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A novel root-to-shoot stomatal response to very high CO_2 levels in the soil: Electrical, hydraulic and biochemical signalling

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Investigations were undertaken in the context of the potential environmental impact of Carbon Capture and Storage (CCS) transportation in the form of a hypothetical leak of extreme levels of CO_2 into the soil environment and subsequent effects on plant physiology. Laboratory studies using purpose built soil chambers, separating and isolating the soil and aerial environments, were used to introduce high levels of CO_2 gas exclusively into the rhizosphere. CO_2 concentrations greater than 32% in the isolated soil environment revealed a previously unknown whole plant stomatal response. Time course measurements of stomatal conductance, leaf temperature and leaf abscisic acid show strong coupling between all three variables over a specific period (3 hrs following CO_2 gassing) occurring as a result of CO_2-specific detection by roots. The coupling of g_s and ABA subsequently breaks down resulting in a rapid and complete loss of turgor in the shoot. Root access to water is severely restricted as evidenced by the inability to counter turgor loss, however the plant regains some turgor over time. Recovery of full turgor is not achieved over the longer term. Results suggest an immediate perception and whole plant response as changes in measured parameters (leaf temperature, g_s and ABA) occur in the shoot, but the response is solely due to detection of very high CO_2 concentration at the root/soil interface which results in loss of stomatal regulation and disruption to control over water uptake.

Introduction

Carbon Capture and Storage (CCS) is currently regarded as a critical mitigation strategy for the global reduction of the atmospheric CO2. It is reported as capable of providing 19% of global CO2 emission reductions by 2050 enabling a smooth transition to a more sustainable energy production and use. (L'Orange Segio et al. 2014). The UK Government is committed under the Climate Change Act 2008

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(http://www.legislation.gov.uk/ukpga/2008/27/contents) to reduce emissions by 80% of 1990s levels by 2050 and has initiated a programme of development of CCS. Inclusive of this is a new area of research focused on the potential environmental impact. The deployment of transportation pipelines carrying captured and compressed CO2 from source (e.g. power stations and industry) to offshore reservoirs for storage is likely to be under agricultural land in areas of intensive land use and therefore requires quantitative assessment of potential effects on agricultural crops of potentially very high concentrations (up to 90%) of CO2 leakage into the soil environment (Lake et al. 2013, Smith et al. 2016).

Previous laboratory studies of plant responses to extreme soil CO_2 (greater than natural soil CO_2 levels) have been intermittent and span many decades. Rudimentary experiments elevating CO_2 in the root environment of tomato and corn were carried out over a century ago (Noyes 1914); a wilting effect on both plant crops was observed with notable differences recorded between corn, which recovered when gassing ceased, and tomato which failed to recover, however, no physiological measurements or CO_2 gas levels were reported (Noyes 1914). A study undertaken in the context of space travel almost 40 years ago also experimented with high levels of CO_2 gas in hydroponic systems and looked at effects on potato tubers (Arteca et al. 1979). This study exposed plant roots to a 40% CO_2 concentration and reported an increase in tuber growth and number when compared to control plants. However, in these experiments (Arteca et al. 1979) it is not clear whether aerial plant parts (leaves) were isolated from elevated levels of gaseous CO_2 which may have had a CO_2 fertilization effect potentially resulting in increased biomass. Furthermore, leaf responses were not reported. Several studies on extreme CO_2 levels in the root zone have been conducted on a number of different crop species including soybean, rice, sorghum, peas, beans, sunflower, barley and cotton and have been reviewed and detailed within a CCS context (Steven et al. 2010). Overall, negative effects on root growth and yield were reported here under high CO_2 together with species-specific effects. None of these studies investigated stomatal responses of leaves. A series of experiments carried out by Cramer and co-workers did measure stomatal conductance and growth rates in tomato seedlings in hydroponic systems with elevated root zone CO_2 of 0.5%. They reported that conductance and transpiration rate was reduced with elevated CO_2 treatment, but only when relative humidity around the shoots was high (80%) (Cramer and Richards 1999). Total dry weight biomass of tomato increased under high CO_2, however similar experiments using white lupin in a sand mix substrate with elevated root zone CO_2 of 0.6% showed no increase in biomass compared to air gassed controls (Cramer et al. 2005) again illustrating species-specific differences. However, these studies did not have ultra-high CO_2 levels, rather concentrations that can be present in agricultural systems (Stolwijk and Thimann 1957, Russell 1973, Glinski and Stepniewski 1985), which in the case of tomato, was in the form of dissolved inorganic carbon. Such conditions may not impose a stress *per se* and as such the studies are not directly comparable to this study utilising CO_2 in gaseous form and in concentrations above 30%.

As gases compete on a volume basis, increases in CO_2 always result in decreased oxygen (O_2) concentration. Plant responses to O_2 depletion (hypoxia and anoxia) have been widely investigated, mainly in the context of flooding (reviewed in Blom and Voesenek 1996, Fukao and Bailey-Serres 2004) however, in the case of CO_2 gassing of the root environment important differences have been noted as separate and distinct from those of O_2 depletion (Chang and Loomis 1945, Boru et al. 2003), including the present experimental system (Lake et al. 2016).

Biochemical root to shoot signalling involved in plant water status under drought is well known (Schachtman and Godger 2008), however integration of biochemistry with hydraulic processes and the relatively under-reported field of electrical signalling in plants is less well understood. Distinct roles for both hydraulic and electrical processes were found to impact on gas exchange in maize (Grams et al. 2007) whilst Malone and Stanković (1991) suggested co-ordination of both in response to wounding-induced changes in water status in wheat. Here a series of experiments was devised to specifically test the effects of extreme soil CO_2 (greater than 30%) completely isolated from aerial leaves, on stomatal physiology and biochemistry and in doing so, investigate the mechanistic basis of CO_2-induced wilting described by Noyes (1914). We report a previously unreported whole plant rapid stomatal response directly emanating from high CO_2 around the roots and propose a hypothetical long-distance mechanistic model orchestrated by a combination of biochemical and electrical signalling together with physical hydraulic processes. Furthermore, this response while synonymous with water stress (defined here as a direct consequence of water deficit) results in a change in water relations that are distinct from other abiotic conditions that affect hydraulic processes such as drought, chilling and flooding.

Material and methods

Experimental protocol

In a laboratory system, soil chambers were constructed of acrylic plastic with pipe inlets and outlets to allow flow-through CO_2 gassing of the soil environment, which is isolated from the above ground environment in a fully replicated experiment (Fig. 1A and B). The experimental system was housed in a controlled environment growth facility (UNIGRO, UK) to standardise all other environmental variables that impinge on plant performance. A maximum of 20 chambers were housed within one walk-in growth room. Gas was supplied from an integral supply (pure CO_2) and separated prior to entering each soil chamber by via step-down primary and secondary manifolds. Gas was fed to and through the soil chamber and exhausted to atmosphere outside the growth facility via a separate manifold. A previous study including air-gassed control plants and non-gassed control plants showed that air-gassed and non-gassed controls were not significantly different in all measured parameters (Table S1 – Supplementary Information, Lake *et al.* 2016) therefore non-gassed control plants grown in the same soil chambers are

reported here.

Plant material

Beetroot (*Beta vulgares* v Pablo F1 – a dicotyledon) seeds were sown and grown in Levington's no.3 compost (to standardise soil conditions) within the growth room for 1 to 2 weeks before being transplanted into the soil chambers (Fig 1B) with the same compost. They were then left to allow sufficient root growth before gassing commenced (approximately 2 weeks). The gassing period lasted for 7 days. After that time, the rapidly growing plants become pot-bound which affects physiology and no longer represents experimental responses to soil CO_2 alone. Soil moisture was maintained by watering to field capacity (free drainage after watering) every 2 to 3 days and the day prior to each experiment to ensure that water deficit did not occur through the experiments.

Standardised growth conditions were set as light level 280 (±20) μmol m^{-2} s^{-1} (at plant height) under fluorescent tube lighting (Osram FQ 54W / 840 HO Lumilux Cool White. Far red supplement: Phillips Clickline 60W G9 240V CL ICT), day/night as 12/12 hrs, temperature 21/18°C and relative humidity 55% and CO_2 within the growth chamber was 425 (± 30) parts per million (ppm). Replication was 6 plants (soil chambers) per treatment in each successive experiment.

CO_2 concentrations in the soil environment

Pure CO_2 was supplied from CO_2 cylinders (BOC, UK) 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 levels at steady state within the soil environment. Gas (CO_2 and O_2) concentration in the rhizosphere was spot measured throughout the course of each experiment by interception of the exhaust pipe (Fig 1A) which was then connected to a GEOTECH GA5000 gas analyser (Geotech, Warwickshire, UK).

Stomatal conductance (g_s)

Stomatal conductance was measured continuously on all plants using a Licor 6400x (Licor Inc, Lincoln, USA) prior to and post-gassing up to 10 hours. CO_2 levels within the plant cuvette for measurement of g_s were the same as conditions in the growth chamber (425 ± 30 ppm.) and therefore the conditions experienced by the whole shoot. Only the isolated soil environment was under high CO_2 treatment, the leaves remained within an ambient CO_2 atmosphere throughout the experiments.

Leaf temperature measurement (T_{leaf})

T_{leaf} was recorded by the Licor 6400x throughout gas exchange measurements. Independent measurements using a calibrated hand held infra-red thermometer reader (Model 68, Fluke, Everett, WA, USA) were made on a target leaf of each plant during the course of one experiment at 30 minute intervals to coincide

with g_s and ABA sampling from separate experimental runs.

% leaf water content

At the end of experiments remaining leaves were harvested, weighed and then dried to constant weight at 80°C. Water content was calculated as fresh weight – dry weight, expressed as a % water loss. (n = 6)

Carbon isotope analysis

Using the same dried leaf material, 0.1 mg of leaf material per plant was ground in a pestle and mortar. Twelve CO_2 gassed plants and eight non-gassed control plants were analysed using the method in Lake et al. (2009). Isotopic composition of the leaf environment was determined by sampling the air inside the growth room using pumps, disposable syringes (Sigma-Aldrich, UK) and 10 mL gas tight collection vials (Labco Exetainer Vials, Labco Ltd, UK), three replicate vials were flushed with N_2 prior to sample collection.

ABA analysis

In a specific experiment leaf samples were taken as times series immediately prior to and following CO_2 gassing of the soil environment for a total of 3 hrs at 30 minute intervals. Separate experimental runs were carried out as removal of leaf samples may have affected measurements of both leaf temperature and g_s. Three samples per time point were taken from both CO_2 gassed and non-gassed control plants. Samples were snap frozen in liquid nitrogen and extracted using the water: methanol: chloroform method (Overy et al. 2005).

A new methodology was developed for ABA analysis using Ultra Performance Liquid Chromotography-Mass Spectrometry (UPLC-MS; Waters Acquity UPLC coupled to Waters Synapt G2 Mass Spectrometer, Waters Ltd, Manchester, UK). The mass spectrometer had an electrospray sample introduction system and data was acquired in negative mode using Waters MassLynx data system. MS conditions were set as Capillary Voltage 2.5 kV; Source Temperature 120°C; Desolvation Temperature 350°C; Sample Cone 20 V; Extraction Cone 4 V; Desolvation Gas Flow 900 L h^{-1}; Cone Gas Flow 20 L h^{-1}. ABA separation using the UPLC was obtained with a 7.6-minute run and a linear gradient system. The column was a Waters Acquity UPLC Peptide BEH C18 column (size = 2.1 × 50 mm, pore size = 130 Å, particle size = 1.7 µm) with a flow rate of 0.6 mL min^{-1}. All solvents used for the analysis were LC-MS Chromasolv Grade from Sigma and ultra-high purity water was obtained from an Elga PureLab system. Gradient UPLC run conditions are reported in Table S2 (Supplementary Information). Samples were not diluted before analysis and 10 µl was injected into the UPLC. A standard curve was produced, with an R^2 value of 0.995, by serially diluting an ABA stock (Sigma-Aldrich, UK) with a concentration of 26 µg mL^{-1} 2-fold down to a concentration of 1.625 µg mL^{-1}. ABA is expressed as mg g^{-1} fresh weight of leaf

sample.

Statistical analyses utilised Student's t-test, 2-sample means (Minitab v12).

Soil pH

Soil samples were taken at the end of each experiment and dried at 40 ± 4°C. A solution of 0.01 M calcium chloride ($CaCl_2 \cdot 2H_2O$ analytical grade) was dissolved in de-ionised water and added to a soil sample to give a final solid to solution ratio of 1:2.5. The mixture was placed on a magnetic stirrer and stirred for at least 5 min. The suspension was allowed to settle for 15 min and measured with a pH electrode (Hanna combination electrode and Jenway PHM6 meter, Fisher Scientific, UK) until readings were stable. 4 replicate samples were taken from pre-gassed soil (before potting and planting) with 4 replicate samples from non-gassed and 6 replicate samples from CO_2-gassed individual soil chambers.

Results and discussion

CO_2 concentrations in the soil environment were between 56% and 32.2% for CO_2-gassed soil chambers and 0.09% and 2.5% for non-gassed control chambers, which is consistent with natural temperate soil levels of CO_2 (Stolwijk and Thimann 1957, Borsato et al. 2015). Mean CO_2 and O_2 concentrations for all experiments are given in Table 1. It is recognised that pure CO_2 gas, as supplied, may also contain impurities such as ethylene at physiologically relevant concentrations, however this was not measured. Ethylene responses induced solely in the root zone which impact on water relations have been reported in the literature as a consequence of flooding and mechanical impedance; no reports of this hormone in response to extreme CO_2 in the root zone were found. We recognise therefore that this may have had some additional effect, which requires further investigation. Fig. 2A shows g_s of an individual representative leaf over the time course of a 10 hr (600+ mins) experiment with mean g_s of control plants remaining at 0.131 (± 0.0178) mol m^{-2} s^{-1} throughout (n = 6). Table 2 shows the mean g_s of CO_2-gassed plants over the time frame of experiments (n = 6). Graphic images record the time course of the response as follows: t_0 is the start of CO_2 gassing in the isolated root environment; t_1 the onset of a wilting response to CO_2 gassing; t_2 shows the CO_2 gassed plant after 180 mins with total loss of turgor; t_3 shows the plant during recovery of turgor and by t_4 the plant appears to regain turgor. Fig. 2B shows the first 180 mins (t_0–t_2) and reports g_s from Fig 2A, the simultaneous time course measurements of changes in leaf temperature from ambient (21°C) using the infra-red reader which coincide with time point samples of leaf ABA from CO_2 gassed and non-gassed control plants.

Highly significant negative correlations measured from t_0 to t_2 between T_{leaf} and g_s (R^2 = 0.74, Pearson's correlation co-efficient and significance test p = 0.0013; GraphPad Prism 6 Analysis) and between leaf ABA and g_s (R^2 = 0.72, Pearson's correlation co-efficient and significance test p = 0.0019) and a positive correlation between T_{leaf} and ABA (R^2 = 0.57, Pearson's correlation co-efficient and

significance test $p = 0.012$) suggest close interaction of all three variables during this initial period of gas delivery (Fig. 3A, B and C respectively). The theoretical relationship between T_{leaf} and both g_s and transpiration rate (Jones 1999) has been previously demonstrated experimentally (Lake and Woodward 2008, Forrai et al. 2012, Janker et al. 2013, Prytz et al. 2003); indeed, T_{leaf} is used to compute total conductance to water vapour in IRGA (infra-red gas analyser) systems (Licor Inc. USA) and is now in widespread use in infra-red thermographic screening equipment for water-related plant traits (Kim et al. 2014).

Closer examination of the integrated order of events show an initial rapid increase in T_{leaf} within 10 mins of root contact with soil CO_2, reaching a maximum of 27.1°C from initial measurement of 23.3°C. This provides evidence of a lack of evaporative cooling at the leaf surface despite g_s being at its highest in gassed plants. Control plants show a steady state g_s and a small and steady rise in T_{leaf} over the same period (from 22.5 to 24.1°C). The rapid rise in T_{leaf} represents an immediate and whole plant perception of an environmental change in the root environment which is initially independent of leaf g_s. This must be in response to a long distance root-to-shoot signal as the shoot (i.e. the leaves) is not subject to any experimental perturbation. Therefore the signal must be solely derived from the CO_2/root interaction. Stomatal closure responds to increases in leaf ABA and is at its lowest when leaf ABA peaks after 30 mins (t_1). T_{leaf} at this time is reduced, which suggests evaporative cooling is now occurring and water is being lost at the leaf surface, despite a reduction is g_s. There is therefore a disparity between an assumed lack of evaporative cooling (high T_{leaf}) and a higher g_s, as T_{leaf} of gassed plants remain higher than control plants throughout (Fig 2B).

These responses are counter-intuitive and suggest that there is a decoupling of evaporative water loss and g_s. However, extremely similar changes to g_s and transpiration have previously been reported. Various abiotic environmental stimuli administered to distant leaves from measured leaves, most notably heat-treatment (burning) of leaves (Grams et al. 2009, Hláváčková et al. 2006, Kaiser and Grams 2006, Herde et al. 1998) and mechanical wounding (Rhodes et al. 1999, Fromm et al. 2013), but also chilling (Fromm et al. 2013), drought (Fromm and Fei 1998) and re-irrigation following drought (Grams et al. 2007), recently reviewed by Choi et al. (2016). All implicate a rapid long-distance electrical systemic signal. Electrical signalling is known to consist of two types; action potential (AP) and variation potentials (VPs). APs are self-propagating, whilst VPs are known to interact with environmental stress and both have been measured to propagate through plant tissues at speeds of up to 105.5 m s^{-1} (Choi et al. 2016). This cited as a plausible explanation of very rapid initiation of systemic responses following perception of environmental perturbation (Hedrich et al. 2016). Furthermore, electrical signals have been demonstrated to impact on primary physiology: photosynthetic ΦPSI and ΦPSII show immediate changes after localised burning-induced VP (Lautner et al. 2005, Sukhov et al. 2015). The full mode of action of electrical

signalling is still unknown. An interpretation of individual responses seen here are discussed and then integrated to allow a mechanistic basis for results to be proposed.

Leaf temperature (T_{leaf})

A study on the thermal resistance of leaves subjected to VP induction following burning of a distant leaf showed that non-burnt leaf temperature of experimental plants are consistently higher than comparable control plants, when external temperatures are kept constant (Sukhov et al. 2015). This suggests that T_{leaf} is a potential physiological perception of stress in response to VPs. In the same study, Sukhov et al. (2015) report depression of both g_s and (*E*) over timescales consistent with the present study. The authors conclude that a drop in *E* following activation by VP is the likely cause of T_{leaf} increases, however, here T_{leaf} is the first response and largely independent of *E* (Fig 4A). Furthermore, IRGA measurements of T_{leaf}, whilst not showing the initial increase in as in infra-red measurements because T_{leaf} is held by the block temperature of the instrument above ambient, does show that high T_{leaf} is sustained for more than 250 mins (Fig 4A). We speculate that T_{leaf} is the initial perception of a 'stress', in this case high CO_2 contact with roots, and is likely to involve VPs given the similarity of g_s and *E* responses with those of Sukhov et al. (2015).

Leaf ABA

Time point measurements of leaf ABA show an initial increase from the onset of gassing to 30 min. Co-ordinated ABA responses with electrical signalling have been reported, again in response to localised burning. Herde et al. (1998) measuring membrane potentials in veins of tomato plants found specific potentials to be affected by the presence or absence of ABA production using ABA-deficient mutants. Hláváčková et al. (2006) directly measured ABA in the fifth leaf of tobacco after burning the tip of the 6th leaf at time points 0, 8, 15, 30 and 60 mins. Together with a rapid induction of electrical signals and a decrease in g_s and *E*, leaf ABA levels rose sharply within the first 15 mins, thereafter reducing to below pre-burning levels after 60 mins. The authors point out that time spot measurements may well miss some information for time points not sampled and suggest that ABA as well as electrical signals may have elicited the stomatal responses reported. It has been shown that radio-labelled ABA translocation can occur throughout the plant shoot when fed to an excised petiole within 5 mins (Malone et al. 1994). The present study has a similar issue with time point measurements, however, both this and the study by Hláváčková et al. (2006) suggest that ABA is controlling stomatal closure up to the 30-minute time point. Interestingly, this study also reported no change in intracellular CO_2 (C_i), commensurate with the present study (data not shown).

Whole plant loss of turgor

Continued loss of water at the leaf surface between t_1 and t_2 causes a complete loss of whole plant turgor which previous studies (referenced above) over similar timescales do not report. The collapse of turgor pressure suggests massive disruption to the hydraulic system and that the roots are unable to access water to counter catastrophic water loss at the leaf surface; i.e. disruption to normal water gating mechanisms has occurred. It has been demonstrated that root-derived ABA plays a role in regulation of aquaporins (water channels in the root at the soil interface) by reversing water channel gating (from closed to open) and increasing plasma membrane permeability (Jiang et al. 2004, Stillwell and Hester 1984) under water deficit conditions (Wan et al. 2004). Furthermore, this mode of action is coupled to a reduction in pH (Gerbeau et al. 2002). It may be speculated that under conditions of high CO_2-gassing weak carbonic acid is formed when CO_2 and water interact lowering pH at the root surface, therefore this ABA response should occur. In the present study this does not occur and suggests a specific and distinctly different response to that of water deficit. Measurements of soil pH after an experimental run are unaffected by the treatment (Table S3) and indeed, plants slightly increase soil pH in both CO_2-gasssed and non-gassed control treatments when compared to soil before plants are introduced to the medium, but this is not statistically significant.

CO_2-specific response

Chang and Loomis (1945) found that high soil CO_2 levels around the roots of wheat, maize and rice in hydroponic solution severely inhibited both water and nutrient uptake, but that a decreased pH solution (equivalent of the CO_2 gassed solution) had little effect, suggesting a specific response to CO_2 gas. They speculated that CO_2 has the ability to alter membrane permeability. This speculation was realised when disruption of cell membrane integrity to CO_2 was elegantly demonstrated by Glinka and Rienhold (1962) who also showed a distinct CO_2 effect compared to both a low pH and a lack of O_2. These authors subsequently showed that CO_2 caused a reduction in turgor pressure of excised hypocotyls of *Helianthus annuus* (Reinhold and Glinka 1966) as seen here in the whole intact plant. To our knowledge these mechanisms remain unknown.

Stomatal response

Between 30 and 60 mins leaf ABA levels drop and control over stomatal conductance is lost; this is evidenced by continued water loss until lack of turgor is complete (180 mins, t_2). This unexpected response may be explained by stomatal 'failure' to close in response to a loss of turgor. This has been reported as a normal hydraulic stomatal response when 'water deficit in angiosperm leaves causes subsidiary cells to lose turgor before the guard cells, and this collapse causes stomata to open "hydro-passively" rather than closing during rapid dehydration' (Brodribb and McAdam 2011). Recent studies on

leaf responses to water deficit elicited by leaf excision (to simulate xylem cavitation) and/or increased vapour pressure deficit (VPD) have reported transient 'wrong-way' stomatal responses to leaf water potential whereby g_s increased in response to decreased RH (Buckley et al. 2011) over time scales and magnitudes similar to the present study. More rapid responses (~2.5 mins) have also been observed in *Helianthus annuus* (Hanson et al. 2013). It may be expected that changes induced by directly manipulating the leaf will produce faster responses than those directed by root responses, however Hanson et al. (2013) invoke a combination of hydraulic disruption (cavitation) and other signal(s) to produce the 'wrong way' stomatal response they observed indicating a response over multiple timescales maybe possible. An even more rapid hydropassive stomatal opening was found in *Mimosa pudica* within 60 seconds of stimulation of a nearby pinna from heat-induced electrical VPs and was interpreted as a sudden loss of epidermal turgor (Kaiser and Grams 2006). The latter study reported g_s to fully recover within twenty minutes in direct contrast to the present study.

Despite an increase in leaf ABA and a corresponding decrease in g_s from 60 to 120 mins suggesting that g_s is still capable of responding to leaf ABA, after 180 mins measured stomatal conductance decreases (Fig. 2A). We suggest that following catastrophic turgor loss and failure of water channels in the roots to open, the leaves have no more water to lose. IRGA measurements record this as extremely low g_s (no evapotranspiration) however, stomata may still be hydropassively open i.e. non-functional.

Turgor recovery

During the period 180 to 630 mins (Fig. 2a, t_2 to t_4), the plant begins to regain turgor following collapse which we interpret as redistribution of remaining plant water because g_s remains close to zero thereafter. Recovery of g_s to pre-experimental levels was observed following leaf excision in *H. annuus* in the study by Hanson et al. (2013) which the authors also explain as water redistribution within the leaf as there is no access to additional water. However, for the remainder of the experiment (6 to 7 days) leaves remained flaccid and full turgor recovery to the pre-gassed state did not occur. Further evidence for a loss of water is shown by a reduce leaf water content and lower carbon isotope discrimination ($\Delta^{13}C$) i.e. a more intrinsic water use efficient plant (Fig 3 a and b). Our conclusion here is that roots remain unable to access sufficient water. Eventually (over several more days) the plant dies under continuous gassing. This finding replicates field experiments (Lake et al. 2013, Smith et al. 2016) which record extremely localised (radius 0.25 m) plant death within a week of elevated soil CO_2, presumably due dehydration and/or nutrient starvation (Sevanto et al. 2014). Table 3 shows fresh weight and dry weight biomass and root to shoot ratio at the end of a 7-day experiment providing evidence for this hypothesis as a dramatic reduction in root biomass, with little effect on shoot biomass. The loss of roots at this stage, however, cannot explain the loss of turgor during the initial phase of CO_2 gassing.

Integrating responses in a mechanistic framework

Our identified root response is initiating subsequent events, and stomatal control by ABA is decoupled during rapid turgor loss, however, the plant appears to utilise hydraulic processes to redistribute water and regain some control of turgor pressure. As such, hydraulic processes are partly responsible for the plant phenotype over the longer term. In Fig. 5 we speculate on a mechanistic framework which integrates electrical, hydraulic and biochemical components to elucidate a whole plant response to root induced CO_2 stress which is distinctly different from drought stress. Clearly, there are still unknown and complex aspects of how hydraulic/mechanical processes interact with biochemical mechanisms in vascular plants, as well as incorporation of electrical signals which provide rapidity of responses but a whole plant approach integrating root and shoot responses is the only way to elucidate these fully.

CO_2 sensing

The results presented here also raise the intriguing question of whether a specific CO_2 sensor in the roots leads to this response. It has long been a goal of plant physiologists to discover a sensing mechanism for CO_2 in leaves, but with limited success. As far back as 1990, Mott identified the need for a CO_2 sensing mechanism to explain overall physiological responses to elevated atmospheric CO_2. 35 years on and despite concerted efforts to locate such a sensor in stomatal guard cells (Vavasseur and Raghavendra 2005) a specific CO_2 sensor in either the leaf or guard cell has remained elusive (Negi et al. 2014). The CO_2 specific nature of the response reported here offers the possibility of elucidating CO_2 sensing, not using the leaves of plants, but the roots. Such a sensing mechanism may be conserved and therefore similar in structure to any found in aerial parts. Furthermore, the interaction between CO_2, ABA, pH status and aquaporin regulation of water uptake requires further investigation and offers a tantalising opportunity to investigate whole plant mechanisms of CO_2 sensing and water regulation. Our results show that plant control over water status possibly holds more elements than water deficit responses alone, indeed mechanisms of a plant's ability to distinguish between different responses are not well known. Revisiting this intriguing topic more than a century after it was first seen exposes the gaps in our knowledge; further research into the physiological dynamics of whole plant responses which have elements in common will elucidate and ultimately allow for a more targeted approach to manipulated water use efficiency.

Author contributions

JAL carried out the work analyses and wrote the manuscript. HW developed the ABA methodology. JAL, DDC and BHL contributed to analyses and the manuscript. We declare we have no conflict of interests.

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Figure legends

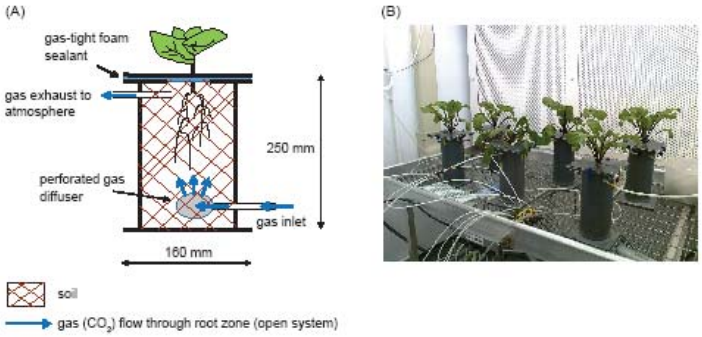

Fig. 1. (A): schematic of soil chamber design, (B): beetroot plants *in situ*.

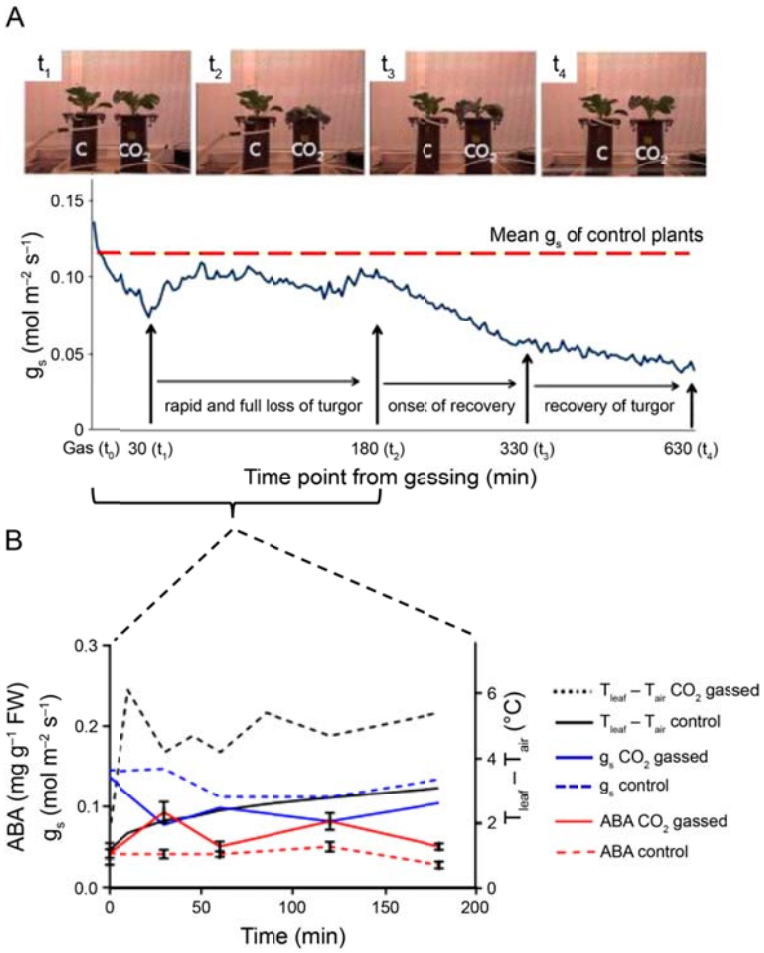

Fig. 2. (A): Stomatal conductance (g_s) of CO_2 gassed plants over at time points of a 10 hr experiment, together with graphic images of the whole plant response (see text for time point definitions; C = non-gassed control plant, CO_2 = CO_2 gassed plant). Mean g_s of control plants is shown as red dashed line (B): Expanded section of (A) showing the change in leaf temperature from ambient temperature ($T_{leaf} - T_{air}$), g_s and leaf ABA levels of CO_2 gassed and non-gassed control plants over 180 mins. [error bars = SE mean for ABA samples, n = 3; no error bars on g_s and T_{leaf}, as these are from one representative leaf]

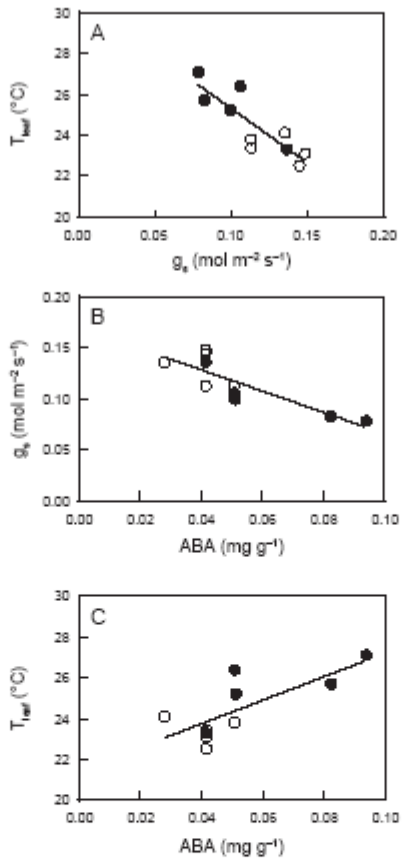

Fig. 3. Correlations between g_s and T_{leaf} (A), leaf ABA (mg g^{-1} fresh weight) and g_s (B) and leaf ABA and

T_{leaf} (C). Measurements taken at 180 mins from onset of gassing. Solid circles are CO_2 gassed; open circles are non-gassed control plants.

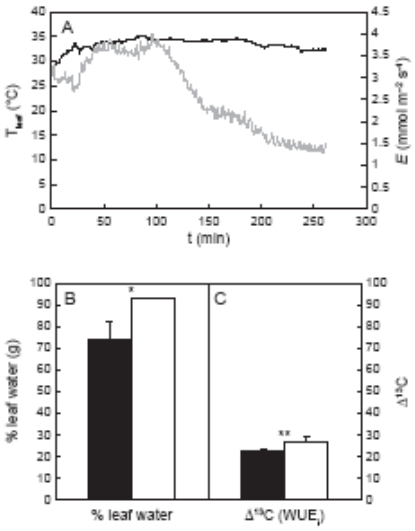

Fig. 4. (A) T_{leaf} (black) and transpiration rate (E) of a representative CO_2 gassed plant measured continuously by IRGA. (B) % water lost from leaves (fresh weight – dry weight) and (C) $\Delta^{13}C$ carbon isotope discrimination (WUE_i – intrinsic water use efficiency). [Black bars= CO_2-gassed plants, white bars = non-gassed control plants. * = p = <0.05, ** = p = <0.005 Student's t-test].

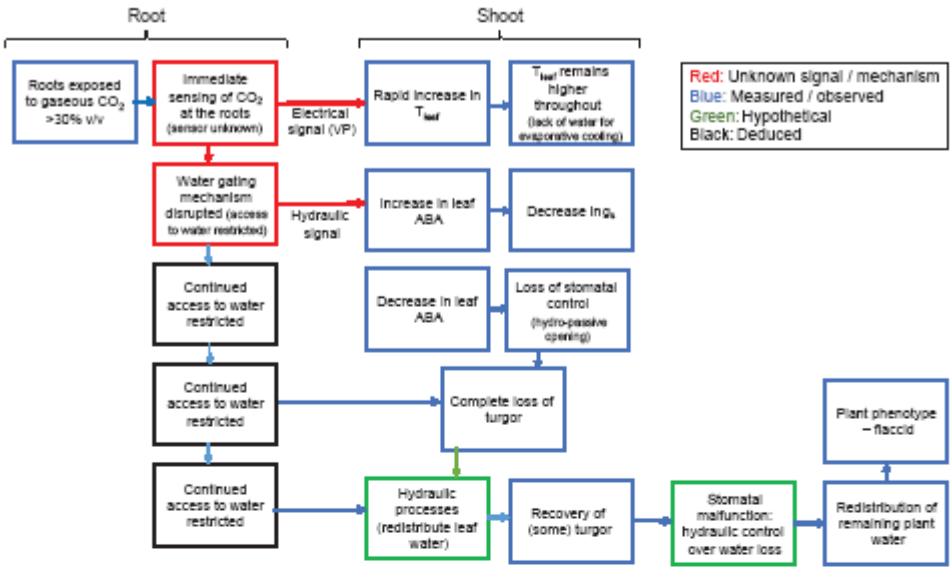

Fig. 5. Putative mechanistic model of the response to high CO_2 gassing in the root zone: involvement of

hydraulic and biochemical signalling. Red highlights represent unknown signals/mechanisms; blue highlights represent measured or observed effects; green highlights represent hypothetical mechanisms; black highlights represent deduced effects from measured/observed effects.

Table 1. Mean CO_2 and O_2 concentrations measured inside soil chambers during laboratory experiments.

Mean gas concentration (%)	**CO_2-gassed**	**Non-gassed control**
CO_2	42.3	1.2
O_2	11.1	19.8

Table 2. Stomatal conductance (g_s) of CO_2-gassed plants over the experimental time frame of 6 experiments. [Mean values (± range of values), n = 6].

Time point	Pre-gassing (t_0)	30 mins (t_1)	180 mins (t_2)	630 mins (t_4)
g_s (mol m^{-2} s^{-1})	0.24 (± 0.07)	0.031 (± 0.02)	0.044 (± 0.04)	0.028 (± 0.05)

Table 3. Mean biomass, root to shoot ratio and % loss of water on drying of beetroot grown with or without CO_2 gassing of the root zone (n = 6).

Experimental treatment	**Non-gassed control**	**CO_2-gassed**	**Non-gassed control**	**CO_2-gassed**	**Non-gassed control**	**CO_2-gassed**
	Fresh weight (g)		**Dry weight (g)**		**% water loss on drying**	
Shoot biomass	41.52	36.78	3.34	4.47	92	88
Lateral root biomass	20.21	2.03	2.57	0.67	87	67
Beet biomass	9.0	1.98	0.65	0.21	93	89
Root biomass (total)	29.21	4.01	3.22	0.88	89	78
Root to shoot ratio	0.70	0.11	0.96	0.19	n/a	n/a