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Phytohormone Profiles of Lettuce and Pepper Grown Aeroponically with Elevated Root-Zone Carbon Dioxide Concentrations

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Abstract: Enhancing root-zone (RZ) dissolved inorganic carbon (DIC) levels of plants grown aeroponically can increase biomass accumulation but may also alter phytohormone profiles *in planta*. These experiments investigated how CO_2 gas (1500 ppm) added to an aeroponic system affected phytohormone concentrations of lettuce (*Lactuca sativa*) and sweet pepper (*Capsicum annuum*) plants. Phytohormonal profiling of root and leaf tissues revealed a solitary treatment difference in lettuce plants, an increased shoot jasmonic acid (JA) concentration under elevated RZ CO_2. Since JA is considered a growth inhibitor, growth promotion of lettuce under elevated RZ CO_2 does not seem related to its phytohormone profile. On the other hand, pepper plants showed changes in foliar phytohormone (aminocyclopropane-1-carboxylic acid, ACC, *trans*-zeatin, tZ and salicylic acid, SA) concentrations, which were correlated with decreased leaf growth in some experiments. Foliar accumulation of ACC alongside decreased leaf tZ concentrations may mask a positive effect of elevated RZ CO_2 on pepper growth. Diverse phytohormone responses to elevated RZ CO_2 between different species may be involved in their different growth responses.

Keywords: root-zone CO_2; hydroponics; aeroponics; phytohormones; lettuce; pepper

1. Introduction

The effects of elevated root-zone CO_2 on plant growth depend on plant species, substrate pH, air temperature, irradiance, mineral nutrition, abiotic stresses such as high irradiance or salinity, the duration of root-zone CO_2 enrichment and the CO_2 concentration applied. In reviewing 358 experiments, Enoch and Olesen (1993) [1] reported that elevated root-zone (RZ) CO_2 significantly increased mean biomass by 2.9%. Furthermore, aerating a hydroponic solution with 5000 ppm CO_2 under high irradiance (1500 μmol m^{-2} s^{-1}) and high air temperatures (37/19 °C (day/night)) at pH 5.8 approximately doubled the biomass of both control and salinized (100 mM NaCl) tomato (*Solanum lycopersicum*) plants, compared to those aerated with 0 ppm RZ CO_2, with a 40% greater effect in control plants [2]. When plants were grown at irradiances less than 1000 μmol m^{-2} s^{-1}, elevated rhizosphere DIC increased growth rates only of control plants grown at high temperatures (35 °C) or salinized plants at more moderate temperature (28 °C) [3]. Two weeks of treatment of elevated RZ CO_2 (50,000 ppm) in aeroponically grown crisphead lettuce increased growth (~1.6 fold) at 36/30 °C, an irradiance of 650 μmol m^{-2} s^{-1} and pH 6.5 compared to plants aerated with ambient (360 ppm) CO_2. Moreover, increasing RZ CO_2 in aeroponically grown lettuce alleviated midday depression of photosynthesis and therefore increased leaf area, shoot and root production [4]. These more recent studies suggest that root-zone CO_2 enrichment might be more effective in promoting growth under stressful conditions, perhaps by improving leaf water and/or nutrient status. However, to our knowledge, root-zone CO_2

enrichment is not applied commercially, perhaps because there is little consensus on the mechanisms by which it affects growth.

Stimulated by the global importance of a continuous increase in atmospheric CO_2 levels, many have studied the effects of high atmospheric CO_2 on plant growth and performance. Although elevated CO_2 (eCO_2) increased total leaf area by promoting leaf expansion [5,6], plant growth is not always enhanced [7,8] and results can be variable even within the same species. Some of these studies have determined phytohormone concentrations *in planta*, as they are important in regulating plant growth and development. In general, elevated atmospheric CO_2 increased leaf indole-3-acetic acid (IAA), gibberellic acid (GA_3), zeatin riboside (ZR), dihydrozeatin riboside (DHZR) and isopentenyl adenosine (iP) concentrations of *Arabidopsis thaliana* [9], increased IAA and ethylene (ETH) of tomato [10] and ETH of rice (*Oryza sativa*) plants [11], while abscisic acid (ABA) and jasmonic acid (JA) concentrations decreased [12,13]. However, applying RZ CO_2 (1500 ppm) to aeroponically grown muskmelon (*Cucumis melo*) had opposite effects, with xylem sap IAA, *trans*-zeatin (tZ) and GA_3 concentrations decreased and ABA increased compared to control plants, but tissue concentrations were not measured [14]. Since there is limited information on RZ CO_2 effects on phytohormone concentrations, information on foliar and root response to eCO_2 are briefly reviewed.

Auxin regulates root and shoot architecture, and tropic growth responses [15] and reactions to environmental changes [16]. Shoots and roots generally grow unequally under eCO_2, such that the shoot-to-root ratios usually increase [17,18]. Leaf size and anatomy changes, often increasing the total leaf area per plant, single leaf area and leaf thickness [19]. IAA regulates the initiation of leaf primordia [20], vascular differentiation [21] and leaf expansion during both the cell division [22] and cell enlargement leaf growth phases [23]. Foliar sugar accumulation under eCO_2 stimulates IAA biosynthesis and promotes its transport from shoot to root [24,25], where it stimulates lateral root growth and root hair development [26,27]. In contrast, with greater shoot and root growth, IAA decreased in roots of sweet pepper exposed to eCO_2 [28]. Although IAA has been proposed to act as a long-distance shoot-to-root signal under eCO_2, whether it acts as a root-to-shoot signal under elevated RZ CO_2 is unknown.

Cytokinins (CKs) promote cell division and regulate embryogenesis, vascular tissue development, root architecture, and light responses [29]. CK concentrations are highest in meristematic regions and continuously growing parts of roots, young leaves, developing fruits, and seeds [30,31]. Elevated CO_2 increased CK delivery from roots to shoots [32], especially at low (2 mM) nitrogen concentrations [33]. However, higher IAA levels under eCO_2 may inhibit CK biosynthesis and signalling [26,34]. While IAA acts as a positive regulator of organ initiation, CKs have complex effects which seem to depend on species, tissue and developmental context [35]. In isolated pumpkin (*Cucurbita pepo*) cotyledons, N6-benzyladenine (BA) activated cell division in palisade mesophyll and upper epidermis without affecting their growth, while stimulating the growth of spongy mesophyll and lower epidermal cells without inducing cell division [36,37]. In addition, adding 5 µM BA to excised leaf discs of pepper plants promoted area expansion after 24 h, but this was not mediated through changes in net uptake of CKs or utilization of carbohydrates [38]. Thus, changes in root CK synthesis under elevated RZ CO_2 may affect leaf growth, but this has not been established experimentally.

Gibberellins (GAs) regulate the elongation of stems, leaves, and reproductive organs [39] and can stimulate leaf expansion by increasing cell length and cell number [40], with enhanced wall extensibility largely promoting cell expansion [41]. Elevated CO_2 increased gibberellin concentrations in several species such as orchids [42], *A. thaliana* [9] and *Populus tomentosa x P.bolleana* [43] and may stimulate growth. Following exogenous GA_3 application, a proteome analysis indicated that rice leaves sensed GA_3 and transmitted a signal to activate cell growth and division [44]. Whether eCO_2 or RZ CO_2 can activate this signal is still unknown.

Jasmonic acid (JA) and salicylic acid (SA) play key roles in regulating plant defence responses to abiotic and biotic stresses [45,46]. Elevated CO_2 enhanced SA-dependent defence and decreased JA-dependent defence [47,48]. On the contrary, eCO_2 increased JA biosynthesis pathway metabolites

in guard cells of *Brassica napus* and Arabidopsis, with jasmonoyl-isoleucine (JA-Ile) and JA signalling believed to play an essential role in the stomatal closure induced by eCO_2 [49]. Abscisic acid (ABA) has also been implicated in stomatal responses to elevated CO_2. Although some ABA-deficient or ABA-insensitive mutants are compromised in CO_2-induced stomatal closure [50], stomatal closure seemed ABA-independent in other cases [49,51,52]. Whether these "stress hormones" are involved in physiological responses to RZ CO_2 has not been established.

Ethylene (ETH) and CO_2 interactions have long been studied, mainly because high CO_2 concentrations can antagonize ETH-induced fruit ripening. ETH is a gaseous hormone that is synthesized in almost all plant tissues in the presence of oxygen [53]. Key enzymes in ETH biosynthesis are 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, which catalyse the reactions from S-adenosylmethionine to ACC, and from ACC to ETH, respectively. CO_2 is an essential cofactor for ACC oxidase [54]. Using ETH inhibitors, or ETH-insensitive mutants lacking the key components of ETH signal transduction [55,56] has established that ETH inhibits leaf growth and development, although effects are dose dependent. Increased ETH production might promote growth when leaf glucose concentrations are high, such as rice growing under eCO_2 [11], but endogenous ETH production decreased leaf expansion of lettuce [57].

While eCO_2 affects the concentrations of hormones (including CKs, IAA and GA_3) that are involved in regulating cell division and cell elongation, the effects of RZ CO_2 enrichment on phytohormone profiles are virtually unknown. Determining whether RZ CO_2 affects phytohormone concentrations *in planta*, and their putative physiological importance in regulating leaf expansion, will further our understanding of the mechanisms by which RZ CO_2 affects growth. Although RZ CO_2 enrichment is not applied commercially to our knowledge, here it was applied to lettuce and pepper plants grown aeroponically under artificially lit, controlled environment conditions (lettuce) or semi-controlled greenhouse conditions (pepper) typical of commercial production.

2. Materials and Methods

To determine the effect of CO_2 enrichment of the rhizosphere, an aeroponic system was built. Crisphead lettuce (*Lactuca sativa* cv. Consul) and pepper (*Capsicum annuum* (L.) cv. Bellboy F1) seeds were purchased from Moles Seeds (Essex, UK). One experiment with lettuce (Experiment 1) and two with pepper (Experiments 2 and 3) were performed. Lettuce were grown in a controlled environment room (CE room), where air temperature ranged between 16 and 22 °C and relative humidity ranged between 60% and 85%. Lights were 102 W LED light strips (B100 series, Valoya Ltd., Helsinki, Finland), providing an average PPFD across the growing area of 189 µmol m^{-2} s^{-1}, with a 16 h photoperiod. Pepper were grown in a naturally lit glasshouse. In Experiment 2, air temperature ranged between 19 and 25 °C and relative humidity between 25% and 65%, while air temperature ranged between 19 and 33 °C and relative humidity between 20% and 60% in Experiment 3. Supplementary Photosynthetic Photon Flux Density (PPFD) of ~500 µmol m^{-2} s^{-1} (at bench height) was supplied with high-pressure sodium lamps (600 W Greenpower, Osram Ltd., St Helens, UK) when PPFD decreased below 400 µmol m^{-2} s^{-1} for a 12 h photoperiod (08.00 hrs to 20.00 hrs). Seeds were individually sown in 150 cell plug trays in 2 cm × 2 cm × 4 cm rockwool cubes (Growell Hydroponics, Ltd., London, UK) and plants were transferred to the aeroponic system at the 4 leaf stage in lettuce and at 2–4 leaf stage in pepper. After transplanting, two different [CO_2], 400 and 1500 ppm, were applied into each bin. The system consisted of an enriched channel supplemented with CO_2 and a non-enriched channel supplied only with compressed air. The air from the enriched channel was completely mixed in a mixing box before entering the aeroponic system. The [CO_2] in the mixing box was monitored continuously using a CO_2 gas analyser (PP Systems, WMA-4). To prevent leakages, the lid was sealed with self-adhesive rubber foam around the rim. The air above the lid and at the shoot base was routinely sampled with a LI-COR 6400, with no significant difference compared to the ambient air.

The reservoir was completely opaque and contained 60 L of half-strength Hoagland solution [58]. Nutrient solution composition was 0.5 mM NH_4NO_3, 1.75 mM Ca $(NO_3)^2{\cdot}4H_2O$, 2.01 mM KNO_3,

1.01 mM KH_2PO_4, 0.5 mM $MgSO_4{\cdot}7H_2O$, 1.57 µM $MnSO_4{\cdot}5H_2O$, 11.3 µM H_3BO_3, 0.3 µM $CuSO_4{\cdot}5H_2O$, 0.032 µM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1.04 µM $ZnSO_4{\cdot}7H_2O$ and 0.25 mM NaFe EDTA. The pH was maintained at between 5.8 and 6.3, with the necessary dropwise application of HCl or NaOH.

2.1. Plant Measurements

Whole shoot (stems, leaves and petioles) fresh weight was determined after 10 days of treatment, along with leaf area using a leaf area meter (LI-COR Model 3100 Area Meter, Cambridge, UK). Roots were collected, rinsed with dH_20 and dried with absorbent paper. Both shoot and root material were then dried at 70 °C for 4 days to record dry weight and stored in airtight containers to provide samples for nutrient analysis.

To determine the leaf expansion rates in aeroponically grown pepper, the length (L) and width (W) of one leaf per plant was measured with a ruler, every day for 12 days. Leaf area was estimated by multiplying W × L. The leaf expansion rate (LER) was calculated as:

$$LER = (\ln LA2) - (\ln LA1)/t2 - t1$$

where LA1 and LA2 are the estimated leaf areas and t2-t1 is time (d) between two consecutive days [59] At harvest, actual leaf area of that leaf was measured with the leaf area meter, in addition to whole plant leaf area (Experiment 3).

2.2. Multi-Hormone Analysis

Aeroponically grown lettuce and pepper root and shoot (Leaf 6, numbering from the base of the plant) samples were taken (Experiments 1 and 2) between 09.00 hrs and 10.00 hrs, immediately frozen in liquid nitrogen and stored at −20 °C before being freeze-dried for 48 h. The samples were then ground and weighed (50 mg) and extracted with 0.5 mL extraction buffer (methanol:water 80:20 *v*/*v*) for 0.5 h at 4 °C. Solids were separated by centrifugation (20,000× *g*, 15 min) and re-extracted for 30 min at 4 °C in an additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C18 cartridge (Waters, Milford, MA, USA) to remove interfering lipids and part of the plant pigments and evaporated at 40 °C under vacuum either to near dryness or until organic solvent was removed. The residue was dissolved in a 1 mL methanol/water (20/80, *v*/*v*) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 µm pore size nylon membranes (Millipore, Bedford, MA, USA). A volume of 10 µL of filtered extract was injected in a U-HPLC-MS system comprising an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). To quantify plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards. Recovery percentages ranged between 92% and 95% [60]. Five out of the 11 hormones (ACC, tZ, ABA, JA and SA) were detected in both leaf and root tissue of lettuce and pepper plants, whereas ZR, iP, GA_3 and IAA were detected just in pepper shoots and roots.

2.3. Leaf Area Expansion in Response to ACC, GA_3 and BA Application

Leaf discs (8 mm diameter) of pepper were harvested using a cork borer from the base and tip of the leaves between 09.00 hrs and 10.00 hrs, and incubated for 1 h on a solution containing 10 mM Mes-KOH (pH 6.5), 10 mM KCl and 10 mM sucrose. Different hormone concentrations were applied based on previous work [38]. After 1 h incubation, the discs were transferred to a similar solution with the following concentrations of each hormone to perform dose–response curve responses:

(1) 0.1, 10 and 100 µM GA_3;
(2) 5, 50 and 500 µM BA;

(3) 0.1, 10 and 100 μM ACC.

Control treatments did not include GA_3, BA or ACC. Two additional experiments with selected concentrations evaluated whether the response was consistent between independent assays. Petri dishes were incubated in the glasshouse under ambient conditions. Two perpendicular diameters were measured on each disc using a ruler and projector with nine-fold magnification.

In another experiment, instead of collecting leaf discs, 100 μM of each hormone was sprayed for 10 days on soil-grown pepper leaves to better understand how these hormones affect leaf expansion in situ. At the end of the experiment, total leaf area was determined using a leaf area meter (LI-COR Model 3100 Area Meter, Cambridge, UK) and shoot fresh weight was recorded, before drying at 70 °C for at least 72 h and then re-weighing.

2.4. Statistics

To determine treatment differences on leaf and root phytohormone concentrations, an Independent Samples Student's *t*-test at the $p < 0.05$ level was performed. One-way analysis of variance (ANOVA) determined differences between hormone concentrations applied in each leaf disc assay followed by LSD post-hoc analysis.

3. Results

3.1. Root-Zone Carbon Dioxide Enrichment of Aeroponically Grown Lettuce and Pepper Plants

In the lettuce experiment, elevated RZ CO_2 (1500 ppm) significantly increased fresh and dry shoot biomass by ~22% compared to those grown with 400 ppm RZ CO_2, consistent with previous experiments [61]. On the other hand, elevated RZ CO_2 did not significantly alter root dry weight. In Experiment 2 with pepper, applying 1500 ppm CO_2 to the root zone did not significantly decrease shoot fresh weight and dry weight, total leaf area and root dry weight, compared to control plants grown at ~400 ppm RZ CO_2 (Table 1). In Experiment 3 with pepper, applying 1500 ppm CO_2 to the root zone tended to decrease (but not significantly) shoot dry weight and total leaf area by 20%. Thus, lettuce and pepper seemed to respond differently to elevated RZ CO_2.

Table 1. Shoot fresh weight, shoot dry weight, total leaf area and root dry weight mean values of lettuce and pepper plants grown aeroponically. Data are the mean ± SE of eight replicates. Asterisks indicate significant differences between treatments (* $p < 0.05$; ** $p < 0.001$). nd means not determined. RZ means root zone. Reproduced from [61].

	Treatment	Shoot Fresh Weight (g) ± SE	Shoot Dry Weight (g) ± SE	Total Leaf Area (cm^2) ± SE	Root Dry Weight (g) ± SE
Experiment 1 Lettuce	**Control**	71.30 ± 4.28	3.40 ± 0.21	nd	0.65 ± 0.17
	RZ CO_2	86.86 ± 2.56 **	4.17 ± 0.12 **	nd	0.56 ± 0.11
Experiment 2 Pepper	**Control**	9.52 ± 0.38	1.10 ± 0.05	215 ± 7	0.64 ± 0.17
	RZ CO_2	9.44 ± 0.39	1.10 ± 0.03	215 ± 9	0.54 ± 0.07
Experiment 3 Pepper	**Control**	10.50 ± 0.75	1.26 ± 0.10	276 ± 21	nd
	RZ CO_2	8.47 ± 0.76	1.01 ± 0.10	219 ± 16	nd

Compared to control lettuce plants grown aeroponically at ambient RZ CO_2, elevated RZ CO_2 had little effect on leaf phytohormone concentrations. ACC was 20% lower and SA 15% higher under RZ CO_2 although these changes were not statistically significant (Figure 1a,e). On the other hand, JA concentrations significantly increased by 30% (Figure 1d). In contrast, tZ and ABA did not show any differences between treatments (Figure 1b,c). Root phytohormone concentrations did not differ significantly between treatments, even though ABA was 30% lower under RZ CO_2 (Figure 1h). Thus, elevated RZ CO_2 had limited effects on phytohormone profiles of lettuce.

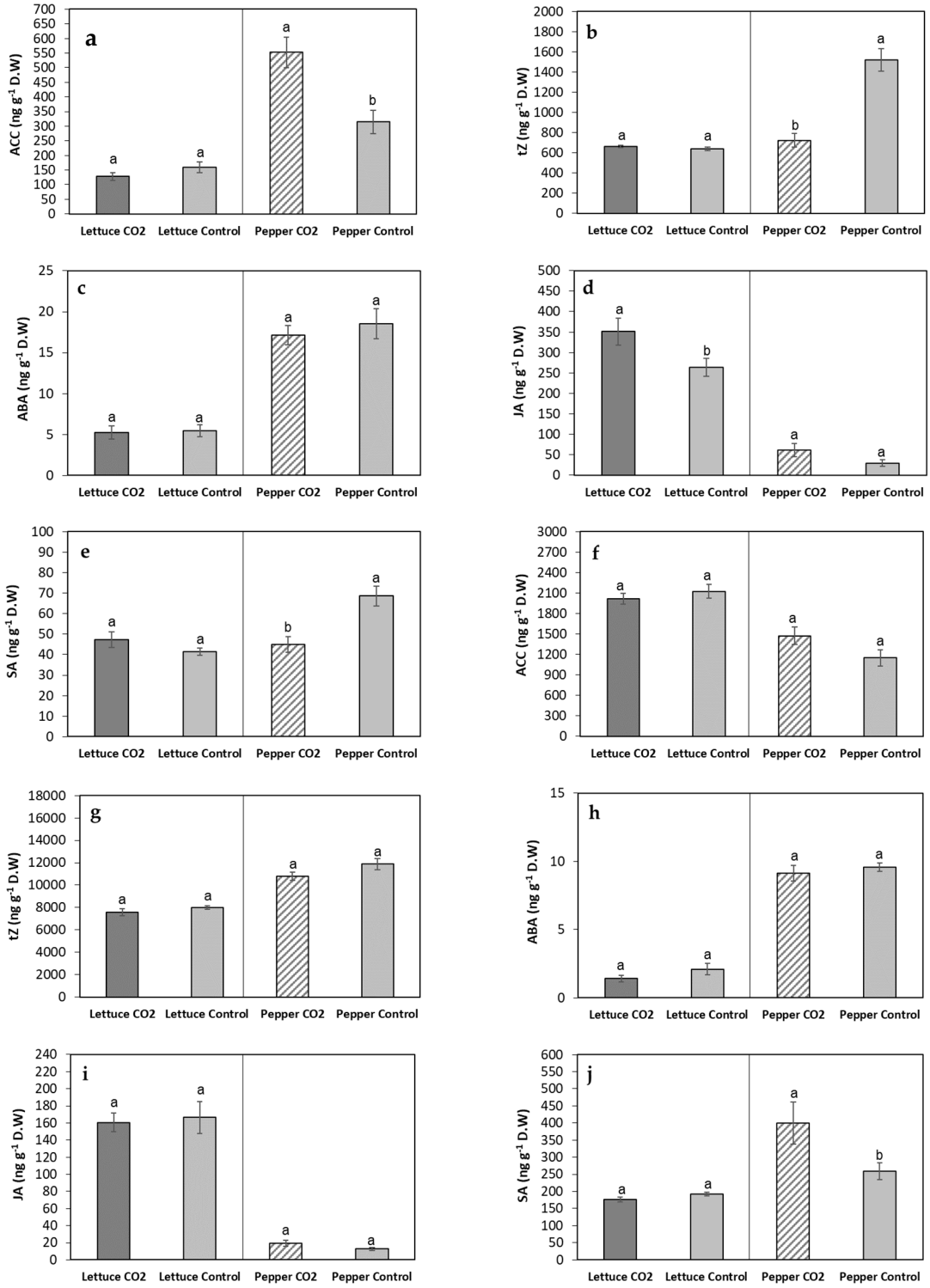

Figure 1. Lettuce and pepper leaf (**a**–**e**) and root (**f**–**j**) phytohormone (and precursor) concentrations under high RZ CO_2 and ambient CO_2. Bars are the mean ± SE of eight replicates, with different letters indicating significant ($p < 0.05$) differences within a species.

In pepper (Experiment 2), RZ CO_2 enrichment increased leaf ACC concentrations by ~60% and decreased leaf tZ concentrations by 50% (Figure 1a,b) Shoot and root SA concentrations showed opposing changes to RZ CO_2 enrichment, with a 35% decrease in leaf SA concentration but a 50% increase in root SA concentration (Figure 1e,j). Root iP and GA_3 concentrations also significantly

differed between treatments. While iP was 30% lower under high RZ CO_2, GA_3 was 10% higher compared to control plants (Figure 2c,d). Pepper was more responsive than lettuce to RZ CO_2 enrichment, with both shoot and root phytohormone profiles changing.

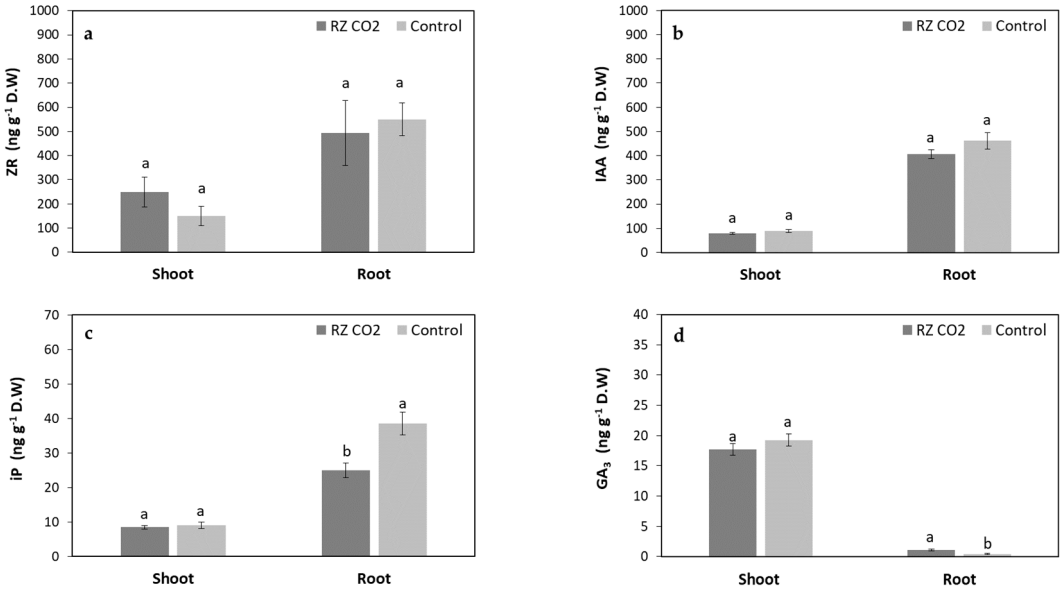

Figure 2. Pepper leaf and root zeatin riboside (ZR) (**a**), indole-3-acetic acid (IAA) (**b**), isopentenyl adenosine (iP) (**c**) and gibberellic acid (GA_3) (**d**) phytohormone (and precursor) concentrations under high RZ CO_2 and ambient CO_2. Bars are the mean ± SE of eight replicates, with different letters indicating significant ($p < 0.05$) differences between treatments.

3.2. Leaf Area Expansion in Pepper Plants Grown Aeroponically

Since there were more changes in phytohormone concentrations in response to RZ CO_2 in pepper than lettuce, leaf expansion was measured over 12 days (Experiment 3). The leaf expansion rate decreased from Days 1 to 7 in both treatments, which could not be attributed to environmental conditions, since temperature and relative humidity were relatively constant during the 12 days (Figure 3a). Nutrient depletion may have caused this decrease as growth ceased on Day 7, the day before the nutrient solution was replaced (Figure 3b). Over the entire experiment, high RZ CO_2 decreased area of the measured leaf by 20% compared to control plants (Figure 3c).

3.3. Leaf Area Expansion in Response to ACC, GA_3 and BA Application

To better understand the impact of different phytohormones on pepper leaf expansion, leaf disc assays were performed as previously described [38]. ACC and GA_3 were selected, because of their role in regulating cell division and elongation and because RZ CO_2 affected these phytohormones. BA was selected as a representative cytokinin to compare with previous experiments [38].

Initially a GA_3 dose–response curve was performed using discs from the tip and the base of the leaf to determine whether leaf disc expansion differed with leaf position. Apical leaf discs expanded little (12% area increase) in comparison with discs taken from the base (25% area increase), meaning that expansion occurs mainly in the basal part of the leaf. In apical discs, increasing GA_3 concentrations from 0.1 to 1 µM increased leaf expansion by 20% more than at 10 µM (Figure 4). In basal discs, GA_3 concentration had no significant effect on leaf expansion.

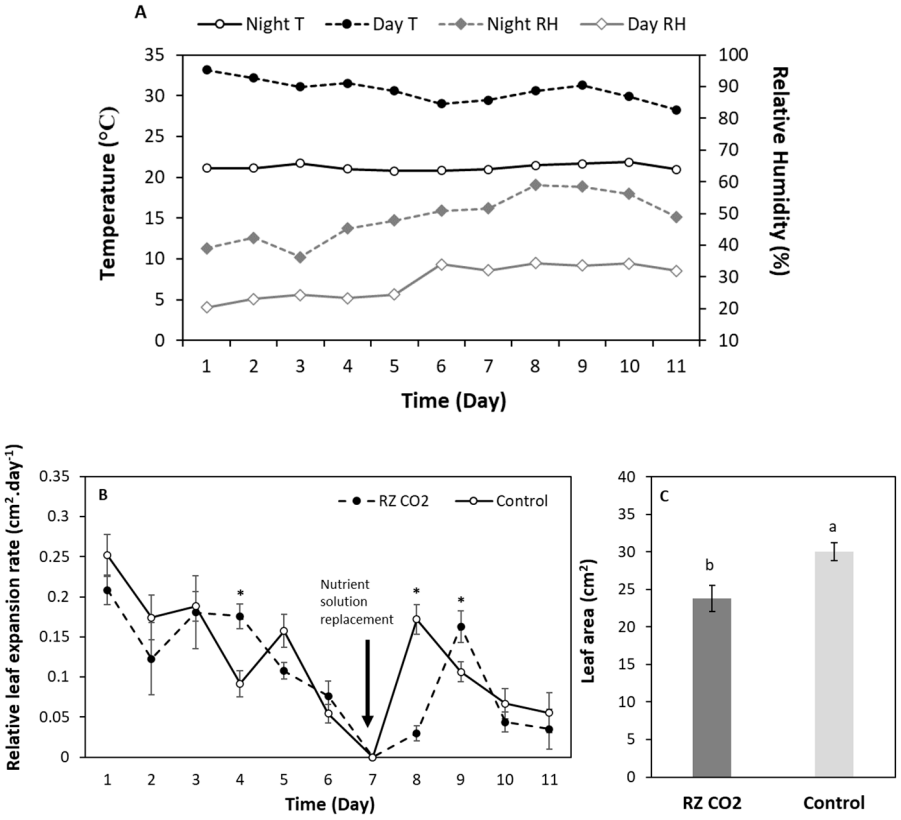

Figure 3. Night/day temperature (T) and relative humidity (RH) variation throughout the measurements (**A**). The daily pepper relative leaf expansion rate (**B**) and final leaf area (**C**) in plants exposed to high RZ CO_2 and ambient RZ CO_2 in plants exposed to high RZ CO_2 and ambient RZ CO_2. Bars are the mean ± SE of eight replicates. Asterisks and different letters indicate significant ($p < 0.05$) differences between treatments.

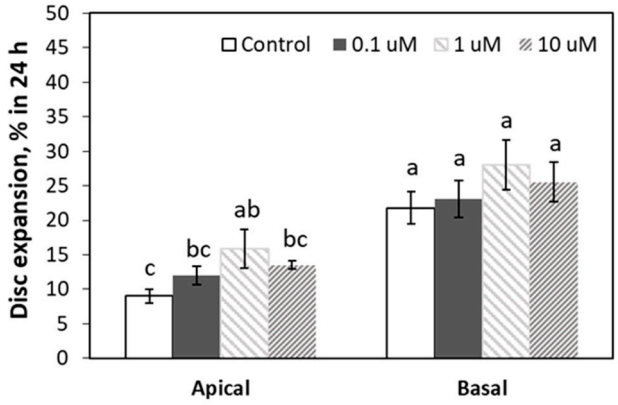

Figure 4. Area expansion of discs excised from the tip and base of pepper leaves after incubation for 24 h on solutions of 0.1, 1 and 10 GA_3. Bars are the mean ± SE of 10 replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

A second assay obtained GA_3, BA and ACC dose–response curves with discs taken from the base of the leaf, which grew more rapidly (50% area increase in control discs over 24 h). Higher GA_3 concentrations (than applied previously) increased leaf expansion by 16% at 100 µM GA_3, with no significant effect at lower concentrations. An intermediate cytokinin concentration (50 µM BA) increased leaf disc expansion by 25% compared to control leaf discs (Figure 5), whereas a higher

concentration (500 μM BA) had no significant effect. Leaf expansion was not affected across a broad range (0.1–100 μM) of ACC concentrations.

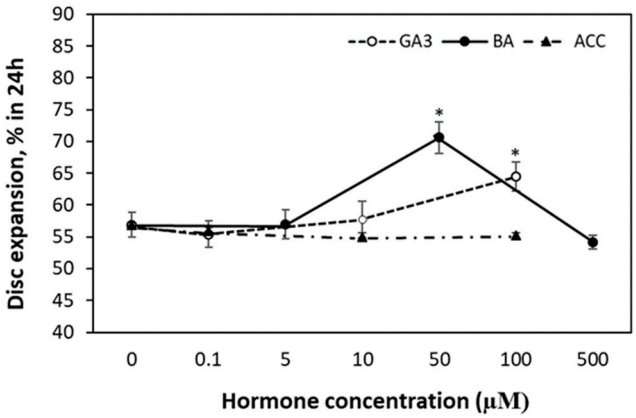

Figure 5. Area expansion of discs excised from the base of pepper leaves after incubation for 24 h on solutions of GA_3: 0.1, 10 and 100 μM; BA: 5, 50 and 500 μM or ACC: 0.1, 10 and 50 μM. Bars are the mean ± SE of 10 replicates. Asterisks indicate significant ($p < 0.05$) differences within hormone treatments.

A third assay checked the consistency of response to GA_3, BA and ACC. In this case, 100 μM GA_3 and 100 μM ACC did not affect leaf expansion, while again 50 μM BA increased leaf disc expansion significantly by 30% compared to control (Figure 6). Therefore, BA consistently stimulated expansion of pepper leaf discs, but not ACC and GA_3.

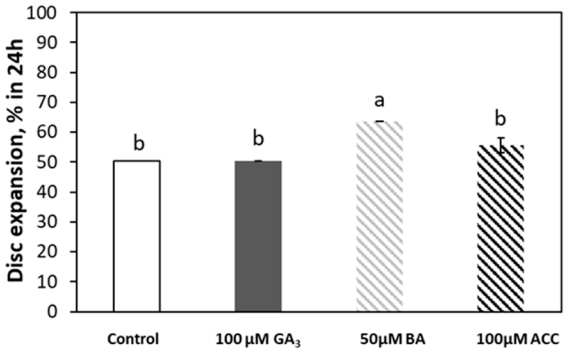

Figure 6. Area expansion of discs excised from the base of pepper leaves after incubation for 24 h on solutions of 100 μM GA_3, 50 μM BA and 100 μM ACC. Bars are the mean ± SE of 10 replicates. Different letters indicate significant ($p < 0.05$) differences between treatments.

To determine whether the effects of these hormones *in vivo* were similar to in excised leaf discs, the same hormones (ACC, BA and GA_3) were individually applied at a single concentration (100 μM). Foliar application of BA or GA_3 to soil-grown peppers had no significant impact on shoot fresh weight, shoot dry weight or total leaf area. ACC application decreased shoot dry weight by 10% and total leaf area by 26% (Table 2).

Table 2. Shoot fresh weight, shoot dry weight and total leaf area of pepper plants sprayed with 100 μM of ACC, GA_3 and BA. Values are the mean ± S.E. of four replicates, with different letters denoting significant differences between means (post-hoc LSD $p < 0.05$).

Treatment	Shoot Fresh Weight (g) ± SE	Shoot Dry Weight (g) ± SE	Total Leaf Area (cm^2) ± SE
ACC	79.93 ± 2.63 ^{a}	8.95 ± 0.15 ^{a}	858 ± 49 ^{c}
GA_3	90.05 ± 6.93 ^{a}	9.83 ± 0.65 ^{a}	1354 ± 126 ^{a}
BA	85.95 ± 2.55 ^{a}	10.13 ± 0.41 ^{a}	1129 ± 39 ^{abc}
Control	84.75 ± 4.48 ^{a}	9.94 ± 0.40 ^{a}	1161 ± 85 ^{ab}

4. Discussion

Phytohormonal profiling revealed a solitary difference between aeroponically grown lettuce plants grown under ambient and elevated RZ CO_2: increased leaf JA concentrations under elevated RZ CO_2 (Figure 1d). JA biosynthesis pathway metabolites increased in guard cells when plants were grown under eCO_2, indicating that the jasmonoyl-isoleucine (JA-Ile) biosynthesis pathway plays an essential role in stomatal closure induced by short-term (1 h) high CO_2 (800 ppm) application [49] while long-term CO_2 exposure decreased JA production. Although RZ CO_2 can induce stomatal closure in lettuce when plants were grown at much higher PPFDs, temperatures and RZ CO_2 concentrations [3] than employed here, stomatal conductance did not differ between treatments here (data not shown). Whereas elevated RZ CO_2 increased the shoot-to-root ratio (Table 1), stomatal closure was associated with decreased shoot-to-root ratios [3], suggesting that their plants experienced leaf water deficit even though the roots were grown aeroponically. Since JA is usually regarded as a growth inhibitor [62], stimulation of lettuce growth under RZ CO_2 enrichment [61] cannot be attributed to this hormonal difference. Nevertheless, since JA is involved in plant defence responses, the importance of these foliar JA decreases should be investigated with factorial experiments imposing RZ CO_2 enrichment and pest/disease assays.

Root-zone CO_2 enrichment did not change lettuce root phytohormone concentrations, but increased root SA concentrations of pepper while decreasing shoot SA concentrations. These opposing tissue-specific responses may reflect enhanced basipetal transport of SA via the phloem from shoots to roots, but this hypothesis can only be assessed by girdling (phloem removal) the stem (e.g., [63]). Research into the signalling pathways initiated by SA has mainly focused on its role in plant defence and immunity [64]. However, SA also mediates responses to abiotic stresses such as drought, low temperature and salinity [65]. Generally, low concentrations (≤10 μM) of applied SA promote plant growth under unfavourable conditions, whereas high SA concentrations (≥100 μM) inhibit growth; the threshold between low and high concentrations depends on plant species and the method of treatment [66–68]. Although comparatively less is known about the role of SA in plant root development, applying exogenous SA to the roots had concentration-dependent effects. Whereas high exogenous SA concentrations (>150 μM) inhibit root growth of Arabidopsis primary and lateral root development [69], lower concentrations (<150 μM) increased root biomass in corn (*Zea mays*) [70] and soybean (*Glycine max*) [71]. Although high RZ CO_2 increased root SA concentrations of pepper by 50%, it was not associated with a significant decrease in root dry weight. Nevertheless, root elongation and architecture should be measured to further evaluate the effects of high RZ CO_2 in this species.

Although in general pepper plants grown aeroponically did not show any significant differences in biomass or leaf area between treatments [61], RZ CO_2 enrichment decreased biomass and leaf area in Experiment 3 (Table 1). Phytohormonal profiling showed significantly higher leaf ACC concentrations and significantly lower leaf tZ and SA concentrations under elevated RZ CO_2 compared to the control treatment. These hormonal changes suggest that RZ CO_2 enrichment induces a long-distance stress response in leaves. Often, but not always, the ETH precursor ACC is transported from the roots to the shoot when the roots are exposed to stress [72]. Tomato plants under flooding or lack of oxygen in the rhizosphere increased ACC transport from the roots to the shoots, where it is converted into ETH [73].

Long-distance transport of ACC has also been suggested to occur with drought [74], nutrient [75] and salinity [76] stresses. Higher respiration and lower oxygen availability in the rhizosphere of plants exposed to high RZ CO_2 did not cause root ACC accumulation, but may have increased ACC transport to the shoot, where it accumulated, although xylem sap composition should be measured to confirm this hypothesis.

Since ETH influences plant growth [77,78], leaf disc assays were performed to understand the effects of ACC (the ETH precursor) on leaf expansion of pepper plants grown without any imposed treatment. While low ETH concentrations (0.02 ppm) can increase growth, high concentrations (1 ppm) inhibit growth [79]. Moreover, ETH inhibits leaf elongation in slow-growing grass species [80] at high concentrations (>1 ppm) compared to fast-growing species. Applying saturating ACC concentrations to the leaves of intact pepper plants decreased leaf area by 26% compared to control plants (Table 2), which could also inhibit leaf growth in pepper grown under elevated RZ CO_2 (Figure 3). While the lack of ACC response in the leaf disc assay (Figures 5 and 6) suggests that wounding during disc excision stimulated additional ETH production, comparable leaf growth promotion by GA_3 in leaf discs and intact plants (cf. Figure 5, Table 2) suggests otherwise. Nevertheless, determining both foliar ETH production and sensitivity to ETH may help explain the regulation of shoot growth under high RZ CO_2.

Applying BA (a synthetic cytokinin) to leaves of intact pepper plants did not stimulate leaf area, while floating leaf discs on 50 µM BA significantly increased area expansion after 24 h. Therefore, a decrease in foliar tZ (Figure 1b) could also be involved in the limited response of pepper plants to high RZ CO_2. Although nitrogen depletion in the plant RZ decreased foliar tZ concentrations [81], the magnitude of N depletion in pepper leaves when grown with high RZ CO_2 (<5%—[61]) is unlikely to significantly alter foliar CK concentrations. Thus, alternative physiological mechanisms (such as rootzone anoxia) must be sought to explain decreased foliar tZ concentration with high RZ CO_2.

Although high RZ CO_2 did not affect endogenous GA concentrations, previous studies indicated that exogenous GA_3 stimulated pepper leaf expansion at high (100 µM) but not low (<100 µM) GA_3 concentrations, especially at higher (75 µmol m^{-2} s^{-1}) light intensities [38]. Our glasshouse-based assays with variable light intensity through the day and between days showed variation in GA_3 response (averaging 12% greater leaf disc expansion compared to the control treatment over three independent experiments—cf. Figures 4–6), consistent with previous assays with this system [38]. Interestingly, intact plants and leaf discs showed similar growth promotion (cf. Figure 5, Table 2) contrary to the hypothesis that leaf excision promoted ETH release thereby antagonising any GA_3-mediated growth increment [82]. Although high RZ CO_2 did not alter shoot GA_3 concentration of pepper (Figure 2d), variation in GA sensitivity of leaf expansion should be investigated.

To conclude, apparent species differences in response to high RZ CO_2 (growth promotion of lettuce and growth limitation of pepper—Table 1 here; [61]) were associated with differences in phytohormone profiles, even if it was difficult to attribute growth regulation to specific hormones. Thus, growth promotion of lettuce under high RZ CO_2 was inconsistent with a solitary phytohormonal change (increased leaf concentrations of the growth inhibitor jasmonic acid), and alternative mechanisms are needed to account for greater growth of lettuce. In contrast, growth inhibition of pepper under high RZ CO_2 was associated with multiple phytohormonal changes (decreased leaf tZ and SA concentrations but increased leaf ACC concentrations). While understanding the physiological role of these changes is likely to require the use of hormonal mutants or transgenics that are deficient in or insensitive to specific hormones, differential root and shoot hormone response to high RZ CO_2 suggests that xylem sap phytohormone profiling is needed to understand root-to-shoot signalling of high RZ CO_2.

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