC and EC but, the rate of uptake was significantly higher in EC grown plants when the concentration of the nitrate in the external medium was low. The Vmax for high affinity nitrate transport system (HATS) was significantly high for the EC grown un-induced seedlings (116.36 µmol g^{-1} fw h^{-1}) compared with AC grown un-induced seedlings (79.55 µmol g^{-1} fw h^{-1}). The pattern of gene expression of the nitrate transporters in roots correlated with the kinetics and uptake. The rate of nitrate uptake was influenced by previous nitrate nutrition and was strongly regulated by external nitrate levels. It is possible that the HATS operates more efficiently under EC as indicated by high Vmax and the plants will be able to take up nitrate more efficiently from the soils low in nitrate in the future high CO_2 world and may have better NUE.

Key words: **Ambient CO_2, elevated CO_2, nitrate uptake, wheat**

INTRODUCTION

Atmospheric CO_2 concentration has increased from pre-industrial concentration of about 280 µl l^{-1} to over 370 µl l^{-1} and is continuously rising. Several responses of higher plants to such changes were not anticipated. For example, a doubling of CO_2 level initially accelerates carbon fixation in C_3 plants by about 30%, yet after days to weeks of exposure to high CO_2 concentrations, carbon fixation declined until it stabilizes at a rate that averages 12% above ambient controls. This general phenomenon, known as CO_2 acclimation, is correlated with a decline in the activity of Rubisco and other enzymes of the Calvin cycle (Moore *et al.* 1998). The changes in Calvin cycle enzyme activities are not necessarily selective, but are often associated with a decline in overall shoot protein and N contents. In wheat, acclimation to elevated CO_2 varies with N supply. Wheat shoots accumulate free NO^-_3 under elevated CO_2, and shoot protein declines (Pal *et al.* 2003, Jain *et al.* 2007). Shoot N contents diminish by an average of 14 % with a doubling of CO_2, a difference that exceeds what would be expected if a given amount of N were diluted by additional biomass. This indicates that nitrate uptake and assimilation often fail to keep pace with photosynthesis and growth in elevated CO_2. Physiological studies using ^{15}N ,^{13}N and electro physical methods have indicated that plants have at least 3 kinetically distinct nitrate uptake systems, low affinity nitrate transport system (LATS), constitutive and inducible high affinity transport systems (CHATS and IHATS) (Siddiqi *et al.* 1990, Glass and Siddiqi 1995,

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Crawford and Glass 1998). While nutrient uptake kinetics may have a regulatory role in plant nutrient budgets, changes in Vmax and Km in response to elevated CO_2 have rarely been examined. Elevated CO_2 may affect whole network of genes that regulate nitrate uptake and assimilation. Our objective was to study the effect of elevated CO_2 concentration on nitrate uptake kinetics and changes in expression of nitrate transporters in wheat.

MATERIALS AND METHODS

Wheat cv. PBW 343 was grown in Hoagland solution devoid of nitrogen (-N) and with 1 mM KNO_3 (+N) under two carbon dioxide levels, viz. ambient (370 µl l^{-1}, AC) and elevated (600±50 µl l^{-1}, EC) for twenty days in growth chambers at National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. The other conditions, viz. temperature (25°C/18°C D/N), photoperiod (14 h/10 h) and light intensity (500 µmol $m^{-2}s^{-1}$) in the growth chambers were exactly similar.

Seedlings were harvested from different growing media (+N or –N, ½ strength Hoagland solution) and incubated in KNO_3 solutions (60 ml) of varying concentrations ranging from (10 µM – 10 mM) for different time periods (30 min – 24 h at a photon flux density of 500 µmol $m^{-2}s^{-1}$). Prior to transfer, the roots of the seedlings were thoroughly rinsed with double distilled water and held in air to drain excess water; the roots were never blotted or touched. After different time periods of incubation, the volume of the solutions in the different beakers were made up to 60 ml by using double distilled water. Care was taken to use the seedlings having similar fresh weights in various replications to avoid error. The nitrate present in the beakers was estimated by Downes (1978) method. The data obtained from the above experiment was used to calculate the rate and kinetics of nitrate uptake.

To confirm the data obtained on nitrate uptake, gene expression studies were conducted. Total RNA was isolated from roots using RNAeasy Plant Mini Kit by Qiagen. The expression of, high affinity nitrate transporter (HAT) gene was analyzed in the present study. The gene-specific primers were designed according to the mRNA sequences and related expressed sequence tag (EST) sequences. *Ta actin* was used as an internal control in the relative quantitative RT-polymerase chain reaction (RT-PCR). The primers for transporter genes were *TaNRT2.2 (*Acc. No. AF 332214) Forward 5' **CCT TCT TCA CCT GCT TCG TC 3',** Reverse 5**' GAAGTG CAC AAG AGC GAC AG3'** and the primers for *Ta Actin* gene (gene Acc. No. TC 246870) were Forward 5' **GAT TAT GAG CAG GAG CTG GA3',** Reverse 5' **CTG GAA AGT GCT AAG AGA G3'.** The PCR amplification was performed using One-step RT-PCR kit by Qiagen. The PCR cycling conditions were as follows : an initial denaturation step at 95°C for 4 min, amplification for 29 cycles with 95°C for 30 s, 58°C for 40 s, and 72°C for 50 s, and a final elongation step at 72°C for 10 min.

RESULTS

Rate of nitrate uptake by seedlings grown in +N and –N Hoagland solution under ambient and elevated atmospheric CO_2 concentrations: With an increase in concentration of nitrate in the incubation medium, the rate of its uptake increased and maximum uptake was at 10 mM and minimum at 0.01 mM in AC as well as EC grown seedlings. The pattern of uptake was similar in seedlings grown under both AC and EC. The rate of nitrate uptake was maximum at 30 min after incubation in low as well as in high external concentrations of nitrate and declined thereafter (Table 1). Among the CO_2 treatments, the rate of nitrate uptake was high in EC grown (+N) seedlings only at low concentrations of nitrate, i.e. upto 0.04 mM. Beyond this concentration, the uptake rate of nitrate was high in AC grown induced (+N) seedlings. In seedlings incubated in 10 mM concentration, rate of nitrate uptake in AC was 60% more as compared to rate of nitrate uptake in EC at same concentration. A sharp decline in the rate of uptake was noticed from 30 min to 2 h at all nitrate concentrations in both the CO_2 treatments (Table 1).

There were significant differences in the rate of nitrate uptake by un-induced seedlings (grown without external N, -N) among CO_2 treatments and concentration of nitrate in the incubation medium (Table 2). In the un-induced (-N) seedlings maximum uptake was at 30 min after incubation, thereafter, it declined. Up to 0.05 mM

Table 1. Effect of elevated atmospheric CO_2 concentration on the rate of nitrate uptake (µmol g^{-1} fw h^{-1}) in the induced (+N grown) wheat seedlings after incubation in the nitrate solutions of various concentration for different time intervals

CO_2 levels	Time	Concentration of nitrate (mM)													
		0.01	**0.02**	**0.03**	**0.04**	**0.05**	**0.08**	**0.1**	**0.5**	**1.0**	**2.0**	**5.0**	**7.5**	**10.0**	**Mean**
AC	30 min	0.130	0.573	0.327	1.270	1.867	2.55	3.00	16.14	45.09	87.23	169.12	262.69	601.56	91.66
	1 h	0.083	0.217	0.280	0.523	0.683	1.12	2.17	9.16	14.97	36.37	75.47	120.76	170.66	33.27
	2 h	0.025	0.120	0.140	0.317	0.400	0.53	0.85	3.59	16.81	18.47	39.31	55.86	74.71	16.24
	24 h	0.002	0.013	0.017	0.018	0.029	0.05	0.04	2.29	0.697	1.43	2.73	5.20	6.84	1.49
	Mean	**0.060**	**0.231**	**0.191**	**0.532**	**0.745**	**1.063**	**1.515**	**7.795**	**19.392**	**35.875**	**71.658**	**111.128**	**213.443**	
EC	30 min	0.160	0.73	0.64	1.17	1.39	2.76	2.27	14.39	31.42	41.34	149.07	271.44	257.16	59.53
	1 h	0.060	0.29	0.29	0.58	0.61	1.35	1.42	9.85	21.08	36.7	108.79	107.82	135.36	32.63
	2 h	0.035	0.10	0.14	0.37	0.34	0.54	0.57	3.65	9.7	14.46	64.62	47.35	78.48	16.95
	24 h	0.002	0.01	0.013	0.024	0.028	0.050	0.046	0.24	0.90	0.70	2.20	4.00	4.33	0.96
	Mean	**0.064**	**0.283**	**0.271**	**0.536**	**0.592**	**1.175**	**1.077**	**7.033**	**15.775**	**23.300**	**81.170**	**107.653**	**118.833**	**-**

CD (5%)	CO_2	Stages	Nutrition	CO_2 x Stage	CO_2 x Nutrition	Nutrition x Stage	CO_2 x Nutrition x Stage
	1.76	2.78	4.48	3.93	6.34	10.03	14.19

AC = 370 µl l^{-1}, EC = 600 ± 50 µl l^{-1}

Table 2. Effect of elevated atmospheric CO_2 concentration on the rate of nitrate uptake (µmol g^{-1} fw h^{-1}) in the uninduced (-N grown) wheat seedlings after incubation in the nitrate solutions of various concentration for different time intervals

CO_2 levels	Time	Concentration of nitrate (mM)													
		0.01	**0.02**	**0.03**	**0.04**	**0.05**	**0.08**	**0.1**	**0.5**	**1.0**	**2.0**	**5.0**	**7.5**	**10.0**	**Mean**
AC	30 min	0.017	0.280	0.407	0.880	0.930	1.173	1.953	8.503	28.89	26.06	146.77	211.40	287.45	**54.98**
	1 h	0.017	0.133	0.243	0.353	0.683	0.740	0.813	4.393	26.43	23.80	68.99	143.42	103.53	**28.73**
	2 h	0.011	0.076	0.147	0.207	0.240	0.300	0.403	1.643	20.42	22.60	39.66	50.50	66.67	**15.61**
	24 h	0.009	0.005	0.031	0.013	0.020	0.030	0.036	0.016	0.700	1.160	2.768	16.43	24.76	**3.54**
	Mean	**0.014**	**0.124**	**0.207**	**0.363**	**0.468**	**0.561**	**0.801**	**3.639**	**19.110**	**18.405**	**64.547**	**105.438**	**120.603**	**-**
EC	30 min	0.022	0.270	0.470	0.763	0.893	1.890	1.343	7.84	23.40	41.72	123.15	188.61	116.37	**16.20**
	1 h	0.020	0.183	0.440	0.540	0.797	0.710	1.043	5.80	11.87	31.23	53.02	74.12	106.01	**8.30**
	2 h	0.011	0.076	0.220	0.243	0.320	0.340	0.460	1.72	8.67	12.32	17.53	24.99	26.06	**3.45**
	24 h	0.002	0.008	0.020	0.019	0.039	0.031	0.047	0.23	0.54	1.20	2.70	4.04	6.98	**0.64**
	Mean	**0.014**	**0.134**	**0.288**	**0.391**	**0.512**	**0.743**	**0.723**	**3.898**	**11.120**	**21.868**	**49.100**	**72.940**	**63.855**	**-**

CD (5%)	CO_2	Stages	Nutrition	CO_2 x Stage	CO_2 x Nutrition	Nutrition x Stage	CO_2 x Nutrition x Stage
	1.13	1.78	2.87	2.52	6.43	4.06	9.09

AC = 370 µl l^{-1}, EC = 600 ± 50 µl l^{-1}

concentration of nitrate incubation solution in the un-induced seedlings, the rate of nitrate uptake was high in EC treatment as compared to AC treatment. The rate of nitrate uptake was 33% more in seedlings grown in EC and incubated in solution of 0.05 mM for 2 h as compared to AC grown seedlings in the solution of same concentration for 2 h. At higher external concentrations of nitrate, the rate of uptake declined in EC plants as compared to AC grown plants (Table 2).

Kinetics and mechanism of nitrate uptake in induced seedlings under AC and EC: The kinetics and mechanisms of nitrate uptake was studied at 2 h after exposure to external nitrate by incubating the seedlings in a range of nitrate concentrations (Fig. 1, a-d). In un-induced seedlings at 2 h after incubation the rate of nitrate uptake increased gradually from 0.02 mM to 0.08 mM in AC as well as in EC. Uptake rate was almost similar in seedlings incubated in 0.05 - 0.08 mM nitrate solutions and at low concentration of nitrate the uptake followed Michaelis-Menten saturation kinetics and this uptake is by high affinity transporter as shown by the expression study. The same pattern was observed in both AC as well EC grown plants. The Line Weaver Burk plot transformation of this Michaelis Menten type of saturation kinetics gave a straight line with an equation of (Fig. 1 a,b). From this, Vmax and Km of the system were calculated found to be 79.55 µmol g^{-1} fw h^{-1} and 0.752 µM, respectively, under AC and Vmax increased to 116.36 µmol g^{-1} fw h^{-1} in the seedlings grown under EC. Vmax and Km from Line Weaver Burk plot were 213.96 µmol g^{-1} fw h^{-1} and 2.06 µM, respectively, under AC and Vmax and Km from Line Weaver Burk plot were 200 µmol g^{-1} fw h^{-1} and 1.50 µM, respectively, for the induced seedlings grown under EC for the uptake system (HATS) at low external concentration (Fig. 1 c, d).

A linear kinetics for nitrate uptake was observed in high concentration range of nitrate used (0.1 – 10 mM). No saturation in the uptake rate was observed at least upto 10 mM concentration of external nitrate. At 2 h after incubation more than 34% increase in the rate of nitrate uptake at 1 mM concentration of external nitrate was observed as compared to the uptake of nitrate at 0.08 mM in both AC and EC. The rate of nitrate uptake increased linearly with the increasing concentration of external nitrate i.e. from 0.1 – 10 mM in both AC and EC. The low affinity component of uptake system of nitrate uptake in un-induced seedlings had the Vmax value of 163.93 µmol g^{-1} fw h^{-1} in AC and Vmax of 227.27 µmol g^{-1} fw h^{-1} in EC, respectively. The Vmax was 294.12 µmol g^{-1} fw h^{-1} and 243.90 µmol g^{-1} fw h^{-1} in induced seedling grown in AC and EC, respectively (Fig. 2 a-d). The Vmax of induced seedlings increased significantly as compared to un-induced seedlings, both for high affinity transport (HATS) system and low affinity transport system (LATS).

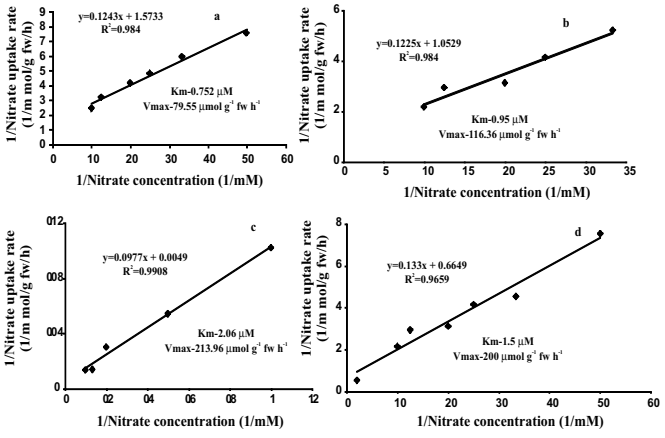

Fig. 1. Line weaver burk plot of high affinity component of nitrate uptake system in (a) uninduced seedlings grown under AC, (b) uninduced seedlings grown under EC, (c) induced seedlings grown under AC and (d) induced seedlings grown under EC.

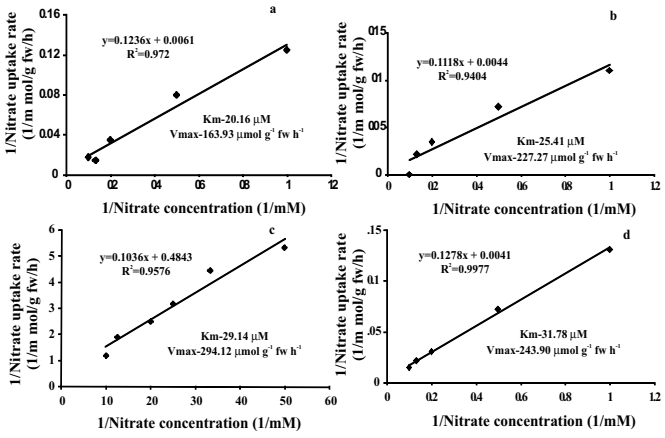

Fig. 2. Line weaver burk plot of low affinity component of nitrate uptake system in (a) uninduced seedlings grown under AC, (b) uninduced seedlings grown under EC, (c) induced seedlings grown under AC and (d) induced seedlings grown under EC.

Expression analysis of high affinity nitrate transporter using RT-PCR: Transcript levels of high affinity nitrate transporter were analyzed in AC and EC grown seedlings. RT-PCR was done using primers corresponding to wheat high affinity nitrate transporter (*TaNRT2.2*; AF 332214). Uninduced seedlings were incubated in solutions of different concentrations of 0.01, 0.1, and 5.0 mM nitrate. RT-PCR was conducted using primer specific for *TaNRT2.2* transporter and controls were *Ta Actin* gene (Fig. 3). The expression of HAT was high in EC grown seedlings incubated at 0.1 mM NO_3^- (Lane 4) as compared to AC grown seedlings incubated at similar concentration (Lane 1). In the case of 0.01 mM NO_3 treatment also the same trend is followed (Lane 2 & 5). Compared to 0.01 mM NO_3 treatment, 0.1 mM treatment led to higher expression of HAT gene in roots of both AC and EC grown seedlings. Whereas, in the roots of nitrogen starved seedlings there was no expression of HAT gene in both AC and EC depicting the inducible nature of HAT. There was also no expression of HAT in the roots of the seedlings incubated for 2h in 5mM nitrate solutions (data not shown). This gene is not expressed when plants are subjected to high external nitrate concentrations.

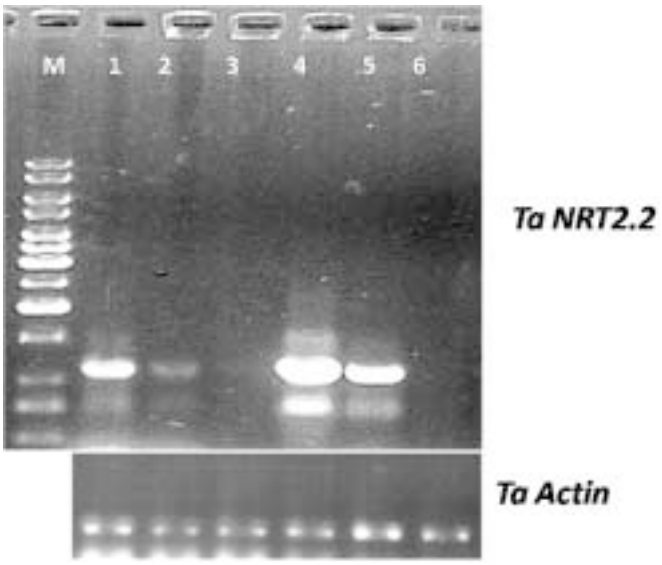

Fig. 3. Expression of high affinity nitrate transporter gene TaNRT2.2 in roots of wheat seedlings, Lane (M) 1Kb DNA Ladder, (1) 0.1mM µM KNO_3, AC, (2) 0.01mM KNO_3, AC, (3) -N, AC, (4) 0.1mM KNO_3 EC, (5) 0.01µM KNO_3, EC, (6) -N, EC

DISCUSSION

The rate of nitrate uptake was studied in AC and EC grown wheat cv. PBW 343 grown under controlled conditions in growth chambers in a range of nitrate concentrations (0.01 mM -10 mM). The nitrate uptake was biphasic in induced as well as uninduced seedlings, i.e. the rate of nitrate uptake saturated at about 0.08 – 0.1 mM and then a sharp increase in the rate of nitrate uptake was noticed in seedlings incubated in solution of 0.5 mM nitrate and the uptake increased linearly in both induced as well as uninduced seedlings in the concentrations beyond 0.08 mM.

The nitrate uptake is active process and transporters are present in root tissues of the crop plants (Glass *et al* 2002). The HAT and LATS are constitutive as well as nitrate inducible (Aslam *et al.* 1992, Glass and Siddiqi 1995). In both the +N and –N grown seedlings, the rate of uptake of nitrate was maximum with in 30 min after incubation. Clarkson (1986) and Warner and Huffaker (1989) reported that exposure to nitrate can increase the uptake rate by 2-5 folds above the constitutive level. In our study, we observed that exposure of roots of un-induced seedlings to external nitrate increased the uptake rate many folds, but at initial stages the uptake of nitrate was high for the induced seedlings. Stimulation of nitrate uptake after preincubation with nitrate has been shown by Aslam *et al.* (1996). In induced (+N) as well as uninduced (-N) seedlings, the rate of nitrate uptake from solutions of low external concentration was high in the seedlings grown in EC as compared to AC. In the induced seedlings, higher uptake was only till the external nitrate concentrations of 0.04 mM in plants from EC and in uninduced seedlings under EC significantly higher amount of nitrate was taken up by roots from the solutions of nitrate concentrations of upto 0.08 mM as compared to AC grown seedlings. These results on the rate of uptake were confirmed by the expression studies of the wheat HAT gene.

In the case of *TaNRT2.2* gene the differential expression can be explained on the basis of nitrate inducible nature of the gene. There was no expression of the gene in plants deprived of NO_3^- for 48 hours after giving treatment with nitrate the expression starts rapidly

within 1 hour and expression was root specific. 0.2 mM and 2 mM NO_3 were able to induce expression. But expression analysis was not done beyond 2 mM nitrate treatment (Zhao *et al.* 2004). TaNRT2 transcripts were accumulated even with 5 µM NO_3^- treatment but expression increased as the NO_3^- concentration increased. The transcript levels increased dramatically when the nitrate concentration shifted from 1.0 to 2.0 mM (Zhao *et al.* 2004). HATS is inducible by nitrate (Filleur and Daniel Vedele 1999, Nazoa *et al.* 2003) and is repressed by N metabolites (Lejay *et al.* 1999). We observed higher expression of HAT gene in the roots of the plants incubated 0.1 mM NO_3^- when compared with the expression of this gene in roots of the seedlings exposed to 0.01 mM NO_3^- in both ambient as well as elevated CO_2 grown plants. Roots of the EC grown seedlings had significantly higher expression compared to AC grown seedlings. This supports the higher nitrate uptake rate observed in un-induced seedlings grown under EC. EC could modify the inorganic nitrogen uptake by altering the rate of uptake per unit of root surface. Plants growing in EC possess larger roots systems (Drake *et al.* 1997) which allows them to exploit larger area of soil this may be beneficial when the nitrate concentrations in the soil are low.

The uptake of nitrate increased linearly when the external concentration of the nutrient was increased (0.5mM -10mM). Uptake of nitrate was higher by the roots of AC grown seedlings as compared to EC grown seedlings. Our results indicate that a linear system became an important component well after saturable system had leveled off. Generally, it has been reported that EC stimulates NO_3^- uptake (Stitt and Krapp 1999) and failure to see stimulation could be low soil nitrate levels. In this experiment, it was observed that nitrate uptake was stimulated by EC, when availability was low but with ample supply of nitrate in the medium roots failed to match the rate of uptake by AC seedlings. EC inhibited nitrate uptake in loblolly pine growing on low nitrate, whereas uptake was stimulated when plants were growing on high nitrate (Larigauderie *et al.* 1994). The results in this study indicate otherwise, viz. low uptake at high N concentrations under EC. These results need to substantiated further by using expression studies of LATS. It has been shown that expression of Nrt1 is affected by the presence of active nitrate reductase and not by N status of plant (Lejay *et al.* 1999) and at EC the NR activity declined at high external concentrations of nitrate.

The change in kinetics of uptake under EC especially the increased Vmax for HATS is of significance as the presence of fully induced HATS might confer advantage when soil N levels are low in the future high atmospheric CO_2 levels.

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