

# **Phytohormonal effects on the regulation of stem elongation of pea (*Pisum sativum*) subjected to drying soil**

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**This thesis is submitted to Lancaster University in partial fulfilment of the  
Requirements for the degree of Doctor of Philosophy**

**Lancaster, March 2019**

## Abstract

To understand whether phytohormones regulate stem elongation of plants grown in drying soil, endogenous phytohormone concentrations in growing internodes were measured along with stem elongation, soil water content ( $\theta$ ) and stem water potential ( $\Psi_{\text{stem}}$ ) in tall pea (*Pisum sativum* cv. Alderman) plants that were well watered or exposed to drying soil. The hormones quantified were abscisic acid (ABA), gibberellins ( $\text{GA}_1$ ,  $\text{GA}_3$ ,  $\text{GA}_4$ ), auxin (IAA), cytokinins (iP, tZ), ethylene (actually its precursor ACC), jasmonic acid (JA) and salicylic acid (SA). After withholding water, stem phytohormone concentrations (ABA, iP,  $\text{GA}_3$ ) changed earlier (Days 3, 4) than changes in stem elongation (Day 5) and  $\Psi_{\text{stem}}$  (Day 7). Furthermore, ABA, tZ, iP and ACC concentrations were negatively related to stem elongation, while IAA,  $\text{GA}_1$  and  $\text{GA}_3$  concentrations were positively related to stem elongation. Since ABA was amongst the first hormone to respond to drying soil, and accumulated to the highest levels with soil drying, its role was further assessed by measuring responses of wild-type (WT) and *wilty* (ABA-deficient) peas to soil drying in a factorial experiment with moderate (50%) and high (95%) relative humidity. Since ABA-deficient plants have higher stomatal conductance, growth at high humidity aimed to attenuate any decline in plant water status. High humidity slowed the soil-drying induced decrease in  $\Psi_{\text{stem}}$ ,  $\theta$ , stem elongation and evapotranspiration rate of both genotypes. With soil drying, *wilty* had a similar leaf expansion rate as WT plants at high humidity, but stem elongation was 28% less. WT plants accumulated more ABA in growing leaves and stems than *wilty* plants, especially at moderate humidity. Although stem and leaf tissue ABA levels

increased with soil drying and were correlated with stem elongation and leaf expansion, respectively, there was no unifying relationship across genotypes and relative humidities. Lower stem elongation of *wilty* indicates that ABA maintains stem elongation and leaf expansion, irrespective of soil drying. Thus, further experiments evaluated the role of endogenous gibberellins and auxin (IAA) concentrations in limiting growth, by independently applying GA<sub>3</sub> (modified to the active form GA<sub>1</sub> in pea) and IAA as foliar sprays to plants grown in drying soil. Although soil drying decreased  $\theta$ ,  $\Psi_{\text{stem}}$  and stomatal conductance ( $g_s$ ), exogenous GA<sub>3</sub> and IAA accelerated the decline in  $\theta$ , while  $\Psi_{\text{stem}}$  decreased (after the 2<sup>nd</sup> spray) and increased (after the 3<sup>rd</sup> spray), respectively. In addition, GA<sub>3</sub> and IAA enhanced  $g_s$  and stem elongation of plants grown in drying soil but did not alter leaf expansion. For plants grown in drying soil, exogenous GA<sub>3</sub> (0.03-0.1 mM) increased stem elongation (although had no effect in well-watered plants), while exogenous IAA (0.05-0.1 mM) increased  $g_s$ , with both GA<sub>3</sub> and IAA increasing  $g_s$  of well-watered plants. This research suggests that different phytohormones have quantitatively different effects on stem elongation,  $g_s$  and leaf expansion, but play an important role in regulating stem elongation responses to soil drying.

## **Declaration**

Except where reference is made to other sources, I declare that the contents in this thesis are my own work and have not been previously submitted, in part or in full, for the award of a higher degree elsewhere.

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Lancaster University  
March 2019

## **Acknowledgement**

I would like to thank my supervisors, Professor Ian Dodd for your precious support of my PhD study. Your patience, encouragement, immense knowledge and wisdom helped me overcome various difficulties and challenges during this research and assisted me to develop as a researcher.

I also would like to thank the lecturer, skilful technicians, scientific researchers and students in the Lancaster Environment Centre for their practical help and meaningful discussions: Shane Rothwell, Jaime Puértolas, Annette Ryan, Carlos José De Ollas Valverde, Rebecca Killick (Breakpoint analysis), Alfonso Albacete (phytohormones analysis) Geoffrey Holroyd, Antje Fiebig, Sarah Donaldson, Pedro Castrovaldecantos, Esti Leibar, Katharina Huntenburg, Vasileios Giannakopoulos, Hend Mandour, Emma Burak, Bailey Kretzler, Robert Kempster, Rachel Baxter, Maureen Harrison and Phillip Nott.

All of my affable colleagues and friends in the Plant and Crop Science Group and in the Malaysian community at Lancaster, I am very glad to meet them all during past four years. Thank you for your enormous help and companionship during my study here and for creating an enjoyable living and research environment.

I give my thanks for the financial support from the Malaysian Rubber Board, thank you for the opportunity given as apart of career development offered to improved knowledge in plant science.

Finally, I would like to express my gratitude to my family: my parents, parents-in-law for all of their unlimited understanding and cheering (even thousand miles away), never failed to give a continuous support. I give special thanks to my great husband, Mohd Syahril Hussin, for always being with me during these years, taking care of me, providing a technical help during the experiment. Last but not least, my little scientist, Muhammad Naqeeb Mohd Syahril (unexpected little man after 7 years of fertility problem) came into my life in the third year of my study. Having you during my PhD life is the greatest things ever happen in my life.

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## Chapter 1

### 1.1 *Pisum sativum* (Pea)

Pea (*Pisum sativum*) belongs to the family of Leguminosae (Genus: *Pisum*, Family: Fabaceae, Subfamily: Faboideae). Economically, the legume family contributes to about 27% of the world's crop production, second only to the grass (Gramineae) family (Graham & Vance 2003). Dry pea currently ranks second only to common bean as the most widely grown grain legume in the world, with primary production in temperate regions and global production of 16.2M tonnes (FAO, 2017). Along with other legumes, pea is significant agronomically as it can fix atmospheric nitrogen through a symbiotic relationship with bacteria housed in the root nodules, thus potentially reducing the fertilizer input required for high yields. Pea can fix nitrogen at rates of up to 86 kg N/ha/year (Peoples et al. 2009), thus it may only require a “starter” dose of N fertilizer, and its N-rich residues become available to the next-planted crop.

Pea has been used as a model plant system since Gregor Mendel's work (Reid & Ross 2011). Gregor Mendel identified seven qualitative characters in peas in 1856, without knowing the function of genes. Recent advancements in molecular biology have identified four of Mendel's genes: *LE*, *R*, *A* and *I* which represent traits for stem length, seed shape, seed coat and cotyledon color. Less well known are the other genes of *GP*, *FA* and perhaps *V*, which represent traits for pod color, position of flowers and pod form (Reid & Ross 2011).

One of *LE*'s functions is to control endogenous gibberellin biosynthesis and turnover (Kohler 1970) and also tissue sensitivity to gibberellins (Kende & Lang 1964). A mutational analysis revealed the wild-type (WT) *LE* allele resulted in the presence of polar gibberellin-like activity in bioassay of stem tissues, which were absent (or present in small amounts) in the dwarf *le* mutant (Potts et al. 1982). Further development of a sensitive physicochemical technique showed that GA<sub>1</sub> levels were 10-fold higher in the tall, wildtype *LE* than the *le* mutant (Ingram et al. 1984; Ross et al. 1989). Since pea has been well characterized genetically and physiologically, it seemed an ideal model system to evaluate the regulation of stem elongation in plants grown in drying soil.

## **1.2 Growth**

### **1.2.1 Definition of growth**

Growth in plants is defined as irreversible changes in volume or weight. This irreversible alteration is due to new cells being added through three growth phases: cell division, cell differentiation and cell enlargement. Irreversible elongation of the plant cell requires cell wall lengthening resulting from turgor pressure exerted on the wall (Taiz and Zeiger, 2002). Total plant growth can be analysed as the total rate of cell expansion/elongation, which may fluctuate over time as turgor pressure changes diurnally. Turgor pressure is a physical force generated equally in all directions and imposed on the plant cell wall and plays a particularly important role in cell expansion. However, variation in cell wall properties (specifically cell wall extensibility) modulates the impact of turgor on cell elongation.

Growth can also be measured as a change of fresh weight over a period of time. However, this technique depends on plant water status, thus measurements of dry weight may prove more appropriate (Taiz and Zeiger, 2002). Biomass accumulation in specific organs (leaves, stem and roots) may occur simultaneously, and partitioning of biomass to these organs can affect the economic value of the crop. In some crop species, biomass allocation to the stem can be particularly important and comprise a major contribution to total plant biomass.

### **1.2.2 Arrangement of shoot apical meristem (SAM)**

New organs form throughout the lifetime of the plant. Following seed germination, the seedling root (radicle) and shoot (plumule) rupture the testa. The tip of the radicle develops a protective root cap which facilitates root penetration of the soil, aided by mucilage (sloughed off cells) that minimize frictional forces. Behind the root cap, cells divide in the root apical meristem, expand in the elongation zone and differentiate into different cell types including xylem and phloem cells to facilitate water export from, and sugar import to, the roots. The tip of the plumule encloses the shoot apical meristem (SAM), which develops primordia (bumps on the side of the apex) that undergo cell division, expansion and differentiation to develop into leaves. The SAM also produces cells that divide, expand in the elongation zone and differentiate into different cell types including xylem and phloem cells to facilitate water export from, and sugar import to, the developing leaves and stems (Wong et al. 2008).

The shoot apical meristem can be divided into radial domains comprising discrete cell layers and concentric zones (Lyndon, 1998; Steeves & Sussex 1989). A common shoot apical meristem has three discrete layers (Satina et al. 1940).

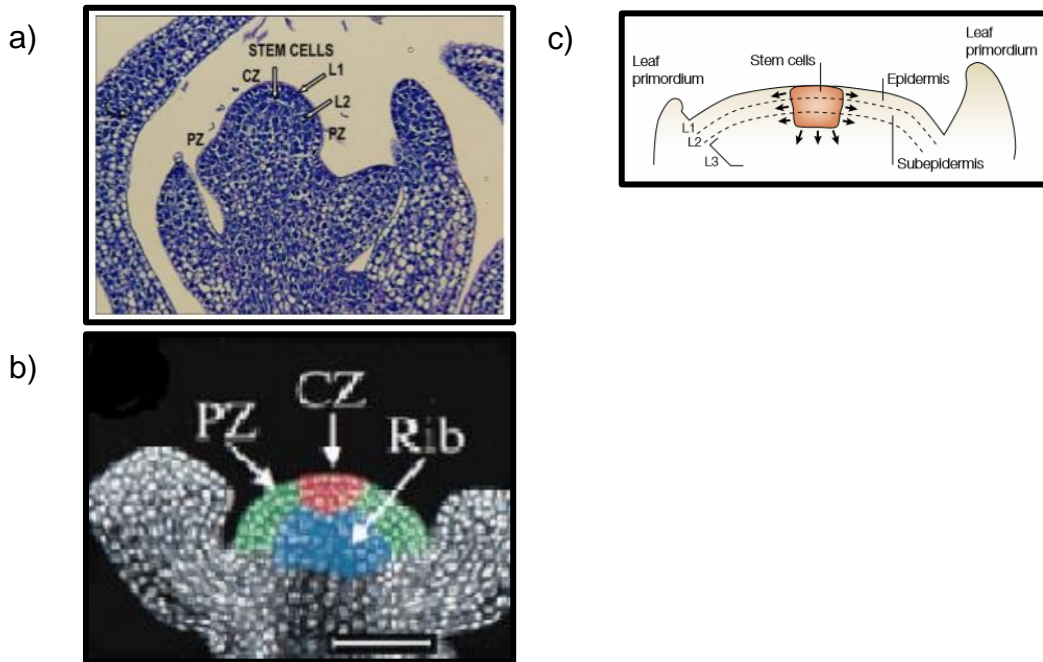


Figure 1.1(a) Organization of shoot apical meristem of pea shoot apex. (CZ, central zone; L1, outer layer; L2, inner layer; PZ, lateral peripheral zone). Stem cells undergo continued division (Bhalla & Singh 2006).

Figure 1.1(b) SAMs and floral meristems of *Arabidopsis* (ecotype *Ws-2*). Laser scanning confocal microscope optical section of SAM and adjacent floral meristems of wild-type *Arabidopsis*. Image colored to show central zone (CZ), peripheral zone (PZ) and rib meristem (Rib).

Figure 1.1(c) Cell layers and cell divisions. A longitudinal section through a shoot meristem, revealing the organization of the meristem into cell layers (L1, L2, L3). The location of the stem cells in each layer is indicated. The flow of cells as a result of cell growth and cell division is indicated with arrows. On the flanks of the meristem, cells form organ primordia, which become apparent (leaf primordia) after rapid cell growth and division (Clark, 2001).

The central zone (CZ), or zone of shoot meristem initials, contains stem cells and surrounding domains where cell divisions are frequent, and then display cell differentiation and organ initiation (Fig. 1.1a). The CZ is surrounded by the lateral peripheral zone (PZ) and the rib meristem is beneath the CZ, with rapid cell divisions in these other zones (Steeves and Sussex, 1989) (Fig 1.1b). The cells in the outermost two layers exhibit cell divisions that are anticlinal; i.e. perpendicular to the surface of the meristem. The anticlinal divisions maintain the continuity during growth of the outer layer (L1) that gives rise to the epidermis. The progeny of layer L2 cells give rise to subepidermal cells that also maintain their continuity by anticlinal cell division. The inner part of the SAM, called the corpus (layer L3), contains cells that divide in both periclinal and anticlinal planes to give rise to the internal tissues of the stem (Fig 1.1c).

The leaves form in the peripheral zone on the flanks of the SAM, while the central tissues of the stem arise from the rib meristem. Cell division in the central zone maintains the meristem itself and also provides new cells to the peripheral zones and rib meristem. Continued division of the cells of the rib meristem and the peripheral zones results in the SAM moving upwards and leaving older cells behind. This process along with cell elongation is how the stem grows taller (Meyerowitz 1997).

Leaf primordia develop into leaves, which are attached to the stem at a node. The stem between two nodes comprises the internode. Near to the SAM, the internodes grow rapidly, facilitated by continued cell division and water uptake into the cells to drive cell expansion. Further from the SAM, cell division ceases and the rate of cell expansion decelerates, until internode expansion ceases. In most herbaceous dicotyledons, only the uppermost internodes undergo expansion. Lignification of non-growing internode tissues enhances their structural rigidity, marking a change in internode function from one of growth to one of support.

The growth and development of the shoot apical meristem (SAM) are composed of elements that interact according to a collective property of patterning. Chemical signals are involved in setting up and maintaining patterning of the SAM (Murray et al. 2012), of which the major ones are auxin and cytokinins (CKs). Generally, auxin is produced by the primary shoot apex cells, which moves basipetally to inhibit the development of axillary buds. However, this inhibition is relieved by CKs produced in the nodal stem to enable the development of the lateral branches (Tanaka et al. 2006). Therefore, growth of the SAM and axillary buds is controlled positively and negatively via the CKs and auxins, respectively.



### **1.2.3 The nature of stem and its primary functions**

The stem serves a number of different primary functions such as a primary support, transport of water and nutrients to the leaves, sugar and other starches to other plant organs and water/carbohydrate storage (Givnish 1986). Stem water transport directly influences leaf water status and thus plant gas exchange and other leaf processes related to whole plant carbon gain (Sperry 1995). Although plant leaves are the dominant photosynthetic organ in most species, photosynthesis can also occur in stems, fruits, flowers, and (aerial) roots that contain chlorophyll. Among these photosynthetic organs, stems are the next most significant contributor (after leaves) to whole plant carbon gain (Nilsen 1995). Thus, stem growth regulation is important in adapting shoot growth to the aerial environment.

### **1.3 Soil moisture deficit and drought**

Drought is defined as a period without significant rainfall, which decreases soil water availability, often in conjunction with high aerial temperatures causing continuous loss of water through evapotranspiration. Thus, soil moisture content declines during drought. Drought stress is probably the most significant environmental constraint to plant productivity (Boyer 1982). Due to water scarcity issues, it has a huge impact on agricultural activity, which is a major user of water resources. The severity of drought effects on plants poses a challenge to better understand the effects of drought on plants, to mitigate its effects on cropping

systems (Passioura 2002). There is a need to understand and exploit plant stress responses to ensure drought tolerance, to maintain plant growth and productivity.

### **1.3.1 Plant responses to drought stress**

Plant water status declines when either the water supply to the roots is limited or the loss of water through transpiration is very high (Benjamin & Nielsen 2006). The severity of the damage caused by the drought is generally unpredictable as it is driven by various factors including, the rainfall patterns, moisture holding capacity of the soil, and water losses through evapotranspiration. Drought interferes with growth, nutrient and water relations, photosynthesis, assimilate partitioning and ultimately significantly reduces crop yields (Farooq et al. 2009; Praba et al. 2009).

Plant responses to drought stress vary between species and plant growth stage. Drought stress limits leaf, stem and root expansion, although the roots are less sensitive to drought than shoots (Westgate & Boyer 2010). Cell expansion may be constrained by insufficient carbohydrate availability (Boyer 1970; Boyle et al. 1991), plant water relations (turgor) (Boyer 1970) and chemical signalling (Gowing et al. 1990). All these factors are discussed further, to better understand the mechanisms by which they regulate plant growth.

## **1.4 What is regulating growth under drought stress?**

### **1.4.1 Carbohydrates**

Water deficits in plants induce stomatal closure and reduce photosynthesis (Chaves et al. 2009), thereby limiting carbohydrate production. However, drought caused leaf growth to cease before carbohydrate accumulation decreased in poplar (Bogeat-Triboulot et al. 2007), sunflower (Boyer 1970), maize (Tardieu et al. 1999) and Arabidopsis (Granier et al. 2006). This suggests that it is not a lack of photosynthate, but rather an inability to use that photosynthate, that is restricting growth. (Boyer 1970) suggested that decreased cell expansion under water deficit conditions was not caused by decreased photosynthesis. Similarly, a mild water stress decreased leaf expansion rate but did not affect photosynthesis (Tardieu et al. 1999). They confirmed that growth is much more sensitive to water limitation than photosynthesis, and consequently carbohydrates often accumulate in stressed plants, showing that growth reduction is not a consequence of carbon deficit (Muller et al. 2011). A different study suggested a higher accumulation of carbohydrate may also arise due to cellular dehydration, but leaves maintained at full turgor as the soil dried (via root pressurization) also had higher carbohydrate concentrations (Munns et al. 2000). All these studies provide evidence that utilization of the carbohydrates (rather than carbohydrate supply) restricts growth.

### 1.4.2 Turgor pressure and cell expansion

For plant cells to expand, their water uptake must exert sufficient turgor pressure to deform the cell walls. Thus, measuring turgor pressure links organ expansion to plant water status (Lockhart, 1965; Genard et al., 2001; Steppe et al., 2006). Lockhart (1965) first described mathematically the relationship between growth rate and turgor pressure. The equation states that the rate of cell wall expansion,  $i$  is equivalent to the difference between the actual pressure  $P$  and a critical threshold value ( $Y$ ) for the pressure if  $P > Y$ , multiplied by a coefficient,  $\phi$ , the cell wall extensibility. Turgor greater than this yield threshold is necessary to overcome mechanical constraints to cellular expansion, determined by the cell wall extensibility  $\phi$ . This is determined by cross-linking of cellulose microfibrils and pectin bonds in the cell wall, and can be mathematically determined as the slope of the relationship between  $i$  and  $P$ .

$$i = \phi(P - Y)$$

Cell expansion occurs when cellular solute concentrations are sufficiently high to extract water osmotically from their surroundings. This water uptake increases cell turgor pressure, causing irreversible cell wall enlargement. However, this enlargement decreases cellular solute concentrations, requiring continued solute import and water uptake to maintain turgor pressure. Low soil water availability can decrease cellular turgor, thereby constraining cell expansion (Westgate and Boyer, 1985).

### 1.4.3 Water potential

Although some studies showed linear relationships between leaf turgor and leaf expansion (Boyer, 1970), other studies provided conflicting results, with soil drying decreasing leaf growth whilst turgor was maintained in the maize leaf elongation zone, due to continued solute accumulation facilitating water uptake (Michelena & Boyer 1982). Such osmotic adjustment allows turgor maintenance in growing cells, even though bulk leaf water potential decreases with soil drying. While leaf water potential ( $\Psi_{\text{leaf}}$ ) may be correlated with leaf growth inhibition, the relationship may not be causative. Nevertheless, the water potential gradient between the growing cells and xylem water potential may prevent growth if xylem tension increases. Further experiments prevented any change in xylem water potential as the soil dried by pressuring the roots until the xylem sap was on the verge of bleeding (Termaat et al. 1985; Passioura 1988). In these studies, leaf elongation declined in response to soil drying in both pressurized and unpressurized plants. This suggests that water relations may not entirely explain the regulation of expansive growth, and instead it has been argued that a chemical message arising in roots may be important.

#### 1.4.4 Gibberellin and Auxin effects on cell wall extensibility

In addition to regulating cell division, phytohormones can regulate cell wall extensibility, which can limit plant growth. The model (Lockhart 1965) suggests that plant cell elongation occurs due to the irreversible, turgor-mediated yielding of cell walls. This concept of turgor-driven wall extension is relevant to the mechanism of action of hormones. Supplying GA<sub>3</sub> significantly increased cell wall extensibility of wild-type (WT) wheat plants but had no effect on isogenic (*Rht – reduced height*) genotypes which are gibberellic acid insensitive. Moreover, further application of ancymidol (inhibitor of gibberellin biosynthesis) diminished cell wall extensibility of WT plants (Keyes et al. 1990). Thus, gibberellin levels can mediate cell wall extensibility.

The walls of expanding tissues possess numerous protein activities that can modify cell wall mechanical properties, including endo-1,4β-glucanase (Hayashi et al. 1984) xyloglucan endotransglycosylase (Fry et al. 1992) and expansins (McQueen-Mason et al. 1992). Expansins are members of a large multigene family of extracellular proteins. Expansins increased *in vitro* cell wall extensibility thus mediating cell expansion (Li et al. 2002). Incubating stem segments in 0.05 mM GA<sub>3</sub> increased acid-induced cell wall extensibility in deep water rice (*Oryza sativa*), thus stimulating expression of *Os-EXP7*, *Os-EXP3*, and *Os-EXP4* genes in stem internodes (Cho & Kende 1997). In (*Lycopersicon esculentum* cv. Moneymaker), *LeEXP9* and *LeEXP2* expression correlated to *in vivo* stem elongation when treated with 1 mM GA<sub>3</sub> or without GA<sub>3</sub> in wildtype (WT) and the GA-deficient mutant

(*gib-1*). This mutant had 50% less expression of *LeExp9* than *LeEXP2*. However, applying GA<sub>3</sub> restored expression of both *LeEXP9* and *LeEXP2* to WT levels, indicating *LeEXP2* expression is GA-dependent (Vogler et al. 2003). These studies indicate that gibberellins mediate expansin gene expression to affect internode growth.

In addition to upregulating expression of expansin genes, (Perrot-Rechenmann 2010) auxin enhances cell wall extensibility via the acid growth hypothesis. Auxin stimulates activity of plasma membrane H<sup>+</sup>-ATPase proton (H<sup>+</sup>) pumps, thereby causing apoplastic acidification (pH 4.5-6) (Rayle & Cleland 1980; Arsuffi & Braybrook 2017). Isolated cell walls from growing cucumber hypocotyls were pre-incubated for 30 min at 4°C in buffers containing various cell wall inhibitors. Low temperatures and short times were used to minimize possible enzymatic activity, and able to examine pure physical effects. Incubating cucumber hypocotyls in pH 6.8 buffer decreased elastic and plastic extensibilities by 20% compared to incubation at pH 4.5 (Cleland 1987). These differences are assumed to be entirely physical, and whether other treatments produce equivalent changes in mechanical extensibilities. Although Al<sup>3+</sup>, Cu<sup>2+</sup> and Hg<sup>2+</sup> strongly inhibited cell wall creep, these cations reduced plastic extensibility by small, statistically insignificant amounts, including Cu<sup>2+</sup> that had little effect in elastic extensibility. Some treatment such as increased pH and Cu<sup>2+</sup> had significant effects on wall viscoelasticity, but others had little or no apparent effects, thus implicating an enzymatic creep mechanism. Sulfhydryl (SH) reducing agents strongly stimulated creep, by stabilizing cell wall

enzymes. The physical effects of these treatments on polymer interaction were examined by instron and stress-relaxation analyses. The sensitivity of creep to SH-reducing agents indicates that thiol reduction of wall enzymes might provide a control mechanism for endogenous cell growth (Cosgrove 1989).

These observations lead to the investigation of pH-dependent wall-loosening enzymes and the discovery of expansins (McQueen-Mason et al. 1992; Li et al. 1993). Relationships between expansins, acid growth and auxin been reported in several experiments, suggesting that auxin and expansin stimulate cell expansion via through the same pathway (Cosgrove 1989). Taken together, auxin and gibberellic acid are proposed to enhance cell wall extensibility, thereby promoting cell growth and expansion. Most of these studies of gibberellic acid (GA<sub>3</sub>) and indole 3 acetic acid (IAA) action were performed in hypocotyls, stem segments and intact, well-watered plants. However, whether GA<sub>3</sub> and IAA regulate stem elongation of pea plants in response to soil drying has not been analysed so far.



## 1.5 Plant hormones and shoot development under drought stress

Various experimental systems have aimed to break the nexus between soil drying and plant water status by maintaining  $\Psi_{\text{leaf}}$  as the soil is allowed to dry. One experimental system grew plants with their roots split between two containers with the soil in one container permitted to dry while the other container was well-watered. Apple plants were grown in divided pots, with half of root system exposed to soil drying, whilst another half was well-watered (Gowing et al. 1990). Withholding water from one of the pots for 24 days decreased daily leaf area increment by 65% and halved leaf initiation rate relative to control plants in which both pots were irrigated. No difference in leaf water status was detected between well-watered plants and those exposed to partial root drying plant. After 24 days, roots of some plants exposed to drying soil were re-watered, in another treatment these roots were excised and the remainder continued half dry. Re-watering and root excision significantly increased leaf growth relative to the group where half of the roots remained dry. Leaf growth inhibition was suggested to result from a positive root to shoot signaling of drying soil (Gowing et al. 1990).

Similarly, when water was withheld from plants grown in long soil columns to ensure roots explored a similar soil volume as in the field, leaf elongation rates decreased as the soil dried while the leaf water potential did not decline, suggesting that chemical signalling may regulate leaf expansion (Zhang & Davies 1989). Such studies have suggested that non-hydraulic signaling in plants can regulate responses to soil drying.

Phytohormones are chemical messengers produced in one part of the plant and translocated to the other parts, where they play critical roles in regulating plant responses to stress at extremely low concentrations. However, in contrast to animal hormones, they can also act locally at the site of their production in regulating plant growth and development, as well as stomatal movement (Davies 1987). The dependency of plant growth on phytohormone levels has been extensively studied (Saab et al. 1992; Sharp et al. 1994). These phytohormones include abscisic acid (ABA), gibberellins (GAs), auxin, cytokinin, ethylene, jasmonic acid and salicylic acid. It has been suggested that ABA is a stress signal that plays a central role in root to shoot hormonal communication in plants grown in drying soils (Zhang & Davies 1987)

### **1.5.1 Abscisic Acid (ABA)**

Although ABA biosynthesis and metabolism occurs in all tissues from roots to leaves, its occurrence in vascular tissues suggests it is transported throughout the plant (Boursiac et al. 2013). ABA can be accumulated in the roots via local biosynthesis, or taken up from soil water surrounding the roots, or delivered to the root from the shoots via phloem (Wilkinson & Davies 2002). ABA in the roots can then be loaded to the xylem (Hartung et al. 1996) with an increase of water flux across the root which promotes the transfer of ABA towards the xylem (Freundl et al. 2000). Xylem-borne ABA enters the leaf, causing a concentration-dependent stomatal closure (Trejo et al. 1995). Laboratory investigations with factorial combinations of ABA and polyethylene glycol (PEG—an osmoticum) showed a high

stomatal sensitivity to ABA in media of low water potential, indicating that the magnitude of stomatal response to ABA may be determined by epidermal water relations (Tardieu & Davies 1992). ABA biosynthesis occurs throughout the plant and is transported bi-directionally via the vascular system.

To better understand ABA's role in affecting plant physiology, and thus shoot and root expansion during water stress, further work has manipulated ABA concentrations *in vivo*. Exposing plants to low water potential decreased root and shoot elongation, while treatment with fluridone (an inhibitor of ABA synthesis) further inhibited root elongation but alleviated shoot growth inhibition (Saab et al. 1990). Thus, ABA accumulation maintains root elongation but inhibits shoot elongation of seedlings grown at low water potential. However, normal levels of endogenous ABA are required to maintain shoot growth (particularly leaf expansion), as the ABA-deficient *flacca* and *notabilis* mutants of tomato have reduced leaf area compared to wild-type (WT) plants, even when grown at a similar leaf water potential as WT plants (Sharp et al. 2000). Taken together, these results demonstrated strong evidence that ABA is necessary to maintain growth in both shoot and root responses to water stress.

However, applying exogenous (0.1  $\mu\text{M}$ ) ABA to intact bean (*Phaseolus vulgaris*) leaves by placing shoots into an ABA solution, or placing leaf discs into an ABA solution, inhibited cell enlargement (Van Volkenburgh & Davies 1983). ABA treated tissues had lower solute concentrations and decreased cell wall extensibility, thus

explaining growth inhibition. While many studies have considered regulation of leaf expansion by ABA, little attention has been given to possible ABA mediation of stem elongation when plants are grown in drying soil.

### 1.5.2 Gibberellins (GAs)

Gibberellins (GAs) are important in regulating cell division, cell elongation, germination, flowering, fruit size (Serrani et al. 2007) and particularly stem elongation (Achard et al. 2009). Higher endogenous GA<sub>1</sub> levels in internodal tissues is positively correlated with tallness. Dwarf mutant peas (*na* allele) have lower gibberellin concentrations than wild-type (*Na* allele) as gibberellin biosynthesis is completely blocked in the conversion of *ent*-Kaurene to GA<sub>12</sub>-aldehyde (Fig. 1.1) (Reid & Howell 1995). However, *nana* plants may still possess an active GA 3 $\beta$ -hydroxylase encoded by *Le* (Fig 1.1, 1.2), and therefore convert GA<sub>20</sub> to GA<sub>1</sub>. If a *nana* (*naLe*) shoot is grafted onto a dwarf *le* plant, the resulting plant is tall because the *nana* shoot tip can convert the GA<sub>20</sub> from the dwarf into GA<sub>1</sub>. The homozygous mutant (*nana*) achieved a height of only 1 cm at maturity suggesting that GA<sub>1</sub> is a biologically active gibberellin that regulates tallness in peas (Ingram et al. 1986; Davies 1995). Grafting the GA-deficient mutant *na-1* onto a wild-type rootstock increased stem length 10 fold relative to *na-1* self-grafts, indicating that root-synthesized GA can move from WT roots to the *na-1* shoot to stimulate stem elongation (Reid et al. 1983). Furthermore, in tomato (*Lycopersicon esculentum*), downregulating the GA biosynthesis pathway of four gibberellin mutants (*W182*, *A70*, *W270*, *W335*) which had a biochemical lesion in GA

biosynthesis pathway (Graebe 1987) prior to GA<sub>12</sub>-aldehyde (Fig. 1.3) causes decreased stem elongation (Koornneef et al. 1990). Thus, GAs have an intrinsic role in promoting pea stem growth.

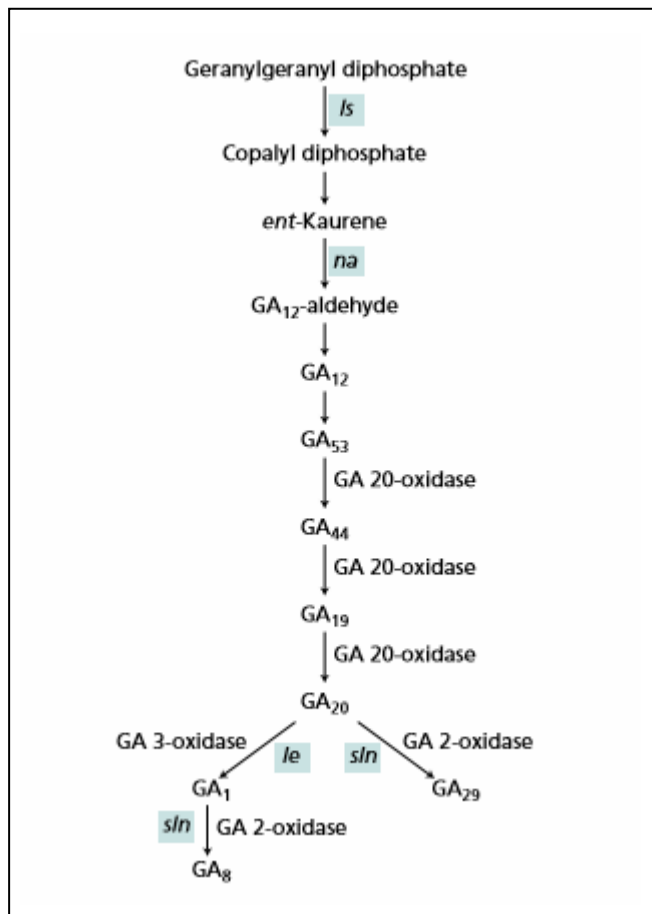


Figure 1.2 A portion of the gibberellin biosynthetic pathway showing the abbreviations and location of the mutant genes (in blue boxes) that block the pathway in pea and the enzymes involved in the metabolic steps after GA<sub>53</sub>.

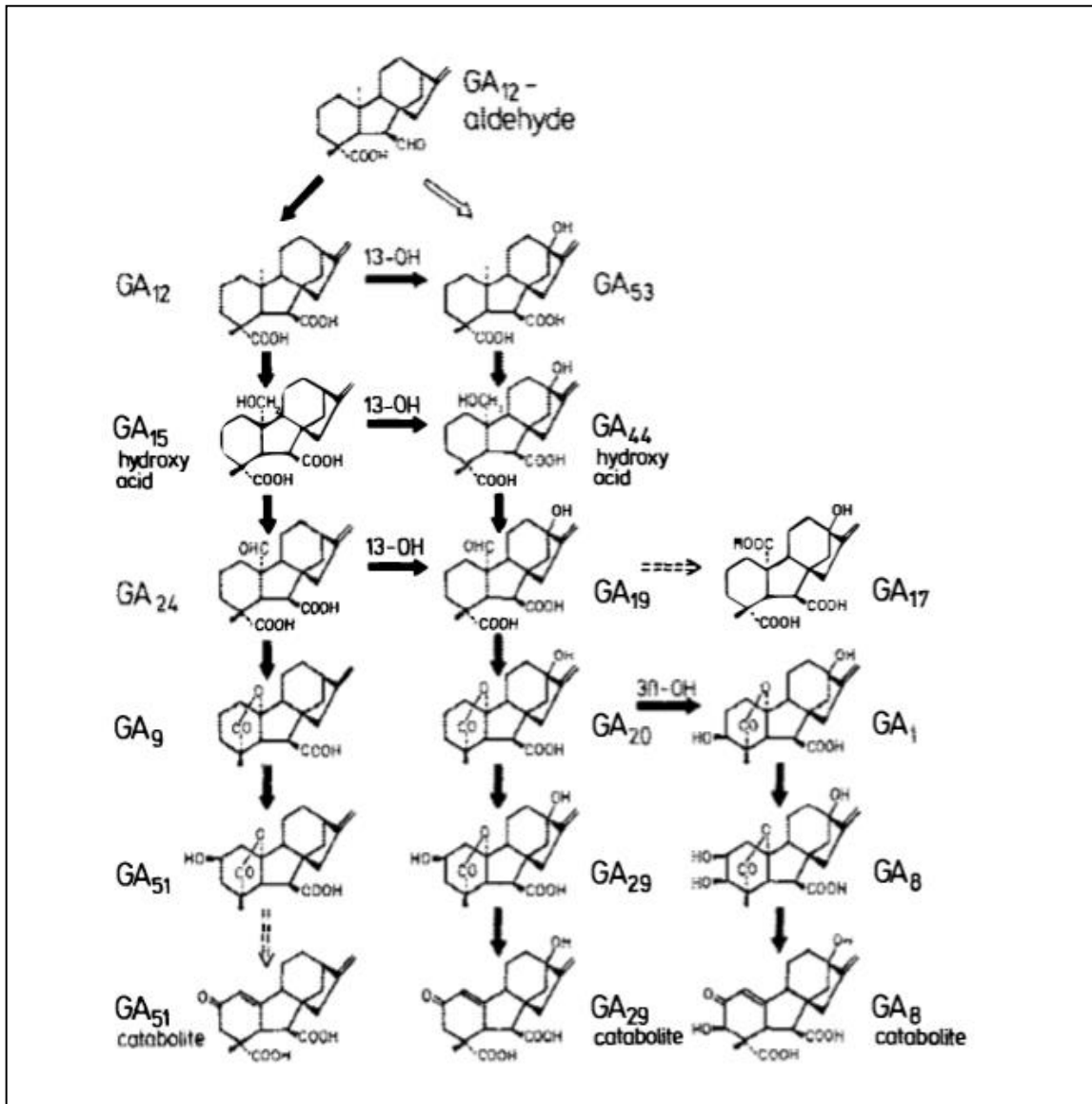


Figure 1.3 GA biosynthesis after GA<sub>12</sub>-aldehyde in *Pisum sativum* immature seeds and shoots. 3β -Hydroxylation only occurs in young, growing regions of the shoots and possibly in very young fruits. Broken line arrows show reactions not yet directly demonstrated (Graebe 1987).

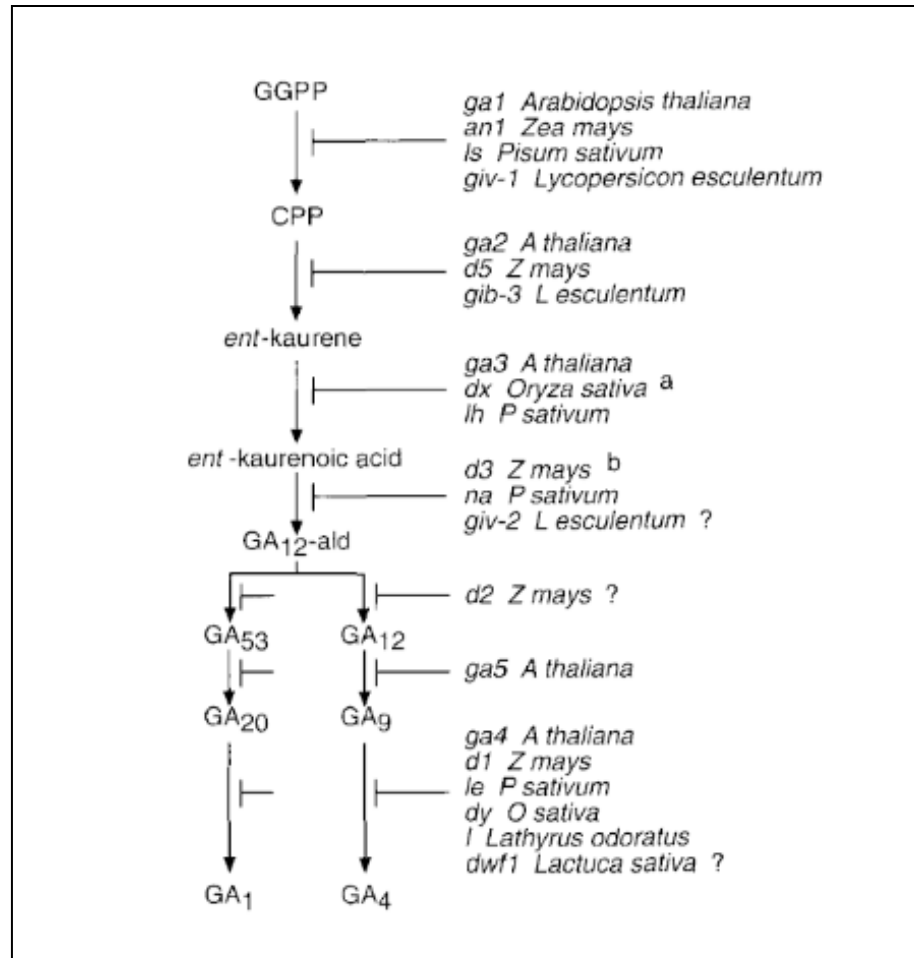


Figure 1.4 GA synthesis mutants and the biochemical steps at which they are blocked. These are single gene recessives in a homozygous state. A 'question mark' indicates that the position of the lesion is uncertain. While the wild-type (WT) gene is written in capital letters and italicized (e.g., *GA<sub>1</sub>*), the corresponding mutant is written in lowercase and italicized (e.g., *ga<sub>1</sub>*) (Koornneef et al. 1990). Note that GA<sub>x</sub> indicates the gibberellin produced where x is a number.

Drying soil decreased endogenous levels of gibberellins (Pandey et al. 2004) which may offer an adaptive advantage as smaller plants are able to tolerate and survive drought conditions (Li et al. 2012). Drought stress and GA deficiency cause similar phenotypes. Dwarfed plants with lower gibberellin concentrations have decreased stem elongation, plant height, leaf development, and limited flowering and fruit set development (Vettakkorumakankav et al. 1999; Olimpieri et al. 2011). Drought down-regulates the expression of genes involved in gibberellin's biosynthesis such as GA<sub>20</sub> oxidase enzymes (Zeevaart et al. 1993). However, it is uncertain whether applying gibberellins to droughted plants can partially reverse the negative effects of a prolonged drought.

### **1.5.3 Indole 3 acetic acid (IAA)**

Auxin is one of the phytohormones that regulates spatial and temporal aspects of plant growth, specifically cell division, enlargement and differentiation (Baker 2000). Auxin is also required as a signal between cells, tissues and plant organs via its basipetal movement from the shoot apex (Haga & Iino 1998). High exogenous auxin concentrations inhibit stem elongation, likely because endogenous concentrations are already optimal for growth (O'Neill & Ross 2002). The lack of IAA biosynthesis mutants makes it difficult to investigate the function of auxin in intact plants (Reid & Ross 2011). Instead, the application of auxin transport inhibitors (Ross 1998), shoot apex decapitation (Ross et al. 2000) or excised segments have been used to investigate the effects of auxin. Exogenous IAA concentrations (>0.1 mM) applied to intact, light-grown tall (cv. Alaska) and



dwarf (cv. Progress no. 9) pea increased internode growth 1- and 6-fold respectively. Interestingly, the initial endogenous auxin levels of tall pea (cv Alaska) were 7-8 fold higher than in dwarf (Progress no. 9) pea, suggesting that endogenous IAA is an important regulator of stem growth with more limiting effects in dwarf than in tall pea (Yang et al. 1993). Similarly, pea lines with differences in plant height show a positive relationship between IAA concentration and stem elongation of intact plants (Law & Davies 1990). Thus, auxin has dose-dependent effects on stem elongation depending on initial endogenous auxin levels.

#### **1.5.4 Hormone interactions and shoot growth**

Different plant hormones can interact to mediate stem elongation in intact *Pisum sativum* (Yang et al., 1996), with one hormone affecting tissue levels or sensitivity to another hormone. Applying 0.1 mM GA<sub>3</sub> to dwarf *lkb* (GA<sub>1</sub> levels are 84% of the WT - Lawrence et al. 1992) pea had limited effects compared to the positive stem elongation response of the *le* mutant (GA<sub>1</sub> levels only 8% of its WT - Ross et al., 1992), indicating that *lkb* is gibberellin-insensitive. GA<sub>3</sub> markedly promoted internode stem growth of *le* up to WT levels. In contrast, applying 0.2 mM IAA to dwarf *lkb* internodes strongly stimulated (8-fold increase) stem elongation to levels comparable to wildtype plants. However, the same IAA concentration applied to *le* had limited impact on stem elongation. Thus, indicating that GA<sub>3</sub> and auxin are essential in regulating stem elongation of intact pea plants. Moreover, even though GA<sub>3</sub> didn't promote internode growth in the absence of IAA in the *lkb* dwarf, combined GA<sub>3</sub> + IAA application enhanced 25% stem elongation more than

applying IAA only (Yang et al., 1996). Thus, auxin levels can mediate GA induction of internode stem elongation.

## 1.6 Aims of thesis

The main hypothesis of this research is that changes in phytohormone concentrations in actively growing tissues alter stem elongation as the soil dries. To test this hypothesis, pea (*Pisum sativum*) plants were used as a model species to study the impact of soil drying on the regulation of stem elongation. Pea was adopted as it has easy-to-measure, clearly defined internodes, and there are several mutants that show genetic variation in stem/internode elongation and/or phytohormone status. Although the literature suggests that phytohormones can control leaf and root growth (as discussed above), the phytohormonal regulation of stem growth as the soil dries has received little attention. This research proposes to close this gap by setting these objectives:

1. To determine whether drought-induced changes in phytohormone concentrations are correlated with stem elongation (Chapter 2).
2. To study whether endogenous ABA levels regulate stem elongation as the soil dries (Chapter 3).
3. To determine whether exogenous GA and IAA hormones can alleviate the inhibition of stem elongation of plants grown in drying soil (Chapter 4).

## Chapter 2

### Do phytohormones regulate stem elongation of droughted pea plants?

#### 2.1 Introduction

Drought can reduce cell expansion and division, limit nutrient uptake and transport, and change plant metabolism, including phytohormone metabolism and signalling (Soroushi et al. 2011). Several processes determine a plant's sensitivity to drought (Hsiao 1973). When plants experience drought, their growth rate slows, most likely due to a decrease in tissue water potential (Boyer 1970b). After this the stomata begin to close, which restricts transpiration and CO<sub>2</sub> assimilation rates (Jarvis & Slatyer 1970). As the stress continues, translocation of photosynthate from source to sink may be inhibited (Kakumanu et al. 2012; Watkinson et al. 2003), accumulation of abscisic acid (ABA) can occur, and cytokinin concentrations may substantially decrease (Pospíšilová et al. 2005). These responses are often considered to be survival mechanisms, though changes in plant biomass allocation (between leaves, stems and roots) can help the plant acclimatize to the severity of the water deficit.

The plant stem serves several primary functions, such as a structural support, bidirectional transport of water and sugars, carbohydrate storage, and even photosynthesis (Thomas & Paul 1995). Stem elongation is facilitated by cell division and expansion, and its time-integrated response (plant height) is often a simple and quantitative measure of drought stress (Alem et al. 2015; Hsiao 1973;

Nuruddin et al. 2003). The reduction in elongation becomes more severe as drought stress increases (Morales et al. 2015) but the regulatory physiological mechanisms are far from resolved.

The fundamental physiological measurement of plant water status is plant water potential ( $\Psi$ ). Plant water status depends on the water status of the soil layers surrounding the root system, the evaporative demand of the atmosphere, and canopy size. Both leaf water potential ( $\Psi_{\text{leaf}}$ ) (Jones 1990); (Meyer & Green 1980; Scholander et al. 1965) and stem water potential ( $\Psi_{\text{stem}}$ ) (Garnier & Berger 1985); (McCutchan & Shackel 1992) can be directly measured to assess the impacts of drought stress on tissue water status. Moreover,  $\Psi_{\text{leaf}}$  and  $\Psi_{\text{stem}}$  are also widely used in determining irrigation schedules (Jones 2004). Different plant species are described as isohydric ( $\Psi$  maintenance with soil drying) or anisohydric (decreased  $\Psi$  with soil drying) yet changes in phytohormone concentrations can occur in representatives of both behavior types. Pea has been described as isohydric (Lecoeur et al. 1995), although the magnitude of the  $\Psi$  response can depend on the severity of stress imposed and plant growth stage, with  $\Psi_{\text{leaf}}$  even increasing with soil drying (Belimov et al. 2009), presumably as a result of stomatal closure. This diversity of changes of  $\Psi$  in response to drying soil calls into question its importance as a regulator of physiological responses, drawing attention to the role of changes in endogenous phytohormone concentrations *in planta*.

Phytohormones are chemical messengers produced in one part of the plant and translocated to the other parts, where they play critical roles in regulating plant responses to stress at extremely low concentrations. They can also be locally synthesized and have local effects, where they affect plant growth, differentiation and development, as well as stomatal movement (Davies 1987), without requiring transport to be active. Changes in endogenous phytohormone concentrations may be proportional to changes of growth rate of the specific plant organ studied (Davies 1995; Trewavas 1991), and they may specifically regulate specific tissue growth rate.

Indole 3 acetic acid (IAA), gibberellins (GAs) and cytokinins (CKs) are known to stimulate shoot growth, while abscisic acid (ABA), ethylene (ETH) and jasmonic acid (JA) can inhibit shoot and root growth. However, in some cases phytohormones can have inconsistent effects, with growth responses being concentration-dependent. For example, both ABA and ethylene at low concentrations can stimulate growth (Nishizawa & Suge 1995; Lehman et al. 1996; Smalle et al. 1997) but be inhibitory at high concentrations (Kieber et al. 1993; Tanaka et al. 2013). Because of this complexity, it has been difficult to identify whether phytohormones regulate plant growth under drought stress. To date, a comprehensive evaluation of endogenous stem phytohormone concentrations and their relationship to stem elongation has not been undertaken. Therefore, this chapter explores the hypothesis that changes in phytohormone concentration are correlated with the sensitivity of stem

elongation to soil drying. A tall pea variety was chosen as a model plant since it has long internodes, allowing more accurate measurements of stem elongation.

## **2.2 Materials and methods**

### **2.2.1 Plant materials**

Pea (*Pisum sativum* cv. Alderman) seeds were germinated on moistened tissue paper and kept in the dark for 5 days. On the emergence of the plumule and radicle, uniform seeds were individually transplanted into round pots of 16 cm height x 13 cm diameter (1 L volume) containing a 1:1 (v:v) mixture of silica sand and growing substrate (John Innes No.2, J. Arthur Bowers). All plants were well watered for 7 days by replacing evapotranspirational losses (determined gravimetrically) daily and maintained in a controlled environment room with supplementary lighting (supplied by Osram 600w daylight bulbs) for 12 hours and 26°C/20°C minimum day/night temperature at the Lancaster Environment Centre.

### **2.2.2 Determination of soil moisture release curve**

Using the substrate described above, 2 treatments were imposed on empty (without a plant) well-watered pots (drainage allowed after watering to the drip point) by either retaining the pots as a well-watered control or withholding water by replacing water to initial value of pot weight. Pots were placed in the controlled environment room described above. Both treatments were imposed for 10 days and 20 mg of soil mixture, 5 cm from the soil surface, were sampled daily. During sampling, the soil mixture was immediately mounted on clean sample holders



and wrapped in aluminium foil to prevent water loss. Seven replications of each treatment were collected, unwrapped and loaded into C52 chambers (Wescor Inc, Logan, UT, USA). Samples were incubated for 2 hours, then the voltages were read by a microvolt meter (Model HR-33T, Wescor Inc, Logan, UT, USA). Voltage data were converted into water potentials based on calibration with salt solutions of known osmotic potential, and a soil moisture release curve fitted as in Fig. 2.1.

To estimate soil water content using a gravimetric method, pot weights were measured every day before irrigation until end of the experiment. Then, the soils (including roots) were removed from the pots, weighed, oven dried for 60-72 hours at 60°C and reweighed. The soil water content was expressed on a mass basis as follows:

Weight of water = Weight of wet soil – Weight of dry soil

$$\text{Soil water content} = \frac{\text{Weight of water (g)}}{\text{Weight of dry soil (g)}}$$

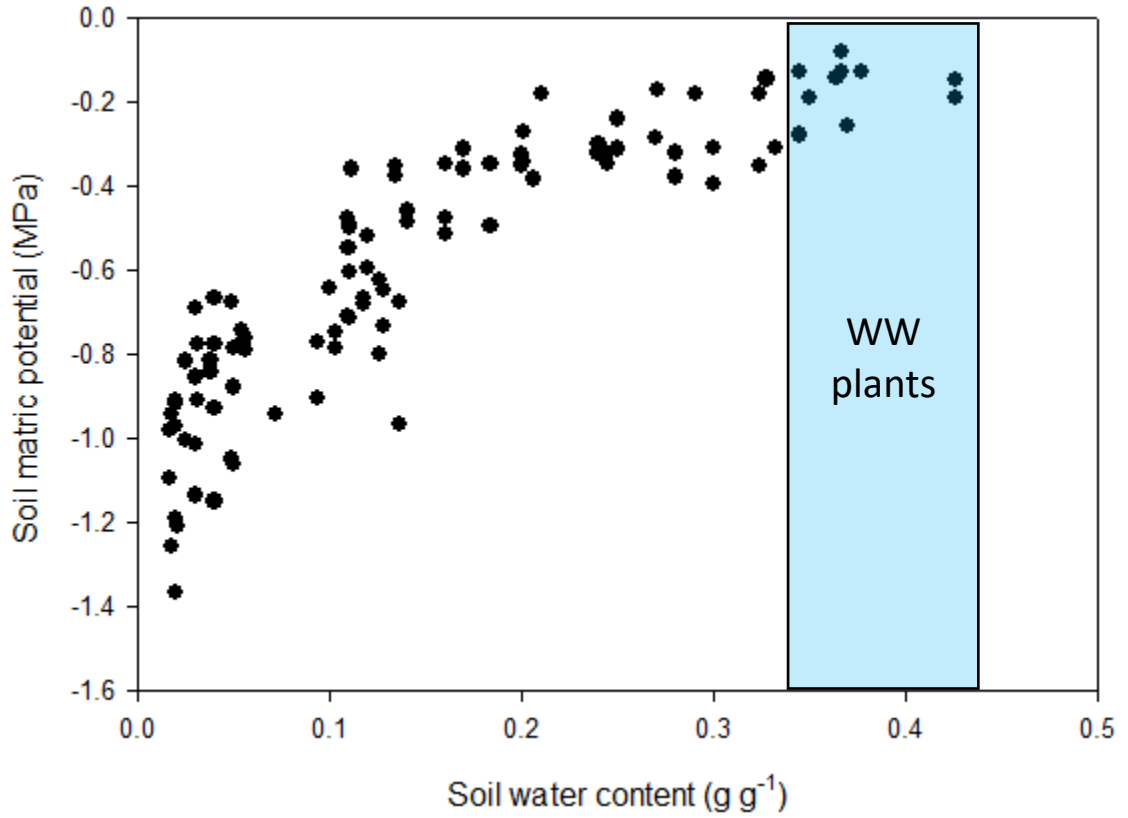


Figure 2.1 Soil moisture release curve. Each point is an individual soil sample. The blue box indicates the range of well-watered (WW) plants over 10 days of treatment with n=10 per day.

### 2.2.3 Comparing leaf water potential and stem water potential values

Leaf water potential ( $\Psi_{\text{leaf}}$ ) was measured on mature, fully expanded transpiring leaves which remained exposed to light for up to 4 hours. Alternatively, stem water potential ( $\Psi_{\text{stem}}$ ) was determined by measuring a non-transpiring, adjacent mature leaf that had been enclosed in aluminium foil and a sealed polyethylene bag for 1, 2, 3 and 4 hours. Enclosing the leaf prior to measuring its water potential ( $\Psi$ ) prevented leaf transpiration and allowed it to equilibrate with the stem water potential (Begg & Turner 1970).

Leaf discs (8 mm diameter) were punched from leaves, and immediately mounted on clean sample holders and wrapped in aluminium foil to prevent water loss. Once nine leaf discs had been collected that had either been enclosed or not (18 in total), they were unwrapped and then loaded into C52 chambers (Wescor Inc, Logan, UT, USA), incubated for 2-3 hours, then voltages were read by a microvolt meter (Model HR-33T, Wescor Inc, Logan, UT, USA). Voltage data were converted into water potentials based on calibration with salt solutions of known osmotic potential. Thus, enclosing the leaf for 2 hours was adequate to achieve an equilibrium  $\Psi_{\text{stem}}$  (Fig. 2.2). Measurements were made between 11:00-2:00 pm.

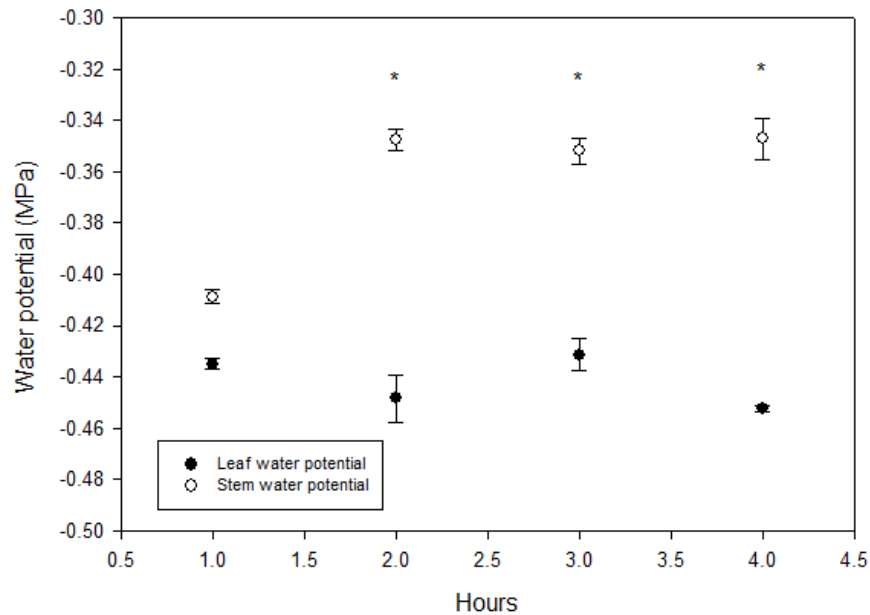


Figure 2.2 Changes in leaf water potential and stem water potential with incubation time. Data are means  $\pm$  S.E of 9 replicate plants. Asterisks indicate significant ( $P < 0.05$ ) differences between leaf water potential and stem water potential.

#### 2.2.4 Stem elongation and stem sampling

Stem elongation was measured daily at 3:00-4:00pm hours by monitoring internode length using a flexible ruler, with stem elongation rates calculated as  $\text{mm day}^{-1}$ . The first internode (at the top of the plant) was measured as the length of stem from the first open stipule to the apical closed stipule and the second internode was the next lowest one (Fig. 2.3). The first and second stem internodes were regularly changed over time as the plants grew, when growth of the second internode ceased. When second internode elongation ceased, the

original first internode was designated as the second internode, while the next uppermost internode became the first internode.

Stem samples were taken every day between 2:00-3:00 pm from the most actively growing internodes (see Fig. 2.3). The first and second internode comprised *circa* 10 mg and 20 mg of fresh weight, respectively.

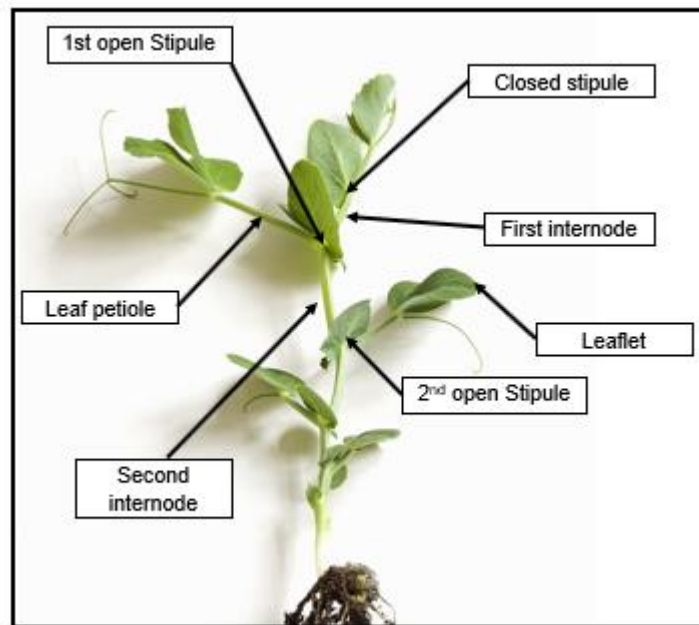


Figure 2.3 Image of a pea plant, showing the first and second internode

### **2.2.5 Irrigation treatment**

Seven days after transplanting, two different treatments were imposed. Each day, well-watered plants received 100% of the previous day's mean evapotranspiration (100% ET) while water was withheld from the other treatment (0% ET=no irrigation). Prior to imposing the treatments, all pots were irrigated (to the drip point) and allowed to drain for 24 hours. Irrigation was applied daily at 4:00-5:00 pm, according to plant requirements. Treatments were randomly arranged in the controlled environment room.

### **2.2.6 Measurement regime**

Each pot was weighed daily, to determine evapotranspiration (to implement well-watered conditions) and to estimate soil water content using a gravimetric method. Pot weights were measured every day before irrigation until the end of the experiment. Then, the soils (including roots) were removed from the pots, weighed, oven dried for 60-72 hours at 60°C and reweighed. The soil water content was expressed on a mass basis as follows:

Weight of water = Weight of wet soil – Weight of dry soil

Soil water content =  $\frac{\text{Weight of water (g)}}{\text{Weight of dry soil (g)}}$

### 2.2.7 Hormone extraction and analysis

Dried stem samples were prepared in Lancaster University, then sent for phytohormonal extraction and analysis conducted by Dr Alfonso Albacete at Department of Plant Nutrition, Campus Universitario de Espinardo, Murcia, Spain. Cytokinins (*trans*-zeatin, *tZ*, zeatin riboside, ZR and isopentenyl adenine, iP), gibberellins (GA<sub>1</sub>, GA<sub>3</sub> and GA<sub>4</sub>), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to Albacete et al. 2008 with some modifications. Plant material (0.1 g FW) was homogenized in liquid nitrogen and dropped in 0.5 ml of cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20 000 *g*, 15 minutes) and re-extracted for 30 minutes at 4°C in an additional 0.5 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C<sub>18</sub> cartridge (SepPak Plus, Waters, 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 membrane (Millipore, Bedford, MA, USA). Ten µl of filtrated extract were injected in a U-HPLC-MS system consisting of 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). For quantification of the plant hormones, calibration curves were constructed for each analyzed component (1, 10, 50, and 100  $\mu\text{g l}^{-1}$ ) and corrected for 10  $\mu\text{g l}^{-1}$  deuterated internal standards. Recovery percentages ranged between 92 and 95%.

### **2.2.8 Statistical Analysis**

Stem elongation, estimated soil water content (from pot weight measurements) and stem water potential were measured from the same plants. Stem samples for hormone analysis were taken periodically (Days 1, 3, 4 and 11 for well-watered plants; Days 1, 2, 3, 4, 5, 6, 8, 9 and 11 for water-stressed plants). Regression analysis determined whether experimental duration affected stem elongation, soil and plant water status, and hormone concentrations (Table 2.1) in both treatment groups. If duration had no significant effect on the variable of interest (which occurred in well-watered plants), an average value was calculated as the well-watered 'baseline'. Measurements from water-stressed plants were compared with this baseline on a daily basis via Student's unpaired t-test. This type of analysis was conducted because it was not economically viable (or practical to grow sufficient plants in a limited floorspace) to analyse hormone concentrations from both treatments on each day of the experiment. Regression analysis was used to determine significant relationships between soil water content, plant water status and plant variables (Table 2.2). Non-linear regressions were done separately using segmented analysis in R Software



(Version 3.4.1) that provided an estimate breakpoint/threshold for the decline in first internode stem elongation in response to decreased soil water content (Fig. 2.6A).

	P value	
	Well-watered	Water stress
First internode stem elongation	0.84	<.0001
Second internode stem elongation	0.94	<.0001
Stem water potential	0.68	<.0001
Soil water content	0.39	<.0001
<b>First internode stem tissue</b>		
ACC (1-Aminocyclopropane-1-carboxylic acid)	0.90	<.0001
Cytokinin (trans-Zeatin)	0.38	<.0001
Cytokinin (Isopentenyladenine)	0.60	<.0001
Gibberellin A1	0.41	<.0001
Gibberellin A3 or gibberellic acid	0.20	<.0001
Gibberellin A4	0.82	0.03
Auxin (Indole-3-acetic acid)	0.21	<.0001
ABA (Abscisic acid)	0.92	<.0001
Jasmonic acid	0.06	0.09
Salicylic acid	0.66	<.0001
<b>Second internode stem tissue</b>		
ACC (1-Aminocyclopropane-1-carboxylic acid)	0.51	<.0001
Cytokinin (trans-Zeatin)	0.11	<.0001
Cytokinin (Isopentenyladenine)	0.17	<.0001
Gibberellin A1	0.67	<.0001
Gibberellin A3 or gibberellic acid	0.78	<.0001
Gibberellin A4	0.18	0.80
Auxin (Indole-3-acetic acid)	0.81	<.0001
ABA (Abscisic acid)	0.14	<.0001
Jasmonic acid	0.16	0.31
Salicylic acid	0.54	0.20

Table 2.1 P-value of regression analysis for different variables (first and second internode stem elongation, soil water content, stem water potential, first and second internode stem hormone concentrations) with time for well-watered plants and those exposed to drying soil.

## 2.3 Results

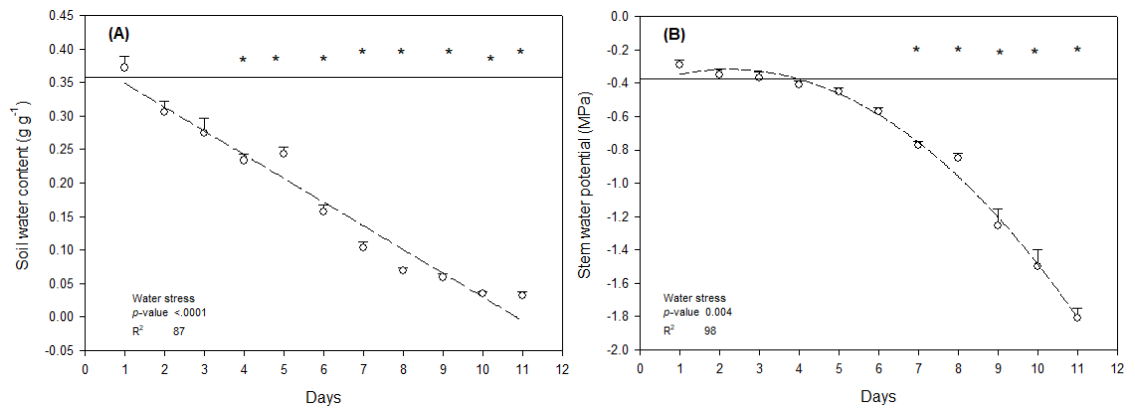


Figure 2.4 (A) Soil water content ( $\theta$ ) and (B) Stem water potential ( $\Psi_{\text{stem}}$ ) of water stressed (hollow circle) pea plants. Data are means  $\pm$  SE of 5 replicates, with linear (A) or second order (B) regressions fitted (dashed lines). The straight lines for well-watered plants represent the average response, when no significant differences were detected over time (Table 2.1). Asterisks indicate significant ( $P < 0.05$ ) differences between well-watered and water stressed treatments.

Soil water content and stem water potential of well-watered plants were stable over time and averaged  $0.36 \text{ g g}^{-1}$  and  $-0.39 \text{ MPa}$ , respectively. After withholding water, soil water content ( $\theta$ ) and stem water potential ( $\Psi_{\text{stem}}$ ) significantly decreased after 4 and 7 days respectively, compared to the well-watered plants (Fig. 2.4A and Fig. 2.4B), reaching  $0.006 \text{ g g}^{-1}$  and  $-1.81 \text{ MPa}$  respectively, at the end of the experiment.

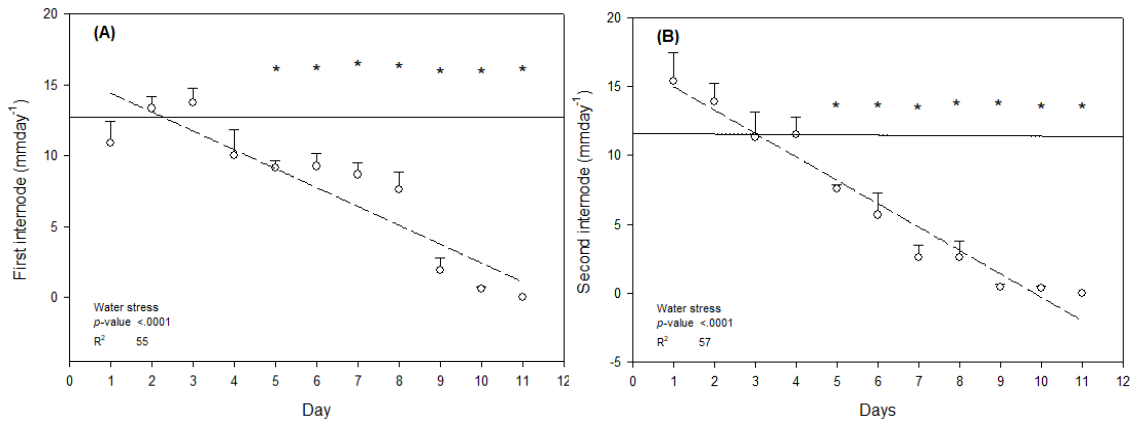


Figure 2.5 (A) First and (B) Second internode stem elongation of well-watered of water stressed (hollow circle) pea plants. Data are means  $\pm$  SE of 5 replicates, with linear regressions fitted (dashed lines). The straight lines for well-watered plants represent the average response, when no significant differences were detected over time (Table 2.1). Dashed lines in (A) and (B) represent linear regressions through the water stressed data. Asterisks indicate significant ( $P < 0.05$ ) differences between well-watered and water stressed treatments.

First and second internode stem elongation of well-watered plants averaged  $12.7 \text{ mm day}^{-1}$  and  $11.7 \text{ mm day}^{-1}$ , respectively. Soil drying significantly reduced first (Fig. 2.5A) and second internode (Fig. 2.5B) stem elongation rate by 28% and 34% respectively, 5 days after withholding water. First and second internode stem elongation ceased after 11 and 9 days of withholding water, respectively.

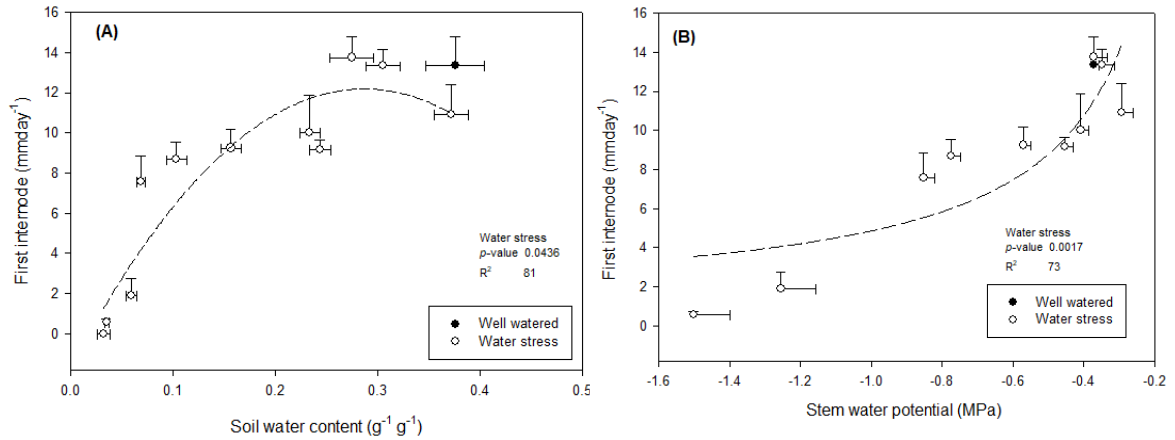


Figure 2.6 First internode stem elongation plotted against (A) Soil water content and (B) Stem water potential for well-watered (filled circle) and water stressed (hollow circle) pea plants. Dashed lines in (A) and (B) represent regressions through the water stressed data. Data are means  $\pm$  SE of 5 replicates (water stress) and 20 replicates (well-watered-average of 4 days (Day 1, 3, 4 and 11) measurement).

For well-watered plants, soil water content averaged  $0.36 \pm 0.03 \text{ g g}^{-1}$ , with primary and secondary stem elongation averaging  $12.7 \pm 1.4$  and  $11.7 \pm 1.9 \text{ mm day}^{-1}$  (Fig. 2.6A and Fig. 2.7A). First internode stem elongation of plants in drying soil showed a curvilinear relationship with soil water content ( $\theta$ ), decreasing rapidly when below  $0.08 \text{ g g}^{-1}$  (Fig 2.6A). First internode stem elongation decreased with little change in stem water potential as determined from a segmented analysis ( $\Psi_{\text{stem}}$ ) (Fig. 2.6B; Table 2.2). Second internode stem elongation decreased with curvilinear relationship with decline in  $\theta$  and  $\Psi_{\text{stem}}$  (Fig. 2.7A, B; Table 2.2). Elongation of first and second internodes was similarly

sensitive to both soil and plant water status, since ANCOVA revealed no significant stem type x water status interactions.

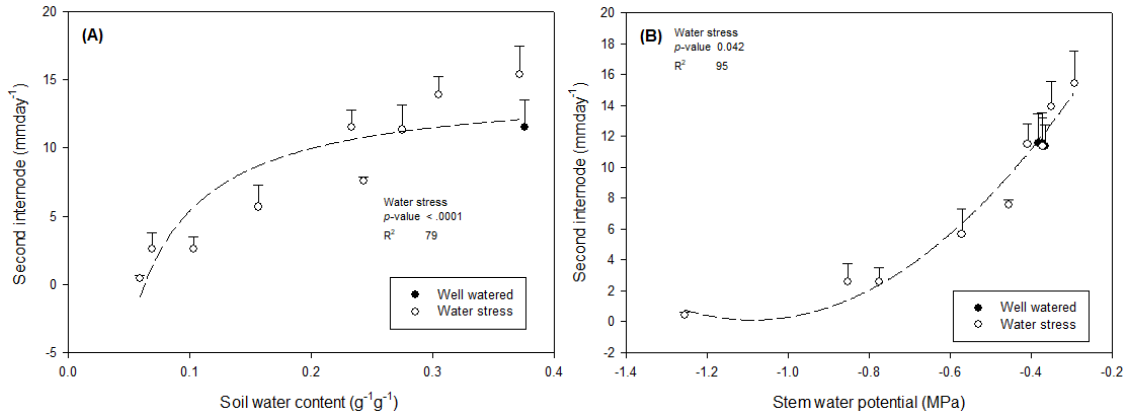


Figure 2.7 Second internode stem elongation plotted against (A) Soil water content and (B) Stem water potential for well-watered (filled circle) and water stressed (hollow circle) pea plants. Dashed lines in (A) and (B) represent regressions through the water stressed data. Data are means  $\pm$  SE of 5 replicates (water stress) and 20 replicates (well-watered-average of 4 days (Day 1,3, 4 and 11) measurement. Results from regression analysis (*P*-values) of water stress treatment are reported in each panel with regressions fitted where significant.

<b>Internode</b>	<b>BP of stem elongation decreased at no difference in stem water potential</b>	<b>Standard error</b>
<b>First</b>	-0.53	0.02
<b>Second</b>	-0.47	0.006

Table 2.2 Results of segmented analysis demonstrating a breakpoint (BP) threshold stem water potential when first and second internode stem elongation significantly decreased as the soil dried. BP values are reported along with the standard error of mean.

	<b><i>P</i>-value</b>	
	Well-watered	Water stress
<b>Soil water content</b>		
First internode stem elongation	0.15	0.04
Second internode stem elongation	0.95	<.0001
<b>Stem water potential</b>		
First internode stem elongation	0.43	0.01
Second internode stem elongation	0.97	0.04

Table 2.3 *P*-value of regression analysis between soil water content and stem water potential against first and second internode stem elongation.

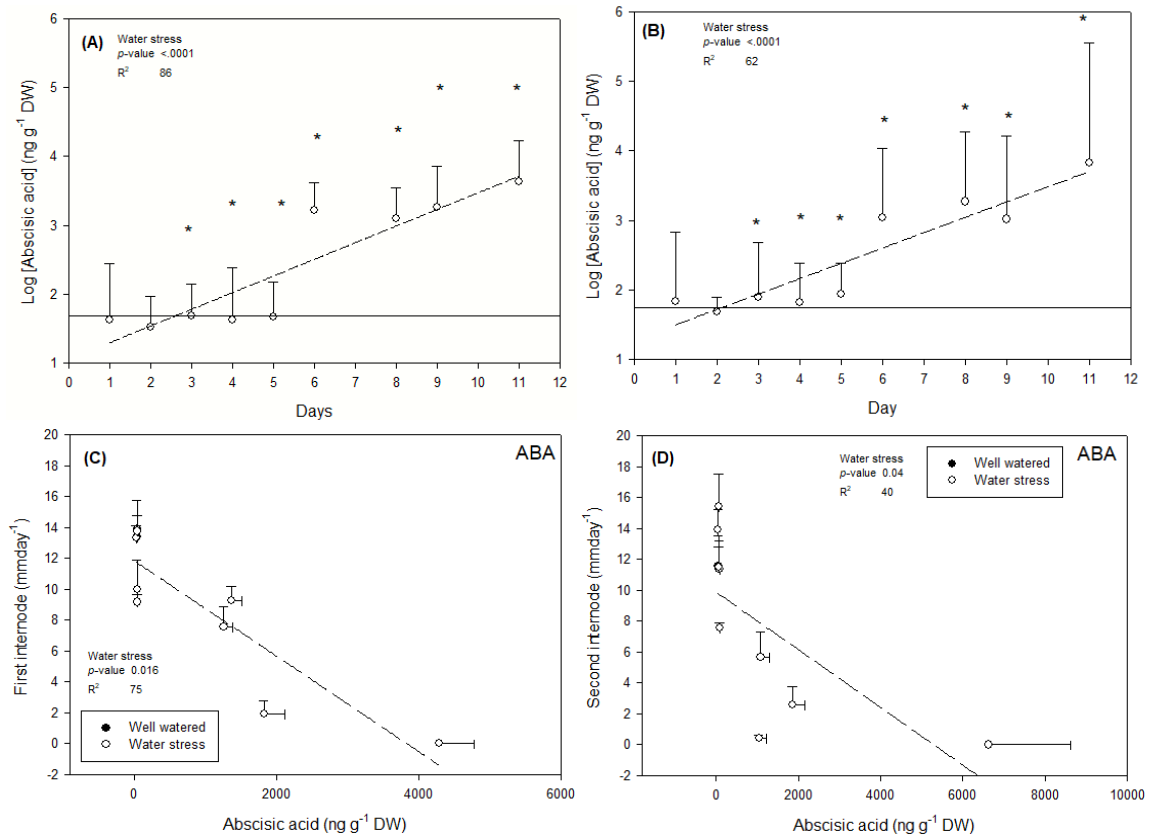


Figure 2.8 First (A) and second (B) internode stem tissue ABA concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue ABA concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.



First and second internodes of well-watered plants maintained stable endogenous ABA levels throughout the 11 days (Table 2.1), averaging  $51 \pm 3$  and  $54 \pm 3$  ng g<sup>-1</sup> DW respectively (Fig. 2.8A and 2.8B). Soil drying significantly increased endogenous ABA levels relative to well-watered plants, as early as Days 3 in first and second internode stems respectively (Fig. 2.8A and 2.8B), and these continued to increase thereafter. Increased first and second internode stem ABA concentrations were correlated with decreased first ( $r^2=75\%$ ) and second internode ( $r^2=40\%$ ) stem elongation respectively (Fig. 2.8C and 2.8D). Elongation of first and second internodes was similarly sensitive to stem ABA concentrations (Table 2.4), since ANCOVA revealed no significant stem type x ABA concentration interaction.

	<b><i>P-value</i></b>		
	<b>Stem tissue</b>	<b>Tissue hormone concentration</b>	<b>Interaction</b>
ACC (1-Aminocyclopropane-1-carboxylic acid)	0.79	<.0001	0.20
Cytokinin (trans-Zeatin)	0.36	0.02	0.21
Cytokinin (Isopentenyladenine)	0.96	<.0001	0.19
Gibberellin A1	0.20	<.0001	0.01
Gibberellin A3 or gibberellic acid	0.74	<.0001	0.31
Gibberellin A4	0.97	0.06	0.36
Auxin (Indole-3-acetic acid)	0.47	<.0001	0.60
ABA (Abscisic acid)	0.36	<.0001	0.28
Jasmonic acid	0.27	0.88	0.24
Salicylic acid	0.24	0.05	0.16

Table 2.4 ANCOVA analysis (*P*-values reported) examined whether first and second internodes showed different relationships between stem elongation and tissue hormone concentrations as in Fig. 2.8 to 2.17. A significant interaction term indicates a difference between stem types.

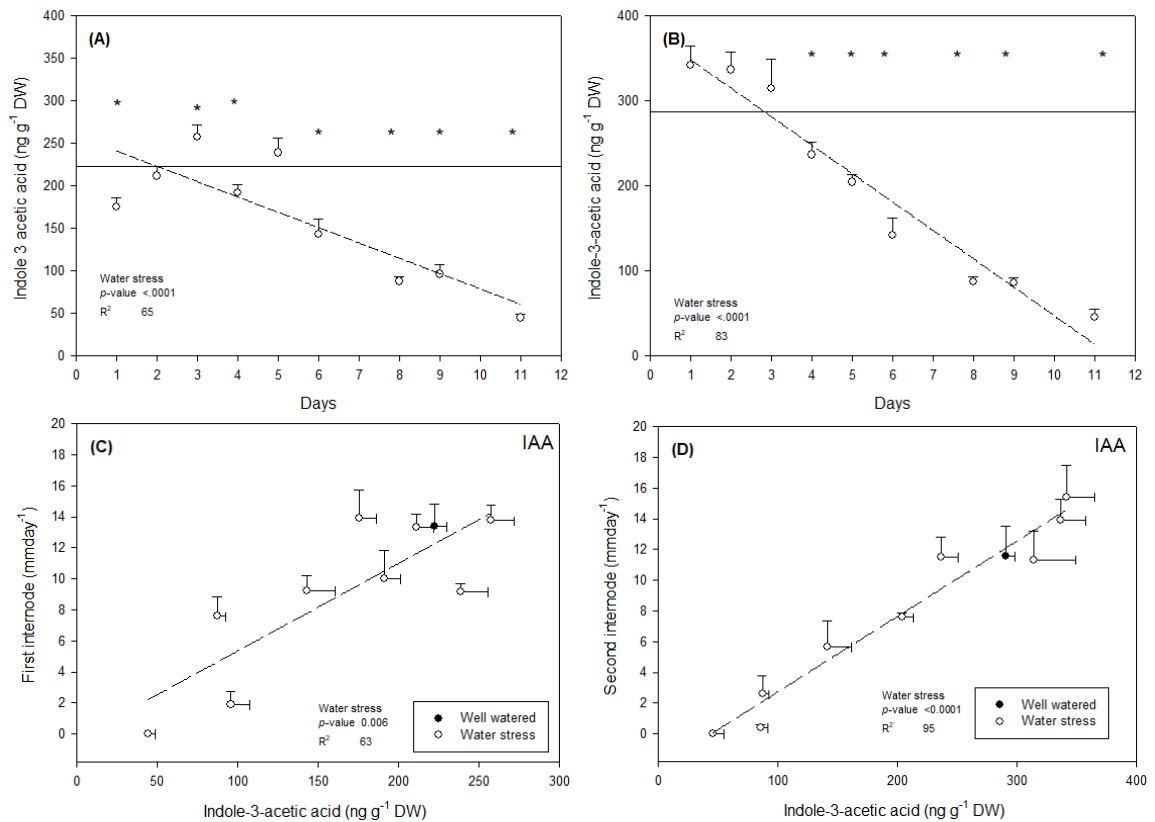


Figure 2.9 First (A) and second (B) internode stem tissue IAA concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue IAA concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internodes of well-watered plants maintained stable endogenous indole 3 acetic acid (IAA) levels throughout the 11 days (Table 2.1), averaging  $226 \pm 8$  and  $291 \pm 8$  ng g<sup>-1</sup> DW respectively (Fig. 2.9A and 2.9B). Soil drying significantly reduced endogenous IAA level concentrations relative to well-watered plants as early as Days 1 and 4 in first and second internode stems respectively (Fig. 2.10A and 2.10B), and these continued to decrease thereafter. Decreased first and second internode stem IAA concentration was correlated with decreased primary ( $r^2=63\%$ ) and secondary ( $r^2=95\%$ ) stem elongation (Fig. 2.9C and 2.9D). Elongation of first and second internodes was similarly sensitive to stem IAA concentrations (Table 2.4), since ANCOVA revealed no significant stem type x IAA concentration interaction.

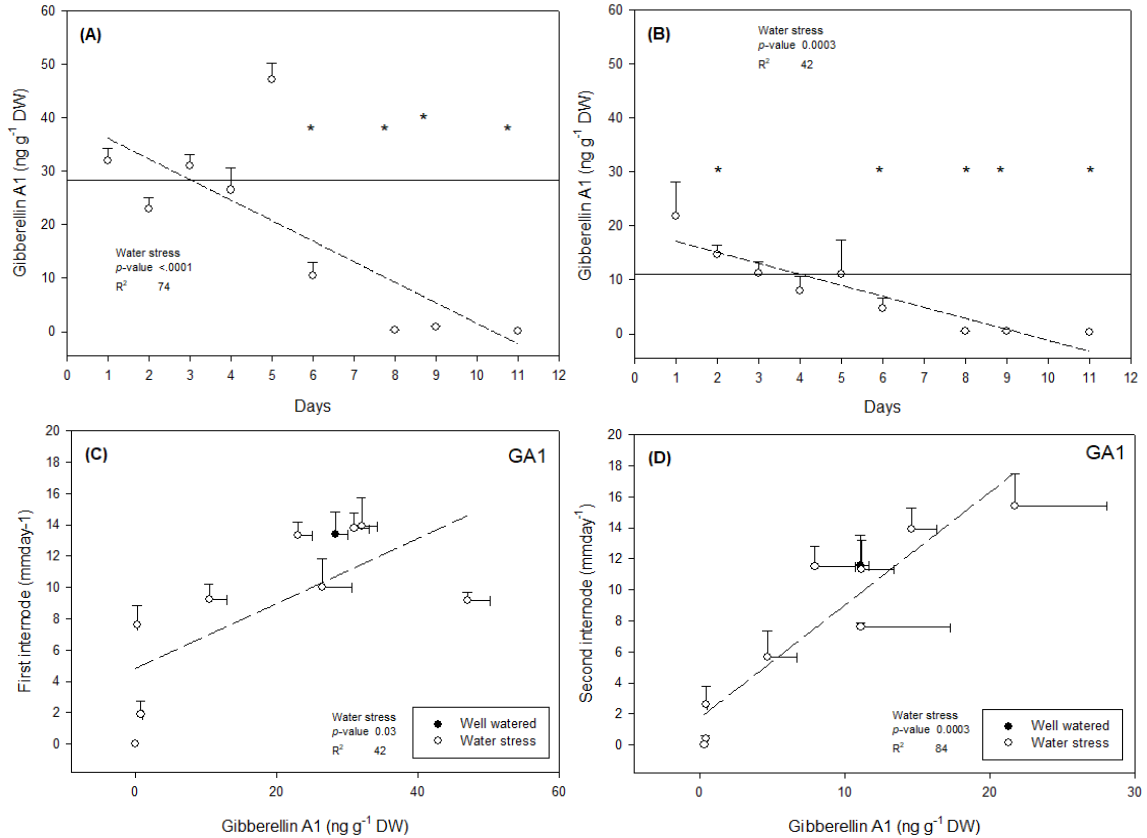


Figure 2.10 First (A) and second (B) internode stem tissue GA<sub>1</sub> concentrations of water stressed (hollow circle) plants over time. Primary (C) and secondary (D) stem elongation plotted against stem tissue GA<sub>1</sub> concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internodes of well-watered plants maintained stable Gibberellin A1 (GA<sub>1</sub>) levels throughout the 11 days (Table 2.1), averaging  $28.4 \pm 1.7$  and  $11.1 \pm 0.5$  ng g<sup>-1</sup> DW respectively. Soil drying significantly decreased endogenous GA<sub>1</sub> levels relative to well-watered plants, as early as Day 6 in both primary and secondary stems respectively (Fig. 2.10A and 2.10B), and these continued to decline thereafter. Decreased primary and secondary stem GA<sub>1</sub> concentrations were correlated with decreased first ( $r^2=42\%$ ) and second ( $r^2=84\%$ ) internode stem elongation respectively (Fig. 2.10C and 2.10D). Elongation of second internodes was more sensitive to declining stem GA<sub>1</sub> concentrations (Table 2.4), since ANCOVA revealed a significant stem type x GA<sub>1</sub> concentration interaction.

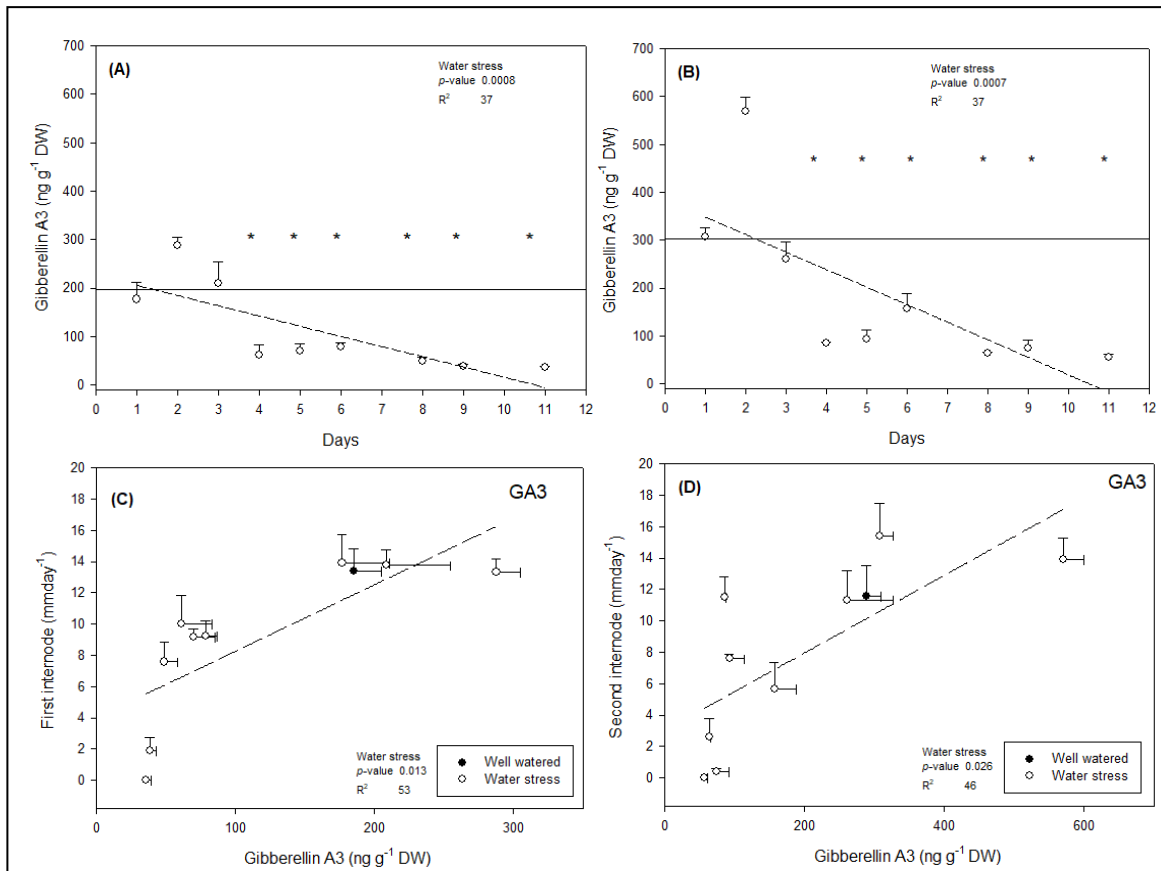


Figure 2.11 First (A) and second (B) internode stem tissue GA<sub>3</sub> concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue GA<sub>3</sub> concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internodes of well-watered plants maintained stable endogenous Gibberellin A3 (GA<sub>3</sub>) concentrations throughout the 11 days (Table 2.1), averaging  $185 \pm 19$  and  $288 \pm 21$  ng g<sup>-1</sup> DW respectively (Fig. 2.11A and 2.11B). Soil drying significantly decreased endogenous GA<sub>3</sub> levels relative to well-watered plants, as early as Day 4 in both first and second stems respectively (Fig. 2.11A and 2.11B), and these continued to decrease thereafter. Decreased first and second internode stem GA<sub>3</sub> concentrations were correlated with decreased first ( $r^2=53\%$ ) and second ( $r^2=46\%$ ) internode stem elongation respectively (Fig. 2.11C and 2.11D). Elongation of first and second internodes was similarly sensitive to stem GA<sub>3</sub> concentrations (Table 2.4), since ANCOVA revealed no significant stem type x GA<sub>3</sub> concentration interaction.



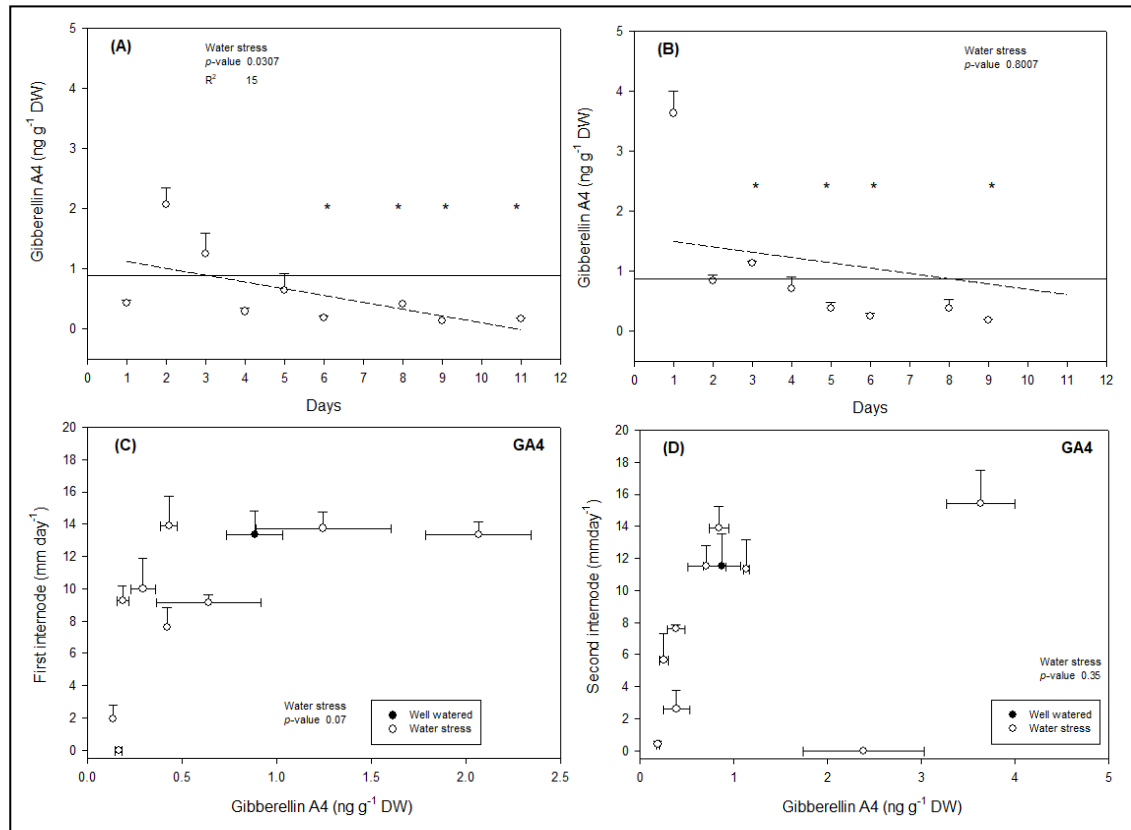


Figure 2.12 First (A) and second (B) internode stem tissue GA<sub>4</sub> concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue GA<sub>4</sub> concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight line for well-watered plants represents an average response where there was no significant change with time. Dashed lines in (A) and (B) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internodes of well-watered plants maintained stable endogenous GA<sub>4</sub> levels throughout the 11 days (Table 2.1), averaging  $0.88 \pm 0.15$  and  $0.87 \pm 0.20$  ng g<sup>-1</sup> DW respectively (Fig. 2.12A and 2.12B). Soil drying significantly decreased endogenous GA<sub>4</sub> levels relative to well-watered plants, as early as Days 6 and 5 in first and second stems respectively (Fig. 2.12A and 2.12B), and these continued to decrease thereafter. First and second internode stem GA<sub>4</sub> concentration were not correlated with first and second internode stem elongation (Fig. 2.12C and 2.12D). Considering the entire data set, there was a tendency ( $P=0.06$ ) for internode elongation to be correlated with stem GA<sub>4</sub> concentrations.

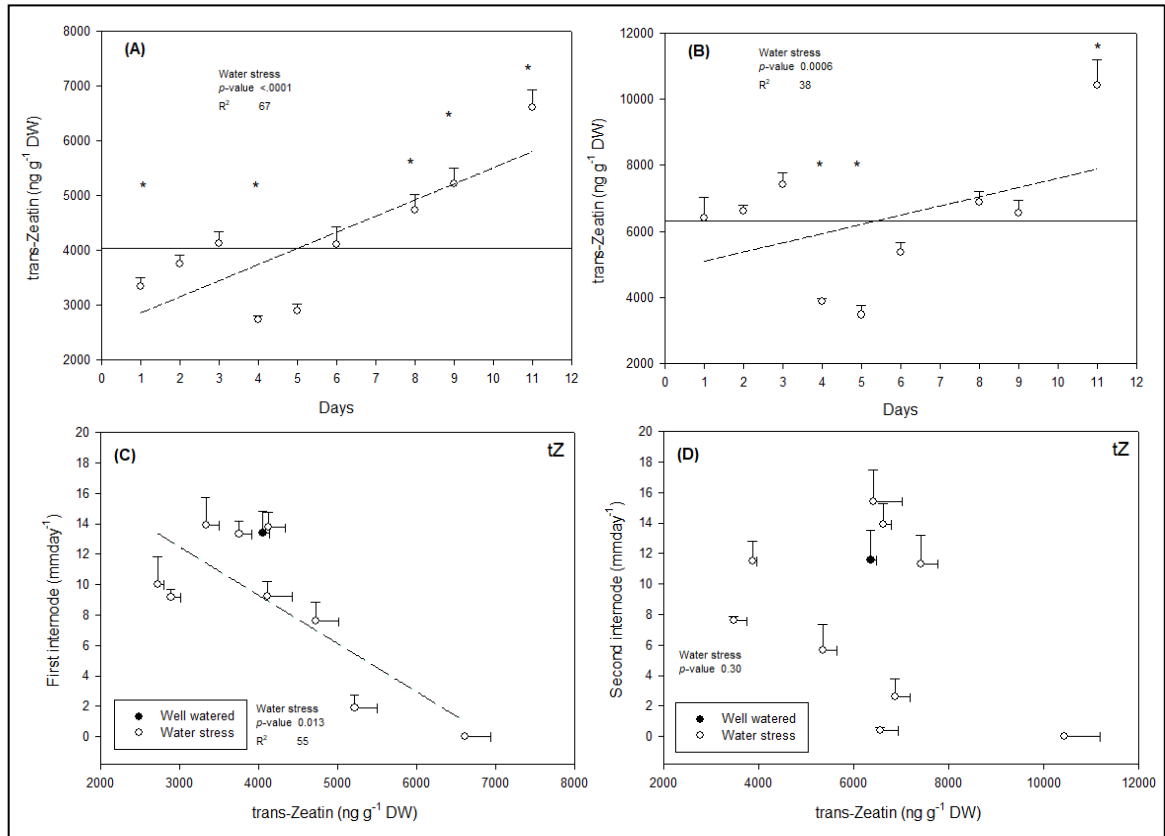


Figure 2.13 First (A) and second (B) internode stem tissue *tZ* concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue *tZ* concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight line for well-watered plants represents an average response where there was no significant change with time. Dashed lines in (A) through (C) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. A linear regression was fitted through the water stressed data in (C). Data are means  $\pm$  SE of 5 replicates.

First and second internode stems of well-watered plants maintained stable endogenous *trans*-Zeatin (*tZ*) levels throughout the 11 days (Table 2.1), averaging  $4054 \pm 80$  and  $6362 \pm 118$  ng g<sup>-1</sup> DW respectively (Fig. 2.13A and 2.13B). Soil drying caused fluctuating endogenous *tZ* levels relative to well-watered plants, in first and second internode stems respectively (Fig. 2.13A and 2.13B) but increased in first internode stems from Day 8 (First internode only). Increased primary stem *tZ* concentration was correlated with decreased first ( $r^2=95\%$ ) internode elongation (Fig. 2.13C) but there was no correlation between second internode elongation and *tZ* concentrations (Fig. 2.13D). Elongation of first and second internode stems were similarly sensitive to stem *tZ* concentrations (Table 2.4), since ANCOVA revealed no significant stem type x *tZ* concentration interaction.

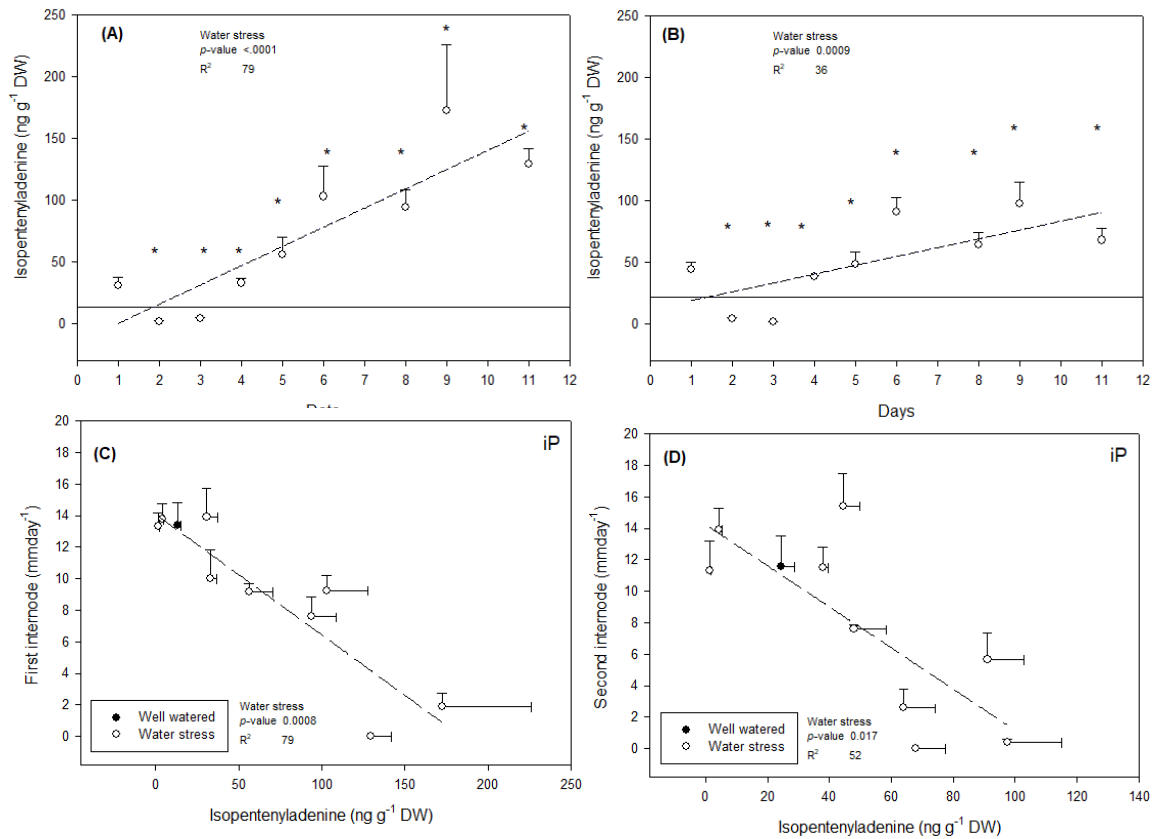


Figure 2.14 First (A) and second (B) internode stem tissue iP concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue iP concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences

between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internode stems of well-watered plants maintained stable endogenous iP levels throughout the 11 days (Table 2.1), averaging  $13.5 \pm 1.6$  and  $24.4 \pm 4.4$  ng g<sup>-1</sup> DW respectively (Fig. 2.14A and 2.14B). After an initial decrease in stem iP levels, (Days 2, 3) soil drying significantly increased endogenous iP levels relative to well-watered plants from Day 4 in both first and second internode stems respectively (Fig. 2.14A and 2.14B), and these continued to increase thereafter. Increased first and second internode stem iP concentrations were correlated with decreased first ( $r^2=79\%$ ) and second ( $r^2=52\%$ ) internode stem elongation respectively (Fig. 2.14C and 2.14D). Elongation of first and second internodes was similarly sensitive to stem iP concentrations (Table 2.4), since ANCOVA revealed no significant stem type x iP concentration interaction.

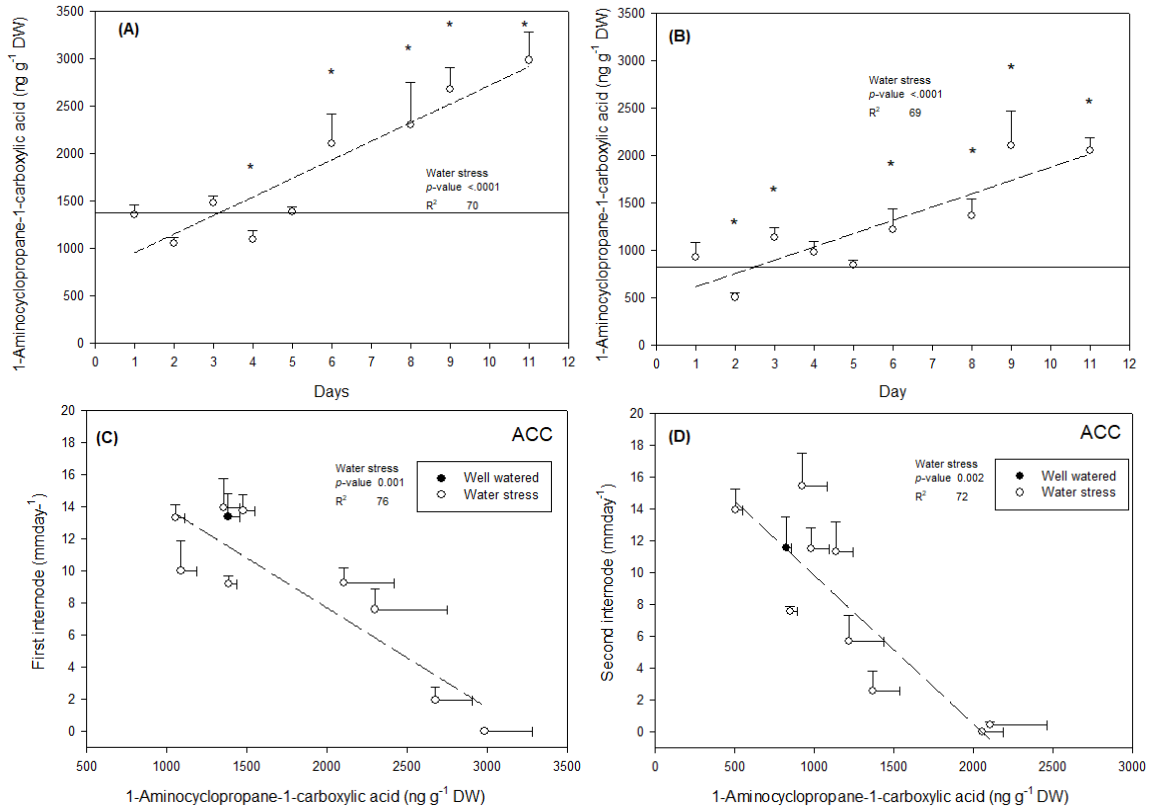


Figure 2.15 First (A) and second (B) stem tissue ACC concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue ACC concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) through (D) represent linear regressions through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internode stems of well-watered plants maintained stable endogenous ACC levels throughout the 11 days (Table 2.1), averaging  $1383 \pm 73$  and  $822 \pm 34$  ng g<sup>-1</sup> DW respectively (Fig. 2.15A and 2.15B). Soil drying significantly increased endogenous ACC levels relative to well-watered plants, from Days 6 and 3 in first and second internode stems respectively (Fig. 2.15A and 2.15B), and these continued to increase thereafter. Increased first and second stem ACC concentrations were correlated with decreased first ( $r^2=76\%$ ) and second ( $r^2=72\%$ ) internode stem elongation respectively (Fig. 2.15C and 2.15D). Elongation of first and second internode stems was similarly sensitive to stem ACC concentrations (Table 2.4), since ANCOVA revealed no significant stem type x ACC concentration interaction.



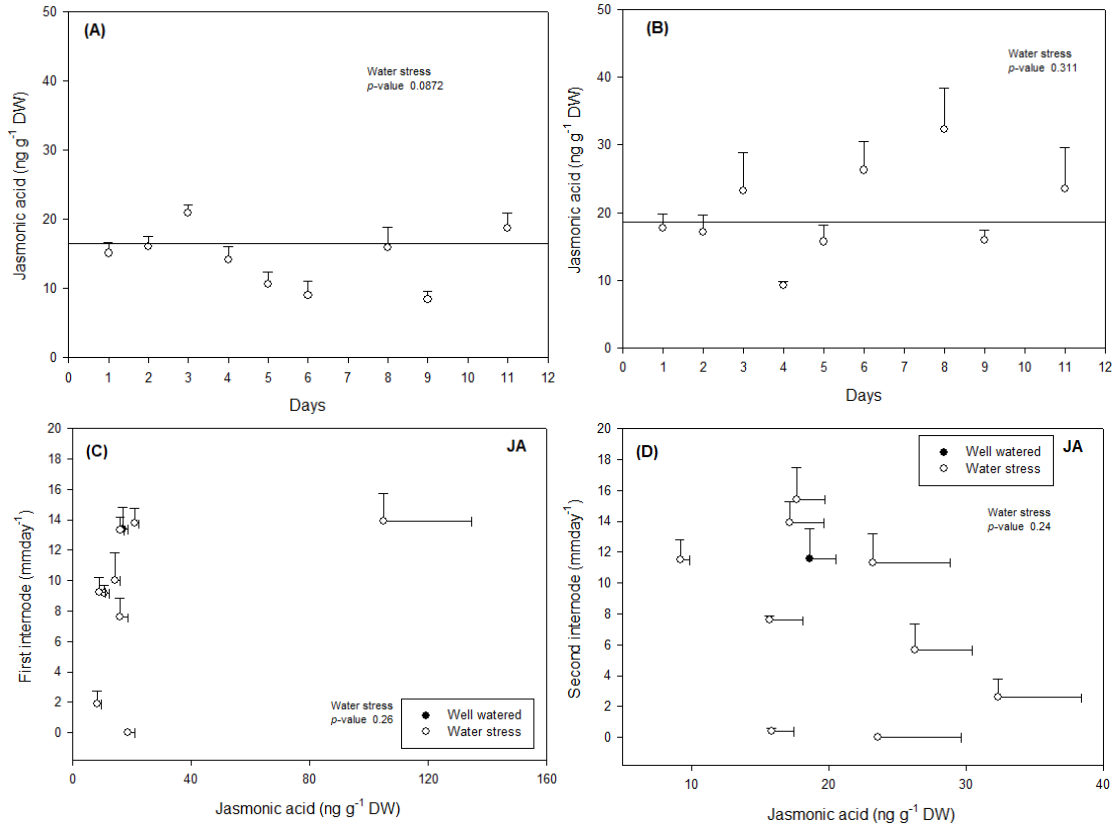


Figure 2.16 First (A) and second (B) stem tissue JA concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue JA concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

Jasmonic acid (JA) concentrations in both first and second stems remained reasonably stable throughout the experiment in both well-watered plants and those from which water was withheld (Fig. 2.16A, B). Stem JA concentrations were not correlated with first and second internode stem elongation in either treatment (Fig. 2.16C and 2.16D).

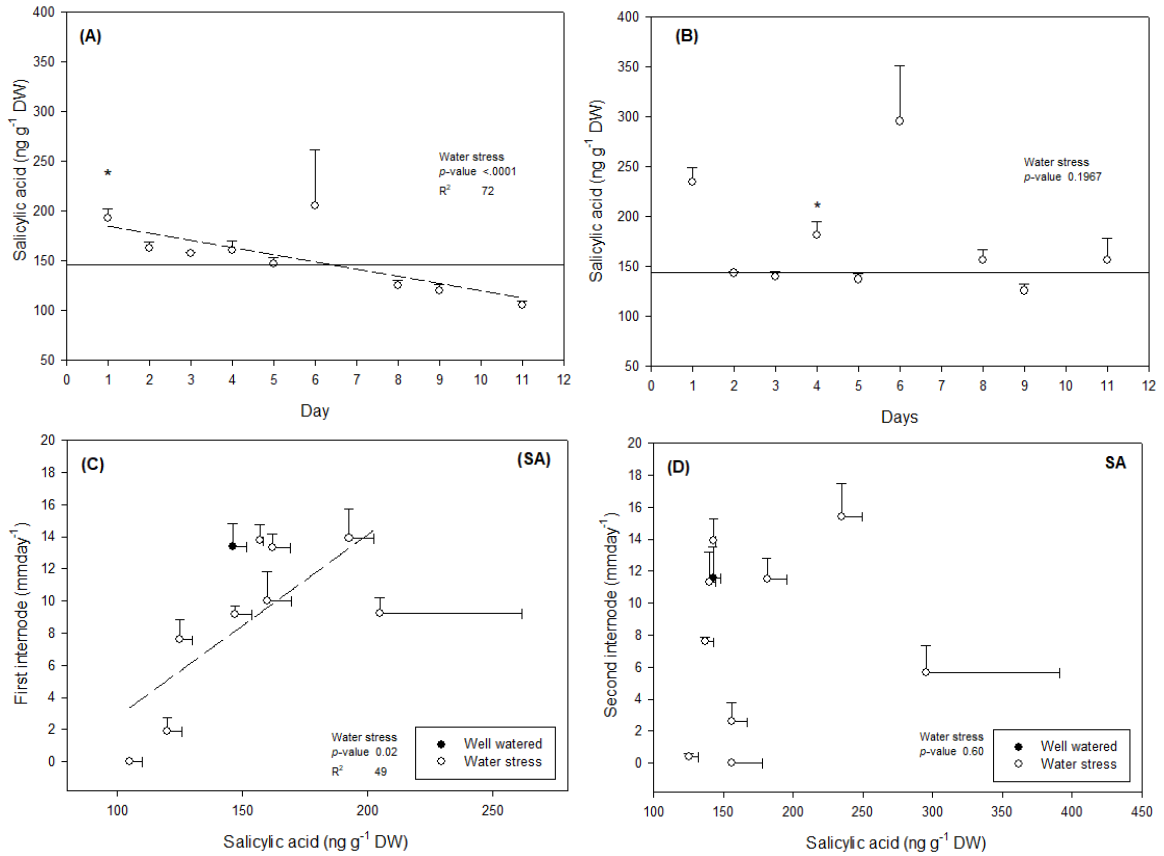


Figure 2.17 First (A) and second (B) stem tissue SA concentrations of water stressed (hollow circle) plants over time. First (C) and second (D) internode stem elongation plotted against stem tissue SA concentrations respectively, in water stressed (hollow circle) plants. In (A) and (B), the straight lines for well-watered plants represent an average response where there was no significant change with time. Dashed lines in (A) and (C) indicate linear regressions fitted through the water stressed data. Asterisks indicate significant differences between (average) well-watered and water stressed plants. Data are means  $\pm$  SE of 5 replicates.

First and second internode stems of well-watered plants maintained stable endogenous SA levels throughout the 11 days (Table 2.1), averaging  $146 \pm 6$  and  $143 \pm 5$  ng g<sup>-1</sup> DW respectively (Fig. 2.17A, B). Although withholding water sometimes resulted in transient peaks in first and second internode stem tissue SA concentrations (Day 1 in first stems, Day 4 in second stems and Day 6 in first and second stems), generally soil drying did not change SA concentrations compared to well-watered plants (Fig. 2.17A and 2.17B). SA concentrations of first stems were weakly ( $r^2=49\%$ ) correlated to the decreased first stem elongation (Fig. 2.17C), but there was no relationship in second stems. Elongation of first and second internodes was similarly sensitive to stem SA concentrations (Table 2.4), since ANCOVA revealed no significant stem type x SA concentration interaction.

## 2.4 Discussion

To date, there have been no reports associating changes in stem hormone concentrations with the regulation of stem elongation as the soil dries. This study provided a comprehensive multi-analyte hormone profiling of stem tissues, identifying soil-drying induced changes in hormone concentrations (Increased ABA, ACC, *iP* and *tZ* and decreased GA<sub>1</sub>, GA<sub>3</sub> and IAA) that preceded any decrease in first and second internode stem elongation. Moreover, many of these changes in phytohormone concentration were correlated with stem elongation (Fig. 2.8 (C-D)-2.18 (C-D)). However, first it was necessary to consider whether soil and/or plant water status were regulating growth.

While many studies have demonstrated that soil drying decreases stem elongation (Bogeat et al. 2007; (Ings et al. 2013), an inhibitory soil moisture threshold is difficult to establish in the absence of soil matric potential data in these studies. Here, first and second internode stem elongation significantly decreased after 5 days, corresponding to  $\Psi_{\text{soil}}$  of -0.32 MPa (Fig. 2.5A, B; Fig 2.1). Nevertheless, stem elongation ceased 2 days earlier in second internode stems, implying differential regulation of growth rates. Taken together, tissue age determines the stem elongation response to soil drying, perhaps by modulating hormone concentrations.

In this study, first and second internode stem elongation decreased (Fig. 2.5A and 2.5B) prior to any change in stem water potential ( $\Psi_{\text{stem}}$ ) (Fig. 2.4B). Decreased plant growth in response to soil water deficits, before any change in  $\Psi_{\text{stem}}$ , was reported in many plant species (Farooq et al. 2009), including pine (De Diego et al. 2012). Furthermore, applying root pressurization to prevent any change in plant water status as the soil dried did not prevent a decline in leaf elongation (Termaat et al. 1985; Passioura 1988). Thus, non-hydraulic factors such as phytohormones potentially control stem elongation in response to soil drying.

### ***Comprehensive changes of stem hormones concentrations as the soil dries***

Several studies have argued that ABA regulates shoot growth as the soil dries (Zhang & Davies 1989) with inhibition of stem elongation highly correlated with ABA accumulation (Fig. 2.8C, D), as in many other studies of vegetative growth of plants grown in drying soil (Valluru et al. 2016). Mechanistically, applied ABA decreased cell wall extensibility (Van Volkenburgh & Davies 1983). Nevertheless, growth responses to ABA vary according to whether it is produced endogenously in response to soil drying or applied exogenously to well-watered plants (Tardieu et al. 2010). Indeed, studies with ABA deficient mutants show that ABA is required to maintain shoot growth of well-watered plants (Sharp et al. 2000). However, increased stem ABA levels under drought (Fig. 2.8A, B) were

associated with decreased stem elongation (Fig 2.8C), suggesting that ABA may inhibit shoot growth as the soil dries. Further experiments in Chapter 3 addressed the physiological significance of stem ABA accumulation, by comparing stem growth of wild-type and ABA-deficient *wilty* pea when grown in drying soil.

The relationship between GA content and stem elongation/internode length has been well studied in *Pisum sativum*, with multiple GA biosynthesis genes affecting internode length in well-watered plants (Reid 1983; Ross et al. 1992; Yang et al. 1996). Soil drying reduced stem GA<sub>3</sub> content 5-fold, which was correlated with decreased stem elongation (Fig. 2.11A-D). Similarly, soil drying (to 60% of field capacity - Pirasteh-Anosheh et al. 2013) or osmotic stress (12% PEG, molecular weight 6000 - Wang et al. 2008) decreased foliar GA<sub>3</sub> concentrations of *Zea mays* (maize). In addition to this, decreased stem GA<sub>1</sub> and GA<sub>4</sub> concentrations were correlated with decreased stem elongation (Fig. 2.10A-D, 2.12A-D). Applying GA<sub>1</sub> to the dwarf mutant *le* (8% GA<sub>1</sub> concentration of its WT) restored internode length to WT levels (Ross et al. 1992; Yang et al. 1996). Taken together, these results provide a strong evidence that endogenous GA concentrations are crucial regulator of stem elongation.

Auxins are also considered to positively regulate stem elongation (McKay et al. 1994), with several studies of well-watered plants demonstrating that applied IAA can increase stem elongation of *Pisum sativum* (Yang et al. 1993; Yang et al. 1996). The auxin biosynthesis inhibitors L- $\alpha$ -aminooxy- $\beta$ -phenylpropionic acid

(AOPP) or (S)-methyl 2-((1,3-dioxoisindolin-2-yl)oxy)-3phenylpropanoate (KOK1101) were applied to (*Solanum lycopersicum* cv. 'Momotaro York'), which significantly reduced endogenous IAA levels, consequently suppressing stem growth (Higashide et al. 2014). In this study, drought stress decreased IAA concentration 7.5 fold compared to control plants (Fig. 2.9A, B) and a similar decrease (2.5-fold) occurred in maize leaves following soil drying (Pirasteh-Anosheh et al. 2013; Wang et al. 2008). Furthermore, under soil drying, IAA content was inversely correlated with stem elongation (Fig. 2.9C, D). IAA is suggested to induce acid growth (apoplast acidification) and loosening of the cell wall, leading to increased cell wall extensibility and rapid cell elongation (Rayle & Cleland 1992; Arsuffi & Braybrook 2017). Therefore, endogenous IAA levels can potentially regulate stem elongation of plants grown in drying soil.

Although the involvement of ethylene in promoting stem elongation has been reported (Poovaiah & Leopold, 1973) (Andel & Verkerke 1978), growth of a transgenic canola containing ACC deaminase activity (Sergeeva et al. 2006) was greater than the WT when exposed to saline stress (200 mM NaCl), suggesting that ethylene inhibited growth. Salinity stress (100 mM NaCl for 3 weeks) increased leaf ACC content of tomato by 10-fold (Albacete et al. 2008), similar to the 8-fold increase in pea stem ACC concentrations, which were negatively correlated to stem elongation (Fig. 2.15A-D). Thus, ACC synthesis and its subsequent conversion to ethylene may decrease stem elongation and shoot growth of plants exposed to drying soil or osmotic stress.



Soil drying increased stem iP and tZ concentration 2 and 4-fold respectively, relative to well-watered plants (Fig. 2.14A, B and 2.13A, B). Similarly, (Havlová et al. 2008) established increased bioactive cytokinin (iP and tZ) accumulation in leaves and roots of *Nicotiana tabacum* L. cv. Wisconsin with increased severity of as soil drying. Although iP and tZ accumulation were correlated with decreased stem elongation (Fig. 2.14C, D and 2.13C, D), overexpressing a cytokinin oxidase gene (*AtCKX2*) in tobacco reduced accumulation of iP and tZ to compared to wildtype plants and retarded internode length (Werner et al. 2001). Since CKX may catabolise several CK species, and the relative sensitivity of stem elongation to different CKs is not known, it is possible that other CKs were also reduced in the *AtCKX2*-transformed plants. CKs are postulated to regulate growth *in-vivo* by inducing cell division (Bakalova et al. 2004). Thus, it is uncertain whether specific cytokinins are involved in regulating stem elongation of plants in drying soil.

Since endogenous concentrations of JA and SA were not significantly correlated with stem elongation, despite a transient soil-drying induced increase in stem SA concentration (Fig. 2.16, 2.17A-D), these hormones are not discussed further in this chapter. However, soil drying can increase tissue JA concentrations (De Ollas et al. 2018), while osmotic stress can increase SA concentrations (Gharbi et al. 2017) in tomato, suggesting that different species may use different phytohormones to respond to below-ground stresses. Nevertheless, stem

concentrations of many hormones changed in pea in response to soil drying (Fig. 2.18).

### ***A possible hormone combination of decreased stem elongation***

While various hormones may regulate stem elongation as discussed above, it is more likely they act together by interacting with each other. Those linked to stem formation will be exported from the tip of the growing shoot, the shoot apical meristem (SAM) (Steeves and Sussex, 1989; Lyndon, 1998), including GAs (Hedden & Phillips 2000; Olszewski et al. 2002), auxin (Vernoux et al. 2010) and cytokinins (Kurakawa et al. 2007). Basipetal transport of multiple hormones from the shoot apical meristem is likely to regulate endogenous concentrations in the internodes, perhaps in conjunction with local synthesis of hormones (O'Neill & Ross 2002).

GAs is abundantly found in actively growing tissues, suggesting that GA biosynthesis is tightly linked to the site of action of bioactive GAs (Hedden and Phillips, 2000; Olszewski et al., 2002). Auxin-mediated promotion of gibberellin A<sub>1</sub> levels was reported in shoots of tall (wildtype) pea line 205+ (Ross et al., 2000). Shoot decapitation (thereby removing an apical auxin source) reduced conversion of GA<sub>20</sub> to GA<sub>1</sub> in stems and subsequently reduced the endogenous GA<sub>1</sub> and *PsGA3ox1* (Mendel's *LE* gene) transcript levels. These effects were reversed by applying auxin to the stump of decapitated plants. In contrast,

decapitation increased *PsGA2ox1* (encoding the enzyme for the conversion of GA<sub>20</sub> to GA<sub>29</sub>) levels, which could be reversed by IAA application to the cut stump. Application of IAA and decapitation together does not considerably affect precursor of GA<sub>1</sub> (*PsGA3ox1*) (Ross *et al.*, 2000). Collectively, these results suggest that auxin basipetally-transported from the shoot apex promotes GA<sub>1</sub> biosynthesis in elongating internodes by maintaining *PsGA3ox1* transcript levels.

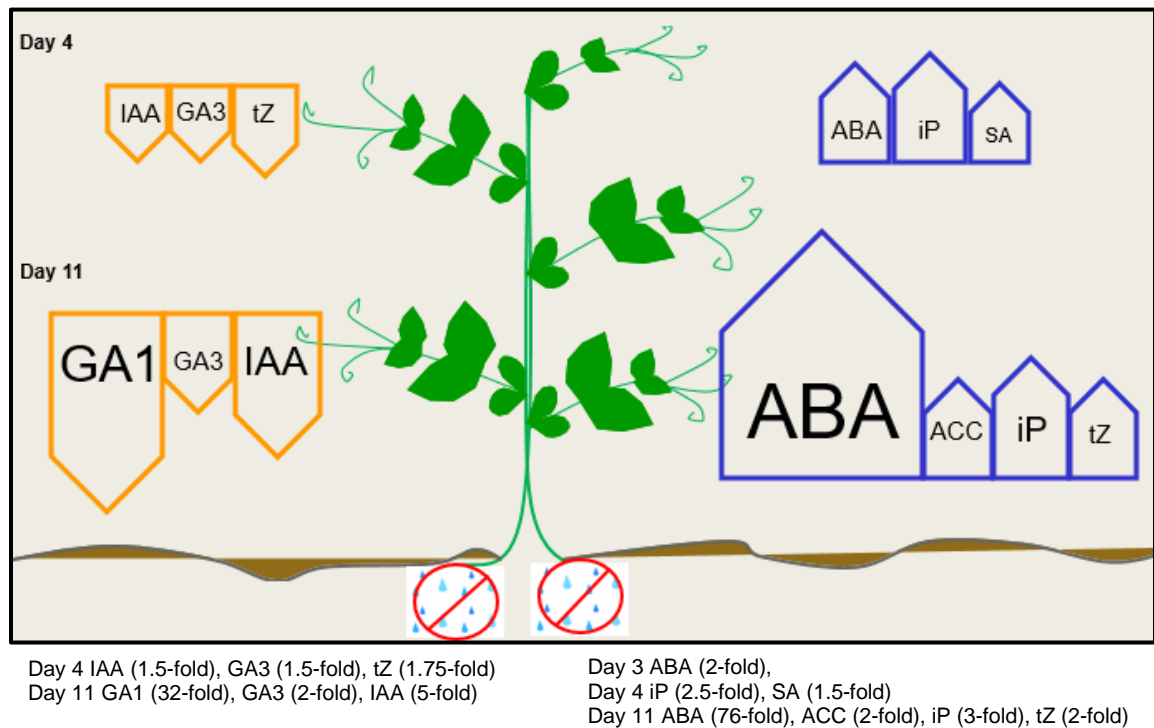


Figure 2.18 A summary of changes in hormone concentrations in pea stems following soil drying. Upward arrows indicate increases and downward arrows indicate decreases in stem hormone concentrations relative to well-watered plants. Font sizes indicate the fold changes in hormone concentration.

## 2.5 Conclusions

Although soil drying ultimately decreased stem water potential ( $\Psi_{\text{stem}}$ ), substantial changes in stem phytohormone concentrations preceded any change in  $\Psi_{\text{stem}}$  (Fig 2.18). While correlations were established between stem elongation and the concentrations of ABA, IAA, GA's,  $\text{Z}$ , iP and ACC, further studies are needed to evaluate the physiological significance of these correlations. Since increased ABA concentration was an early response to soil drying which potentially inhibited stem elongation, Chapter 3 will investigate the function of ABA in controlling stem elongation by measuring the responses of a wildtype pea and its ABA deficient mutant (*wilty*). Since decreased IAA and GA concentrations may limit stem elongation, Chapter 4 will investigate their function by exogenous application to plants in drying soil.

## Chapter 3

### **Does abscisic acid (ABA) regulate stem elongation and leaf expansion under drought?**

#### **3.1 Introduction**

Abscisic acid is an important phytohormone regulating abiotic stress responses, including drought stress in crop plants (Shinozaki & Yamaguchi-Shinozaki 2000; Schroeder et al. 2001). Under drought stress, endogenous ABA concentrations increase considerably throughout the plant, an effect that helps plants to survive stressful environments (Ng et al. 2014). ABA plays an important role in maintaining root and shoot growth (Munns & Cramer 1996; Sharp 2002), tissue hydraulic conductivity (Hose et al. 2000; Parent et al. 2009), and stomatal regulation (Assmann 2003; Christmann et al. 2007).

In determining the role of ABA in controlling growth, efforts should be made to disentangle direct effects of ABA from its effects on plant water relations. This complex situation has been investigated using several approaches, including feeding plants synthetic ABA (or inhibitors that diminish ABA concentrations), and analyzing the physiological responses of mutant or transgenic plants with altered endogenous ABA concentrations (Dodd et al. 2009; Parent et al. 2009). , Various experimental designs have been used to observe ABA's effects independently of changes in water status (by independently regulating plant water status by changing evaporative demand of the atmosphere and/or shoot

misting (Sharp et al. 2000; Dodd et al. 2009) and/or maintaining water status as the soil dries by pressurising the root system or applying partial root drying), resulting in different explanations of ABA's function in regulating plant growth.

ABA's effects (on growth) can be dose-dependent (Tal & Nevo 1973). Abscisic acid deficient mutants of tomato *notabilis* (*not*), *flacca* (*flc*) and *sitiens* (*sit*) had lower ABA concentrations (74%, 83% and 88% of WT plants respectively) and less leaf area than wild-type plants (Tal and Nevo, 1973), suggesting that ABA was needed to maintain leaf area expansion. With soil drying, decreased leaf growth was correlated with increased xylem ABA concentrations in maize (Zhang & Davies 1990). Detached barley shoots fed with a range of ABA concentrations supplied in an artificial xylem solution showed a concentration-dependent decrease in leaf elongation (Dodd & Davies 1996). Thus, different approaches of manipulating ABA concentrations *in vivo* have resulted in different conclusions on the importance of ABA in regulating leaf expansion under normal and drying soil conditions.

Moreover, several researchers have investigated the role of ABA in regulating stem elongation (height) under well-watered (optimal) conditions, independently of plant water status, by growing ABA-deficient mutants at different relative humidities. When ABA-deficient tomato mutants *not*, *flc* and *sit* (which show increasing severity of ABA deficiency) were grown under high relative humidity (92% RH) to moderate the effect of ABA deficiency on leaf water potential ( $\Psi_{\text{leaf}}$ ),

they were 23%, 27% and 26% taller, respectively, than wild-type (WT) plants (Jones et al. 1987). This height increment occurred even though WT  $\Psi_{\text{leaf}}$  was higher (-0.52 MPa) than the *flc* and *sit* mutants (-0.86 MPa and 0.76 MPa respectively) and only *not* had a higher  $\Psi_{\text{leaf}}$  (-0.30 MPa) than WT plants. Similarly, in another study conducted in a greenhouse without humidity control, double and triple mutants of *not*, *flc* and *sit* were taller than the WT 25 days after propagation (Tarr 1993). Similarly, at different humidities (50/70% for WT plants, 92/95% RH for *flc* plants) aiming to equalise  $\Psi_{\text{leaf}}$  (average values of -0.45 MPa for WT and *flc* plants), stem elongation of *flc* plants was 20% taller than WT plants after 21 days (Sharp et al. 2000). These three experiments suggest that although differences in  $\Psi_{\text{leaf}}$  may confound direct impacts of ABA status on stem elongation, ABA-deficient plants were taller at high humidities.

Nevertheless, these experiments do not assess the contribution of ABA in regulating shoot growth of plants in drying soil. Thus, in this study, the ABA-deficient *wilty* pea (Donkin et al. 1983) and its WT were grown under well-watered conditions and in drying soil. Since ABA deficiency increases stomatal conductance and transpiration rates which decrease leaf relative water content and water potential (Dodd 2003; Rothwell et al. 2014), plants were grown in chambers at two different relative humidities (50% and 95%). The aim was to compare the sensitivity of stem elongation to soil drying in the two genotypes, but at a similar plant water status, to avoid possible regulatory effects of stem elongation by stem water potential. Since it was hypothesized that ABA

accumulation during soil drying inhibits stem elongation, the *wilty* pea mutant was expected to maintain stem elongation as the soil dried.



## 3.2 Materials and Method

### 3.2.1 Plant culture

Seeds of wild-type (WT) line (A10) and *wilty* pea (De Bruijn et al. 1993) were pre-germinated on tissue paper and kept in the dark. On the emergence of the plumule and radicle, uniform seeds were individually transplanted into round pots of 16 cm length x 13 cm diameter (1L) containing a 1:1 (v:v) mixture of silica sand and growing substrate (John Innes No.2, J. Arthur Bowers). All plants were well watered for 7 days by replacing evapotranspirational losses (determined gravimetrically) daily. Plants were initially grown in a controlled environment room with a Photosynthetic Photon Flux Density (PPFD) at bench height of (needs a value in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) with supplementary lighting (supplied by Osram 600w daylight bulbs) for 12 hours and 26°C/20°C minimum day/night temperature and relative humidity between 45-50% at Lancaster Environment Centre. After ten days, the plants were transferred to two controlled environment growth chambers (Snijder Microclima 1750, Snidjer Scientific, Tilburg, The Netherlands). The photoperiod was 12 hours (0600-1800 hours), with temperatures day and night of 26°C and 20°C with a PPFD of 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at canopy height. The first chamber was set at 50% relative humidity to replicate greenhouse conditions, and a second chamber at 92-95% relative humidity. Temperature in both chambers were similar at 26°C and 20°C during day and night. Average VPD during the day and night periods at 50%RH were 1.45(+/-0.02) and 1.15(+/-0.01) kPa, respectively, while average VPD during the day and night periods at 95%RH were 0.33(+/-0.01) and 0.12(+/-0.01) kPa, respectively.

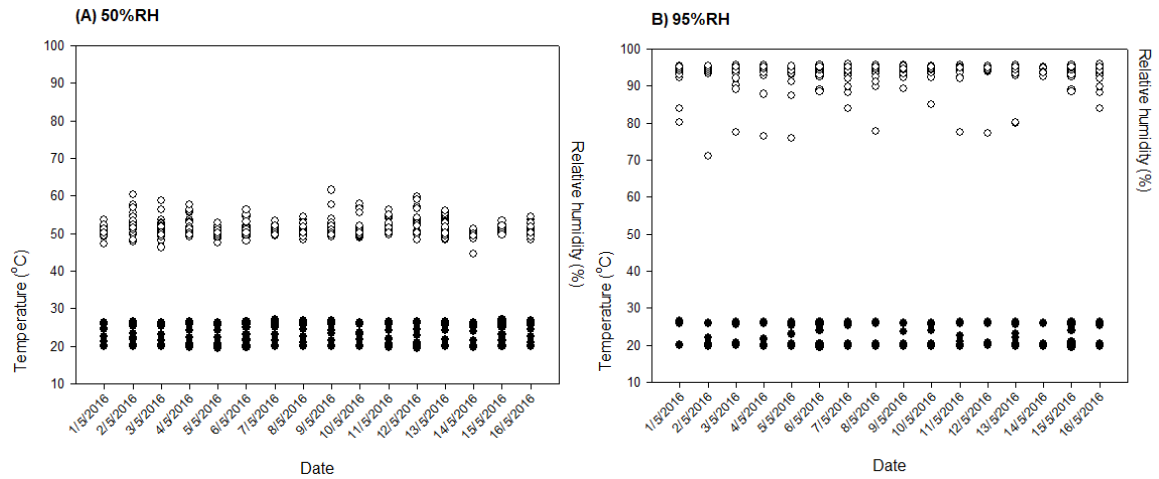


Figure 3.1 Relative humidity (RH) and temperature of growth chambers (A) 50% RH (B) 95% RH, recorded every 30 minutes from 27<sup>th</sup> April 2016-12<sup>th</sup> May 2016. Filled and hollow circles represent chamber air temperature and relative humidity, respectively.

Six days after transplanting, two different water treatments were imposed on 8 replicates per treatment. Each day, the control plants (100% ET) received 100% of the previous day's mean evapotranspiration while the other treatment received no irrigation. Prior to imposing the treatments, all pots were irrigated (to the drip point) and allowed to drain for 24 hours. Irrigation was applied daily from 4:00-5:00 pm, according to the plant requirements. Treatments were randomly arranged in the controlled environment growth chambers.

### 3.2.2 Leaf expansion

After ten days (when plants had two to three whorls of leaves), leaf expansion was measured daily at 3.00-4.00pm hours by determining the length and width of each leaf using flexible ruler. When length and width of a leaf was constant between successive measurements (indicating that the leaf had stopped growing), it was no longer measured, and the measurements continued with the expanding leaves further up the stem.

### 3.2.3 Measurement regime

Each pot was weighed daily, to determine evapotranspiration (to implement well-watered conditions) and to estimate soil water content using a gravimetric method. Pot weights were measured every day before irrigation until the end of the experiment. Then, the soils (including roots) were removed from the pots, weighed, oven dried for 60-72 hours at 60°C and reweighed. The soil water content was expressed on a mass basis as follows:

Weight of water = Weight of wet soil – Weight of dry soil

Soil water content =  $\frac{\text{Weight of water (g)}}{\text{Weight of dry soil (g)}}$

Stem elongation and stem water potential were measured daily as explained in Sections 2.2.4 and 2.2.3, respectively.

Stem water potential measurements in this chapter were restricted to 5 replications per treatment due to limited psychrometer availability but were fully randomised between treatments. The measurements made in two batches at between 9:00-11:00 am and 1:00-3:00 pm.

After 11 days, leaf and stem samples were oven-dried at 80°C to obtain the dry weight. Prior to being oven-dried, the excised leaves were measured individually using a leaf area meter (Li-Cor Inc., Lincoln, Nebraska, USA) to determine leaf area.

#### **3.2.4 Abscisic acid quantification**

Secondary stems and leaves (see Fig. 2.3) were collected on alternate days. Daily measurements were impractical due to limited number of plants during the experiment. At least 3 plants were required for 1 replicate of 5 mg of stem tissue sample (in this study n=4). Average dry weight of these stem and leaf samples were 5 mg and 10 mg, respectively. Stem internode abscisic acid (ABA) concentration was measured via a radioimmunoassay (RIA) using a monoclonal antibody, AFRC MAC 252 (based on the method described in Quarrie *et al.* 1988). Stem internodes were harvested, weighed, snap frozen in liquid nitrogen, freeze-dried for 48 h, then finely ground and diluted with deionized, distilled water (1:25). Samples were then placed on a shaker in a cold room (4°C) overnight to extract ABA. A standard curve was constructed using standards from a serial dilution of synthetic unlabelled ( $\pm$ ) -cis, trans-ABA (Sigma Let., UK). ABA

concentration of samples was calculated by reference to this standard curve after linearization using the “logit” transformation.

### **3.2.5 Statistical Analysis**

All treatments were arranged in a randomized complete block design (CRD). Data were analyzed using SAS 9.3 version and Least Significant Differences (LSD) ( $P \leq 0.05$ ) were used to discriminate significant differences between treatment means. Three-way ANOVA was used to determine the effects of genotype (*WT versus wilt*), irrigation treatment (*well-watered versus drying soil*) and growth environment (*50% versus 92-95% RH*). ANCOVA was used to determine genotypic differences in the sensitivity of stem elongation to soil water content, stem water potential, stem and leaf tissue ABA concentration. Non-linear regressions were done separately using segmented analysis in R Software (Version 3.4.1), that provided an estimate breakpoint/threshold for the decline in stem elongation and leaf expansion as stem water potential decreased (Fig. 3.11C,D).

### 3.3 Results

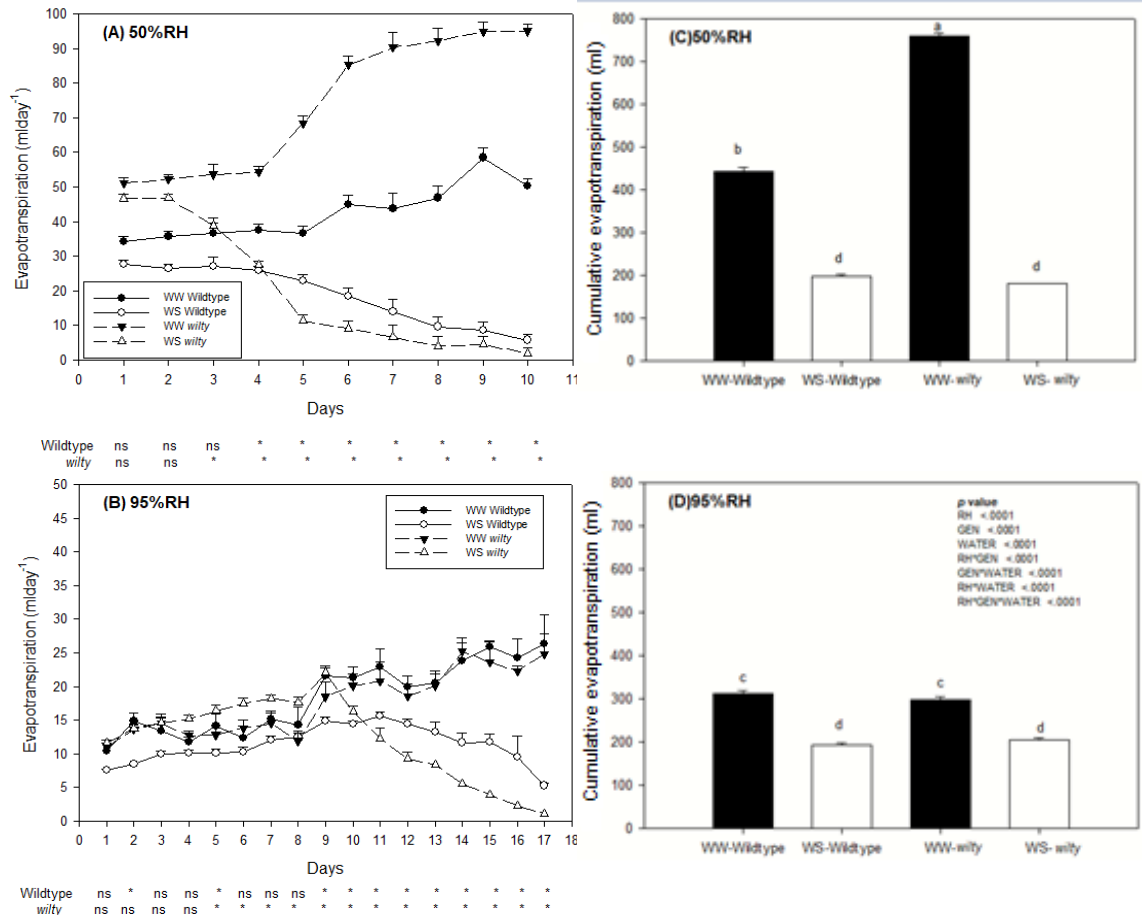


Figure 3.2 Daily (A, B) and cumulative (C, D) evapotranspiration of wildtype (WT - circles) and *wilty* (triangles) pea grown under well-watered (WW - filled symbols) and water stress conditions (WS - hollow symbols) at 50% (A, C) and 95% (B, D) relative humidity, RH. Data are means  $\pm$  SE of 6 replicates. Asterisks beneath panels A, B indicate significant ( $P < 0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Different letters in C, D indicate significant ( $P < 0.05$ ) differences between humidity x water x genotype

combinations for cumulative evapotranspiration. P-values for 3-way ANOVA of cumulative evapotranspiration reported in panel D.

When grown at 50% relative humidity (RH), the evapotranspiration (ET) of well-watered controls of both genotypes increased with time (Fig. 3.2A). Cumulative evapotranspiration of well-watered *wilty* plants during the experiment was 1.7-fold higher than in WT plants (Fig. 3.2C). Withholding irrigation from WT and *wilty* gradually decreased ET from Days 4 and 3, respectively, before reaching 0 mL day<sup>-1</sup> in some replicates on Day 10. Cumulative evapotranspiration of water-stressed *wilty* and WT plants during the experiment was equivalent.

When grown at high humidity (95%), ET of well-watered WT and *wilty* plants did not significantly differ during the experiment (Fig. 3.2B). Withholding irrigation of WT increased until Day 11 before decreasing ET, while *wilty* increased from Day 5 until Day 9 before decreasing from Day 9, with some *wilty* replicates reaching 0 mL day<sup>-1</sup> by Day 17. Cumulative evapotranspiration of both well-watered and water stress treatments did not differ WT and *wilty* (Fig. 3.2D). Although withholding water transiently increased evapotranspiration of *wilty* in both 50%RH (Days 1-3) and 95%RH (Days 1-9), both genotypes used the same amount of water in both humidities in the water stress treatments.

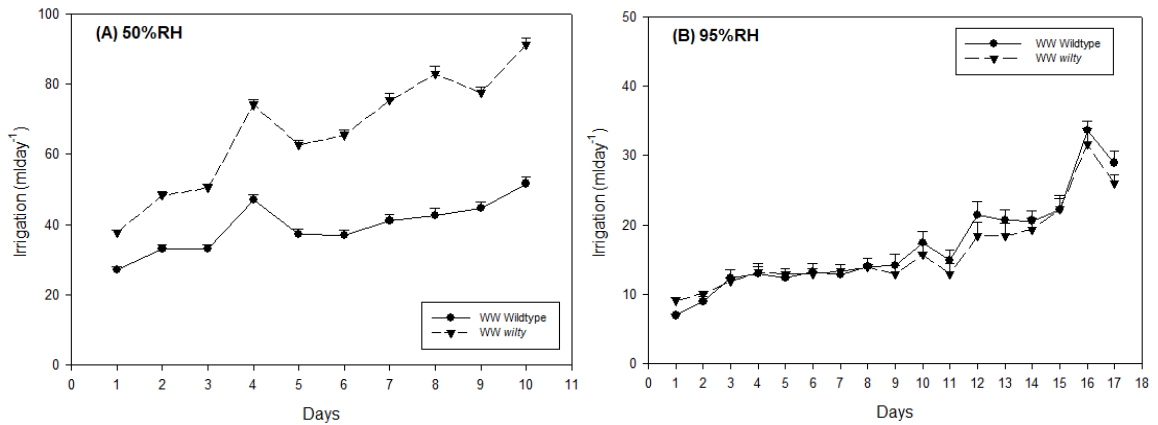


Figure 3.3 Irrigation rate of well-watered wildtype (WT-circles) and *wilty* (triangles) pea grown at (A) 50% and (B) 95% relative humidity (RH). No water was supplied to the water stress treatments. Data are means  $\pm$  SE of 6 replicates.

To ensure the control treatments remained well-watered, irrigation volumes increased with time in both WT and *wilty* plants grown at both relative humidities (Fig. 3.3A, 3.3B). Although irrigation volumes applied to WT and *wilty* plants did not differ when grown at 95%RH (Fig. 3.3B), *wilty* plants grown at 50%RH received 60% more irrigation over the course of the experiment than WT plants grown at 50%RH. (Fig. 3.3A).



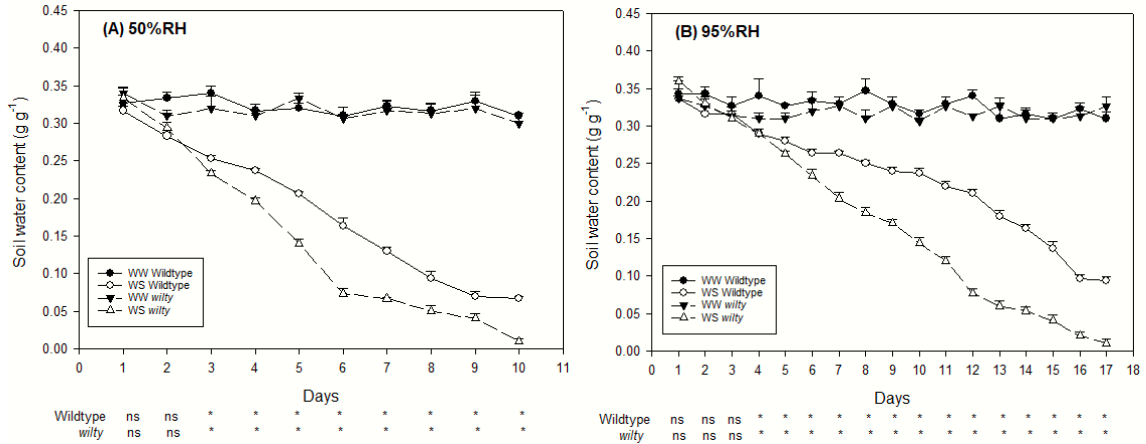


Figure 3.4 Daily soil water content ( $\theta$ ) of wildtype (WT-circles) and *wilty* (triangles) pea grown under well-watered (WW-filled symbols) and water stress (WS-hollow symbols) at (A) 50% and (B) 95% RH. Data are means  $\pm$  SE of 6 replicates. Asterisks beneath panels A, B indicate significant ( $P < 0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”.

At both RH%, irrigation of well-watered controls-maintained soil water contents ( $\theta$ ) of WT and *wilty* between 0.30 g g<sup>-1</sup> to 0.35 g g<sup>-1</sup>, and similar between genotypes, throughout the experiment (Fig. 3.4A, B). When grown at 50%RH, withholding irrigation significantly decreased  $\theta$  in both genotypes from Day 3 until the end of the experiment. From Day 3,  $\theta$  remained higher in WT plants (than in *wilty*) of stressed plants (Fig. 3.4A) due to their lower evapotranspiration (Fig. 3.2A). When grown at 95%RH, withholding irrigation significantly decreased  $\theta$  of WT and *wilty* genotypes from Day 4 until the end of the experiment. From Day 4,  $\theta$  remained higher in WT plants (than *wilty*) due to their lower evapotranspiration

(Fig. 3.2B). Soil water depletion was slower at 95%RH in both genotypes due to lower evapotranspiration rates.

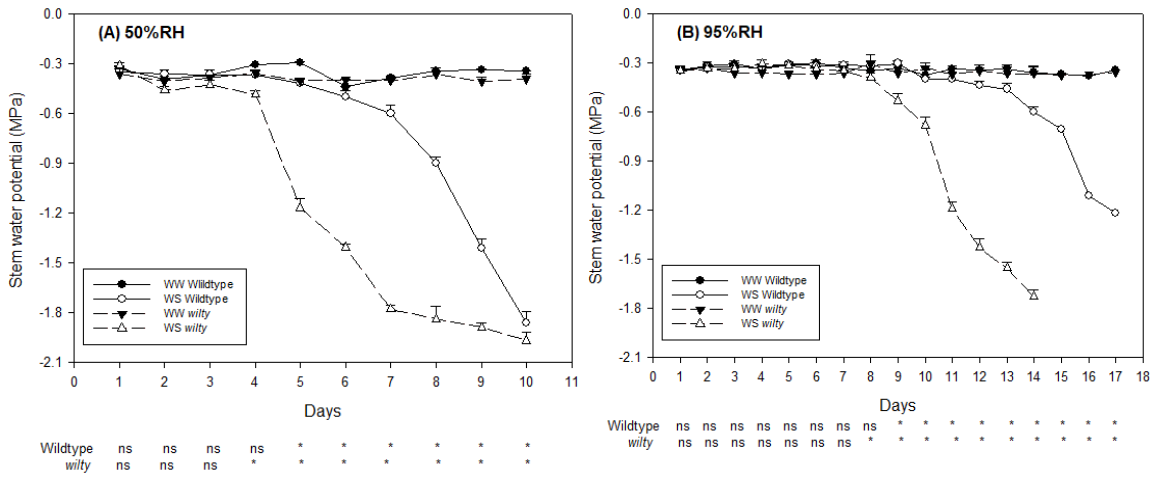


Figure 3.5 Stem water potential ( $\Psi_{stem}$ ) of wildtype (WT-circles) and *wilty* (triangles) pea grown under well-watered (WW-filled symbols) and water stress conditions (WS-hollow symbols) at (A) 50% and (B) 95% relative humidity, RH. Data are means  $\pm$  SE of 5 replicates. Asterisks beneath panels A, B indicate significant ( $P < 0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Measurements of  $\Psi_{stem}$  of *wilty* plants at 95%RH were suspended on Day 14 when stem elongation ceased.

At both RH%*s*, stem water potentials ( $\Psi_{stem}$ ) of well-watered plants of both genotypes averaged  $-0.37 \pm 0.008$  MPa ( $n=5$ ) throughout the experiment (Fig. 3.5A, 3.5B). Although  $\Psi_{stem}$  was generally independent of RH%, well-watered controls grown at 95%RH had a significantly higher  $\Psi_{stem}$  than plants grown at 50%RH on Days 2 and 6. When grown at 50%RH, withholding irrigation significantly decreased  $\Psi_{stem}$  of WT and *wilty* plants on Days 5 and 6 respectively

(Fig. 3.5A). When grown at 95%RH, withholding irrigation significantly decreased  $\Psi_{\text{stem}}$  of *wilty* and WT plants on Days 8 and 9, respectively (Fig. 3.5B). At 50%RH, *wilty* and WT reached a similar  $\Psi_{\text{stem}}$  at the end of the experiment. At 95% RH, when stem elongation of *wilty* plants ceased on Day 14,  $\Psi_{\text{stem}}$  reached  $-1.78 \pm 0.003$  MPa (n=5). Thereafter  $\Psi_{\text{stem}}$  measurements of *wilty* were suspended, whereas in WT plants  $\Psi_{\text{stem}}$  declined further to reach  $-1.20 \pm 0.006$  MPa (n=5) on Day 17. Thus, withholding water decreased  $\Psi_{\text{stem}}$  more rapidly in *wilty* plants, independent of relative humidity.

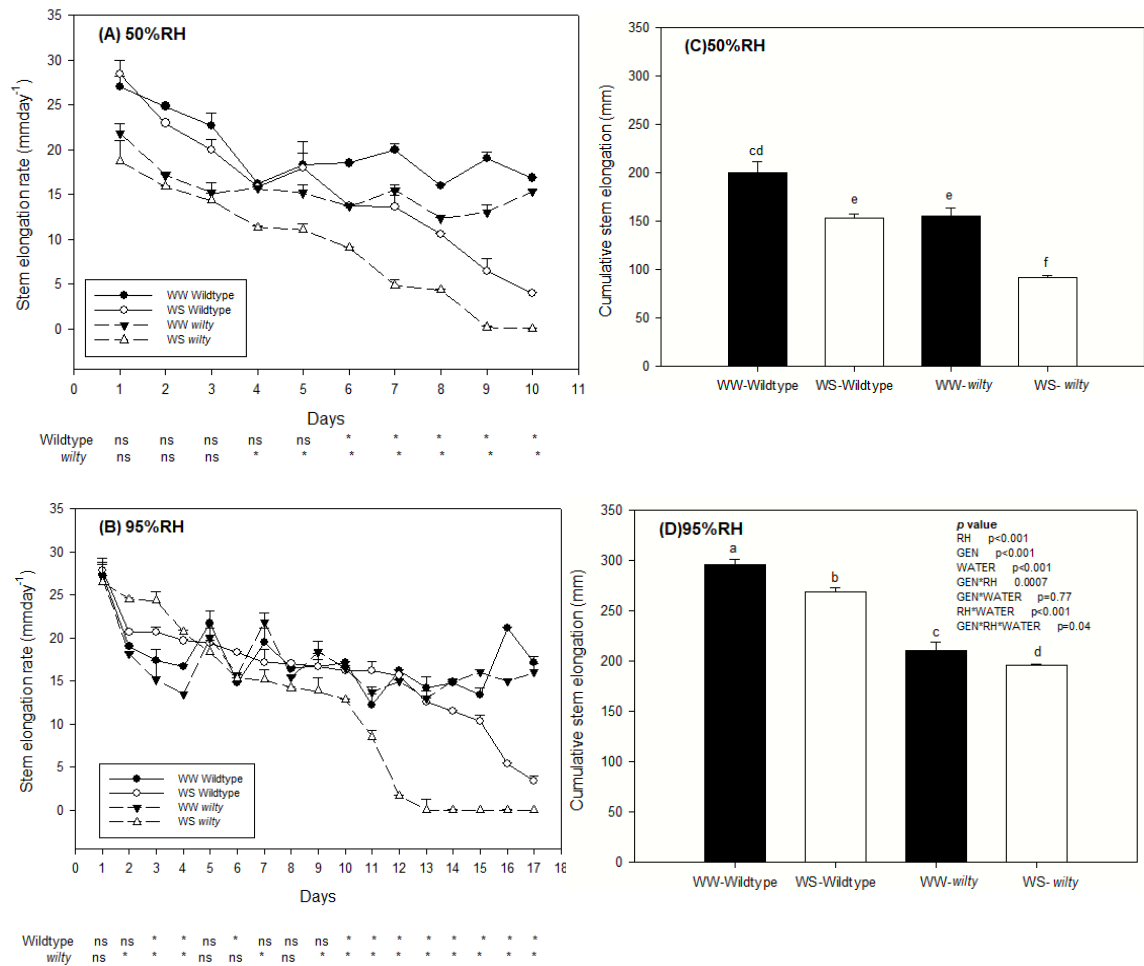


Figure 3.6 Daily (A, B) and cumulative (C,D) stem elongation of wildtype (WT-circles) and *wilty* (triangles) pea grown under well-watered (WW-filled symbols) and water stress conditions (WS-hollow symbols) at 50% (A,C) and (B) 95% (B, D) relative humidity, RH. Data are means  $\pm$  SE of 6 replicates. Asterisks beneath panels A, B indicate significant ( $P<0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Different letters in C, D indicate significant ( $P<0.05$ ) differences between humidity x water x genotype combinations for cumulative evapotranspiration.  $P$ -values of cumulative stem elongation reported in panel D.

At both relative humidities, stem elongation rate of well-watered WT plants averaged 17 mm day<sup>-1</sup> over the entire experiment. In *wilty* plants, stem elongation rate averaged 15 and 16 mm day<sup>-1</sup> at 50%RH and 95%RH respectively (Fig. 3.6A, 3.6B). When grown at 50% RH, withholding irrigation significantly decreased stem elongation of *wilty* and WT (compared to well-watered controls) on Days 4 and 6, respectively and elongation ceased on Days 9 and 10, respectively (Fig. 3.6A). When grown at 95% RH, withholding irrigation consistently decreased stem elongation of both genotypes on Day 3, and elongation ceased on Days 13 and 17 in *wilty* and WT, respectively (Fig. 3.6B). Thus, stem elongation declined earlier in *wilty* plants, irrespective of the relative humidity.

Cumulative stem elongation was 62% higher at 95%RH (averaged across genotypes and water treatments), mostly due to the longer experimental duration at 95%RH. Nevertheless, *wilty* had less stem elongation in both humidities (as indicated by a significant humidity x genotype interaction). Soil drying decreased cumulative stem elongation by 32% at 50%RH (averaged across genotypes), and 8% at 95%RH, as indicated by a significant water x humidity interaction. Wild-type plants had 1.4-fold greater cumulative stem elongation (averaged across both humidities and treatments), but a similar sensitivity to drought as *wilty* plants (no significant water x genotype interaction) (Fig. 3.6C, D). Although *wilty* plants elongated less during the experiment, they were equally responsive to soil drying.

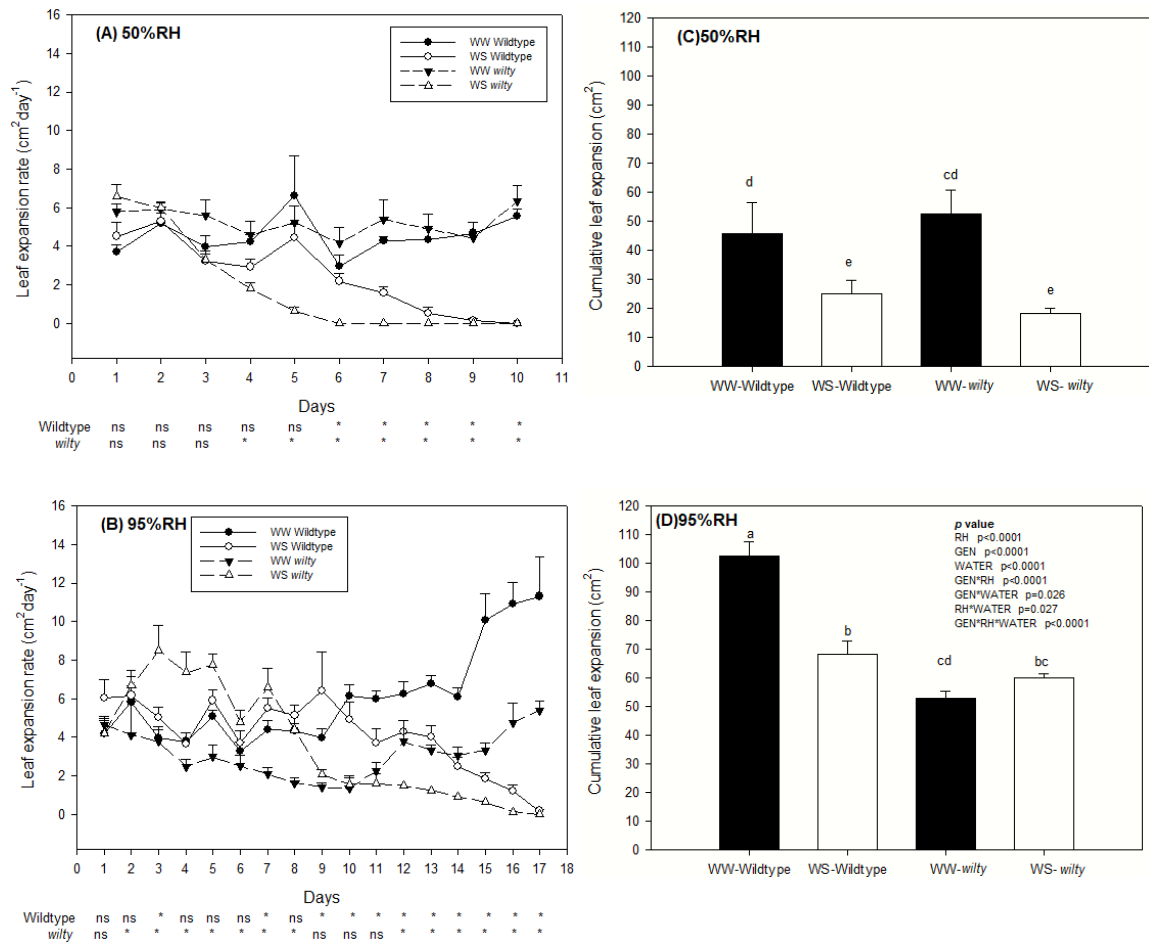


Figure 3.7 Daily (A,B) and cumulative (C,D) leaf expansion of wildtype (WT-circles) and *wilty* (triangles) pea grown under well-watered (WW-filled symbols) and water stress conditions (WS-hollow symbols) at 50% (A, C) and 95% (B, D) relative humidity, RH. Data are means  $\pm$  SE of 6 replicates. Asterisks beneath panels A, B indicate significant ( $P<0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Different letters indicate significant differences in humidity x genotype x water interaction for cumulative

leaf expansion. *P*-values for 3-way ANOVA of cumulative leaf expansion reported in panel D.

At 50% RH, leaf expansion rates of well-watered WT and *wilty* plants at 50%RH were similar (averaging 5 cm<sup>2</sup> day<sup>-1</sup> over the entire experiment) (Fig. 3.7A), At 95%RH, leaf expansion rate of well-watered WT and *wilty* plants had the same pattern from Day 10 (Fig. 3.7B). When grown at 50% RH, withholding irrigation significantly decreased leaf expansion of *wilty* and WT plants (relative to well-watered controls) on Days 4 and 6, respectively and expansion ceased on Days 6 and 10 respectively (Fig. 3.7A). When grown at 95% RH, withholding irrigation increased leaf expansion of *wilty* between Days 2 to 8, while in WT plants leaf expansion significantly decreased from Day 9 and gradually decreased before ceasing on Day 17 (Fig. 3.7B). Since 95%RH caused very low leaf expansion rates of well-watered *wilty* plants until Day 10, it was more appropriate to consider when soil drying decreased leaf expansion compared to the maximum values achieved in these treatments. Thus, soil drying decreased leaf expansion of *wilty* earlier than in WT plants at 95%RH, as in plants grown at 50%RH.

Cumulative leaf expansion was 50% higher at 95%RH (averaged across genotypes and water treatments), mostly due to the longer experimental duration at 95%RH. Although *wilty* and WT had a similar pattern of response at 50%RH, leaf expansion of *wilty* was less than WT at 95%RH (indicated by a significant humidity x genotype interaction). Soil drying decreased cumulative leaf expansion by 58% at 50%RH (averaged across genotypes) and 38% at 95%RH

(WT only), as indicated by significant water x humidity interaction. Wildtype plants had 1.5 folds greater cumulative leaf expansion (averaged across both humidities and treatments), but a similar sensitivity to drought as *wilty* at 50%RH, but not at 95%RH. Cumulative leaf expansion of *wilty* was similar at 95%RH in both well-watered and water stressed plants, while in WT, stressed plants 34% lower than well-watered (significant water x genotype interaction) (Fig. 3.7C, D). Although *wilty* had a similar response to soil drying as WT at 50%RH, cumulative leaf expansion of *wilty* plants at 95%RH was independent of soil drying, and much lower than WT plants.



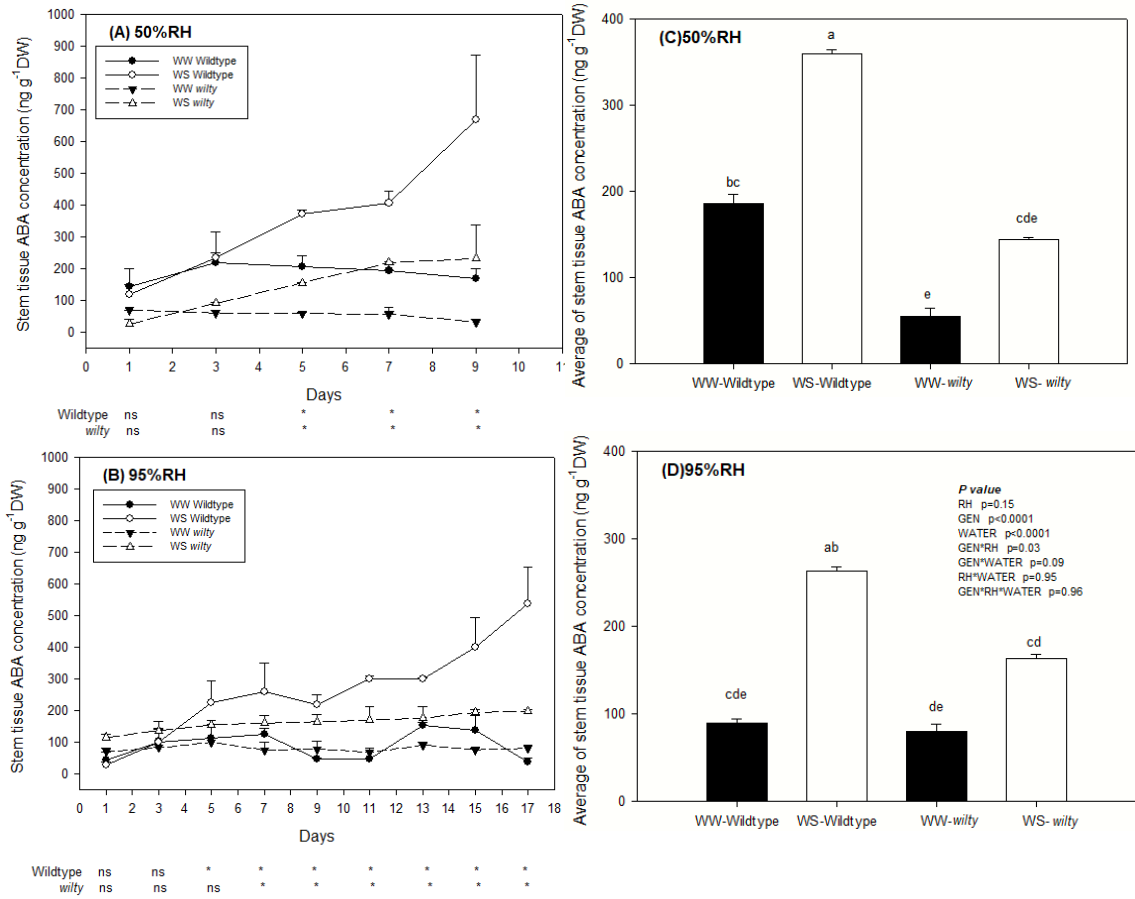


Figure 3.8 Punctual (A, B) and average (C, D) stem tissue ABA concentration of wildtype (WT-circles) and *wilty* (triangle) pea grown under well-watered (WW-filled symbols) and water stress (WS-hollow symbols) at 50% (A, C) and 95% (B, D) relative humidity, RH. Data are means  $\pm$  SE of 4 replicates. Asterisks beneath panels A, B indicate significant ( $P < 0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Different letters in C, D indicate significant ( $P < 0.05$ ) differences between humidity x water x genotype combinations for average of stem ABA tissue concentration.  $P$ -values for 3-way ANOVA average of stem ABA tissue concentration reported in panel D.

When grown at 50%RH, stem tissue ABA concentrations of well-watered *wilty* and WT plants were  $53 \pm 12.1 \text{ ng g}^{-1} \text{ DW}$  and  $172 \pm 13.3 \text{ ng g}^{-1} \text{ DW}$ , respectively (Fig. 3.8A, 3.8B). Thus, WT plants had 3-fold higher ABA concentrations. Withholding irrigation significantly increased stem tissue ABA concentration of both genotypes on Day 5 (Fig. 3.8A). When grown at 95%RH, stem tissue ABA concentrations of well-watered *wilty* and WT plants were statistically equivalent, averaging  $85 \pm 4.2 \text{ ng g}^{-1} \text{ DW}$  across both genotypes. Withholding irrigation significantly increased stem tissue ABA concentration of *wilty* and WT plants from Days 7 and 5 respectively (Fig. 3.8B).

Surprisingly, there was no significant effect of relative humidity on stem tissue ABA concentration, when considering the entire data set. Genotypic differences in stem ABA concentration were accentuated at 50% RH (as indicated by a significant genotype x RH interaction), in part because well-watered plants of both genotypes had similar ABA concentrations at 95%RH. Otherwise, stem ABA concentrations of WT plants were approximately doubled compared to *wilty* plants (averaged across both humidities and treatments) (Fig. 3.8C, D). As expected, soil drying significantly increased stem ABA concentrations, to a similar magnitude in both relative humidities and genotypes. Stem ABA accumulation in response to soil drying tended to be restricted in *wilty* ( $P=0.09$  for genotype x water interaction).

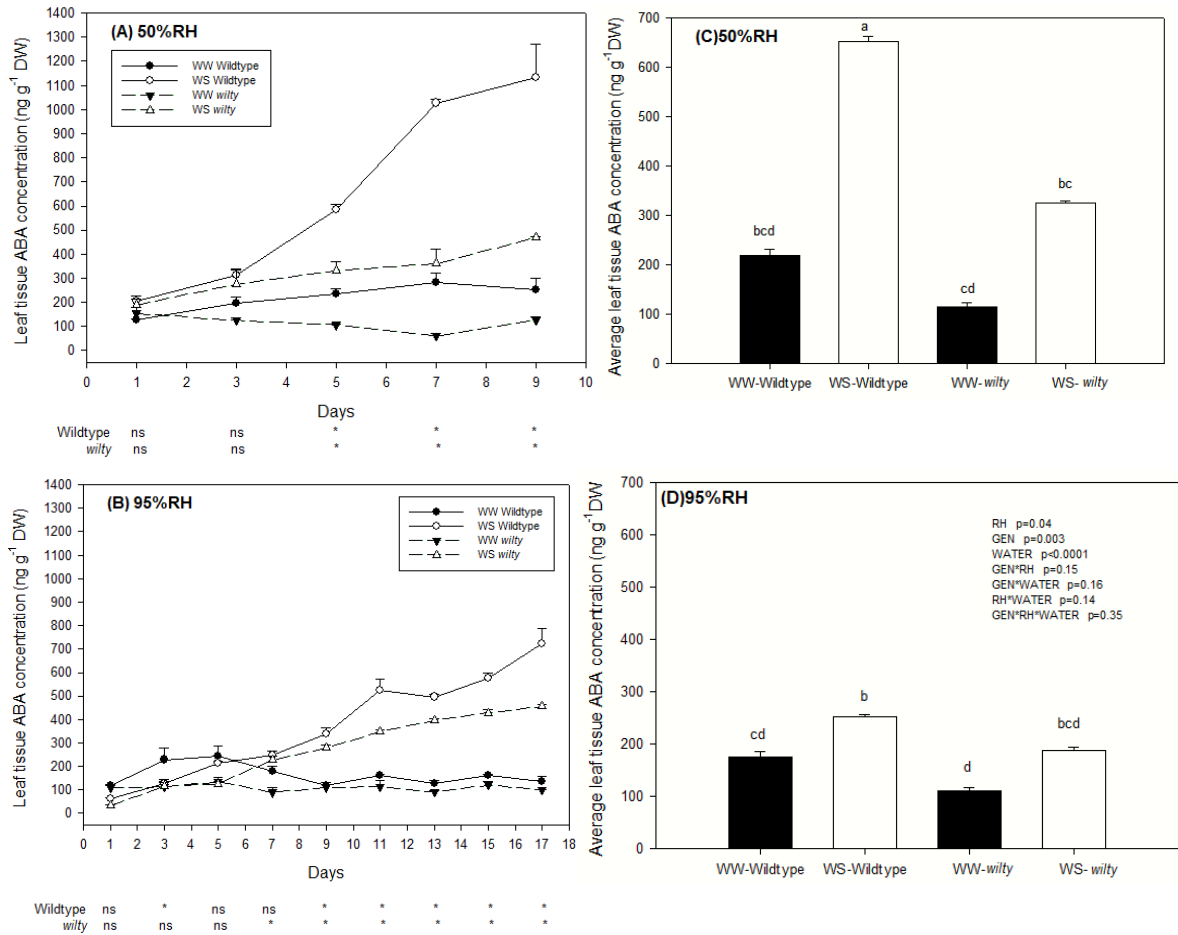


Figure 3.9 Punctual (A, B) and average (C, D) leaf tissue ABA concentration of wildtype (WT-circle) and *wilty* (triangle) pea grown under well-watered (WW-filled symbols) and water stress (WS-hollow symbols) at 50% (A, C) and 95% (B, D) relative humidity, RH. Data are means  $\pm$  SE of 4 replicates. Asterisks beneath panels A, B indicate significant ( $P<0.05$ ) differences between water treatments for each genotype, with ns meaning “not significant”. Different letters in C, D indicate significant ( $P<0.05$ ) differences between humidity x water x genotype combinations for average leaf tissue ABA concentration.  $P$ -values for 3-way ANOVA of average leaf tissue ABA concentration reported in panel D.

When grown at 50%RH, leaf tissue ABA concentrations of well-watered *wilty* and WT plants averaged  $110 \pm 9.5$  and  $210 \pm 13.1$  ng g<sup>-1</sup> DW respectively (Fig. 3.9A, B). Withholding irrigation significantly increased leaf tissue ABA concentration of both genotypes on Day 5, with a greatly magnified response of WT plants by Day 9 (Fig. 3.9A). At 95%RH, leaf tissue ABA concentrations of *wilty* and wildtype plants averaged  $110 \pm 8.8$  and  $170 \pm 12.3$  ng g<sup>-1</sup> DW respectively. Withholding irrigation increased leaf tissue ABA concentration of *wilty* and WT plants from Days 7 and 9 respectively (Fig. 3.9B).

When considering the entire data set, leaf ABA concentration was higher at 50%RH, mostly due to substantial soil-drying induced ABA accumulation at this humidity. Genotypic differences in leaf ABA concentration were similar at both humidities (no significant genotype x RH interaction). Leaf ABA concentrations of WT plants were approximately doubled compared to *wilty* plants (averaged across both humidities and treatments). (Fig. 3.9C, D). Soil drying significantly increased leaf ABA concentrations, to a similar magnitude in both relative humidities and genotypes.

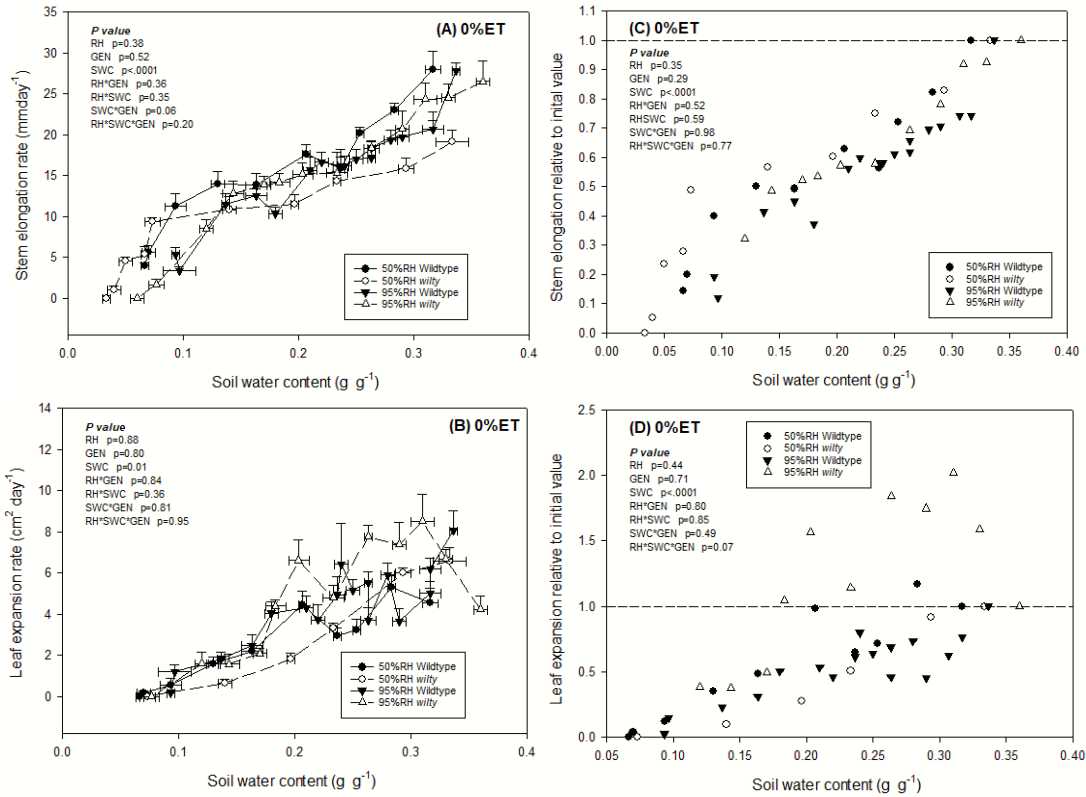


Figure 3.10 Changes in absolute (A, B) and relative [to their initial values] (C, D) stem elongation (A, C) and leaf expansion (B, D) of wildtype (WT-filled symbols, solid lines) and *wilty* (hollow symbols, dashed lines) plants from which irrigation was withheld, plotted against soil water content. Symbols are means  $\pm$  SE of data presented in Figures 3.4, 3.6 (A, B) and 3.7 (A, B), with error bars removed for clarity in C, D. P-values for 3-way ANCOVA of each relationship reported in their respective panel.

Withholding water decreased stem elongation (Fig. 3.9A) and leaf expansion (Fig. 3.9B) as soil water content decreased. Irrespective of humidity, both wildtype and *wilty* showed similar trends of stem elongation and leaf elongation decreasing as the soil dried (Fig. 3.9 C, D).

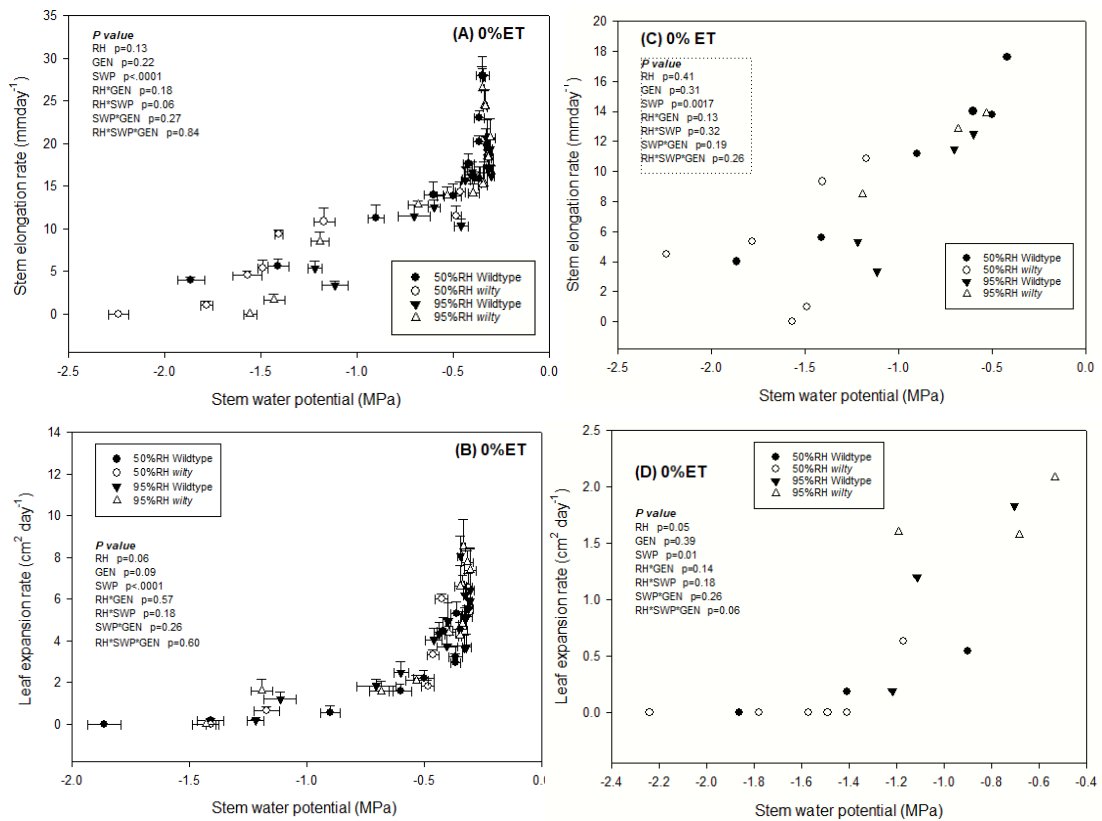


Figure 3.11 Changes in stem elongation (A, C) and leaf expansion (B, D) of wildtype (WT-filled symbols) and *wilty* (hollow symbols) plants from which irrigation was withheld, plotted against stem water potential. Relationships in (C, D) represent data once stem elongation and leaf expansion had decreased after breakpoint in all treatments. Points and bars are means  $\pm$  SE of data presented in Figures 3.5, 3.6 (A, B) 3.7 (A, B), with error bars removed for clarity in (C, D). P-values for 3-way ANCOVA of each relationship reported in their respective panel.

	Humidity	Genotype	BP of stem elongation decreased at no difference in stem water potential	Standard error
<b>Stem</b>	50%RH	<i>wilty</i>	-0.49	0.39
		WT	-0.37	0.004
	95%RH	<i>wilty</i>	-0.42	0.13
		WT	-0.47	0.16
			BP of leaf expansion decreased at no difference in stem water potential	Standard error
<b>Leaf</b>	50%RH	<i>wilty</i>	-0.64	0.14
		WT	-0.68	0.14
	95%RH	<i>wilty</i>	-0.44	0.05
		WT	-0.64	0.22

Table 3.1 Results of segmented analysis demonstrating a breakpoint (BP) threshold stem water potential, when stem elongation and leaf expansion significantly decreased as the soil dried. BP values are reported along with the standard error of mean.

Withholding water decreased stem elongation (Fig. 3.11A) and leaf expansion (Fig. 3.11B) as stem water potential decreased. Irrespective of humidity effects, both wildtype and *wilty* shown similar trends of stem elongation decreasing as stem water potential declined, even when excluding the part of the relationship after breakpoint when both variables declined with no change in stem water potential (Fig. 3.11C, D; Table 3.1).



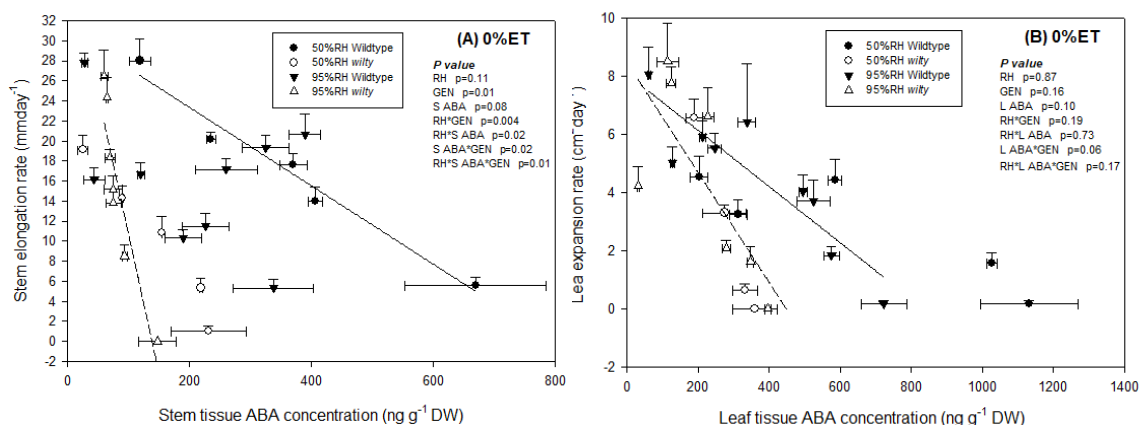


Figure 3.12 Changes in (A) stem elongation and (B) leaf expansion rate plotted against stem and leaf ABA concentrations respectively, of wildtype (WT-filled symbols, solid lines) and *wilty* (hollow symbols, dashed lines) plants from which water was withheld, when grown at 50% and 95% relative humidity, RH. Symbols are means  $\pm$  SE of data presented in Figures 3.6, 3.7, 3.8, 3.9, with linear regressions fitted to each genotype. *P*-values for 3-way ANCOVA of each relationship reported in their respective panel.

After withholding water, stem elongation and leaf expansion decreased as stem and leaf ABA concentrations increased (respectively) in both genotypes, independent of RH% (Fig. 3.12A, B). Nevertheless, stem elongation decreased more sensitively with stem ABA concentration at 95%RH (significant RH x stem ABA interaction). Moreover, stem elongation of *wilty* decreased at lower stem ABA concentrations, and more sensitively as ABA concentrations increased, compared to WT plants (Fig. 3.12A), as indicated by a significant genotype x ABA interaction. Although a similar pattern existed when comparing leaf expansion with ABA concentration (Fig. 3.12B), the statistical interaction only

approached significance ( $P=0.06$ ). For both tissues, there was no unifying relationship between tissue expansion and tissue ABA status.

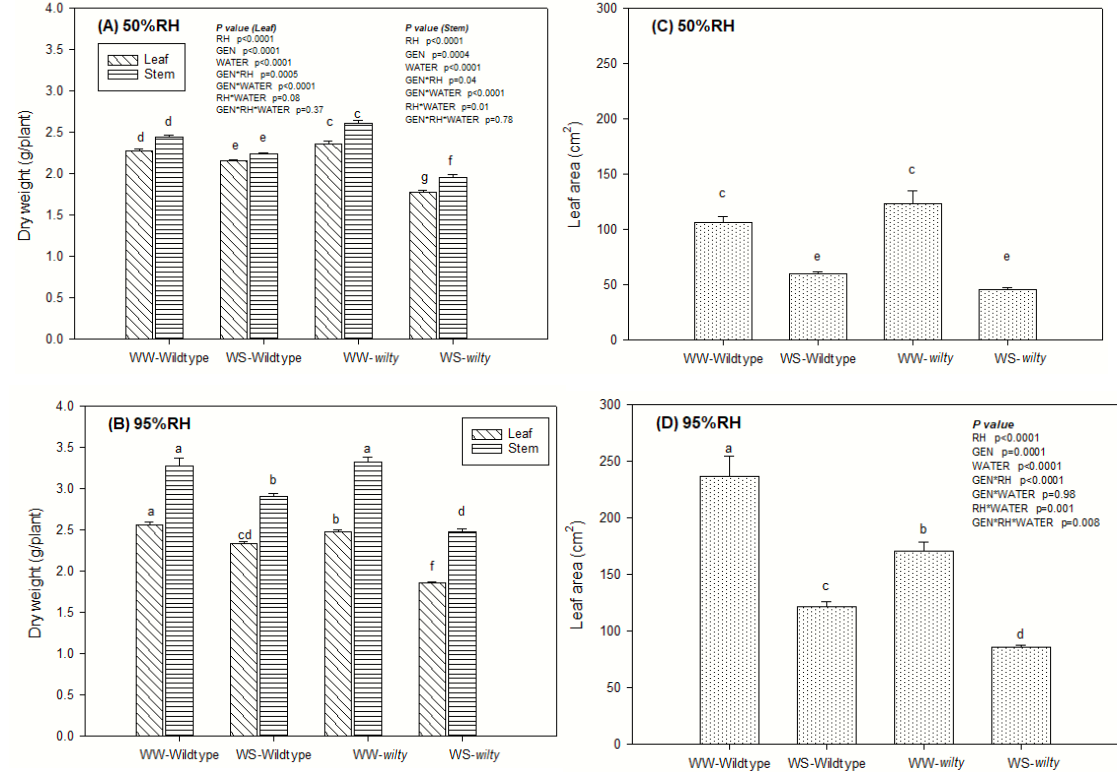


Figure 3.13 Final leaf and stem dry weight of wildtype (WT) and *wilty* pea grown at (A) 50% and (B) 95% relative humidity (RH) at well-watered and water stress; and total leaf area of WT and *wilty* pea grown at (C) 50% and (D) 95%RH at well-watered and water stress. Bars are means  $\pm$  SE of 6 replicates. Different letters indicate a significant difference of humidity\*genotypes\*water at  $P<0.05$ . P-values for 3-way ANOVA of dry weight at 50%RH and 95%RH as reported in panel A and leaf area in panel D.

Final stem and leaf dry weights, and leaf area were 7%, 23% and 45%, respectively higher at 95%RH (averaged across genotypes and water treatments). This was mainly due to the longer experimental duration at 95%RH. Nevertheless, *wilty* and WT had similar responses in both humidities, with *wilty* showing greater decreases than WT (indicated by significant humidity x genotype interaction). Soil drying decreased final stem, leaf dry weight and leaf area by 15%, 17% and 54% at 50%RH (averaged across genotypes) and 17%, 18%, 49% at 95%RH (as indicated by significant water x humidity interaction). Wildtype plants had 11%, 10% and 12% higher stem and leaf dry weights and leaf area by (averaged across both humidities and water treatments), but a similar sensitivity to drought as *wilty* in both humidities, but not leaf area at 50%RH. At 50%RH in both well-watered and water stress treatments, *wilty* and WT had a similar leaf area a (no significant genotype x water interaction). Thus, *wilty* had similar responses to soil drying as WT.

### 3.4 Discussions

Since this study aimed to determine the role of ABA in regulating stem elongation, genotypes differing in ABA concentration (the ABA-deficient mutant *wilty* and its wild-type, WT) were used. This mutant was isolated (described in Donkin et al. 1983) as a spontaneous mutation causing wilting in mature plants and was recently identified as a lesion affecting xanthoxin dehydrogenase activity in the ABA biosynthesis pathway (McAdam et al., 2015). Although it has been used to investigate the role of ABA in regulating stomatal responses to differing environmental conditions (Donkin et al. 1983; Leymarie et al., 1998; McAdam et al. 2015; Rothwell et al. 2015), its shoot growth has scarcely been reported (Dodd 2003b) and not in response to drying soil.

#### ***wilty was shorter than WT plants irrespective of soil moisture***

Although high humidity previously increased stem elongation of ABA-deficient tomato mutants to a greater extent than their respective WT (Bradford 1983; Jones et al., 1987; Tarr, 1993; Sharp et al. 2000), the *wilty* pea mutant was shorter (less cumulative stem elongation) irrespective of humidity (Fig. 3.6C, D). Thus, in pea endogenous stem ABA concentrations seem to maintain stem elongation irrespective of soil or atmospheric water availability (Fig. 3.8C, D), in contrast to reported observations in tomato. The reason(s) for these species differences in the role of ABA in mediating stem elongation are not clear. ABA seems necessary to maintain stem elongation in well-watered plants by antagonizing the growth inhibitory effects of ethylene (Sharp et al., 2000). In

addition, *wilty* in drying soil had less cumulative stem elongation than WT in both 50%RH and 95%RH (Fig.3.6 A-D). Thus, to our knowledge, this is the first study indicating that cumulative stem elongation of *wilty* was less than WT.

As the soil dried, stem elongation decreased similarly in both WT and *wilty* genotypes (Fig 3.10A, C), especially when data were expressed as percentage of initial values (Fig 3.10C), implying that ABA has a limited role in regulating stem elongation as the soil dries. Even though stem elongation was highly correlated with ABA accumulation in internode tissues in both genotypes (Fig 3.12A), the different slopes (sensitivities) between genotypes implies that stem elongation was more sensitive to ABA accumulation in *wilty*. However, stem water potential of *wilty* was also much lower in drying soil (Fig 3.5), and low water potential can sensitise stomata (Tardieu and Davies 1992) and growing coleoptiles (Dodd and Davies 1996) to ABA. Evaluating the impact of osmotic stress and exogenous ABA on excised stem elongation would allow the sensitivity of the two genotypes to these two potential growth inhibitory agents (and their interaction) to be evaluated. Nevertheless, the consistency (across genotypes) in stem elongation responses to stem water potential (Fig 3.11A, C) and divergent relationships of stem elongation response to endogenous ABA concentrations (Fig 3.12A) makes it difficult to argue for a growth regulatory role of ABA. However, since stem elongation halved before any change in stem water potential (Fig 3.11A), it is also difficult to advocate that stem water potential was regulating growth, consistent with the results of Chapter 2.

***Similar leaf expansion of wilty and wildtype (WT) in drying soil, but increased leaf expansion in well-watered WT plants***

While few studies have examined the role of ABA in regulating stem elongation, there has been more interest in evaluating its impact on leaf expansion. Leaf expansion rate of WT and *wilty* plants decreased similarly as the soil dried, irrespective of relative humidity (Fig. 3.7 A,B). The large genotypic differences in ABA accumulation following soil drying (Fig. 3.9 C, D) implies that it is not involved in regulating leaf expansion. Similarly, soil drying caused similar leaf growth inhibition in WT and the ABA deficient tomato mutant *flacca* (Coleman & Schneider 1996). Moreover, salinity stress (0-75 mM NaCl) similarly decreased leaf area of various ABA-deficient and ABA-insensitive mutants (*sitiens* (*sit*) tomato and the *abi1-1*, *abi2-1*, *abi3-1* and *aba1-3* Arabidopsis mutants) and their respective WT (Mäkelä et al. 2003; Cramer, 2002). This similarity of leaf growth inhibition is perhaps surprising, since ABA-deficient mutants are less able to regulate their water status in response to edaphic stresses (Fig. 3.7A, B), resulting from a lack of stomatal control caused by ABA deficiency which may decrease turgor (Mäkelä et al. 1998; Nagel et al. 1994). Although plants were grown at 95%RH to moderate water relations and stem water potential (which eliminates transpiration before measurement), well-watered *wilty* plants had much less leaf area than WT plants, but no genotypic difference occurred at 50%RH. Thus, endogenous ABA accumulation seems important in maintaining leaf growth (irrespective of soil drying), via non-hydraulic mechanisms (Sharp et al. 2000).

Furthermore, *wilty* had less leaf area than WT under well-watered conditions (Fig. 3.7C, D) as reported previously (Dodd 2003). Consistent with this result, Sharp *et al.*, 2000 established *flacca* (*flc*) and *notabilis* (*not*) had less leaf area than WT plants even when these genotypes were compared at the same leaf water potential (WT plants were growing at lower %RH than the mutants). Comparable leaf growth inhibition of *flc* was reported when plants were grown in the same environment as WT plants, with *flc* having lower leaf water potentials (Bradford 1983; Jones *et al.*, 1987), suggesting that differences in leaf water status were not regulating growth. Thus, all abovementioned studies and present results indicates that non-hydraulic effects of ABA deficiency under optimal conditions are the major cause of leaf growth inhibition, irrespective of plant water status.

Surprisingly, *wilty* and WT plants both accumulated ABA (in both stems and leaves) in response to drying soil (no significant effect of genotype x water interaction) (Fig. 3.12A, B). In contrast, the ABA-deficient tomato mutant *flc* failed to accumulate ABA in the leaves as the soil dries (Holbrook *et al.* 2002). It is uncertain whether the degree of soil drying and/or the lesion in ABA biosynthesis (*flc* is perturbed in the penultimate step of ABA biosynthesis) are responsible for these contrasting results. The above-mentioned studies and the present results suggest that ABA is maintaining leaf expansion as the soil dries, irrespective of endogenous ABA levels.

***High humidity slowed the decline in soil water content and stem water potential as the soil dried***

High humidity delayed a soil drying induced decrease in soil water content (1 day) and stem water potential (4 days), especially in *wilty* plants (Fig. 3.4A, B; Fig. 3.5A, B), which had a greater evapotranspiration (ET) than WT plants. Although stomatal conductance was not directly measured, greater ET agrees with enhanced stomatal conductance of *wilty* (Dodd 2003; Rothwell et al. 2015) and other ABA-deficient mutants (Dodd et al. 2009). Thus, it was necessary to compare plant growth responses to different humidity conditions at the same soil and plant water status (Fig. 3.14).



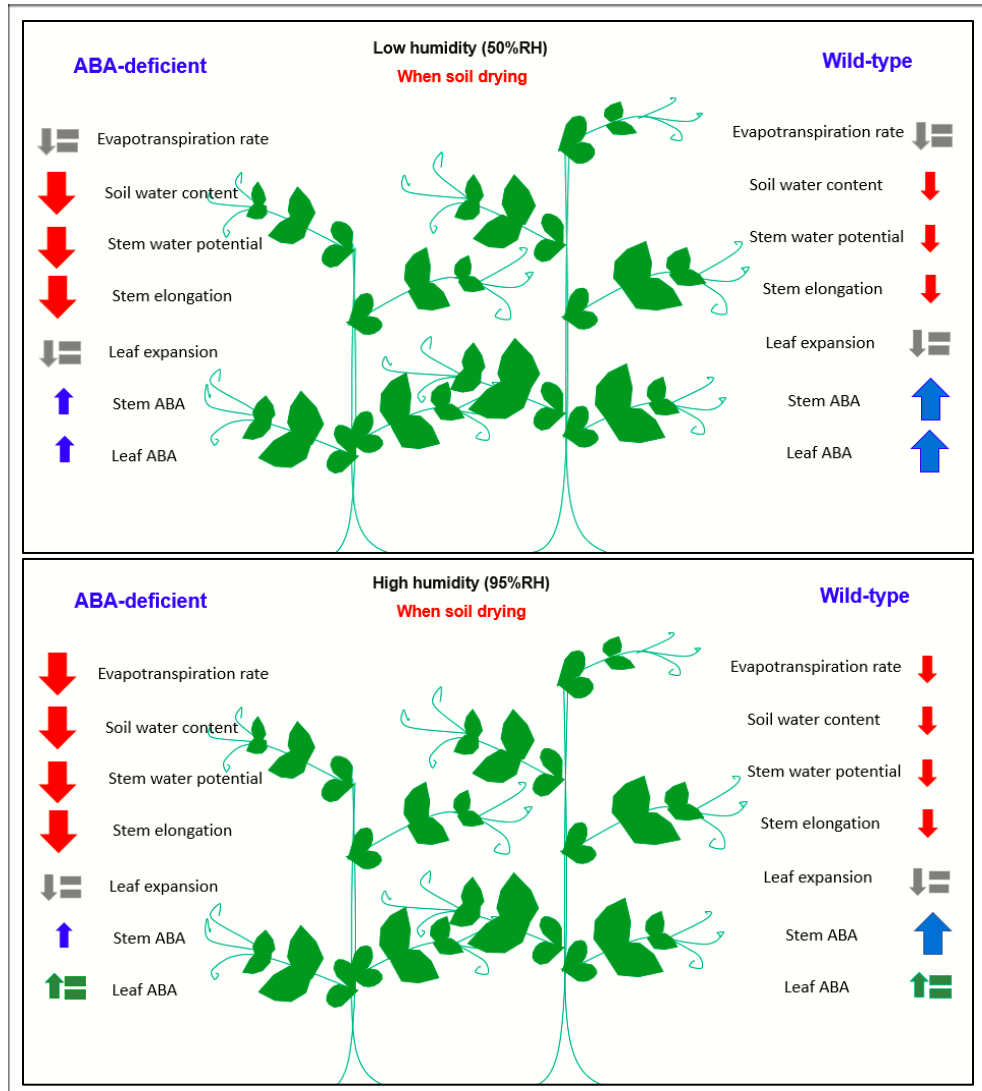


Figure 3.14 A model of the role of stem and leaf ABA concentrations at high and low humidity, proposing that ABA maintains stem elongation and leaf expansion in response to drying soil. Upward- and downward-facing arrows indicate increases and decreases in the respective parameter; the size of arrow indicates the magnitude of changes; the equal sign indicates similar response

### **3.5 Conclusion**

Taken together, higher stem and leaf tissue ABA concentrations (irrespective of atmospheric and soil water status) seems to maintain stem elongation and leaf expansion. With soil drying, growth of both ABA-deficient and WT genotypes declined despite similar relative changes in ABA status, suggesting that absolute endogenous ABA concentrations may not be responsible for regulating shoot growth.

## Chapter 4

### Do indole-3-acetic acid (IAA) and gibberellic acid (GA<sub>3</sub>) promote stem elongation in *Pisum sativum* exposed to drying soil?

#### 4.1 Introduction

In *Pisum sativum* L., gibberellins (GAs) and indole-3-acetic acid (IAA) are the two major endogenous plant growth regulators controlling stem elongation of well-watered plants (Yang et al. 1993b; Yang et al. 1996; Yang & Davies 1999). This understanding has been revealed via five major genes (*WT/mutant* alleles) that cause substantial phenotypic changes of dwarf and slender (hyper-elongated) peas: dwarf (*Le/le*), crypto dwarf (*Cry/cry<sup>c</sup>/cry<sup>s</sup>*), nana (*Na/na*), micro (*Lm/lm*) and slender (*Sl/sln*) mutant (*La/la*). *LE* encodes a GA<sub>3</sub>-oxidase in pea shoots (Lester et al., 1997; Martin et al., 1997) and *SLN* controls GA<sub>2</sub>-oxidation in seeds (Ross et al., 1995), of which mutations in *SLN* produce hyper-elongated plants. The five loci are known to directly influence distinct internode (length) phenotypes (Reid et al. 1983). The mutant allele of the *Le* locus, first identified by (Mendel, 1866), has 40-60% lower endogenous GA<sub>1</sub> levels in growing shoots, resulting in much shorter internodes than tall wild-type plants (Ross et al. 1992). Furthermore, the dwarf pea cultivar Progress No.9 (*le*, *SLN*) has lower levels of endogenous IAA, but a greater relative response to exogenous IAA, compared to tall cultivars (Yang et al., 1993). One barrier to establishing the role of auxin in regulating stem extension is the lack of dwarf mutants specifically attributable to auxin deficiency. Nevertheless, endogenous IAA content was closely positively

correlated with internode growth in a range of genetically distinct pea lines differing in height (Law & Davies 1990). Thus, gibberellin and auxin seemingly play an important role in regulating stem elongation in intact pea plants.

Gibberellins (GAs) are involved in many aspects of plant development, particularly stem elongation. GA<sub>1</sub>, which is biosynthesized by the early 13-hydroxylation pathway, is the principal GA regulating stem length in pea (Ingram et al. 1984). GA-mediated growth promotion has generally been attributed to an increase in both cell number and cell length (Arney & Mancinelli, 1966 ; Arney & Mancinelli, 1967; Reid *et al.*, 1983), with enhanced wall extensibility promoting cell elongation (Cosgrove & Sovonick-Dunford 1989; (Behringer et al. 1990). Moreover, continuously supplying exogenous IAA to intact light-grown dwarf cv. Progress No. 9 maintained stem elongation rate between 6 to 8 µm/min as compared to elongation rate at 2 µm/min when IAA was withdrawn (Behringer & Davies 1992; Yang et al., 1993). Since elongation of isolated stem segments is stimulated by exogenous auxin, it has been inferred that endogenous auxin concentrations may also regulate stem elongation of intact plants (Cleland 1995). Nevertheless, stimulation of stem elongation by exogenous GA<sub>3</sub> and IAA concentration depends on the concentration applied, plant species and methods of application.

In addition to this, GA<sub>3</sub> and IAA may regulate stomatal responses to environmental changes (Dodd 2003b; Pospisilová 2003). Drought stress decreased leaf endogenous gibberellin and auxin concentrations in maize (Wang et al., 2008), and these changes effect stomatal closure to minimize water loss from leaves (Pandey et al., 2003). Moreover, foliar sprays or soil application of concentration of 5 µM GA<sub>3</sub> and IAA decreased stomatal conductance of cotton (*Gossypium hirsutum L.*) (Kumar et al. 2001). In addition, observation of *Commelina communis* epidermal strips incubated for 3 hours increased concentration of IAA (0.000001mM to 0.1mM induced stomata opening, in contrast response to ABA (Snaith and Mansfield 1982). Thus, GA<sub>3</sub> and IAA may be involved in mediating stomatal opening and closure as the soil dries. Although the roles of GA<sub>3</sub> and IAA in regulating stem growth have been extensively studied, their role in regulating stomatal conductance of droughted plants has received little attention.

Consequently, this chapter aimed to examine stem elongation responses of a tall pea under different soil water availabilities and in response to exogenous GA<sub>3</sub> and IAA applications, to elucidate the relative roles played by both hormones. The hypotheses tested were

- Drought decreased endogenous GA<sub>3</sub> and IAA levels and stem elongation (Chapter 2), thus exogenous GA<sub>3</sub> and IAA application would rescue stem elongation

- Exogenous GA<sub>3</sub> and IAA application increased stomatal conductance, thereby accelerating soil drying

## **4.2 Materials and Method**

### **4.2.1 Plant Materials and growth conditions**

Pea (*Pisum sativum* cv. Alderman) seeds were germinated on moistened tissue paper and kept in the dark for 5 days. On the emergence of the plumule and radicle, uniform seeds were individually transplanted into round pots of 16 cm length x 13 cm diameter (1 L volume) containing a 1:1 (v:v) mixture of silica sand and sieved growing substrate (John Innes No.2, J. Arthur Bowers). After being transplanted, seedlings were watered daily to maintain soil water content at 100% of field capacity for seven days. To minimize soil evaporation, the top of each pot was covered with black tape, leaving a 2 cm<sup>2</sup> opening to allow watering. The day before the experiment started, plants were selected for the experiment based on similar phenological development and plant height.

The experiment was conducted in a controlled environment room with supplementary lighting (supplied by Osram 600w daylight bulbs) supplying 300  $\mu\text{mol m}^{-2}\text{s}^{-1}$  photosynthetic photon flux density (PPFD) at bench level for 12 hours per day. Day and night temperatures were 26°C maximum and 20°C minimum, with 50±5% relative humidity (RH).

#### **4.2.2 Irrigation treatments**

The well-watered group of plants were watered daily between 4.00-5.00pm by replacing evapotranspirational losses (determined gravimetrically) over 24 hours. After watering at 100% field capacity on the first day of the experiment, water was withheld from the drought-stressed treatment during the next 11 days.

#### **4.2.3 Stem elongation and stem water potential ( $\Psi_{\text{stem}}$ )**

Stem elongation and stem water potential was measured as explained in Sections 2.2.4 and 2.2.3, respectively.

#### **4.2.4 Leaf expansion**

After ten days (when plants had two to three whorls of leaves), leaf expansion was measured daily at 3.00-4.00pm by determining the length and width of each leaf using flexible ruler. When length and width of a leaf was constant between successive measurements (indicating that the leaf had stopped growing), it was no longer measured and the measurements continued with the expanding leaves further up the stem. The cumulative leaf expansion graph of each concentration of gibberellin and auxin applied were based on the total leaf expansion over 11 days of drought stress.

#### **4.2.5 Stomatal conductance**

Stomatal conductance was measured daily between 9:00 and 11:00am (photoperiod started at 8:00am) with an AP4 porometer (Delta-T Devices, Cambridge, UK). Leaves at the second node from the base of the plant were measured. Abaxial leaf surfaces were measured on both sides of the midrib in each leaflet, with the four readings averaged to represent the stomatal conductance of an individual plant.

#### **4.2.6 Auxin (IAA), Gibberellic acid (GA<sub>3</sub>) and lanolin preparation and application**

The plant growth regulator compounds used in this study were gibberellic acid (GA<sub>3</sub>; Sigma Aldrich) and indole-3-acetic acid (IAA; Sigma Aldrich). The GA<sub>3</sub> and IAA powder were first diluted in 100 µL of 70% (v/v) ethanol before being made up to working concentrations of 0.03 mM, 0.05 mM and 0.1 mM with deionized water.

Prior to spraying the different hormone concentrations, lanolin was applied with a small soft brush around the two uppermost stem internodes with a layer of 1 cm height (Fig. 4.1). First, lanolin paste (Sigma Aldrich) was placed in a beaker and the bottom of the beaker immersed in warm water (maximum of 50°C). The lanolin was stirred until it changed from a light-yellow solid to a darker yellow semi-solid. Hormonal treatments were applied 3 times (two days apart) in the morning to both drought-stressed and well-watered plants (Fig. 4.1).



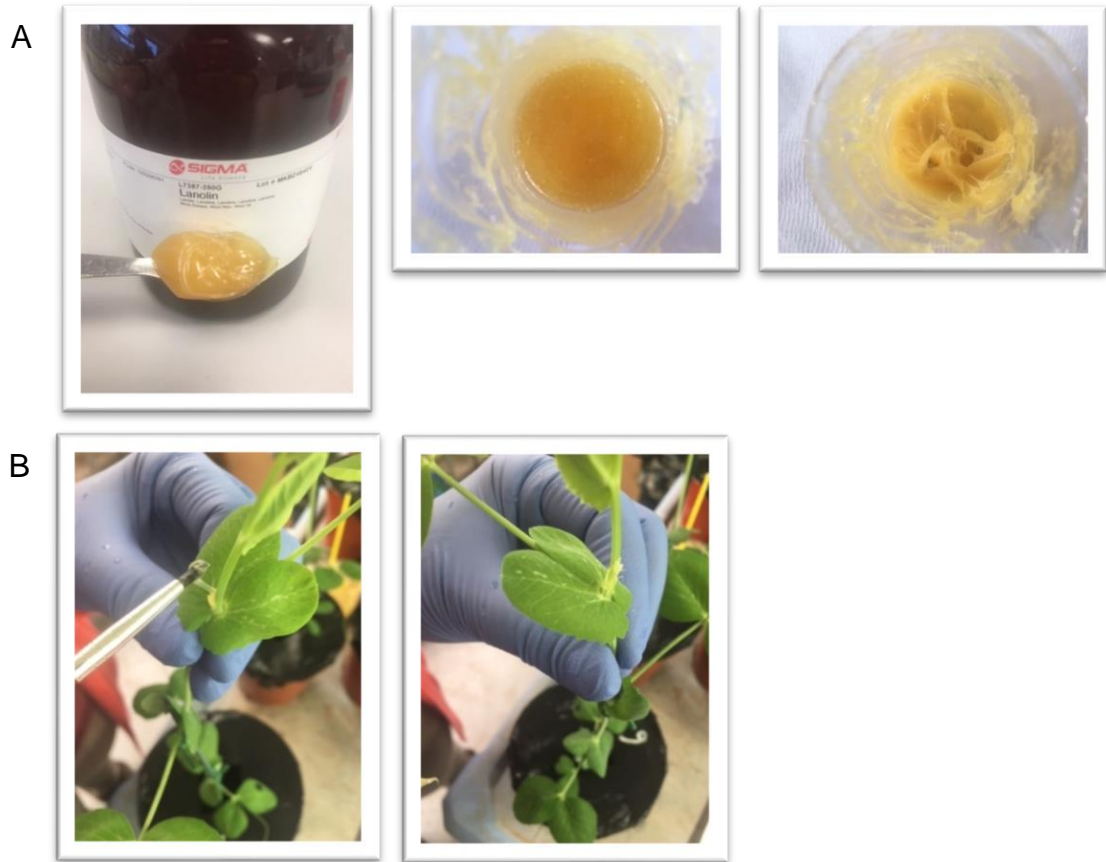


Figure 4.1 (A) Lanolin paste warmed and change from solid to semi-solid state prior to application around the stem. (B) A layer of lanolin applied around the stem using a soft brush.

#### **4.2.7 Stem sampling for phytohormone analysis**

Samples of stem tissues from the two uppermost internodes were collected and immersed in liquid nitrogen before being stored in -20°C. Frozen stem tissues were then freeze dried (Martin Christ Freeze Dryer, Alpha 1-2 LDplus, Germany) for 48 hours and ground using a ball mill machine (Retsch, Mixer Mill MM400, Germany) to transform stem tissues into powder form. For phytohormone analysis, 10 mg of stem tissue powder was weighed in an eppendorf tube before continuing with the extraction procedure.

#### 4.2.8 Hormone extraction and analysis

Gibberellic acid (GA<sub>3</sub>) and indole-3-acetic acid (IAA) were analysed according to Albacete et al., 2008 with some modifications. Analyses were conducted by Dr Alfonso Albacete at Department of Plant Nutrition, Campus Universitario de Espinardo, Murcia, Spain. Briefly, 0.1 g of freeze-dried material was homogenized in liquid nitrogen and dropped in 0.5 ml of a cold (-20°C) extraction mixture of methanol/water (80/20, v/v). Solids were separated by centrifugation (20 000g, 15 min) and re-extracted for 30 minutes at 4°C in an additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C<sub>18</sub> cartridge (SepPak Plus, Waters, USA) to remove interfering lipids and part of the plant pigments, and evaporated at 40°C in a vacuum, either to near dryness or until the organic solvent was removed. The residue was dissolved in 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 membrane (Millipore, Bedford, MA, USA).

Ten µL of filtrated extract were injected in a U-HPLC-MS system consisting of 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). For quantification of the plant hormones, calibration curves were constructed for each analyzed component (1, 10, 50, and 100 µg L<sup>-1</sup>) and

corrected for 10  $\mu\text{g L}^{-1}$  deuterated internal standards. Recovery percentages ranged between 92 and 95%.

#### **4.2.9 Statistical Analysis**

All treatments were arranged in the randomised complete block design (CRD). The fully factorial experiment was analyzed using SAS 9.2 version and Least Significant Differences (LSD) ( $P \leq 0.05$ ) used to discriminate significant treatment and interaction means. Two-way ANOVA was used to determine the effects of the three hormone concentrations (auxin and gibberellin) and irrigation treatment (well-watered *versus* drying soil). The effects of individual hormone concentrations and their interactions on dependent variables (stem elongation) were determined in identifying the most influential dosage. Analysis of covariance (ANCOVA) was used to analyse the linear relationship between soil water content or stem water potential, and the decrease of stem elongation, leaf expansion and stomatal conductance under drought stress; Least Square Means (LSM) with an adjustment using Tukey Kramer, was used to discriminate the effects of three different hormone concentrations of gibberellin and auxin. For non-linear relationships, regression analysis was done separately using segmented analysis in R Software (Version 3.4.1) that provided an estimate of the breakpoint/threshold for the decline in stem elongation and leaf expansion. Further, a significance test was used to discriminate the regression slope before and after the breakpoint of three different hormone concentrations of gibberellin

and auxin relative to the control treatment. Null hypothesis: No treatment effect on slope (coefficient of treatment  $GA_3/IAA * Soil\ water\ content = 0$ ) of the physiological response to soil/plant water status, Alternative hypothesis: There is treatment effect on slope (coefficient of treatment  $GA/IAA * Soil\ water\ content \neq 0$ )

### 4.3 Results

<b>Parameter</b>	<b><i>P</i> value</b>
Soil water content	0.68
Stem water potential	0.95
Stem elongation	0.61
Leaf expansion	0.33
Stomatal conductance	<0.0001

Table 4.1 The effect of time on soil and plant variables (*P*-values reported) for well-watered control plants during the 11 days.

Irrespective of whether hormone treatments were applied, well-watered plants showed no change in soil water content, stem water potential, stem elongation and leaf expansion throughout the 11 days of the experiment, as indicated by non-significant ( $P > 0.05$ ) *P*-values when determining the dependence of these variables on time (Table 4.1). However, stomatal conductance increased over time.

	GA ( <i>P-value</i> )			IAA ( <i>P-value</i> )		
	Treatment	Day	Treatment* Day	Treatment	Day	Treatment* Day
Stem elongation	<.0001	0.49	0.29	<.0001	0.61	0.98
Leaf expansion	<.0001	0.66	0.53	<.0001	0.31	0.46
Soil water content	<.0001	<.0001	<.0001	<.0001	<.0001	0.02
Stem water potential	<.0001	0.05	0.06	<.0001	0.01	0.14
Stomatal conductance	<.0001	<.0001	0.09	<.0001	<.0001	0.25

Table 4.2 The effect of gibberellin and auxin treatment on changes of soil and plant variables with time in well-watered plants (*P-values* reported).

GA<sub>3</sub> and IAA application to well-watered plants altered soil water content, stem elongation, leaf expansion, stem water potential and stomatal conductance, as indicated by significant treatment *P-values*, when determining the dependence of these variables on time (Table 4.2).

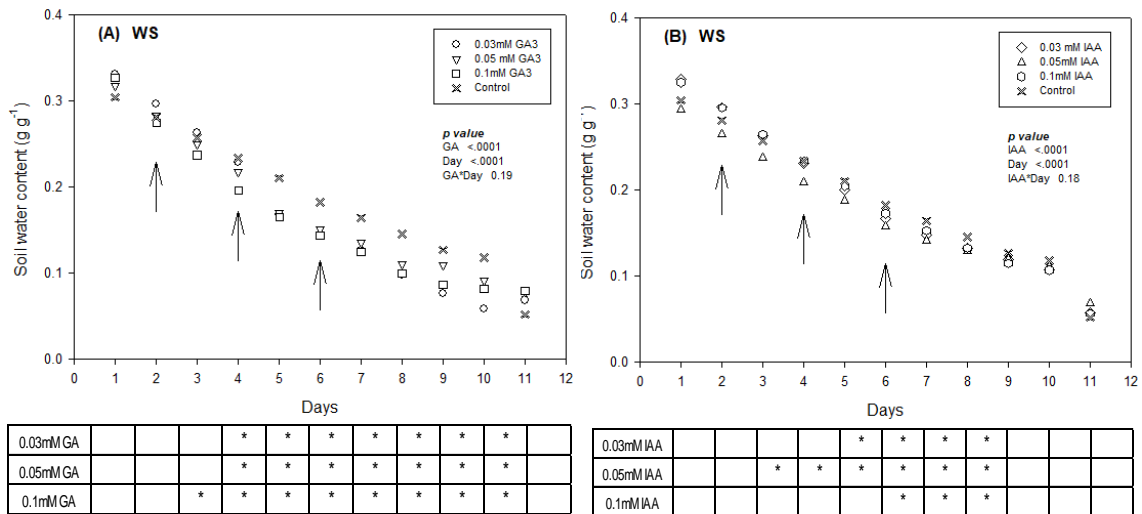


Figure 4.2 Effects of applying (A) gibberellic acid (GA<sub>3</sub>) and (B) auxin (IAA) on soil-drying induced changes in soil water content. The treatments are 0.03 mM, 0.05 mM and 0.1 mM of GA<sub>3</sub> and IAA respectively. Arrows indicate when GA<sub>3</sub> and IAA were applied. \* symbol in the tables below figure A and B denote when GA<sub>3</sub> and IAA treatments significantly (LSD,  $P < 0.05$ ) differ from control plants. Data are means of four replicates for all treatments, with error bars omitted for clarity. Effects of (A) GA<sub>3</sub> and (B) IAA application were consistent over time, as indicated by no significant treatment x time interactions.

Irrigation (supplying a controlled amount of water calculated from the daily rate of evapotranspiration) maintained soil water content ( $\theta$ ) of the well-watered plants throughout the experiment (Table 4.1). Applying different concentrations of gibberellic acid and auxin significantly decreased  $\theta$  of well-watered plants and significantly change over the 11 days (Table 4.2). Under drought stress conditions, the  $\theta$  declined steadily over the 11 days, with gibberellic acid (GA<sub>3</sub>)-sprayed plants drying faster than the control group from Day 3 (0.1 mM GA<sub>3</sub> only)



until Day 10 (Fig. 4.2A). Similarly, auxin (IAA)-sprayed plants dried the soil faster than the control plants (Fig. 4.2B), but not as fast as the GA<sub>3</sub>-sprayed plants (Fig. 4.2A, B). Thus, applying GA<sub>3</sub> and IAA increased the rate of water loss, particularly after the second and third sprays, which was noticeable after Day 4.

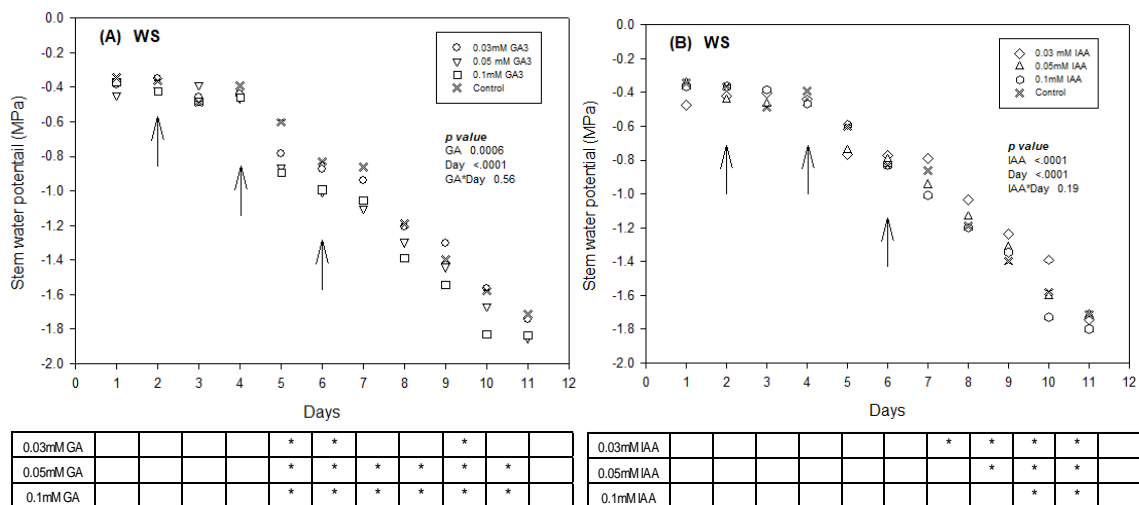


Figure 4.3 Effects of applying (A) gibberellic acid (GA<sub>3</sub>) and (B) auxin (IAA) on soil-drying induced changes in stem water potential ( $\Psi_{\text{stem}}$ ). The treatments are 0.03 mM, 0.05 mM and 0.1 mM of GA<sub>3</sub> and IAA respectively. Arrows indicate when GA<sub>3</sub> and IAA were applied. \* Symbol in the tables below figure A and B denote when GA<sub>3</sub> and IAA treatments significantly (LSD,  $P < 0.05$ ) differ from control plants. Data are means of four replicates for all treatments, with error bars omitted for clarity. Effects of (A) GA<sub>3</sub> and (B) IAA application were consistent over time, as indicated by no significant treatment x time interactions.

The stem water potential ( $\Psi_{\text{stem}}$ ) of well-watered plants remained consistent throughout the 11 days of treatments (Table 4.1). Applying different concentrations of gibberellic acid and auxin caused significant fluctuations in  $\Psi_{\text{stem}}$  of well-watered plants, but no consistent differences over the 11 days (Table 4.2). With soil drying, hormone spraying had no effect on  $\Psi_{\text{stem}}$  on Days 1, 4 and 6 of the experiment. After the second gibberellic acid ( $\text{GA}_3$ ) application,  $\Psi_{\text{stem}}$  of  $\text{GA}_3$  treatments decreased more rapidly than the untreated control plants (Fig. 4.3A). In contrast, the third application of auxin increased  $\Psi_{\text{stem}}$ , beginning with 0.03 mM IAA (Day 7), followed by rapid increase in all concentrations until Day 9 (Fig. 4.3B). Adding  $\text{GA}_3$  and IAA tended to decrease or increase  $\Psi_{\text{stem}}$  respectively, during the later stages of drought stress (Fig. 4.3A, 4.3B).

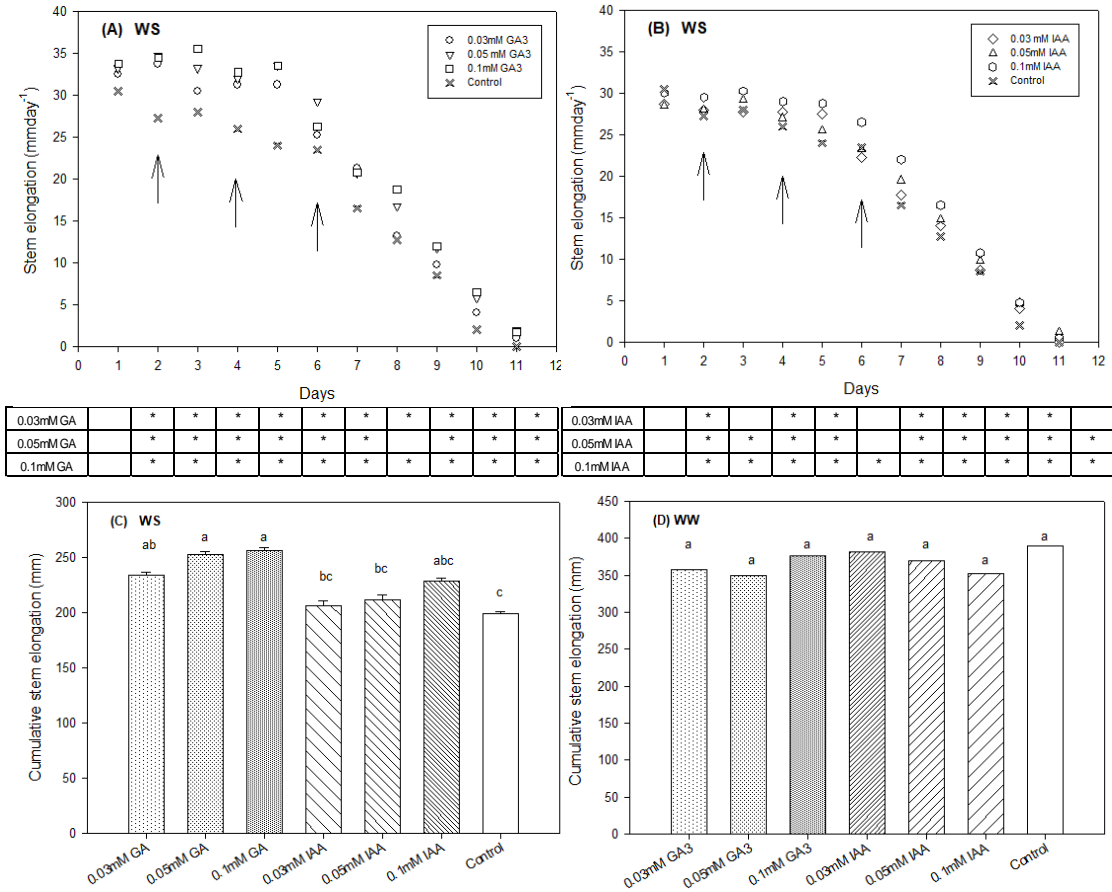


Figure 4.4 Effects of (A) gibberellic acid (GA<sub>3</sub>) and (B) auxin (IAA) on stem elongation rates relative to control plants in drying soil and cumulative stem elongation of plants grown in drying (C) and well-watered (D) soil over the 11 days of the experiment. The treatments are 0.03 mM, 0.05 mM and 0.1 mM of GA<sub>3</sub> and IAA respectively. Arrows indicate when GA<sub>3</sub> and IAA were applied. \* symbol in the tables below Figure A and B denote when GA<sub>3</sub> and IAA treatments significantly (LSD,  $P < 0.05$ ) differ from control plants. Data are means of four replicates for all treatments, with error bars omitted for clarity. In (C) and (D), different letters denote significant ( $P < 0.05$ ) differences according to LSD test.

Stem elongation of well-watered plants remained steady over the 11 days of treatment (Table 4.1). Applying different concentrations of gibberellic acid and auxin significantly increased stem elongation of well-watered plants, which were consistent over the 11 days (Table 4.2). Soil drying decreased stem elongation of control plants (relative to well-watered plants) on Day 4. Relative to control plants exposed to drying soil, gibberellic acid ( $GA_3$ ) (Fig. 4.4A) and auxin (IAA) sprays-maintained stem elongation on the first day that  $GA_3$  and IAA were applied (Fig. 4.4B), an effect that was maintained throughout the experiment. Compared to control plants exposed to drying soil,  $GA_3$ -treated plants had a significantly higher cumulative stem elongation (Fig. 4.4C). However, IAA-treated plants showed no significant differences in cumulative stem elongation as the soil dried. Thus, the day when stem elongation decreased with soil drying varied from Days 5 to 6 (with a breakpoint separating two segments) in  $GA_3$ , IAA and control-treated plants (Table 4.3). Although hormone treatments did not affect cumulative stem elongation of well-watered plants, for plants grown in drying soil IAA treatments tended to increase cumulative stem elongation while  $GA_3$  treatments significantly increased cumulative stem elongation.

<b>Treatment</b>	<b>BP of stem elongation decrease</b>	<b>Standard error</b>
0.03mM GA <sub>3</sub>	5.00	0.39
0.05mM GA <sub>3</sub>	5.02	0.28
0.1mM GA <sub>3</sub>	4.76	0.34
0.03mM IAA	4.98	0.12
0.05mM IAA	5.49	0.26
0.1mM IAA	5.66	0.13
Control	5.84	0.44
<b>Treatment</b>		
	<b>BP of leaf expansion decrease</b>	<b>Standard error</b>
0.03mM GA <sub>3</sub>	5.00	0.39
0.05mM GA <sub>3</sub>	5.02	0.28
0.1mM GA <sub>3</sub>	4.76	0.34
0.03mM IAA	4.98	0.12

Table 4.3 Results of segmented analysis demonstrating a breakpoint (BP) threshold (in days) when stem elongation and leaf expansion significantly decreased as the soil dried. BP Values are reported along with the standard error of the mean.

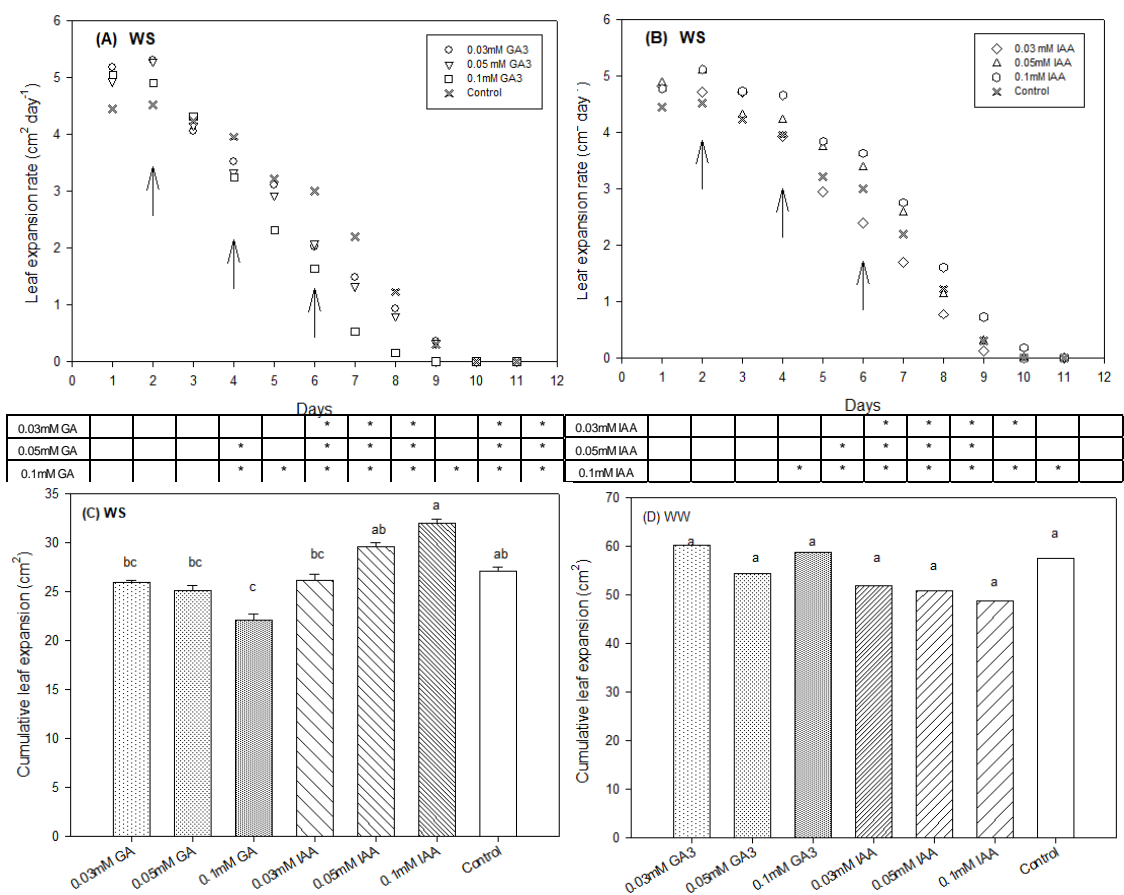


Figure 4.5 Effects of (A) gibberellic acid (GA<sub>3</sub>) and (B) auxin (IAA) on leaf expansion rate relative to control plants in drying soil and cumulative leaf expansion of plants grown in drying (C) and well-watered (D) soil over the 11 days of the experiment. The treatments are 0.03 mM, 0.05 mM and 0.1 mM of GA<sub>3</sub> and IAA respectively. Arrows indicate when GA<sub>3</sub> and IAA were applied. \* Symbol in the tables below figure A and B denote when GA<sub>3</sub> and IAA treatments significantly (LSD,  $P < 0.05$ ) differ from control plants. Data are means of four replicates for all treatments, with error bar omitted for clarity. In (C) and (D), different letters denote significant ( $P < 0.05$ ) differences according to LSD test.

Leaf expansion rate of well-watered control plants was stable over the 11 days of treatment (Table 4.1). Applying different concentrations of gibberellic acid and auxin significantly increased leaf expansion of well-watered plants, which were consistent over the 11 days (Table 4.2). With soil drying, on the day of the second application of GA<sub>3</sub> (Fig. 4.5A) and IAA (Fig. 4.5B), leaf expansion of non-irrigated plants slowed compared to well-watered plants, decreasing to zero between Days 9 and 11 (according to the treatment). High GA<sub>3</sub> concentrations (0.1 mM) tended to inhibit total leaf expansion, whereas there was no significant auxin impact relative to control plants (Fig. 4.5C). Although leaf expansion fluctuated over Days 1 to 4, leaf expansion significantly decreased from Day 5 (Table 4.3). For plants grown in drying soil, cumulative leaf expansion tended to decrease with increasing GA<sub>3</sub> dose but tended to increase with increasing IAA dose.

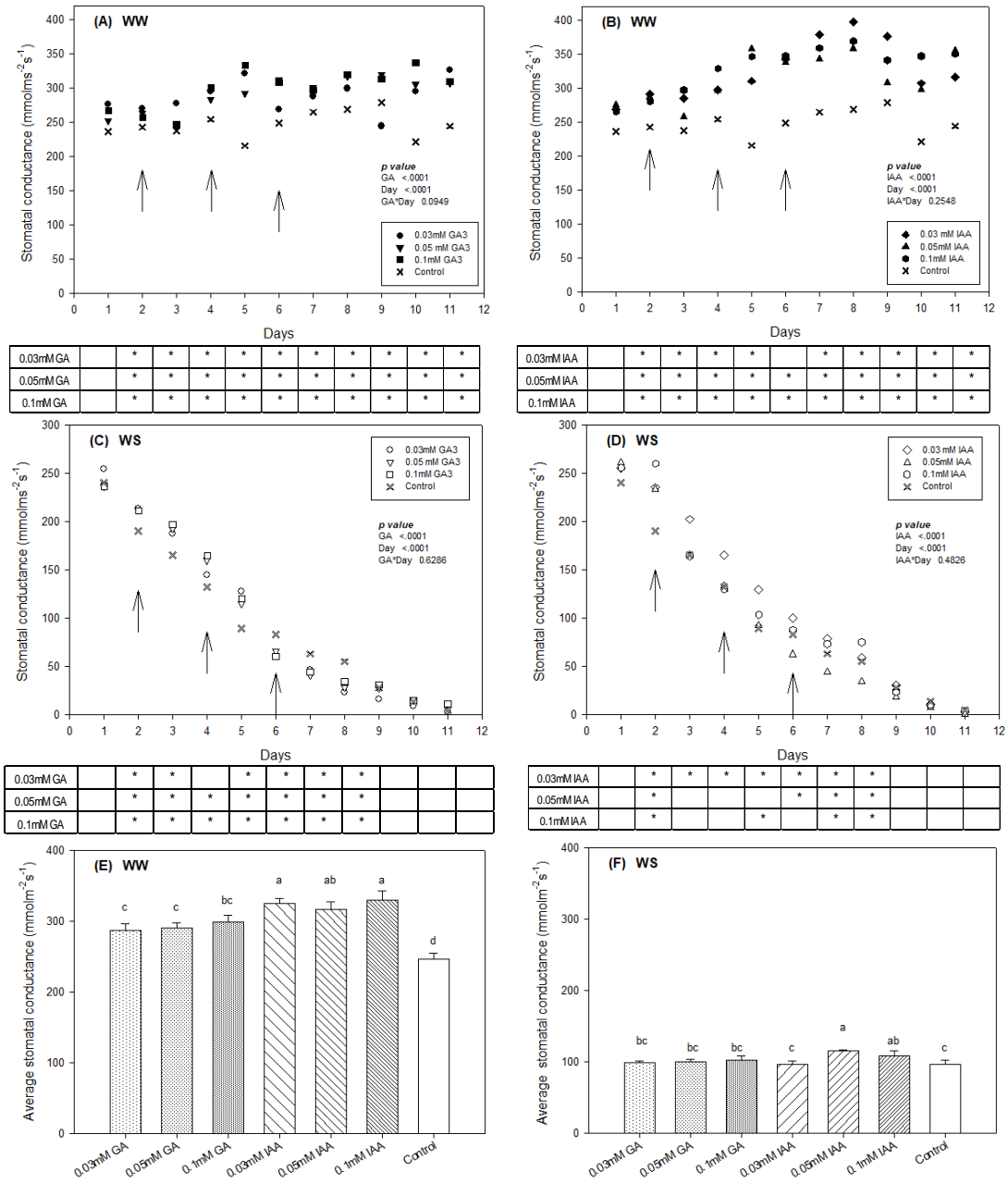


Figure 4.6 Changes in stomatal conductance and the effects of gibberellic acid (GA<sub>3</sub>) and auxin (IAA) on well-watered plants (A-B) and those exposed to water stress (C-D). Average stomatal conductance of (E) well-watered plants and (F) those exposed to water stress for gibberellin, auxin and control treatments.



Arrows in the figure indicate the day of the application of GA<sub>3</sub> and IAA. *P*-values from the ANCOVA are shown for each variable. \* symbol in the tables below panels A, B, C and D denote significant differences between GA<sub>3</sub> and IAA treatments and control (LSD, *P*<0.05). Different letters in E, F denote significant differences between GA<sub>3</sub>, IAA and control treatments on average stomatal conductance within well-watered (WW) and water-stressed (WS) plants during 11 days of drought stress (LSD, *P*<0.05). Data are means of four replicates for all treatments.

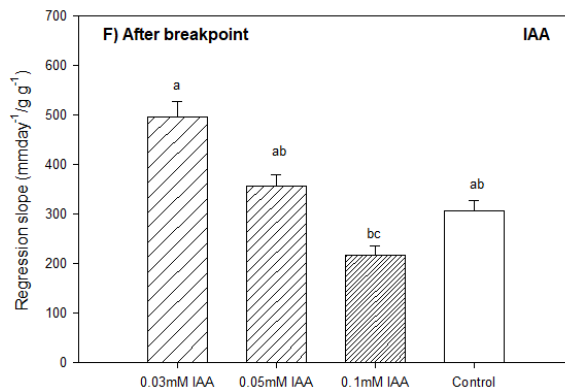
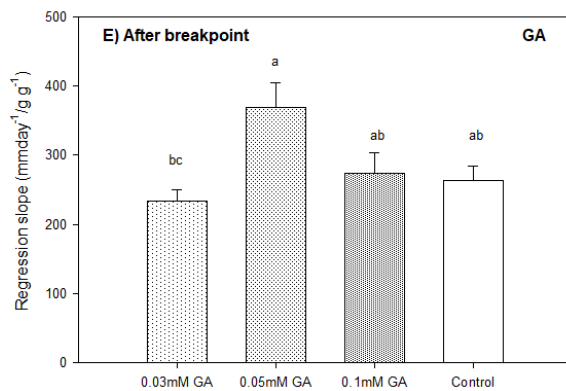
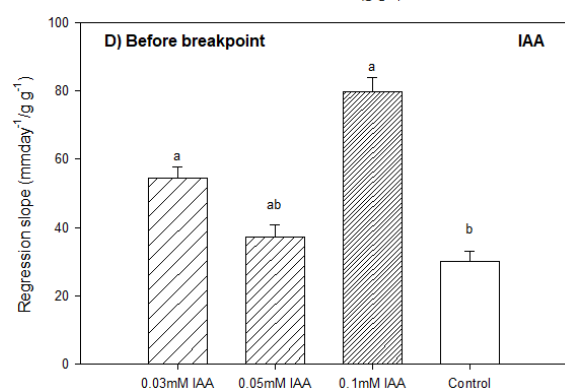
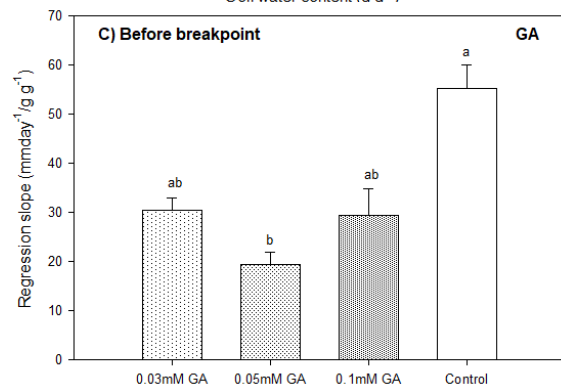
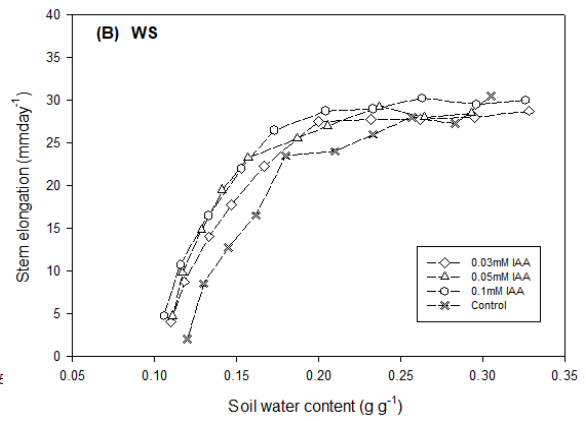
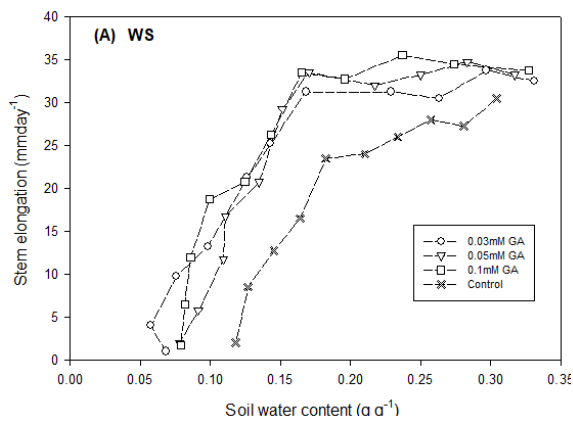
Gibberellic acid (GA<sub>3</sub>) and auxin (IAA) applications to well-watered plants increased *g<sub>s</sub>* immediately after the first application (Day 2), which remained consistently higher than control plants thereafter (Fig. 4.6A, B). Soil drying decreased *g<sub>s</sub>* of all treatments from Day 2 (Fig. 4.6C, D). When plants were grown in drying soil, GA<sub>3</sub> treatment accelerated stomatal closure over Days 1-5, but this response slowed from Days 6-11 (Fig. 4.6C). IAA treatment (0.03 mM) delayed stomatal closure over Days 1-8, while 0.05 mM IAA accelerated stomatal closure from Day 5 until the end of experiment (Fig. 4.6D). Thus, hormone treatments affected the stomatal response to soil drying.

Although stomatal conductance changed over time in both well-watered and water-stressed plants, the response was similar between treatments, so it was convenient to average the data from all measurement occasions. In well-watered conditions, control plants had the lowest  $g_s$ , with GA<sub>3</sub> treatment increasing  $g_s$  by 18% (averaged across all GA<sub>3</sub> concentrations) and IAA increasing  $g_s$  by 31% (averaged across all IAA concentrations) (Fig. 4.6E). These phytohormone effects on  $g_s$  were concentration-independent. Averaged over time and phytohormone treatments, soil drying decreased  $g_s$  by 48% (Fig. 4.6F). Only 0.03 mM IAA increased  $g_s$  of water-stressed plants compared to the control, while other GA<sub>3</sub> and IAA treatments had no effects on  $g_s$ . GA<sub>3</sub> and IAA consistently improved  $g_s$  under well-irrigated conditions, while water stress maintained  $g_s$  similar to control plants, except for the lowest concentration of IAA. Thus soil water status determined stomatal responses to phytohormone application.

	<b>Soil water content</b>	<b>Stem water potential</b>	<b>Stomatal conductance</b>	<b>Stem elongation</b>	<b>Leaf expansion</b>
<b>Soil water content</b>	1				
<b>Stem water potential</b>	-0.68 <i>0.08</i>	1			
<b>Stomatal conductance</b>	0.68 <i>0.09</i>	-0.61 <i>0.13</i>	1		
<b>Stem elongation</b>	-0.07 <i>0.87</i>	0.01 <i>0.97</i>	0.26 <i>0.56</i>	1	
<b>Leaf expansion</b>	0.47 <i>0.28</i>	-0.11 <i>0.81</i>	0.57 <i>0.17</i>	-0.35 <i>0.42</i>	1

Table 4.4 Correlation matrix (reporting  $r^2$  and  $P$ -values) for relationships between soil water content, stem water potential, stomatal conductance, stem elongation and leaf expansion in well-watered plants.

Under well-watered conditions, none of the measured variables (soil water content, stem water potential, stomatal conductance, stem elongation and leaf expansion) were significantly correlated (Table 4.4).



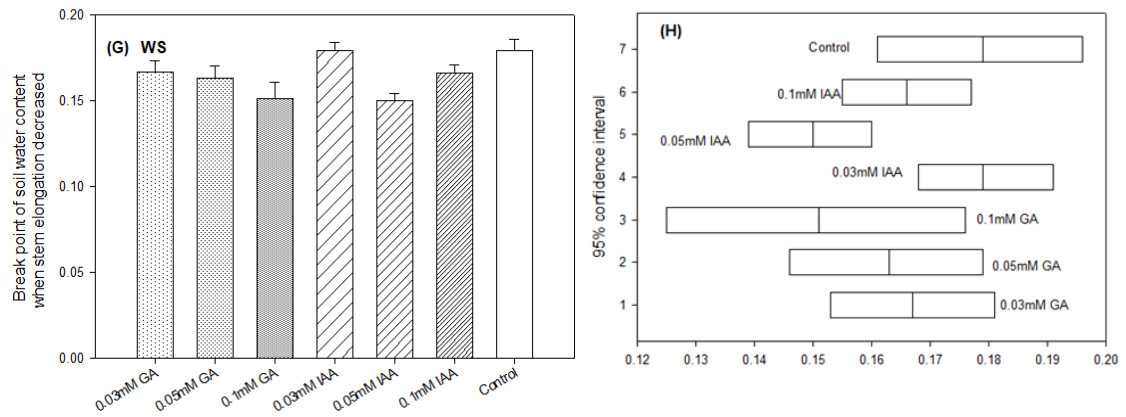
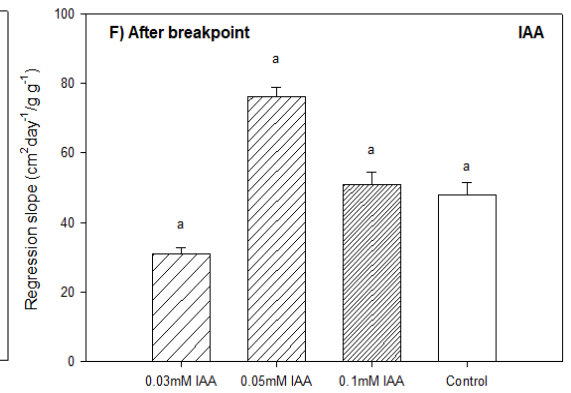
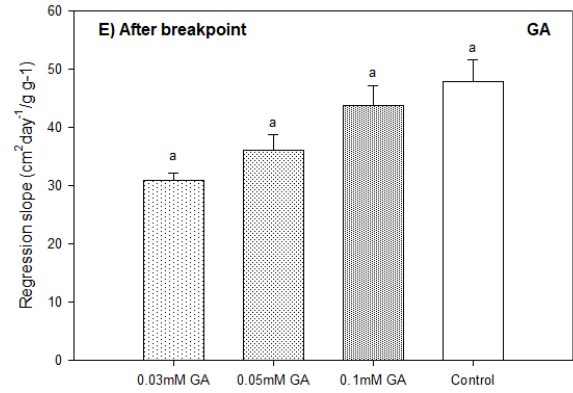
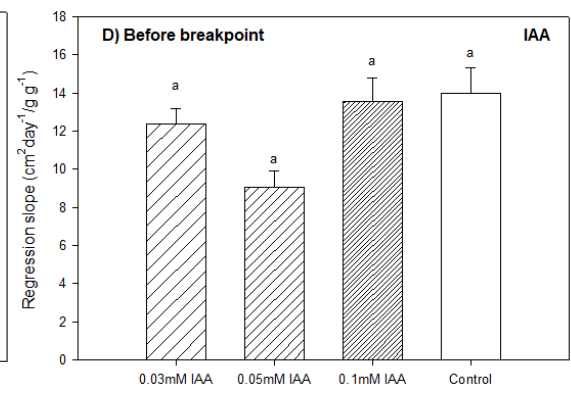
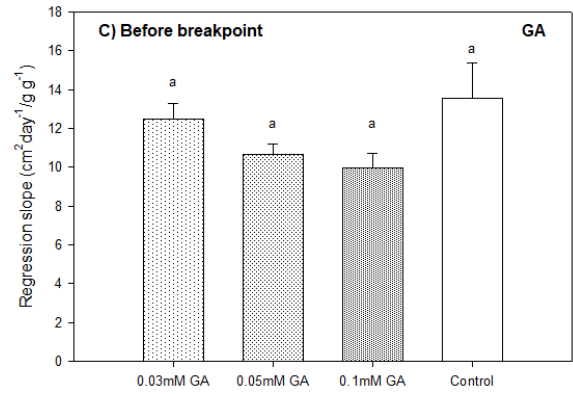
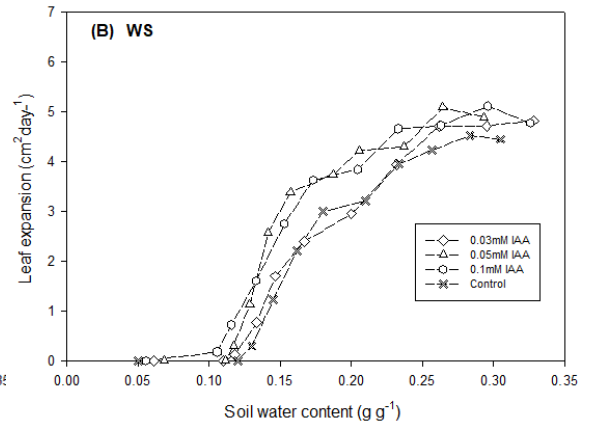
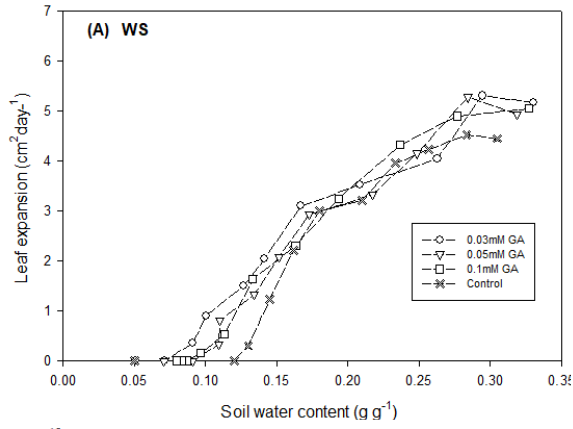


Figure 4.7 Relationship between soil water content ( $\theta$ ) and stem elongation and the effects of (A) gibberellic acid ( $GA_3$ ) and (B) auxin (IAA) during water stressed conditions. The regression slope of stem elongation versus soil water content before (C, D) and after (E, F) the breakpoint for plants treated with  $GA_3$  (C, E) and auxin (D, F) is given along with breakpoint values (G) and (H) confidence intervals. Bars are slopes  $\pm$  standard error, with values with the same letter across adjacent panels not significantly different at  $P < 0.05$  (ANCOVA) with adjustment using Tukey Kramer. Variation in BPs are plotted  $\pm$  standard error (G) and with 95% confidence intervals (H), with overlapping bars representing no significant difference between treatments.

Control plants showed a prominent biphasic response of stem elongation to soil moisture, with any decrease in soil moisture inhibiting stem elongation but an accentuated response below a soil moisture of  $0.17 \text{ g g}^{-1}$ .  $GA_3$  application changed this response, with variation in soil moisture  $> 0.15 \text{ g g}^{-1}$  having no significant effect on stem elongation rate. In drier soil, stem elongation of  $GA_3$ -treated plants was similarly sensitive to soil drying as control plants (Fig. 4.7A,

E). IAA treatments did not affect the sensitivity of stem elongation to drying soil (Fig. 4.7B) except before the breakpoint where 0.03 and 1.0mM IAA desensitised stem elongation to soil drying (Fig. 4.7D). Segmented analysis discriminated the response into two linear relationships, based on a breakpoint (BP) or threshold value (Table 4.3) of  $\theta$  determining when stem elongation began to decrease. Before the BP, GA<sub>3</sub> and IAA application attenuated the sensitivity of stem elongation to soil drying relative to control plants (Fig. 4.7C, D). After the BP, stem elongation was also less sensitive to soil drying when treated with GA<sub>3</sub>, as compared to IAA and control (Fig. 4.7E, F).

Although the BP value of GA<sub>3</sub>, IAA and control treatments seemed to vary (Fig. 4.7G), overlapping confidence intervals (Fig. 4.7H) suggested no significant differences. All GA<sub>3</sub> and IAA treatments had a similar  $\theta$  threshold to the control plants (overlapping confidence intervals), except the 0.05 mM application of IAA, which had a lower  $\theta$  threshold when stem elongation decreased. Therefore, these results indicate a variation in  $\theta$  when plants are treated with GA<sub>3</sub> and IAA before and after breakpoint in soil drying conditions. At intermediate soil moisture ( $\theta$  is 0.15 g g<sup>-1</sup>) GA<sub>3</sub> treated plants had a greater stem elongation (35 mm day<sup>-1</sup>) than control (25 mm day<sup>-1</sup>) plants. IAA-treated plants showed a small (non-significant) increase in the stem elongation rate compared to control plants.



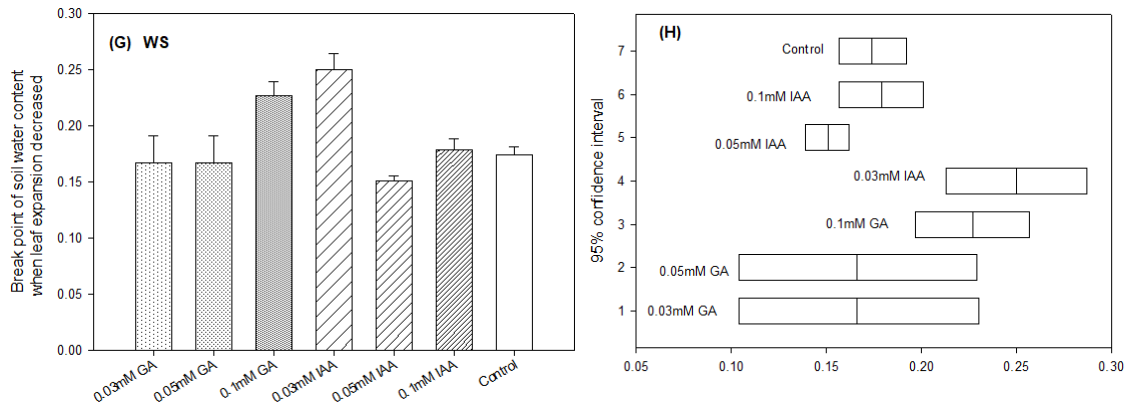


Figure 4.8 Relationship between soil water content and leaf expansion rate following (A) gibberellic acid ( $GA_3$ ) and (B) auxin (IAA) treatment of plants grown in drying soil. The regression slope of leaf expansion versus soil water content before (C, D) and after (E, F) the breakpoint for plants treated with  $GA_3$  (C, E) and auxin (D, F) is given along with breakpoint values (G) and (H) confidence intervals. Bars are slopes  $\pm$  standard error, with values with the same letter across adjacent panels are not significantly different at  $P < 0.05$  (ANCOVA) with adjustment using Tukey Kramer. Variation in BPs is plotted  $\pm$  standard error (G) and with 95% confidence intervals (H), with overlapping bars representing no significant difference between treatments.



Control plants showed a prominent biphasic response of leaf expansion to soil moisture, with any decrease in soil moisture gradually reducing leaf expansion. Applying GA<sub>3</sub> or IAA to the plants had no significant effect on the sensitivity of leaf expansion to drying soil (Fig. 4.8A, B). Segmented analysis discriminated the responses into two linear relationships based on a breakpoint (BP) or threshold value (Table 4.3) of  $\theta$ , when there was a significant change in the sensitivity of leaf expansion to drying soil. Although the sensitivity of leaf expansion to soil drying was much greater after the BP (Fig. 4.8 C, D *versus* E, F), there was no significant effect of hormone treatments before and after the BP (Fig. 4.8 C-F). Likewise, there was limited treatment variation in the BP values when leaf expansion started to decrease (Fig. 4.8 G, H). However, 0.1 mM GA<sub>3</sub> and 0.03 mM IAA had a higher  $\theta$  threshold value relative to the control, indicating leaf expansion started to decrease at a higher soil water content. Nevertheless, there was much less effect of the hormone treatments on the sensitivity of leaf expansion to drying than the sensitivity of stem elongation to drying soil (cf. Fig. 4.7; 4.8).

Hormone concentration (ng g <sup>-1</sup> DW)				
		GA <sub>3</sub>	IAA	Control
<b>First spraying gibberellic acid (GA<sub>3</sub>)</b>	Well-watered	10502 (a, A)	1030 (b, A)	1307 (b, A)
	Water stress	3494 (a, A)	199 (b, B)	869 (b, A)
<b>Third spraying gibberellic acid (GA<sub>3</sub>)</b>	Well-watered	8912 (a, A)	571 (b, A)	620 (b, A)
	Water stress	4308 (a, A)	146 (b, A)	120 (b, A)
		GA <sub>3</sub>	IAA	Control
<b>First spraying indole-3-acetic acid (IAA)</b>	Well-watered	7489 (b, A)	10362 (a, A)	8782 (b, A)
	Water stress	10484 (b, A)	32175 (a, A)	7518 (b, A)
<b>Third spraying indole-3-acetic acid (IAA)</b>	Well-watered	8951 (b, A)	152352 (a, A)	9807 (b, A)
	Water stress	9902 (b, A)	77553 (a, B)	8259 (b, A)

Table 4.5 Effects of soil drying and well-watered treatments on endogenous stem hormone concentrations (A) GA<sub>3</sub> and (B) IAA after first and third exogenous application of GA<sub>3</sub> and IAA. Values with the same lower-case letters are not significantly different within a row at each spraying time, and those with the same capital letters are not significantly different between well-watered and soil drying treatments, at  $P < 0.05$  (ANOVA) with mean comparison test using LSD.

Soil drying decreased GA<sub>3</sub> and IAA concentration, similar to previously reported in Chapter 2. Further applications of GA<sub>3</sub> and IAA increased *in vivo* GA<sub>3</sub> and IAA concentrations (Table 4.5). After the first and third sprays of GA<sub>3</sub>, well-watered plants had 8- and 14-fold higher stem GA<sub>3</sub> concentrations respectively, relative to untreated plants. In droughted plants, these increases were 4- and 36-fold higher respectively. After the first and third sprays of IAA, well-watered plants had 1- and 16-folds higher stem IAA concentration respectively, relative to untreated plants. In droughted plants, these increases were 4- and 9-folds after the first and third applications of IAA, respectively.

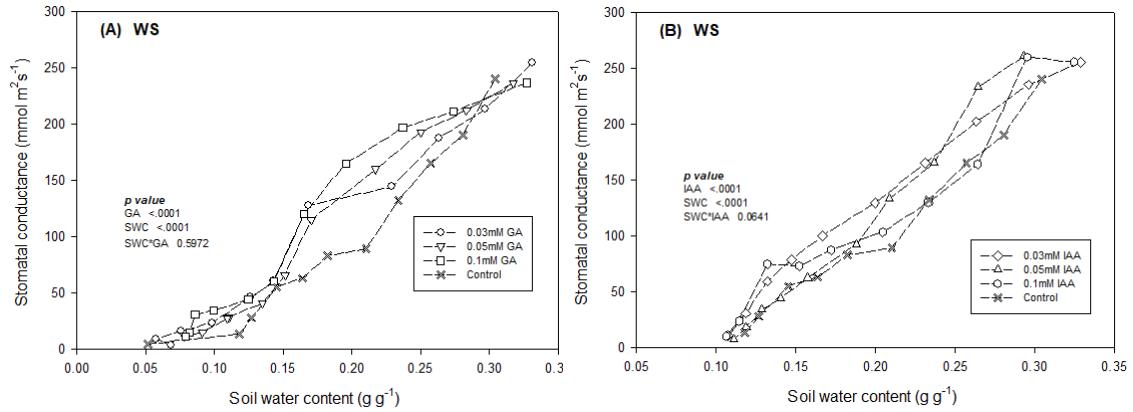


Figure 4.9 Relationships between stomatal conductance ( $g_s$ ) and soil water content ( $\theta$ ) following application of (A) gibberellic acid ( $\text{GA}_3$ ) and (B) auxin (IAA) to plants grown in drying soil. Values are means of  $n=4$ , with error bars omitted for clarity.

Stomatal conductance ( $g_s$ ) declined linearly with soil water content ( $\theta$ ) in all treatments, with hormone applications having no significant effect (as indicated by no significant hormone  $\times \theta$  interactions) (Fig. 4.9A; 4.9B). At intermediate  $\theta$  ( $0.17\text{-}0.23 \text{ g g}^{-1}$ ),  $g_s$  tended to be higher in  $\text{GA}_3$ -treated and IAA-treated plants.

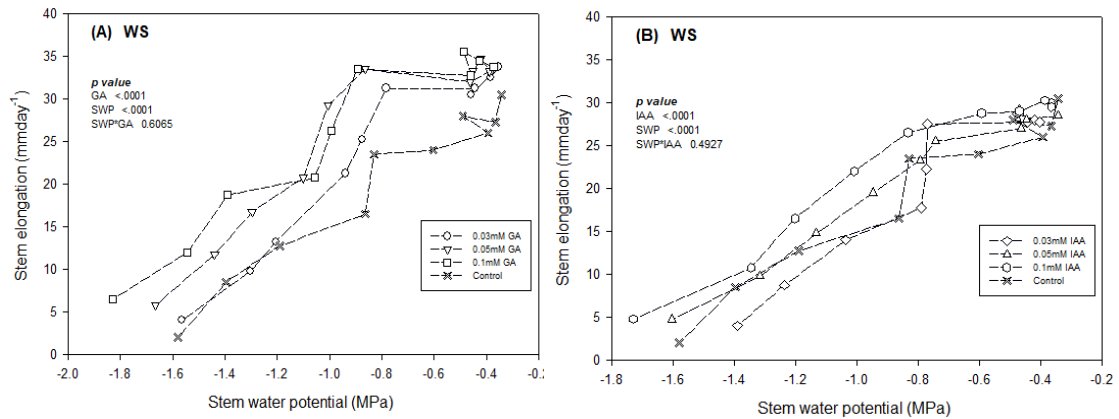


Figure 4.10 Relationship between stem elongation as stem water potential ( $\Psi_{\text{stem}}$ ) following application of (A) gibberellic acid ( $\text{GA}_3$ ) and (B) auxin to plants grown in drying soil. Values are means of  $n=4$ , with error bars omitted for clarity.

Stem elongation dropped linearly with stem water potential ( $\Psi_{\text{stem}}$ ) in all treatments, with hormone applications having no significant effect (as indicated by no significant hormone  $\times$   $\Psi_{\text{stem}}$  interactions) (Fig. 4.10A; 4.10B).  $\text{GA}_3$  treatments increased stem elongation at  $\Psi_{\text{stem}}$  between -0.6 and -1.0 MPa.

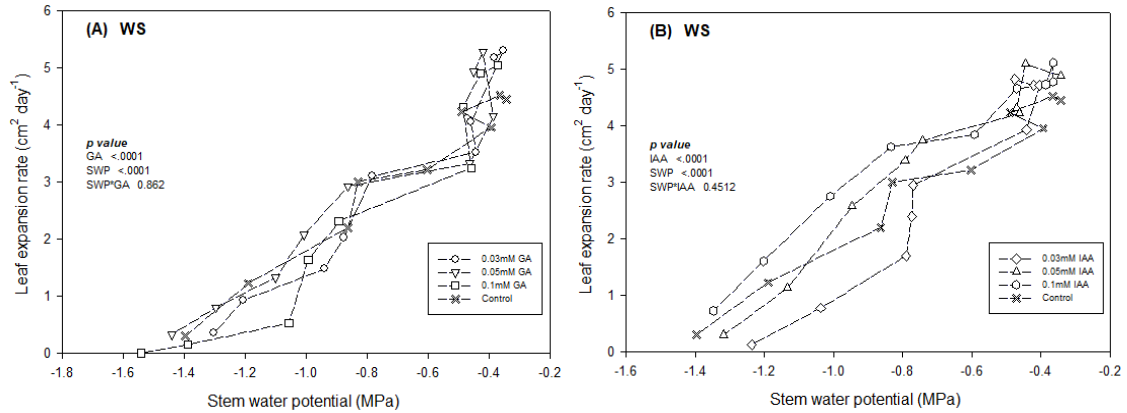


Figure 4.11 Relationship between leaf expansion and stem water potential ( $\Psi_{\text{stem}}$ ) following the application of (A) gibberellic acid ( $\text{GA}_3$ ) and (B) auxin under drought stress. Values are means of  $n=4$ , with error bars omitted for clarity.

Leaf expansion reduced linearly with stem water potential ( $\Psi_{\text{stem}}$ ) in all treatments, with hormone applications having no significant effect (as indicated by no significant hormone  $\times \Psi_{\text{stem}}$  interactions) (Fig. 4.11A; 4.11B). IAA treated plants increased (0.05 and 0.1 mM) and decreased (0.03 mM) leaf expansion at  $\Psi_{\text{stem}}$  between -0.8 and -1.2 MPa.

## 4.4 Discussion

### ***Gibberellic acid and auxin promote stem elongation and attenuate sensitivity to soil drying***

In well-watered plants, GA<sub>3</sub> application increased stem elongation compared to non-treated (control) plants (Fig. 4.4A), as did IAA which was less effective (Fig. 4.4B). Similarly, continuously applying 0.01 mM IAA to the uppermost internode of *Pisum sativum* cv Alaska (tall pea) plants approximately doubled stem elongation relative to control untreated plants during the first 6 hours (Yang et al., 1993). Growth responses to these treatments are likely concentration, time and genotype dependent. Applying exogenous (0.2 mM) IAA to the GA-deficient dwarf *le* pea mutant induced 50% greater stem elongation than applying half that concentration within the first 10 hours post-application, and thereafter (until 20 hours post-application) both concentrations caused similar elongation rates (Yang et al., 1996). Applying 4 mg of the GA biosynthesis inhibitor paclobutrazol to *Pisum sativum* cv Torsdag (tall pea), when the cotyledenary hook was fully straightened, decreased stem elongation by 20-32% after 14 days (Grindal et al. 1998). Applying the auxin transport inhibitor TIBA in a lanolin ring around elongating internodes decreased stem elongation by 14% relative to untreated plants (McKay et al. 1994). These studies suggest that endogenous GA<sub>3</sub> and IAA levels are important in regulating stem elongation, but whether these exogenous hormones mediate physiological responses to drying soil has not been investigated.

Gibberellic acid (GA<sub>3</sub>) and to a lesser extent indole-3-acetic acid (IAA) treatments attenuated the deleterious effects of soil drying on stem elongation (Fig. 4.4A; 4.4B). Since soil drying decreased endogenous GA<sub>3</sub> and IAA concentrations by 50% and 44% respectively (Fig. 2.9C-D, 2.11C-D), as confirmed here (Table 4.5), it is perhaps not surprising that exogenous GA<sub>3</sub> and IAA application stimulated stem elongation. Although endogenous GA<sub>3</sub> and IAA concentrations were strongly positively correlated with stem elongation (Fig. 2.9C-D, 2.11C-D), the concentrations detected following exogenous applications of GA and IAA were much (1.3-fold and 3-fold) higher respectively than occurring in well-watered plants. For plants grown in drying soil, very high (0.1 mM) phytohormone concentrations were required to provide moderate (23%) stimulation of stem elongation (Fig. 4.4C). In contrast, plants grown in well-watered soil had 34% higher growth rates with much lower endogenous hormone concentrations (Fig. 4.4D; Table 4.5), implying that soil drying decreases tissue sensitivity to GA<sub>3</sub> and IAA. Nevertheless, these results provide strong evidence that GA<sub>3</sub> and IAA can partially reverse the effects of soil drying on stem elongation by enhancing stem GA<sub>3</sub> and IAA concentrations.

These results with foliar hormone sprays are consistent with studies of mutant and transgenic lines where hormone-related genes have been up or down regulated (Hedden 1999). Constitutive overexpression of a GA biosynthesis gene (35S::*PdGA20ox1*) in five transgenic *Pinus densiflora* lines enhanced height and stem diameter, stem biomass and endogenous GA concentrations



relative to non-transgenic plants (Park et al. 2015). Overexpressing two different GA catabolism genes (*AtGA2ox2* and *AtGA2ox8*) in different parts of the vascular tissue of hybrid aspen (*P. tremula x tremuloides*) decreased internode length compared to non-transformed plants (Mauriat et al. 2011). Similarly, in tomato (*Solanum lycopersicum*), overexpression of *SlDREB* restricts stem elongation by downregulating two GA biosynthesis genes (*SlCPS* and *SlKS*) and lowering endogenous GA concentrations. Nevertheless, spraying GA<sub>3</sub> increased stem elongation of both WT and the dwarf *SlDREB*-OE genotypes (Li et al. 2012). Thus, downregulating GA biosynthesis inhibits stem elongation, which could be reversed by GA application. Whether overexpression of GA biosynthesis genes can maintain stem elongation when plants are exposed to drying soil was not directly assessed in these studies.

However, *DELLA* (a key gene involved in GA signalling) transcript accumulation occurred as the soil dried (Litvin et al. 2016), presumably GA and expansin play a role in regulating stem elongation under drought stress independently of their *DELLA* response. Expansin is the primary factor in the cell wall that mediates pH-dependent wall loosening, which can disrupt the non-covalent binding between the cell wall polysaccharides, thereby allowing turgor-driven wall extension (Cosgrove 1998). When stem sections of deepwater rice (*Oryza sativa*) were incubated for 48 hours and treated with 50 µM gibberellic acid (GA<sub>3</sub>), expression of expansin genes (*Os-EXP2* and *Os-EXP4*) greatly increased which

were correlated to rapid internodal growth (Lee & Kende 2002). Thus expansins are suggested to play a role in GA-mediated growth.

Auxin is another hormone that can regulate stem elongation of intact plants (Yang et al., 1993; Yang et al., 1996). Applying TIBA and HFCA (that inhibit polar auxin transport) to the base of internode 6 or 7 of WT and (semi-erectoides) *ikb* mutant of *Pisum sativum* (2-3 fold less free IAA than WT) induced endogenous auxin accumulation and restored internode elongation to 50% of WT above the application site in *ikb*, but had no effect in WT. Below the application site, TIBA reduced both IAA content and internode elongation (McKay et al., 1994). Furthermore, continuously applying 0.2 mM auxin promoted stem elongation of the dwarf *ikb* mutant, achieving comparable rates to WT plants after 20 hours. Thus, the dwarf phenotype of *ikb* is attributed to IAA deficiency (Yang et al., 1996). Similarly, soil drying decreased IAA concentrations and stem elongation, which could be rescued with IAA (Fig. 4.4B; Table 4.5). Applying 0.1 mM auxin to the uppermost internodes increased stem elongation of the pea cultivars Progress No. 9 (semi-dwarf-low IAA content) and Alaska (tall) by 6.5-fold and 2-fold respectively, within 15 hours of treatment (Yang et al., 1993). These studies suggest that effects of exogenous IAA application depend on their effects on endogenous IAA levels.

Mechanistically, auxin enlarges cells by increasing cell wall extensibility, with the acid growth hypothesis (Rayle & Cleland 1992b) proposing protons (H<sup>+</sup>) as a mediator between auxin and cell wall loosening that lead to the discovery of expansins. Expansins are proteins that promote cell wall extension in an *in vitro* assay, which disrupt the hydrogen bonds between cellulose microfibrils and the matrix polymers (McQueen-Mason et al. 1993; Cosgrove & Durachko 1994). Expansins regulate cell wall loosening activity at an optimal pH between 3 and 5.5 (Cosgrove 1989; Li *et al.*, 1993; McQueen-Mason et al. 1992), with auxins causing such apoplastic acidification (Perrot-Rechenmann 2010). Thus auxins stimulate cell expansion via promoting expansin activity (Cosgrove et al. 2002). Furthermore, expansin gene expression (*LeExp2* and *LeExp18*) was correlated with tomato (*Lycopersicon esculentum* cv Moneymaker) hypocotyl elongation rate, suggested growth regulation by expansin. *LeExp2* (but not *LeExp18*) was strongly expressed in the hypocotyl elongation zone and rapidly growing stems. Furthermore, applying 0.01 mM IAA strongly stimulated *LeExp2* expression in etiolated hypocotyls and weakly promoted *LeExp18* expression in stem tissue (Caderas et al. 2000). Thus, stem and hypocotyl elongation is probably regulated by auxin stimulation of expansin activity. Irrespective of the regulatory mechanisms, GA<sub>3</sub> and to a lesser extent IAA application changed the soil water content threshold at which stem elongation decreased, in a concentration-dependent manner (Fig. 4.7A-B, G-H). Mechanistically, GA and IAA can facilitate solute translocation to elongating cells (Reid & Ross 1993), relax cell walls thereby decreasing the yield threshold (Cosgrove and Sovonick-Dunford, 1989;

Behringer et al., 1990) mechanism and loosen the cross-linking between polymers (Potter & Fry 1993). Cell wall stiffening occurs with soil drying (Bacon et al. 1997), which seems to be ameliorated by GA and IAA application.

***Gibberellin and auxin don't stimulate leaf expansion per se, but maintain leaf expansion in drying soil***

Applying 0.3 mM IAA to droughted plants enhanced leaf expansion (until Day 4), while plants were essentially well-watered (Fig. 4.5B). Furthermore, IAA spraying stimulated *Epipremnum aureum* leaf area in a concentration-dependent manner, saturating at 1.6 mM (Di Benedetto et al. 2015). As with stem elongation, exogenous (1  $\mu$ M) IAA application transiently increased leaf expansion but had no net effect over 24 hours (Keller, 2011). Thus, exogenous auxin transiently maintained leaf expansion in drying soil, presumably by affecting level of endogenous IAA.

Meanwhile, overexpressing a GA catabolism gene in *Jatropha curcas* (*JcGA2ox6*) decreased endogenous GA<sub>4</sub> concentrations and caused smaller and dark-green leaves (Hu et al. 2017). Moreover, overexpression of *SIDREB* in cultivated (*Solanum lycopersicum*) and wild (*S. pennellii*) tomato restricted leaf expansion, perhaps by downregulating genes (*SIGA20ox1, 2, 4* and *SICPS*) that are involved in GA biosynthesis (Jinhua et al. 2012). While these observations may explain why drought decreased leaf expansion (due to low GA<sub>3</sub> levels –Fig 4.5A), it is not clear why applied GA<sub>3</sub> did not promote leaf expansion. Either

exogenous GA<sub>3</sub> did not enhance foliar gibberellin accumulation (only stem GA<sub>3</sub> concentrations were measured – Table 4.5), the leaves were insensitive to any such accumulation or perhaps other phytohormones are important.

***Gibberellic acid and auxin induced stomatal opening and more rapid soil drying but didn't affect plant water status***

Gibberellin effects on stomatal conductance are contradictory and varied with duration of soil drying in this experiment (Fig. 4.6C, F). Foliar sprays of 0.05 mM GA<sub>3</sub> enhanced soil-drying induced stomatal closure of *Gossypium hirsutum* L. cv H-777 (Kumar et al. 2001), consistent with the effect following the final GA<sub>3</sub> application (Fig. 4.6C, F) when GA supplementation was maximal (Table 4.5). Similarly, transgenic tomato overexpressing an *Arabidopsis thaliana* GA METHYLTRANSFERASE1 (*AtGAMT1*) gene maintained plant water status by reducing stomatal conductance, which increased soil water availability (Nir et al. 2014). Likewise, overexpressing an *Arabidopsis S-della* gene (*rgaΔ17*) in tomato plants promoted soil-drying induced stomatal closure thereby maintaining leaf relative water content as the soil dried (Nir et al. 2017). In contrast, the DELLA loss of function mutant *procera* (*pro*) had increased transpiration, presumably because of greater stomatal conductance, which decreased leaf water potential relative to WT plants (Nir et al., 2017). Applying exogenous GA<sub>3</sub> enhanced stomatal opening at intermediate soil moisture content (Fig. 4.9), likely accelerated soil drying (Fig. 4.2A) and exacerbating the decrease in plant water

status (Fig. 4.3A). Similarly, GA<sub>3</sub>-treated plants had higher g<sub>s</sub> than untreated plants under well-watered conditions (Fig. 4.6C-D). These results suggest that GA levels play an important role in adjusting stomatal response according to soil moisture status. Exogenous IAA increased g<sub>s</sub> at all IAA concentrations (0.03 mM to 0.1 mM) in well-watered plants relative to untreated control plants (Fig. 4.6B, E), as in okra (*Abelmoschus esculentus*) plants sprayed with 0.5 mM IAA (Khandaker et al. 2018). Incubating detached epidermis of *Commelina communis* L. in the presence of IAA (0.0001-0.000000001 mM) induced stomata opening, in contrast response of ABA (Snaith & Mansfield, 1982). IAA and ABA also had antagonistic effects on stomatal aperture in *Vicia faba* observed on abaxial epidermis (Dunleavy & Ladley 1995). Thus, IAA may be involved in regulating stomatal responses to water deficits in conjunction with ABA.

#### **4.5 Conclusion**

Taken together, applying gibberellic acid (GA<sub>3</sub>) and auxin (IAA) to plants exposed to drying soil partially reversed a decline in stem elongation, but effects on leaf expansion were minimal. Moreover, GA and IAA may be involved in mediating stomatal closure, thereby affecting the sensitivity of plants to changes in soil and plant water status.

## Chapter 5

### General discussion and conclusion

Since a survey of the available literature identified that very little information was available on the regulation of stem elongation in drying soil (Chapter 1), this thesis aimed to determine the importance of plant water status and phytohormones in mediating this response. Initially, the effects of soil drying on soil and plant water status, stem elongation and phytohormone concentrations were measured in a correlative approach (Chapter 2). Different hormones showed positive (Gibberellins (GA<sub>1</sub>, GA<sub>3</sub>, GA<sub>4</sub>), auxin) and negative (ABA, the ethylene precursor ACC, cytokinins (iP)) relationships with stem elongation. Changes in endogenous concentrations of some of these hormones preceded any change in soil water content ( $\theta$ ) or stem water potential ( $\Psi_{\text{stem}}$ ), suggesting they may be important in regulating stem elongation.

Since ABA concentration was the earliest response to soil drying, Chapter 3 investigated the function of ABA in controlling stem elongation via testing the responses of *wilty* (ABA-deficient mutant) and wildtype (WT) peas to soil drying. Since ABA deficiency increases stomatal conductance thereby lowering plant water status, experiments were conducted under moderate and high humidity (to allow genotypic effects to be compared at the same plant water status). Although *wilty* (ABA-deficient) showed similar leaf expansion as the WT when the soil was

allowed to dry, it was shorter, indicating that ABA is needed to maintain stem elongation. Further analysis reveals no unique relationships between stem and leaf ABA accumulation and growth inhibition across genotypes and relative humidities. Thus, it seems unlikely that ABA accumulation is the key mechanism that diminishes stem height as the soil dries.

Thus, Chapter 4 evaluated the effect of applied gibberellic acid ( $GA_3$ ) and auxin (IAA) on the stem elongation response to soil drying.  $GA_3$  and IAA partially reversed an inhibitory effect of soil drying on stem elongation but did not change leaf expansion. Thus, different hormones seem involved in regulating different growth responses to soil drying.

### **5.1 Stem elongation decreased before stem water potential ( $\Psi_{\text{stem}}$ ) changed**

Soil drying significantly decreased stem elongation rate on Day 5 before stem water potential declined on Day 7 (cf. Fig. 2.6B, 2.7B). Although decreased stem water potential and stem elongation were correlated, detailed diurnal observations disagree with this relationship (Fig 2.4B; 2.5A; B). Decreased growth such as stem elongation (De Diego et al., 2012) and leaf elongation (Van Volkenburgh & Boyer 1985) has been reported prior to any decrease in plant water status. Consequently, decreased stem water potential cannot cause the decrease in stem elongation in response to soil drying. Experiments that applied



root pressurization to prevent any change in plant water status as the soil dried also did not prevent a decline in leaf elongation (Termaat et al. 1985; Passioura 1988). Thus non-hydraulic factors such as phytohormones potentially control stem elongation in response to soil drying.

## **5.2 Stem elongation decreases with soil drying independent of stem ABA status**

In Chapter 3, the shorter *wilty* plants accumulated less ABA in response to water stress when grown at similar soil and plant water status (Fig. 3.6C, D). Furthermore, the decrease in stem elongation as ABA accumulated with soil drying was more sensitive in *wilty* (Fig 3.12 A, B). In contrast, stomatal sensitivity to ABA concentrations applied to detached epidermal peels showed no genetic variation between *wilty* and wildtype (Donkin et al. 1983). These genetic differences in apparent sensitivity of different processes (stem elongation, stomatal closure) to ABA suggests that another factor might regulate stem elongation.

### **5.3 Exogenous gibberellin enhances stem elongation of plants grown in soil drying**

Gibberellins applied to intact pea (*Pisum sativum* cv. Alderman) stems partially reversed the effects of soil drying on stem elongation (Fig. 4.4 C, D). Gibberellin-mediated promotion of stem elongation (in either intact or excised internodes) via several mechanisms has been extensively studied (Yang et al., 1993; Sauter et al. 1993). GA enhances cell wall extensibility (without apoplastic acidification) by increasing osmotically driven water uptake (Kaufman & Dayanandan 1983). Pea genotypes differing in gibberellin content showed that gibberellin decreases the minimum force required cause wall extension (the cell wall yield threshold) (Behringer et al. 1990). Nevertheless, soil drying can either tighten or loosen growing cell walls according to their position in the growing tissue. Tissues that are distal to the growing zone are tightened (made inextensible) whereas tissues that are essential to maintain growth such as shoot apices are loosened, allowing continued growth at lower turgor pressures (Wu & Cosgrove 2000). It is likely that the threshold turgor pressure in these growing regions is modified to permit wall growth. Thus, gibberellin seems to exert its effects by modifying cell wall properties.

Spraying gibberellic acid (GA<sub>3</sub>) on droughted plants enhanced stem elongation (relative to untreated plants) as early as the first application (Fig. 4.4C). Another mechanism by which gibberellins can stimulate stem elongation is increased activity of the enzyme xyloglucan endotransglycosylase (XET). XET activity regulates cell wall expansion by incorporating newly synthesized xyloglucan into the wall matrix (Darley et al. 2001). A single foliar spray of 0.1 mM GA<sub>3</sub> increased internode length of a GA-responsive dwarf pea (cv. Feltham First) by 40%, while a 70% increase occurred in tall pea (cv. Pilot). XET activity increased in both tall and dwarf genotypes in parallel with internode growth (Potter and Fry 1993). Similar changes in XET activity may have occurred here in response to foliar GA spraying, thereby enhancing stem elongation (Fig 4.4A, C). However, further work is needed to determine whether XET is involved in GA's promotive effect on stem elongation in response to soil drying.

Ideally, the effects of GA<sub>3</sub> application to plants grown in well-watered and drying soil (as conducted in Chapters 2-4) would be examined with the *lkb* mutant (GA insensitive mutant) and its WT. Daily stem elongation and XET activity in the first and second internodes (Figure 2.1) would be measured throughout the experimental duration, with GA<sub>3</sub> application unable to promote stem elongation or XET activity in the *lkb* mutant.

## **FUTURE PERSPECTIVES**

In general, the findings of this study may be important to improve plant productivity (of biomass crops) in drought conditions. Exogenous gibberellic acid (GA<sub>3</sub>) application attenuated the decrease of stem elongation under water stress. The use of plant hormones at low concentrations is cost effective in the agricultural sector (Erviö et al. 1994), but limited efforts have aimed to modulate plant responses to drying soil (Dodd & Ryan 2016). Therefore (breeding and GM) approaches to boost biological active GA levels may mitigate the impact of soil drying on GA-mediated stem elongation, maintaining plant productivity. This will be especially important in crops (such as tree crops grown for biomass) where the stem is the harvested portion.

How GA affects plant traits related to drought tolerance is worth evaluating. The plant traits for drought tolerance includes stem height, xylem size, cambium activities, leaf size, aboveground biomass (Eriksson et al. 2000) and lignin biosynthesis (Biemelt et al. 2004). Crops in the field reported a positive correlation between xylem size and tree height growth, implying that crops with larger xylems are able to transport more water, facilitating more rapid growth (Cochard & Tyree 1990; Sperry et al. 1994). However, xylem size is also well correlated with the occurrence of dieback (dying shoots), with crops with smaller xylem vessels less susceptible to cavitation and therefore more drought tolerant (Dixon et al. 1984). This emphasizes the importance (and potential tradeoffs) of

considering the alteration of GA in plants which can improve morphological traits and stimulate secondary growth (Eriksson et al., 2000) under soil drying conditions.

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