Running title

Growth and hormone physiology of maize

Title

Stomatal and growth responses to hydraulic and chemical changes induced by progressive soil drying

Xiaoqing Li[1, 3], Sally Wilkinson[1], Jianbo Shen[2], Brian G. Forde[1], William J. Davies[1]

1 Lancaster Environment Centre, Lancaster University, Bailrigg, Lancaster, LA1 4YQ, UK

2 Permanent address: Key Laboratory of Plant-Soil Interactions, Department of Plant Nutrition, Ministry of Education, China Agricultural University, Beijing 100193, China

3 Present address: CSIRO Agriculture and Food, GPO Box 1700, Canberra, ACT 2601, Australia

Author for correspondence

William J. Davies w.davies@lancaster.ac.uk

Telephone number: +44 (0)1524 510203

Emails for authors

Xiaoqing Li: lixqymail@gmail.com

Sally Wilkinson: sally@levitycropscience.com

Jianbo Shen: jbshen@cau.edu.cn

Brian Forde: b.g.forde@lancaster.ac.uk

Date of submission: 02/06/17

Number of table: 0

Number of figure: 7, Figure 1 should be in colour in print

Word count: 6910

Supplementary data

Number of table: 1

27 Number of figure: 4

Title

Stomatal and growth responses to hydraulic and chemical changes induced by progressive soil drying **Highlight**

This study synchronously investigated maize growth and physiological responses to progressive soil drying. It indicate hydraulic and chemical changes may regulate plant development and functioning during the onset of drought.

Abstract

A better understanding of physiological responses of crops to drought stress is important for ensuring sustained crop productivity under climate change. Here we studied the effect on 15 d-old maize (*Zea mays* L.) plants of a 6-d non-lethal period of soil drying (soil water potential (SWP) decreased from –0.20 to –0.81 MPa). Root growth was initially stimulated during drying (when SWP decreased from –0.31 to –0.38 MPa, c.f. –0.29 MPa in well-watered pots), followed by inhibition during Days 5–6 (SWP from –0.63 to –0.81 MPa). Abscisic acid (ABA) in the root began to accumulate as the root water potential declined during Days 2–3. Leaf elongation was inhibited from Day 4 (SWP < –0.51 MPa), just after leaf ABA content began to increase, but coinciding with a decline in leaf water potential. The stomatal conductance was restricted earlier in the younger leaf (4th) (on Day 3) than in the older leaf (3rd). The ethylene content of leaves and roots decreased during drying, but after the respective increase in ABA contents. This work identified critical timing of hydraulic and chemical changes at the onset of soil drying, which can be important in initiating early stomatal and growth responses to drought.

Keywords: Abscisic acid (ABA), drought, ethylene, hormone, maize, physiological responses, root, shoot

Abbreviations

ABA: abscisic acid; CE: controlled-environment; GC: gas chromatography.

Introduction

Drought is a major factor restricting crop production in many regions of the world (Boyer, 1982; Boyer *et al*., 2013). Whilst maize (*Zea mays* L.) is among the top three staple crops worldwide (Varshney *et al*., 2012), its production is likely to suffer more from drought stress in the future under a changing climate with increased risk of high temperatures and more variable precipitation (Battisti and Naylor, 2009; Challinor *et al*., 2014; Tardieu, 2012). Therefore, it is important to breed plants that are more drought resistant and to improve current irrigation management for agricultural systems. Both of these requirements can depend upon a better understanding of the physiological responses to drought stress of shoots and roots (Tuberosa *et al*., 2007).

Unfortunately the term 'drought', as used in agriculture, is imprecise and does not have a universal definition (Wilhite and Glantz, 1985; Gilbert and Medina, 2016; McDaniel *et al.*, 2017). However, it is valuable to use a combination of indices to characterise a specific drought stress event (e.g. onset, severity and duration), which can facilitate comparison and interpretation of specific plant drought responses (Lawlor, 2013). A non-lethal drought stress is common in the field and is considered to be an important target for the improvement of plant performance in droughted environments (Tuberosa *et al*., 2007; Skirycz *et al*., 2011).

Plants use different strategies to cope with different degrees of drought (avoidance and tolerance), including numerous responses to avoid water loss, continue water uptake at low soil moisture contents or tolerate a low tissue water content, and thereby minimise the reduction of crop growth and yield under drought (Lawlor, 2013). These avoidance and tolerance strategies are accomplished through a range of physiological responses, such as reducing stomatal conductance and development of leaf area, changing root and shoot growth to enhance root to shoot ratio and maintaining turgor pressure by reducing cellular solute potential (osmotic adjustment) etc. (Lawlor, 2013; Gilbert and Medina, 2016). Plant shoots and roots may respond differently to the same drought stress by means of development, growth and other physiological changes (Munns and Cramer, 1996; Romero *et al*., 2017; Zhang *et al*., 2017). Shoot growth is generally more inhibited by drought than root growth (Sharp and Davies, 1979; Durand *et al*., 2016). In some cases, under mild drought, root growth may be promoted by soil drying, which is of great importance in maintaining sufficient water supply for the plant (Sharp and Davies,

1979; Kano *et al*., 2011). Westgate and Boyer (1985) showed that the maize nodal root could continue its elongation when the water potential in its growing region was –1.4 MPa, while the elongation of the stem, silks and leaves from the same plant was completely inhibited when the water potentials in their growing regions were –0.50, –0.75 and –1.0 MPa respectively. Similarly, the primary root elongation rates of maize, soybean, cotton and squash were reduced but maintained when the substrate water potential was –1.6 MPa, while the shoot growth was completely inhibited at –0.8 MPa (Sharp, 2002).

Phytohormones have been shown to regulate plant development and growth under drought stress (Santner *et al*., 2009; Pierik and Testerink, 2014). The concentration of abscisic acid (ABA), one of the most important drought-relevant hormones, increases under drought stress in many plant species (e.g. Arabidopsis, maize and potato) (Zhang and Davies, 1989; Huang *et al*., 2008; Puértolas *et al*., 2015). It is also suggested that the concentration of ABA in the root could be an indicator of a local change in soil water availability (Zhang and Davies, 1989). Furthermore, the accumulation of ABA under drought stress is reported to be responsible for stomatal closure and the inhibition of shoot and root growth (Chen *et al.*, 2013; Harris, 2015). Mild drought can stimulate root growth, while severe drought can inhibit it (Sharp and Davies, 1979; Creelman *et al*., 1990). Accordingly, stimulatory and inhibitory effects on root growth were shown when ABA was applied to plants at low and high concentrations respectively (Xu *et al*., 2013; Li *et al*., 2017).

Ethylene is a gaseous plant hormone, which is probably also involved in plant drought responses (Sharp and LeNoble, 2002; Kazan, 2015). Previous studies have indicated that drought stress may promote, restrict or not affect the ethylene production in various plant species (Morgan *et al*., 1990; Sharp and LeNoble, 2002; Arraes *et al*., 2015). Morgan *et al*. (1990) reported that intact cotton and bean plants showed reduced ethylene production during slow soil drying in contrast to the responses shown by detached leaves under rapid desiccation. Therefore the types of drought stress and sampling methods could affect the ethylene production result. Ethylene has been shown to be an inhibitor of shoot growth, root elongation and lateral root initiation (Pierik *et al*., 2006; Muday, 2012). A series of studies have suggested that significant accumulation of ABA is necessary to prevent extra ethylene production and thus ameliorate its inhibition of maize shoot and root growth

under low water potentials (Saab *et al*., 1990; Sharp and LeNoble, 2002). Hence, it has been assumed that the interaction between ABA and ethylene plays an important role in regulating plant drought response (Sharp and LeNoble, 2002; Tanaka *et al*., 2005). Nevertheless, there is also good evidence for a controlling influence of plant hydraulics in the regulation of plant development and functioning under drought (e.g. Brodribb, 2009) and more precise estimation and measurement of intra-organ variation in hydraulic and chemical status of plant cells (e.g. Buckley *et al*., 2017) highlights the difficulty of ruling in or out hydraulic and/or chemical control in individual studies. However, few studies have simultaneously investigated the gradual changes of hormone levels and leaf and root growth in response to a gradual soil drying, let alone the timing of these changes, which is prerequisite if we are to elucidate the complex signalling pathways which are important components of the plant drought response.

By subjecting 15-d old maize plants to a 6-d non-lethal soil drying episode, the responses of leaf and root growth and physiological variables, such as endogenous ABA and ethylene accumulation, were investigated synchronously in this study. The results from this work imply the important involvement and the timing of hydraulic and hormonal changes in regulation of shoot and root growth during soil drying and could provide useful plant physiological information for improving crop management under drought.

Materials and methods

Plant growth

The maize cultivar *Earligold* F1 (VSW041, Moles Seeds, UK) was used. In experiment one, 280 seeds (0.15–0.19 g seed^{-1}) were soaked in deionized water for 48 h and then pre-germinated on wet paper towels for 72 h in a controlled-environment (CE) room in the dark (temperature: 24˚C/18˚C; photoperiod:14 h/10 h; relative humidity: 40%; light density: 350 µmol m^{-2} s^{-1}). Then seedlings with a root length of 4–10 cm were transplanted into 155 pots (height: 24 cm; diameter: 6.4 cm; with stainless wire mesh at the bottom) with one seedling per pot. Each pot was filled with 785 g of moist soil (ca. 628 g dry soil) to make a 22-cm tall soil column. The soil was sieved (1-cm sieve) John Innes No.2 (Foremost, UK). After transplanting, each pot was watered thoroughly by adding 200 ml water. Seedlings became visible on

the next day and another 20 ml water was added to each pot. The soil column was then drained for 1 h and weighed to determine the pot capacity for water (54% of soil water content, w/w soil dry weight). All pots were weighed and watered to the pot capacity every day until the 15th day, except on the 7th day after transplantation when 50 ml Hoagland's nutrient solution (pH = 5.8–6.0) was given to each pot. The third leaf was expanded fully (the leaf collar became visible) by the 15th day after transplantation, which was set as the last watering day (Day 0) for the soil drying treatment.

One hundred and four plants at a similar growth stage were selected: 48 plants for the soil drying treatment and another 48 plants as the well-watered control during the following 6 d, in addition to these, 8 plants were sampled on Day 0 as the starting reference. Control plants were watered daily to pot capacity. Eight pots of each treatment were destructively harvested every day during Days 1–6. All of the pots were moved every other day to ensure a uniform growth environment.

This experiment was repeated once (experiment two). In experiment two, 170 seeds (0.15–0.19 g seed^{-1}) were pre-germinated and 95 seedlings were transplanted into pots. On the last watering day (the 15th day, Day 0), 65 plants at a similar growth stage were selected: 30 plants for each treatment (soil drying and well-watered) and 5 plants were sampled on Day 0. The growth condition and other process in these two experiments were the same. Similar results were seen in these two experiments. The data presented in this paper were combined results by treating every sample in either experiment as one replicate.

Soil water content and soil water potential

After removing the shoot from the soil surface, the soil column was cut into top and bottom halves from the middle (Figure 1A). After root tissue was removed, each part of the column was weighed ($W_{original}$), oven dried at 80˚C for about a week and weighed again for dry weight (W_{dry}). Then the soil water content (%, w/w) was calculated by $[(W_{orignial} – W_{dry})/ W_{dry}] \times 100\%$.

A soil water characteristic curve can be found in Supplementary Data Figure S1. The soil water potential was measured by thermocouple psychrometer (Wescor Inc., Utah, USA) when the soil water content was above 25% (water potential higher than –0.37 MPa) and by the WP4-T Dewpoint Potentiometer (Decagon Devices,

Washington, USA) when the water content was between 5–25%. The soil water potential result was estimated from this soil water characteristic curve based on soil water content values.

Leaf elongation rate and root growth measurements

From the day before Day 0, the length of four growing leaves (the 4th–7th leaves) was measured daily once visible. The leaf elongation rate (mm h^{-1}) was calculated. After the incubation for root ethylene (see below), the entire root system was scanned and analysed for total root length and root surface area with the WinRHIZO Pro system (Regent Instruments Inc., Quebec, Canada). In each treatment, the mean of root length or surface area in the previous day was treated as the root length or surface area for that day for calculation of the daily increase rates of these parameters (units: m d^{-1}, cm^2 d^{-1}).

Leaf and root water potential and solute potential

Leaf and root water potential (Ψ_{leaf} and Ψ_{root}) were measured with thermocouple psychrometers. Leaf discs (5 mm diameter) were punched from the middle of the 3rd leaf (avoiding the midrib). The leaf disc was immediately wrapped in aluminum foil to minimize water loss and loaded into a C52 sample chamber (Wescor Inc., Utah, USA) within minutes for a 3 h incubation. The voltage was then recorded on a HR-33T Dew Point Microvolt meter (Wescor Inc., Utah, USA). The water potential in MPa was converted from the recorded voltage based on the calibration with salt solutions of known osmotic potentials. A few roots (no root tips) were collected from the outer surface of top two-third soil columns after the root tips were collected for ABA assay (see below). The roots were cut into small segments (5–8 mm). Ten to fifteen root segments were wrapped in aluminum foil and used to measure the water potential in the same way as for the leaf samples. During Days 0–6, leaf and root tissues were sampled from 10:00 am till 18:00 pm in the light period of the CE room (6:00 am to 20:00 pm) when a plant was destructively harvested on each day. Plants from well-watered and soil drying treatments were harvested alternately within each day (except Day 0).

The same leaf and root samples were then used to measure solute potentials ($\Psi_{s\text{-}leaf}$ and $\Psi_{s\text{-}root}$) by the same psychrometer. Samples were frozen by submergence into liquid nitrogen and then stored in a –20˚C freezer, defrosting before use. The voltage

was record after 30 min incubation of samples and then converted to solute potential in MPa. Leaf and root turgor pressure ($\Psi_{t\text{-leaf}}$ and $\Psi_{t\text{-root}}$) were then calculated for every sample according to the equation $\Psi_t = \Psi - \Psi_s$.

Stomatal conductance

Stomatal conductance was measured daily between 7:00 and 9:00 am (photoperiod started at 6:00 am) with an AP4 porometer (Delta-T Devices, Cambridge, UK). The 3rd (fully expanded on Day 0) and the 4th (fully expanded on Day 2 or 3) leaves of each plant were measured. The measurement was on the abaxial leaf surfaces from both sides of the midrib in the middle one-third of each leaf. Two positions on each side of the midrib were measured and the mean value of the four readings was used to represent the stomatal conductance for an individual plant.

ABA assay for leaf and root tissues

In experiment one, the 3rd leaves of every two of the eight plants from the same treatment were pooled as one replicate. In experiment two, the 3rd leaf of each plant was treated as one replicate. The leaves were cut at the collars, folded into one 15 ml centrifuge tube and submerged into liquid nitrogen immediately. Around 100 root tips (ca. 3 cm) were collected from the top two-third of the soil column of the same two pots used for leaf sampling in experiment one. Similarly, around 40 root tips were collected from one plant in experiment two. The root tips were quickly washed with tap water, transferred into a 1.5 ml centrifuge tube and submerged into liquid nitrogen. All samples were stored at –20°C before being freeze-dried for 48 h. The samples were then ground, and ca. 30 mg leaf tissue and all root tips were extracted with deionised water at 1:25 mg:µl ratio in a 1.5 ml centrifuge tube and shaken at 4°C overnight. Then the competitive radioimmunoassay (Quarrie *et al*., 1988) was used to determine ABA concentrations (ng g^{-1} DW). The extract was centrifuged at 12 000 g for 4 min and then 50 µl supernatant was pipetted into the reaction buffer. This buffer contained 200 µl of 50% 50 mM PBS buffer (pH = 6.0), 100 µl diluted antibody MAC 252, and 100 µl diluted [^{3}H] ABA. The mixture was then incubated for 45 min at 4°C. The bound radioactivity of [^{3}H] ABA was measured with a liquid scintillation counter (Packard TriCARB 1600TR liquid scintillation analyser, Canberra, CT, USA). A standard curve with 8 ABA solutions (0, 62.5, 125, 250, 500, 1000, 2000 and 2×10^6 pg 50 μl^{-1} (+)-ABA), which was made from (±)-ABA (A1049, Sigma-

248 Aldrich) and was measured with samples and used for calculating the ABA
249 concentrations of samples.

250 *Ethylene release rates from leaf and root*

251 In experiment one, four of the eight plants in each treatment were used for ethylene
252 incubation every day during Days 1–6, while every plant was used in experiment two.
253 The 5th leaf and the entire root system of a plant were used to quantify the ethylene
254 release rate respectively. The entire root system was washed out of the soil (within
255 30 min) after root tips were collected. Leaf and root samples were incubated in glass
256 test tubes sealed with rubber stoppers for 1.5 h under light and dark respectively. To
257 prevent water loss from the sample, a piece of wet filter paper was enclosed. After
258 the incubation, 1 ml gas was taken with a syringe and injected into a gas
259 chromatography system (GC) fitted with a FID detector (6890N, Agilent
260 Technologies, California, USA) (Chen *et al*., 2013). A 20 ppm ethylene/nitrogen
261 standard gas (BOC Limited, Surrey, UK) was used to check the ethylene peak time
262 and also for calibration. The leaf and root samples (after root scanning, see above)
263 were oven dried and weighed. Then ethylene release rates (nl g^{-1} DW h^{-1}) were
264 calculated for leaves and roots.

265 *Statistical analysis*

266 The statistical software SPSS 21.0 (IBM, USA) was used to perform either one-way
267 ANOVA with Tukey's *post hoc* test or *t*-test at the $P < 0.05$ level.

268 Results

269 *Soil water content during soil drying*

270 To establish a non-lethal progressive soil drying episode and to investigate maize
271 root and shoot physiological responses during this process, several preliminary
272 experiments were conducted and this 6 d drying treatment was chosen for this study.
273 On the 6th day of soil drying, maize plants started to wilt, but this wilting
274 phenomenon can be eliminated quickly by rewatering (data not shown). To
275 determine the drought intensity of the soil drying treatment during the 6 d after last
276 watering, soil water contents of top and bottom halves of soil columns were
277 measured. The top half of the column had a lower soil water content than the bottom
278 half of the column in both well-watered and drying treatments (Figure 1B). The well-
279 watered pots had a soil water content of 38% (soil water potential: –0.30 MPa) and

44% (soil water potential: –0.26 MPa) in the top and bottom soils on average during the 6 d, respectively (Figure 1B). In contrast, the water content in the drying treatment declined from 37% (soil water potential: –0.30 MPa) to 10% (soil water potential: –0.95 MPa) in the top half soil and from 43% (soil water potential: –0.27 MPa) to 12% (soil water potential: –0.73 MPa) in the bottom half soil (Figure 1B). Soil water contents in both top and bottom halves of the drying treatment were significantly lower than those in the well-watered pots from Day 2 (Figure 1B). The average water content of the soil columns in the drying treatment dropped gradually from pot capacity (54%, just after watering) on Day 0 to 11% on Day 6 (Figure 1B), corresponding to water potentials of –0.20 and –0.81 MPa respectively (Figure 1B, Supplementary Data Figure S1).

Effects of soil drying on leaf and root growth

Maize leaf elongation rate, total root length and total surface area were measured to indicate plant growth responses during soil drying. Results showed that soil drying significantly reduced the leaf elongation rate after Day 4 (the average soil water potential in drying pots: –0.51 MPa) (Figure 1B, 2 and Supplementary Data Figure S1). More than 30% and around 80% reduction was seen respectively during Days 4–5 (the average soil water potential in drying pots decreased from –0.51 to –0.63 MPa) and Days 5–6 (from –0.63 to –0.81 MPa) (Figure 1B, 2 and Supplementary Data Figure S1). Other older (the 4th leaf) or younger leaves (the 6th and 7th leaves) showed similar reduction in elongation rate during soil drying (Supplementary Data Figure S2).

Maize in the soil drying treatment showed a larger total root length and surface area than the well-watered plants on Day 3 (the average soil water potential in drying pots: –0.38 MPa) (Figure 1B, 3 and Supplementary Data Figure S1), which was caused by a greater root growth rate during Days 2–3 (the average soil water potential in drying pots decreased from –0.31 to –0.38 MPa) of the soil drying treatment, when drought was mild (Figure 1B, Supplementary Data Figure S1 and S3). However, maize subjected to the soil drying treatment had a smaller root system on Day 6 (the average soil water potential in drying pots: –0.81 MPa) (Figure 1B, 3 and Supplementary Data Figure S1), which was due to the reduced root growth rate after Day 3 when the drought became more severe (Supplementary Data Figure S3).

Physiological responses to soil drying

Changes in water potential and turgor pressure of leaf and root

Leaf water potential and solute potential of the 3rd leaf were monitored as an indicator of leaf water status during soil drying. The leaf water potential in well-watered maize was between –0.34 to –0.37 MPa during the 6-d period, while in the drying treatment it dropped to a significant lower value on Day 5 (leaf water potential: –0.86 MPa; the average soil water potential in drying pots: –0.63 MPa) and it decreased further to –1.10 MPa on Day 6 (Figure 1B, 4A and Supplementary Data Figure S1). The leaf turgor pressure of both well-watered and droughted plants was lower than starting values of the respective treatments from Day 4 (Figure 4B). However, the soil drying treatment did not reduce leaf turgor during the 6-d period when compared with controls (Figure 4B).

The root water status was determined by measuring root water potential and calculating root turgor pressure. The root water potential was always around –0.30 MPa in the well-watered plants over the 6 d (Figure 4C), which was close to the average soil water potential (Figure 1B and Supplementary Data Figure S1). In contrast, the root water potential in the soil drying treatment decreased from –0.26 to –1.37 MPa between Day 1 and Day 6 (the average soil water potential in drying pots decreased from –0.29 to –0.81 MPa) and was significantly lower than that in the well-watered plants from Day 3 (the average soil water potential in drying pots: –0.38 MPa) (Figure 1B, 4C and Supplementary Data Figure S1). It is notable that the root water potential decreased along with, but remained lower than, the average soil water potential in the drying treatment from Day 2 (Figure 1B, 4C and Supplementary Data Figure S1). Root turgor pressure was maintained and even increased in the treated plants over the 6 d (Figure 4D), but was not significantly increased during the early stages of soil drying when increases in root growth were detected (Figure 3, 4D).

Changes in leaf stomatal conductance

The stomatal response to soil drying was monitored on a mature leaf (the 3rd) and a younger one (the 4th). The stomatal conductance of the 3rd leaf decreased along with soil drying from Day 5 (the average soil water potential in drying pots: –0.63 MPa) and decreased by 43% and 75% compared with the well-watered maize plants

on Day 5 and 6 respectively (Figure 1B, 5A and Supplementary Data Figure S1). However, the 4th leaf showed a higher stomatal conductance than the 3rd leaf, by around 30% on average over the 6 d (Figure 5). In addition, an earlier response of stomata to soil drying was seen in this younger leaf; a significant reduction in stomatal conductance (by 12%) was seen on Day 3 (the average soil water potential in drying pots: –0.38 MPa) in drying plants (Figure 1B, 5B and Supplementary Data Figure S1). On the last two days of soil drying, the stomatal conductance in the 4th leaf decreased further (by 39% and 62% respectively) (Figure 5B).

Changes of ABA concentrations and ethylene release rates in leaf and root

During the 6 d of the experiment, ABA concentrations in the 3rd leaf of well-watered plants ranged between 80–119 ng g^{-1} DW (Figure 6A), while in the soil drying treatment the concentrations increased to around twice this value on Day 4 (the average soil water potential in drying pots: –0.51 MPa) and more than twenty times this value from Day 5 (the average soil water potential in drying pots: –0.63 MPa) (Figure 1B, 6A and Supplementary Data Figure S1). By contrast, the ethylene release rate of the 5th leaf only showed a reduction with soil drying treatment on Day 6 (by 35%, P = 0.064; the average soil water potential in drying pots: –0.81 MPa) (Figure 1B, 6B and Supplementary Data Figure S1). In one preliminary 5-d soil drying experiment, ethylene release rates of the 5th and 6th leaves showed significant reduction during soil drying from Day 4, which was one day later than the increase of leaf ABA concentration (Supplementary Data Table S1, Figure S4).

The ABA concentration in the root tips of well-watered maize ranged between 66–123 ng g^{-1} DW, which was similar to ABA concentrations in the 3rd leaf (Figure 6A, C). In response to soil drying, the ABA concentration in root tips significantly increased by 95% on Day 3 (the average soil water potential in drying pots: –0.38 MPa), earlier than an increase in ABA concentration in the 3rd leaf of these plants, which increased significant only from Day 4 (Figure 1B, 6A, C and Supplementary Data Figure S1). In root tips, soil drying continued to stimulate the ABA concentration on Days 4, 5 and 6, when the concentration was 3, 9 and 12 times of that in well-watered plants, respectively (Figure 6C). It has to be noted that the root tips were sampled for ABA assay while the entire root system was used for ethylene analysis. From Day 4, the root ethylene release rate in the drying treatment was significantly lower than that of the watered treatment (Figure 6D). In roots of the well-watered

controls the rate of ethylene release increased by 23–54% on Days 4–6 compared with Day 1 (Figure 6D).

Discussion

Different responses of maize leaf and root growth during soil drying

Previous studies have reported that shoot and root growth in maize respond differently during soil drying (Sharp and Davies, 1979; Watts *et al*., 1981). Shoot growth can be inhibited during soil drying (Sharp and Davies, 1979, 1985; Westgate and Boyer, 1985), while root growth can be stimulated under mild drought and inhibited when the drought becomes severe (Sharp and Davies, 1979; Watts *et al*., 1981; Creelman *et al*., 1990). Similarly in this study, roots of maize plants under the soil drying treatment showed higher growth rates under mild drought (Days 2–3, the average soil water potential in drying pots decreased from –0.31 to –0.38 MPa), but lower growth rate once the drought became more severe (after Day 3) (Figure 1B, 3, 7A and Supplementary Data Figure S1, S3). In contrast, leaf elongation was inhibited by soil drying, but only when the drought became more severe, during Days 4–5 (the average soil water potential in drying pots decreased from –0.51 to –0.63 MPa) (Figure 1B, 2 7A and Supplementary Data Figure S1). Modification of shoot and root growth rates can be an important drought avoidance strategy for plants (Lawlor, 2013). Notably, the increase of root growth was the earliest detected developmental change. It has been shown that such stimulation of root growth (especially in deeper soil) under mild drought exerted a positive effect on crop production since it helps maintain water uptake (Manschadi *et al*., 2006; Kano *et al*., 2011). However, when the soil volume is limited, or there is little water stored in deep soil layers, there may be little benefit from increased root growth or a deeper root system (Tardieu, 2012; Wasson *et al*., 2012). Under such conditions, the increased root growth can quickly deplete the small amount of extractable water that remains and then root growth will soon be significantly inhibited (Kamoshita *et al*., 2004; Tardieu, 2012). Additionally, apart from the severities of drought stress, the plant developmental stages will also affect its shoot and root responses to drought (Boonjung and Fukai, 1996a, b; Tardieu, 2012).

In previous studies on maize, roots showed earlier responses to drought (water potential decrease) than shoots (Sharp and Davies, 1979; Westgate and Boyer,

1985; Saab and Sharp, 1989). In the present study, the root water potential started to decrease during Days 2–3 of soil drying (when the average soil water potential in drying pots decreased from –0.31 MPa to –0.38 MPa), while the leaf water potential did not decline until Days 4–5 (when the average soil water potential in drying pots decreased from –0.51 MPa to –0.63 MPa) (Figure 1B, 4A, C, 7B and Supplementary Data Figure S1). The later response in the leaf than in the root may be attributable to the early stimulation of root growth under mild drought, allowing the root to take up sufficient water to maintain leaf elongation and leaf water relations for a number of days. In addition, the water potential gradient between leaves and roots/soil was increased during Days 2–3 of soil drying due to a decrease in the water potentials of root and soil while the leaf water potential was sustained. This result suggests that the root hydraulic conductance was increased by mild soil drying, since the stomatal conductance of the 3rd leaf was maintained (Scoffoni and Sack, 2017). It has also been reported that root proliferation under drought was able to increase whole root system hydraulic conductance and supply more water for transpiration in grape (Alsina *et al*., 2011).

The decrease in leaf water potential only after the decrease in root and soil water potential supports the view that while leaf water potential can be an indicator of plant water status, but it does not always represent the water status of the soil or the root (reviewed in Davies and Zhang, 1991). Because leaf water potential may not change synchronously with reductions in soil water potential, and other physiological responses may have already been activated in roots and perhaps in leaves also (e.g. reduced stomatal conductance and leaf elongation) (Sharp and Davies, 1979; Bahrun *et al*., 2002). Some studies suggest that leaf growth inhibition and stomatal closure are the earliest plant responses to drought and the former is earlier than the latter (Hsiao, 1973; Chaves, 1991; Osório *et al*., 1998). But these conclusions are often reached in studies where changes in root growth and physiology are not quantified. It is worthy of note that, to avoid the effect of growth-induced water potential in leaves and roots samples (Cavalieri and Boyer, 1982; Boyer, 2017), growing tissue (e.g. root tips and young leaves) was not used for water potential measurements.

The calculated leaf and root turgor pressures were maintained during the 6 d period of soil drying (Figure 4B, D), which resulted from a reduced solute potential in tissues

through osmotic adjustment. The maintenance of turgor pressure is important for tissue to continue growing despite the decrease of tissue water potential (Boyer, 2017). Interestingly, the root turgor pressure in droughted plants increased from 4 days after last watering when the soil drying became more severe (Figure 4D), but this was after the increase in root growth in droughted plants. Similar increase in leaf turgor pressure under drought has been seen in two out of seven pearl millet accessions included in the study of Kusaka *et al*. (2005). This may be an adaptation of plants to maintain tissue growth under soil drying when tissue water potential is reduced.

In this study, stomatal conductance in the 3rd leaf was reduced by soil drying from Day 5 (the average soil water potential in drying pots: –0.63 MPa), when the leaf water potential dropped (Figure 1B, 4A, 5A, 7B, C and Supplementary Data Figure S1). This is different from previous reports that stomata can start to close before leaf water potential is reduced by soil drying (Bahrun *et al*., 2002; Tardieu *et al*., 2010). Reduced stomatal conductance is a typical drought avoidance strategy in many plant species because it prevents continued high rates of water loss from leaves and thereby postpones or minimises potential damage by more severe decreases in water potential and turgor (Lawlor, 2013).

Interestingly, in our experiments, the younger leaf (the 4th) showed lower stomatal conductance on Day 3 (the average soil water potential in drying pots: –0.38 MPa) when only the water potential of the root was significantly reduced by soil drying (Figure 1B, 4C, 5B, 7B, C and Supplementary Data Figure S1). This could be explained if stomata of the younger leaves were more sensitive to soil drying than those of the older leaves, but there is still a question of how the stomata respond to a change in root water potential while the water potential of the leaves is not affected by soil drying. Stomata of the 4th leaf may be responding to an ABA-based root signal but if this is the case, why do stomata of the 3rd leaf not respond to this signal? Stomata in older leaves have been found to be less sensitive to ABA than those of relatively younger leaves (Chen *et al*., 2013).The results also indicates that the stomata of the growing leaf responded more quickly to soil drying than did its elongation rate. Leaf water potential in the 4th leaf was not measured, so it is not clear whether soil drying reduced both the water potential and stomatal conductance in the 4th leaf at the same time or not. Bajji *et al*. (2001) found that the decreases of

leaf water potential and solute potential were larger in younger growing leaves than those in relatively older leaves in three wheat cultivars when subjected to a same 15 day-progress soil drying. It was suggested that this phenomenon may be associated with the higher capacity of younger leaves for osmotic adjustment and maintenance of cellular water content and turgor (Morgan, 1984; Bajji *et al*., 2001). Water potential in younger leaves could also be more depressed than in mature leaves due to possible hydraulic limitation in the growing zone at the base of the younger leaves. If this was the case, such a decrease in leaf water potential of the 4th leaf (younger leaf) (not measured) might have stimulated ABA production here. As highlighted above, intra organ variation in water status can be a complication in analysis of the kind attempted here (Buckley *et al*., 2017).

The literature reports that older leaves can provide ABA to sustain higher ABA concentrations in younger leaves (Zeevaart and Boyer, 1984; Chater *et al*., 2014), but there is no evidence of this here. Thus, these results indicated that earlier root physiological responses to soil drying and stomatal closure in younger leaves may be better indicators to define the onset and severity of a drought event than leaf growth inhibition and other later responses in leaves. Furthermore, stomatal closure in young leaves will be easier to measure than root responses when plants are grown in soil.

The relationship between the ABA concentration, ethylene release rate and the leaf and root growth during soil drying

It is often unclear from the literature at which stage plant hormone levels start to change following the initiation of a soil drying episode and whether these changes are synchronous with other root or leaf physiological changes. In this study, it was found that ABA concentrations in both root tips and leaf tissues of maize increased under soil drying (Figure 6A, C), which is in accordance with previous studies (Davies and Zhang, 1991). Where the extra ABA came from in those samples of droughted plants cannot be determined in this study but extra ABA is detected in the root before a decline in leaf water potential is detected (although a possible decrease in water status of younger leaves is discussed above). It may be newly synthesised or released from stored inactive glucose ester conjugate either in sampled tissues or circulated from other tissues (Wasilewska *et al*., 2008). Interestingly, the accumulation of ABA in the roots triggered by soil drying was accompanied by a

stimulation of root growth on the same day (Days 2−3, mild drought, the average soil water potential in drying pots decreased from –0.31 MPa to –0.38 MPa), (Figure 1B, 7A, D and Supplementary Data Figure S1). After Day 3, as the soil moisture content declined further, ABA continued to accumulate in roots and this was accompanied by slower rates of root growth (Figure 7A, D). Exogenous ABA has been found to both stimulate and inhibit root growth in maize, rice and also in Arabidopsis, depending on its concentration (Watts *et al*., 1981; Xu *et al*., 2013; Li *et al*., 2017). Therefore, this suggests that increased ABA levels in roots may have either stimulated or inhibited root growth, depending on the magnitude of ABA accumulation under a mild or a more severe drought. In contrast to the root, the ABA concentration in the leaf increased later, during Days 3–4 (Figure 7D). However, the leaf elongation rate was inhibited later, during Days 4–5 (Figure 7A). This indicates that a small increase of leaf ABA (around two-fold increase) was not related to a change in leaf elongation rate, while a large increase in leaf ABA level coincided with the inhibition of leaf elongation, which is consistent with previous reports that ABA is an inhibitor of shoot growth (Sharp and LeNoble, 2002; Meguro and Sato; 2014).

In this study, root tips were sampled only from the top two-thirds of the pot to analyse ABA concentration, because the root sampling method can be important if we want to argue that root ABA increase occurred together with the decrease of root water potential. Soil water was distributed heterogeneously in the pot (Figure 1B), so that when the top part of the soil column is dry enough to trigger an increase of ABA concentration in the root, the lower part may still be too wet to see any enhanced root ABA level. Thus, if root tips are collected from the entire soil column, this may make it difficult to see an early increase of ABA concentration in the root even when the average soil water content had dropped to 22% in a preliminary experiment (data not shown). Puértolas *et al*. (2015) reported a similar finding in potato plants, which were grown in a vertical partial root-zone drying system, that roots sampled in the lower wetter part of a soil column had a lower ABA concentration than roots in the upper, drier soil.

The present study showed that soil drying inhibited ethylene release from both maize leaves and roots (Figure 6B, D), which is in accordance with the finding that maize ethylene emission was inhibited under low water potentials when the ABA level was increased (Sharp and LeNoble, 2002). However, the inhibitory effects of soil drying

on leaf and root ethylene occurred at a later stage of the soil drying than the ABA accumulation (on Day 6 and 4 respectively) (Figure 7E). Thus, the ABA concentrations in leaf and root were more susceptible to soil drying than ethylene release rates. Furthermore, both the leaf and root growth responses had occurred prior to the detected changes of ethylene level during soil drying (Figure 7A, E). These non-synchronous effects suggest that changes in ethylene level do not play an important role in the regulation of leaf elongation and root growth under drought (at least before Day 4 in the current experiment). Similarly, Voisin *et al*. (2006) found that leaf elongation rate was not affected in moderately drought-stressed ABA-deficient maize plants that showed high ethylene levels. One further possibility is that the ethylene emissions may have been affected by the soil drying in the first few days of soil drying, but the GC equipment may not be sufficiently sensitive to detect such small changes (Cristescu *et al*., 2013).

A possible explanation for the increase in root ethylene levels of well-watered plants from Day 4 is that the container has constrained the growing volume of root system and caused stress (Poorter *et al*., 2012) (Figure 6D). Ethylene has been reported to be a stress-induced hormone. Mechanical impedance can enhance the ethylene production without changing ABA level, while phosphorus deficiency can also promote ethylene emissions (Moss *et al*., 1988; Li *et al*., 2009).

Results from this work indicate when and how the hydraulic and chemical (hormonal) changes in maize leaves and roots could regulate stomatal conductance and plant growth in response to initially very small changes in soil water status during a 6-d non-lethal drying. It is suggested that ABA accumulation may play important roles in regulating both root growth promotion and inhibition during different stages of soil drying, while a reduced ethylene content may not be involved in regulating leaf and root growth at an early stage of drying. These early developmental and physiological responses may be key to crop establishment. However, plants are complex systems, and different results could be seen with different time scales of drought treatments (short-term *vs.* long-term), plant genotypes or soil conditions (e.g. soils with different depths) (Tardieu and Parent, 2017). The identification of the critical point at which soil water status affects root growth (either positively or negatively), along with the other observed physiological responses (e.g. stomatal conductance reduction in different leaves and changes in leaf and root water potential) focusses attention of

physiological and developmental changes that can influence both agronomy and crop improvement strategies for establishment of crops in dryland environments. It is clear that considerable precision in both chemical and hydraulic status of different plant parts is important if we are to understand which are the controlling influences for growth, development and functioning of plants under drought.

Supplementary Data

Table S1: Soil water content data from a preliminary 5-d soil drying experiment.

Figure S1: Soil water characteristic curve: soil water potential against soil water content.

Figure S2: Leaf elongation rate of (A) the 4th leaf (leaf was fully expanded on Day 2 or 3), (B) the 6th leaf (leaf was expanding and visible from Day 1), (C) the 7th leaf (leaf was expanding and visible from Day 4).

Figure S3: (A) Root growth rate, (B) total root surface area increase rate during the 6-d soil drying treatment.

Figure S4: Leaf ABA concentration and ethylene release rate results from a preliminary 5-d soil drying experiment.

Acknowledgements

The authors would like to thank Ms Maureen Harrison for her helpful maintenance for the CE room. This work was supported by the National Natural Science Foundation of China (No. 31330070 and 31421092), the EU FP7-KBBE-2011-5 project EURoot, the China Scholarship Council (CSC) and the Science Bridge Project EP/G042683/1.

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Figure legends

Figure 1: (A) Soil columns from the well-watered and soil drying treatments on Day 6 after the last watering; (B) soil water content in top and bottom parts of well-watered (WW) and soil drying (SD) treatments (Days 0−6). Pre-germinated maize seeds (*Earligold* F1) were transplanted into pots filled with sieved soil (John Innes No.2). Seedlings germinated from the soil surface after one day. All pots were weighed and watered to the pot capacity every day until the 15th day, except on the 7th day after transplantation when 50 ml Hoagland's nutrient solution (pH = 5.8−6.0) were given to each pot. The third leaf was fully expanded on the 15th day after transplantation, and this day was set as the last watering day (Day 0). Plants at a similar growth stage were selected. The same experiments were conducted twice and data presented here is the combined result. After Day 0, control plants were watered daily to the pot capacity while watering was ceased in the soil drying treatment for 6 d. Pots of each treatment were destructively harvested every day during Days 1−6. Each soil column was cut into top and bottom halves from the middle to measure the soil water content in top and bottom parts. Points and bars are means ± standard error. Data was analysed using one-way ANOVA with Tukey's *post hoc* test and different letters indicate significant difference on the same day at $P < 0.05$ (n = 13 on Day 0 and n = 9 on other Days). Values in the brackets are estimated soil water potentials (MPa) based on the soil water content values and the soil water characteristic curve (Supplementary Data Figure S1).

Figure 2: Leaf elongation rate of the 5th leaf of maize seedlings (leaf was expanding and visible before the start of soil drying), replication n = 13. Points and bars are means ± standard error. Data was analysed using *t*-test and stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$.

Figure 3: (A) Total root length and (B) total root surface area during the experimental period (Days 0–6). During the 6-day soil drying treatment (Figure 1), the roots that were used for ethylene incubation in each treatment were scanned and analyzed for total root length and root surface area using the WinRHIZO Pro system. Columns and bars are means ± standard error. Data was analysed using *t*-test and stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 9).

Figure 4: (A) Leaf water potential and (B) leaf turgor pressure of the 3rd leaf during the experimental period (Days 0–6). (C) Root water potential and (D) root turgor pressure during the experimental period (Days 0–6). During the 6-day soil drying (Figure 1), a leaf disc (5 mm diameter) from the middle of the 3rd leaf (avoiding the midrib), or a root sample (10−15 root segments, 5−8 mm in length and without root tips) from the top two-third of the soil columns was incubated for 3 h in a C52 sample chamber in the thermocouple psychrometer. The voltage was then recorded on a HR-33T Dew Point Microvolt meter. The leaf and root samples were then frozen and defrosted before they were used to measure the solute potentials, which were also measured by the same thermocouple psychrometer used for water potential measurement. Each sample was incubated for 30 min and the voltage was recorded. The voltage readings were then converted to water potentials and solute potentials respectively. Columns and bars are means ± standard error. Data was analysed using *t*-test and stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 13).

Figure 5: Leaf stomatal conductance of (A) the 3rd leaf (leaf was fully expanded before soil drying), (B) the 4th leaf (leaf was fully expanded on Day 2 or 3) in response to soil drying. During the 6-day soil drying (Figure 1), the 3rd and 4th leaves of each plant were measured for stomatal conductance using an AP4 porometer. The measurement was on the abaxial leaf surface from both sides of the midrib in the middle one-third of each leaf. Two positions on each side of the midrib were measured and the mean value of the four readings represented the stomatal conductance of the respective leaf. Columns and bars are means ± standard error. Data was analysed using *t*-test and stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 8).

Figure 6: (A) Leaf ABA concentration in the 3rd leaf (fully expanded before soil drying), (B) leaf ethylene release rate of the 5th leaf (expanding), (C) ABA concentrations in root tips, (D) ethylene release rate of the entire root system. During the 6-day soil drying (Figure 1), leaf samples were cut at the collars and root tips (ca. 3 cm each) were collected from the top two-third of the soil column. These samples were submerged into liquid nitrogen immediately and then stored at −20˚C before being freeze-dried for 48 h. Dry samples were then ground and extracted with water. The extract was then used to determine the ABA concentration by the

radioimmunoassay. The 5th leaf was cut from the soil surface and then incubated for 1.5 h (under light in the CE room) with a piece of wet filter paper in a sealed glass tube. A whole root system of a plant was then washed out and incubated similarly as the leaf sample but under dark. Then 1 ml gas was taken with a syringe and measured with a GC system fitted with a FID detector. The leaf or root sample was then oven dried for dry weight and the ethylene release rate was calculated. Points and bars are means ± standard error. Data was analysed using *t*-test and stars indicate significant difference between well-watered and soil drying treatments on the same day at $P < 0.05$ (n = 9).

Figure 7: Relative differences in growth and physiology responses of plants exposed to soil drying compared to that were well-watered during the 6-d experimental period. The relative changes in (A) leaf and root growth rates, (B) leaf and root water potentials, (C) stomatal conductance of the 3rd and 4th leaves, (D) leaf and root ABA concentrations, (E) ethylene release rate of leaf and root. Points and bars are means ± standard error. Arrows and Day indicate the time when the two treatments became significantly different.