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Plant responses to elevated CO_2 levels in soils: distinct CO_2 and O_2-depletion effects.

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Running Title

extreme CO_2 in soils

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Key Words**:** extreme CO_2, soils, gas exchange, O_2 depletion, hypoxia, crops, carbon capture and storage, CCS, roots

Abstract

To investigate potential environmental effects in the context of carbon dioxide (CO_2) leakage from Carbon Capture and Storage (CCS) schemes, the University of Nottingham ASGARD (Artificial Soil Gassing And Response Detection) facility, was used to inject CO_2 into the soil in replicated open-air field plots over several seasons to measure the effects on UK crop species. However, this system lacked a way of distinguishing the concomitant effects of oxygen (O_2)-depletion (occurring as a consequence of high CO_2 levels in the soil). As plants are aerobic, they require O_2 for functional integrity of root processes. Here a complementary laboratory system was used to specifically identify distinct CO_2 and O_2-depletion effects on two crop species, beetroot and wheat. Parameters measured (photosynthetic rate, transpiration rate, stomatal conductance and biomass) between CO_2-gassed, nitrogen (N_2)-gassed (O_2-depletion control) and non-gassed control plants showed distinct differences in response to CO_2 gassing and O_2-depletion. Differences between field and laboratory studies illustrate effects of variable meteorological conditions in the field, whilst more stable laboratory conditions show differences between crop species. Results show that the interactions of these two stresses (very high soil CO_2 and O_2 depletion on crop physiology are discrete and complex.

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Introduction

Rising atmospheric carbon dioxide (CO_2) levels and links with climate change have led to the development of innovative technologies to facilitate Carbon Capture and Storage (CCS). CCS is currently regarded as a critical mitigation strategy for the global reduction of the atmospheric CO_2 accumulation (IPCC 2007) with the UK Government committed to reducing emissions by 80% of 1990s levels by 2050 under the Climate Change Act of 2008. CCS is reported as being capable of providing 19% of the global CO_2 emission reductions required by 2050 to facilitate a smooth transition to sustainable energy production and use (L'Orange Segio *et al.* 2014). Many high CO_2 emitting industries (e.g. power stations) in the UK are distant from potential carbon storage sites (offshore geological reservoirs) and therefore an infra-structure of CO_2 transportation must be initiated to carry the CO_2 to safe storage. As such there is a need to understand the risks involved and mitigation of potential leaks associated with CCS and dense-phase CO_2 transportation networks into the environment. As most transportation pipelines are likely to be routed through agricultural land, assessment of the impacts in the unlikely event of a leak on the environment and in particular on economically grown vegetation (crops) is required from the outset to inform stakeholders, industry and policy makers with the aim of providing industry best practice.

Although other studies have been carried out with regard to potential CCS leakage of CO_2 (Zhou *et al.* 2013, Sharma *et al.* 2014), these studies utilised a non-replicated

CO_2-gradient experiment with soil CO_2 levels of between 1 and 16%. Previous replicated field studies, the first of their kind, specifically designed to assess impacts of a hypothetical CO_2 pipeline leak were carried out at the ASGARD (Artificial Soil Gassing And Recovery Detection) facility (details in Smith *et al*. 2016 - this issue) over several crop seasons. Various crops and species assemblages were investigated including winter bean (*Vicia faba* cv. Clipper) (Patil *et al*. 2010), field bean (*Vicia faba*), maize (*Zea mays*) (Al-Traboulsi *et al*. 2012a,b, 2013), commercial turf (Pierce and Sjörgesten 2009) and a cover of grass/clover mix (Smith *et al*. 2013). These studies investigated germination, biomass and root production and reported varied responses to the effects of high CO_2 within the rooting zone from no change, through to moderate and severe. These studies, however, could not differentiate between effects directly caused by CO_2 or by hypoxia (a lack of oxygen (O_2). As gases compete on a volume basis, increases in CO_2 result in substantial decreases in O_2 (Gal *et al*. 2012) (Zhou *et al.* 2013); severe O_2-depletion in the root zone is a consequence of the experimental design at ASGARD and therefore, two stresses are imposed simultaneously. As plants are aerobic organisms there is a requirement for O_2 to be present in the root zone for functional integrity. Hypoxia responses in plants have been widely reported as a consequence of waterlogging; with a recent notable review specifically on wheat varieties (Herzog *et al.* 2016). Here we report the results of a comparative study of the impacts on two crop species grown both in the field and in the laboratory to isolate responses to both high soil CO_2 and low soil O_2.

Materials and methods

Field studies

ASGARD is a purpose-built facility located at the University of Nottingham's Sutton Bonington campus in the UK (location, N52°, 49'60; W01°, 14'60) for the study of agro-ecosystem responses to elevated soil CO_2 concentrations. This was the same facility as used and described previously (Al-Traboulsi *et al*. 2012a, b 2013) but with newly prepared test sites for the current investigation. Briefly, CO_2 gas is delivered to up to 16 field plots via 20 mm (Inside Diameter (ID)) medium density polyethylene (MDPE) gas pipes. The pipes are sealed at the end, perforated over the final 210 mm and inserted into the ground at an angle of 45° to the vertical so that the CO_2 is delivered into the soil 0.5-0.6 m below the centre of each gassed plot. Food-grade, CO_2 is delivered by 16 individual mass flow controllers (Alicat, Tucson, USA) to individual experimental plots. The mass flow controllers are operated, and the system data logged, by a PC-based control system (TVC, Great Yarmouth, UK).

The experimental area was divided by crop type into three blocks of eight replicated 2.5 m × 2.5 m plots. In each block, four randomly selected plots were treated with injected CO_2 and four were left as untreated controls for each crop species. CO_2 was supplied to each plot at a constant rate of 1 L min^{-1}. The single point injection scheme generates a distribution of CO_2 in the soil ranging from high concentrations, sometimes above 50%, in the plot centre down to values approaching control levels at the plot edges.

Gas measurement

Soil CO_2 and O_2 concentrations were measured using a GA5000 landfill gas analyser (Geotech, Warwickshire, UK) on a weekly basis via permanently installed tubes located at 0.15 and 0.70 m from the centre of the plot. Sampling areas within the plots were zoned into low, medium and high CO_2, corresponding to soil concentrations of approximately 0-4%, 4-10% and >10% respectively.

Crop species

Studies were carried out on spring wheat (*Triticum aestivum* v Tybault - a monocotyledon, grass) and beetroot (*Beta vulgaris* v Pablo F1 - a dicotyledon, vegetable). These crops were chosen to examine any differential effect on monocotyledonous and dicotyledonous plant forms as well as differences in root structure; grasses have fibrous roots, whilst beetroots form storage roots (the beet). Following establishment of the crop, CO_2 gas was delivered continuously to the gassed plots until harvest.

Plant gas exchange

Plant gas exchange (photosynthetic rate, stomatal conductance and transpiration rate) was measured using an infra-red gas analyser (Licor 6400x, Licor Inc., Utah, USA). A minimum of 3 replicate plants in each plot in areas of high CO_2 (>10%) were measured respectively.

Laboratory studies

Plant material and methods

The same crop species (and varieties) grown in field trials were used in laboratory studies to examine potential differences between field and laboratory plant responses measured under both varied and standardised conditons respectively. Crops were sown and grown in Levington's no. 3 multipurpose compost within the growth room for 1 to 2 weeks before being transplanted into the soil chambers. They were then left to allow sufficient root growth before gassing commenced (approximately 2 weeks). The gassing period lasted for up to 7 days. After that time, plants become pot-bound which affects physiology and plant responses no longer reflect those under field conditions.

Soil chambers were constructed of acrylic plastic with pipe inlets to allow CO_2 or N_2 gassing of the soil environment exclusively, which was isolated from the above ground environment to reduce the effects of physiologically relevant atmospheric CO_2 (Fig. 1A & B). The experimental system was housed in a controlled environment growth facility (UNIGRO, UK) to standardise all other environmental variables: irradiance was 300 μmol m^{-2} s^{-1} (at plant height), day/night as 12/12 hours; temperature 21/18^{o}C; relative humidity 60%. Gas was supplied from either an integral supply (pure CO_2) or a gas cylinder (nitrogen - N_2) and separated prior to entering each individual soil chamber by 2 flow rate step-down manifolds. Gas was delivered to each individual chamber at a rate of 30 (±15) mL min^{-1} to maintain CO_2 and N_2 levels at steady state. Gases were exhausted to atmosphere via a separate manifold to prevent build up within the growth room. Gas concentrations (CO_2 and O_2) were measured daily using the GEOTECH GA5000 gas analyser (Geotech, Warwickshire, UK). Each experiment consisted of 3 levels of control: CO_2-gassed soil (experiment), N_2-gassed soil (O_2-depleted control), air-gassed soil and non-gassed soil. Replication for each species was 24, 24, 16 and 16 respectively.

Plant gas exchange

Gas exchange was measured on each replicate plant prior to and then daily during gassing until harvest using a Licor 6400x IRGA (Licor Inc, Utah, USA).

Biomass (shoot and root)

Plants were harvested between days 5 and 7. Shoots were taken from each plant, washed and dried at 80^o C for 2 days. Biomass was measured as fresh and dry weight.

Roots were carefully removed from the chambers, washed, patted dry, weighed and dried for 4 days at 50°C. They were then re-weighed. The beet (storage root) was separated from the lateral roots from beetroot plants and analysed independently. Beets were dried until the constant dry weight was measured. Wheat roots were measured as dry weight only.

Statistical analyses were all carried out using Minitab v 12 (USA). One-way ANOVA and Student's t tests of each treatment from each other (comparison of means).

Results

Gas concentrations

In the field study, CO_2 injection caused elevated concentrations of soil CO_2 which were highest above the delivery point and rapidly decreased radially towards the edge of the gassed plots. Concentration varied in each plot due to the variability of the soil conditions. Table 1 shows the mean soil CO_2 and O_2 concentration achieved in the plots measured from the permanently installed gas measurement tubes.

There was a strong negative correlation (R^2=0.95 P=<0.001) between the CO_2 and O_2 concentration measured at 150 mm from the centre of the plot as O_2 was displaced by CO_2.

In the laboratory studies, mean gas concentrations in both CO_2-gassed and N_2-gassed chambers, also in Table 1, showed a reduction in O_2 levels comparable to the field conditions, with the N_2-gassed chambers being generally slightly lower in O_2 concentration than the CO_2 chambers. Air-gassed plants were not statistically different to the non-gassed controls (Table S1 – Supplementary Information) and so data is shown for non-gassed controls only (as comparable to the field study).

Gas exchange

Fig 2A-L shows the mean gas exchange parameters in both the field and laboratory for both species over time. Both were measured from the onset of gassing, however measurements continued in the field for 15 days (weather permitting) whilst the laboratory studies were terminated after 6/7 days. Photosynthetic rate (*A*) (Fig. 2A-D) for both species differed in magnitude between the field and laboratory; measurements were normally higher in the field due to higher light levels, but measurements varied according to the prevailing weather conditions on the day. Both experimental sets show an initial effect of CO_2 gassing on *A*, however this difference diminishes in field grown crops. By day 15, wheat showed a reduction in *A* compared to non-gassed controls, but beetroot remained the same as control plants.

Stomatal conductance (g_s) levels were comparable for both species in the laboratory and the field (Fig. 2E-H). Again an immediate and sustained reduction in g_s is recorded under both CO_2-gassing and O_2-depletion. Transpiration rate (E) (Fig. 2I-L) was also lower in the laboratory than the field for beetroot, but comparable in wheat. Both species showed an immediate and sustained effect of CO_2 gassing on E compared to non-gassed controls. N_2-gassed (O_2-depletion) showed an intermediate effect in beetroot for *A*, g_s and E (Fig 2A, E & I), but in wheat there is no statistical difference for *A* (Fig 2C). E is recorded as higher in N_2-gassed plants compared to controls from days 1 to 3 (Fig 2K).

Laboratory studies show greater differences between crop species than field measurements. This is a consequence of both larger error rates under field conditions and greater stability in laboratory conditions. Percentage (%) change from non-gassed controls at the end of experimental gas exchange measurements is shown to allow comparison between the field and laboratory results (Table 2). Fig 3A-C graphically shows the relative effect of O_2-depletion. CO_2-gassing has a separate and greater effect on reducing all three gas exchange parameters in the laboratory, with only *A* remaining higher (lower % reduction) in the field in beetroot over the measured time course (Fig 3A). Wheat is more sensitive to CO_2-gassing under field compared to lab conditions (Fig 3A, B & C).

Whilst a one-way ANOVA for each gas exchange parameter between all treatments reports highly significant differences (p=>0.000), Table 3 is more useful in demonstrating the differences between CO_2-gassed and N_2-gassed plants via individual Student's t-test results for individual treatments (comparison of means). CO_2 versus N_2-gassed plants all show highly significant differences.

Shoot biomass

Table 4 gives the dry weight (g) for the total shoot and total root. Beetroot has a greater shoot biomass (after drying) under CO_2-gassing than non-gassed controls, while wheat has the smallest shoot biomass when CO_2-gassed.

Root biomass

Root biomass is severely affected by both CO_2 and N_2-gassed O_2-depletion, with wheat roots affected more by O_2-depletion than CO_2 gas.

Root to shoot ratio

Table 4 also gives the root to shoot ratio (R/S). Non-gassed control plants show healthy root to shoot ratios of 0.96 (beetroot) and 0.51 (wheat). Wheat has more shoot to root biomass, whereas beetroot at this developmental stage has an equal amount of both. CO_2-gassing has an effect on roots only in beetroot, while in wheat both leaves and roots are affected. Wheat R/S is most severely affected under O_2-depletion.

Discussion

There are differences in time series responses of gas exchange measurements between the field and laboratory studies for both species. Field conditions varied due to the dynamic weather conditions and therefore changes in air temperature, vapour pressure deficit and water availability would all impact on measurements of A, E and g_s on daily basis. In the laboratory, CO_2 is delivered directly and efficiently to the roots, whereas in the open field system lateral diffusion may take the CO_2 away from any individual plant, so that responses in the laboratory may be expected to be more severe. Nevertheless, the impacts of CO_2 gassing were immediate (within 1 day) in both species for all parameters in both field and laboratory settings. Both g_s and E exhibit similar responses in the laboratory as the field, with significant reductions under elevated CO_2 soil levels. This is in contrast to g_s measured for both dandelion and orchid grass leaves in a study carried out at the ZERT site (Montana, USA) where stomatal conductance was recorded as higher under the highest CO_2 level (16%) (Sharma *et al.* 2014) with near-normal O_2 levels (recorded separately) of ~19% (Zhou *et al*. 2013), despite localised death of vegetation over time. It may be that higher CO_2/lower O_2 levels recorded in the field at ASGARD here (Table 1) produce a more severe stomatal response.

N_2-gassed O_2-depletion responses are more complex. Although each species responded differently to all gassing scenarios the % reduction (Fig. 3) shows that O_2-depletion effects are always less severe than CO_2 effects, illustrating that O_2 depletion and CO_2 responses are clearly separate and distinct. Whilst not exactly the same growth conditions and developmental stage to the present study, several wheat varieties were found to show similar decreases in *A* and g_s after 1 to 3 days of waterlogging imposed O_2-depletion. Other varieties showed no response to this treatment (Herzog *et al* 2016), suggesting that both variety and age of the plant can have differential effects on root responses to O_2-depletion.

Shoot biomass as dry weight is not affected in beetroot and only slightly affected in wheat with either CO_2 or N_2-gassing (Table 4). Examination of dry root biomass shows that the effect of both CO_2 gassing and O_2-depletion is severe. Comparison of % change in dry weight against non-gassed control plants in the laboratory, reductions for wheat are 71% and 75% for CO_2-gassed and N_2-gassed, respectively. The same measurements for beetroot record a reduction of 71% and 65% respectively.

The root to shoot ratio (Table 4) is considered a measure of plant health, with a balanced amount of both roots and shoots contributing to below ground resources (nutrients, water) and carbon acquisition respectively. A change in this ratio suggest that an unfavourable environment (stress) has had an effect on either or both the root or shoot. The ratio is different for different plant forms and for different age classes of the same plant (Werger 1998, Kozlowski *et al.* 2012). Here, only comparisons between treatments are taken into account; previous studies on wheat show R/S for non-experimental control plants of between 1.32 and 0.33 comparable to a control for wheta here of 0.51. Changes in R/S under O_2-depleted waterlogging experiments decreased from 0.4 to 0.2 (Herzog *et al.* 2016) which also is comparable to a reduction reported here to 0.22 under N2-gassing. This suggests that O_2-depletion is having a greater effect than CO_2-gassing and that it is largely an effect on root biomass; wheat is known to be sensitive to low O_2 in the root zone (Herzog *et al.* 2016). Little information is available about beetroot in terms of O_2-depletion sensitivity, but two values for R/S have been previously reported; the first in non-stressed hydroponic systems of between 0.41 and 0.57 (Egilla 2012), which suggests that beetroot in the present study is healthy at 0.96 under non-gassed conditions. The second gives an R/S for non-treated beetroot as 2.57, but the plants were 75 days old, so it is expected that the storage organ would have been much bigger at that stage and contributed to a larger root biomass.

A more detailed analysis of root fresh weight versus dry weight for beetroot (Fig. 4) shows that most losses occur in the form of true roots; the beet (storage root) showing a greater loss under CO_2-gassing than O_2 depletion. Furthermore, the difference between control plants (fresh weight to dry weight) shows that CO_2-gassed plants are severely short of water at the end of the experiment. This is in agreement with the time course measurements of E and g_s, which show greater reductions under CO_2 gassing than either control or N_2-gassed plants in this crop. This suggests that stomatal function and normal hydraulic mechanisms of water transport are disrupted under CO_2-gassing for both species, and constitutes a specific CO_2 response. As the aerial organs are isolated from treatment in the laboratory studies, the effects can only be due to changes imposed on the root zone i.e. increases in CO_2 and decreases in O_2; all other variables in the root zone are the same and therefore standardised for each treatment (sufficient water availability, temperature and growth medium) which allows for our interpretation of results. It is noted that each species responds in a specific and different way. This may reflect the differences in root architecture, however, as both crops are severely affected in the root zone, such differences are subtle and don’t impact hugely on the end result of CO_2-gassing.

The aim of this study was to determine the differential effects of high CO_2 and low O_2 levels in the soil. Data presented clearly demonstrate a separate and distinct effect of elevated levels of CO_2 in the root zone. However, aspects of CO_2-gassed and concomitant O_2-depletion effects show that both environmental stresses interact in a complex manner. Gas exchange characteristics for beetroot show an intermediate effect of O_2-depletion between non-gassed and CO_2-gassed plants, suggesting that CO_2 and O_2-depletion effects may potentially be additive. Wheat was more sensitive to CO_2-gassing under field conditions than in the lab, suggesting that field conditions may contribute to the degree of sensitivity in the species. Roots were affected differentially with beetroot more sensitive to CO_2-gassing (or an additive effect of both CO_2 and O_2-depletion) whereas wheat was more severely affected by O_2-depletion. Further investigations are required to elucidate the specific mechanisms of each species to each stress.

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Figure legends:

Figure 1. Schematic diagram of the soil chamber showing CO_2 diffusion in the root zone and isolation from the aerial environment and graphic *in situ* of beetroot (A) and wheat (B).

Figure 2. Gas exchange parameters for laboratory (left hand panels) and field (right hand panels) experiments: photosynthetic rate (*A*) beetroot A, B; wheat C, D: stomatal conductance (g_s) beetroot E, F; wheat G, H: transpiration rate (E) beetroot I, J; wheat K, L. (n = 24, 24 and 16 for CO_2-gassed, N_2-gassed and non-gassed control laboratory experiments respectively , n = 12 for CO_2-gassed and non-gassed control in field experiments. Error bar = SEmean).

Figure 3. Comparison of % change from non-gassed controls in photosynthetic rate (A), stomatal conductance (B) and transpiration rate (C) showing relative effects and clear differences of CO_2-gassing and O_2-depletion (as N_2-gassing) in both field and laboratory experiments.

Figure 4. Effects of CO_2-gassing and N_2-gassing root biomass for beetroot comparing fresh and dry weight of separated lateral and storage (beet) roots.

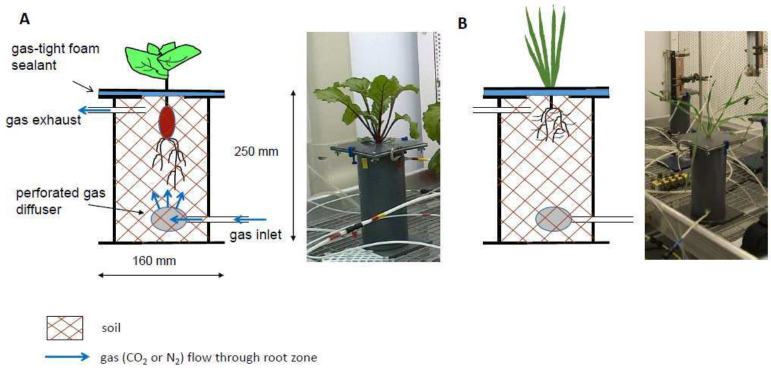
A
gas-tight foam sealant
gas exhaust
perforated gas diffuser
250 mm
gas inlet
160 mm
B
soil
gas (CO_2 or N_2) flow through root zone

Figure 1

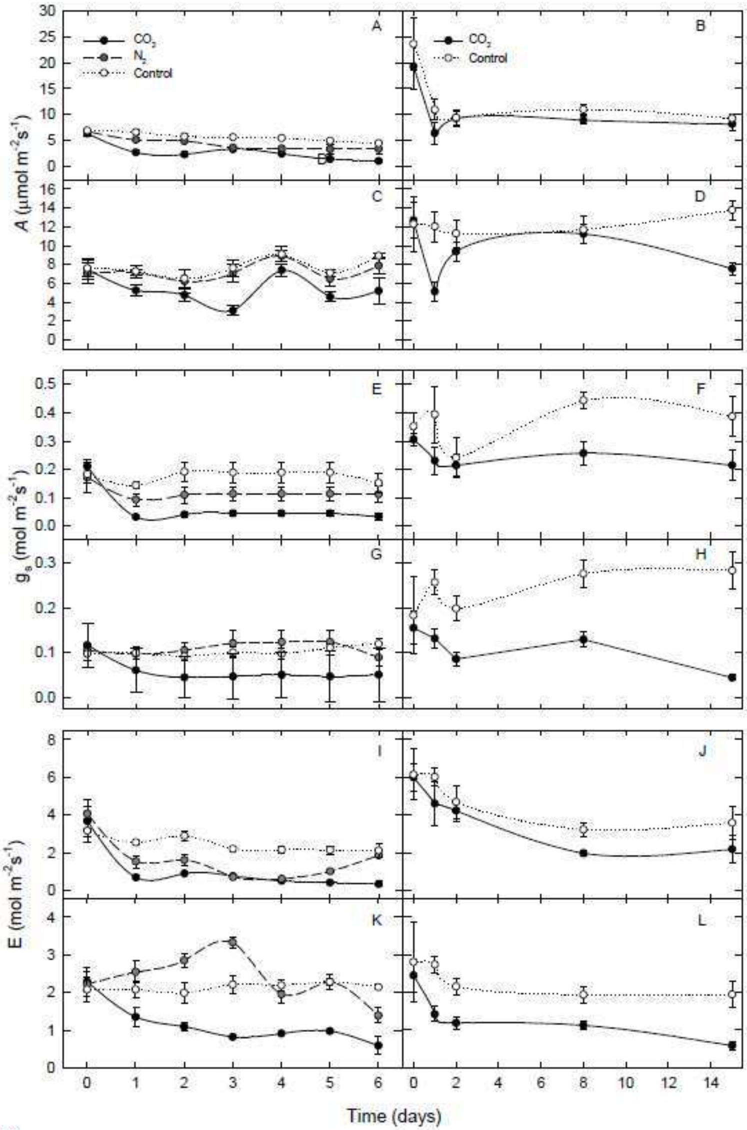

Fig.2.
Lake *et al.* (2015).

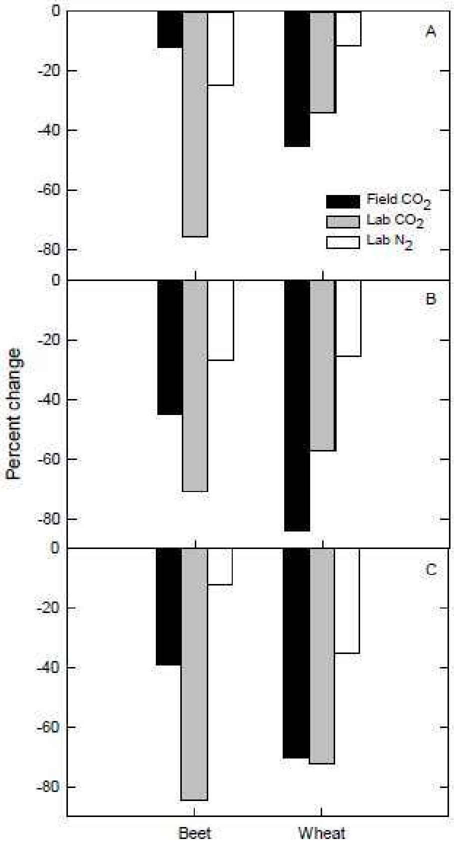

Fig. 3.
Lake *et al.* (2015).

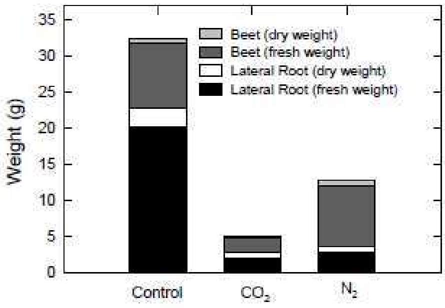

Fig. 4.
Lake *et al.* 2016

Table 1. Mean CO_2 and O_2 concentrations measured in both field and laboratory experiments. Laboratory experiments replicate the highest mean values measured in the field.

Mean gas level	**CO_2 concentration (%)**		**O_2 concentration (%)**	
	CO_2-gassed	control	CO_2- gassed	control
field	42.2	0.7	12.7	19.6
laboratory	42.3	0.4	11.1	9.4 (N_2-gassed)

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Table 2. Mean % changes in gas exchange parameters from non-gassed control plants

Crop species	**beet**									**wheat**								
	A (photosynthetic rate)			**E (transpiration rate)**			**g_s (stomatal conductance)**			**A (photosynthetic rate)**			**E (transpiration rate)**			**g_s (stomatal conductance)**		
Day	field CO_2	lab CO_2	lab N_2	field CO_2	lab CO_2	lab N_2	field CO_2	lab CO_2	lab N_2	field CO_2	lab CO_2	lab N_2	field CO_2	lab CO_2	lab N_2	field CO_2	lab CO_2	Lab N_2
0	-18.3	-8.5	-2.3	-2.4	+16.4	+28.9	-13.1	+22.7	-5.9	+3.3	+6.9	-7.5	-12.7	+10.2	+6.9	-15.3	+19.4	+6.9
1	-40.7	-58.7	-22.2	-23.4	-73.3	-39.9	-41.7	-66.5	-35.7	-57.4	-26.6	-1.7	-48.3	-34.9	+22.6	-48.6	-38.5	-2.4
2	-1.4	-59.1	-15.1	-9.8	-69.4	-44.4	-11.4	-63.8	-42.8	-17.0	-23.7	-4.4	-45.2	-45.1	+43.1	-56.6	-50.7	+14.4
3		-40.4	-35.4		-66.1	-67.9		-61.5	-40.2		-56.0	-7.1		-62.9	+50.7		-52.9	+20.2
4		-54.5	-36.4		-76.7	-72.1		-61.5	-40.2		-17.0	-2.2		-58.4	-10.8		-48.2	+20.2
5		-70.0	-32.0		-81.2	-53.1		-61.5	-40.2		-29.5	-8.7		-57.1	0		-57.5	+11.3
6		-75.6	-24.6		-84.4	-12.2		-71.0	-26.8		-34.0	-11.7		-72.5	-35.2		-57.1	-25.3
8	-19.0			-39.2			-42.1			-4.0			-42.1			-53.0		
15	-12.0			-39.4			-44.7			-45.2			-70.2			-84.2		

Table 3. Student's t-test *p* values between gassing treatment and controls and between CO_2-gassing and N_2-gassing. (>0.05 is significantly different; * = test variables). Non-significant results are highlighted.

species	**beet**			**wheat**			**beet**			**w**	
study	**lab**			**lab**			**field**			**f**	
parameter **treatment**	***A***	$\mathbf{g_s}$	**E**	***A***	$\mathbf{g_s}$	**E**	***A***	$\mathbf{g_s}$	**E**	***A***	
CO_2*control	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	**0.21**	0.02	0.04	<0.000	<
N_2* control	**0.095**	0.049	<0.000	0.028	**0.86**	**0.53**					
CO_2* N_2	<0.000	<.0000	<0.000	<0.000	<0.000	<0.000					

Table 4. Dry weight (g), total shoot and total root and root to shoot ratio (R/S) of beet and wheat (n = 6 per treatment, SEmean in parentheses).

crop	**beetroot**			**wheat**		
	shoot biomass	**root biomass (total)**	**root to shoot ratio**	**shoot biomass**	**root biomass**	**root to shoot ratio**
non-gassed control	3.34 (0.35)	3.22 (0.75)	0.96	1.68 (0.8)	0.87 (0.32)	0.51
CO_2-gassed	4.47 (1.0)	0.88 (0.21)	0.19	1.33 (0.24)	0.34 (0.12)	0.26
N_2-gassed	3.42 (0.6)	1.44 (0.22)	0.42	1.62 (0.1)	0.22 (0.1)	0.14