Final Report

CP 81

Detection and amelioration of root-zone ethylene production in protected crops

Project title	Detection and amelioration of root-zone ethylene production in protected crops
Project number:	CP 81
Project leader:	Dr Ian Dodd Lancaster University
Report:	Final Report Year 3, December 2014
Previous report	Annual Report Year 2, October 2013
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Location of project:	Lancaster Environment Centre
Project coordinator:	Debbie Wilson, HDC
Date project commenced:	1 October 2011
Date project completed	18 November 2014
Key words:	Over-irrigation, root-zone ethylene, tomato, nitrogen deficiency, soil moisture sensors

The results and conclusions in this report are based on investigations conducted over a 3 year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Dr Antje Fiebig

Former PhD Student

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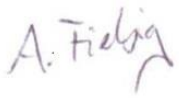
Signature:  Date: 15/12/2014

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GROWER SUMMARY

Headlines

- Automatic irrigation via soil moisture monitoring provides a useful tool to water plants according to their actual water needs, and may decrease variation in soil moisture compared to hand watering.
- Over-irrigating containerised pot plants grown in a peat-based substrate significantly reduces crop fresh weight, height and leaf area.
- Over-irrigation significantly decreases leaf nitrogen concentration, and adding small doses of calcium nitrate to over-irrigated soil can ameliorate over-irrigation-induced foliar ethylene production and growth inhibition.

Background and expected deliverables

Watering in ornamental nurseries may not be especially well controlled, as irrigation can still be based mainly on the grower's experience. It is possible to misjudge the plant's actual needs, causing under- or over-irrigation which impacts on crop quality. Although the effects of flooding (acute, short-term stress) on plant growth and stomatal behaviour have been well studied, effects of suboptimal soil aeration caused by over-irigation (chronic, long-term stress) have not, despite its likely commercial significance.

Even though flooding limits photosynthesis, growth and yield, the mechanisms behind these effects are not completely clear. Changes in foliar concentrations of plant hormones like ethylene or abscisic acid (ABA) could act as signals and initiate plant physiological responses to flooding. Furthermore, flooding causes changes in the soil environment, especially lack of oxygen (hypoxia) and roots are the first organs to sense these changes. Therefore, ethylene produced in the root-zone might be an important factor in plant sensing of stress. Excessive ethylene production can cause flower and foliage senescence and abscission, and limit yield and quality of protected crops. Until now, root-zone ethylene production has not been measured and its role in plant response has not been assessed. Flooding can also change the concentration of mineral nutrients in plants, but the impact of over-irrigation on nutrient deficiency (which may also stimulate foliar ethylene emission) and possible growth amelioration through adding nutrients to the soil are not clear.

This project aims to:

- Determine if automatic irrigation scheduling according to soil moisture is a useful tool to irrigate plants according to their actual water needs,
- assess whether short-term (flooding) and long-term stresses (over-irrigation) induce different changes in soil properties and plant physiology,

- understand the effects of excessive soil moisture (over-irrigation) on plant growth and physiology,
- understand the physiological mechanism(s) causing growth reduction induced by over-irrigation, which may help design mitigation strategies, and
- exploit recent developments in ethylene measurement technology.

Summary of the project and main conclusions

Plants automatically irrigated according to defined soil moisture thresholds (feedback irrigation control based on continuous soil moisture monitoring) showed less variation in soil moisture than hand watered plants, which showed (alternately) insufficient and excessive soil moisture. Feedback irrigation control was implemented in a controlled environment room, with different numbers of drippers per pot allowing different soil moisture treatments (over-irrigation vs well-drained control). Furthermore, effects of flooding as an acute stress and over-irrigation as a chronic stress on plant physiology and soil properties were compared. Short-term flooding induces more pronounced changes in soil oxygen concentration than chronic over-irrigation does. Over-irrigating tomato plants for four weeks significantly reduces fresh weight and total leaf area compared to well-drained plants. 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. Although over-irrigation significantly increases foliar ethylene emission and the ethylene precursor ACC increases in leaf xylem sap of over-irrigated plants, root-zone ethylene production does not differ between well-drained and over-irrigated tomato plants. However, over-irrigating the partially ethylene-insensitivite genotype *Never ripe* (*Nr*) does not inhibit growth as much as in the wild type, suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to some extent. Furthermore, over-irrigation decreased foliar nitrogen concentration and daily supplementation of small volumes of 10 mM $Ca(NO_3)_2$ to over-irrigated soil restores foliar nitrogen concentrations, ethylene emission and shoot fresh weight and total leaf area of over-irrigated plants to control levels. Thus decreased plant nitrogen uptake plays an important role in over-irrigation-induced growth inhibition.

Financial benefits

It is difficult to assess the full impact of over-irrigation on the “hidden” costs (to growers) of decreased crop quality causing wastage prior to offering plants for retail. Nevertheless, 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 (prices correct 2014)), successful implementation of automatic irrigation scheduling according to soil moisture can decrease labour costs involved in hand-watering

as well as costs for excessive water and energy. Further work is needed to assess the likely impact of such irrigation treatments on crop quality and retail value.

Action points for growers

- To note that automatic irrigation scheduling regulated by soil moisture sensors can adequately irrigate plants according to their actual water needs.
- To note experimental results which show that over-irrigation severely decreases crop biomass.
- To note that over-irrigation induces foliar nitrogen deficiency, which may limit foliage quality (of bedding plants).

SCIENCE SECTION

Introduction

Heavy rainfall, poor drainage or irrigation practices can induce waterlogged soil or flooding, 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 flooding 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). 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. ABA accumulated in leaves only hours after flooding (Bradford and Hsiao 1982, Else et al. 1996, Jackson et al. 1978, Zhang and Davies 1987). 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, the ethylene pre-cursor 1-aminocyclopropane-l-carboxylic acid (ACC) seems to play an important role in root-to-shoot signalling during flooding, as 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). Interestingly, the role of ethylene produced in the root-zone has not yet been studied.

Flooding can also change soil nutrient availability and plant nutrient uptake. Flooding of barley for seven days reduced foliar N, P and K concentrations by 51, 60 and 58 %, respectively (Leyshon and Sheard 1974) and flooding rape 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 flooding (Steffens et al. 2005). Whether changes in plant nutrient status occur following over-irrigation has not yet been studied.

Flooding can constrain crop yields in open field agriculture (FAO 2011), 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. 2007).

Thus, the aims of this work are:

- To understand whether automated irrigation scheduling via soil moisture sensors provides a useful tool to water plants according to their actual needs.
- To examine whether the above mentioned physiological responses to soil flooding also occur in over-irrigated plants.
- To identify the underlying mechanism(s) of a possible growth inhibition due to over-irrigation

Materials and methods

Monitoring manual watering habits

A Delta-T DL6 Data Logger with three ML2x ThetaProbes soil moisture sensors was installed at a commercial nursery to monitor soil moisture of three potted Coleus (*Solenostemon*) plants watered by hand according to personnel experience (Figure 1).

Figure 1 Soil moisture sensors installed at a commercial nursery to understand how manual irrigation of potted Coleus (*Solenostemon*) affects soil moisture

Plant material

Tomato (*Solanum lycopersicum* Mill. cv Ailsa Craig) was used as a model species, as it is one of the most important greenhouse vegetables, grows rapidly from seeds and offers a range of different genotypes to test the physiological significance of various metabolic pathways. Seeds were sown individually in 1 cm diameter plastic pots filled with Levington M3 compost, Scotts Company (Ltd), UK (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.

Irrigation setup and soil moisture monitoring

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 (Figure 2).

Figure 2 Experimental set-up

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 1.

Table 1 Sampling times for ABA, ethylene, stomatal conductance (g_s), photosynthesis rate (Pn), leaf water potential (Ψ_{leaf}), 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

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.

In another set of experiments, the partial ethylene-insensitive genotype *Never ripe* (*Nr*) in the 'Ailsa Craig' background was used. To verify relative ethylene-sensitivity of *Nr* and wild type (WT) 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 %). Furthermore, the classical "triple response" to ethylene was seen in the wild type, but *Nr* showed normal hypocotyl extension.

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 (θ_v) over time (Figure 6E). Soil oxygen concentration and soil temperature in the middle of the soil profile 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).

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, 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 another set of experiments, 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 previous experiments, plants for root xylem sap collection were grown in special pots which fit into a Scholander pressure vessel (23 cm height x 6.5 cm diameter, 632 ml volume, Figure 4.1). Plants were grown under the two different irrigation treatments (well-drained and over-irrigated) described earlier and harvested after only 3 weeks of treatment due to the smaller pot volume. To match sap flow with evapotranspiration, 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), 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, frozen in liquid nitrogen and stored at -80°C for further analysis. Samples were collected between 09:00 h and 12:00 h (3 to 6 hours after the beginning of the photoperiod).

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.

Plant hormone analysis

Bulk leaf ABA concentrations was measured on the youngest fully expanded leaflet (10 – 15 mg dry weight, DW) using a radioimmunoassay (RIA) using the monoclonal antibody, AFRC MAC 252 (Quarrie et al. 1988).

Ethylene evolution rate was measured on the same leaves (but different leaflets) as sampled for ABA analysis. Each sample was immediately placed in a 28 ml glass vial with moist tissue, which was then sealed with a rubber puncture cap (Pyrex). After incubating for 60 min under light (determined precisely for each measurement), 4 ml of the air in the glass vial was stored in evacuated 3.7 ml soda glass vials (so-called exetainers, Labco Ltd., High Wycombe, UK). Samples were then stored at 4 °C (for no longer than four weeks) until injection and analysis via gas chromatography (GC).

The ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was extracted and purified according to the method of Dobrev and Kaminek (2002) and analysed via HPLC-MS according to Albacete et al. (2008). For quantification of ACC, calibration curves were constructed (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 ACC was 0.1 µg l^{-1}. ACC delivery in root xylem sap was calculated as the product of ACC concentration and sap flow rate.

Root-zone ethylene measurement

To detect ethylene produced in the root-zone online, a portable ethylene analyser EASI-1 (Figure 3) was used (Absoger Atmosphere Controlee, Les Barthes, France). Ethylene oxidises on a gold electrocatalyst which produces an amperometric signal. The analyser samples at a flow rate ~250 ml/min via an internal pump with a fixed flow rate and the user can specify the sampling time interval. Furthermore, a multi-position microeletric valve (multiplexer) was used (10 multi-position dead-end path, integrated actuator/RS-232, valve diameter 1/4"-28*.75mm, VICI Valco Instruments Co. Inc., Houston, USA). This multiplexer, connected via USB with a PC (software Microelectric actuator, Thames Restek UK Limited, Saunderton, UK), can switch between different channels, where each channel represents one plant or surrounding air (control value). The user can again specify the sampling time interval and which channels to choose through the software. The outlet of the ethylene

analyser is connected with a PTFE tube (outer diameter 6.35 mm x inner diameter 3.18 mm 、Polyflon, Staffordshire, UK) to the main multiplexer outlet.

For measurements, the analyser was calibrated with 20 ppm ethylene/nitrogen gas (BOC Gases, UK). The surface of each plant pot was covered with cling film to assure that the sampled air came directly from the root-zone (Figure 4). Polyflon tubes with a filter to prevent dirt/water getting sucked in to the analyser were vertically inserted in the pots and each tube was fitted to one channel of the multiplexer. An appropriate program was written for the multiplexer and an appropriate sampling interval was chosen for the ethylene analyser (each plant was sampled once every hour). For each experiment, surrounding air of the growth room was measured as a control value. The analyser automatically logged each data point, which was downloaded by USB port after the experiment. Results are presented as raw data and as ethylene in ppm per gram root dry weight to account for different root weight between individual plants. Root-zone ethylene production was also plotted against volumetric soil moisture. The experiment was repeated several times and a representative result is given.

Figure 3 and 4 (from left to right): Portable ethylene analyser EASI-1 and root-zone ethylene measurement set up. Soil moisture sensors (thetaprobes) and teflon tubes (connected to the ethylene analyser, indicated by arrows) were inserted in each pot.

Nutrient analysis

Macro- and micronutrients (Ca, K, Mg, Na, P and S) were analysed in oven-dried leaf tissue via nitric acid microwave digestion followed by Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). To validate the digestion, tomato leaf NIST (SRM 1573a, National Institute of Standards and Technology, USA) samples with known nutrient concentrations were run as well and the recovery detected through the ICP-OES was used to calculate final sample concentration. Leaf nitrogen in % was analysed in both wild type and *Nr* plants via EA combustion using an Elemental Analyser (VARIO Micro Cube, Germany).

Nitrate application

Because no treatment differences in soil moisture were observed until Day 14 of treatment (Figure 6E), 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. Table 2 gives an overview for sampling times of ABA, ethylene, g_s, Pn, Ψ_{leaf}, shoot fresh weight and total leaf area.

Table 2 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

Statistical analysis

For each sampling day, treatment differences were determined via an Independent Samples T-test (SPSS 19, IBM). Furthermore, 2-way ANOVA was performed to test both the individual effects of parameters (e.g. Ψ_{leaf}, g_s, Pn, ABA and ethylene) and treatment and any interactions. 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 (Figure 8B). The *Nr* genotype could not be grown together with the wild type (WT) at the same time (due to difficulty of scheduling irrigation independently), so interpretations of the results where *Nr* and WT are compared require some caution. 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. Furthermore, when parameters were sampled on different days throughout the treatment period (g_s, Pn, ABA and ethylene) and showed no significant variation with time, values were integrated over time (Figure 14).

Results and Discussion

Comparing manual watering with automatic irrigation scheduled according to soil moisture

To illustrate the advantages of automatic irrigation, soil moisture sensors installed at a commercial nursery showed that manual irrigation of three potted Coleus (*Solenostemon*) plants resulted in significant variation in volumetric water content (θ_v, Figure 5A). Vertical increases in θ_v indicate manual watering, while plant transpiration decreased θ_v gradually. Ideally, all lines should be between 0.2 and 0.35 m^3 m^{-3}, whereas here, θ_v lies between 0.09

and 0.3 m^3 m^{-3}, indicating that manual irrigation may not always meet the plant's water needs.

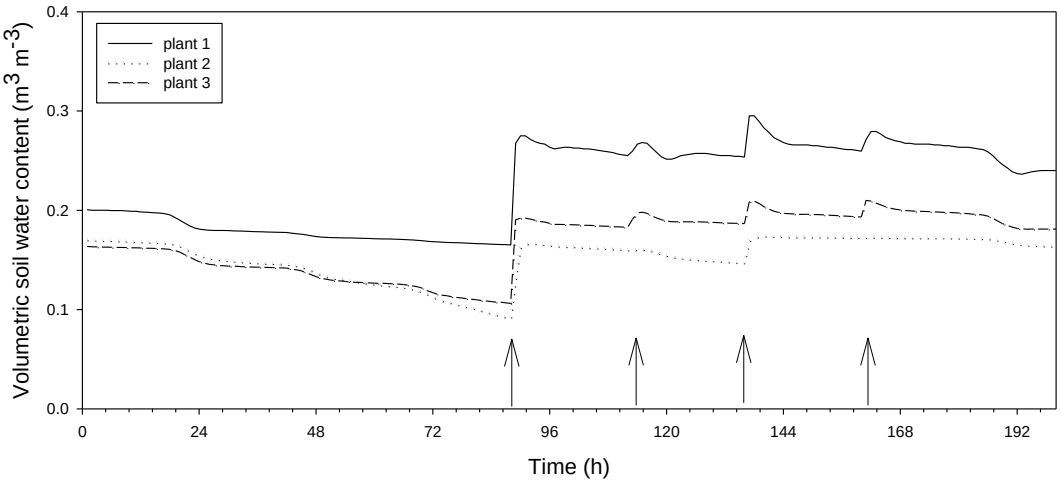

Figure 5A Volumetric soil water content of 3 manually watered Coleus (*Solenostemon*) maintained at commercial nursery over a period of 200 hours (recorded by Delta-T thetaprobes connected to a DL6 datalogger), arrows indicate approximately daily irrigation events.

In contrast to manual watering, irrigating tomato plants according to a set threshold (0.23 m^3 m^{-3} volumetric soil water content) allows soil moisture to remain between 0.22 and 0.37m^3 m^{-3} (Figure 5B, based on duration of pre-determined irrigation) with more frequent watering events (but applying less water at each event) to prevent under-irrigation. Significant differences between their coefficient of variation (12.7% and 24.9% for automatic and manual irrigation, respectively, Table 3) suggest that automatic irrigation scheduling according to feedback monitoring of soil moisture allows more precise watering according to actual plant water needs.

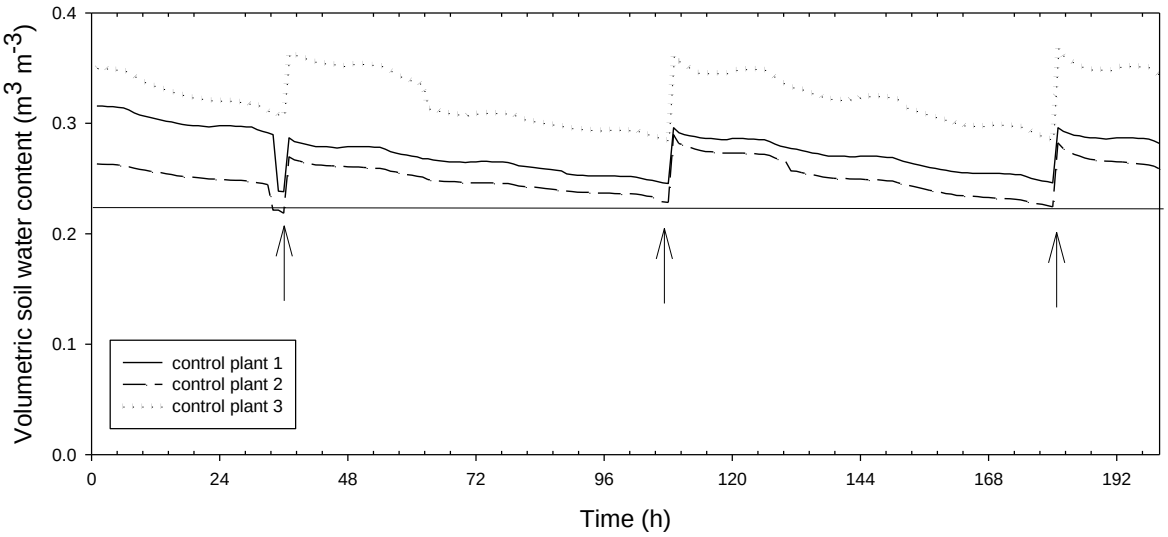

Figure 5B Volumetric soil water content of 3 automatically irrigated tomato plants over a period of 200 hours (recorded by Delta-T thetaprobes connected to a DL6 datalogger), arrows indicate irrigation events. Horizontal line indicates minimum threshold soil moisture setting .

Table 3 Mean, minimum, maximum, standard error and coefficient of variation for volumetric soil moisture of automatically and manually irrigated plants. Data are means ± SE of 600 measurements of 3 plants per treatment. Different letters indicate significant differences for coefficient of variation (Independent-Samples T-Test, p-value < 0.05).

Irrigation	Mean	Min	Max	Std error	Coefficient of variation (%)
Automatic	0.28	0.22	0.37	0.0015	12.7[a]
Manual	0.18	0.09	0.30	0.0018	24.9[b]

Comparing soil properties between short-term (flooding) and long-term (over-irrigation) stresses

Flooding steadily decreased soil oxygen concentration from 23% to 17% within 26 hours (Figure 6A), 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 % (Figure 6B). In contrast, soils that were well-drained had consistent oxygen values (23 %, Figure 6A 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 (6C-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 (Figure 6E). 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.

Generally, oxygen deficiency (hypoxia) in the soil decreases cellular oxygen, which results in root tissue damage, inhibits vegetative and reproductive growth, changes plant anatomy and causes premature senescence and plant mortality (Drew 1997). Due to soil hypoxia (oxygen deficiency) or anoxia (oxygen absence), root respiration changes from aerobic to anaerobic which in turn leads to less ATP production and adenosine diphosphate (ADP) oxidative phosphorylation. The lack of ATP 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). Those metabolites can accumulate in the plant during anaerobic root respiration and cause cell death (Jackson 2002). The more severe changes in soil oxygen suggest that even as an acute stress, flooding has a greater impact on soil properties than over-irrigation as a chronic stress, and in turn might also more severely change physiology of flooded than over-irrigated plants.

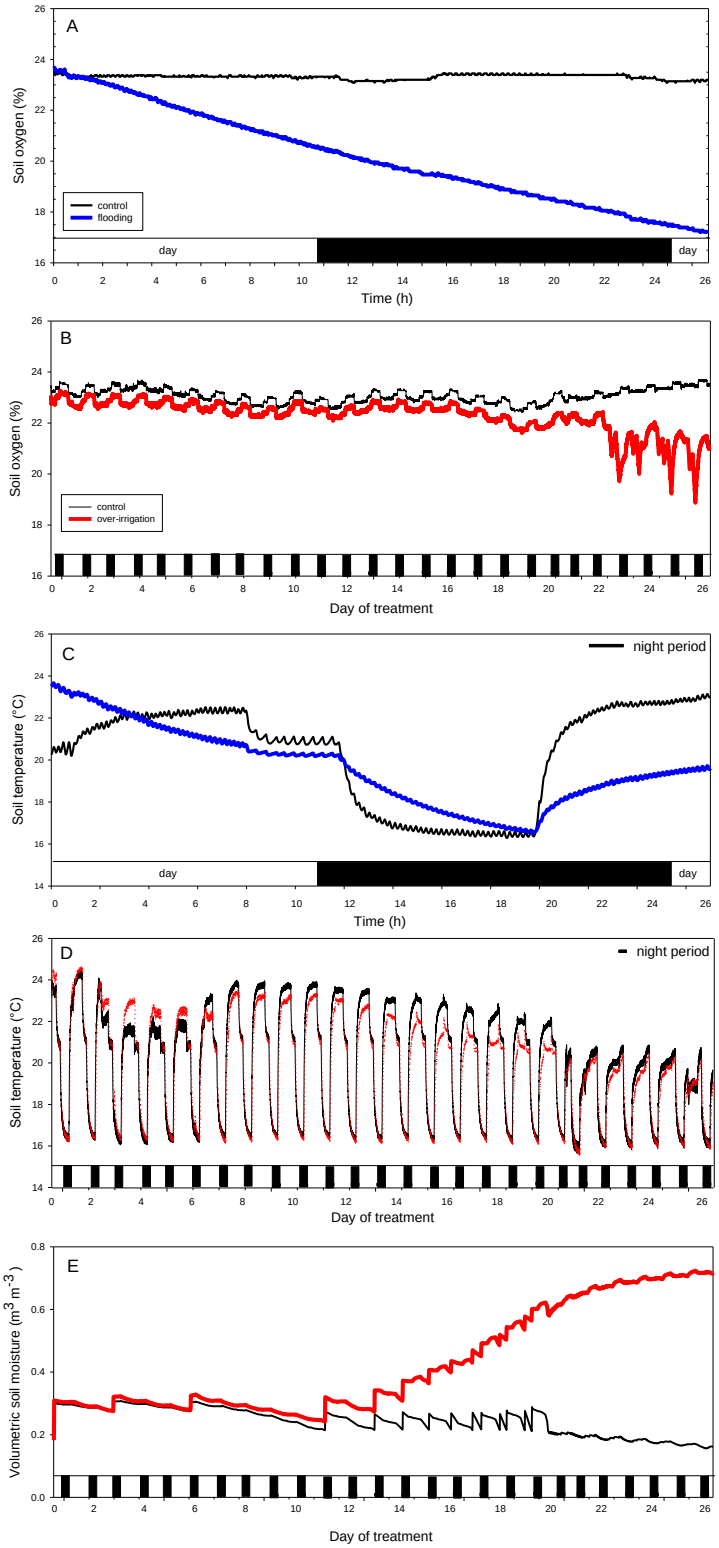

Figure 6 (A) Soil oxygen concentration for well-drained (black line) and flooded tomato plants (blue line) over 26 h and (B) for over-irrigated (red line) and well-drained (black line) plants throughout the experimental period; (C) soil temperature for well-drained (black line) and flooded tomato plants (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.

Effect of over-irrigation on plant growth

Over-irrigation significantly ($P < 0.001$) decreased shoot fresh weight (by 62 %) compared to control (well-drained) plants (Table 4 and Figure 7). Plant height and whole plant leaf area were also decreased by 27 % and 70 % respectively for over-irrigated plants compared to the control (Table 4). Epinasty was only seen in flooded plants (data not shown), but not in over-irrigated plants.

Figure 7 Control (blue tag, left) and over-irrigated tomato plants (red tag, right)

Table 4 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

Comparing plant physiological responses to short-term and long-term stresses

Short-term stress (26 hours flooding) caused more severe plant physiological changes than long-term stress (4 weeks over-irrigation) did. 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 (Figure 8A). In contrast, leaf water potential (Ψ_{leaf}) did not significantly differ between over-irrigated and control plants (Figure 8B), suggesting that growth inhibition of over-irrigated plants was not hydraulically regulated.

Flooding decreased stomatal conductance (g_s) by 25 % within 6 h (Figure 8C), whereas stomatal conductance of over-irrigated plants varied throughout the experiment period

(Figure 8D). On some occasions, both flooding and over-irrigation reduced photosynthesis rate (Pn) when compared to well-drained plants (Figure 8E, F). 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).

Flooding doubled foliar ABA concentration within 26 hours (Figure 8G), but 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 (Figure 8H). ABA does not necessarily regulate leaf growth under environmental stresses, since 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 2003). Furthermore other hormones, such as ethylene, could be of importance in regulating plant responses to over-irrigation.

Flooding increased foliar ethylene emission by 34 % after 26 hours (Figure 8I). 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 (Figure 8J). Treatment differences were more pronounced as the duration of over-irrigation increased. Ethylene seems to promote growth during early stages of seedling development, but later inhibits growth (Sharp and LeNoble 2002). It also constrains cell division, mitosis, DNA synthesis and growth of root and shoot meristems (Burg 1973) and oxygen deprivation of roots increased ethylene emission from tomato shoots (Bradford and Dilley 1978).

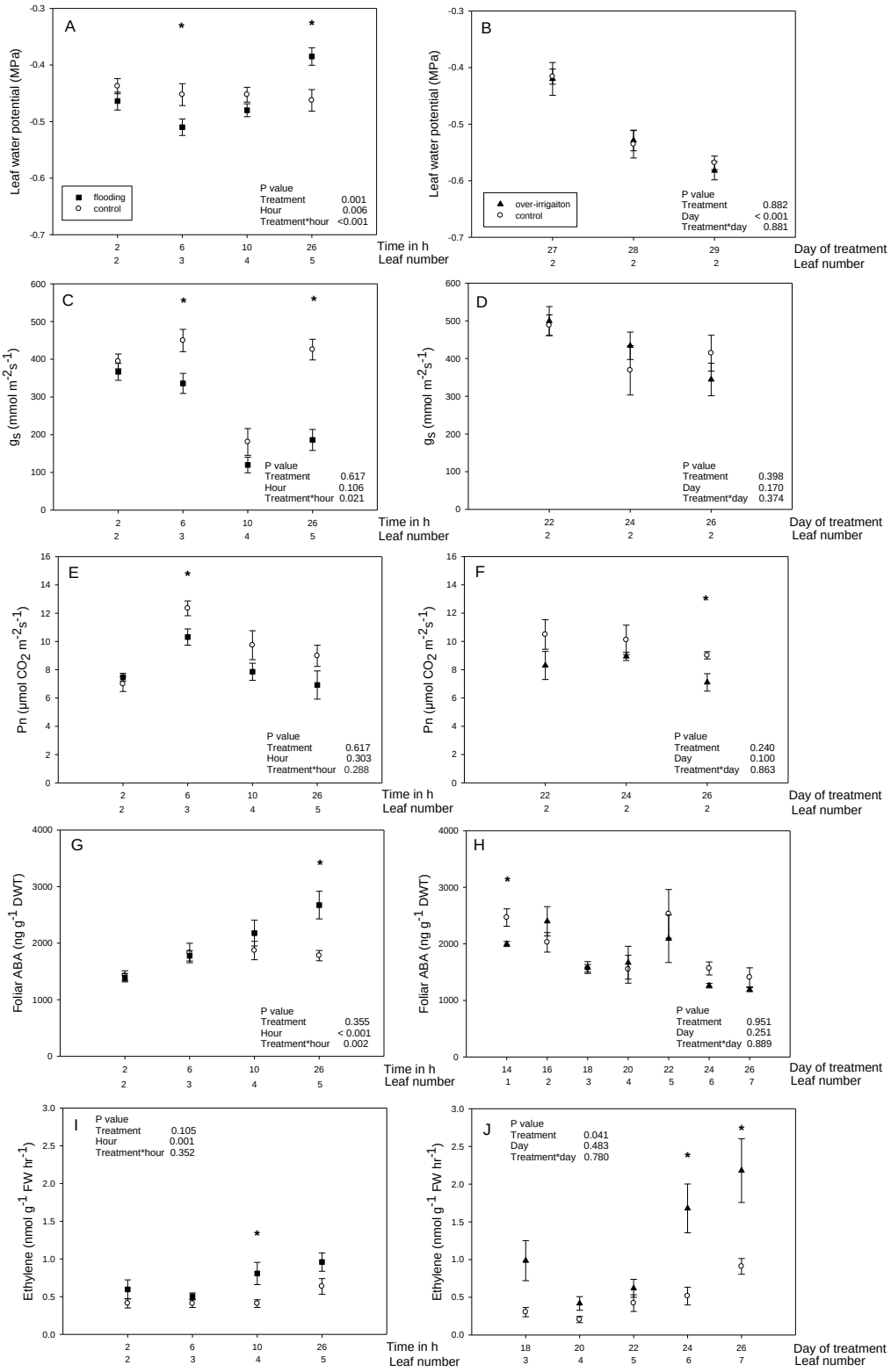

Figure 8 (A, B) Leaf water potential (Ψ_{leaf}), (C, D) stomatal conductance (g_s), (E, F) photosynthesis rate (Pn), (G, H) foliar abscisic acid (ABA) concentration and (I, J) foliar ethylene evolution of flooded (closed square) and well-drained (open circle) tomato plants and over-irrigated (closed triangle) and well-drained (open circle) tomato plants throughout the experimental period. Data are means ± SE of 5-8 replicates, asterisk indicates significant treatment differences (Independent Samples T-test, p-value < 0.05). Two way ANOVA (P

Values presented) for main effects of treatment, time and their interaction are presented in each panel.

ACC xylem sap analysis and root-zone ethylene production

Due to increased foliar ethylene emission in both flooded and over-irrigated plants, the ethylene precursor was measured in root and leaf xylem sap to understand its possible role in root-to-shoot signalling. ACC was not detected in root xylem sap, presumably because concentrations *in vivo* were below the detection limit of the instrument. Although not statistically significant, over-irrigation increased leaf xylem ACC concentration (Table 5). This increase could explain higher ethylene emission from shoot. Observed fluxes of ACC might be sufficient enough to support ethylene production rates assuming mole to mole conversion of ACC to ethylene (Else and Jackson 1998).

Table 5 Root and leaf xylem sap ACC delivery/concentration of over-irrigated and well-drained (control) tomato plants. Data are means ± SE of 4-5 replicates. Different letters indicate significant differences (Independent-Samples T-Test, P-value < 0.05). NF – not found.

ACC	Over-irrigation	Control
Root xylem sap delivery (ng/s)	NF	NF
Leaf xylem sap concentration (ng/ml)	0.49 ± 0.09[a]	0.29 ± 0.04[a]

Over-irrigation is a stress which mainly changes soil properties and therefore, roots are the first organs to sense these different conditions (Sauter 2013). Measuring ethylene produced in the root-zone might help further understand how over-irrigation influences plant growth and possibly whether online root-zone ethylene measurement could be a reliable tool to detect stressful environmental conditions. Until now, there has not been an accurate method for analysing endogenous ethylene concentrations in root systems (Visser et al. 1996). However, in this work, a portable ethylene analyser EASI-1 (Absoger Atmosphere Controlee, Les Barthes, France) was used as a non-damaging technique which allows fast and continuous (online) root-zone ethylene detection over a longer period of time.

Root zone ethylene production did not differ between well-drained and over-irrigated tomato plants and neither dark/light period or irrigation events had a significant effect on ethylene produced in the root-zone (Figure 9A-D). Possibly, lack of ACC oxidation in the root-zone of over-irrigated plants is responsible, as oxygen is needed to convert the precursor ACC to ethylene (Vriezen et al. 1999). However, highest ethylene concentration was measured in

soil without plants (Figure 10), suggesting that plants are affecting rhizosphere bacteria and thereby decreasing bacterial ethylene production.

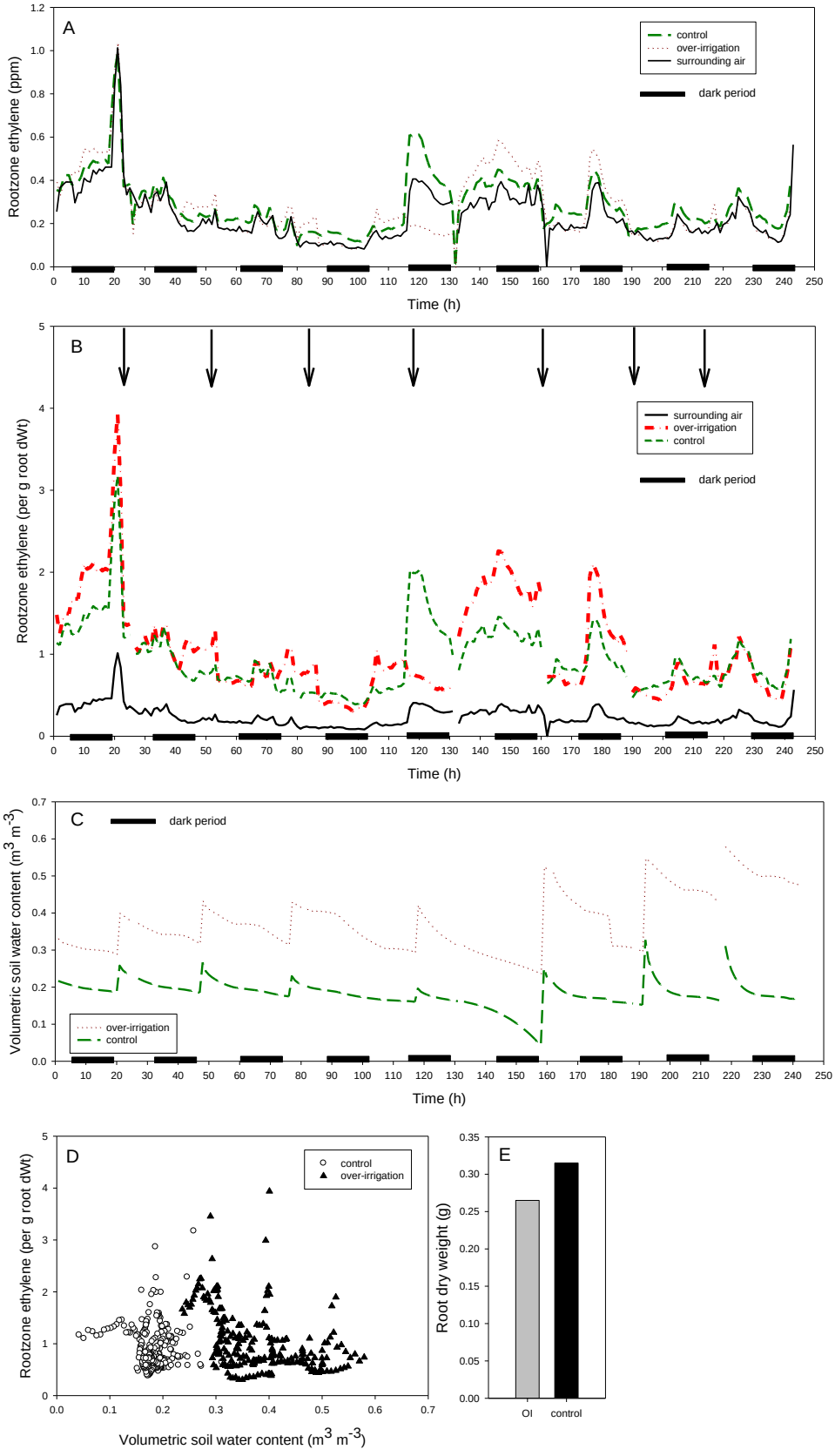

Figure 9 (A) Root-zone ethylene production in ppm and (B) per g root dry weight, (C) soil moisture throughout the experiment, (D) soil moisture plotted against root-zone ethylene production of over-irrigated (closed triangle) and well-drained (control, open circle) tomato

plants, (E) root dry weight of over-irrigated (OI, grey bar) and well-drained (control, black bar) tomato plants and surrounding air. Black arrows indicate time of watering, dark bars indicate night period.

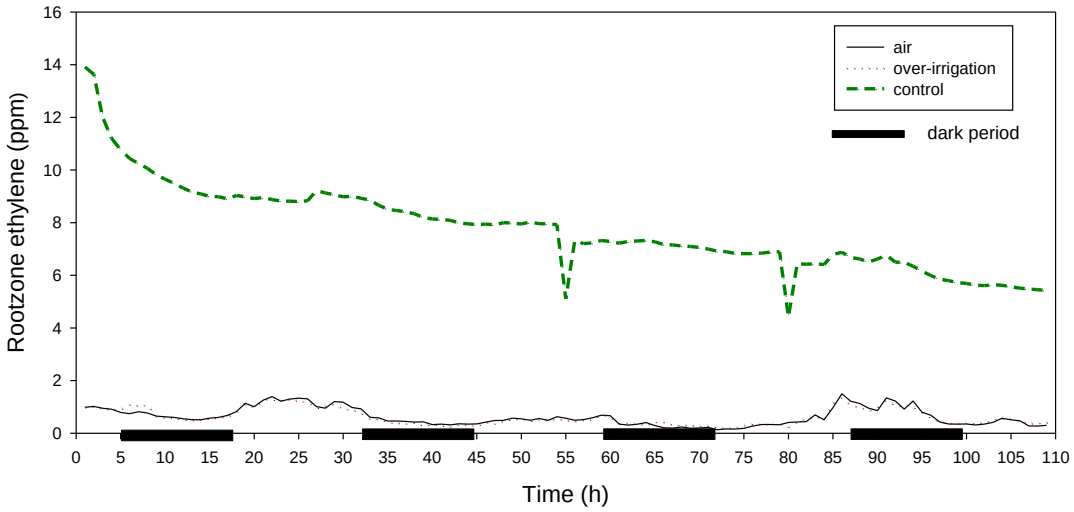

Figure 10 Root-zone ethylene production in over-irrigated (red line) and well-drained (green line) soil without plants.

Evaluating the effects of partial ethylene-insensitivity on physiological responses

Over-irrigation-induced growth suppression increases with the duration of treatment in wild type plants (Figure 11). Although shoot fresh weight of well-drained *Nr* plants was less than that of wild type well-drained plants grown for the same length of time, over-irrigated *Nr* plants did not show such a dramatic growth inhibition as comparable over-irrigated wild type (WT) plants (Figure 11). Thus partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to some extent.

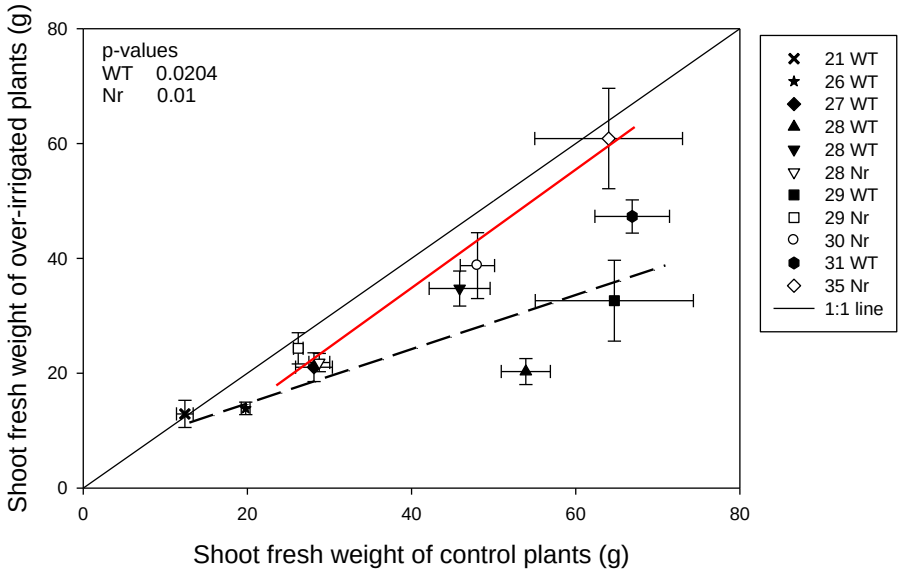

Figure 11 Well-drained (control) shoot fresh weight plotted against over-irrigated shoot fresh weight of wild type (WT, closed symbols) and *Nr* (open symbols) tomato plants. Data are means ± SE of 5-10 replicates. Different symbols indicate duration of treatment in days and genotype. P-values and regression lines (red solid line – *Nr*, dashed black line – WT) given. The 1:1 (solid) line is also indicated, with points on this line indicating that over-irrigation did not inhibit growth.

Nutrient analysis

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 %; Figure 12A and B). Only sulphur showed a statistically significant ($P < 0.05$) change.

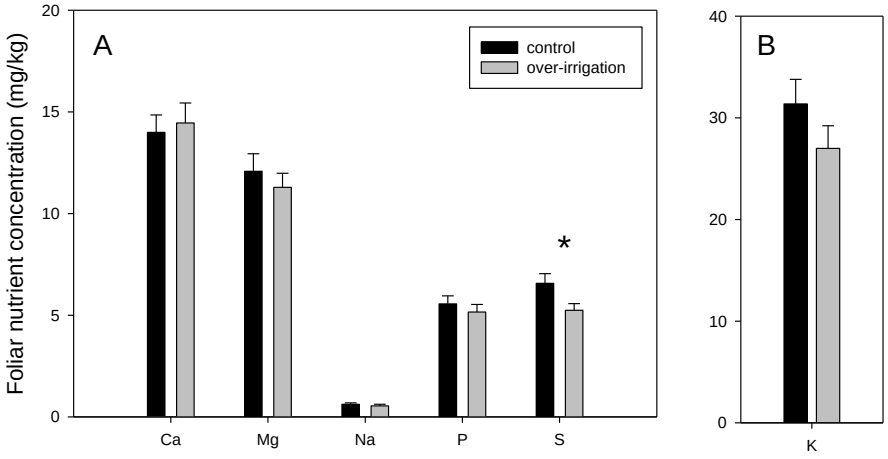

Figure 12 (A) Foliar calcium, magnesium, sodium, phosphorus, sulphur, and (B) potassium concentration of over-irrigated (grey bar) and well-drained (black bar) tomato plants. Data are means ± SE of 10 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, 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 (Figure 13A and B). Furthermore, increasing leaf nitrogen concentration tended to correlate with higher shoot fresh weight in both wild type (P=0.060) and *Nr* (P=0.055) and there was no significant interaction between genotype and leaf N concentration (Figure 13C), suggesting that the effect of nitrogen concentration on shoot fresh weight is not affected by partial ethylene-insensitivity. 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. 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 %. 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 nitrogen was the only nutrient that was significantly decreased during over-irrigation. Decreases in foliar nitrogen concentrations can be induced through de-nitrification in waterlogged soil or because of reduced root N uptake (Hamonts et al. 2013).

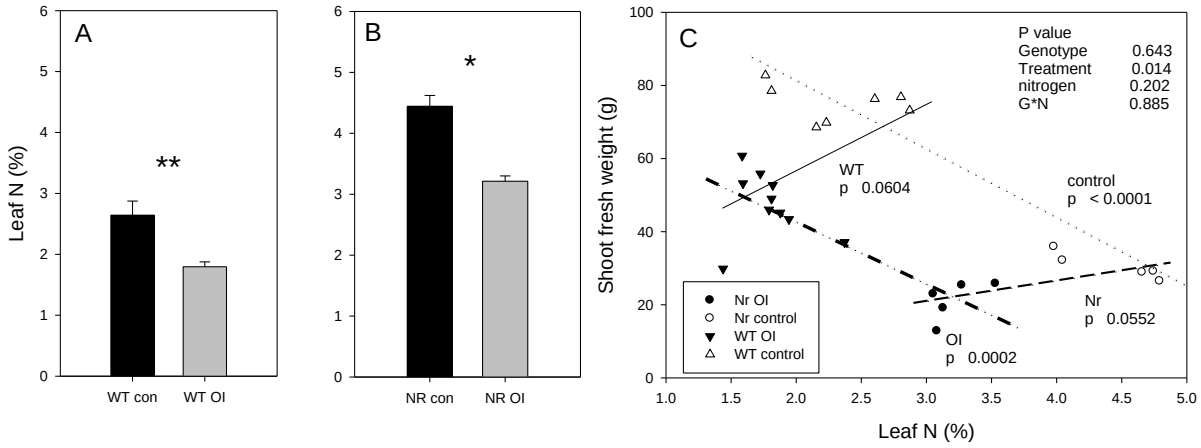

Figure 13 (A) Leaf nitrogen concentration of over-irrigated (OI – grey bars) and well-drained (con – black bars) wild type (WT) and (B) *Nr* tomato plants. Data are means ± SE of 5-10 replicates, asterisk indicates significant differences between treatments (Independent Samples T-test, p-value < 0.05*, 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 ANOVA and linear regression (black line – WT, dashed line – *Nr*, dotted line – control, dash-dotted line – over-irrigated) given.

Nitrate application

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

(Figure 14A). Similarly, total leaf area was highest in control and over-irrigated plants treated with 10 mM $Ca(NO_3)_2$ (90 % of control, Figure 14B). 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 (Figure 14C).

Ψ_{leaf} did not significantly differ between the four treatments (Figure 14D), suggesting that physiological responses to over-irrigation and nitrogen-induced growth recovery of over-irrigated plants are unlikely to be hydraulically regulated. No significant differences in stomatal conductance were detected (Figure 14E), 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 (Figure 14F).This bolsters the earlier argument that changes in photosynthesis were not due to stomatal limitation, but could rather be attributed to reduced mesophyll activity (Ciompi et al. 1996, Flexas and Medrano 2002).

Foliar ABA concentration did not vary significantly between treatments (Figure 14G). Although foliar ABA concentrations increased when tomato was transferred to a nitrate-free nutrient solution (Chapin et al. 1988), a slowly developing chronic N deficiency did not alter foliar ABA concentration (Dodd 2003). 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 2003). Thus, it seems unlikely that ABA mediates growth of over-irrigated plants.

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 (Figure 14H). 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). Possible interactions between ethylene and nitrogen remain unclear, but because the slope of the relationship between shoot fresh weight and leaf N did not differ in wild type and *Nr* plants (P=0.97, Figure 13C), it seems unlikely that ethylene is the key growth regulator of over-irrigated tomato plants. Instead, experiments presented here suggest that both hypoxia and reduced nitrogen uptake negatively affect metabolic and transport processes, thus explaining why calcium nitrate supplementation of overirrigated tomato plants increased shoot growth to the level of control (well drained) plants.

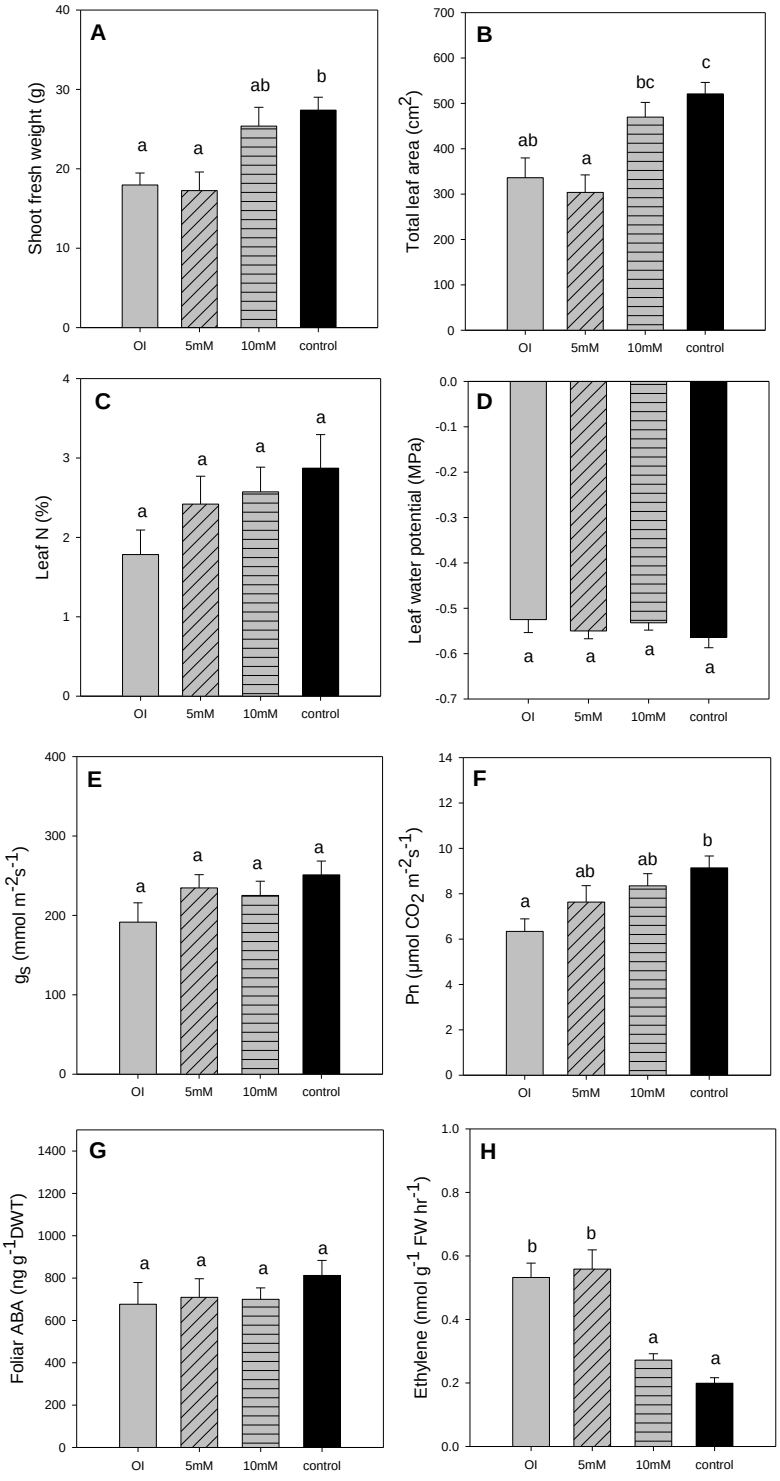

Figure 14 (A) Shoot fresh weight, (B) total leaf area, (C) leaf nitrogen concentration, (D) leaf water potential (Ψ_{leaf}), (E) stomatal conductance (g_s), (F) photosynthesis rate (Pn), (G) foliar abscisic acid (ABA) and (H) foliar ethylene evolution for over-irrigated (OI), over-irrigated + 5 mM $Ca(NO_3)_2$, 10 mM + $Ca(NO_3)_2$ over-irrigated and well-drained (control) tomato plants. Data are means ± SE of 4-30 replicates, different letters above the bars indicate significant differences between treatments (ANOVA, p-value < 0.05).

Conclusions

Understanding the physiological impacts of 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, this project provides new insights on understanding the effects of chronic over-irrigation on tomato plant growth and physiology.

Key findings are out-lined below:

- Comparing variation of soil moisture between hand-watered plants and plants automatically irrigated based on measurements of soil moisture sensors suggests that the latter allows more precise watering according to actual plant water needs.
- 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.
- Over-irrigation significantly increases foliar ethylene emission compared to well-drained plants. The ethylene precursor ACC increases in leaf xylem sap of over-irrigated plants and could be sufficient for extra foliar ethylene produced during over-irrigation. In contrast, root zone ethylene production does not differ between well-drained and over-irrigated tomato plants. However, over-irrigating the partial ethylene-insensitivite genotype *Nr* inhibits growth less than the wild type, suggesting that partial ethylene-insensitivity can ameliorate over-irrigation induced growth-inhibition to some extent.
- Over-irrigation induces significant foliar nitrogen deficiency and daily supplementation of small volumes of 10 mM $Ca(NO_3)_2$ to over-irrigated soil restores 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 inhibiting growth of over-irrigated plants.

Future work

Future projects should mainly focus on the likely impact of current irrigation management on crop quality and retail value and whether the automatic irrigation system could improve crop quality.

This could be implemented through:

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

Using the EASI-1 analyser to detect the impacts of pests and pathogens on root-zone ethylene production may be a fruitful future line of enquiry (especially plant/disease interactions known).

Technology transfer

Meetings attended and output

Event	Date
HDC/BPOA Poinsettia Meeting at Roundstone Nurseries, Chichester (Oral presentation – **article in HDC News Issue)**	07.11.2012
HDC Exploratory Day ("Novel technologies for future pest and disease control in herbs") at Lancaster Environment Centre	28.11.2012
BPOA meeting at Heyrose Golf Club, Cheshire	22.11.2011, 26.11.2013
News article about studentship project in HDC magazine	June 2014
Society of Chemical Industry blog about attending the International Horticultural Congress in Brisbane, Australia http://www.soci.org/News/Awards/David-Miller/Antje-Fiebig	September 2014
Lancaster Environment Centre blog about attending the International Horticultural Congress in Brisbane, Australia and	November 2014

receiving the David Miller Travel Award http://www.lancaster.ac.uk/lec/news-and-events/blog/antje-fiebig/talking-to-top-horticulturalists-in-brisbane/

PhD thesis submission (before 3 year deadline)	22.09.2014
Viva	24.10.2014
Award of PhD degree	18.11.2014

Presentations

- Oral presentation at 29th International Horticultural Congress, 17th-22nd August 2014, Brisbane, Australia: *Feedback regulation of irrigation via soil moisture monitoring and its implication on plant growth and physiology* **(received £250 David Miller Travel Bursary Award from the Society of Chemical Industry and £350 Faculty for Science and Technology, Lancaster University travel)**
- Poster presentation at Society of Experimental Botany (SEB) Manchester Annual Meeting, 1st-4th July 2014: *Xylem ionomic and phytohormonal responses to over-irrigation*
- Poster presentation at Fruit and Roots, 6th-7th November 2013, East Malling: *A biphasic response of leaf ethylene emission to soil moisture status in tomato*
- Poster presentation at 11th International Conference on Plant Anaerobiosis, 6th-10th Ocotober 2013, Los Banos, Philippines: *Acute versus chronic stress: Flooding causes more severe changes of soil environment and physiological responses in tomato than over-irrigation* **(received £350 Faculty for Science and Technology, Lancaster University travel grant and £500 British Soil Science Society travel grant)**
- Poster presentation at HDC Studentship Conference, 9th-10th September 2013, Worcestershire: *Detection and amelioration of root-zone ethylene production in protected crops*
- Poster presentation at BSSS Annual meeting, 3rd-5th September 2013, Lancaster: *Acute versus chronic stress: Flooding causes more severe soil and plant changes than over-irrigation*
- Poster presentation at Faculty of Science and Technology, Lancaster University 2012 Christmas Conference, UK: *Over-irrigation suppresses tomato growth*
- Poster presentation at Lancaster Environment Centre 2012 Poster Day, UK: *Effects of over-irrigating pot plants on growth, stomatal conductance and hormone balance in tomato* **(Won prize for best poster in year group 2011)**

- Poster presentation at Stomata 2012 Conference, Manchester, UK, 02. – 04.07.2012: *Effect of over-irrigating pot plants on growth, stomatal conductance and hormone balance in tomato*
- Poster presentation at HDC Studentship 2012 Conference, Winchester, UK: *Do nurseries overwater their pot plants? – Effect of over-irrigation on plant growth and hormone balance*

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