Effects of over-irrigation on tomato (*Solanum lycopersicum* Mill.) plant growth and physiology

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Abstract

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Antje Fiebig, Lancaster University

By automatically scheduling irrigation according to soil moisture thresholds, this work aims to understand how over-irrigation (applying 50 % more than full water requirements) affect soil properties (volumetric water content, soil oxygen and temperature) and plant physiological responses. Four weeks of over-irrigation significantly decreased shoot fresh weight and total leaf area of tomato plants (*Solanum lycopersicum* Mill. cv. Ailsa Craig) compared to well-drained plants. Since over-irrigation did not alter leaf water potential, stomatal conductance or foliar concentrations of the plant hormone abscisic acid (ABA), it seems unlikely that ABA accumulation or leaf water status mediate growth. In contrast, over-irrigation significantly increased foliar ethylene evolution. Over-irrigating the partial ethylene-insensitivite genotype *Never ripe* did not lead to such a dramatic growth inhibition as in the wild type, suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to a degree. Although the ethylene precursor ACC was not detected in root xylem sap, over-irrigation increased leaf xylem sap ACC concentration. Shoot fresh weight correlated with increased *t*Z, GA3, ABA and JA leaf xylem sap concentration, but was not correlated with concentrations of any root xylem sap phytohormone. Over-irrigation significantly decreased foliar nitrogen concentrations by 32 % and daily additions of 10 mM $Ca(NO_3)_2$ to over-irrigated soil did not significantly change leaf water potential, stomatal conductance or foliar ABA

concentration, but restored foliar nitrogen concentrations, ethylene emission and growth of over-irrigated plants to control levels. Thus, over-irrigation-introduced foliar nitrogen deficiency may be key to limiting growth of over-irrigated tomato plants.

Zusammenfassung (German abstract)

Einfluss von Überbewässerung auf das Wachstum und die Physiologie von Tomatenpflanzen (*Solanum lycopersicum* Mill.)

Antje Fiebig, Lancaster University

Mit Hilfe von automatisch gesteuerter Bewässerung anhand von Bodenfeuchteschwellenwerten versucht diese Arbeit zu verstehen, wie sich Überbewässerung (um 50 % erhöhte Bewässerung gegenüber vollem Wasserbedarf) auf Substrateigenschaften (volumetrischer Wassergehalt, Bodensauerstoff und -temperatur) und pflanzenphysiologische Reaktionen auswirkt. Vier Wochen Überbewässerung verminderten signifikant Blattfrischgewicht und Gesamtblattfläche von Tomatenpflanzen (*Solanum lycopersicum* Mill. Cv. Ailsa Craig) im Vergleich zu gut gewässerten (und drainierten) Pflanzen. Da Überbewässerung weder das Blattwasserpotenzial noch stomatäre Leitfähigkeit oder die Konzentration des Pflanzenhormones Abscisinsäure (ABA) im Blatt veränderte, erscheint es unwahrscheinlich, dass ABA-Akkumulation oder Blattwasserstatus das Wachstum beeinflussen. Im Gegensatz dazu erhöhte Überbewässerung signifikant die Ethylenkonzentration in Blättern. Überbewässerung des partiell Ethylen-unempfindlichen Genotyps *Never ripe* führte zu weniger drastischer Wachstumseinschränkung als im Wildtyp, woraus sich schließen lässt, dass Ethylen-Unempfindlichkeit bis zu einem gewissen Grad Wachstumshemmung durch Überbewässerung ameliorieren kann. Obwohl das Ethylen-Vorprodukt ACC nicht im Wurzelxylemsaft festgestellt werden konnte, erhöhte Überbewässerung die Konzentration von ACC im Blattxylemsaft. Frischegewicht korrelierte mit erhöhten

Konzentrationen von *t*Z, GA3, ABA und JA im Blattxylemsaft, aber mit keinem Pflanzenhormon im Wurzelxylemsaft. Überbewässerung verringerte signifikant Stickstoffkonzentrationen im Blatt um 32 % und tägliche Gaben von 10 mM $Ca(NO_3)_2$ auf überbewässertes Substrat veränderte weder Blattwasserpotenzial, stomatäre Leitfähigkeit noch Konzentrationen von ABA, aber stellte Stickstoffkonzentrationen, Ethylengehalt und Wachstum von überbewässerten Pflanzen ähnlich gut gewässerten Pflanzen wieder her. Somit könnte Stickstoffmangel durch Überbewässerung entscheidend zur Wachstumseinschränkung in überbewässerten Tomatenpflanzen beitragen.

Declaration

I hereby declare that this work has been originally produced by myself for the present thesis and it has not previously been submitted for the award of a higher degree at any other institution. Inputs from co-authors and collaborators are acknowledged throughout.

Antje Fiebig

Lancaster, UK, November 2014

Acknowledgement

‘The world isn’t just the way it is. It is how we understand it, no? And in understanding something, we bring something to it, no? Doesn’t that make life a story?’

Life of Pi, Yann Martel

Conducting experiments in natural science and writing a PhD thesis is something that cannot be done just by one person. I have been fortunate enough to receive a lot of support, assistance and kind words during the journey of doing a PhD and would like to take this chance and express my thankfulness in a mixture of English and German.

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Chapter 1.

1 Introduction

‘Classical hormone theory confuses playing “a” role with playing “the” role’.

(Trewavas 1986)

‘... There is the air of an “unfinished project” about the phytohormone research field... The reasons why plants have hormones is not fully understood and doubts remain as to how they exert control over plant actions at the whole organism level’.

(Weyers and Paterson 2001)

‘In addition, recent work confirms that hormone signalling interacts at multiple levels during plant growth and development. In the future, a major challenge will be to understand how the information conveyed by these simple compounds is integrated during plant growth.’

(Santner et al. 2009)

1.1 Waterlogging

1.1.1 Importance of waterlogging

The human population is expected to grow from 7.2 billion in 2013 to 8.3 billion in 2030 and to assure food security, energy and crop production has to double, which will result in 30 % more fresh water demand (Beddington 2009). This adds another challenge to recent climate changes, which include increased risks of temperature extremes, storms, droughts, but also floods all over the world (Fig 1.1). Models and data show that a shift to more intense rainstorms can increase the frequency of flooding (Van Der Ploeg et al. 2002) and as a result of melting polar ice caps, the sea level is predicted to rise up to 20 cm over the next 50 years, increasing the frequency of coastal flooding (IPCC 2007). Furthermore, higher fluxes of river water due to mountain deforestation are already happening now in many parts of the world. Examples for increased flooding of lowland regions are the River Rhine in Europe and the Euphrates delta of Bangladesh and West Bengal, which all contain productive farmland (Jackson 2011). The increase in earth's average temperature (IPCC 2007) and unreliable and fluctuating rainfall may greatly magnify the problem of waterlogged soil in the near future. Waterlogging, drought and variable seasonal water supply are of equal importance in the agricultural sector and represent the most severe global problems for crop production (Akhtar and Nazir 2013, FAO 2011).

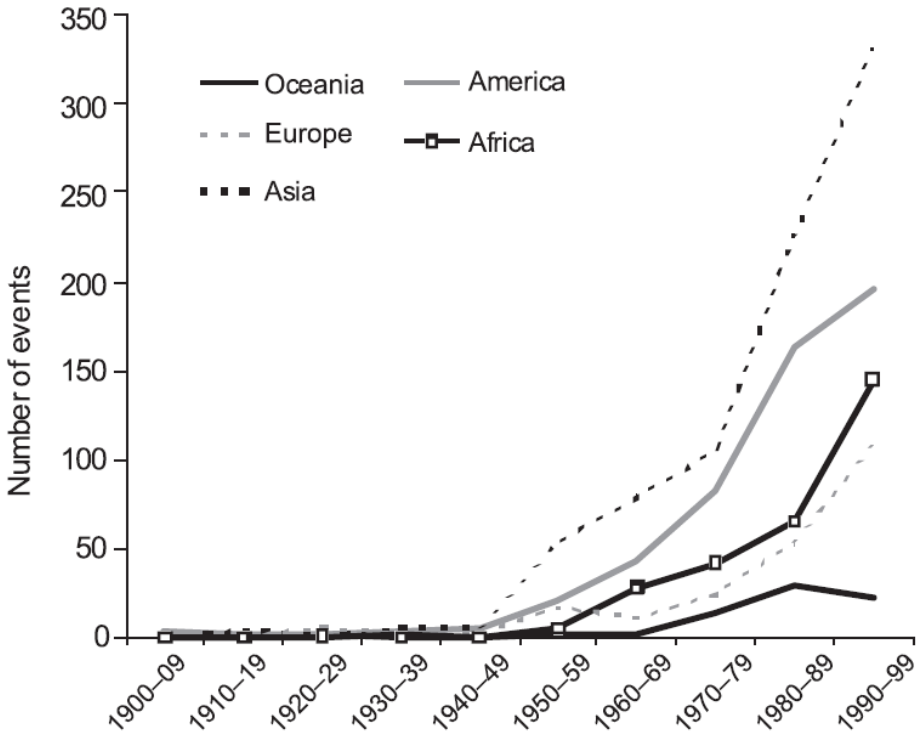

Fig 1.1 Numbers of recorded flood events have increased since 1940 across the globe (adapted from OFDA/CRED 2002)

In addition to climate-changed induced flooding, other factors, such as poor irrigation management or drainage, can lead to waterlogged soil, which can affect more than 17 million km² land area in all continents in certain years, an area about twice the size of the USA. Approximately 10 % of irrigated farmlands worldwide suffer from prolonged waterlogging and this value can increase to 20 % in regions like Eastern Europe and the Russian Federation (FAO 2011). Based on a hydrological model, agricultural production along the Lower Arkansas River of Colorado was negatively affected by improper irrigation leading to waterlogging and soil salinization, with average annual losses of US$4.3 million (£2.5 million, Houk et al. 2006). Salinization is linked to waterlogging, as capillary rise, for example due to high groundwater tables, brings salts into the root-zone (Datta and de Jong 2002). In Western Australia,

waterlogging occurs on 1.3 million ha of pastures and 500 000 ha of cultivated land. Barley is grown on 250 000 ha in Western Australia and waterlogging can reduce yields up to 20-25 %. If waterlogging appears in 2 out of 5 years, an estimated lost yield of US$6.4 million (£3.7 million) each year has been calculated (McFarlane and Cox 1992). Thus, waterlogging is an important factor in crop losses and actions need to be taken to secure food production. Furthermore, waterlogging cannot only occur in open field agriculture, but also in protected plant production systems (e.g. nurseries), where improper irrigation management can lead to over-irrigation due to poor drainage and excessive watering due to insufficient understand of the plants' actual water needs. Currently, 80 % of the world's available fresh water resource is used for irrigated agriculture, including outdoor and greenhouse vegetable production (Condon et al. 2004). Agriculture accounts for 70 % of the worldwide increased water demand and this sector is competing for water more and more with the world's growing cities (Beddington 2009). Through enhancing irrigation management, water and costs can be saved and crop uniformity, quality and ultimately yield can be improved.

This chapter will define and provide an overview of the scale and reasons for waterlogging, and consider techniques to improve irrigation management. The impact of excess water on soil properties (changes in the root-zone) as well as plant growth and development will be reviewed. Particular attention is given to metabolic and physiological responses of plants to waterlogging, focusing on gas exchange, plant hormones, root-to-shoot signalling and nutrient concentrations, as these represent likely parameters influencing plant growth under waterlogged conditions.

1.1.2 Definition of waterlogging

Surprisingly, the term “waterlogging” has been described rather loosely, is still not well-defined and has been used interchangingly with “submergence” and “flooding”. Waterlogging can be the “addition of excessive water to a soil resulting in loss of structure of soil and a soggy condition” (Singh and Yadava 2003) or simply the “flooding of the root system” (Bailey-Serres et al. 2012). Submergence can be defined as “waterlogging and partial to complete immersion of the aerial system” (Bailey-Serres et al. 2012). Field capacity is determined as the soil water content after the soil has been saturated and allowed to drain freely for 24 to 46 hours. Due to the force of gravity pulling on the water, free drainage occurs and when water stops draining, the remaining water is held in the soil with a force greater than that of gravity. Whenever the soil water content of the surface layer is more than 20 % higher than the field’s carrying capacity, water stands on the soil surface (Agarwal and Grover 2006). Jackson and Colmer (2005) call both soil waterlogging and submergence “flooding”. A schematic overview of well-drained, waterlogged and submerged soil is given in Fig 1.2.

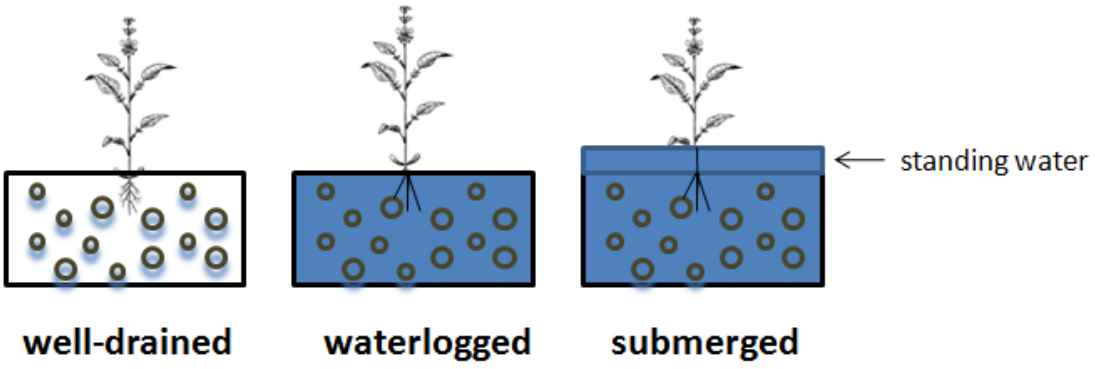

Fig 1.2 Well-drained, waterlogged and submerged soil

A better approach to define waterlogging might be through its physical meaning. During waterlogging, soil pores are saturated by water and therefore, the term "waterlogging" could be replaced by "saturated soil". Waterlogging is influenced by the water flow around the root-zone and the water-absorbing capacity of the soil, which in turn is affected by the soil structure. The size range of the pores, their interconnectivity, stability and the relative proportions of each size class are especially important in determining how much water the soil can hold (Jackson 2011). Drainage in pots will only occur while the soil at the bottom is saturated (zero water suction) and water suction varies with height (Passioura 2006).

Since soil water relations, temperature and structure will vary between substrates used in the nursery industry and field soil (Passioura 2006), some understanding of the physical environment for root growth is necessary. Soil holds water by capillary forces and in unsaturated soil, the pressure in the soil water is usually less than that of the atmosphere, but can be higher than zero if water is coming out of soil under pressure. The difference in pressure between the water and the atmosphere is described as the suction in the soil water (Passioura 2006).

Freshly watered and drained soil has a water suction between 10 – 30 kPa (Passioura 2006). In the growing industry it is often assumed that for potted plants, the water suction is zero at the base of the pot and 10 H elsewhere (where H equals the height of the pot in mm). Thus a freshly watered and drained pot of 100 mm height has a water suction of 1 kPa at its top (even though this value also depends on the structure of the soil), which is very different from that of soil in fields (Passioura 2006). Taller pots lower the risk of limited air-filled porosity (AFP) and oxygen deficiency (hypoxia). However, the possibility of hypoxia depends not only on the height of the pot, but also

on the substrate used and its air filled porosity. Air filled porosity (or free air space – FAS) is defined as "the ratio of gas filled pore volume to total sample volume." Peat-based substrates (used in the experiments for this thesis) usually have an AFP around 0.3 m^3 m^{-3} (Allaire et al. 1996, Nkongolo and Caron 2006). Air spaces in the soil provide oxygen for both bacteria and plants. Insufficient air spaces (low AFP) can lead to anaerobic conditions.

1.1.3 Improving irrigation management

Waterlogging severely affects soil oxygen concentration. An optimal oxygen level, termed normoxia, typically occurs around 20.6 % oxygen at 1 atm and 20°C (Bailey-Serres and Voesenek 2010) and oxygen deficiency, termed hypoxia, occurs when oxygen levels are below the critical oxygen pressure for mitochondrial oxidative phosphorylation for the cell (or organ) and limit adenosine triphosphate (ATP) production. Anoxia (oxygen absence) develops when ATP is produced only through fermentative glycolysis and respiration is completely inhibited (Parent et al. 2008). Oxygen diffuses into plants from the soil pores to a water film surrounding plant root hairs. When these pores are filled with air, this process is facilitated; however, as oxygen cannot easily diffuse in water due to a slow diffusion rate of gases (10^{-4} less), oxygen uptake is hindered when the pores are filled with water. Therefore, waterlogging can restrict gas exchange between the soil and the atmosphere (Singh and Yadava 2003) and anoxic soil can occur within a few hours. The diffusion coefficient of oxygen into soil is affected by soil porosity, soil water content, temperature and root density. Generally, flooding and waterlogging will lead to circumstances close to anoxia, whereas improper irrigation management (over-irrigation) will rather induce hypoxic soil conditions.

Poor irrigation implementation and management cause misallocations and/or waste of water (Hamdy et al. 2003). Because water is a renewable, yet not inexhaustible resource and cannot be substituted, possible over-irrigation especially in greenhouses and for the production of protected crops has to be ameliorated. If water use efficiency can be increased, more food can be produced and in addition, the competition for water might decrease (Hamdy et al. 2003). Reducing or stabilising the use of water for agriculture down to 70 % could increase water availability for other needs (domestic, municipal, industrial and environmental purposes) up to 50 % (Hamdy et al. 2003).

Several types of irrigation scheduling exist and include irrigation for a fixed time each day, irrigation to replace crop evapotranspiration and/or measuring plant water status (e.g. leaf water potential, stomatal conductance [g_s]) to determine when to irrigate (Jones 2004). Another alternative is to manage irrigation according to the soil water status, for example by using soil moisture sensors, which allow a rapid, repeatable and relatively non-destructive measurement of volumetric soil water content (θv, Schmutz and Namikas, 2011). By setting soil moisture thresholds depending on the substrate's drained capacity, the irrigation will be automatically switched on when the soil moisture falls below this threshold, without any need for adaption to developmental stage of the plant. With this automatic irrigation system, plant growth and crop uniformity can be increased (DEFRA Hortlink Report HL0132LHN/HNS 97b).

When containerised bedding plants were irrigated via dielectric soil moisture sensors, volumetric water content was maintained close to the set-point for several weeks, independent of plant developmental stage, although the effects of sensor-based irrigation were not contrasted with other irrigation systems (Nemali and van Iersel 2006). Using soil moisture sensors to irrigatedcontainerised hydrangea reduced water

use by 82 % (standard irrigation practice controlled by nursery personnel, van Iersel et al. 2009).

However, studies on automatic irrigation via soil moisture sensors involving containerised plants are rare and more are needed to fully understand their suitability for this sector. Growers worry about possible water wastage, run-off or nutrient leaching (Nemali and van Iersel 2006) and initial costs of the irrigation equipment. More research (which includes demonstration pilots at nurseries) might help convince growers to embed soil moisture sensors in their growing systems.

Interestingly, many farmers seem willing to use new irrigation technology (Zotarelli et al. 2009). Research and demonstration activities in Tuscany, Italy, significantly reduced farmers' scepticism on the use of irrigation management of container ornamental plants (Zotarelli et al. 2009). Investment costs of vegetable drip irrigation, consisting of a well, pump, power unit and distribution system (drip tubing costs not included) in 1991 were estimated to be US$3 390/ha (£2100). Total annual fixed costs were US$640/ha (£397) and total variable costs/season US$811/ha (£503) with total costs US$587 (£343, Prevatt et al. 1992). Developments in computer science may reduce prices for technical equipment in the near future (Pardossi et al. 2009). Following an initial investment of soil moisture sensors and datalogger (minimum requirement of GP1 datalogger costing £285, 2 x SM200 sensors costing £334 and irrigation timer costing £180 for one species at the same developmental stage), successful implementation of automatic irrigation scheduling according to soil moisture can decrease labour costs involved in hand-watering and costs for water and energy.

While much work has focused on field crop responses to waterlogged soils (Ahmed et al. 2006, Cowie 1996, Rodriguez-Gamir et al. 2011, Yordanova and Popova 2007), effects of constant over-irrigation of pot plants (in contrast to flooding or submergence where the entire substrate is immersed in water) have not been well studied. Of the crops produced, tomato is one of the most consumed vegetable, with fresh tomato trade rising by 45 % in the last five years (Vidoz et al. 2010). In England and Wales, the total greenhouse area for all protected edible crops and leafy salads covers 3 750 ha.. 75 000 tonnes tomatoes per year are produced on 220 ha (personal communication with Barry Mulholland, 2014)). The retail value of British tomato production is around £600 million, of which UK growers own a 20 % market share. Approximately 2 500 people are currently employed in the British tomato industry (personal communication with Barry Mulholland, 2014), making tomato the second most important vegetable crop (after potato) and emphasizing the importance of studies on environmental stress on tomato (Else et al. 2009). Waterlogging of tomato is not an industry concern, as it is usually grown hydroponically. However, tomato is a good model species, as it grows relatively quickly and different genotypes are readily available. Therefore, tomato is used as a tool to understand the effects of over-irrigation in this thesis. Over-irrigation is much more relevant to nurseries, as flooding is rather unlikely to occur. Plants grown in pots under controlled environments are especially suitable to investigate the physiological responses to soil moisture, because treatments are relatively easy to apply and repeatable. While technology exists to improve irrigation management, this has not been widely implemented and performance of irrigation systems needs to be upgraded (Augier et al. 1996).

1.2 How do plants respond to waterlogging?

1.2.1 Metabolic changes in the root-zone

Plant life developed 460 million years ago in an aquatic environment (Beerling 2007). Photosynthetic macrophytes, previously living in water, invaded land and were able to survive in air while securing water and minerals through using a non-photosynthetic, foraging root system. Even though the progenitors of the more than 300,000 plant species, which can be found in almost every terrestrial niche nowadays, were aquatic, land plants are relatively intolerant of free water in their surroundings (Jackson 2011).

Land plants, in contrast to aquatic plants, are aerobes and need freely diffusing oxygen, which can be limited under waterlogged conditions. Actual soil CO_2 and O_2 concentrations during flooding have only been measured in very few studies (Eberling et al. 2011, Araki 2006), making it hard to compare effects of flooding on plants. CO_2 subsurface concentrations of wetlands in Denmark were > 5000 µM, but varied with depth (Eberling et al. 2011). Means of O_2 partial pressure of a clay loam paddy field varied between 12 to 20 kPa and CO_2 partial pressure ranged from 1 to 5 kPa, but varied according to season (Araki et al. 2006). In addition, the influence of over-irrigation on oxygen levels in containerised soil has not been analysed, yet. Even though it is important to understand whether substrate and cellular oxygen concentrations are equivalent, to our knowledge soil and cellular oxygen levels during waterlogging have not been directly compared. Low oxygen in cells results in inhibition of vegetative and reproductive growth, changes in plant anatomy, premature senescence and plant mortality (Drew 1997), but the actual oxygen depletion needed for causing such responses is not known.

For substituting oxygen, cells might use nitrate for the electron transport pathway and as an alternative respiratory pathway (Drew 1997, Parent et al. 2008). In this case, nitric oxide (NO) is oxidized to nitrate (Parent et al. 2008). Due to soil hypoxia or anoxia, root respiration changes from aerobic to anaerobic which leads to a reduction in ATP production and adenosine diphosphate (ADP) oxidative phosphorylation. Plants need to find other ways to generate energy without using oxygen as terminal electron acceptor (Drew 1997), so plants shift their metabolism from aerobic respiration to anaerobic fermentation (Parent et al. 2008). However, only 2 moles of ATP per mol glucose can be produced in that way, compared to 36 moles of ATP per mole of glucose during aerobic respiration, depleting plant carbohydrate reserves. Involved in this process are anerobic proteins, which are induced during hypoxia and include enzymes of glycolysis, ethanolic fermentation, processes related to carbohydrate metabolism, formation of aerenchyma and cytoplasmic pH control (Parent et al. 2008). Sucrose does not accumulate in leaves, but in roots of flooded poplar seedlings, suggesting that this form of carbohydrates is transported from leaves to roots during flooding (Kreuzwieser et al. 2009).

The lack of ATP and carbohydrates leads to less energy for plant metabolic processes, such as ion uptake, root growth and secondary metabolism (Bailey-Serres and Voesenek 2010). Furthermore, some substances can be reduced from their normally oxidized states to toxic metabolites (ethanol, lactic acid, acetaldehyde, cyanogenic compounds, Fe^{2+}, Mn^{+}, sulphide and ammonia) (Drew 1997, Singh and Yadava 2003). Those metabolites can accumulate in the plant during anaerobic root respiration and cause cell death (Jackson 2002, Liao and Lin 2001). Organic matter is anaerobically decomposed and at the same time, nitrification is prevented (Singh and Yadava 2003).

The soil pH generally tends to increase towards neutrality upon waterlogging, possibly due to dissolution of carbonate and bicarbonate early during waterlogging (Lu et al. 2004).

In conclusion, changes in the root environment (especially the decrease in oxygen) through waterlogging decrease ATP production. The lack of ATP leads to less energy for plant metabolic processes and could be a likely cause of growth limitation. Plants shift their metabolism from aerobic respiration to anaerobic fermentation to find other ways to generate energy without using oxygen.

1.2.2 General plant responses to waterlogging

In plants adapted to well-aerated soil conditions, waterlogging severely inhibits shoot growth and development (Jackson et al. 1978). Generally, changes in the root environment (differences in gases like ethylene, CO_2 and O_2 inside and outside of the plant) result in decreased crop growth and yield (Yordonova and Popova 2007) due to changes in water, carbohydrate, nutrient and hormone concentrations and interactions, starch-degradation and fermentation enzymes and scavenging mechanisms for reactive oxygen species (Kozlowski 1984). Waterlogging can slow root extension (Jackson 2011) or cause severe root damage (Visser et al 1996). Flooding intolerant cultivars are at high risk of suffering from severe defoliation and plant death even several days or weeks after returning the plants to un-flooded conditions (Gil 2007, Jackson 1990). Waterlogging at or after flowering leads to large yield loses, for example in chickpea, where 10 days of waterlogging caused leaf necrosis and abscission and death of roots and nodules (Cowie et al. 1996).

Responses to waterlogging can be classified as metabolic (reductions in the metabolic activity and the translocation of photoassimilates and metabolites such as amino acids, carbohydrates and phytohormones), physiological (decreased hydraulic conductivity [Lp], root water potential [Ψ_{root}], leaf water potential [Ψ_{leaf}], stomatal conductance [g_s], and photosynthesis rate [Pn], leaf chlorophyll content, early leaf senescence and a reduction in leaf area) and adaptive (hypertrophied lenticel in woody species, development of adventitious roots or development of lacunae gas spaces [aerenchyma] in the root cortex, Fig 1.3). Waterlogging has also been reported to decrease the concentration of macronutrients, such as N, P and K in leaves (Leyshon and Sheard 1974). Waterlogging can lead to similar responses in plants as drought and salt stress, such as stomatal closure, decreased photosynthetic rate, and photochemical efficiency (Ahmed et al. 2006, Hsu et al. 2011). Flooding also increases concentrations of the plant hormones ABA (Else et al. 1996), ethylene and auxin in shoots (Wample and Reid 1979), whereas gibberellin and cytokinin concentrations in stems and roots are reduced (Burrows and Carr 1969, Reid and Railton 1974). Not only the concentration of individual phytohormones, but also their ratio and interactions are important, as the production of each hormone is influenced by the concentration of other ones (Kozlowski 1984, Wilkinson et al. 2012). Initial plant responses to stress are determined by the sensitivity of the sensing tissue. Reactions are regulated by plants metabolism and can lead to induction, up- or down-regulation of the expression of involved genes and enzymes (Irfan et al. 2010). An appropriate balance between all these compounds might be the key for surviving waterlogging (Bailey-Serres and Voesenek 2010).

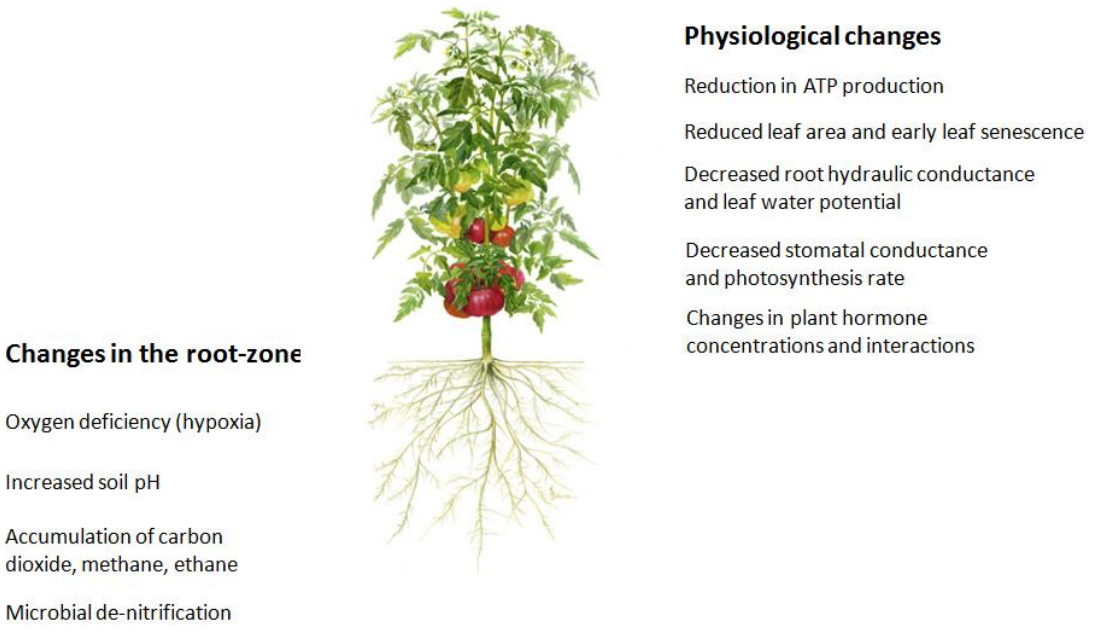

Fig 1.3 Changes in the root-zone and in the plant during waterlogging

1.2.3 Leaf gas exchange and water status

Combined processes of water potential, osmosis and evaporation lead to plants losing water from leaves (Ayres 1976). Because the gradient between soil and leaves is smaller than the gradient between leaf and air, the latter one is mainly responsible for water movement. Cells in the epidermis are covered by a hydrophobic cuticle resistant to water loss, which is punctuated by stomatal pores with guard cells. Carbon dioxide (CO_2) needed for photosynthesis can enter and water vapour can leave those pores. Guard cells control stomatal aperture and the rate at which water is lost from the leaf. This process depends on many different environmental factors, like diurnal cycle, temperature, CO_2 concentration, but also stresses such as drought or flooding (Grimmer et al. 2012). Altering water use strategy and decreasing transpirational

water loss causes changes in leaf water potential (Ψ_{leaf}), stomatal conductance (g_s) or photosynthesis rates (Pn) (Bradford 1983b, Else et al. 2001).

Leaf photosynthesis rate (Pn) of tomato decreased during the first few hours after flooding, but only dropped significantly below those of well-drained plants after 24 to 28 h of flooding. Decreased Pn was not due to stomatal limitation, even though internal CO_2 (C_i) decreased. Carboxylation efficiency, accompanied by a rise in the CO_2 compensation point of almost 40 % was reduced as well (Else et al. 2009). Limited CO_2 due to stomatal closure could decrease amounts of electron-accepting NADPH due to slower carbon reduction cycle. Else et al. (2009) reported a decrease in operating efficiency of the photosystem II photochemistry (Fv/Fm) within 8 h after inundation, which is associated with unrepaired damage to the photosystem II, possibly due to the increase of damaging superoxide anions like O_2^- and H_2O_2 (Yan et al. 1996, Yordanova and Popova 2007).

Several studies have shown that stomatal conductance (g_s) decreases during flooding (Else et al. 1995a, 1995b, 2009, Rodriguez-Gamir et al. 2011, Yordanova and Popova 2007). Stomatal closure is initially associated with decreased leaf water potential (Ψ_{leaf}) and root hydraulic conductance (Lp) of oxygen-deficient roots. It can arise within 2 to 6 hours of flooding (Else et al. 2001). Ψ_{leaf} decreased within 4 to 7 hours of a 12 h photoperiod from -0.55 MPa to -0.80 MPa. Towards the end of the photoperiod, Ψ_{leaf} recovered and exceeded those of well-drained plants (Else et al. 2009). In accordance, Jackson et al. (1978) and Else et al. (2009) report that flooded plants show increased stomatal closure accompanied by higher leaf water potentials especially during the first few days of flooding. Decreased Lp, possibly combined with stomatal closure, limits water uptake and internal water deficit during flooding of

tomato plants (Else et al. 2009). Other studies suggest that plants close their stomata in order to prevent leaf water deficit rather than being a response to low Ψ_{leaf} (Bradford 1983b, Rodriguez-Gamir et al. 2011). Rodriguez-Gamir et al. (2011) concluded that a decrease in Ψ_{leaf} is not necessarily responsible for stomatal closure, since Ψ_{leaf} of flooded citrus plants showed similar values to well-drained plants, even though g_s was reduced. In accordance, a temporarily decreased Ψ_{leaf} was not sufficient enough to initiate stomatal closure or have an effect on leaf expansion in flooded tomato plants (Else et al. 1995b). These results suggest that rather than a negative hydraulic message, chemical substances (such as plant hormones) generated by oxygen-deficient roots or chemical signals in the transpiration stream are sensed by stomatal guard cells and expanding leaf cells during waterlogging (Meinzer et al. 1991). Changes in the internal environment of guard cells (for example hormone concentrations) can determine stomatal response. However, evidence for this usually comes from exogenous phytohormone application to detached plant tissue instead of correlating endogenous plant hormone concentration with stomatal conductance (Dodd 2003a) and more studies on the latter would be needed to understand how chemical signals can regulate stomatal behaviour.

1.2.4 Plant hormones

Results from studies undertaken as early as in the nineteenth century by Julius von Sachs (1880) and Charles Darwin (1880) proved that “substances” (later identified as plant hormones) moving from one plant part to the other can regulate plant growth and development as well as responses to stress conditions (Gaspar et al. 2013). Plant hormones are natural compounds and can be synthesized in any type of living cell. They can influence growth and development processes in trace (nanomolar) quantities both at their site of synthesis but also at a distance. These processes depend on changes in hormone concentrations at the site of action and changes in the tissue’s sensitivity to the substance. Furthermore, transport and metabolism as well as ratios and antagonism with other compounds at level of synthesis or sensitivity, for example in their signalling pathways, play an important role in determining physiologically activity of plant hormones.

The plant hormone abscisic acid (ABA) is synthesized through oxidative cleavage of zeaxanthin, a C_{40} epoxycarotenoid precursor in plastids by the enzymes zeaxanthin epoxidase (ZEP), neoxanthin synthase and 9-cis-epoxycarotenoid dioxygenase (NCED). This C_{15} intermediate called xanthoxin is exported to the cytosol and converted to ABA via ABA-aldehyde and ABA aldehyde oxidase 3 (AAO3) (Fig 1.4, Finkelstein and Rock 2002, Schwartz et al. 2003, Seo and Koshiba 2002, Taylor et al. 2000, Xiong and Zhu 2003), making it an oxygen dependent process.

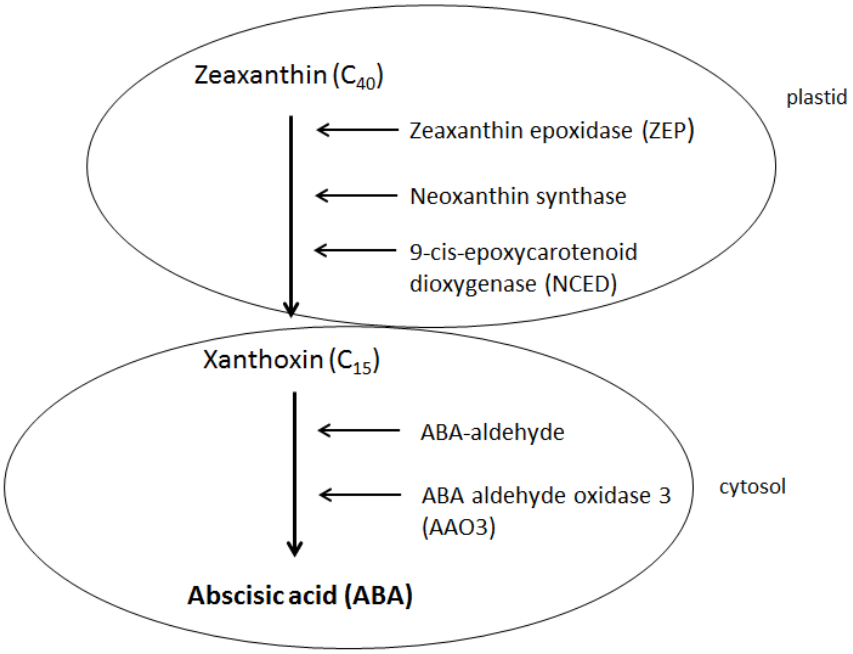

Fig 1.4 Biosynthetic pathway of ABA (redrawn after Xiong and Zhu 2003)

Stomatal response is extremely sensitive to changes in ABA concentrations and only small amounts of the plant hormone are sufficient to effect stomatal closure within minutes (Weyers and Paterson 2001). However, responses of foliar ABA concentrations during flooding seem inconsistent. Flooding increased foliar ABA concentrations of tomato plants within 4 to 24 h (Else et al. 1996) and up to 8-fold after 24h of flooding in pea shoots and roots (Zhang and Zhang 1994). Else et al. (1995a) suggest that foliar ABA concentrations increased in flooded tomato plants due to hindered export and internal redistribution. Increased foliar ABA concentrations of waterlogged mungbean coincide with decreased stomatal conductance (Ahmed et al. 2006). In contrast, 6 days of flooding tomato plants did not significantly alter foliar ABA concentrations (Calvo-Polanco et al. 2014) and ABA levels in leaves of flooded Citrus started to increase only after 3 weeks of flooding (Rodriguez-Gamir et al.

20011), suggesting that ABA might only play a secondary role in signalling physiological responses to flooding.

Ethylene (C_2H_4) is a "special hormone" because it is gaseous (Bradford and Yang 1980). Its biosynthesis is well understood (Fig 1.5): Methionine is converted to S-adenosyl methionine via SAM synthetase, which is converted to 1-aminocyclopropane-l-carboxylic acid (ACC) through ACC synthase. ACC is converted to ethylene by ACC oxidase (Wang et al. 2002). Ethylene synthesis and the response of plants to ethylene differ according to tissue, plant species and internal and environmental factors (Ahmed et al. 2006). Even though ethylene is widely known as a plant hormone involved in the ripening process of fruits, higher ethylene production can also be regarded as a stress signal. Initial small peaks of ethylene emission in stressed plants might be a protective reaction, where the already existing amount of ACC in plant tissue is consumed, ACS gene transcription is activated and more ACC is accumulated for the second larger peak of ethylene (Robison et al. 2001).

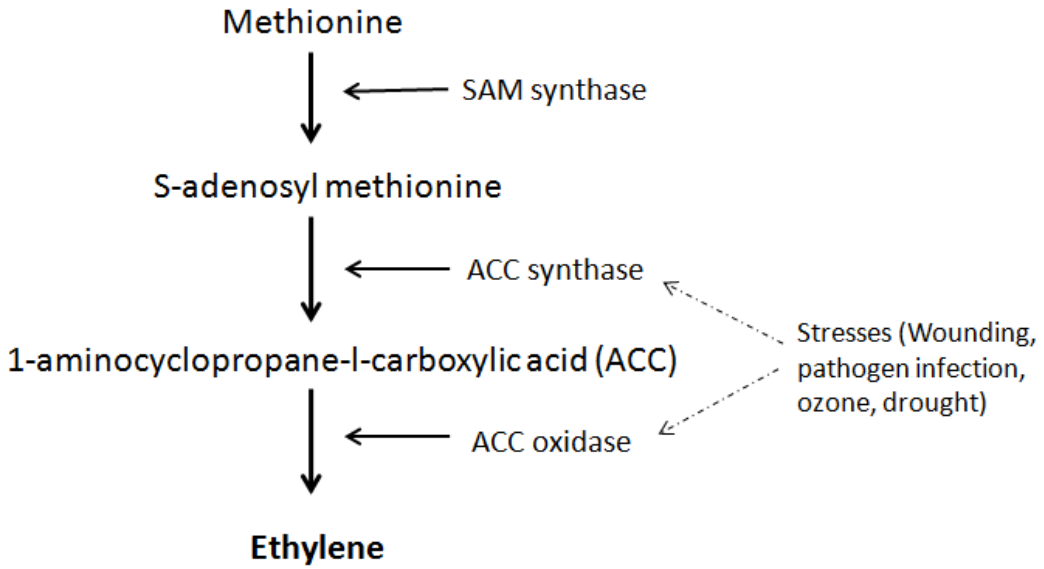

Fig 1.5 Biosynthetic pathway of ethylene (redrawn after Wang et al. 2002)

Ethylene concentration increases in submerged plants since it becomes trapped in tissues as its release into water (compared to air) from the root is ten times slower. In many plants, ethylene leads to growth retardation under hypoxia (Irfan et al. 2010). Ethylene production by petioles, main stem and shoot apex of tomato increased 4-6 fold after flooding the soil for 24 h (English et al. 1995, Jackson et al. 1978). Furthermore, ethylene accumulation was associated with inhibition of stem and root growth, development of aerenchyma and adventitious roots, epinasty, chlorosis, leaf abscission and premature fruit drop during flooding (Vartapetian and Jackson 1997). Ethylene also regulates apoptosis, a form of programmed cell death (Subbaiah and Sachs 2003), nodal adventitious rooting, formation of air chambers, and metabolic changes under anaerobiosis. Ethylene or ACC do not seem to directly affect stomatal behaviour, as applying ACC at concentrations detected in xylem of flooded tomato plants to excised shoots via the transpiration stream did not significantly change leaf conductance (Bradford and Hsiao 1982). ACC can also be accumulated in roots, as

lack of oxygen prevents the conversion of ACC to ethylene and accumulated ethylene during flooding causes epidermal cell death at the tip of the root. ACC is then transported in the xylem to the shoots where it is oxidized to ethylene and can, for example, induce epinastic leaf curvature (Bradford et al. 1982).

Ethylene can be bound by 2-component regulators (receptors), consisting of the sensor and the response regulator (Stock et al. 2000). Ethylene receptor genes in tomato consist of six members (LeETR1, 2, 4, 5, 6 and NR) (Lashbrook et al. 1998, Tieman and Klee 1999, Wilkinson et al. 1995, Zhou et al. 1996). Several mutations in tomato are readily available to test different hypotheses concerning ethylene signalling and sensing. The *never ripe* (*Nr*) genotype is a naturally occurring mutation in the ethylene-binding domain of the NR receptor (Wilkinson et al. 1995) and therefore cannot bind ethylene. Analysis showed that *Nr* is ethylene-insensitive at all stages of development (Lanahan et al. 1994), is defective in floral abscission and leaf and flower petal senescence (Klee 2002), but does not show developmental and morphological abnormalities (apart from delayed fruit ripening). So far, *Nr* has mainly been used as a tool to assess the role of ethylene in a range of developmental and gene expression processes such as differential expression of ethylene receptor genes in tomato fruit during development and ripening (Nakatsuka et al. 1998, Rose et al. 1997), but few studies exist on the influence of environmental stresses on *Nr*.

1.2.5 Root-to-shoot-signalling

Roots are the first organs to sense changes in soil conditions and physiological responses in stressed roots usually occur before apparent changes in shoots. Therefore, there must be some form of signal transmission from the stressed roots to shoots which converts the physical stress (flooding) into a biochemical response (Else et al. 2001). Furthermore, physiological changes in the shoot due to flooding or drought can arise even when shoot water status does not alter (Else et al 1995a, Passioura 1988, Sharp and LeNoble 2002) which suggests some sort of communication between roots and shoots, so-called root-to-shoot signalling. Developmental or environmental influences can lead to the release of those signals and have positive or negative effects on the source and/or the target tissue. Long-distance communication can help understand the effect of above mentioned influences on plant growth and responses.

Root-to-shoot signalling has been described as a stop-gap mechanism, which reduces the need for root-sourced supplies (water, minerals) when root activities are decreased, for example due to low oxygen availability (Jackson 2011). Numerous studies propose a system in plants which forms a systemic communication between flooded (roots) and non-flooded tissues (shoots) (Hsu et al. 2011). Chemical substances are transported in the xylem sap (transpiration stream) primarily to the sites of transpiration. Rapid signalling from roots to shoots during waterlogging could slow water loss from the foliage due to decreased stomatal aperture, leaf expansion, sometimes epinasty, stem elongation and faster leaf senescence. The amounts of nutrients, hormones or their precursors and/or their delivery rates entering the shoots via the transpiration stream can change during flooding (Bradford and Yang 1980, Jackson and Campbell 1975).

For estimating root export of solutes, solute concentrations in xylem sap can be used, though measuring delivery rates (product of concentration and flow rate) seems more suitable (Else et al. 1995a). Soil flooding for 24 h increased delivery of ACC and phosphate from roots into shoots in tomato. In contrast, soil flooding decreased delivery of ABA from roots to shoots (Else et al. 1995a, 1996). Root-to-shoot signalling of flooded soil may also include changes in root export of gibberellins (GA) and cytokinins (CK) to the shoot in the xylem sap (Else et al. 2009). Cytokinins can promote stomatal opening and antagonise ABA-induced stomatal closure (Pospisilova 2003) and gibberellins can regulate stem elongation (Reid et al. 1969). However, the number of studies on the effect of environmental stresses on xylem sap delivery rates of hormones and nutrients is still limited and more research would be needed to better understand the composition of sap and its function in signalling.

1.2.6 Nutrient status

Mineral nutrients, such as nitrogen, potassium, phosphorus, magnesium, calcium, sulphur or sodium, have important roles in plant metabolism and physiology (Rubio et al. 2009). Waterlogging changes the concentration of macronutrients in leaves and xylem sap (Else et al. 1995b, Jackson et al. 1996, Leyshon and Sheard 1974). Decreased N, P, K, Mg, Cu, Zn and Mn concentrations were found in wheat and barley shoots after 15 days of waterlogging (Steffens et al. 2005) and flooding of barley for seven days reduced foliar N, P and K concentrations by 51, 60 and 58 %, respectively (Leyshon and Sheard 1974). These deficiencies can occur due to decreased ion concentrations in the soil solution (Steffens et al. 2005) and decreased uptake and/or transport of mineral ions by roots, which leads to redistribution of nutrients within the plant and/or premature leaf senescence (Drew et al 1979, Trought

and Drew 1980). Flooding can reduce nutrient delivery from roots to shoots, possibly through loss over control of ion uptake (Jackson et al. 1996), but can also increase nutrient delivery, which might be due to release of stored ions from root cell vacuoles (Lee and Ratcliffe 1983) or degeneration of the cell plasma membranes resulting in solute leaking (Else et al. 1995b).

Plants can adapt to changes in mineral nutrient availability and have developed a system involving sensing and signalling mechanisms to monitor and regulate external and internal nutrient concentrations. Plant hormones play an important role is this network, but at the same time, changes in nutrient concentrations can influence hormone biosynthesis. Few reports exist on the involvements of plant hormones in mediating plant responses to nutrient deprivation (Chapin et al. 1988, Dodd et al. 2004, Rengel and Kordan 1988), but the impacts of waterlogging-induced nutrient deficiency on foliar phyotohormone relations have not been studied.

1.3 Aims of thesis

Flooding and in consequence waterlogged soil can affect more than 17 million km² of land area in all continents in certain years and approximately 10 % of irrigated farmlands worldwide suffer from prolonged waterlogging due to poor drainage or irrigation management (FAO 2002). Waterlogging results in physico-chemical changes in the soil environment such as differences in gas diffusion, soil pH and the accumulation of possibly toxic products due to anaerobic processes (Parent et al. 2008). Lack of oxygen and carbon dioxide influences plants energy metabolism and can decrease crop growth and yield or ultimately lead to death of the inundated organs or the whole plant (Jackson 1990). On the physiological level, waterlogging can lead

to changes in leaf water potential and decrease transpiration and photosynthesis rate (Bradford 1983b, Else et al. 2001). Plant hormones might be key signals regulating growth and responses to oxygen-deficiency, since waterlogging can increase foliar ABA and ethylene concentrations (Else et al. 1996, English et al. 1995, Jackson et al. 1978). The changes of their concentrations can happen either in the flooded tissue or in a more distant, non-flooded part due to root-to-shoot signalling (Jackson 1990), but a comprehensive, multi-analyte study on the effect of over-irrigation on root-to-shoot signalling of several plant hormones has not been conducted. Waterlogging can also change nutrient concentration in sap and leaves (Else et al. 1995b, Jackson et al. 1996, Leyshon and Sheard 1974), but the impacts of waterlogging-induced nutrient deficiency on foliar phyotohormone relations have not been well studied.

This thesis focuses on understanding the effects of over-irrigation on tomato plant growth and physiology and how the underlying mechanism(s) of over-irrigation-induced changes in plant growth can be explained. Specifically, it aims to answer the following questions:

- How does over-irrigation affect soil properties, such as soil oxygen, temperature and moisture? Does over-irrigation (negatively) affect tomato plant growth and do over-irrigation and flooding alter plant physiology in similar ways? (Chapter 2 – *Comparing effects of acute flooding and chronic over-irrigation on soil properties and growth and physiological responses of tomato*)

- What is the role of ethylene during over-irrigation? Does over-irrigation change growth and physiological responses of a partial ethylene-insensitive genotype similar to the wild type? (Chapter 3 – *Partial ethylene-insensitivity reverses over-irrigation-induced growth inhibition to some extent*)

- Does over-irrigation have an effect on root-to-shoot signalling? Do xylem sap concentrations and delivery rates of plant hormones change during over-irrigation? (Chapter 4 – *Influence of over-irrigation on phytohormonal root-to-shoot signalling*)

- What are the foliar and xylem sap nutrient responses to over-irrigation? Can calcium nitrate addition to the soil of over-irrigated tomato plants ameliorate the effects of over-irrigation? (Chapter 5 – *Over-irrigation decreases xylem nutrient and foliar nitrogen concentrations*)

- How can growth regulation of plants exposed to abiotic stress be best assessed and can it be pinned down to one factor only? (Chapter 6 – *Concluding remarks*)

Chapter 2.

2 Comparing effects of acute flooding and chronic over-irrigation on soil properties and growth and physiological responses of tomato

Fig 2.1 Well-drained (left, blue tag) and over-irrigated tomato plants (right, red tag) at harvest day

2.1 Introduction

Heavy rainfall, poor drainage or irrigation practices can induce waterlogging, which in turn affects plant growth. During waterlogging, pores in the soil are filled with water and become saturated, which leads to slower gas diffusion rates and decreased soil oxygen concentrations (Drew 1997). However, free exchange of gases like oxygen and carbon dioxide in the growing medium is important for root respiration and growth and its indirect effects on shoot development and, ultimately, crop productivity (Visser et al. 2003).

Early physiological responses to waterlogging include stomatal closure to reduce water loss, which also decreases photosynthesis (Arbona et al. 2008, Domingo et al. 2002). In a series of papers (Else et al. 1995a, 1995b, 1996, 2009), Else and colleagues report rapid (within hours) inhibition of leaf elongation and gas exchange associated with decreased leaf water potential (Ψ_{leaf}) when tomato plants were flooded (the entire pot and surface of the growing medium was submerged in water). However, Else et al. (1995a) suggest that hydraulic signals such as changes in leaf water status are not sufficient to induce these flooding responses, as applying a balancing pneumatic pressure to prevent any flooding-induced decrease in leaf water status did not ameliorate stomatal closure and leaf growth inhibition. Instead, it was suggested that these physiological processes were regulated by chemical messages.

The plant hormones abscisic acid (ABA) and ethylene (and the ethylene precursor 1-aminocyclopropane-l-carboxylic acid - ACC) can play important roles in sensing of low oxygen availability in the growing medium by the root system. Thus, ABA accumulated in leaves only hours after flooding, accompanied by transient leaf water deficits (Bradford and Hsiao 1982, Else et al. 1996, Jackson et al. 1978, Zhang and

Davies 1987). However, this ABA is not the only factor involved in stomatal closure, as stomata remain closed even when ABA concentrations decline during later stages of flooding (Else et al. 1996), suggesting that another chemical substance may be implicated in stomatal closure of flooded plants.

Ethylene production by petioles, main stem and shoot apex of tomato increased 4-6 fold after flooding the soil for 24 h (English et al. 1995, Jackson et al. 1978). However, ethylene's role in stomatal closure is still controversial, as ethylene has been reported to induce (Desikan et al. 2006) or inhibit stomatal closure in well-drained Arabidopsis (Tanaka et al. 2005). Possibly, the effect of ethylene during flooding is more related to inhibition of stem and root growth, development of aerenchyma and adventitious roots, epinasty, chlorosis, leaf abscission and premature fruit drop (Vartapetian and Jackson 1997) rather than its influence on stomatal regulation. Most of the early physiological responses of the shoot system to flooding (reduced photosynthesis and stomatal closure as well as decreased Ψ_{leaf}) are associated with oxygen deficiency at the roots. This in turn can raise the production of ethylene thought to induce symptoms such as leaf epinasty, abscission and decreased extension growth (Kozlowski 1984).

Waterlogging can constrain crop yields in open field agriculture (FAO 2002, McFarlane and Cox 1992), but should be of limited importance in nurseries or greenhouses, when plants are grown in containers, often in highly porous substrates to maximise drainage (Passioura 2006). However, the actual water requirements of such containerised plants are often misjudged and could lead to over-irrigation (Thompson et al. 2007b). Whether the above mentioned physiological responses to soil flooding also occur in over-irrigated plants has not yet been tested. Physiological differences

between plant responses to long term, chronic (over-irrigation) *versus* short term, acute (flooding) stress have also not been analysed. Thus, this chapter aims to determine whether acute soil flooding and chronic over-irrigation induce similar changes in soil properties and physiological responses (leaf water status, gas exchange and phytohormone balance).

2.2 Materials and Methods

2.2.1 Plant treatments

Tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) seeds (Moles Seeds, Colchester, UK) were sown individually in seedling compartments (22 ml) filled with a peat-based substrate (Levington's M3, Scotts Company Ltd, UK; added nutrients in base fertilizer: 218.5 mg/l total N; 133.5 mg/l NO_3^-N; 85 mg/l NH_4/N; 102.3 mg/l P; 338.6 mg/l K; pH 5.3-5.7 and 310/420 S/m conductivity) and covered with black plastic to assure high humidity and darkness to promote germination. After 5 to 7 days, the plastic was removed to prevent etiolation of the seedlings. After a further week, seedlings were potted into cylindrical 1.14 l (11 cm diameter x 12 cm high) pots, filled with the same growth medium and initially watered with 300 ml. Pots were placed on a saucer in a walk-in controlled environment room with a day/night temperature of 22/16°C and a 12 h photoperiod (06:00 to 18:00 h). Day/night relative humidity was 42/54 %, CO_2 concentration was 440/390 ppm and light intensity at plant height between 400-640 µmol $m^{-2}s^{-1}$ PPFD.

After potting up into the same substrate (Levington's M3, Scotts Company Ltd, UK), irrigation was scheduled using a Delta-T GP1 Data Logger with two Delta T SM200 soil moisture sensors each placed in a different pot coupled with an irrigation timer (TORO, type MC-212) and a solenoid valve attached to a hose with drippers. The irrigation timer was set to allow irrigation every two hours for 2 minutes, if the soil moisture of either pot was below the set threshold. A pot (same volume as above) was filled with 360 g of the substrate and watered with 300 ml to full capacity. The volumetric soil moisture content remaining after 24 hours drainage was used as the threshold for the well-drained treatment and determined to be 0.23 m^3 m^{-3}. The irrigation program on the GP1 was set to activate if either of the SM200 sensors measured soil moisture below 0.23 m^3 m^{-3} and to deactivate if both were above 0.3 m^3 m^{-3}. The SM200 sensors were placed in the control treatment. The irrigation treatments lasted ~26-28 days. No additional fertilizer was added throughout the course of the experiment. Two treatments were used in the experiment: over-irrigation (150 % - three drippers inserted in to the pot) and well-drained (100 % - two drippers) and each treatment consisted of 5 to 10 plants. An overview of sampling times for different parameters within this experiment is given in Table 2.1. To verify measurement techniques and compare results with previous work, a third treatment (flooding) was imposed on some plants that had been grown in well-drained conditions for ~26-28 days. Tomato plants each were placed, one hour after the photoperiod had started, in larger pots (volume 21 l) which were filled with warm tap water (20°C) which was maintained 10 mm above the substrate surface. This experiment was repeated several times to measure multiple plant variables and all plant variables were measured 2, 6, 10 and 26 hours after the treatment had started.

Table 2.1 Sampling times for ABA, ethylene, g_s, Pn, leaf water potential, shoot fresh weight and total leaf area during the over-irrigation experiment

Measurement	**Day of sampling (after treatment had begun)**
Soil moisture, oxygen and temperature	Continuously (every 15 min) from day 0 to 27
Foliar ABA concentration	14, 16, 18, 20, 22, 24, 26
Foliar ethylene emission	18, 20, 22, 24, 26
g_s, Pn	22, 24, 26
Ψ_{leaf},	27, 28, 29
Shoot fresh weight, total leaf area	28

2.2.2 Soil measurements

A DL6 soil moisture logger (Delta-T Devices, Cambridge, UK) connected to ML2x ThetaProbes (Delta-T Devices, Cambridge, UK) independently monitored volumetric soil moisture content over time (Fig 2.2E). Soil oxygen concentration and soil temperature at middle height of the pot were measured with SO-110 soil oxygen thermistor sensors (Apogee Instruments, Utah, USA) connected to a CR1000 Campbell data logger (Campbell Scientific, Inc., Utah, USA).

2.2.3 Plant measurements

Plants at the five- to seven-leaf stage (~40 days old, after 26-28 days of treatment) were harvested to measure area of each individual leaf using a leaf area meter (Licor Model 3100 Area Meter, Cambridge, UK), main stem height and shoot fresh weight.

Leaf water potential (Ψ_{leaf}) was routinely measured on Leaf 2 (numbering from the base of the plant) with a Scholander type pressure chamber on different harvest days, since Leaf 1 had begun to senesce. Leaf 2 of each plant was excised and placed in a plastic bag to minimise transpiration during transport to a Scholander pressure vessel (Soil Moisture Equipment Corp., Model 3000F01, Santa Barbara, CA, USA) in an adjacent laboratory (time from excision to sealing in the chamber was < 90 seconds), then Ψ_{leaf} was measured.

In addition to Ψ_{leaf}, stomatal conductance (g_s) and photosynthesis rate (Pn) were routinely measured on Leaf 2 with a LiCOR LI-6400XT (Lincoln, NE, USA) portable photosynthesis system equipped with a sensor head which has two infrared gas analysers to measure absolute concentrations of CO_2 and H_2O. Since measurements were made with a 2 cm^2 cuvette (environmental conditions inside the cuvette: 390 µM CO_2, 20°C, 250 µml PAR), leaves had expanded sufficiently to permit measurement 22 days after the start of the experiment.

2.2.4 Plant hormone analysis

Bulk leaf abscisic acid (ABA) concentration of the youngest, fully expanded leaflet was measured via a radioimmunoassay (RIA) using a monoclonal antibody, AFRC MAC 252 (based on method described in Quarrie et al. 1988). Leaflets were harvested, weighed, snap frozen in liquid nitrogen, freeze-dried for 48 h, then finely ground and diluted with deionized, distilled water (1:70). Samples were then placed on a shaker in a cold room (4°C) overnight to extract ABA. A standard curve was constructed with standards in a serial dilution of synthetic unlabelled (±)-cis, trans-ABA (Sigma Let., UK). ABA concentration from samples was calculated by reference to this standard curve after linearization using the "logit" transformation. Since only a small amount of plant tissue (10 – 15 mg DWt) was needed for this analysis, ABA concentration was measured from Day 14 (after start of treatment) on every second day at the same time each day (9:30 am) to avoid diurnal effects on ABA concentration.

Ethylene evolution rate was measured on the same leaves (but different leaflets) sampled for ABA analysis. Each sample (0.1 g minimum fresh weight required for detection by the gas chromatograph - GC) was immediately placed in a 28 ml transparent glass vial with moist tissue, which was then sealed with a rubber puncture cap (Subaseal). After incubating for 60 min under light (Tungsten 60 W light bulb, PAR 80 µmol m^{-2} s^{-1}), 1 ml of the air in the glass vial was injected with a gas-tight syringe and ethylene determined using a GC (model 6890N Agilent Technologies UK Ltd, Wokingham, UK; Networked GC System, method: Ethylenesplit.M, software: Enhanced Chemstation Online GC) equipped with a J&W HP-AL/S (50 m x 0.537 mm x 15.0 mm) column *HiChrom Ltd, Reading, UK). The initial column

temperature was 100°C for resolving ethylene and then increased to 150°C (rate 15°C/min) and held for 1.5 min to remove the water vapour introduced into the column by sample injection (injector and detector oven temperatures were 250°C and 300°C, respectively). The helium carrier gas was set at a flow rate of 5.7 ml min^{-1} and detection by flame ionization. Injection of 20 ppm ethylene (BOC Special Gases, Manchester, UK) confirmed the timing of the ethylene peak and was also used for quantitation. To allow storage of headspace samples, the exetainer method previously described (Glatzel and Well 2007, Laughlin and Stevens 2003) was used. Samples were incubated in the same way as above, but 4 ml was taken out of each vial and inserted with a glass syringe into evacuated 3.7 ml soda glass vials with flat bottoms and fitted with septum caps, so-called exetainers (Labco Ltd., High Wycombe, UK). Samples were then stored at 4°C (for no longer than two weeks) until injection and analysis via GC.

2.2.5 Statistical analysis

For each sampling day, treatment differences were determined via an Independent Samples T-test (SPSS 19, IBM). Furthermore, a univariate (2-way) ANOVA was performed to test both the individual effects of parameters and treatment and any interactions between parameters and treatment. If no significant treatment interaction was found, a single linear regression was fitted to data from both treatments. Experiments were repeated several times. Generally, data from a representative experiment are presented, except where measurements could only be made destructively on a specific whole leaf (Fig 2.4B and C), explaining the different soil moisture range.

2.3 Results

Flooding steadily decreased soil oxygen concentration from 23% to 17% within 26 hours (Fig 2.2A), whereas soil oxygen concentration of over-irrigated soil did not change until Day 21 and then continuously dropped from 23 % to between 21 and 19 % (Fig 2.2B). In contrast, soils that were well-drained had consistent oxygen values (23 %, Fig 2.2A and B). Soil temperature varied according to day/night temperature in the controlled environment room, and did not differ between the over-irrigated and flooding treatment, but was ~1°C lower when compared to the well-drained treatment (2.1C-D). It was not possible to measure soil water content in flooded soil, as the sensors rapidly (~ 15 minutes) recorded out of range values. Soil moisture for the over-irrigated treatment did not increase linearly, but slowly accumulated over time. Hence, it took 14 days of treatment for soil moisture to significantly differ compared to well-drained soil (Fig 2.2E). From then on, soil moisture of over-irrigated plants continued to increase until it reached relatively stable values (~0.7 m^3 m^{-3}) by Day 21.

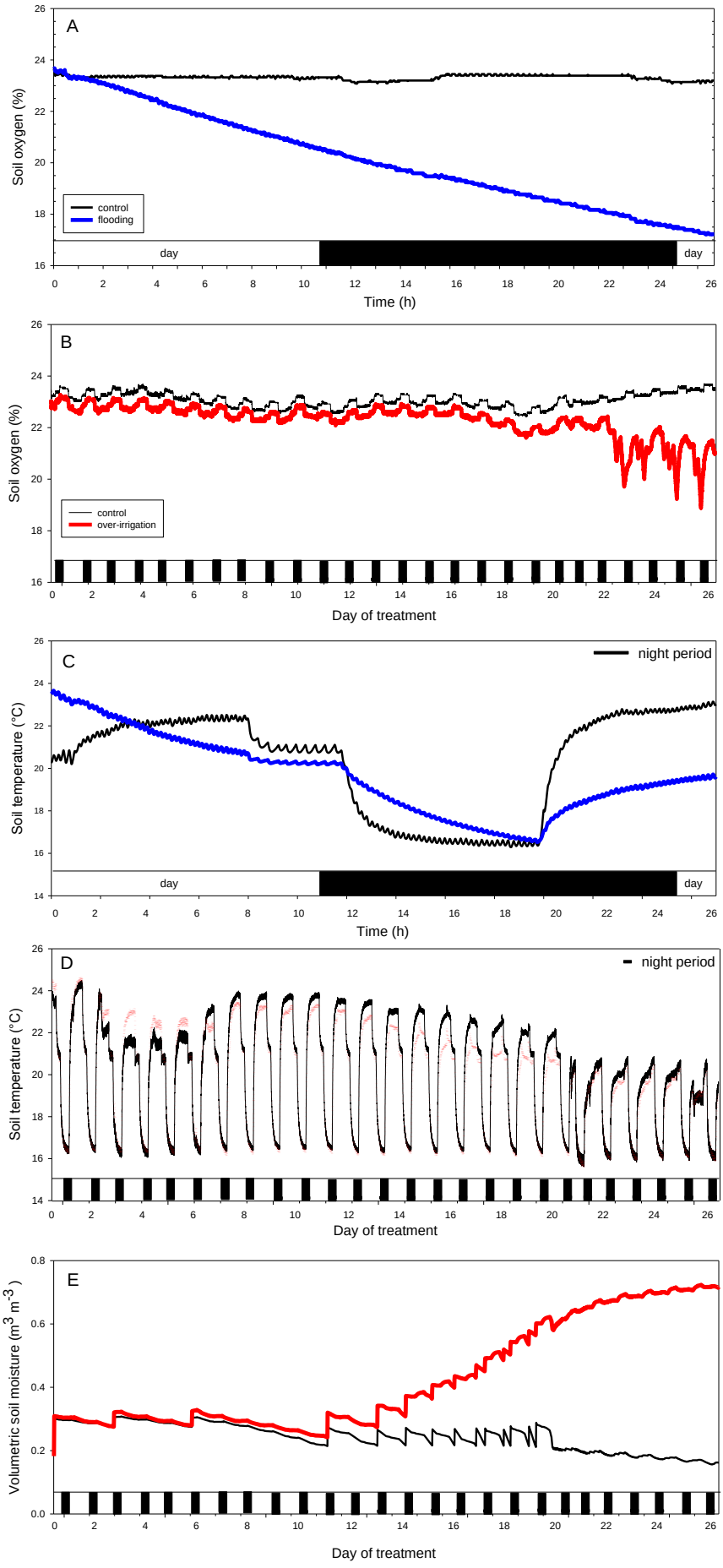
A
Soil oxygen (%)
control
flooding
day
day
Time (h)
B
Soil oxygen (%)
control
over-irrigation
Day of treatment
C
night period
Soil temperature (°C)
day
day
Time (h)
D
night period
Soil temperature (°C)
Day of treatment
E
Volumetric soil moisture (m3 m-3)
Day of treatment

Fig 2.2 (A) Soil oxygen concentration for well-drained (black line) and flooded tomato plants (blue line) over 26 h and (B) for over-irrigated (red line) and well-drained (black line) plants throughout the experimental period; (C) soil temperature for well-drained (black line) and flooded tomato plants (blue line) over 26 h and (D) for over-irrigated (red line) and well-drained tomato plants (black line) throughout the experimental period, (E) continuous soil moisture changes (determined with ML2x ThetaProbes) over time for over-irrigated (red line) and well-drained tomato plants (black line) throughout the experimental period; black bars (C and D) indicated dark periods. Data are from a single representative sensor.

Over-irrigation significantly ($P < 0.001$) decreased shoot fresh weight (by 62 %) compared to control (well-drained) plants (Table 2.2 and Fig 2.1). Plant height and whole plant leaf area were also decreased by 27 % and 70 % respectively for over-irrigated plants compared to the control (Table 2.2). Plotting leaf area against leaf node indicates that the effects of over-irrigation on leaf growth were consistent throughout development (Fig 2.3), justifying the use of leaves at any node for other physiological measurements. Epinasty was only seen in flooded plants (data not shown), but not in over-irrigated plants.

Table 2.2 Shoot fresh weight, height and total leaf area for over-irrigated and well-drained (control) tomato plants. Data are means ± SE of 5 replicates. Different letters indicate significant differences (Independent-Samples T-Test, P-value < 0.05).

	Over-irrigation	**Control**	**% of control plants**
Shoot fresh weight (g)	20.3 ± 2.5^{a}	53.9 ± 3.4^{b}	37.6
Height (cm)	16.3 ± 1.4^{a}	22.2 ± 1.4^{b}	73.4
Total leaf area (cm^2)	258 ± 60^{a}	859 ± 62^{b}	30.0

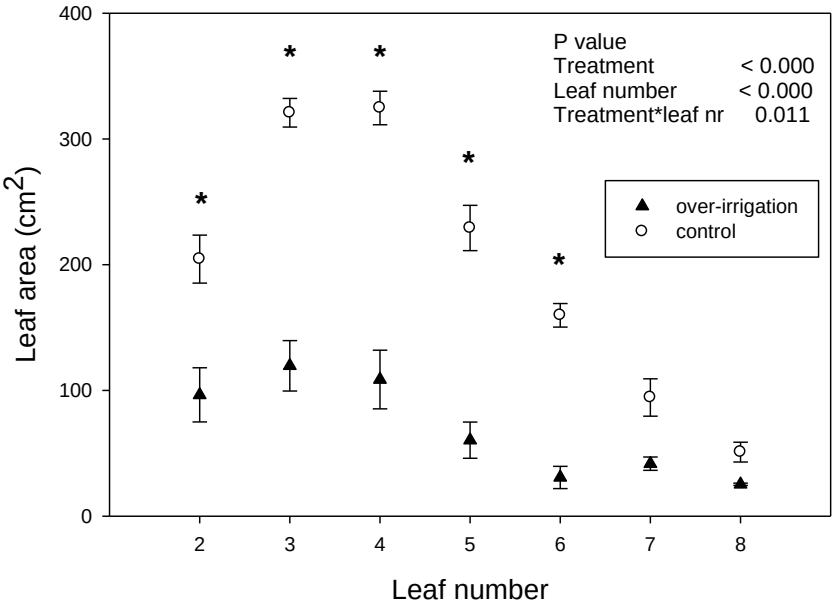

Fig 2.3 Leaf area profile of over-irrigated (triangle) and control (open circle) tomato plants at harvest day. Data are means ± SE of 5 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, P-value < 0.05). P-values for 2-way ANOVA given.

Flooding decreased leaf water potential (Ψ_{leaf}) 2, 6 and 10 h after the treatment had started (by 0.03, 0.06 and 0.03 MPa, respectively), but increased Ψ_{leaf} by 0.04 MPa after 26 h (Fig 2.4A). Leaf water potential (Ψ_{leaf}) did not significantly differ between over-irrigated and control plants in three replicated experiments (Fig 2.4B) and soil moisture did not have an effect on Ψ_{leaf} (Fig 2.4C). Ψ_{leaf} increased with soil oxygen concentration in flooded plants but decreased with soil oxygen concentration in over-irrigated plants (Fig 2.4D).

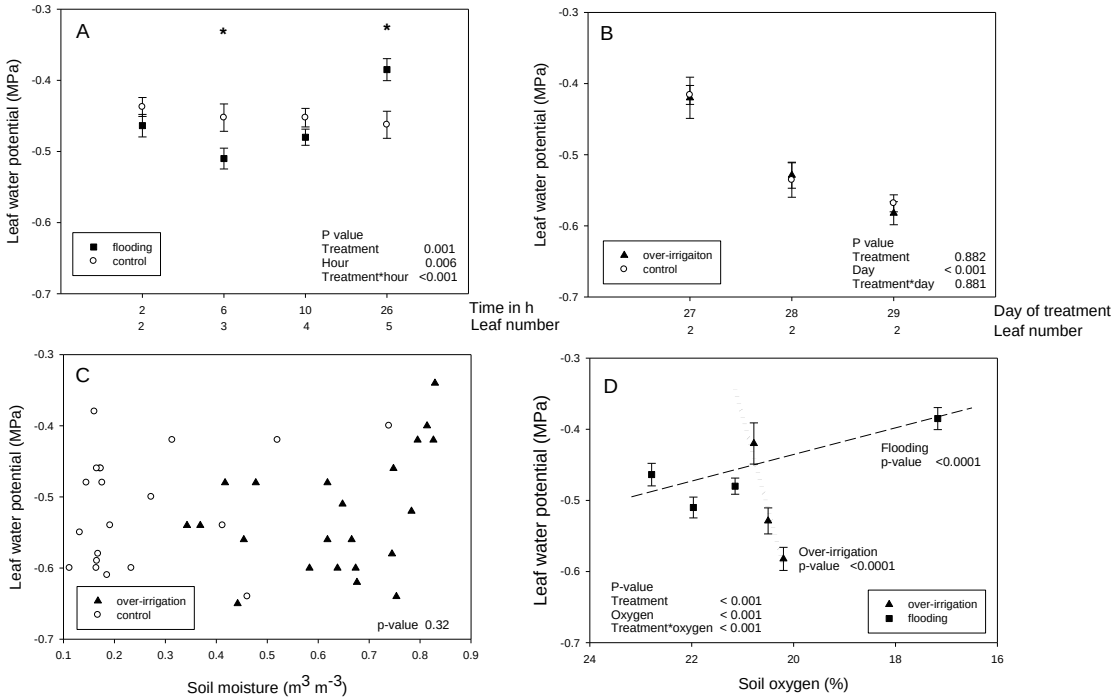

Fig 2.4 (A) Leaf water potential (Ψ_{leaf}) of flooded (closed square) and well-drained (open circle) tomato plants and (B) over-irrigated (triangle) and well-drained (open circle) tomato plants on different harvest days; (C) Ψ_{leaf} plotted against soil moisture for over-irrigated (closed triangle) and well-drained tomato plants, (D) Ψ_{leaf} plotted against soil oxygen for over-irrigated (closed triangle) and flooded (closed square) tomato plants (with linear regressions fitted to each treatment). Data are means ± SE of 5-8 replicates except in (C) where each point is an individual plant, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05, [Fig A/B]). P-values for 2-way ANOVA and linear regressions for flooded (dashed line) and over-irrigated (dotted) plants given [Fig C].

Flooding decreased stomatal conductance (g_s) by 25 % within 6 h. Although flooded and control plants had a statistically similar g_s at the end of the photoperiod (after 10 h), g_s of flooded plants had declined by 56 % after 26 hours (Fig 2.5A). Stomatal conductance of over-irrigated plants varied throughout the experiment period (Fig 2.5B) and soil moisture did not affect g_s (Fig 2.5C). g_s decreased similarly with oxygen concentration in both flooded and over-irrigated plants and no significant interaction between treatment and soil oxygen concentration was observed (Fig 2.5D).

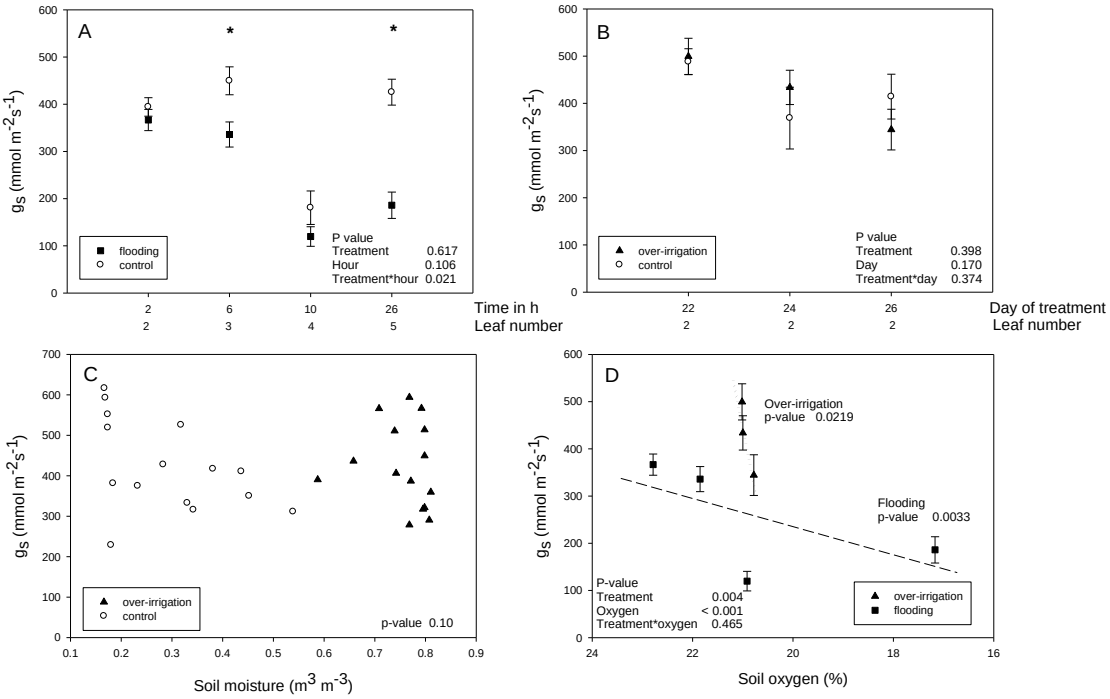

Fig 2.5 (A) Stomatal conductance (g_s) of flooded (closed square) and well-drained (open circle) tomato plants and (B) over-irrigated (triangle) and well-drained (open circle) tomato plants throughout the experimental period; (C) g_s plotted against soil moisture for over-irrigated (closed triangle) and well-drained tomato plants, (D) g_s plotted against soil oxygen for over-irrigated (closed triangle) and flooded (closed square) tomato plants (with linear regressions fitted to the flooding treatment). Data are means ± SE of 5-8 replicates except in (C) where each point is an individual plant, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05, [Fig A/B]). P-values for 2-way ANOVA and linear regressions for flooded (dashed line) and over-irrigated (dotted) plants given [Fig C].

On some occasions, both flooding and over-irrigation reduced photosynthesis rate (Pn) when compared to well-drained plants (Fig 2.6A-B). Pn significantly decreased with higher soil moisture ($P=0.0034$, Fig 2.6C) and decreasing soil oxygen concentration was correlated with decreased Pn for flooded and over-irrigated plants (Fig 2.6D).

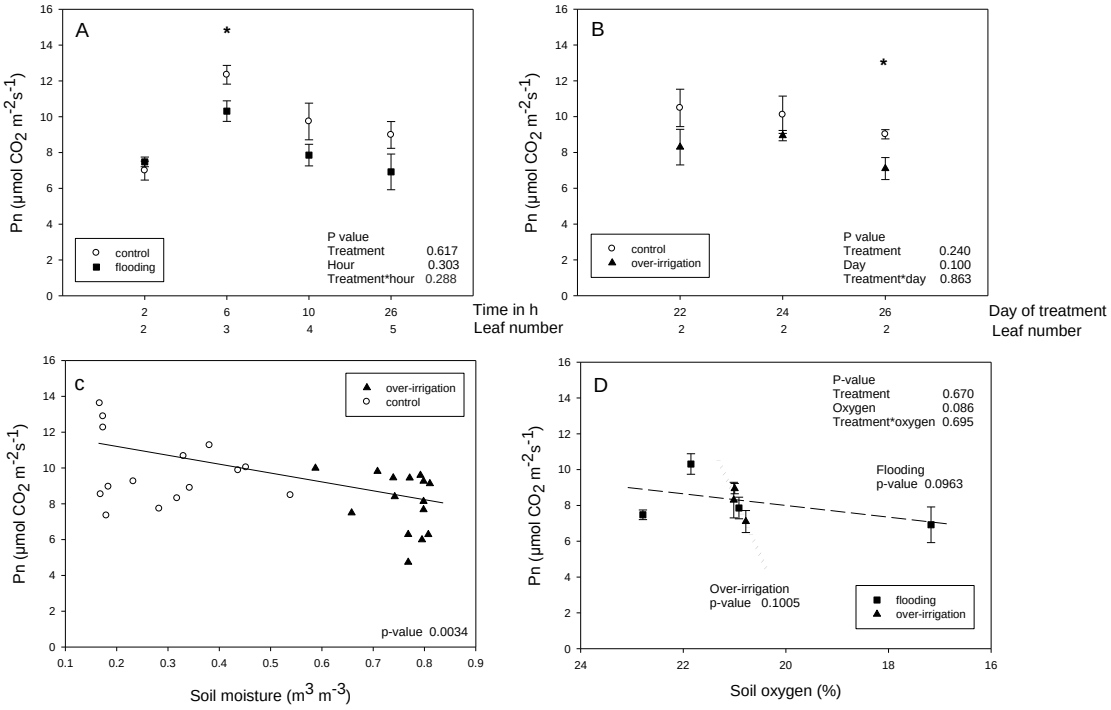

Fig 2.6 (A) Photosynthesis rate (Pn) of flooded (closed square) and well-drained (open circle) tomato plants and (B) over-irrigated (triangle) and well-drained (open circle) tomato plants throughout the experimental period; (C) Pn plotted against soil moisture for over-irrigated (closed triangle) and well-drained tomato plants, (D) Pn plotted against soil oxygen for over-irrigated (closed triangle) and flooded (closed square) tomato plants (with linear regressions fitted to each treatment). Data are means ± SE of 5-8 replicates except in (C) where each point is an individual plant, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05, [Fig A/B]). P-values for 2-way ANOVA and linear regressions for flooded (dashed line) and over-irrigated (dotted) plants given [Fig C].

Flooding doubled foliar ABA concentration within 26 hours (Fig 2.7A). Over-irrigated plants had variable leaf ABA concentrations throughout the measurement period and were lower than control plants on Day 14, 22, 24 and 26, but higher on the other days measured (Fig 2.7B). However, higher soil moisture correlated with lower ABA concentrations (P=0.009, Fig 2.7C). In flooded plants, foliar ABA levels increased as soil oxygen concentration decreased, while there was no clear relationship in over-irrigated plants (Fig 2.7D).

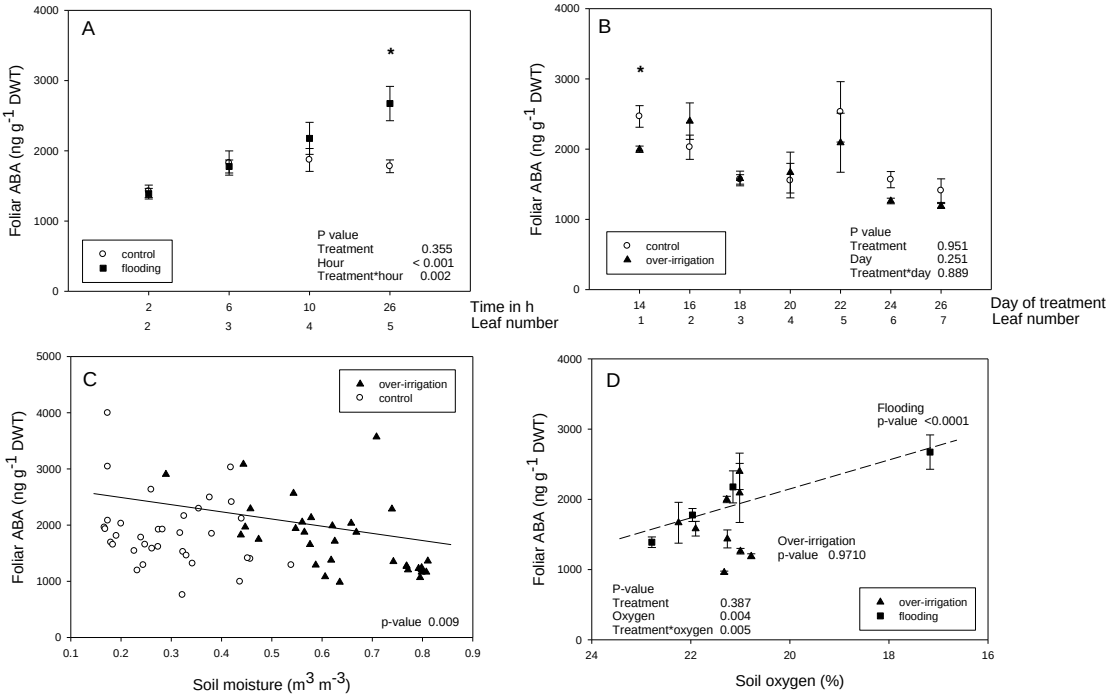

Fig 2.7 (A) Foliar abscisic acid (ABA) concentration of flooded (closed square) and well-drained (open circle) tomato plants and (B) over-irrigated (triangle) and well-drained (open circle) tomato plants throughout the experimental period; (C) ABA plotted against soil moisture for over-irrigated (closed triangle) and well-drained tomato plants, (D) ABA plotted against soil oxygen for over-irrigated (closed triangle) and flooded (closed square) tomato plants (with linear regressions fitted to the flooding treatment). Data are means ± SE of 5-8 replicates except in (C) where each point is an individual plant, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05, [Fig A/B]). P-values for 2-way ANOVA and linear regressions for flooded (dashed line) and over-irrigated (dotted) plants given [Fig C].

Flooding increased foliar ethylene emission by 34 % after 26 hours (Fig 2.8A). Similarly, foliar ethylene emission was elevated in over-irrigated plants when compared to the controls on all days measured and significantly ($P < 0.05$) higher on 2 out of 5 days (Fig 2.8B). Treatment differences were more pronounced as the duration of over-irrigation increased. Increasing soil moisture correlated with increased ethylene emissions ($P<0.0001$, Fig 2.8C). The relationship between foliar ethylene evolution and soil oxygen concentration differed between flooding and over-irrigation (Fig 2.8D), with over-irrigated plants showing a more sensitive response.

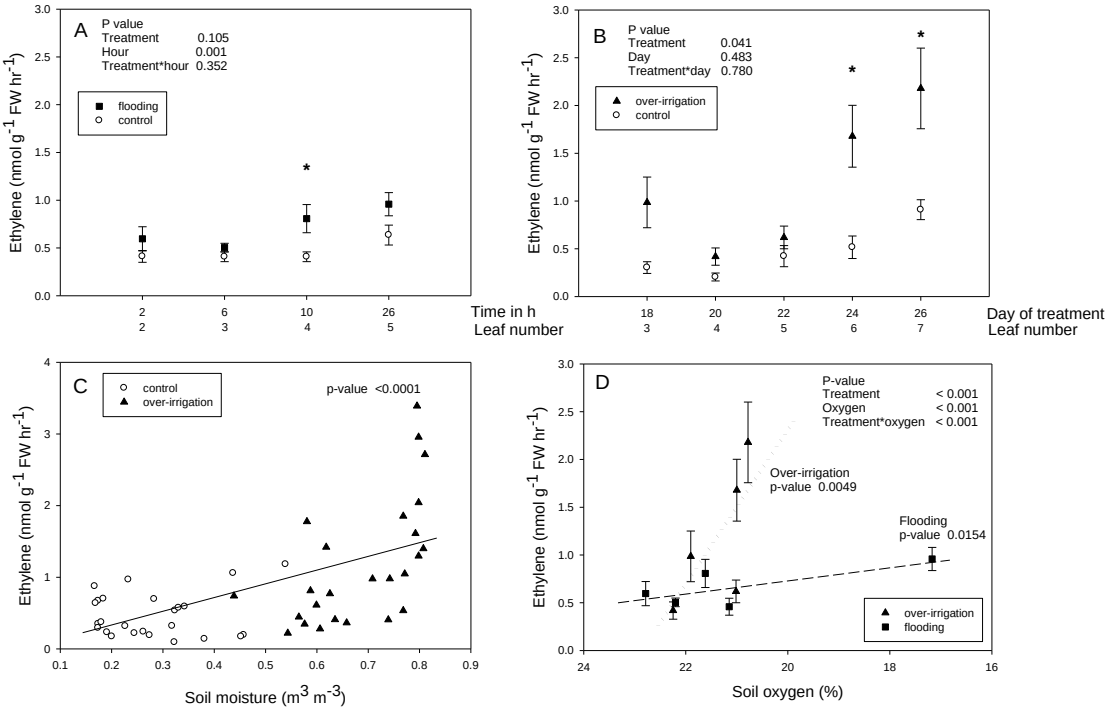

Fig 2.8 (A) Foliar ethylene evolution of flooded (closed square) and well-drained (open circle) tomato plants and (B) over-irrigated (triangle) and well-drained (open circle) tomato plants throughout the experimental period; (C) ethylene plotted against soil moisture for over-irrigated (closed triangle) and well-drained tomato plants, (D) ethylene plotted against soil oxygen for over-irrigated (closed triangle) and flooded (closed square) tomato plants (with linear regressions fitted to each treatment). Data are means ± SE of 5-8 replicates except in (C) where each point is an individual plant, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05, [Fig A/B]). P-values for 2-way ANOVA and linear regressions for flooded (dashed line) and over-irrigated (dotted) plants given [Fig C].

There was no correlation between Ψ_{leaf} and g_s in the flooding experiment (Fig 2.9A), whereas increased Ψ_{leaf} tended to correlate with decreased g_s for over-irrigated plants (P=0.14, Fig 2.9B). Higher photosynthesis rate correlated with increased g_s for both flooding (P=0.0007, Fig 2.9C) and over-irrigation (P=0.038, Fig 2.9D). Higher ABA concentrations were correlated with decreased g_s in the flooded treatment (P=0.0005, Fig 2.9E), but the opposite occurred in over-irrigated plants (P=0.0048, Fig 2.9F). Furthermore, as ethylene levels increased, g_s decreased in flooded plants (Fig 2.9G), but seemingly had no effect on over-irrigated plants (Fig 2.9H). There was no significant relationship between ABA:ethylene ratio and g_s for either treatment (Fig 2.9I and J). In flooded plants, foliar ethylene emission significantly (P=0.013) increased as ABA concentration increased (Fig 2.9K). In the over-irrigation experiment, the highest ethylene emission occurred in plants with the lowest ABA concentration and ethylene emission declined exponentially as ABA concentrations increased (P=0.002, Fig 2.9L). Fig 2.9A-L combine data from three to five different sampling times (day 27 to 29 for Ψ_{leaf}, day 22 to 26 for correlations involving g_s, day 18 to 28 of treatment for foliar ABA concentration and foliar ethylene emission, Table 2.1) within a single representative experiment, therefore providing an integrated assessment of plant responses during the treatment period.

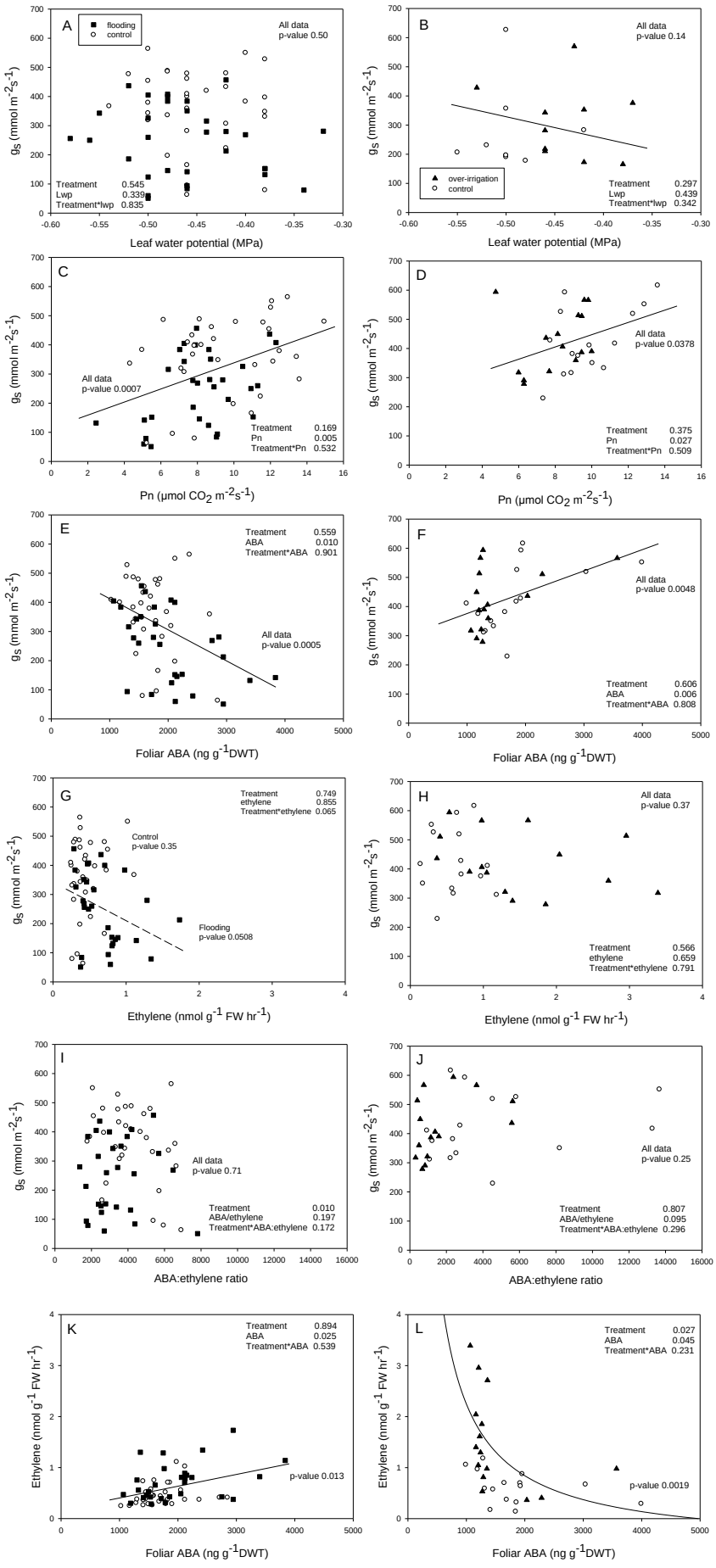
A
flooding
control
All data
p-value 0.50
Treatment 0.545
Lwp 0.339
Treatment*lwp 0.835
gs (mmol m-2s-1)
Leaf water potential (MPa)
B
All data
p-value 0.14
over-irrigation
control
Treatment 0.297
Lwp 0.439
Treatment*lwp 0.342
gs (mmol m-2s-1)
Leaf water potential (MPa)
C
All data
p-value 0.0007
Treatment 0.169
Pn 0.005
Treatment*Pn 0.532
gs (mmol m-2s-1)
Pn (µmol CO2 m-2s-1)
D
All data
p-value 0.0378
Treatment 0.375
Pn 0.027
Treatment*Pn 0.509
gs (mmol m-2s-1)
Pn (µmol CO2 m-2s-1)
E
Treatment 0.559
ABA 0.010
Treatment*ABA 0.901
All data
p-value 0.0005
gs (mmol m-2s-1)
Foliar ABA (ng g-1DWT)
F
All data
p-value 0.0048
Treatment 0.606
ABA 0.006
Treatment*ABA 0.808
gs (mmol m-2s-1)
Foliar ABA (ng g-1DWT)
G
Treatment 0.749
ethylene 0.855
Treatment*ethylene 0.065
Control
p-value 0.35
Flooding
p-value 0.0508
gs (mmol m-2s-1)
Ethylene (nmol g-1 FW hr-1)
H
All data
p-value 0.37
Treatment 0.566
ethylene 0.659
Treatment*ethylene 0.791
gs (mmol m-2s-1)
Ethylene (nmol g-1 FW hr-1)
I
All data
p-value 0.71
Treatment 0.010
ABA/ethylene 0.197
Treatment*ABA:ethylene 0.172
gs (mmol m-2s-1)
ABA:ethylene ratio
J
All data
p-value 0.25
Treatment 0.807
ABA/ethylene 0.095
Treatment*ABA:ethylene 0.296
gs (mmol m-2s-1)
ABA:ethylene ratio
K
Treatment 0.894
ABA 0.025
Treatment*ABA 0.539
p-value 0.013
Ethylene (nmol g-1 FW hr-1)
Foliar ABA (ng g-1DWT)
L
Treatment 0.027
ABA 0.045
Treatment*ABA 0.231
p-value 0.0019
Ethylene (nmol g-1 FW hr-1)
Foliar ABA (ng g-1DWT)

Fig 2.9 Relationships between Ψ_{leaf} and g_s (A, B), Pn and g_s (C, D), foliar ABA concentration and g_s (E, F), foliar ethylene emission and g_s (G, H), ABA:ethylene ratio and g_s (I, J) and foliar ABA concentration and ethylene emission (K, L) for flooded (square) and over-irrigated tomato plants (triangle). In all panels, well-drained controls are hollow circles. P-values for 2-way ANOVA and linear regressions for all data (black line) or flooded plants (dashed) are given.

Shoot fresh weight was not correlated with Ψ_{leaf}, g_s or foliar ABA concentration (Fig 2.10A, B and D), but higher shoot fresh weight tended to correlate with increased Pn and ABA/ethylene ratio (Fig 2.10C and F). The highest ethylene emission occurred in plants with lower shoot fresh weight (Fig 2.10E). All parameters apart from Ψ_{leaf} (measured on the harvest day) were taken one day prior to the harvest.

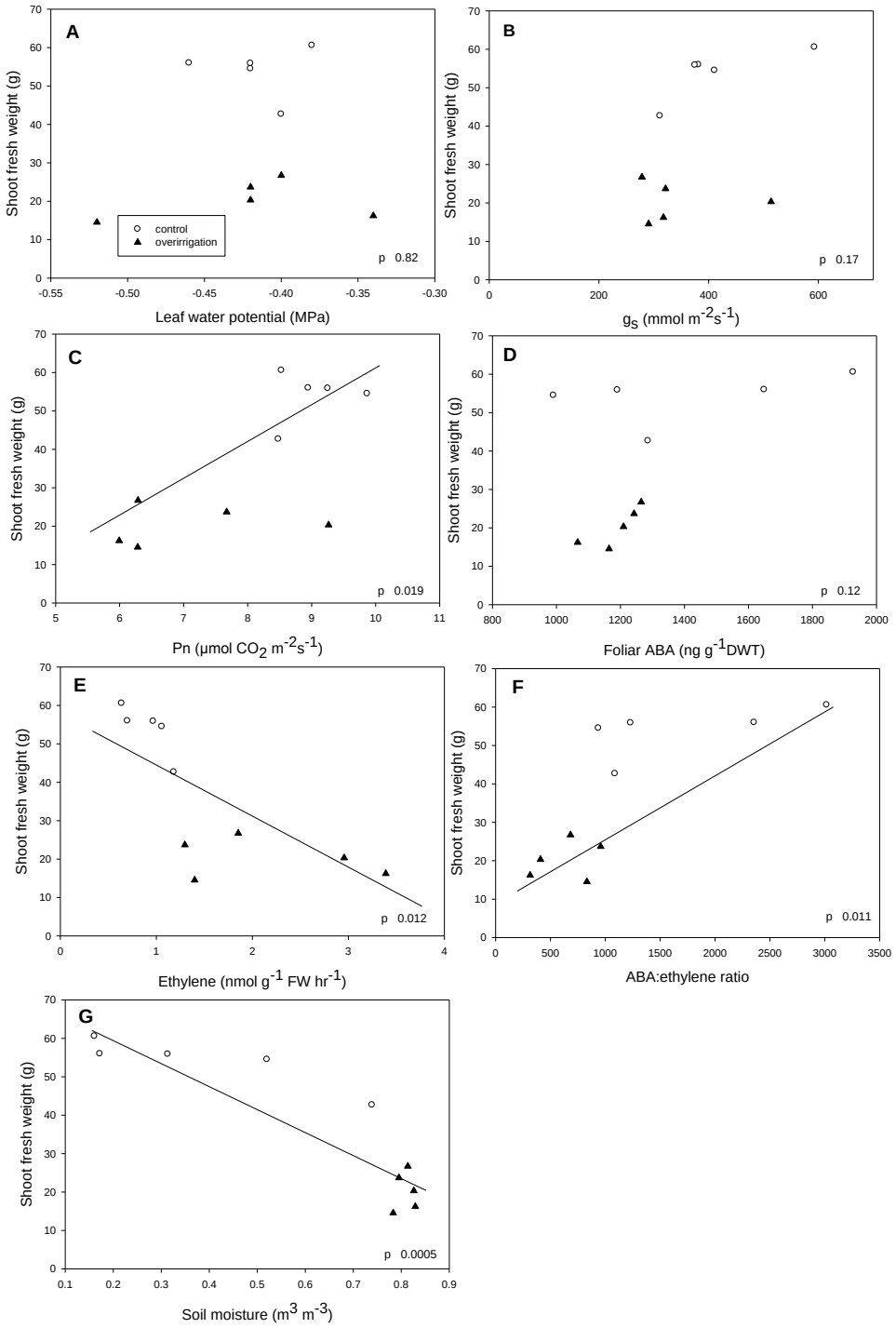

Fig 2.10 Shoot fresh weight plotted against (A) $\Psi_{leaf,}$ (B) g_s, (C) Pn, (D) foliar ABA concentration, (E) ethylene emission, (F) ABA:ethylene ratio and (G) soil moisture for over-irrigated (closed triangle) and well-drained (open circle) tomato plants within a representative experiment. P-values for linear regressions are given.

2.4 Discussion

Over-irrigating tomato plants for 4 weeks significantly decreased shoot growth in comparison to well-drained plants (Table 2.2 and Fig 2.1) and this chapter aims to understand the mechanisms by which shoot growth was limited and whether changes in the soil and physiological responses differ between chronic over-irrigation and acute flooding.

Flooding had a greater effect on soil oxygen concentration than over-irrigation did, decreasing it from 23 % to 17 % (Fig 2.2A). An optimal oxygen level, termed normoxia, typically occurs around 20.6 % oxygen at 1 atm and 20°C, though it was not specified whether this referred to soil or cellular oxygen (Bailey-Serres and Voesenek 2010). Oxygen deficiency, termed hypoxia, occurs when oxygen levels are below the critical oxygen pressure for mitochondrial oxidative phosphorylation for the cell (or organ) and limit ATP production. However, 40 % external oxygen was thought necessary to avoid internal hypoxia in the rapidly respiring maize root tip (Saglio et al. 1984) and even small decreases in external oxygen concentration can slow root elongation without clearly decreasing root respiration. In addition to soil oxygen concentrations, measurements of oxygen flux to an oxygen sink would better estimate the oxygen supply required for root respiration (Blackwell and Wells 1983). Even though it is important to understand the relationship between substrate and cellular oxygen concentrations, to our knowledge these have not been directly compared during waterlogging, possibly due to difficulties in measuring subcellular oxygen concentrations. Different studies on plant adaption to flooding and waterlogging only assume that hypoxic growing substrates decrease cell oxygen concentration, but have not actually measured it (Branco-Price et al. 2005, Jackson

and Armstrong 1999, Liu et al. 2005). Usually, oxygen consumption is only estimated through measuring oxygen partial pressure reduction in a sealed vessel. Because conventional electrodes are too big for oxygen measurements within small plant cells, O_2 microsensors have been suggested (Ober and Sharp 1996). Recently, a needle-borne oxygen probe was inserted into the airspace between the tassel (pollen-producing flower) and the innermost leaf to understand the effect of hypoxia on germ cell fate in maize (Kelliher and Walbot 2012), but most uses of this probe are still limited to studies in animal biology. Nevertheless, the relatively high substrate oxygen (Fig 2.2B) suggests that growth inhibition during over-irrigation is unlikely to be induced by oxygen deficiency.

In addition to oxygen deficiency, root zone temperature changes in the range of 5-10°C can affect plant growth and development. Tomato shoot dry weight decreased by 22 % when root zone temperature changed from 30 to 35°C and 56 % when temperature changed from 30 to 24°C (Jaworski and Valli 1964). In this work (Fig 2.2C and D), soil temperature ranges did not vary between over-irrigated and flooded treatment and thus cannot explain physiological differences. Furthermore, the 1°C root zone temperature difference between over-irrigation and well-drained plants is unlikely to explain the 60 % reduction in shoot fresh weight of over-irrigated plants.

Due to the infrequent irrigation at the beginning of the experiment (Fig 2.2E), soil moisture for the over-irrigated treatment did not increase linearly, but slowly accumulated over time. Hence, it took 14 days of treatment for soil moisture to differ significantly from well-drained soil and physiological measurements (such as ABA) were taken from this point. Other parameters, such as Ψ_{leaf} and growth measurements were only taken at the end of the treatment period.

Flooding decreased Ψ_{leaf} during the first photoperiod, and then increased it during the second photoperiod (Else et al. 1995a; Fig 2.4A here), whereas Ψ_{leaf} did not differ between over-irrigated and well-drained plants (Fig 2.4B). Consequently, flooded and over-irrigated plants showed differing relationships between Ψ_{leaf} and soil oxygen (Fig 2.4C), likely since flooding increased Ψ_{leaf} during the second photoperiod due to stomatal closure (Fig 2.4A, 2.5A). In over-irrigated plants, Ψ_{leaf} did not correlate with shoot fresh weight (Fig 2.10A), suggesting that growth inhibition was not hydraulically regulated.

Photosynthesis supplies carbon, which is important for synthesis and mobilization of proteins (Paul and Pellny 2003) and 'acute' and 'acclimatory' responses to changes in carbon supply are important for sustaining growth during stress conditions (Smith and Stitt 2007). In contrast to the decreased g_s following flooding (Bradford and Hsiao 1982, Fig 2.5A here), over-irrigation did not alter stomatal conductance (Fig 2.5B) even though Pn was reduced for both flooded and over-irrigated plants (Fig 2.6A and B). This suggests that changes in photosynthesis of over-irrigated plants were not due to stomatal limitation, but could rather be attributed to reduced mesophyll activity (Ciompi et al. 1996). Higher shoot fresh weight tended to correlate with increased Pn one day before the harvest (Fig 2.10C). In monocotyledons, carbohydrates accumulate in the growing zone when environmental stresses (e.g. drought, salinity) inhibit leaf elongation, suggesting that carbon utilisation and not its availability limits growth (Pheloung and Barlow 1981). Whether this applies to dicotyledons (where growing cells are photosynthetic) has not been studied yet and there is limited knowledge about the levels of carbon availability (and their changes) that affect leaf expansion, but results here suggest that decreased Pn could limit plant growth during over-irrigation.

Plant hormones such as ABA, ethylene and its precursor 1-aminocyclopropane-l-carboxylic acid (ACC) are related to environmental stress and might also regulate physiological responses to flooding and over-irrigation. Although flooding increased foliar ABA concentrations (Fig 2.7A), foliar ABA concentrations of over-irrigated plants varied throughout the experiment with no systematic pattern compared to control plants and foliar ABA accumulation generally did not occur in over-irrigated plants (Fig 2.7B). Furthermore, foliar ABA concentrations increased only in flooded plants when Ψ_{leaf} was higher than in control plants (Fig 2.4A, 2.7A), indicating that leaf water deficits were not the cause of ABA accumulation. Molecular oxygen and NADPH are needed for ABA hydroxylation (Xiong and Zhu 2003), though hypoxic conditions in roots did probably not occur in the over-irrigated treatment. Higher ABA concentrations were correlated with stomatal closure in the flooded treatment (Fig 2.9E), but an opposing trend was found in over-irrigated plants (Fig 2.9F).

In addition, foliar ABA might only have a limited role in regulating growth of over-irrigated plants, as foliar ABA concentrations were not correlated with shoot fresh weight in this set of experiments (Fig 2.10D). Under non-stressed conditions, higher concentrations of exogenous ABA can hinder plant growth (Tardieu et al. 2010), but ABA can maintain growth by limiting production of stress-induced ethylene (Sharp 2002) and environmental stresses usually inhibit growth of ABA-biosynthesis-defective mutants more than wild type plants (Xiong et al. 2002). Nevertheless, ABA does not necessarily regulate leaf growth under environmental stresses, since Arabidopsis ABA-deficient or ABA-insensitive mutants show a similar leaf growth inhibition as wild type plants under salinity or different nitrogen levels (Cramer 2002, Dodd 2003b), and other hormones, such as ethylene, could be of importance in regulating plant responses to over-irrigation.

Whole maize seedlings increased ethylene biosynthesis within 4 h of hypoxic conditions (He et al. 1996) and oxygen deprivation of roots increased ethylene emission from tomato shoots (Bradford and Dilley 1978). Even though ethylene emission was always higher in over-irrigated and flooded plants when compared to the well-drained treatment (Fig 2.8A-B), ethylene emission increased more sensitively in response to decreased soil oxygen in over-irrigated plants (Fig 2.8D). While the reasons for this more sensitive response are not yet clear, apparent impacts of ethylene accumulation on g_s varied between treatments, as ethylene emission was correlated with stomatal closure in flooded plants (Fig 2.9G), but not in over-irrigated plants (Fig 2.9H). Ethylene can induce (Desikan et al. 2006 – 99.9 % pure ethylene gas) or inhibit stomatal closure in well-drained Arabidopsis (Tanaka et al. 2005 – 100 µl l^{-1} ethylene gas), suggesting that other compounds apart from ethylene are involved in stomatal regulation. Possibly, ethylene does not have a role in stomatal regulation at the concentrations existing endogenously, or acts via interactions with other hormones.

Plant growth and development is usually regulated by more than one hormone and responses to environmental conditions usually arise due to the combined interplay or ratio of several hormones (Wang and Irving 2011). Particular focus has been paid to ABA:ethylene ratio, which when higher was correlated with greater grain-filling (Yang et al. 2007, Zhao et al 2007). Here, the ratio of ABA to ethylene was not correlated with g_s in either flooded or over-irrigated plants (Fig 2.9I, J), but with shoot fresh weight (Fig 2.10F). Further evidence is needed that ethylene or its ratio to ABA is involved in modulating stomatal or growth responses to either flooding or over-irrigation.

ABA can inhibit the synthesis (Sharp 2002, Spollen et al. 2000) or the signalling pathway of ethylene and vice versa (Ghassemian et al. 2000). Conversely, ethylene has been reported to regulate cellular sensitivity to ABA (Wilkinson and Davis 2010) and stress-ethylene-induced reductions in ABA accumulation could decrease stress sensitivity, as ethylene accumulation can antagonize effects of drought and ABA on gas exchange and leaf growth (Tanaka et al. 2005, Wilkinson and Davies 2010). In the experiments presented here, increased ABA was correlated with increased ethylene for flooded plants (Fig 2.9K), but increased ABA was correlated with decreased ethylene in the over-irrigated treatment (Fig 2.9L). The latter results agree with other studies suggesting that higher ABA levels are necessary to prevent ethylene accumulation due to environmental stresses such as water deficit and ABA could therefore maintain shoot growth instead of hindering it (Sharp and LeNoble 2002).

Ethylene seems to promote growth during early stages of seedling development, but later inhibits growth (Sharp and LeNoble 2002, Fig 2.10E here). It also constrains cell division, mitosis, DNA synthesis and growth of root and shoot meristems (Burg 1973). Oxygen deprivation of roots increased ethylene emission from tomato shoots (Bradford and Dilley 1978), suggesting that shoot ethylene production can be regulated by root-to-shoot signalling of ACC. Xylem ACC delivery of tomato plants increased after 24 h of flooding to levels sufficient to produce extra ethylene in shoots (Else and Jackson 1998). Whether increases in ACC delivery and foliar ethylene production limit growth of over-irrigated plants should be tested by comparing the growth responses of ethylene-insensitive and wild-type genotypes to over-irrigation (Chapter 3) and analysing root-to-shoot ACC signalling during over-irrigation (Chapter 4).

2.5 Conclusion

By automatically scheduling irrigation according to soil moisture thresholds, effects of chronic over-irrigation and acute flooding on leaf growth, gas exchange and phytohormone (ABA, ethylene) relations of tomato (*Solanum lycopersicum* Mill. cv. Ailsa Craig) were studied. Flooding induced more pronounced changes in soil oxygen concentration (23 % to 17 % within 26 hours) than over-irrigation (23 % to 19 % over 4 weeks). Flooding also induced stomatal closure (g_s), increased leaf water potential (Ψ_{leaf}) and increased foliar abscisic acid (ABA) concentrations and ethylene evolution. Although over-irrigation significantly reduced shoot growth, this was apparently not due to altered leaf water status or a stomatal limitation of photosynthesis, as neither g_s or Ψ_{leaf} differed from control plants. Over-irrigation did not alter bulk leaf abscisic acid (ABA) concentration, but increased foliar ethylene evolution, provoking the question of whether ethylene is the principal growth regulator during over-irrigation.

Chapter 3.

3 Partial ethylene-insensitivity reverses over-irrigation-induced growth inhibition to some extent

Fig 3.1 Experimental set-up

3.1 Introduction

The gaseous plant hormone ethylene influences many different plant growth processes, but also regulates responses to environmental stimuli, for example pathogen attack, wounding or waterlogging (Abeles et al. 1992). Numerous studies have reported that waterlogging leads to entrapment and accumulation of ethylene in the plant (Bradford and Yang 1980, Else et al. 1995b, English et al. 1995). The ethylene precursor ACC can also be accumulated in roots (Singh 1998), as lack of oxygen prevents the conversion of ACC to ethylene. Ethylene also inhibits longitudinal extension through promoting longitudinal deposition of cellulose microfibrils, which exist at the inner surface of primary cell walls and increase cell strength (Lang et al. 1982).

Ethylene regulates plant growth by alterations in its biosynthesis and changes in intracellular signalling which involves proteins called receptors (Klee 2002, Wilkinson et al. 1995). Ethylene receptors can bind ethylene and are 2-component regulators, consisting of the sensor and the response regulator (Stock et al. 2000). Ethylene receptor genes in tomato consist of six members (LeETR1-6) (Lashbrook et al. 1998, Tieman and Klee 1999, Wilkinson et al. 1995, Zhou et al. 1996). LeETR1 and LeETR2 are expressed at constant levels in all tissues throughout development; however, LeETR1 is expressed at higher levels (Lashbrook et al. 1998). Concentrations of LeETR3 (historically NR), LeETR4, LeETR5 and LeETR6 are highly regulated depending on environmental and developmental stimuli. For example, LeETR4, LeETR5 and LeETR6 are highly expressed in flowers and fruits, but less abundant in vegetative tissues (Tieman et al. 2000). LeETR3 is highly expressed during flower anthesis and fruit ripening, but not at stages in between

(Wilkinson et al. 1995). During shoot elongation and later plant growth (until 5-leaf stage), LeETR3 is expressed at levels similar to LeETR1 and 2 (Lashbrook et al. 1998).

Several mutations in tomato are readily available to test different hypotheses concerning the physiological importance of ethylene signalling and sensing. The *Never ripe* (*Nr*) genotype was first described in 1956 by Rick and Butler, with fruits showing impaired colour change and softening. Possibly homologous to ETR1 in *Arabidopsis thaliana*, *Nr* is a naturally occurring mutation in the ethylene-binding domain of the NR receptor in tomato (Wilkinson et al. 1995) and therefore cannot bind ethylene. Analysis showed that *Nr* is ethylene-insensitive at all stages of development (Lanahan et al. 1994), is defective in floral abscission and leaf and flower petal senescence (Klee 2002), but does not show developmental and morphological abnormalities (apart from delayed fruit ripening).

So far, *Nr* has mainly been used as a tool to assess the role of ethylene in a range of developmental and gene expression processes such as differential expression of ethylene receptor genes in tomato fruit during development and ripening (Nakatsuka et al. 1998, Rose et al. 1997), but only a few studies have determined the influence of environmental stresses on *Nr*. Ciardi et al. (2000) report that *Nr* shows increased tolerance to the virulent strain of *Xanthomonas campestris* pv. *vesicatoria* in tomato, but not the avirulent strain and conclude that reduced ethylene sensitivity may limit the spread of necrosis. Similarly, *Nr* showed significantly reduced disease symptoms after inoculation with *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* as well as *Fusarium oxsporum* f sp *lycopersici* (Lund et al. 1998). In another study, Firon et al. (2012) show that *Nr* pollen grains are more sensitive to heat-stress

(32/26°C day/night) than wild-type (WT) grains, and suggest that ethylene plays a key role in tomato pollen thermo-tolerance. Sodium and cadmium stresses (100 mM NaCl and 0.5 mM $CdCl_2$) reduced leaf chlorophyll content and increased H_2O_2 production in *Nr* fruits compared to the wild type, suggesting that ethylene signalling of the *Nr* receptor can influence biochemical pathways of oxidative stress (Monteiro et al. 2011). Until now, *Nr* growth analysis and studies examining the effect of over-irrigation on growth and plant hormone concentrations of *Nr* and its WT do not exist.

In Chapter 2, over-irrigation severely decreased vegetative growth of tomato when compared to the well-drained treatment (Table 2.2). Furthermore, flooding and over-irrigation increased foliar ethylene emission (Fig 2.8A and B) and lower fresh weight was correlated with higher ethylene emission (Fig 2.10E), suggesting that ethylene could play a key role in regulating physiological responses in tomato plants. Thus, the effects of ethylene during over-irrigation were studied by comparing the growth and physiological responses of the *Never ripe* (*Nr*) genotype and wild-type plants, to determine if partial ethylene-insensitivity could ameliorate the growth inhibition caused by over-irrigation.

3.2 Materials and Methods

For details of irrigation treatments and physiological measurements, see Chapter 2

3.2.1 Ethylene insensitivity test

For this set of experiments, the *Never ripe* (*Nr*) genotype in the 'Ailsa Craig' background was used. To verify relative ethylene-sensitivity of *Nr* and wild type plants, seeds from both genotypes were germinated on a 20 µM ACC solution in a petri dish in the dark and germination rate was compared (*Nr* 100 %, WT 45 %; Fig 3.1). Furthermore, the "triple response" was seen in the wild type, but *Nr* showed normal hypocotyls extension.

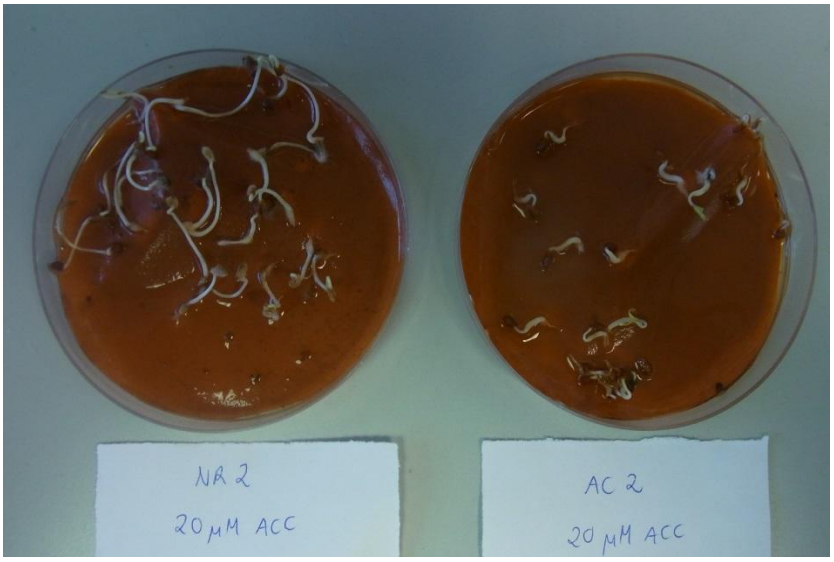

Fig 3.2 Germination of ethylene insensitive genotype (*Nr*, left) and wild type (AC, right) after 14 days on 20 µM ACC

3.2.2 Statistical analysis

For each sampling day, treatment differences were determined via an Independent Samples T-test (SPSS 19, IBM). Furthermore, a univariate ANOVA or ANCOVA was performed to test both the individual effects and any interactions. Linear regressions

were fitted to data from both treatments. The data presented is a compilation of two separate experiments that were conducted in the same controlled environment room, and comparison with the wild type was performed with data from Chapter 2 (plants grown for the same treatment duration). As both genotypes could not be grown together at the same time (due to difficulty of scheduling irrigation independently), interpretations of the results where *Nr* and WT (data from Chapter 2) are compared requires some caution.

3.3 Results

Over-irrigating the ethylene-insensitive *Nr* genotype significantly ($P < 0.05$) reduced shoot fresh weight and total leaf area by 21.4 % and 21.5 % when compared to the well-drained control (Table 3.1). Height and Ψ_{leaf} did not change significantly in over-irrigated *Nr* plants (Table 3.1). Furthermore, shoot fresh weight, total leaf area and Ψ_{leaf} varied significantly between genotypes and significant interactions between treatment and genotype suggest that the genotype influences the effect of the treatment on shoot fresh weight, height and total leaf area (Table 3.2).

Table 3.1 Shoot fresh weight, height, total leaf area and Ψ_{leaf} for over-irrigated and well-drained (control) *Nr* and WT (Chapter 2) tomato plants. Data are means ± SE of 9 replicates. Different letters indicate significant differences (Independent-Samples T-Test, p-value < 0.05).

	Over-irrigation	**Control**	**% of control plants**	
			Nr	**WT**
Shoot fresh weight (g)	22.6 ± 1.7[a]	28.8 ± 1.2[b]	78.6	37.6
Height (cm)	18.0 ± 0.7[a]	17.7 ± 1.1[a]	101.9	73.4
Total leaf area (cm^2)	397 ± 28[a]	506 ± 31[b]	78.5	30.0
Ψ_{leaf} (MPa)	-0.48 ± 0.01[a]	-0.51 ± 0.02[a]	95.6	98.8-102.4

Table 3.2 P-values for 2-way ANOVA comparing significant interactions between treatment, genotype (WT from Chapter 2) and treatment*genotype interaction.

	Shoot fresh weight	**Height**	**Total leaf area**	**Ψ_{leaf}**
Treatment	<0.001	0.012	<0.001	0.602
Genotype	<0.001	0.180	0.005	<0.001
T*G	<0.001	0.006	<0.001	0.454

Plotting well-drained shoot fresh weight against over-irrigated shoot fresh weight for wild type and *Nr* plants grown for different lengths of time indicates that over-irrigation-induced growth suppression increases with length of treatment in wild type plants (Fig 3.3). Furthermore, shoot fresh weight of well-drained *Nr* plants is smaller than that of wild type well-drained plants grown for the same length of time. However, over-irrigated *Nr* plants did not show such a dramatic growth inhibition as comparable over-irrigated WT plants (Table 3.1), as *Nr* plants were closer aligned to the 1:1 line (Fig 3.3), suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to a degree.

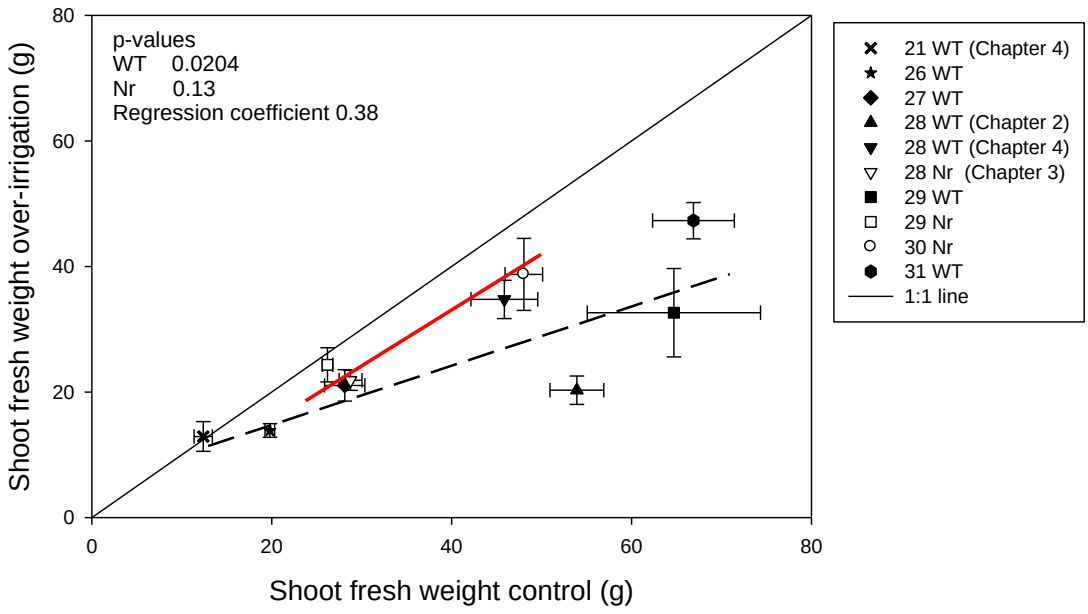

Fig 3.3 Well-drained (control) shoot fresh weight plotted against over-irrigated shoot fresh weight of WT (closed symbols) and *Nr* (open symbols) tomato plants. Data are means ± SE of 5-10 replicates. Different symbols indicate length of treatment period and genotype. P-values and regression lines (red solid line – *Nr*, dashed black line – WT) given.

Over-irrigation did not have an effect on stomatal conductance (g_s) of *Nr* plants on all days measured when compared to well-drained plants (Fig 3.4A). Similarly, photosynthesis rate (Pn) did not significantly differ between treatments in *Nr* (Fig 3.4B). Foliar ABA concentration tended to increase in over-irrigated *Nr* plants from Day 20 of the treatment on and was significantly higher on Day 24 when compared to the well-drained control (Fig 3.4C). Foliar ethylene emission varied throughout the treatment period and was only significantly elevated in over-irrigated *Nr* plants on Day 24 (Fig 3.4D).

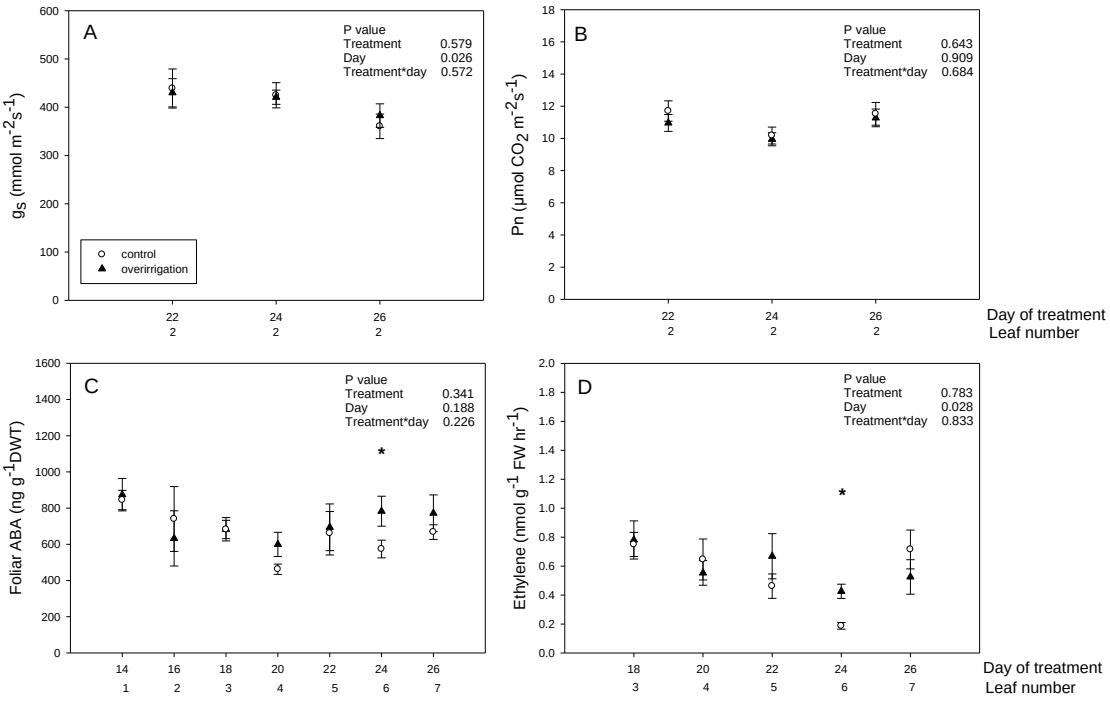

Fig 3.4 (A) Stomatal conductance (g_s), (B) photosynthesis rate (Pn), (C) foliar abscisic acid (ABA) concentration and (D) foliar ethylene evolution of over-irrigated (closed triangle) and well-drained (open circle) *Nr* tomato plants throughout the experimental period. Data are means ± SE of 9 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, P-value < 0.05), P-values for 2-way ANOVA given.

Because no significant interactions between treatment and day of sampling were found for all variables, data from sampling times were combined and compared to the wild type (Fig 3.5A-D). There was no significant difference in g_s between treatments and genotypes (Fig 3.5A). Over-irrigation significantly decreased Pn in over-irrigated wild type plants, but had no effect in *Nr* (Fig 3.5B). Furthermore, foliar ABA concentrations were significantly decreased in the *Nr* genotype, independently of irrigation treatment, though this effect could have been due to calibration, as samples from Chapter 2 and Chapter 3 were not run together on the same analysis batch (Fig 3.5C). Over-irrigating the wild type significantly increased foliar ethylene emission when compared to well-drained wild type plants and both over-irrigated and well-drained *Nr* plants (Fig 3.5D).

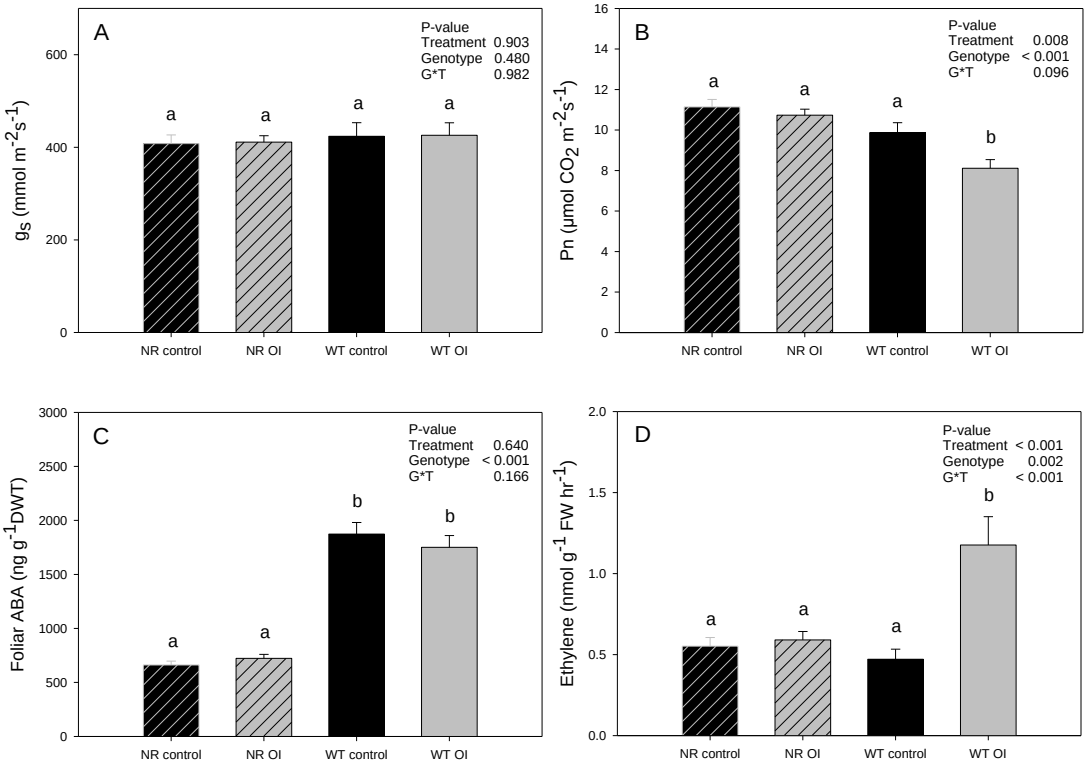

Fig 3.5 (A) Stomatal conductance (g_s), (B) photosynthesis rate (Pn), (C) foliar abscisic acid (ABA) and (D) foliar ethylene evolution for over-irrigated (OI, grey bar) and well-drained (control, black bar) wild type (WT – Chapter 2, grown for same length of treatment period) and *Nr* (coarse bar) tomato plants. Data are means ± SE of 27-61 replicates, different letters indicate significant differences between treatments (ANOVA, P-value < 0.05).

Pn was correlated with g_s independently of genotype (P=0.009) (Table 3.3), even though the effect of Pn on g_s was not as apparent in *Nr* (P=0.18) as in the wild type (P=0.0378). Furthermore, foliar ABA concentration was significantly correlated with g_s (P=0.027), though the relationship varied according to genotype, as increasing ABA concentrations were correlated with increased g_s in the wild type (P=0.0048), but decreased g_s in *Nr* (P<0.0001). Ethylene did not have a significant effect on g_s in

either genotype (P=0.86 and 0.37 for *Nr* and WT, respectively). In addition, the relationship between ABA and ethylene varied according to genotype, where higher ABA concentrations were associated with decreased ethylene evolution in the wild type (P=0.0019), but not in *Nr* (P=0.20).

Table 3.3 P-values for 2-way-ANCOVA for interactions between parameters for wild type (WT – Chapter 2) and *Nr* tomato plants throughout the experimental period, along with P-values and regressions.

	Pn - g_s	**ABA - g_s**	**Ethylene -g_s**	**ABA - ethylene**
***Nr* correlation**[1]	0.18	<0.0001	0.86	0.20
WT correlation[2]	0.0378	< 0.0048	0.37	0.0019
Parameter[3]	0.009	0.027	0.513	0.405
Genotype[3]	0.507	< 0.001	0.431	< 0.001
parameter*G[3]	0.288	< 0.001	0.149	0.027

[1] regression line for *Nr* data (for example regression line for Pn vs g_s)

[2] regression line for WT data (same as above)

[3] 2way ANCOVA for *Nr* and WT data (for example effect of Pn, genotype and Pn*genotype on g_s)

Shoot fresh weight was not correlated with Ψ_{leaf}, g_s, and Pn, but increased foliar ABA concentrations correlated with decreased shoot fresh weight in *Nr* (P=0.0284, Table 3.4). Furthermore, higher soil moisture correlated with lower shoot fresh weight (P=0.0305). Genotypic differences were detected for the correlation of shoot fresh weight with Pn, ABA, ethylene and soil moisture.

Table 3.4 Correlations of different parameters with shoot fresh weight for *Nr* and WT (Chapter 2) tomato plants.

	Ψ_{leaf}	g_s	Pn	ABA	Ethylene	Soil moisture
***Nr* correlation**[1]	0.32	0.996	0.68	0.0284	0.59	0.0305
WT correlation[2]	0.82	0.17	0.019	0.12	0.012	0.0005
Parameter[3]	0.988	0.109	< 0.001	0.198	0.046	< 0.001
Genotype[3]	0.430	0.302	0.010	0.036	< 0.001	0.002
parameter*G[3]	0.570	0.110	0.001	0.012	0.013	< 0.001

[1] regression line for *Nr* data (for example regression line for Pn vs shoot fresh weight)

[2] regression line for WT data (same as above)

[3] 2way ANCOVA for *Nr* and WT data (for example effect of Pn, genotype and Pn*genotype on shoot fresh weight)

Highest shoot fresh weight was correlated with low ethylene concentrations in the wild type (P=0.0119, Fig 3.6), but no correlation was detected in the *Nr* genotype (P=0.59) and genotype affected the relationship between shoot fresh weight and ethylene (significant genotype x ethylene interaction, P=0.013).

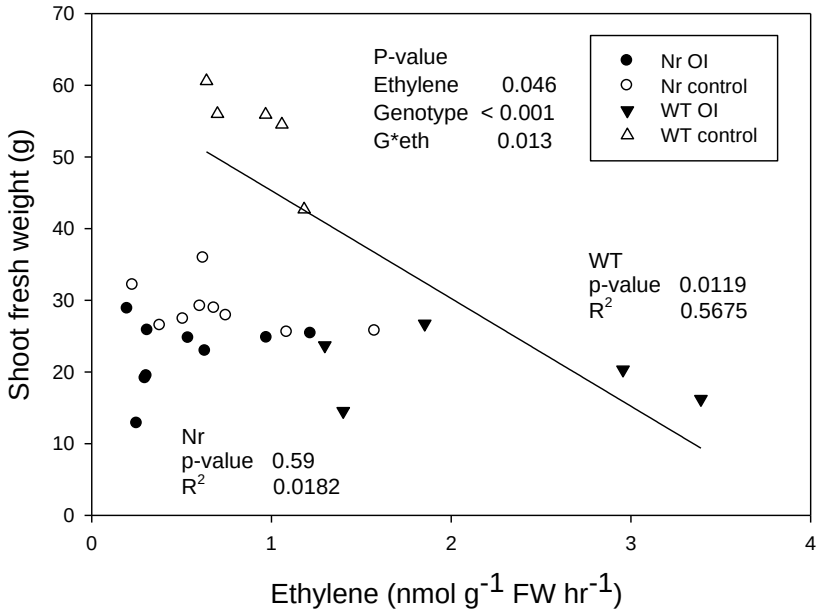

Fig 3.6 Foliar ethylene plotted against shoot fresh weight of over-irrigated (OI) and well-drained wild type (WT – Chapter 2) and *Nr* tomato plants. P-values for 2-way ANOVA and linear regression for WT (black line) given.

3.4 Discussion

To test whether the *Nr* mutation was leaky in the Ailsa Craig background (possibly due to leaky *LeEIL* genes, Lanahan et al. 1994), a germination test was performed. In contrast to wild type seeds, germination of *Nr* in the dark on 20 μM ACC was not inhibited (Fig 3.2), verifying relative ethylene-insensitivity. Similarly, germination on a 20 μM ACC solution did not induce the triple response (inhibition of stem elongation, radial swelling of the stem, and absence of normal geotropic response) in etiolated *Nr* hypocotyls (Lanahan et al. 1994).

Over-irrigation significantly reduced shoot fresh weight and total leaf area of *Nr* plants (Table 3.1), though not as drastically as in the wild type (Fig 2.1, Fig 3.3, Table 2.1 and Table 3.1), as indicated by significant treatment x genotype interactions affecting shoot fresh weight and total leaf area (Table 3.2). To our knowledge, this is the first study reporting decreased shoot growth under non-stressed conditions in *Nr* compared to its wild type (Fig 3.3). Under optimal conditions, ethylene insensitivity did not alter total leaf area of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Petunia x hybrida* compared to their wild type equivalents (Tholen et al. 2004). Cultivating *Nr* and the wild type (Micro-Tom) on either 100 mM NaCl or 0.5 mM $CdCl_2$ resulted in similar growth reductions in both genotypes (Monteiro et al. 2011) and phosphorus deficiency reduced growth at similar rates in both wild type (Pearson) and *Nr* tomato plants (Kim et al. 2008). However, the *Nr* data presented here is a compilation of two separate experiments (grown for the same length of time). Furthermore, *Nr* and wild type plants were not grown together at the same time, due to difficulty with scheduling the automatic irrigation according to possible differences in plant water

requirements. Therefore it is difficult to reconcile whether differences in growth between *Nr* and wild type are due to genotype-related growth inhibition.

Ethylene can play an important role in regulating physiological responses, such as Ψ_{leaf} and gas exchange, to environmental stress conditions. Decreases of leaf water potentials from 20 to 50 % caused by diurnal changes over 24 h were accompanied by a 7-fold increase in ethylene emission from leaves of *Vicia faba* (El-Beltagy and Hall 1974) and endogenous ethylene production significantly increased when Ψ_{leaf} decreased outside of the normal diurnal Ψ_{leaf} range in excised wheat leaves (Wright 1977). These results suggest that ethylene may affect plant water relations, but to our knowledge, studies of Ψ_{leaf} in the ethylene-insensitive genotype *Nr* under stress conditions do not exist. Similar to the wild type, over-irrigating *Nr* did not alter Ψ_{leaf} when compared to well-drained plants (Table 3.1) and shoot fresh weight was not correlated with Ψ_{leaf} in *Nr* or the wild type (P=0.32 and P=0.82 for *Nr* and WT, respectively, Table 3.4), suggesting that ethylene-insensitivy does not play a role in regulating Ψ_{leaf} during over-irrgation and that over-irrigation-induced growth inhibition is not hydraulically regulated. Even though treatment did not have a significant effect on leaf water potential (Table 3.2), Ψ_{leaf} was lower in *Nr*, which could be due to genotypic effects or a developmental artefact, as *Nr* plants were smaller than wild type plants grown for the same period of time (Fig 3.3).

Ethylene can induce (Desikan et al. 2006 – 99.9 % pure ethylene gas) or inhibit stomatal closure in well-drained wild type Arabidopsis (Tanaka et al. 2005 – 100 µl l^{-1} ethylene gas), but was not responsible for partial stomatal closure in flooded tomato plants (Bradford 1983a). The role of ethylene-insensitivity on g_s seems inconsistent, as g_s increased in an ethylene-insensitive *Petunia* genotype compard to its wild type,

but reduced by 40 % in ethylene-insensitive *Arabidopsis* (Tholen 2005). No genotypic differences in g_s were detected here (Fig 3.5A). Similar to the wild type, there was no significant effect of over-irrigation on g_s of *Nr* plants on any day measured (Fig 3.4A) and no significant differences in g_s between treatments or genotypeswas detected (Fig 3.5A). Furthermore, g_s was not correlated with shoot fresh weight in either genotype (P=0.996 and P=0.17 for *Nr* and WT, respectively, Table 3.4), supporting previous arguments (Chapter 2) that stomatal limitation of photosynthess is not the key cause of growth inhibition during over-irrigation. Furthermore, g_s was not correlated with foliar ethylene evolution in either genotype (P=0.86 and P=0.37 for *Nr* and WT, respectively, Table 3.3), suggesting that endogenous ethylene concentrations do not have a role in stomatal regulation.

Leaf photosynthesis per unit area decreased slightly (and nonsignificantly) in ethylene insensitive tobacco plants compared to the wild type (Tholen et al. 2007), consistent with the similar Pn of WT and *Nr* plants (Fig 3.5B). Pn did not change in response to over-irrigating *Nr* plants (Fig 3.4B) and did not correlate with shoot fresh weight (P=0.68, Table 3.4). Despite maintenance of Pn by over-irrigated *Nr* plants, shoot growth was still inhibited to a degree, suggesting that other factors, such as plant hormones, might need more consideration.

Foliar ABA concentrations in *Nr* decreased by 44 to 70 % compared to wild type tomato plants independently of soil moisture (Fig 3.5C), in contrast to other studies where foliar ABA evolution increased by 47 % in the ethylene-insensitive *etr1-1* Arabidopsis genotype compared to its wild type (LeNoble et al. 2004). These genotypic differences could be due to calibration effects, as samples from Chapter 2

and Chapter 3 were not run together on the same analysis batch and calibration curves differed according to genotype (Fig 3.7).

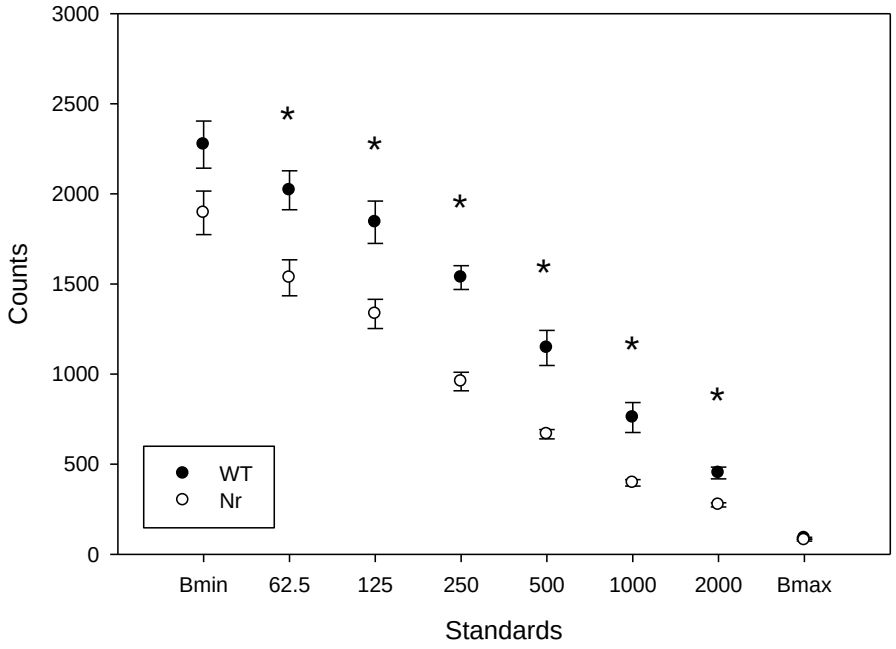

Fig 3.7 ABA calibration curves for wild type (closed circle) and *Nr* (open circle) tomato plants. Data are means ± SE of 5-13 assays. Asterisks indicate significant differences between genotypes (Independent-Samples T-Test, p-value < 0.05).

Despite increases in foliar ABA concentration of over-irrigated *Nr* plants (compared to the well-drained treatment) from Day 20 of the treatment on (Fig 3.4C), ABA accumulation over time did not significantly vary between treatments in *Nr* (Fig 3.5C). Shoot fresh weight decreased with increasing foliar ABA concentrations in the *Nr* genotype (P=0.0284, Table 3.4), though this effect was not seen in the wild type (P=0.12).

Furthermore, increasing foliar ABA concentrations significantly increased g_s in the wild type (P=0.0048), whereas increasing ABA significantly decreased g_s in *Nr* plants (P<0.0001, Table 3.3.). In addition, the relationship between ABA and ethylene varied according to genotype, where higher ABA concentrations were correlated with decreased foliar ethylene evolution in the wild type (P=0.0019), but did not have a significant effect in *Nr* (P=0.20, Table 3.3). Due to the variable relationships with shoot fresh weight, g_s and ethylene, foliar ABA concentrations might not be biologically significant in understanding the mechanisms behind over-irrigation-induced growth inhibition.

Foliar ethylene emission significantly increased in over-irrigated wild type plants (Fig 2.8B), but apart from Day 24, there was no significant difference in ethylene emission between the treatments in the ethylene-insensitive genotype (Fig 3.4D and 3.5D). Possibly, fully functional ethylene sensing is needed for auto-catalytic ethylene production (Nakano et al. 2002, Yokotani et al. 2009). Furthermore, ethylene emission rates of well-drained *Nr* plants were similar to that of the wild type (Fig 3.5D), consistent with previous studies where the ethylene-insensitive *Arabidopsis* genotype *etr1-1* was used (Bleecker et al. 1988, LeNoble et al. 2004). Because the *Nr* gene expression in tomato is developmentally regulated, even if one receptor is non-functional, the other receptors can compensate (Tieman et al. 2000).

In contrast to the wild type, there was no correlation between shoot fresh weight and foliar ethylene evolution in *Nr* plants (P=0.59, Table 3.3 and Fig 3.6), an expected result as ethylene cannot be perceived by *Nr* plants. However, not only leaf hormone concentrations, but also levels of plant hormones in the xylem sap should be considered and analysing long-distance communication via root-to-shoot signalling

might help to understand the effect of above mentioned influences on plant growth and responses during over-irrigation.

3.5 Conclusion

The partially ethylene-insensitive tomato genotype *Nr* was used to examine whether effects of over-irrigation on plant physiology and hormonal balance could be attributed to ethylene signalling. Four weeks of over-irrigation significantly reduced shoot fresh weight and total leaf area in contrast to well-drained *Nr* plants. However, over-irrigating the partial ethylene-insensitivite genotype *Nr* did not lead to such a dramatic growth inhibition as in the wild type, suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to a degree. Similar to the wild type, Ψ_{leaf} and g_s did not significantly differ between the two treatments and both parameters did not correlate with shoot fresh weight, fortifying the argument that over-irrigation-induced growth inhibition is not hydraulically regulated or due to stomatal limitation. Furthermore, Pn did not change in response to over-irrigation in *Nr*, even though over-irrigating the wild type significantly decreased Pn. These results suggest that maintenance of leaf photosynthesis cannot prevent the over-irrigated phenotype. Foliar ABA concentrations did not differ between treatments in *Nr* plants and relationships between ABA and shoot fresh weight, g_s and ethylene varied between genotypes, suggesting that foliar ABA concentration might not be informative in understanding the mechanisms behind over-irrigation-induced growth inhibition. In contrast to the wild type, foliar ethylene emission did not sigificantly differ between treatments and shoot fresh weight did not correlate with foliar ethylene emission in *Nr*. To better understand the involvement of plant

hormones during over-irrigation, root-to-shoot signalling should be assessed, as roots are the first organs to sense stress changes in the soil and therefore should have an important function in over-irrigation-imposed stress conditions.

Chapter 4.

4 Influence of over-irrigation on phytohormonal root-to-shoot signalling

Fig 4.1 (left) Preparation of pressure pots to insert soil moisture sensors, (right) root xylem sap collection

4.1 Introduction

Growth and development of roots and shoots are interconnected, since water and minerals taken up by roots are exchanged for photosynthetic assimilates from the shoots (Jackson 1994). During environmental stress, roots can produce different signals which can be transported to shoots in the transpiration stream (xylem sap) (Jackson 2002). Experiments about coordination between roots and shoots were undertaken already in the 18thcentury, when du Monceau (1758) proposed that plant growth is controlled by movement of sap. Now it is known that long-distance communication (so-called root-to-shoot signalling) includes export and translocation of chemical signals such as ions and phytohormones. Many different signals occur in stressed plants, interact with each other and can be positive (increased delivery from stressed to responding part), negative (decreased delivery) or accumulative (decreased movement out of the responding part). Those signals include hydrogen and nutrient ions, ethanol as well as water and solutes from outside the roots and plant hormones (Jackson 2002).

Plant hormones are commonly classified into the following groups: auxins, cytokinins (CK), gibberellins (GA), abscisic acid (ABA), ethylene, jasmonates, salicylic acid (SA), brassinosteroids (BR) (Covarubbias et al. 2006) and strigolactones (SL) (Xie et al. 2010). Many of these hormones show altered xylem sap concentrations or delivery rates (product of concentration x flow rate) in response to flooding. Flooding of tomato plants decreased root to shoot delivery rate of ABA by 89 %, but increased ACC delivery 3-fold (Else et al. 1995b). Furthermore, the decline in gibberellic acid (GA) export from root to shoots of flooded tomato plants was associated with reduced growth (Reid et al. 1969). A foliar spray of the cytokinin benzyladenine in

combination with GA3 (10 µg ml^{-1} each) on flooded tomato plants can ameliorate stem elongation inhibition (Jackson and Campbell 1979). Not much is known about concentrations/delivery of jasmonic acid (JA) and SA in xylem sap during low oxygen conditions and so far, a comprehensive, multi-analyte study on the effect of over-irrigation on root-to-shoot signalling of several plant hormones has not been conducted.

Xylem sap pH might act as a signal, for example in regulating leaf growth, since higher xylem sap pH decreased barley leaf elongation via an ABA-dependent mechanism (Bacon et al. 1998). Flooding can increase xylem sap pH during the first few hours by at least 0.5 pH units, but the pH stabilised after 24 h (Else et al. 1995b). However, changes in anti-transpirant activity in xylem sap, which can cause stomatal closure, cannot be attributed to pH in Commelina (Else et al. 2006). Increased sap pH is unlikely to initiate stomal closure in flooded tomato plants when transpiration assays were performed, possibly because the change in pH did not raise xylem sap ABA sufficiently to reach active concentrations (Jackson et al. 2003). In contrast, manipulating apoplastic pH of tomato can control stomatal conductance, leaf growth rate and plant water loss (Wilkinson and Davies 2008). Further studies on the impacts of xylem sap pH changes are necessary. Therefore, this chapter aims to understand the effects of over-irrigation on xylem sap pH and concentrations and delivery rates of plant hormones.

4.2 Materials and Methods

For details of plant and irrigation treatments and physiological measurements, see Chapter 2.2

4.2.1 Sap collection

In contrast to previous experiments, plants for root xylem sap collection (Experiment 1) were grown in special pots which fit into a Scholander pressure vessel (23 cm height x 6.5 cm diameter, 632 ml volume, Fig 4.1). Plants were grown under the two different irrigation treatments (well-drained and over-irrigated) described earlier (Chapter 2.2) and harvested already after 3 weeks of treatment due to the smaller pot volume. To match sap flow with evapotranspiration (= (plant weight at start – plant weight at finish)/(time at finish - time at start)*24*60*60), plants were weighed one hour after watering and again immediately before collecting root xylem sap, to calculate evapotranspiration during the time interval. Plants were then detopped with a razor blade just below the cotyledonary node and root systems (still in the pot) were placed inside a Scholander pressure vessel (Soil Moisture Equipment Corp., Model 3000F01, Santa Barbara, CA, USA, Fig 4.1), then root water potential (Ψ_{root}) was measured. At certain pressures (0.2 – 0.5 MPa), sap flow rate was calculated during a 20 sec interval (by weighing sap collected in an eppendorf vial). Once sap flow matched evapotranspiration (94-97 %), more sap (~ 250 µl) was collected at that pressure and sap pH was measured with a B-212 pH twin compact meter electrode (Horiba Scientific Ltd., United Kingdom). The remaining sap was frozen in liquid nitrogen and stored at -80°C for further analysis. Delivery of substances in the sap was calculated by multiplying measured solute concentration by sap flow rate. Samples

were collected between 09:00 h and 12:00 h (3 to 6 hours after the beginning of the photoperiod).

In a separate experiment (Experiment 2), leaf xylem sap was extracted after 4 weeks of treatment from plants grown as in Chapter 2.2. Xylem sap from Leaves 3 and 4 was collected more or less simultaneously as two operators each placed those leaves in separate Scholander pressure vessels (Soil Moisture Equipment Corp., Model 3000F01, Santa Barbara, CA, USA) and applied 0.4 (Leaf 3) and 0.5 MPa (Leaf 4) overpressure above the balancing pressure (Ψ_{leaf}). Plants were sequentially harvested between 09:00 h and 15:00 h (3 to 9 hours after the beginning of the photoperiod), alternating between well-drained and over-irrigated plants. A 50:50 mix of xylem sap from Leaf 3 and Leaf 4 was used for further analysis. In contrast to root xylem sap collection, plants for leaf xylem sap collection were grown for 4 weeks to assure older, more structurally rigid leaves to insert into the pressure chamber.

4.2.2 Hormone extraction and analysis

Cytokinins (*trans*-zeatin - *t*Z, zeatinriboside – ZR and isopentenyladenine - iP), auxin (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 extracted and purified according to the method of Dobrev and Kaminek (2002) and analysed according to Albacete et al. (2008). Prior to injection, 100 µl of xylem sap were filtered through 13 mm diameter Millex filters with 0.22 µ pore size nylon membrane (Millipore, Bedford, MA, USA). 10 µl of filtered xylem sap, dissolved in mobile phase A, was injected onto a Zorbax SB-C18 HPLC column (5 lm, 15030.5 mm, Agilent Technologies, Santa Clara, CA, USA), maintained at 40°C, and eluted at a flow rate of 10 µl min^{-1}. 8 µl of each sample 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. Mobile phase A, consisting of water/ acetonitrile/formic acid (94.9:5:0.1 by vol.), and mobile phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1 by vol.), were used for the chromatographic separation. The elution programme maintained 100 % A for 5 min, then a linear gradient from 0 % to 6 % B in 10 min, followed by another linear gradient from 6 % to 100 % B in 5 min, and finally 100 % B maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analytical run. The UV chromatogram was recorded at 280 nm with a DAD module (Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 22 000 m/z s^{-1} from 50–500 m/z. The nebulizer gas (He) pressure was set to 30 psi, whereas the drying gas was set to a flow of 6.0 l/min at a temperature of 350°C. 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 analysed component (1, 10, 50, and 100 µg l^{-1}) and corrected for 10 µg l^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95%. The detection limit of the instrument for all plant hormones was 0.1 µg l^{-1}. Hormone delivery in root xylem sap was calculated the following:

$$\text{Delivery rate} = \text{concentration} \times \text{sap flow}$$

4.2.3 Statistical analysis

Treatment differences were determined via an Independent Samples T-test (SPSS 19, IBM). Furthermore, a univariate (2-way) ANOVA was performed to test both the individual effects and any interactions between plant hormone and treatment or genotype and treatment. If no significant treatment interaction was found, one linear regression was fitted to data from both treatments.

4.3 Results

Over-irrigation increased whole plant absolute transpiration (by 49 %) and transpiration rate per unit leaf area (by 62 %) when compared to the well-drained treatment (Fig 4.2A, B). There was no significant difference between treatments in Ψ_{leaf} of Leaf 3 and 4 and between the two leaf positions, but root water potential (Ψ_{root}) decreased by 31 % in the over-irrigated treatment (Table 4.1). Over-irrigation decreased leaf xylem sap by 0.26 pH compared to the well-drained treatment, but did not significantly change root xylem sap pH (Table 4.1). Over-irrigation reduced shoot fresh weight and total leaf area in both experiments (Table 4.2), though only significantly in Experiment 2.

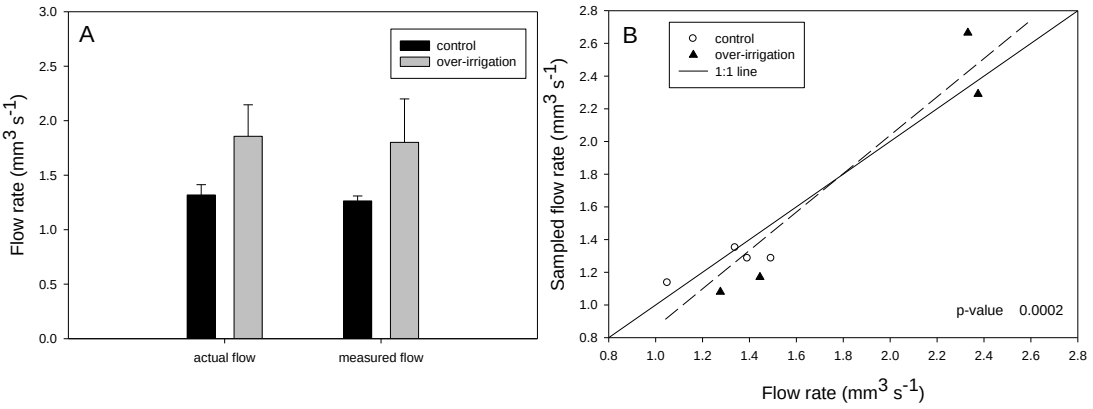

Fig 4.2 (A) Actual (absolute transpiration) and sampled sap flow rate and whole plant transpiration rate per unit leaf area of over-irrigated (grey bar) and well-drained (control, black bar) tomato plants, (B) calculated flow rate plotted against sampled flow rate of over-irrigated (closed triangle) and well-drained (control, open circle) tomato plants. Data are means ± SE of 4 replicates from Experiment 1. P-value for linear regression for all data (dashed line) given.

Table 4.1 Leaf and root water potential and leaf and root xylem sap pH of over-irrigated and well-drained (control) tomato plants from 2 separate experiments. Data are means ± SE of 4-8 replicates, different letters indicate significant differences between treatments (Independent-Samples T-Test, P-value <0.05).

	Ψ_{root} (MPa) Exp 1	**Ψ_{leaf} Leaf 3 (MPa) Exp 2**	**Ψ_{leaf} Leaf 4 (MPa) Exp 2**	**Root xylem sap pH Exp 1**	**Leaf xylem sap pH Exp 2**
Over-irrigation	-0.21 ± 0.03[a]	-0.46 ± 0.01[a]	-0.56 ± 0.01[a]	5.75 ± 0.03[a]	6.26 ± 0.12[a]
Control	-0.16 ± 0.01[a]	-0.47 ± 0.01[a]	-0.57 ± 0.02[a]	5.68 ± 0.06[a]	6.52 ± 0.05[a]

Table 4.2 Shoot fresh weight and total leaf area of over-irrigated and well-drained (control) tomato plants from 2 separate experiments. Data are means ± SE of 4-8 replicates, different letters indicate significant differences between treatments (Independent- Samples T-Test, P-value <0.05).

	Shoot fresh weight (g) Exp 1	**Shoot fresh weight (g) Exp 2**	**Total leaf area (cm^2) Exp 1**	**Total leaf area (cm^2) Exp 2**
Over-irrigation	11.5 ± 2.4^a	34.7 ± 3.3^a	269 ± 55^a	670 ± 46^a
Control	13.1 ± 1.1^a	45.9 ± 4.0^b	343 ± 23^a	894 ± 80^b

Over-irrigation reduced *t*Z, iP, GA3, IAA and ABA concentration in root xylem sap by 44, 50, 50, 24 and 37 %, respectively when compared to well-drained plants, although not to statistically significant values (Fig 4.3A). In contrast, JA and SA concentrations increased by 31 and 43 % respectively in over-irrigated root xylem sap, though not significantly (Fig 4.3A and B). When delivery was calculated, trends were the same for both treatments (Fig 4.3C and D). ACC, ZR, GA1 and GA4 were not detected, presumably because concentrations *in vivo* were below the detection limit of the instrument.

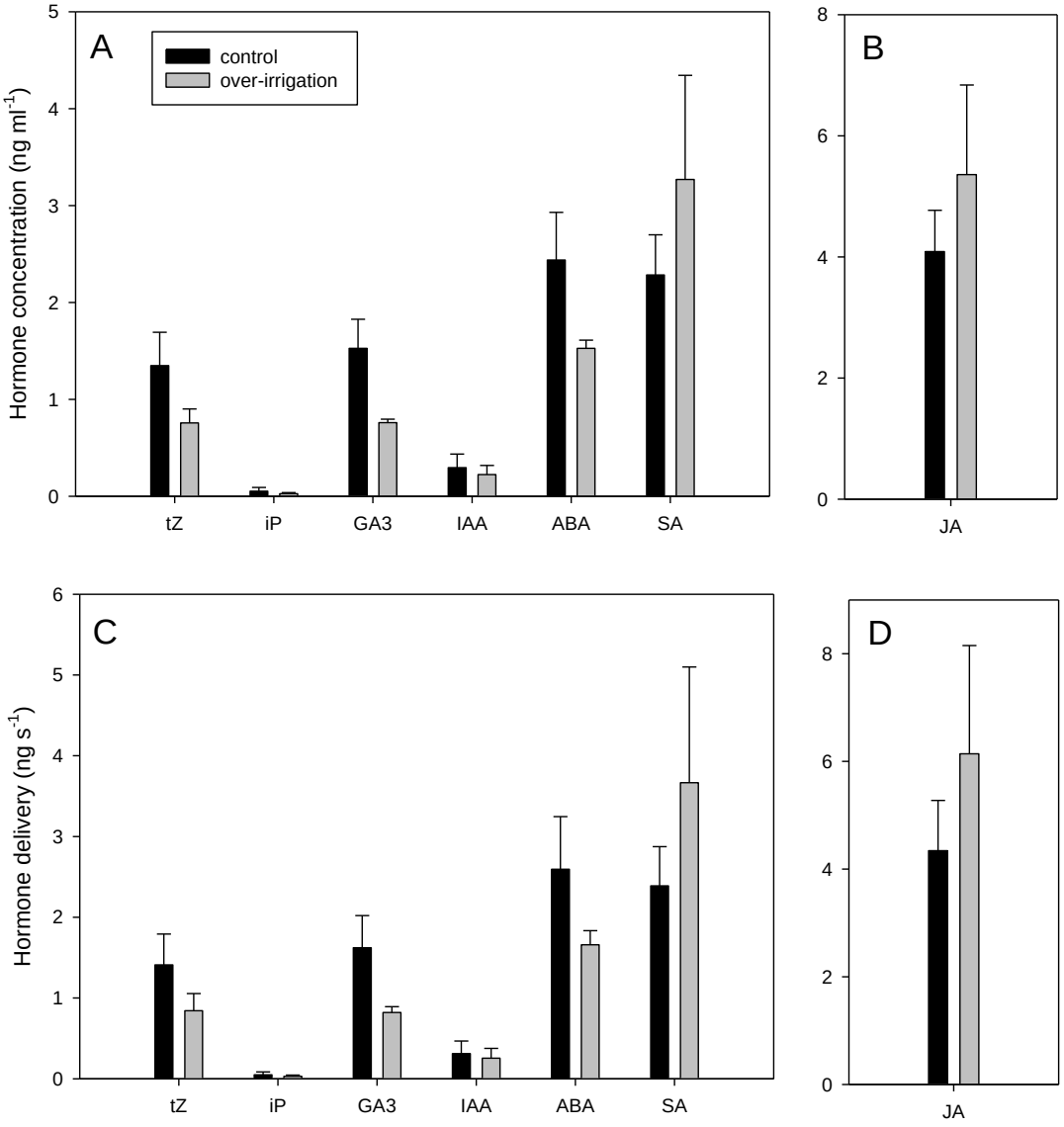

Fig 4.3 (A,B) *t*Z, iP, GA3, IAA, ABA, SA and JA concentration and (C,D) delivery in root xylem sap of over-irrigated (grey bars) and well-drained (control, black bars) tomato plants. ACC, ZR, GA1 and GA4 were not detected. Data are means ± SE of 3-4 replicates from Experiment 1. Asterisk indicates significant differences (Independent-Samples T-Test, P-value < 0.05).

Over-irrigation significantly reduced *t*Z, GA3, ABA, JA and SA concentrations in leaf xylem sap by 34, 33, 24, 43 and 43 %, respectively compared to well-drained plants (Fig 4.4A and B). Though not statistically significant, over-irrigation increased ACC, iP and IAA concentrations (Fig 4.4A). The plant hormones ZR, GA1 and GA4 could not be detected.

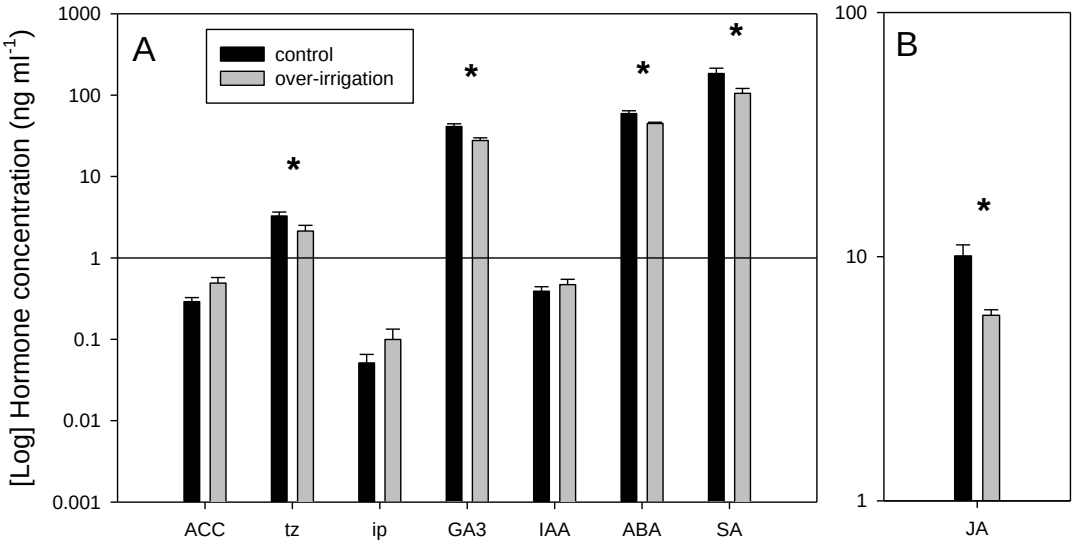

Fig 4.4 (A) ACC, *t*Z, iP, GA3, IAA, ABA, SA and (B) JA concentration (log) in leaf xylem sap of over-irrigated (grey bar) and well-drained (control, black bar) wild type tomato plants. ZR, GA1 and GA4 were not detected. Data are means ± SE of 8 replicates from Experiment 2. Asterisk indicates significant differences (Independent-Samples T-Test, P-value < 0.05).

Higher JA delivery in root xylem sap tended to correlate with decreased fresh weight (P=0.051), but no other hormone correlated with fresh weight (Table 4.3). Higher *t*Z root xylem sap delivery correlated with increased GA3, IAA and ABA delivery (P=0.014; 0.0022 and 0.0112, respectively). Furthermore, increased GA3 delivery correlated with increased ABA (P<0.0001) and tended to correlate with higher IAA delivery (P=0.093). In addition, increased JA correlated with higher SA root xylem sap delivery (P=0.0228, Table 4.4).

Table 4.3 Correlations of root xylem hormone delivery with shoot fresh weight (using data from both well drained and over-irrigated plants from Experiment 1). P-values and R^2 for linear regressions are given.

	p-value	**R^2**
***t*Z**	0.93	0.001
iP	0.48	0.99
GA3	0.43	0.11
IAA	0.79	0.01
ABA	0.53	0.07
SA	0.17	0.30
JA	0.051	0.50

Table 4.4 Correlation matrix for root xylem hormone delivery (using data from both well drained and over-irrigated plants from Experiment 1). P-values (and R^2 in brackets) reported.

	***t*Z**	**iP**	**GA3**	**IAA**	**ABA**	**SA**	**JA**
***t*Z**		0.27	0.0144	0.0022	0.0112	0.45	0.31
		(0.19)	(0.66)	(0.81)	(0.69)	(0.10)	(0.17)
iP			0.86	0.27	0.79	0.65	0.90
			(0.005)	(0.20)	(0.01)	(0.04)	(0.003)
GA3				0.0926	<0.0001	0.85	0.65
				(0.40)	(0.97)	(0.03)	(0.03)
IAA					0.0549	0.0851	0.11
					(0.49)	(0.41)	(0.36)
ABA						0.65	0.42
						(0.04)	(0.11)
SA							0.0228
							(0.61)
JA							

In comparing data from individual plants, higher shoot fresh weight was correlated with increased *t*Z, GA3, ABA and JA leaf xylem sap concentration (Fig 4.5B, D, F and H), whereas there was no correlation between leaf xylem sap ACC, iP, IAA and SA concentration and shoot fresh weight (Fig 4.5A, C, E and G).

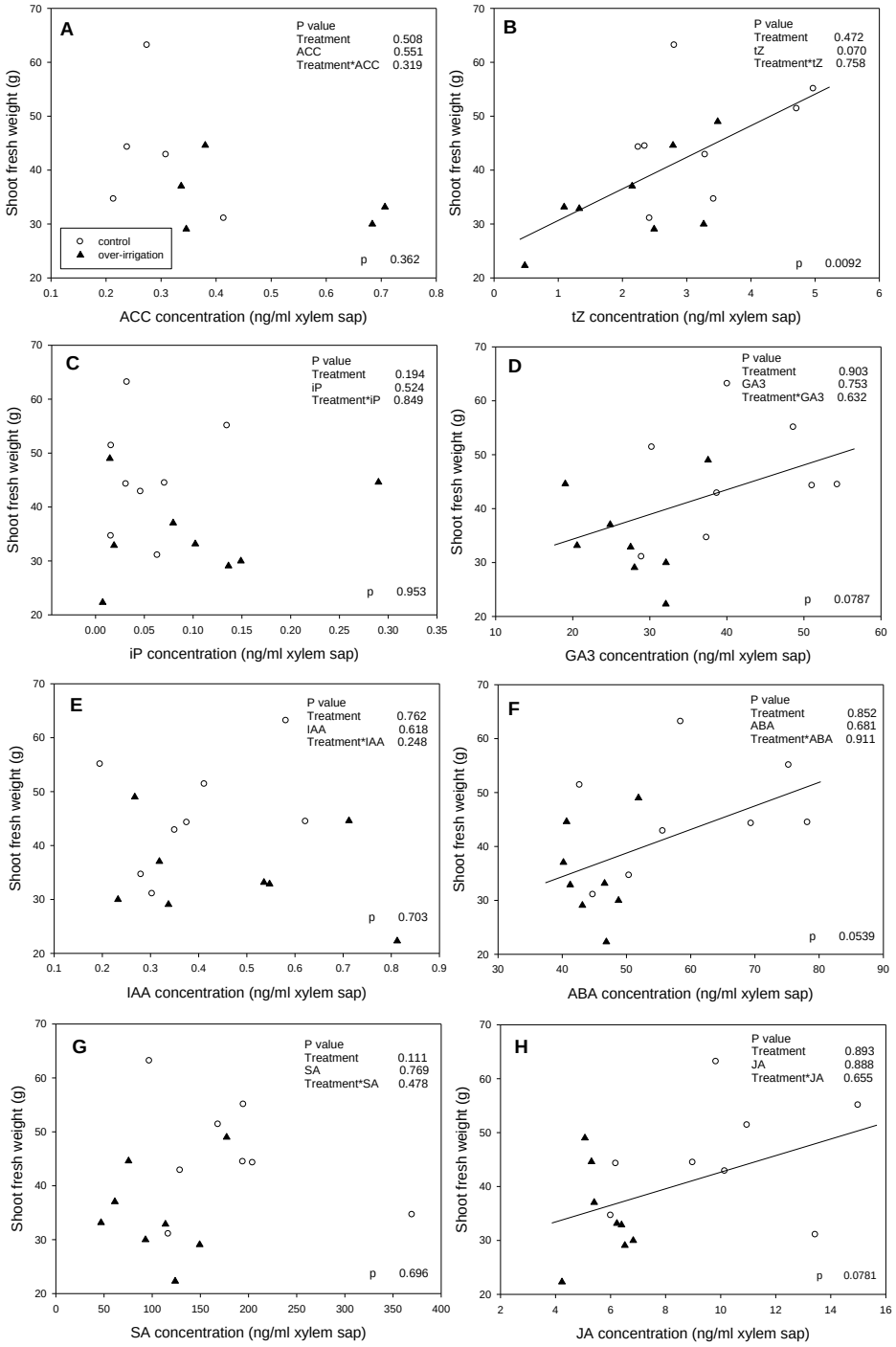

Fig 4.5 Leaf xylem sap (A) ACC, (B) *t*Z, (C), iP, (D) GA3, (E) IAA, (F) ABA, (G) SA and (H) JA concentrations plotted against shoot fresh weight of over-irrigated (closed triangle) and well-drained (control, open circle) tomato plants from Experiment 2. P-values for 2-way ANOVA (upper right part of each panel) and linear regressions (lower part of each panel) for all data (black line) given.

When hormone concentrations were plotted against each other, increased *t*Z concentration was significantly correlated with decreased IAA concentration (P=0.0099), but increased JA concentration (P=0.0177, Table 4.5.). Furthermore, increased GA3 concentrations were significantly correlated with increased ABA (P<0.0001) and SA concentrations (P=0.0236, Table 4.5).

Table 4.5 Correlation matrix for leaf xylem hormone concentrations (using data from both well drained and over-irrigated plants from Experiment 2). P-values (and R^2 in brackets) reported.

	ACC	*t*Z	iP	GA3	IAA	ABA	SA	JA
ACC		0.75	0.32	0.16	0.88	0.37	0.10	0.78
		(0.01)	(0.13)	(0.24)	(0.003)	(0.10)	(0.30)	(0.01)
***t*Z**			0.66	0.17	0.0099	0.29	0.12	0.0177
			(0.01)	(0.13)	(0.39)	(0.07)	(0.16)	(0.34)
iP				0.17	0.79	0.67	0.16	0.95
				(0.13)	(0.005)	(0.01)	(0.14)	(0.0003)
GA3					0.47	<0.0001	0.0236	0.18
					(0.04)	(0.87)	(0.32)	(0.13)
IAA						0.70	0.21	0.17
						(0.01)	(0.11)	(0.13)
ABA							0.13	0.11
							(0.16)	(0.17)
SA								0.76
								(0.007)
JA								

4.4 Discussion

Over-irrigation increased absolute transpiration by 49 % and plant transpiration rate per unit leaf area by 62 % in this work (Fig 4.2A). In contrast, 24 hours of flooding tomato plants reduced absolute transpiration by 30 to 40 % (Bradford and Hsiao 1982). Flooding can decrease transpiration due to stomatal closure (Jackson 2002), but it is important to note whether studies have reported absolute transpiration or transpiration rate, which is normalized for leaf area. As reported previously, stomatal closure does not occur in over-irrigated plants (Fig 2.5B) and the results presented here suggest that neither stomatal closure nor reduced transpiration are responsible for growth inhibition during over-irrigation. Changes in stomatal conductance and plant physiology are usually correlated with changes in soil water status (during both drought and waterlogging), but are only weakly or only in some species well-correlated with changes in Ψ_{leaf} (Comstock 2002). As the root is the first organ to sense differences in the soil environment, root water potential (Ψ_{root}) may better indicate how plants react to changes in soil moisture (Comstock 2002). Over-irrigation decreased Ψ_{root} by 31 %, though this change was not significantly different from the well-drained treatment (Table 4.1). This is in accordance with other studies, where Ψ_{root} did not change in flooded tomato plants after 24 hours (Jackson et al. 1996), suggesting that Ψ_{root} does not regulate plant growth during over-irrigation.

Another aim of this chapter was to assess the role of plant hormones in root-to-shoot signalling during over-irrigation. An overview of changes in plant hormone delivery/concentration in root and leaf xylem sap of over-irrigated tomato plants compared to well-drained plants is given in Fig 4.6.

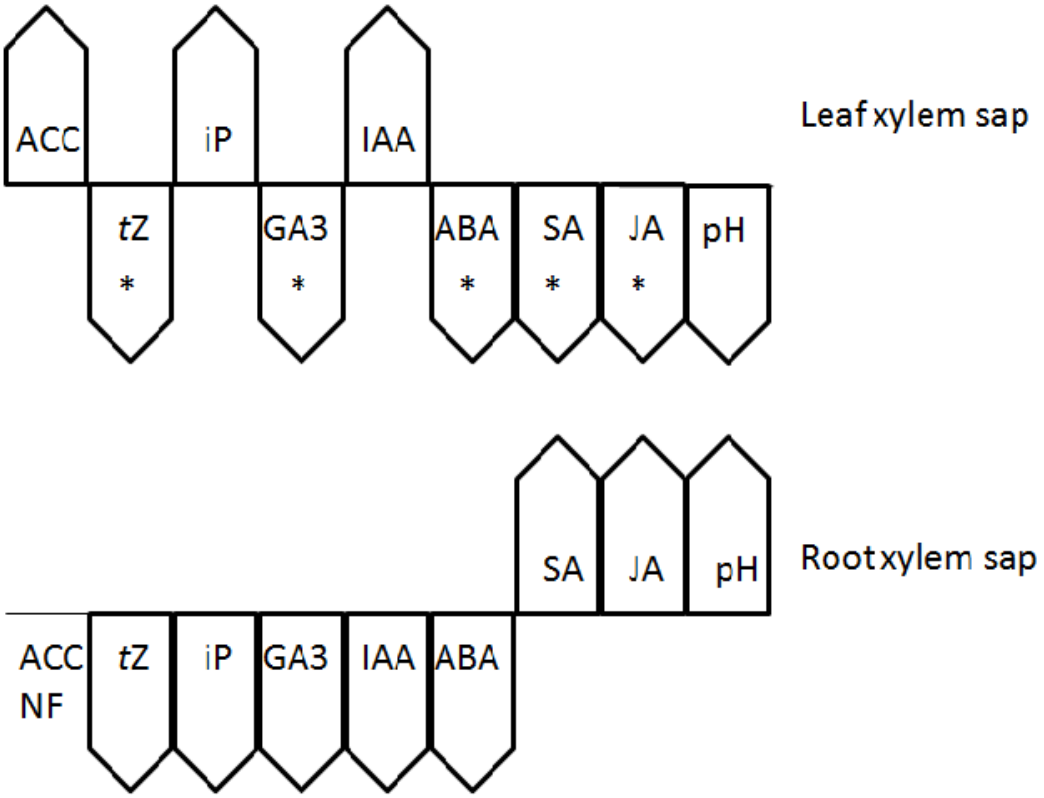

Fig 4.6 Overview of changes in plant hormone concentration in root and leaf xylem sap of over-irrigated tomato plants compared to well-drained plants. Arrow upwards indicates increase, arrow downwards indicates decrease, NF – not found. Asterisk indicates significant difference between over-irrigated and well-drained plants (Independent-Samples T-Test, p-value <0.05).

It is important to note that solute concentrations in sap change depending on the flow rate (Else et al. 1995b, Jackson 1994) and this flux of solutes from roots to shoots determines the concentration at the site of action. If the transpiration rate changes (for example due to environmental stress), solute concentrations can change even though the solute output from the roots remains the same. A root signal therefore is not defined as a change in solute concentration, but rather a change in delivery rate, which is calculated by concentration multiplied by flow rate of the xylem sap (Jackson 2002). Accordingly, it is essential to see if studies have taken sap flow rate into

account when reporting xylem hormone concentrations. If the sap analysed flowed at slower rates than the actual whole plant (absolute) transpiration, hormone concentrations are usually overestimates (Jackson 1994). When hormone delivery from the root system was compared with hormone concentrations in root xylem sap in the experiments presented here, trends remained the same (Fig 4.3A-D). Solute concentration should only be used when considering events at or near to the site of action. Therefore, delivery of hormone concentrations in leaf xylem sap was not calculated here. In addition, the overpressure applied to the leaf, which affects sap flow rate, does not affect xylem ABA concentrations (Dodd et al. 2009).

Due to previous experiments involving ethylene (Chapter 2 and 3), its precursor ACC was of primary interest. During hypoxia, ACC cannot be converted to ethylene in roots as ACC oxidase activity is inhibited under oxygen-deficiency (Vriezen et al. 1999). Once in leaves and in the presence of oxygen, ACC is converted to ethylene by ACC oxidase (Bradford and Yang 1980). Accordingly, flooding tomato plants increased ACC delivery from roots to shoots and this delivery was sufficient to support extra ethylene production by leaves of flooded tomato plants (Bradford and Yang 1980, English et al. 1995, Jackson et al. 1996). However, in the results presented here, ACC was not detected in root xylem sap of either over-irrigated or well-drained plants. As described previously (Fig 2.2B), over-irrigation does not decrease soil oxygen concentrations as severely as flooding and therefore, oxygen might still be available for ACC conversion into ethylene. Though not significantly, over-irrigation increased ACC concentration in the leaf xylem sap (Fig 4.4A), which could explain higher ethylene emission from shoot. Observed fluxes of ACC (0.49 nmol/l) might be sufficient enough to support ethylene production rates of 2.18 nmol/g fresh weight (Fig 2.8B) assuming mole to mole conversion of ACC to ethylene (Else and Jackson

1998). However, ACC was not detected in all samples, explaining the lack of correlation between ACC and shoot fresh weight (P=0.362, Fig 4.5A). Possibly, detection limits were not low enough with the volumes of xylem sap used in the LC-MS method. Albacete et al. (2008) report that leaf and xylem ACC concentrations increased after 3 weeks of salinization of tomato. However, growth was inhibited already during the first week when ACC concentration was below the detection limit, suggesting that ACC might not be the primary growth-limiting factor during salinization (Albacete et al. 2008).

In Chapters 2 and 3, the physiological role(s) of foliar ABA concentration during over-irrigation was assessed. Here, over-irrigation decreased ABA delivery/concentrations in both root and leaf xylem sap (Fig 4.3C and Fig 4.4A), possibly because ABA biosynthesis requires oxygen (Else et al. 1995b). In accordance, flooding tomato plants decreased ABA delivery in root xylem sap (Else et al. 1995b, Jackson et al. 1996). Decreased ABA delivery (Fig. 4.3C) might be responsible for the lack of foliar ABA accumulation in leaves of over-irrigated tomato plants (Fig 2.7B) and subsequent maintenance of g_s (Fig 2.5B). However, higher shoot fresh weight was correlated with increased leaf xylem sap ABA concentration (Fig 4.5F), suggesting that increased ABA concentrations assist plant growth. Increased ABA can have a positive effect on leaf growth through increased tissue hydraulic conductivity, but the overall effect of higher ABA concentrations depend on the environmental conditions, such as light, water and nutrient availability (Tardieu et al. 2010). Highly compacted soils present a stress comparable to flooding, as hypoxia is likely to occur due to reduced available air capacity and decreases in aerobic micro-organism activity (Gebauer et al. 2012). Soil compaction can significantly decrease leaf expansion in barley plants without detectable changes in foliar ABA

concentrations. However, feeding synthetic ABA to the root-zone (100 nM) or to the xylem sap (5 pg μl^{-1}) increased leaf expansion to levels of uncompacted plants, suggesting that xylem sap ABA can maintain leaf growth during compaction (Mulholland et al. 1996). However, the role of both foliar and xylem sap ABA on growth during over-irrigation remains unclear in the absence of further experiments specifically manipulating ABA concentrations.

Concentrations of total CK in xylem sap of sunflower significantly decreased after 24 h of flooding, and waterlogging might reduce root CK production (Burrows and Carr 1969). So far, it was unclear whether this decrease of CK in xylem sap was due to lower biosynthesis or hindered transport from roots to shoots, which would allow root CK accumulation. Here, over-irrigation reduced *t*Z delivery and concentration in both root and leaf xylem sap (Fig 4.3C and 4.4A). In addition, delivery of the cytokinin iP decreased in root xylem sap of over-irrigated plants (Fig 4.3C), but concentrations increased in leaf xylem sap (Fig 4.4A). These results could suggest that iP transport down to the roots is interrupted, though analysing phloem iP concentrations would be required to further test this hypothesis. However, leaf xylem sap iP concentration was not correlated with shoot fresh weight (Fig 4.5C), even though higher shoot fresh weight correlated with increased *t*Z leaf xylem sap concentrations (Fig 4.5B), similar to increased xylem CK (Z and ZR) concentrations in tomato which were identified as one of the key long-distance signals in meditating shoot responses to NO_3^--induced leaf growth stimulation (Rahayu et al. 2005). Increased root cytokinin synthesis (*t*Z, iP and the corresponding ribosides *t*ZR and iPR) ameliorated salinity-induced tomato growth and yield reductions (Ghanem et al. 2011). *t*Z and foliar iP concentrations of over-irrigated plants would be of interest to further determine possible physiological significance of root-to-shoot CK delivery,

even though there was no significant change in CK leaf concentration during soil drying in *Phaseolus* despite decreased xylem cytokinin (ZR and dihydrozeatin riboside [DHZR]) concentrations (Neumann et al. 1990). A spray with exogenous cytokinins (single or in combination to determine which one is the most effective) might give more insight in understanding the possible influence of CK on growth-inhibition during over-irrigation.

Furthermore, CK and ABA can interact with each other in regulating stomatal behaviour, but possibly also their synthesis (Popsilova et al. 2005, Subbiah and Reddy 2010). There was no significant correlation in their leaf xylem sap concentrations between *t*Z or iP and ABA (P=0.29 and 0.67, respectively, Table 4.5), even though increasing *t*Z root xylem sap delivery was correlated with increased ABA delivery (P=0.0112, Table 4.4), suggesting that any interaction between *t*Z and ABA is more important at the level of root delivery.

GA3 delivery and concentration decreased in both root and leaf xylem sap of over-irrigated plants (Fig 4.3C and 4.4A) and higher shoot fresh weight correlated with increased GA3 leaf xylem sap concentration (Fig 4.5D). GAs are synthesized in the shoot apex and transported to elongating internodes (Covarubbias et al. 2006) and can stimulate stem elongation. Decreased GA concentrations in the xylem sap could act as a growth inhibitor (Burrows and Carr 1969) and reduce stem height of over-irrigated plants (Table 2.1). Furthermore, exogenous application of GAs to some dwarf (GA biosynthesis) mutants which were GA-sensitive reversed their phenotype, demonstrating an effect of GA on internode elongation (Koornneef and van der Veen 1980). These results suggest that a spray with exogenous GAs might ameliorate over-irrigation-induced growth inhibition. A combined spray with an unspecified GA and

the cytokinin benzyladenine ameliorated flooding-induced inhibition of stem elongation in tomato (Jackson and Campbell 1979), suggesting that shortage of root-derived GAs and CKs in plants subjected to flooding could be a negative signal to slow shoot growth (Jackson 1990). In the results presented here, there was no significant correlation between either *t*Z or iP and GA3 leaf xylem sap concentration (P=0.22 and 0.17, respectively, Table 4.5), but increasing *t*Z root xylem sap delivery was correlated with increased GA3 (P=0.0144, Table 4.4), suggesting that *t*Z root-sourced delivery might be more important in signalling during environmental stress conditions. Furthermore, higher GA3 leaf xylem sap concentration and root xylem sap delivery correlated with higher ABA (P<0.0001, Table 4.4 and 4.5) concentration/delivery. GA and ABA can interact in regulation root growth (Achard et al 2006). Possibly, the balance between GA and ABA (rather than their absolute levels) are important in regulating growth during over-irrigation.

In addition to cytokinins, auxin and ethylene can influence GA concentrations (Yamaguchi 2008). Auxins are distributed within the plants through a cell-to-cell transport system (Vieten et al. 2007) and developmental and environmental influences affect cellular localization of auxin (Santner et al. 2009). Auxin is probably transported away from the source tissue (young leaves and flowers) throughout the plant towards the root apex (Petrasek and Friml 2009). Over-irrigation decreased IAA (auxin) delivery in root xylem sap (Fig 4.3C), but increased its concentration in leaf xylem sap (Fig 4.4A), suggesting that transport from leaves to roots is disrupted during over-irrigation. Similarly, flooding can interrupt auxin movement down the shoot (Jackson 1990), therefore leading to increased auxin concentrations in the shoot and salinity decreased leaf growth as well as the auxin IAA by 50 to 90 % in xylem sap (Albacete et al. 2008). As with cytokinins, gibberellins and auxins have been

associated with promoting cell elongation and division, suggesting that these plant hormones could regulate leaf initiation and growth under salinity and other environmental stresses (Albacete et al. 2008). Though leaf xylem sap iP concentration did not correlate with IAA, increasing *t*Z leaf xylem sap concentration was correlated with decreased IAA concentration (P=0.0099, Table 4.5), whereas increasing *t*Z root xylem sap delivery was correlated with increased IAA concentration (P=0.0022, Table 4.4). Few studies (Phillips 1964, Wample and Reid 1979) using bioassays show that shoot auxin activity increases after waterlogging sunflower, but more specific quantification (using physico-chemical techniques) and possible spray of exogenous auxin is needed to determine the role of auxin in the xylem sap during environmental stress conditions.

Studies of jasmonic acid (JA) have mainly focused on response to wounding, herbivore or insect feeding and fungal infection (Wasternack 2007). Foliar JA concentrations increased in salt-stressed tomato (Pedranzani et al. 2003) and in drought-stressed papaya (Mahouachi et al. 2007), but reports about JA concentration and/or delivery in xylem sap of stressed plants are rare. In this experiment, JA delivery increased in root xylem sap of over-irrigated plants (Fig 4.3D), but its concentration significantly decreased in leaf xylem sap (Fig 4.4B). Waterlogging citrus seedlings transiently increased foliar JA concentration and was followed by an accumulation of foliar ABA, suggesting that JA might be an early mediator of stress responses (Arbona and Gomez-Cadenas 2008). Jasmonates can inhibit both shoot and root growth (Dathe et al. 1981, Staswick et al. 1992) and promote senescence (Parthier 1990, Ueda and Kata 1980), but the mechanisms behind these responses are still unclear. Furthermore, cytokinins can regulate endogenous JA levels especially during wounding responses, where higher CK (benzylaminopurine) levels increase JA

concentration (Sano et al. 1996). A similar interaction could exist during over-irrigation, where higher *t*Z xylem sap concentration was correlated with higher JA concentration (P=0.0177, Table 4.5).

Over-irrigation increased SA delivery in root xylem sap (Fig 4.3C), but decreased concentrations were found in leaf xylem sap (Fig 4.4A), suggesting that SA transport to the shoots is hindered during over-irrigation or greater SA metabolism in transit, possibly regulated by ROS and Ca^{2+} (Chen and Kuc 1999, Chen et al 2001). Exogenously applied SA (aqueous spray, 10^{-2} to 10^{-8} M) increased root and shoot growth in soybean (Gutierrez-Coronado et al. 1998). These results suggest that SA is a growth promoter and reduced concentrations could be one of the signals for growth inhibition during over-irrigation.

An abundant literature suggests that all plant hormones interact with one or several other hormones both at the level of synthesis and sensitivity and influence developmental processes and responses to environmental stresses together. It is difficult to resolve independent effects of plant hormones on over-irrigation, as hormones in this experiment were measured only once. However, foliar ABA and ethylene concentration in response to over-irrigation were consistent over time (Fig 2.7B and 2.8B) and this might be the case for phytohormones measured here as well.

Though over-irrigation induced differences in root xylem sap hormone concentrations compared to well-drained tomato plants, these were not significant, in contrast to results obtained from leaf xylem sap analysis, where significant changes were found. These results might be due to a lower replication number in root xylem sap (n=3-4) than in leaf xylem sap (n=8). The process of matching root xylem sap flow rate with plant transpiration requires more time than leaf xylem sap collection. Furthermore, a

different growing system (compared to the remainder of this thesis) was used for root xylem sap collection and root xylem sap phytohormone analysis (Experiment 1) to assure that pots can be inserted in the pressure chamber. Due to the smaller pot volume (0.632 l instead of 1.14 l), plants used in this experiment were relatively young and small, so sap collection took more time. Given the different treatment duration, root xylem sap delivery and shoot xylem sap concentrations might not be comparable. However, as stated above, foliar hormone concentrations measured previously (Chapter 2 and 3) showed consistent responses to over-irrigation over time, suggesting that measurement timing is not critical. Differences in hormone concentrations between root and leaf xylem sap have not been reported before except for ABA (Dodd et al. 2008) and could be a methodological artefact, as these do not occur when the same xylem sap sampling methodology was applied (Netting et al. 2012). However, if the source of the leaf xylem sap is partially symplastic, then differential hormone responses of roots and shoots are consistent with the observed xylem sap phytohormone concentration.

Another signal during waterlogging/over-irrigation might be the xylem sap pH. Changes in sap pH are followed by cell death and sap pH can regulate water channel proteins (aquaporins) during hypoxia (Drew et al. 2000). To our knowledge, differences in sap pH between roots and leaves have not been reported previously. Here, over-irrigation decreased leaf xylem sap by 0.26 pH compared to the well-drained treatment, but increased root xylem sap pH by 0.07 pH units (Table 4.1). However, none of these changes were statistically significant, making it difficult to untangle possible signalling mechanisms. Alkaline pH did not decrease transpiration of leaves detached from an ABA-deficient mutant, unless low concentrations of ABA were fed (Wilkinson et al. 1998), suggesting that low sap pH changes (until 0.5 pH

units) on their own are not sufficient enough to induce for example changes in transpiration.

Jackson (1997) proposed three phases of root-to-shoot signalling during environmental stress conditions (Fig 4.7). In Phase 1, the stress is perceived through the root system and signal transduction, and gene expression and/or metabolism is altered, which can increase or decrease substance (i.e. hormone) production. Transport of these substances as a positive or negative signal represents Phase 2. During Phase 3, substances have reached the shoot system and target cells, where the signals are perceived and transduced and again, gene expression and/or metabolism is altered as a consequence, followed by the specific developmental response and possible acclimation (to help the plant adjust to the altered conditions). As reported previously, root-to-shoot signalling involves many different substances apart from plant hormones, such as nutrients in both sap and tissue (Jackson 2002) and analysing these might give more insight into their role during over-irrigation.

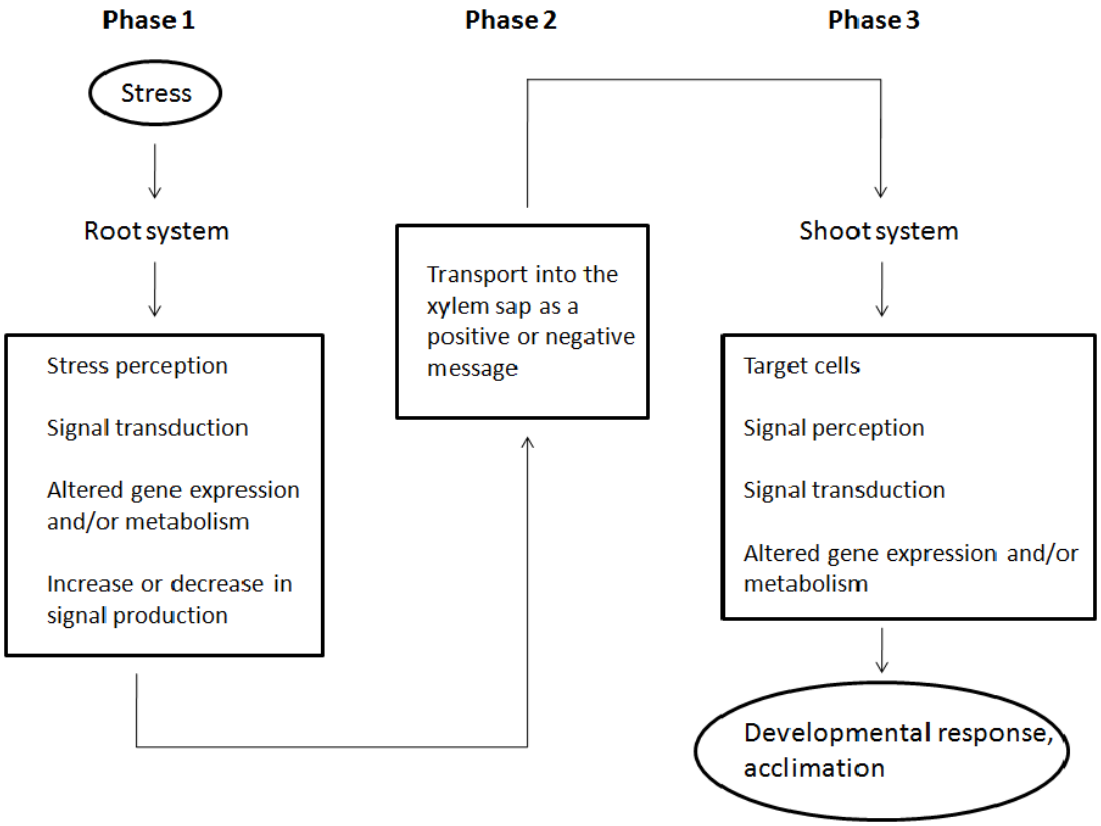

Fig 4.7 Root-to-shoot signalling involving three temporal phases (re-drawn after Jackson 1997)

4.5 Conclusion

This chapter aimed to understand the effect of over-irrigation on hormonal root-to-shoot signalling. ACC was not found in root xylem sap, possibly due to detection limits of the HPLC method, but increased in leaf xylem sap of over-irrigated plants and could be sufficient for extra foliar ethylene produced during over-irrigation. Decreased xylem ABA delivery might be responsible for the lack of ABA accumulation in leaves of over-irrigated tomato plants (Fig 2.7B) and why no effect on g_s was observed (Fig 2.5B). Concentration and delivery of the cytokinin *t*Z were reduced in both root and xylem sap, whereas iP delivery decreased in root xylem sap.

However, its concentration increased in leaf xylem sap of over-irrigated plants, possibly through hindered transport down to the roots, thus allowing accumulation. Previous studies have shown that a cytokinin spray could not ameliorate growth inhibition, suggesting that cytokinins alone are not responsible for reduced growth during environmental stress. GA3 delivery and concentration were reduced in both root and xylem sap of over-irrigated plants, possibly explaining shorter stems (Table 2.1), as GAs have been associated with stem elongation and cell division. Likewise, auxins like IAA can promote cell division and its delivery decreased in root xylem sap of over-irrigated plants. However, higher IAA concentrations were found in leaf xylem sap during over-irrigation. Restricted auxin transport between roots and shoots could be responsible for shoot growth retardation. Over-irrigation increased JA and SA delivery in root xylem sap, but decreased their concentrations in leaf xylem sap and higher shoot fresh weight was correlated with increased JA leaf xylem sap. It is difficult to dissect possible independent effects of plant hormones on over-irrigation, and other signals, such as changes in xylem or foliar nutrient status could be responsible for over-irrigation-induced growth inhibition.

Chapter 5.

5 Over-irrigation decreases xylem nutrient and foliar nitrogen concentrations

Fig 5.1 ICP-OES for analysing macronutrients

5.1 Introduction

Waterlogging can change soil nutrient availability and plant nutrient uptake. Due to changes in ion-exchange reactions, flooding can increase Fe^{2+} and Mn^{2+} concentrations in the soil and these ions can displace sodium, potassium, calcium and magnesium from substrates, thereby reducing their levels in the soil (Leyshon and Sheard 1974). Accordingly, flooding of barley for seven days reduced foliar N, P and K concentrations by 51, 60 and 58 %, respectively (Leyshon and Sheard 1974) and waterlogging *Brassica napus* L. for 7 or 14 days reduced N, P, K and Ca uptake (Gutierrez Boem et al 1996). Decreased N, P, K, Mg, Cu, Zn and Mn concentrations were found in wheat and barley shoots after 15 days of waterlogging (Steffens et al. 2005). Decreased availability of ion concentrations in the soil solution can lead to nutrient deficiency (Steffens et al. 2005) as well as decreased uptake and/or transport of mineral ions by roots, which leads to redistribution of nutrients within the plant or premature leaf senescence (Drew et al 1979, Trought and Drew 1980). Flooding can also reduce nutrient delivery from roots to shoots and flooding tomato plants decreased nitrate delivery in the xylem sap (Jackson et al. 1996), possibly through loss over control of ion uptake (Jackson et al. 1996). However, 24 h of flooding increased delivery of phosphate from roots to shoots 2.3 fold (Else et al. 1995b) as well as sulphate, calcium, potassium and sodium (Jackson et al. 1996), which might be due to release of stored ions from root cell vacuoles (Lee and Ratcliffe 1983) or degeneration of the root cell plasma membranes resulting in solute leakage (Else et al. 1995b).

Nitrogen, combined with other elements, is assimilated into essential amino acids such as glutamine, glutamate, asparagine and aspartate (Lam et al. 1996), which are necessary for forming proteins, and nitrogen is therefore essential for plant growth and development (Silva and Uchida 2000). Nitrogen is also needed for enzymatic reactions in plants and is part of the chlorophyll molecule and of ribulose biphosphate carboxylase (Rubisco – an enzyme which catalyzes the first step in net photosynthetic CO_2 assimilation and photorespiratory carbon oxidation), making it an important factor for photosynthesis and carboxylation efficiency (Barneix and Causin 1996, Zhao et al. 2005). Insufficient nitrogen can rapidly (within days) decrease leaf elongation by decreasing turgor and/or cell wall extensibility (Chapin 1990, Palmer et al. 1996, Radin et al. 1982). However, hydraulic signals such as changes in leaf water status are not the primary cause of physiological responses to nitrogen deprivation, as applying a balancing pneumatic pressure to prevent any N deprivation-induced decrease in water status did not prevent leaf growth inhibition (Dodd et al. 2002). Instead it was suggested that chemical signals such as increased ABA accumulation or decreased cytokinin concentrations were involved in mediating plant responses to nutrient deprivation (Chapin et al. 1988, Dodd et al. 2004). Moreover, ethylene production and sensitivity seems to be influenced by N and P deficiency, even though the interaction between nitrogen deficiency and ethylene is still unclear. Ethylene production decreased in maize roots during N, P or K deficiency (He et al. 1996, Rengel and Kordan 1988). In contrast, nitrogen deficiency increased ethylene production of 5-day old wheat seedlings (Tari and Szen 1995).

Adding calcium nitrate solution daily to the soil restored N concentrations, leaf extension and shoot weight of waterlogged barley plants, possibly through replacing nitrate lost by de-nitrification and microbiological fixation in the anaerobic soil to allow nutrient uptake (Drew et al. 1979), but the impacts of waterlogging-induced nutrient deficiency on foliar phyotohormone relations have not been studied. Plants obtain essential mineral nutrients primarily from the soil and nutrients are involved in plant metabolism and physiology (Rubio et al. 2009). Plants have developed a sensing and signalling mechanism to monitor these external and internal nutrient concentrations and phytohormones are a part of this signalling network and at the same time, nutrient concentrations influence hormone concentrations (Rubio et al. 2009). Therefore, hormones can be seen as a link between nutrient concentrations and their effects on growth and development.

This chapter describes nutrient responses (nitrogen, calcium, potassium, magnesium, sodium, phosphorus and sulphur) to chronic over-irrigation and aims to determine whether calcium nitrate addition to the soil of over-irrigated plants can mitigate the effects of over-irrigation on tomato plants.

5.2 Materials and Methods

For details of plant and irrigation treatments and physiological measurements, see Chapter 2.2

5.2.1 Nutrient analysis

In a separate experiment, macronutrients (Ca, K, Mg, Na, P and S) were analysed via acid microwave digestion followed by ICP-OES (Fig 5.1). Nitric acid (HNO_3) was used to decompose all organic matter to CO_2. Ball-milled (MM400, Retsch, Haan, Germany) oven dried leaf tissue (0.25 g – a mixture of all leaves, stems not included) was weighed in acid-washed and rinsed reaction vessels. Five ml of 100 % HNO_3 (Aristar grade) was added and left for 15 min in a fume hood until the initial reaction subsided. Vessels were sealed and weighed and then placed in the rotor in a MARS 6 microwave (CEM, Buckingham, UK). Vessels were heated to 200°C over 15 min and then held at 200°C for another 15 min. After cooling down, vessels were weighed again to note weight loss. Samples and blank solutions were then diluted in two steps to first 20 % HNO_3 and second to the final concentration of 2 % HNO_3 by using MilliQ water. For root xylem sap analysis, 250 µl of sap collected at the right flow rate (see Chapter 4.2) was diluted to the same final concentration. To analyse nutrients, an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermo Scientific, Massachusetts, USA) with axial view configuration was used. To validate the digestion, tomato leaf NIST (SRM 1573a, National Institute of Standards and Technology, USA) samples with known nutrient concentrations were run and the recovery detected through the ICP-OES was used to calculate final sample concentration. The element reference standard solutions were prepared daily from 1000 mg/l stock solutions.

5.2.2 Leaf nitrogen analysis

Leaf nitrogen in % was analysed via EA combustion using an Elemental Analyser (VARIO Micro Cube, Germany). Oven-dried leaf tissue samples (1.2 mg) were dropped into a furnace held at 905°C onto CuO with a pulse of O_2 and a constant flow of Helium carrier gas. N was converted to gas (N_2) and a pure copper reduction unit after the furnace reduced any conversion of NO_x to N_2. N_2 was measured in a TCD (total dissolved carbon) detector positioned at the end of the elemental analyser and peak areas were compared to standards and amounts of N calculated. For comparison with the wild type, leaf nitrogen concentrations were also measured in the partial ethylene-insensitive genotype *Nr*.

5.2.3 Nitrate application

Because no differences in soil moisture were observed until Day 14 of treatment (Fig 2.2E), 10 ml of either distilled water (to over-irrigated and well-drained soil), 5 mM or 10 mM $Ca(NO_3)_2$ were added to the soil daily from 14 days after the over-irrigation treatment started until harvest day in additional experiment. Table 5.1 gives an overview for sampling times of ABA, ethylene, g_s, Pn, Ψ_{leaf}, shoot fresh weight and total leaf area.

Table 5.1 Sampling times for ABA, ethylene, g_s, Pn, Ψ_{leaf}, shoot fresh weight and total leaf area during $Ca(NO_3)_2$ supplementation experiment.

Measurement	**Day of sampling**
ABA, ethylene	16, 18, 20, 23, 25, 27
g_s, Pn	23, 25, 27
Ψ_{leaf}, shoot fresh weight, total leaf area	28

5.2.4 Statistical analysis

Similar to Chapter 3 (Fig 3.5), parameters, which were sampled on different days throughout the treatment period (g_s, Pn, ABA and ethylene), were accumulated over time (Fig 5.5). Treatment (over-irrigated *versus* well-drained) differences were determined via an Independent Samples t-Test (SPSS 19, IBM). When $Ca(NO_3)_2$ was re-supplied to some plants, one-way ANOVA and a post-hoc Tukey-Test were used to separate means and to compare any significant difference between the treatments. Linear regressions were fitted to data in Fig 5.3C and 5.6.

5.3 Results

After 28 days of treatment, over-irrigation decreased foliar potassium (14 %), magnesium (6.5 %), sodium (13.5 %), phosphorus (7.2 %) and sulphur concentration (20.2 %), but increased calcium concentrations (3.3 %; Fig 5.2A and B). Only sulphur showed a statistically significant ($P < 0.05$) change.

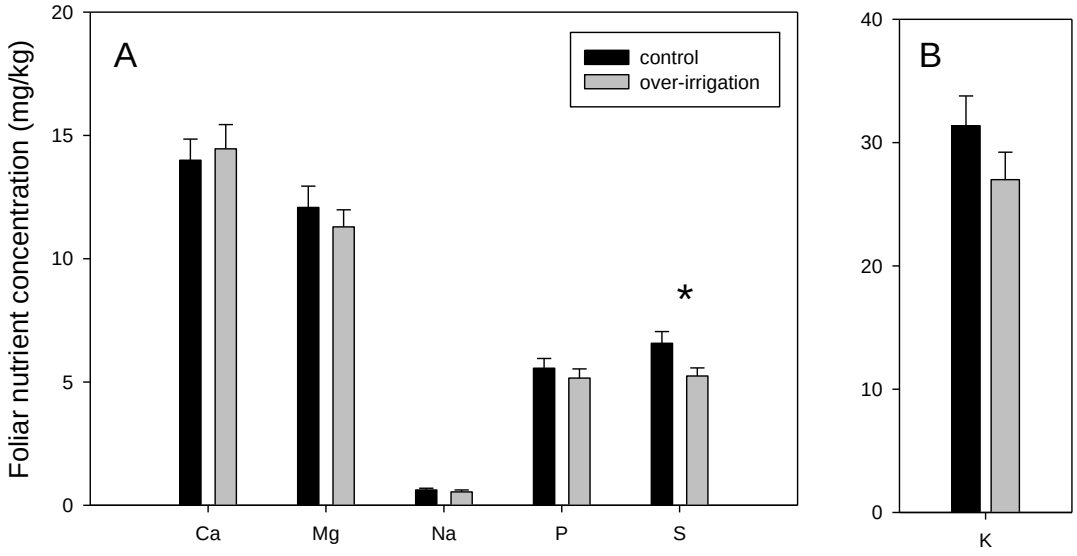

Fig 5.2 (A) Foliar calcium, magnesium, sodium, phosphorus, sulphur, and (B) potassium concentration of over-irrigated (grey bar) and well-drained (black bar) tomato plants. Data are means ± SE of 10 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, p-value < 0.05).

Over-irrigation significantly decreased foliar nitrogen concentrations by 32 % in wild type and 28 % in *Nr* compared to well-drained plants (Fig 5.3A and B). Furthermore, increasing leaf nitrogen concentration tended to correlate with higher shoot fresh weight in both wild type (P=0.0604) and *Nr* (P=0.0552) and there was no significant interaction between genotype and leaf N concentration (Fig 5.3C). However, within an irrigation treatment, higher shoot fresh weight of wild type plants was correlated with lower leaf nitrogen concentration, likely as a result of dilution of nitrogen taken up in biomass growth.

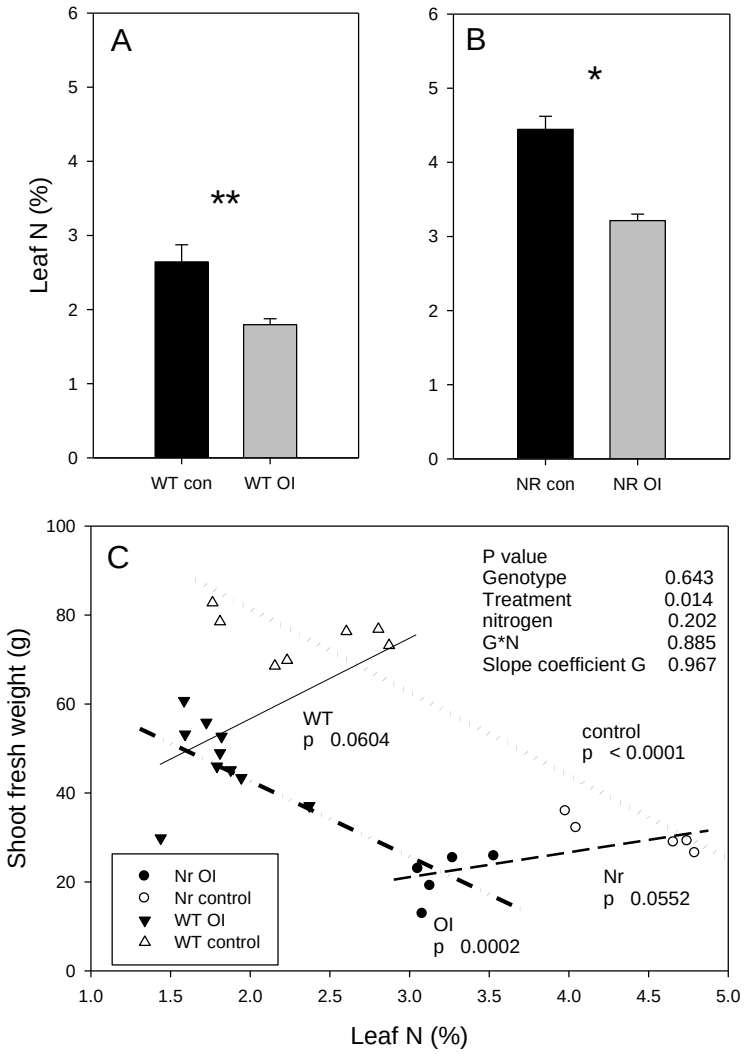

Fig 5.3 (A) Leaf nitrogen concentration of over-irrigated (OI – grey bars) and well-drained (con – black bars) wild type (WT) and (B) *Nr* tomato plants. Data are means ± SE of 5-10 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, p-value < 0.05*, p-value < 0.001**), (C) Leaf nitrogen concentration plotted against shoot fresh weight for over-irrigated (OI) and well-drained (control) wild type (WT) and *Nr* tomato plants. P-values for 2-way ANOVA and linear regression (black line – WT, dashed line – *Nr*, dotted line – control, dash-dotted line – over-irrigated) given.

Over-irrigation significantly reduced root xylem sap concentrations of calcium (by 41 %), magnesium (44 %), sodium (36 %), sulphur (34 %) and potassium (31 %) when compared to the well-drained control (Fig 5.4A and B). These trends were the same when nutrient delivery (the product of concentration and flow rate) was calculated, even though only Ca and Mg delivery were still significantly reduced (by 32 and 36 % respectively) in xylem sap of over-irrigated plants (Fig 5.4C and D).

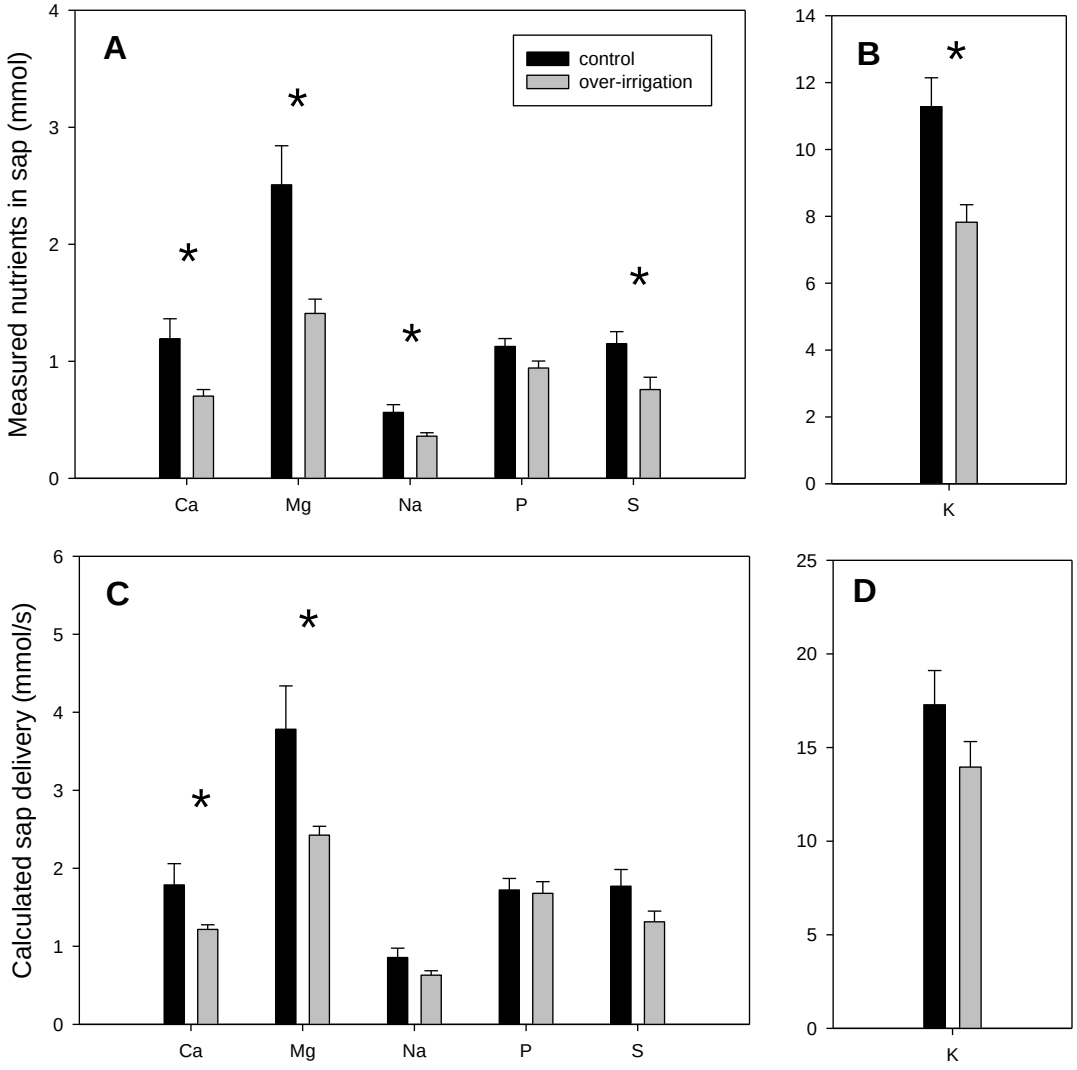

Fig 5.4 (A, B) Measured and calculated (C, D) calcium, magnesium, sodium, phosphorus, sulphur and potassium concentration/delivery in root xylem sap of over-irrigated (OI - grey bar) and well-drained (control, black bar) tomato plants. Data are means ± SE of 7-9 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, p-value < 0.05).

To determine whether physiological responses to over-irrigation could be ameliorated by improving plant nutrition, over-irrigated plants were irrigated with small volumes of either a 5 mM or 10 mM $Ca(NO_3)_2$ solution. Adding 10 mM $Ca(NO_3)_2$ restored shoot fresh weight of over-irrigated plants to control levels, whereas over-irrigated plants treated with 5 mM $Ca(NO_3)_2$ showed a statistically similar growth reduction as non-treated over-irrigated plants (Fig 5.5A). Similarly, total leaf area was highest in control and over-irrigated plants treated with 10 mM $Ca(NO_3)_2$ (90 % of control, Fig 5.5B). Over-irrigation decreased leaf nitrogen by 38 % compared to well-drained plants, but this effect was minimized by adding 5 and 10 mM $Ca(NO_3)_2$, such that leaf nitrogen decreased by 16 and 10 %, respectively (Fig 5.5C). Ψ_{leaf} did not significantly differ between the four treatments (Fig 5.5D). No significant differences in stomatal conductance were detected (Fig 5.5E), even though photosynthesis rate (Pn) was reduced by 31 % for over-irrigated plants, but only by 17 and 9 % for over-irrigated plants treated with 5mM and 10 mM $Ca(NO_3)_2$, respectively (Fig 5.5F). Foliar ABA concentration did not vary significantly between treatments (Fig 5.5G), but over-irrigation increased ethylene emission by 1.7 fold (even when 5 mM $Ca(NO_3)_2$ was applied to over-irrigated plants). Adding 10 mM $Ca(NO_3)_2$ to over-irrigated plants reverted ethylene emission to the levels of well-drained plants (Fig 5.5H).

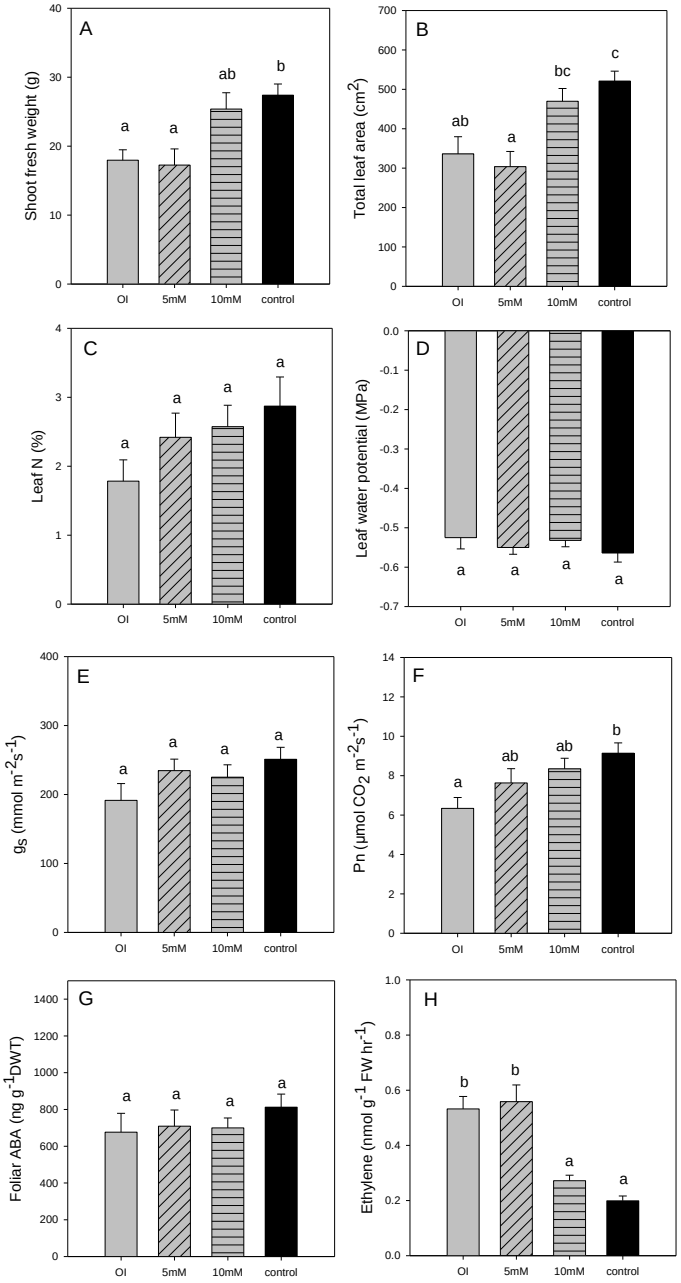

Fig 5.5 (A) Shoot fresh weight, (B) total leaf area, (C) leaf nitrogen concentration, (D) leaf water potential (Ψ_{leaf}), (E) stomatal conductance (g_s), (F) photosynthesis rate (Pn), (G) foliar abscisic acid (ABA) and (H) foliar ethylene evolution for over-irrigated (OI), over-irrigated + 5 mM $Ca(NO_3)_2$, 10 mM + $Ca(NO_3)_2$ over-irrigated and well-drained (control) tomato plants. Data are means ± SE of 4-30 replicates, different letters indicate significant differences between treatments (ANOVA, p-value < 0.05).

In the experiment that supplied $Ca(NO_3)_2$ to over-irrigated plants, higher g_s was highly correlated with higher photosynthesis rate ($P<0.0001$, Fig 5.6A). Although g_s was not correlated with either foliar ABA concentration (Fig 5.6B) or ethylene emission (Fig 5.6C), an increased ABA to ethylene ratio tended to correlate with increased g_s ($P=0.0733$, Fig 5.6D). Furthermore, increased ABA concentrations were correlated with decreased ethylene emission (Fig 5.6E, $P=0.0024$).

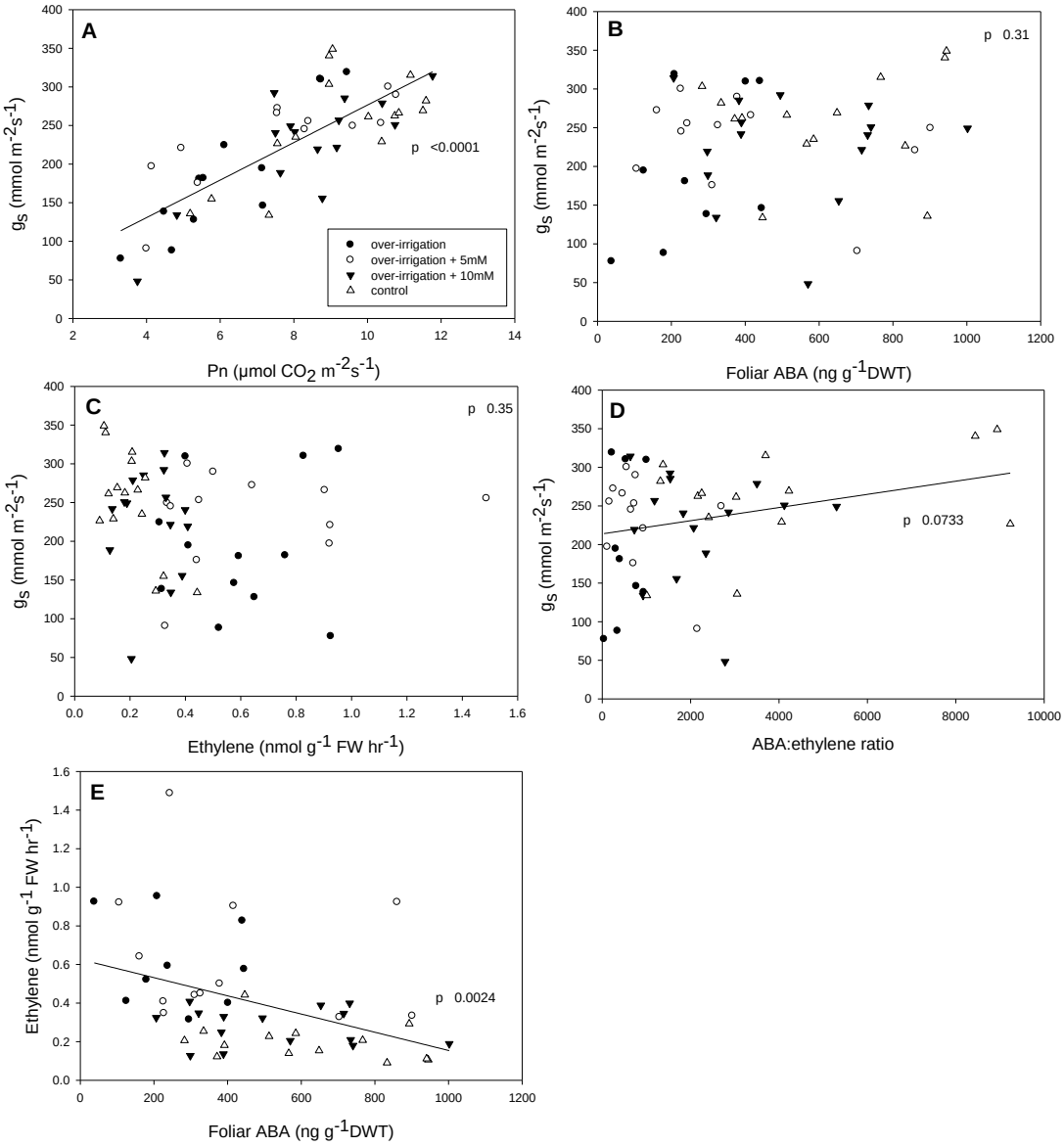

Fig 5.6 Relationship between stomatal conductance and (A) Pn, (B) ABA, (C) ethylene and (D) ABA to ethylene ratio, (E) ABA plotted against ethylene for over-irrigated (closed circle), over-irrigated + 5 mM $Ca(NO_3)_2$ (open circle), over-irrigated + 10 mM $Ca(NO_3)_2$ (closed triangle) and well-drained (open triangle) tomato plants. P-values for linear regressions (all data) are given.

As leaf nitrogen increased, shoot fresh weight (P=0.094, Table 5.2), foliar ABA concentration (P=0.0058) and ABA:ethylene ratio (P=0.0215) increased, whereas ethylene emission tended to decrease (P=0.081). There was no correlation between leaf nitrogen and Ψ_{leaf}, Pn and g_s (Table 5.2).

Table 5.2 Correlation matrix for leaf nitrogen concentration with shoot fresh weight, Ψ_{leaf}, g_s, Pn, foliar ABA concentration, foliar ethylene emission and ABA:ethylene ratio. P-values and R^2 for linear regressions are given.

	p-value	**R^2**
Shoot fresh weight	0.094	0.17
Ψ_{leaf}	0.19	0.11
g_s	0.26	0.08
Pn	0.41	0.04
ABA	0.0058	0.43
Ethylene	0.0811	0.18
ABA:ethylene	0.0215	0.32

5.4 Discussion

A constant nutrient availability is essential for optimal plant growth and development (Iqbal et al. 2013) and environmental stresses, such as waterlogging, can decrease the concentration of macronutrients in leaves (Gutierrez Boem et al 1996, Leyshon and Sheard 1974, Steffens et al. 2005) and xylem sap (Else et al. 1995a,b, Jackson et al. 1996). In the results presented here, over-irrigation did not significantly change leaf calcium, potassium, magnesium, sodium and phosphorus concentrations when compared to well-drained plants (Fig 5.2A and B). Measuring foliar nutrient concentration at different leaf levels (for example Leaf 1-3, Leaf 4-6 and so on) could indicate possible nutrient deficiency more sensitively than analyzing a mixture of all leaves, however, not enough dry plant material was available for such an analysis. Furthermore, loss of nutrients usually affects older leaves first, partly due to hindered supply from oxygen-stressed roots to shoots (Drew et al. 1979).

In contrast to foliar nutrient concentrations, over-irrigation significantly reduced total Ca and Mg delivery in root xylem sap (Fig 5.4C). Decreased xylem sap calcium concentrations might not have a key role in directly regulating responses to flooding, but can lead to calcium deficiency in shoot tissue (Marschner 1995, White and Broadley 2003). What caused reduced total calcium delivery from roots to shoots and whether it can be physiologically significant in this experiment remains unclear. Additions of only calcium to the soil could clarify its role during over-irrigation.

Furthermore, over-irrigation significantly decreased Mg delivery of root xylem sap (Fig 5.4C). Magnesium is the central atom of the chlorophyll molecule and therefore plays an essential role in regulating photosynthesis (Shaul 2002). However, magnesium deficiency does not impair photosynthesis per se, but rather decreases photosynthate export in the phloem, therefore resulting in accumulation of carbohydrates in the source leaves and feedback limitation of photosynthesis (Fischer and Bremer 1993) as well as decreasing root-shoot dry weight ratio (Marschner 1995). Possibly, Mg deficiency could play a role in decreasing photosynthesis rates (Fig 2.6B) and growth of over-irrigated plants. Furthermore, the results presented here suggest that measuring nutrient delivery in sap might detect a possible nutrient deficiency more sensitively than analysing foliar nutrient concentration.

Although xylem nitrogen fluxes were not measured, over-irrigation significantly decreased leaf nitrogen concentration by 32 % (Fig 5.3A). Critical levels of N in most plants are stated as ~3 % (Plank and Kissel 1999), yet over-irrigation decreased foliar N of wild type plants to less than 2 % (Fig 5.3A) and higher leaf nitrogen concentrations tended to correlate with increased shoot fresh weight within a genotype ($P=0.0604$, Fig 5.3C). Plants have high N requirements and nitrogen seems to be the most important nutrient for growth and developmental processes (Drew et al. 1979), possibly explaining why only nitrogen was significantly reduced during over-irrigation. Decreases in foliar nitrogen uptake can be induced through de-nitrification in waterlogged soil or because of reduced root N uptake (Hamonts et al. 2013). During flooding and low oxygen soil conditions, oxygen is replaced by nitrate as the terminal electron acceptor in microbial respiration, which leads to de-nitrification (Laanbroek 1990). Nitrous oxide analysis could indicate bacterial de-nitrification from over-irrigated soils (Jackson et al. 1984). Furthermore, bacterial nitrate reduction decreases

nitrogen availability in soils and induces competition for nitrate between root and bacteria (Drew 1988, Pelmont 1993). Decreases in nitrate reductase activity roots and shoots caused by flooding can reduce plant nitrate assimilation or the import capacity of amino acids from source organs, ultimately reducing plant growth (Alaoui-Sosse et al. 2005). These results can explain the nitrogen-induced growth inhibition seen in over-irrigated plants.

To evaluate the physiological impacts of a (possible) over-irrigation-induced nitrogen deficiency, some over-irrigated plants were supplemented with calcium nitrate applied to the substrate (as in Drew et al. 1979). A liquid application of N to the substrate was chosen, as foliar sprays of nitrogen (equalling 0.64 g/pot) did not increase pod fresh weight of waterlogged bush beans as much as applying nitrogen as a solution (same final concentrations and amounts) did (Reed and Gordon 2008). Previous experiments showed that supplying 5 mM $Ca(NO_3)_2$ daily to the soil restored leaf extension and shoot weight of waterlogged two days old barley plants (Drew et al. 1979). Although this $Ca(NO_3)_2$ concentration was ineffective at reversing the physiological effects of over-irrigation in tomato (likely due to greater N demands of tomato), a higher concentration (10 mM $Ca(NO_3)_2$) almost completely restored shoot fresh weight, total leaf area and leaf nitrogen status to the levels of well-drained plants (Fig 5.5A-C). As seen before, shoot fresh weight tended to positively correlate with leaf nitrogen concentration within a genotype ($P=0.094$, Table 5.2). While increased nitrogen uptake from the substrate is obviously important, transfer of mineral nutrients from older to younger leaves during waterlogging might also enhance growth and could be triggered by changes in hormonal concentrations (Drew et al. 1979).

Flooding can decrease Ψ_{leaf} during the first photoperiod, and then increase it during the second photoperiod (Else et al. 1995a), due to sequential changes in root hydraulic and stomatal conductance. In contrast, Ψ_{leaf} was similar in over-irrigated and control plants and did not change in response to $Ca(NO_3)_2$ supplementation (Fig 5.5D), suggesting that physiological responses to over-irrigation and nitrogen-induced growth recovery of over-irrigated plants are unlikely to be hydraulically regulated. Similarly, plant water potential in N-deficient cotton did not change and therefore was not the direct cause of reduced growth (Chapin et al. 1990).

$Ca(NO_3)_2$ supplementation did not significantly change g_s (Fig 5.5E) and even though higher foliar ABA concentrations were paradoxically correlated with increased g_s in Chapter 2 (Fig 2.9F), results here show no significant effect of foliar ABA on g_s (Fig 5.6B). In both experiments, ethylene emission did not significantly correlate with g_s (Fig 2.9H and Fig 5.6C), suggesting that g_s might be regulated by other factors apart from foliar ABA and ethylene concentrations during over-irrigation.

However, flooding can decrease photosynthesis (Bradford 1983b, Yordanova and Popova 2007), as in over-irrigated tomato plants (Fig 2.6B), possibly through decreased CO_2 assimilation rates of carboxylating enzymes and ribulose biphospate carboxlase (Rubisco) (Barneix and Causin 1996, Yordanova and Popova 2007). Stomatal behavior and photosynthetic capacity might be independently regulated, as photosynthesis rate was not reduced as much as g_s in flooded tomato plants (Bradford 1983b), even though opposite trends have been found in over-irrigated plants (Fig 2.5B and 2.6B). Although $Ca(NO_3)_2$ supplementation recovered photosynthesis (Fig 5.5F), g_s did not significantly differ between the four treatments (Fig 5.5E), suggesting

that changes in photosynthesis were not due to stomatal limitation, but could rather be attributed to reduced mesophyll activity (Ciompi et al. 1996).

Plant hormones such as ABA and ethylene might directly regulate expansive growth of over-irrigated plants independently of any effect on plant carbon gain. Foliar ABA concentrations did not change in response to nitrogen supplementation during over-irrigation (Fig 5.5G), consistent with the similar leaf water status of well-drained and over-irrigated plants (regardless of nitrogen supplementation). Although foliar ABA concentrations increased when tomato was grown in a nitrate-free nutrient solution (Chapin et al. 1988), a slowly developing chronic N deficiency did not alter foliar ABA concentration (Dodd 2003b). Further evidence that ABA is not involved in regulating shoot growth of N-deficient plants comes from studies with ABA-deficient mutants, which responded similarly to wild type plants in response to nitrogen deficit (Chapin 1990, Dodd 2003b). Thus, it seems unlikely that ABA mediates growth of over-irrigated plants and instead, changes in other phytohormones such as gibberellins, cytokinins or ethylene should be considered (Chapin 1990, Else et al. 2009).

Ethylene emission was always higher in over-irrigated plants when compared to the well-drained treatment (Fig 5.5H), in accordance with other studies of hypoxic and/or flooded plants. The effect of N-deficiency on ethylene production is still unclear. Whereas ethylene production decreased in maize roots when nitrate or phosphate was excluded from the nutrient solution (Rengel and Kordan 1988), N deficiency increased ethylene production of 5-day old wheat seedlings (Tari and Szen 1995). N-shortage might enhance plant sensitivity to ethylene, as N-deficiency promoted aerenchyma formation in maize roots (Drew et al. 1989). Supplying 10 mM $Ca(NO_3)_2$ daily to the

soil of over-irrigated plants restored ethylene concentrations back to levels of well-drained plants (Fig 5.5H), and as leaf nitrogen concentration increased, ethylene emission tended to decrease in wild type plants (P=0.0811, Table 5.2). It remains unclear whether (and why) nitrogen and ethylene are linked to each other, for example due to an active signalling system or a passive feedback system through changes in nitrogen assimilatory pathways during over-irrigation. However, significantly reduced foliar nitrogen concentrations even in the partial ethylene-insensitive *Nr* genotype (Fig 5.3B) suggest that instead of the plant metabolism being responsible for decreased leaf nitrogen concentration, over-irrigation reduces available nitrogen in the soil and therefore causes foliar nitrogen deficiency and growth inhibition. Because the slope of the relationship between shoot fresh weight and leaf N did not differ in wild type and *Nr* plants (P=0.967, Fig 5.3C), it seems unlikely that ethylene is the key growth regulator of over-irrigated tomato plants.

5.5 Conclusion

Over-irrigation significantly reduced xylem calcium and magnesium delivery to the leaves, but did not significantly change foliar Ca, Mg, K, P or Na concentration, suggesting that xylem sap analysis indicates current nutrient uptake, while foliar concentrations possibly reveal nutrient uptake over the life of the plant. Although over-irrigation induced foliar nitrogen deficiency, daily supplementation of small volumes of 10 mM $Ca(NO_3)_2$ to over-irrigated soil did not significantly change Ψ_{leaf}, g_s or ABA concentration, but restored foliar nitrogen concentrations, ethylene emission and shoot fresh weight and total leaf area of over-irrigated plants to control levels, suggesting that N deficiency is the principal cause of growth limitation during

over-irrigation. Similar relationships between shoot fresh weight and leaf nitrogen concentration in the wild type and the partial ethylene-insensitive genotype *Nr* make it unlikely that ethylene is the principal growth regulator during over-irrigated soil conditions.

Chapter 6.

6 Concluding remarks

‘Hormones are what physiologists call hormones.’

(Leopold 1987)

‘Plant hormones are what we eventually find them to be.’

(Weyers and Paterson 2001)

6.1 Growth regulation of plants exposed to abiotic stress

In contrast to animals, plants are sessile organisms and therefore have to adapt to changes in their environment. Plants are also able to repeatedly cease and resume growth, and phytohormones (also called growth regulators) are involved in determining almost every aspect of plant growth and development and responses to stress conditions. One hormone can regulate many different processes, but multiple hormone interactions can also influence just one process (Gray 2004). For example, the phytohormone ethylene is involved in regulating seed germination, flower initiation, fruit ripening, tissue senescence and organ abscission (Abeles et al. 1992). While it is well-known that ABA regulates stomatal opening, jasmonates (Suhita et al. 2003) and brassinosteroids (Haubrick et al. 2006) can also promote stomatal closure under water deficit. During drought, ABA can interact synergistically with jasmonic acid to promote stomatal closure (Daszkowska-Golec and Szarejko 2013).Cytokinins (Tanaka et al. 2006) and auxins (Lohse and Hedrich 1992) can induce stomatal opening in low concentrations, whereas high concentrations inhibit it. Furthermore, both cytokinins and auxins can antagonise ABA-induced stomatal closure (Dodd 2003a). Lastly, ethylene can stimulate both closing and opening of the stomata (Nemhauser et al. 2006).

To cope with abiotic stresses, plants can change their phytohormone concentrations, which affect developmental processes such as plant growth (Finkelstein et al. 2002). However, plant responses to stress occur at different scales of organization (cellular, tissue and organ) and it is questionable whether growth regulation can be attributed to only one single factor. During soil drying, root growth is usually less inhibited than shoot growth (Sharp and Davies 1989), likely because roots can elongate even at low

water potentials whereas shoot growth is completely inhibited. In maize primary roots grown under low water potential, turgor was maintained (albeit lower than in well watered plants) throughout the growing zone, suggesting that enhanced cell wall loosening in specific tissues was important in allowing growth (Sharp et al. 2004). Phytohormones can regulate cell wall loosening (Cosgrove 2000) and ABA accumulation can play an important regulatory role during root growth in drying soil (Sharp 2002) by limiting ethylene production to prevent further growth reduction (Spollen et al. 2000). In ABA-deficient mutants, ABA accumulation in roots at low water potentials is insufficient to prevent ethylene-induced growth inhibition (Sharp 2002). Furthermore, ethylene inhibitors increased root elongation of ABA-deficient mutants when grown at low water potential (Sharp 2002) and ABA accumulation influences auxin transport in the root tip to maintain root growth at low tissue water status (Xu et al. 2013). Therefore measuring multiple phytohormones within growing tissues seems necessary to explain growth of primary roots under water deficit.

Since there has been little work on growth regulation of plants gradually exposed to over-irrigation for a prolonged period (in contrast to experiments that have abruptly flooded the entire root system), initial studies sought to understand both the environmental and endogenous factors regulating growth. Four weeks of over-irrigation significantly reduced shoot fresh weight, height and total leaf area (Table 2.2), but did not drastically alter soil oxygen concentration and temperature, apparently excluding these variables as the cause(s) of growth inhibition. Furthermore, in contrast to flooding, over-irrigation did not alter leaf water potential, stomatal conductance, or bulk ABA concentration, suggesting that hydraulic mediation of growth was unlikely. Furthermore, shoot fresh weight was not correlated with Ψ_{leaf}, g_s or foliar ABA concentration (Fig 2.10A, B and D). However, shoot ethylene emission

increased in both flooded and over-irrigated plants and higher shoot fresh weight tended to correlate with increased Pn, ABA/ethylene ratio and ethylene (Fig 2.10C, F and G) in over-irrigated plants. Thus both carbon capture and/or phytohormones may be involved in regulating shoot growth of over-irrigated tomato, making it difficult to attribute growth-reduction to only one (hormonal) factor (Chapter 2).

In trying to understand growth regulation of over-irrigated plants, the value of single leaf measurements at specific times (the sampling strategy within Chapter 2) has to be critically assessed. To avoid diurnal rhythms affecting physiological parameters such as Ψ_{leaf}, g_s, Pn or hormone concentrations, these measurements were always performed on the same time of the day and all samples were taken within a short time period (within 1-3 hours). Diurnal changes occur in flooded plants, where photosynthesis rate of tomato decreased during the first few hours after flooding, but only dropped significantly below those of well-drained plants after 24 to 28 h of flooding (Else et al. 2009). Furthermore, Ψ_{leaf} decreased within 4 to 7 hours of a 12 h photoperiod from -0.55 MPa to -0.80 MPa, but recovered and exceeded those of well-drained plants towards the end of the photoperiod (Else et al. 2009). These results suggest that individual parameters and relationships between variables change according to time of day. While sampling should occur within as short a period as possible at the same time each day, correlations may change according to the time of day, limiting the usefulness of this approach.

Even though the over-irrigation treatment lasted between three to four weeks, most physiological parameters, such as g_s, ABA and ethylene were frequently measured (every 2-4 days) as soon as enough plant material for analysis was available. However, total leaf area was only measured at the end of the experiment rather than

daily measurements of leaf growth. High resolution growth measurements (e.g. daily) might better correlate with foliar hormone concentrations (Dodd and Davies 1996, Thompson et al. 2007a), but are difficult to achieve in dicotyledons (e.g. tomato) due to pronounced spatial and temporal gradients in leaf expansion (Walter et al. 2009).

Furthermore, due to their destructive nature, some measurements such as leaf and root water potential, xylem sap pH and hormone analysis, as well as foliar and sap nutrient concentrations, could only be undertaken once at the end of the treatment period. Therefore, these measurements might not be representative of changes occurring throughout the treatment period and may disguise significant initial treatment differences. Furthermore, the lack of treatment differences at the end of the treatment period may indicate that plants might have adjusted to over-irrigated conditions. Long-term flooding (for 35 days) transiently increased foliar JA concentration in citrus plants until Day 11 of the flooding treatment (Arbona and Gomez-Cadenas 2008), but dropped back to levels of control plants after this date. Therefore higher resolution studies about possible transient phytohormone changes in herbaceous plants during long-term over-irrigation seem necessary.

Furthermore, the time-consuming nature of some analyses means they cannot be performed on all leaves, thus young expanding leaves were selected for ABA and ethylene analysis. However, effects of over-irrigation on leaf growth were consistent throughout development (Fig 2.3), justifying the use of leaves at any node for physiological measurements. In contrast, g_s and Pn had to be measured on older leaves, as the available leaf gas exchange cuvette required a minimum leaf area (2 cm^2). It is questionable whether correlations between leaf gas exchange and foliar hormone concentrations indicate potential regulatory processes. Vertical gradients in

foliar hormone concentrations within the canopy exist in trees, with higher concentrations of leaf cytokinins found in the upper canopy of mature sugar maples (Reeves et al. 2007). However, stomatal conductance did not vary, suggesting that stomatal behaviour was decoupled from hormonal regulation (Reeves et al. 2007). Simultaneous measurements of plant hormones and gas exchange on different leaves in herbaceous plants are necessary to fully understand possible regulation of leaf gas exchange by hormone signalling.

Jacobs' rules for hormone action critically evaluate whether a hormone controls a given developmental process (Jacobs 1959). In reviewing these rules, Jackson (1987) suggested that observing simultaneous changes in development and hormone concentration is necessary to substantiate a case for hormonal involvement in a given process. Furthermore, Jackson proposes duplication, deletion and re-instatement of the hormone(s) of interest as essential criteria in determining its significance in influencing physiological responses. These responses should be reproduced or annulled through manipulating (adding or removing/ decreasing) internal hormone concentrations (Jackson 1987). In attempting to satisfy these criteria and substantiate the argument that ethylene evolution limited growth of over-irrigated plants, the ethylene-insensitive tomato mutant *Nr* was grown in over-irrigated soil.

Over-irrigating the partial ethylene-insensitivite genotype *Nr* did not lead to such a dramatic growth inhibition as in the wild type (Table 3.1 and Fig 3.3), suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to some extent (Chapter 3).

While the genetic material used to test the relationship betwene ethylene and growth differed between Chapter 3 (*Nr*) and previous literature ($ACO1_{AS}$; Hussain et al. 1999; Sobeih et al. 2004), there are clear parallels between the nature of this response. In compacted soil, leaf expansion and shoot growth of tomato was more closely correlated to endogenous ethylene concentration than with xylem sap ABA concentration. A transgenic ($ACO1_{AS}$) tomato genotype, which has a reduced capacity to produce ethylene, did not show reduced growth when part of the root-zone was exposed to compacted soil in a split-pot treatment. However, growth was reduced in the wild type (WT), which was accompanied by increased foliar ethylene evolution, suggesting ethylene levels inhibited shoot growth (Hussain et al. 1999). Furthermore, when WT tomatoes were grown with part of their roots in drying soil, leaf growth inhibition was temporally correlated with foliar ethylene evolution. Soil drying decreased leaf growth in wild type tomato plants, but not in an ACC oxidase (ACO) antisense genotype (Sobeih et al. 2004).

Given the apparent importance of ethylene in regulating shoot growth of over-irrigated tomato, further studies sought to establish whether the ethylene precursor ACC acted as a root-sourced signal of over-irrigated soil. Although ACC was not found in root xylem sap in this study due to technical difficulties, over-irrigation increased ACC concentration in leaf xylem sap, which might be sufficient to increase ethylene emission from shoots of over-irrigated tomato plants. In accordance, increased ACC export from flooded tomato roots could be accounted for higher ethylene production in shoots (compared to well-drained plants) and occurred at the same time as ACC accumulation in leaves (Else and Jackson 1998). However, leaf xylem sap ACC concentration was not correlated with shoot fresh weight (Fig 4.5A),

suggesting that other root-sourced phytohormones might be involved in regulating other processes.

No plant hormone found in the root xylem sap was correlated with shoot fresh weight, but higher shoot fresh weight correlated with increased *t*Z, GA3, ABA and JA leaf xylem sap concentration (Chapter 4). Salinity reduced xylem sap cytokinin and auxin concentration in tomato and both hormones (and their ratios) were correlated with shoot growth and leaf area (Albacete et al. 2008). Xylem sap ABA and ACC concentrations were not correlated with tomato leaf biomass during salinity, but the xylem sap zeatin (cytokinin) concentration was positively correlated with leaf weight and leaf area (Albacete et al. 2009). However, it is difficult to dissect possible independent effects of plant hormones on over-irrigation (or other abiotic stresses) in the absence of further experiments. Thus over-irrigating rootstocks that export different levels of the hormone of interest, or scions with different sensitivity to these plant hormones, will be necessary to test putative relationship between xylem hormone concentrations and growth.

More advanced statistical techniques (than the simple correlations used in Chapters 2, 3) seem necessary to unravel the putative physiological significance of different phytohormones when multiple changes in xylem sap composition occur. A principal component analysis (PCA) identifies directions (principal components), which are linear combinations of the original variables and along which the variation in the data is maximal (Joliffe 2002). As a mathematical algorithm, it provides an appropriate technique to understand hormone profiling experiments even with a low sample size. To gain further understanding about the contribution of hormonal and ionomic xylem sap constitution to shoot fresh weight, a PCA was performed (Fig 6.1A and B). Leaf

xylem *t*Z, JA, ABA, GA3 and SA were strongly and positively loaded into the first principal component determining shoot fresh weight (Fig 6.1A), whereas ACC, IAA and iP were negatively correlated. This fortifies the significant correlations between shoot fresh weight and *t*Z, GA3; ABA and JA (Fig 4.5B, D, F and H). A PCA for root xylem sap revealed that all plant hormones and nutrients analysed were within the same cluster apart from iP (Fig 6.1B). This suggests little discriminatory value of the PCA and root xylem sap hormone concentration might not help explaining over-irrigation induced growth inhibition. Thus measuring xylem sap hormone composition as close as possible to the sites of action (eg. growing leaves) provides a better indication of possible physiological significance of hormone action. Nevertheless, analysis of other compounds, specifically xylem sap and foliar nutrient status, may also be valuable in explaining physiological responses.

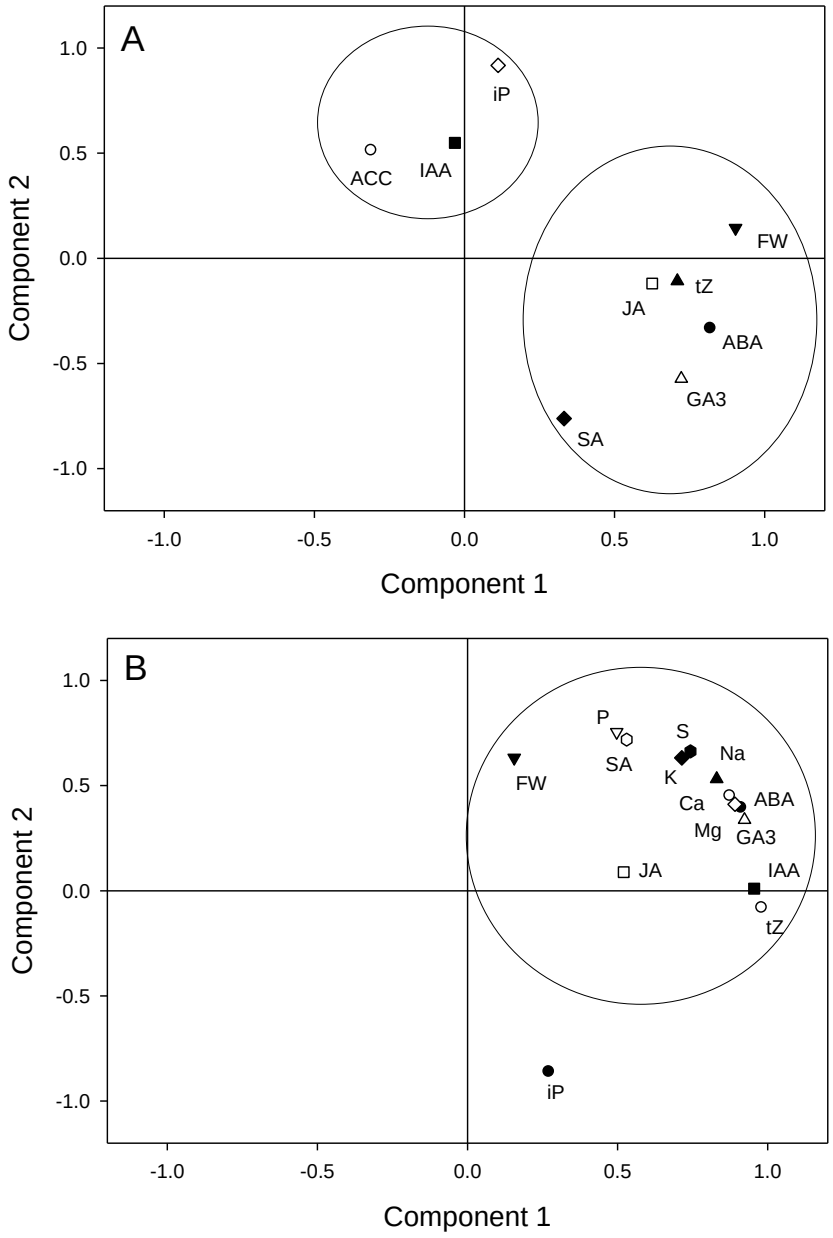

Fig 6.1 Two axes of a principal components analysis (PCA) showing shoot fresh weight (FW) and the position of various hormonal and ionomic variables (denoted by abbreviations) in (A) leaf xylem sap and (B) root xylem sap for over-irrigated and well-drained tomato plants. The circles enclose those variables that fall into the same cluster (95 % confidence level).

Waterlogging can change soil nutrient availability and plant nutrient uptake. Flooding of barley for seven days reduced foliar N, P and K concentrations (Leyshon and Sheard 1974) and waterlogging *Brassica napus* L. for 7 or 14 days reduced N, P, K and Ca uptake (Gutierrez Boem et al 1996). Decreased N, P, K, Mg, Cu, Zn and Mn concentrations were found in wheat and barley shoots after 15 days of waterlogging (Steffens et al. 2005). To cope with nutrient deficiencies, plants have evolved a signalling cascade which involves plant hormones (Rubio et al. 2009). Cytokinins can act as a long-distance signal in nitrate re-supply (Rahayu et al. 2005) and nitrogen deficiency can lead to shoot ABA and root IAA accumulation (Teplova et al. 1998).

Nitrogen for plants is mainly available as NO_3^- and NH_4^+ and has been suggested to have a signalling role in triggering plant growth responses. Leaf growth of hydroponically grown tomato increased through NO_3^- application to NH_4^+-pre-cultured plants (Rahayu et al. 2005). However, foliar and xylem sap ABA concentrations did not correlate with leaf growth rates of wild type plants. Despite excessive foliar ethylene production in the ABA-deficient mutant *flacca*, NO3- supply stimulated leaf growth, suggesting that neither ABA nor ethylene are directly regulating the effect of N supply on tomato leaf growth (Rahayu et al. 2005). Leaf growth was correlated with increased xylem and foliar concentrations of cytokinins, suggesting a role as long-distance signal (Rahayu et al. 2005), but more work would be necessary to understand the interactions between nitrogen and phytohormones on growth regulation, especially during environmental stress.

Over-irrigation significantly reduced xylem calcium and magnesium delivery to the leaves, but did not significantly change foliar Ca, Mg, K, P or Na concentration, suggesting that xylem sap analysis is a more sensitive indicator of instantaneous nutrient deficiency, while foliar concentrations integrate nutrient uptake over the life of the plant. Furthermore, over-irrigation significantly decreased leaf nitrogen concentration (Fig 5.3A) while supplementary application of small volumes of 10mM $Ca(NO_3)_2$ restored shoot fresh weight, total leaf area, leaf nitrogen status and ethylene concentration close to the levels of well-drained plants, and shoot fresh weight was positively correlated to leaf nitrogen concentration (Chapter 5). Calcium nitrate application therefore seems to be a viable mitigation strategy in horticulture, even though this should not be necessary if appropriate automatic irrigation according to the plants' actual water needs is used.

Taken together, the results presented herein indicate there is considerable complexity in the regulation of shoot growth, which depends on the interaction of multiple signals and physiological responses over different time-scales. It is unlikely that growth during environmental stress conditions can be attributed to only one causal factor. Interpreting hormone-growth relationships needs to separate cause and effect (Ober and Sharp 2007): Do altered environmental conditions directly regulate growth (cause) thereby affecting hormone concentrations (effect) or do altered environmental conditions directly regulate hormone concentrations (cause) thereby directly affecting growth (effect)? However, it is difficult to separate cause and effect and possibly, only molecular analysis of interactions and links between plant hormones, genes and growth-related process will provide clarity. Similar relationships between shoot fresh weight and leaf nitrogen concentration in the wild type and the partial ethylene-insensitive genotype *Nr* (Fig 5.3C) make it unlikely that ethylene is the principal

growth regulator during over-irrigated soil conditions. Therefore, it seems plausible that nitrogen deficiency can directly limit growth during over-irrigation.

Even though significant relationships between some analysed parameters and shoot fresh weight were found, it is difficult to attribute over-irrigation-induced growth inhibition to one factor. The most obvious finding of this work is foliar nitrogen deficiency in over-irrigated plants, which was ameliorated through $Ca(NO_3)_2$ supplementation of over-irrigated plants. Lower oxygen concentrations in the soil, for example through over-irrigation, can lead to bacterial de-nitrification, making nitrogen unavailable for plant uptake (Laanbroek 1990). Because no measurements of xylem nitrate concentrations were made (ICP-OES analysis uses nitric acid, so nitrate cannot be detected), it is not certain whether nitrogen uptake was inhibited. Soil compaction significantly decreased leaf xylem nitrate concentrations (measured spectrophotometrically according to the procedure of Larsson et al. 1989) in barley plants and this decrease was strongly correlated with reductions in leaf area, suggesting that nitrate might play an important role in root-to-shoot signalling and mediating effects of environmental stresses on plant growth (Mulholland et al. 1999). Since decreased nitrate reductase activity in roots and shoots can reduce plant nitrate assimilation or the import capacity of amino acids from source organs during flooding, ultimately reducing plant growth (Alaoui-Sosse et al. 2005), additional measurements of nitrate reductase activity may give more insight into their role during over-irrigation.

A major challenge for plant biologists will be to improve plant performance under less favourable environmental conditions (O'Brien and Benkova 2013). Understanding interactions between phytohormones during developmental processes and stress

conditions may help improve plant growth in horticultural systems. It is still unclear why plants have evolved multiple hormone response pathways for regulating the same process (Depuydt and Hardtke 2011). Possibly, plants can achieve a more flexible development and better fitness (Depuydt and Hardtke 2011), but a more thorough study of hormone responses and interactions both under non-stressed and stressed conditions is necessary to understand their role during growth and cell elongation.

Because peat based substrates contain relatively few bacteria, reduced leaf nitrogen concentrations cannot only be attributed to denitrification. Instead, experiments presented here suggest that hypoxia and reduced nitrogen uptake negatively affect metabolic and transport processes, explaining the mitigation of shoot growth reduction when tomato plants were supplied with calcium nitrate fed as a solution to the roots under low oxygen conditions.

6.2 Key findings and ideas for the future

Over-irrigation is much more relevant to nurseries, as flooding is rather unlikely to occur. In contrast to the extensive literature on adaptive features of plants to waterlogging/flooding (over-wet soils primarily involving flooding with excess water), this thesis takes a more applied approach, providing new insights on understanding the effects of chronic over-irrigation on tomato plant growth and physiology. They key findings are out-lined below:

- Over-irrigating tomato plants for four weeks significantly reduces fresh weight and total leaf area compared to well-drained plants. Short-term flooding induces more pronounced changes in soil oxygen concentration than chronic over-irrigation does. In contrast to flooding, over-irrigation does not alter

stomatal conductance, leaf water potential or foliar ABA concentrations, suggesting that over-irrigation induced growth inhibition is not hydraulically regulated or dependent on stomatal closure or changes in ABA. (Chapter 2 – *Comparing effects of acute flooding and chronic over-irrigation on soil properties and growth and physiological responses of tomato*)

- Over-irrigation significantly increased foliar ethylene emission from shoot. However, over-irrigating the partial ethylene-insensitivite genotype *Nr* did not lead to such a dramatic growth inhibition as in the wild type, suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to some extent. (Chapter 3 – *Partial ethylene-insensitivity reverses over-irrigation-induced growth inhibition to some extent*)

- The ethylene precursor ACC increased in leaf xylem sap of over-irrigated plants and could be sufficient for extra foliar ethylene produced during over-irrigation. Decreased xylem ABA delivery might be responsible for the lack of ABA accumulation in leaves of over-irrigated tomato plants and why no effect on stomatal conductance was observed. Shorter stems of over-irrigated plants might be explained by reduced gibberellin root-to-shoot delivery. (Chapter 4 – *Influence of over-irrigation on phytohormonal root-to-shoot signalling*)

- Over-irrigation induced significant foliar nitrogen deficiency and daily supplementation of small volumes of 10 mM $Ca(NO_3)_2$ to over-irrigated soil restored foliar nitrogen concentrations, ethylene emission and shoot fresh weight and total leaf area of over-irrigated plants to control levels, suggesting that reduced nitrogen uptake plays an important role in over-irrigation induced

growth inhibition. (Chapter 5 – *Over-irrigation decreases xylem nutrient and foliar nitrogen concentrations*)

- The findings of this thesis suggest that regulation of shoot growth is complex and depends on the interaction of multiple signals and physiological responses over different time-scales. It is unlikely that growth during environmental stress conditions can be attributed to only one causal factor. (Chapter 6 – *Concluding remarks*)

Future work could mainly focus on the likely impact of current irrigation management on crop quality and retail value and whether the system used in this thesis would be of improvement, if used in a larger scale. This could be implemented through:

- Meetings with farmers/greenhouse or nurseries managers to understand their needs and interests in research in the irrigation area
- Deploying soil moisture sensors in greenhouse and nurseries to directly assess irrigation habits and management
- Usage of different species (e.g. herbs or ornamental plants) or substrates (e.g. rockwool or coir)

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