

**Ethylene-mediated plant responses to severe
water deficit in strawberry**

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I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged

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Abstract

The research described in this thesis focussed on the potential effect of ethylene produced under severe water deficit conditions (SWD) on the concentration of phenolic compounds in fruit and leaves of commercial strawberry (*Fragaria x ananassa* Duch.). An increased ethylene production rate was detected in green fruit and leaves at midday of the second day of wilting in response to SWD. The stress-induced rise in ethylene production was successfully inhibited by applications of aminoethoxyvinylglycine (AVG) and subsequently re-instated by 1-aminocyclopropane-1-carboxylic acid (ACC) or replaced by abscisic acid (ABA) in fruits. Initially, fruit total ellagic acid concentration increased along with ethylene production but conclusive evidence for the causal role of ethylene in the accumulation of individual phenolics in fruit from the inhibition, re-instatement and replacement of the ethylene signal was not obtained. The quantitative expression of selected genes in leaves was investigated, and a significant reduction in the relative expression of *FaACS1* (coding for ACC synthase), an increase in *FaACO1*, *FaACO4* (coding for ACC oxidase) expression and no change on the expression *FaPAL* (coding for Phenylalanine ammonia lyase) was observed. Increased leaf ethylene production rate was likely due to increased expression of genes regulating ACC oxidase activity. However, no evidence was found from these genetic studies to confirm the role of stress-induced ethylene production in accumulation of phenolics in fruit or leaves.

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List of abbreviations

1-MCP	1-Methylcyclopropene
ABA	Absciscic acid
ACC	1-Aminocyclopropane-carboxylate
ACF	Autocorrelation function
ADAS	Agricultural Development Advisory Service
(+)-AdoMe	(+)-S-adenosyl-L-methionine
Aib	Aminoisobutyric acid
AOA	Aminooxyacetic acid
APRI	Alternate Partial Root Zone Irrigation
ATP	Adenosine triphosphate
AVG	Aminoethoxyvinylglycine
BME	2-Mercaptoethanol
CAPRI	Controlled Alternate Partial Root Zone Irrigation
CCA	Cyclopropanecarboxylic acid
CDA	Chiral derivatizing agent
cDNA	Complementary deoxyribonucleic acid
CHS	Chalcone synthase
CK	Cytokinins
CTAB	Cetrimonium bromide
CTR1	Constitutive Triple Response
DACP	Diazo-cyclopentadiene
DEFRA	Department for Environment, Food & Rural Affairs
DI	Deficit irrigation
DMSO	Dimethyl sulfoxide
DPPH	Diphenylpicrylhydrazyl
DW	Dry weight

EDTA	Ethylenediaminetetraacetic acid
EIN	Ethylene Insensitive Protein
ERF	Ethylene Responsive Factor
ERS	Ethylene Response Sensor
ET	Evapotranspiration
ET _c	Crop evapotranspiration in standard conditions
ET _{c.adj}	Crop evapotranspiration in non-standard conditions
ETR	Ethylene Response Mediator
FAO	Food and Agriculture Organization
Fm'	Light saturation pulse
FPRD	Fixed Partial Root Zone Drying
FRAP	Ferric Reducing Antioxidant Power
Ft	Saturating pulse
FW	Fresh weight
GA	Gibberellins
GC	Gas chromatograph
g _s	Stomatal conductance
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
JA	Jasmonic acid
LSD	Least significant difference
MACC	ACC N-malonyl-transferase
MAPK	Mitogen Activating Protein Kinase
MAS	Marker Assisted Selection
MAT	5'-Methylthioadenosine
MCA	2-Methyl cyclopropanecarboxylic acid
MPK	Mitogen Protein Kinase
MPQ	Maximal photochemical quenching

MTR	5'-Methylthioribose
ORAC	Oxygen Radical Absorbance Capacity
p	p -value
PAL	Phenylalanine ammonia lyase
PCCA	<i>trans</i> -2-Phenylcyclopropane-1-carboxylic acid
PIN	Auxin Efflux Carrier
POD	Peroxidase
PPO	Polyphenoloxidase
PRD	Partial Rootzone Drying
PVMC	Peat volumetric moisture content
PVP	Polyvinylpyrrolidone
PW	Pot-and-plant weight
QTL	Quantitative trait <i>locus</i>
R^2	Coefficient of determination
RDI	Regulated Deficit Irrigation
RH	Relative humidity
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	S-Adenosyl-L-methionine
SAT	Single electron transfer
<i>SD</i>	Standard deviation
<i>SEM</i>	Standard error of the mean
STS	Silver thiosulfate
SWD	Severe Water Deficit
T	Temperature
TBA	<i>tert</i> -Butyl alcohol
TCEP	<i>tris</i> (2-Carboxyethyl) phosphine
TDI	Transient deficit irrigation

TEAC	Trolox equivalent antioxidant capacity
TFA	Trifluoroacetic acid
WUE	Water use efficiency
WW	Well-watered
Yield (II)	Efficiency of the photosystem II

Chapter 1. Literature Review

1 Importance of fruit quality and research for strawberry production

The strawberry, *Fragaria x ananassa* Duch., is extensively cultivated worldwide and is consumed by millions of people. Figures from the Food and Agriculture Organization of the United Nations (FAO) show a steady increase in the annual worldwide strawberry production, reaching 4.5 million tonnes in 2012 (FAO, 2015). Since 1985 production in the United Kingdom (UK) has fluctuated, but accelerated rapidly at the turn of the millennium showing a sharp increase of some 39% in 12 years, reaching 100,000 tons in 2014 (**Figure 0.1**). Data from the UK Department for Environment, Food and Rural Affairs (DEFRA) highlight the importance of this figure in the UK: in 2012 the annual strawberry production was 74% of soft fruit and 25% total fruit production (DEFRA, 2013a). However, up to 4.5% of total yield is lost during storage or on the shelf, prior to being sold to the consumer. Around 17,000 tonnes are additionally wasted in households each year (Terry, 2008). These losses could be reduced by increasing fruit phytonutrient contents and therefore extending shelf-life (Jiang et al., 2001; Bassolino et al., 2013; Zhang et al., 2013). In 2014 chief buyers for major UK retailers reported that strawberries were their top-selling products, especially around the Wimbledon Championship (Gilbert, 2014). The strawberries vibrant red colour and aroma, fruity flavour and large size for a soft fruit are the main reasons for their popularity. These characteristics define strawberry fruit quality and the level of scientific interest in this area is reflected in the wide range of studies on the different organoleptic traits of the strawberry such as flavour (Song & Forney, 2008; Schwieterman et al., 2014), aroma (Zabetakis & Holden, 1997), colour, firmness (Aguilheiro-Santos, 2008; Gunness et al., 2009), size (Grant et al., 2010) and shelf-life (Jiang et al., 2001; Pelayo et al., 2003).

The improvement of strawberry fruit quality has been the target of numerous studies investigating the use of a) improved agronomic practices such as precision and deficit irrigation (Dodds et al., 2007; Ripoll et al., 2014), fertilization (Keutgen & Pawelzik,

2007), pests and disease (Terry et al., 2007), the use of plastic covers (Ordidge et al., 2010) and mulches (Atkinson et al., 2006) among others; and b) development of new cultivars (Hummer et al., 2011) by classical breeding techniques (EUberry, 2010) and by using new molecular tools such as quantitative trait loci (QTL) in marker assisted selection (MAS) (Sargent, 2005; Weebadde et al., 2008; Zorrilla-Fontanesi et al., 2011). In all, these efforts aim to maintain and perhaps increase the success of strawberries in the market.

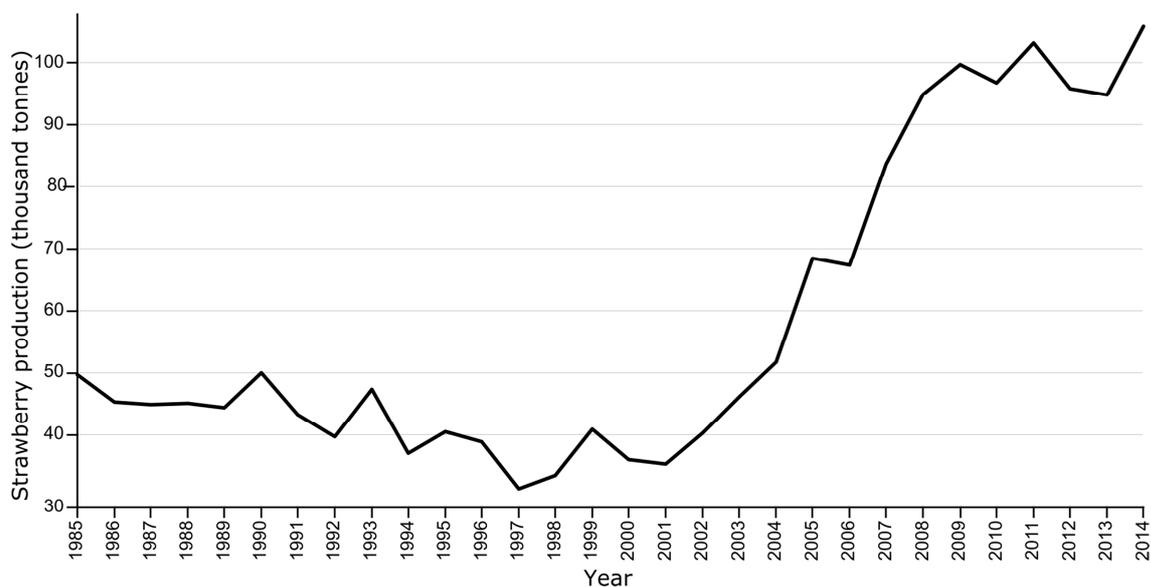


Figure 0.1. Annual strawberry production in the UK, in thousands of tonnes for the period 1985 to 2014 (DEFRA, 2015).

2 Importance of water availability for strawberry production

Plant water content typically constitutes 80 to 95% of the mass of its growing tissues. Most of this water within the cell is located in vacuoles whereas in the plant up to 75% of the water is located in the xylem and phloem. Because of the transient hydrogen bonds formed between the oxygen and hydrogen atoms of neighbouring molecules, water is an essential solvent and transporter and a reactant or substrate in many important processes such as respiration and photosynthesis (Kramer & Boyer, 1995; Nobel, 2005).

If water supply is limited at critical stages of plant and fruit development, the plant will suffer a water deficit stress which, depending on its intensity and duration, can lead to loss of turgor and wilting, cessation of cell growth, partial or complete stomatal closure, reduction of photosynthetic rate as well as interference with many other basic metabolic processes. If the deficit stress persists, yields can be suppressed and the plant could eventually die (Kramer & Boyer, 1995; Bray, 2001; Araya & Keynes, 2007). A major concept linking the water used by the plant and its biological responses is water use efficiency (WUE). WUE can be defined as the ratio of photosynthesis to transpiration or yield to transpiration; these have different biological significance and uses. The first expression of WUE is more useful for MAS since it is less affected by environment, whereas the second form is generally used when yield is a target of the study (Kramer & Boyer, 1995; Tardieu, 2013). For this reason, when comparing different cultivars or more interestingly, deficit irrigation (DI) treatments, the ratio of photosynthesis to transpiration can be more relevant (Kang & Zhang, 2004; Dodds et al., 2007; Grant et al., 2010; Foday et al., 2012).

There are two main factors that could limit the availability of vital resources for future strawberry production. Firstly, non-climate risks. Increasing competition for limited water supplies from the manufacturing industry and households will be exacerbated by the need for irrigation in the production of other crops that have not traditionally relied on irrigation to deliver the yields and quality demanded by growers and retailers. Demand for food is increasing rapidly due to population growth and greater incomes per capita (Tilman et al., 2011), and is expected to double by 2050 (Mueller et al., 2012). Currently, the minimum daily dietary intake per capita is 2,700 kilocalories (FAO, 2009), which is estimated to require 2,700 litres of water to produce. Potential water savings would enable considerable gains in food/energy production (Hanjra & Qureshi, 2010); for example, a reduction of 12% of the water needed per calorie would suffice to supply 3,500 kcal/capita/day. Experiments at East Malling Research have shown that it is possible to obtain equivalent strawberry yields and quality with up to

70% less irrigation water applied than the current recommended “best practice” volume (ADAS, 2003; Else & Atkinson, 2010).

Secondly, there are climate risks. Climate change will impact greatly on the economic feasibility of strawberry production in the next decades, mainly through impacts on the availability of fresh water for irrigation in the major strawberry growing regions. On 25th January 2012, a Government report (UK Climate Change Risk Assessment) presented to Parliament highlighted potential challenges for agriculture in the future, particularly beyond 2050; including increasing summer soil moisture deficits and demand for irrigation (DEFRA, 2012). Subsequently, the Government published their plan of action (The National Adaptation Programme), one of the major points of which was “Building resilience in agriculture through effective water management” (DEFRA, 2013b). These publications were ultimately based on climate projections predicting summer rainfall decreases of 6 to 65%, alongside increases in temperature from 2.2 to 6.8 °C by the 2080s. Strawberry production in East Anglia and Southeast England will be among those most severely affected by climate change (UK Climate Projection, 2010). In consequence, new legislation such as the Water Act 2014 has been enacted in order to increase the regulation of water resources.

Strawberries in the UK are cultivated under cover to protect them from changeable weather and to ensure a reliable supply of high quality berries to the market. Consequently, rainfall is excluded and they must be irrigated. According to the ADAS “best practice” grower guide, fruiting strawberries grown in substrate require 0.5 litres of water per plant on a sunny day, equivalent to 17.3 m³ per ha per day (ADAS, 2003). In 2012, there were 4,536 ha harvested in the UK in 2014 (DEFRA, 2015) which required an estimated 73,698 m³ of water per day. Much of this water is obtained from areas classified by the Environment Agency (the EA) as “over-abstracted” and/or “over licenced” (Knox et al., 2009). Consequently, the soft fruit industry is keen to support studies focussing on the efficient use of vital resources such as water (Ferrerres et al.,

2003; Knox et al., 2009; Else & Atkinson, 2010; Hanjra & Qureshi, 2010; Ripoll et al., 2014).

3 Deficit irrigation as a tool to manage strawberry production

The combination of two separate processes, evaporation from the soil or substrate surface and transpiration from the plant, are known as evapotranspiration (ET). The ET of a crop in non-stressed conditions is termed crop evapotranspiration under standard conditions (ET_c) and under stressed conditions is referred to as crop evapotranspiration under non-standard conditions ($ET_{c,adj}$) (Allen & Pereira, 1998). Deficit irrigation techniques aim to improve WUE, in terms of the ratio of yield to transpiration, compared to well-watered conditions (WW) supplying the crop with $ET_{c,adj}$ volumes of water, including effective rainfall, and maximising yields. This is normally achieved via trickle irrigation to deliver small amounts of water to target areas on demand (Jones, 2004). The main scheduling irrigation methods are Regulated Deficit Irrigation (RDI), Partial Root Zone Drying (PRD) and Transient Deficit Irrigation (TDI). Their correct management requires knowledge of when and how much water to apply, which is normally done by measuring a) soil water or matric potential, volumetric soil moisture content, or soil water balance; and b) plant physiological responses such as those measured by gas exchange (photosynthetic rate and stomatal conductance), stem or leaf water potential, thermal sensing, sap-flow sensing or growth rate (Jones, 2004; Tardieu, 2013). Since these irrigation techniques can reduce vegetative growth, leading to improved fruit quality, they have been particularly successful in fruit crops (Kriedemann & Goodwin, 2003; Else & Atkinson, 2010; Ripoll et al., 2014). Timing is an important factor to consider in the use of these techniques. Imposing the correct degree or intensity of stress at the optimum stage of plant or fruit development and maintaining it for an appropriate length of time will maximize the chances of obtaining the desired outputs i.e. improved resource use efficiency, maintenance of marketable yields and improved fresh produce quality.

Regulated deficit irrigation was probably the simplest deficit irrigation (DI) technique, at times also referred to just as DI (Jones, 2004; Geerts & Raes, 2009) or also mild drought stress (Bahrun et al., 2002). Regulated deficit irrigation is applied only during a specific stage of crop development and to the whole root-zone (Geerts & Raes, 2009). When expressed as a percentage of ET_c supplied by irrigation, reductions of yield in strawberry appear around 60% (Dodds, 2007). However some authors report reduced yields under RDI probably because the degree of stress is too high (Kriedemann & Goodwin, 2003; Klamkowski & Treder, 2008; Else & Atkinson, 2010). Independently of the effect the level of stress has on yield, RDI has the potential to improve fruit quality in strawberry, particularly sugars, acids, antioxidants and phenolics (Terry et al., 2007; Dodds, 2007; Giné Bordonaba & Terry, 2010). However, it is important to note the effect that fruit water content has on these findings: Terry et al. (2007) reported an increase in pelargonidin 3-glucooside in secondary fruits, total phenolics, antioxidant capacity (FRAP), sucrose, fructose, glucose, citrate, malate, sugar:acid ratio but only when concentrations were calculated on a fresh weight (FW) basis. When these were calculated on a dry weight (DW) basis, only total phenolic concentration was higher under the deficit irrigation treatment.

Partial root zone drying is an irrigation technique by which two differentiated parts of a plant root system are alternated from a wet to a dry state so shoots and leaves are simultaneously supplied with water and nutrients from well-watered roots, and water-stress signalling compounds produced in roots exposed to drying soil (Kriedemann & Goodwin, 2003). PRD is also referred to in the literature as Controlled Alternate Partial Root Zone Irrigation (CAPRI) (Kang & Zhang, 2004)) and Alternate Partial Root Zone Irrigation (APRI) (Du et al., 2008). Fixed Partial Root Zone Drying (FPRD) is a variation of PRD in which the wet and dry zones are not alternated (Li et al., 2010). Irrigation can be supplied to half of the root system by dividing the root zone by planting in split pots (Dodds et al., 2007), separate compost bags (Grant et al., 2004; Savić et al., 2009), using soil-less conditions (Dasgan et al., 2009) or by tilting pots so receive drip

irrigation at an angle and the substrate is irrigated at an angle,. In addition, it is possible to obtain an effect without physically separating plant roots just by directing the irrigation to half of the root system; i.e. irrigating only one side of a row of vines (Du et al., 2008).

The switching between the dry and wet zones seem to be important to increase the transport of root-sourced ABA to the shoots in *Lycopersicon esculentum* (Dodd et al., 2006). In strawberry, in turn, ABA seems to be essential for fruit ripening (Chai et al., 2011; Jia et al., 2011). This may be the reason why when supplying 80% of ET_c by PRD management, a 55% and 270% increase in concentrations of ascorbic and ellagic acids (expressed on frozen weight basis), respectively were reported, whereas fruit yields did not vary significantly (Dodds et al., 2007). However, when the volume of water applied was only 60% of that needed to maintain soil at full capacity, yields were reduced compared to plants grown in fully irrigated soils (Liu et al., 2007).

Transient Deficit Irrigation was developed by Dr Mark Else at East Malling Research from observations made in plants under RDI or PRD. The potential gains in fruit quality such as improved phytonutrient content are higher when using PRD than when using RDI, and it was hypothesised that this effect was linked to the switching of the irrigation between dry and wet zones (Else & Atkinson, 2010), and the resulting transient loss in shoot turgor. The TDI treatment consists of withholding irrigation, or applying a reduced volume of irrigation, until transient wilting is first observed, then applying small volumes of irrigation at intervals to keep the plant on the verge of wilting for a period of time. Finally the plant is returned to WW conditions. In basil, TDI can increase antioxidant concentrations beyond WW and RDI controls and still maintain yields (Else et al., 2013). Quality increase by TDI has been also observed in strawberry (Mark Else, personal communication)

It is clear that DI can increase fruit quality in strawberry but different cultivars will respond differently (Bordonaba & Terry, 2010; Grant et al., 2010). In addition, timing

when to start the treatment and how long maintain it, can also influence both yield and fruit quality (Geerts & Raes, 2009; Ripoll et al., 2014).

4 Nutritional and organoleptic composition of the strawberry fruit

The true fruit of the strawberry is botanically termed an achene, and is often but erroneously referred to a “seed”. The main commercial interest in this plant lies in the tissue on which these achenes rest, the receptacle. This complex structure is commonly known as the “strawberry fruit” and it is highly appreciated for its organoleptic and nutritional composition (Hancock, 2000). Carbohydrates, organic acids and phenolics are the most studied compounds in strawberry and have been found to relate both to nutritional and organoleptic quality. Glucose, fructose and sucrose are the main sugars; ascorbic, malic and especially citric are the main acids found in the strawberry (Terry et al., 2007; Bordonaba & Terry, 2010; Mikulic-Petkovsek et al., 2012). Sugars are a source of energy (Mann, 2004) whereas short chain organic acids have been shown to contribute to gastrointestinal health (Dibner & Buttin, 2002). Ascorbic acid in particular has been associated with a reduced risk of chronic diseases, arguably by an antioxidant mechanism (Carr & Frei, 1999). It is worth noting, however, that there is very little evidence to show that the antioxidant mechanism is responsible for the reduction of the risk of disease in the human body (Pandey & Rizvi, 2009). Synthetic antioxidants are found to be harmful to health (Krishnaiah Bono, 2007) and purified extracts are not as effective as when taken directly from their natural source (Jin et al., 2009). The sugar to acid ratio is an important indicator of fruit taste (Bordonaba & Terry, 2010; Akhatou & Fernández Recamales, 2014; Ripoll et al., 2014).

Phenolics are a range of compounds comprising phenolic acids, flavonoids and tannins (Winkel-Shirley, 2001). Anthocyanins, a type of flavonoid, are the most abundant phenolics in strawberry, being responsible for their characteristic red colour, in addition to enhancing antioxidant power (Mas et al., 2007). Most phenolics express relevant

antioxidant properties *in vivo* and *in vitro* (Giampieri et al., 2012) and are related to organoleptic features of the fruit such as colour, aroma, flavour and texture. It is important to note that definitive evidence regarding the potential benefits of strawberries for human health is still weak. However compound-specific studies and work in model species seem to indicate that strawberries, for their particular nutritional composition, are likely to be beneficial for human health (Giampieri et al., 2012).

4.1 Carbohydrates

Carbohydrates or sugars are organic compounds formed of carbon, hydrogen and oxygen. The sugars found in strawberry leaves and roots are pentoses (xylose and lyxose) and hexoses (fructose, glucose, galactose and mannose) with their corresponding hexose-phosphates. These play a major role in cold acclimation (Rohloff et al., 2012). Glucose, fructose and sucrose are the three main water-soluble carbohydrates found in the berry (Bordonaba & Terry, 2010) where they mainly accumulate in the cell vacuole and are derived from the Calvin-Benson cycle (John & Yamaki, 1994). Sugar concentrations accumulate as the fruit develops (Macías-Rodríguez et al., 2002). Reported sugar concentrations in berries range from 16.2 to 27.3 mg of glucose, 14.7 to 30.2 mg of fructose and 2.48 to 33.5 mg of sucrose per gram of fresh weight (FW), and total sugars can be 325.1 mg g⁻¹ FW (Basson et al., 2010; Giné Bordonaba & Terry, 2010; Mikulic-Petkovsek et al., 2012).

The stage of ripeness (Basson et al., 2010), the age of plants, the soil or substrate quality, the strawberry production system, the genotype and the geographical location have been shown to affect the berry sugar content (Kallio et al., 2000). Additionally, storage conditions can alter sugar levels in strawberry (Pérez & Sanz, 2001). Sugars are significant quality factors (Kallio et al., 2000) and a source of energy for humans (Mann, 2004). The contributions of the different sugars to berry sweetness are not equivalent: fructose and sucrose are respectively 2.3 and 1.35 times sweeter than glucose (Keutgen & Pawelzik, 2007).

4.2 Organic acids

Organic acids are low-molecular weight carbohydrates which are found in all organisms and which are characterised by the possession of one or more carboxyl groups (Jones, 1998). Citric and malic are the main organic acids in the strawberry berry (Mikulic-Petkovsek et al., 2012). Other organic acids detected are tartaric, fumaric, shikimic, oxalic and ascorbic. Citric, malic and fumaric acids are produced in mitochondria via the citric acid cycle and to a lesser extent in the glyoxysome as part of the glyoxylate cycle (López-Bucio et al., 2000). Concentrations reported in the literature range from 5 to 20.27 g kg⁻¹ FW for citric; 0.98-5.34 g kg⁻¹ FW for malic; 150 mg kg⁻¹ FW for oxalic; 0.9 g kg⁻¹ FW for tartaric; 51.7 mg kg⁻¹ FW for fumaric; 7.9 to 8.7 mg kg⁻¹ FW for shikimic; 0.37 to 4.73 g kg⁻¹ FW for ascorbic acid. The total organic acid content can range from 7.26 to 57.4 g kg⁻¹ FW (Sturm et al., 2003; Kafkas et al., 2007; Basson et al., 2010; Giné Bordonaba & Terry, 2010; Koyuncu & Dilmaçunal, 2010; Mikulic-Petkovsek et al., 2012). Organic acid contents in the strawberry are known to be affected by the stage of ripeness (Kallio et al., 2000; Sturm et al., 2003; Kafkas et al., 2007; Basson et al., 2010), irrigation regime (Bordonaba & Terry, 2010), genotype (Cordenunsi et al., 2002; Kafkas et al., 2007; Mikulic-Petkovsek et al., 2012), and storage conditions (Pérez & Sanz, 2001). Additionally, the age of plants, genotype and geographical origin have been reported to influence fruit organic acid contents (Kallio et al., 2000).

4.3 Phenolic compounds

Chemically, the most abundant classes of phenolic compounds in strawberry derive from phenylalanine; this aromatic amino acid originates from the shikimic acid pathway which converts simple carbohydrate precursors into phenylalanine. The phenylpropanoid pathway starts at this point. Phenylalanine ammonia lyase (PAL) catalyses the reaction whereby phenylalanine loses an ammonia molecule forming cinnamic acid. PAL is situated at the branching point between primary and secondary

metabolism and the reaction it catalyses is an important regulatory step in the formation of phenolics. Chalcone synthase (CHS) is located at the end of the phenylpropanoid pathway and is rate limiting in the formation of most phenolic compounds, excluding phenolic acids that derive from the previous stages of the pathway (Cheng & Breen, 1991; Winkel-Shirley, 2001). However, the profile of phenolics will depend not only on these two enzymes but also on other enzymes involved in intermediate steps (Shahidi & Naczk, 2003; Winkel-Shirley, 2004).

Concentrations of phenolics reported in the literature are shown in **Table A.1**.

Table 0.1. Concentration of total phenolics determined by the Folin-Ciocalteu method described in previous studies of commercial strawberry fruit (*Fragaria x ananassa* Duch.)

Total Phenolics	Units	Comments	Reference
11.7 – 22.8	mg GAE g ⁻¹ DW	cv 'Pavana', 'Cavendish', 'Dania', 'Korona', and 'Honeoye' grown organically and conventionally	(Olsson et al., 2006)
1.7 - 3	mg GAE g ⁻¹ FW	Four selections of the interspecific cross between <i>F. x ananassa</i> and <i>F. virginiana glauca</i> . Dry matter ranging from 5.3 to 12.5%.	(Tulipani et al., 2008)
~650	mg GAE 100g ⁻¹ FW	cv 'Chandler'. Different storage temperature applied.	(Ayala-Zavala et al., 2004)
22.4 – 22.10	mg GAE g ⁻¹ DW	cv 'Elsanta.' Different irrigation regimes and abiotic stress (<i>Botrytis</i>)	(Terry et al., 2007)
187.6 – 271.3	mg GAE 100g ⁻¹ FW	cv 'Korona'. Different mulches, planting dates and fruit order	(Anttonen et al., 2006)
~288	mg GAE 100g ⁻¹ FW	cv 'Selva' grown in organic and conventional conditions	(Crecente-Campo et al., 2012)
~863	mg GAE kg ⁻¹ FW	-	(Mikulic-Petkovsek et al., 2012)
2.64- 3.36	mg GAE g ⁻¹ FW	cv 'Elsanta' planted at different dates under different plastic films.	(Ordidge et al., 2010)
3.6 – 4.4	mg GAE g ⁻¹ FW	cv 'Elsanta'. Different deficit irrigation regimes applied.	(Dodds, 2008)

Currently, concentrations of phenolics (total phenolics) are commonly determined by the Folin-Ciocalteu assay (Ainsworth & Gillespie, 2007). Phenolics in strawberry are

represented by phenolic acids, flavonoids and tannins (Giampieri et al., 2012). These are further discussed under the following sub headings.

4.3.1 Phenolic acids

Phenolic acids contain a single aromatic ring in their structure, occurring free and mostly in a diverse array of bound forms. Depending on the number of bonds between the hydroxyl group and the aromatic ring, phenolic acids can be grouped as hydroxycinnamic or hydroxybenzoic acids (Robbins, 2003; Mattila et al., 2006). The most common hydroxycinnamic acids in strawberry are caffeic, *p*-coumaric, ferulic and sinapic acids whereas the most common among the hydroxybenzoic acids are gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and benzoic acids (Mas et al., 2007). Concentrations of total phenolic acids, *p*-coumaric, caffeic, ferulic and *p*-hydroxybenzoic acids reported in the literature range from 8 to 67, 30 to 170, 0.5 to 14, 6 to 21 and 10 to 36 mg kg⁻¹ of fresh weight, respectively (Häkkinen & Heinonen, 1999; Buendía et al., 2010).

Hydroxycinnamic acids derive from phenylalanine. Cinnamic acid synthesis is catalysed by phenylalanine ammonia lyase, and then cinnamate 4-hydroxylase produces *p*-coumaric acid. These two are part of the phenylpropanoid pathway. Subsequent hydroxylations and methylations along a different pathway starting from *p*-coumaric acid lead to caffeic, ferulic and sinapic acids. The hydroxybenzoic acids have several proposed origins. The main route is the side chain degradation of the corresponding hydroxycinnamic acid (Robbins, 2003); an alternative route is postulated to start from an intermediate in the shikimic acid pathway: 3-dehydroshikimate, forming several hydroxybenzoic acid derivatives (Herrmann, 1995).

4.3.2 Flavonoids

Flavonoids are the major class of phenolic compounds (Giampieri et al., 2012). They are considered polyphenols (Robbins, 2003) as the basic carbon skeleton of a

flavonoid contains 15 carbons arranged in two aromatic ring connected by a three-carbon bridge (Shahidi & Naczki, 2003). The most common flavonoids in strawberry fruit are anthocyanins, flavonols and flavanols (Giampieri et al., 2012).

Anthocyanins are glycosides of anthocyanidins. Cyanidin and pelargonidin are the most commonly occurring anthocyanidins in strawberry (Lopes-da-Silva et al., 2007). The majority of anthocyanins are glucosides but galactosides, rhamnosides and arabinosides are also commonly found in strawberry berries (Clifford, 2000; Giampieri et al., 2012). Total anthocyanin content has been reported to be from 85 to 600 mg/kg fresh weight (Lopes-da-Silva et al., 2007; Aaby et al., 2012). Pelargonidin 3-glucoside is the major anthocyanin in strawberries constituting from 60 to 95% of the total (Lopes-da-Silva et al., 2002, 2007; Giampieri et al., 2012).

Flavonols provide a minor contribution to flavonoids in strawberry (Määttä-Riihinen et al., 2004; Giampieri et al., 2012). Kaempferol is the most common flavonol in strawberry followed by quercetin and myricetin (Häkkinen & Törrönen, 2000) accumulating in the vacuoles as flavonol glycosides (Aharoni et al., 2001; Griesser et al., 2008). Kaempferol and quercetin contents have been reported to be respectively 6 to 9 mg/kg of fresh weight and 3 to 12 mg/kg of fresh weight (Hollman & Arts, 2000; Häkkinen & Törrönen, 2000) whereas for quercetin 3-glucuronide and kaempferol 3-glucuronide, amounts are 11 and 6 mg/kg of fresh weight, respectively (Määttä-Riihinen et al., 2004). Total flavonol content can be 36 mg/kg of fresh weight (Almeida et al., 2007)

Flavanols are monomers of condensed tannins (Schofield et al., 2001; Almeida et al., 2007). Their content in strawberry is represented by catechin and epicatechin (Hannum, 2004; Määttä-Riihinen et al., 2004; Seeram et al., 2006; Aaby et al., 2012). Yet catechin is virtually the only flavanol present in strawberry fruit as epicatechin is found in very small amounts (Almeida et al., 2007). Although the glycosidic form is a general feature of flavonoids, making them more water soluble (Hannum, 2004), flavanol glycosides are rare (Hollman & Arts, 2000). Total flavanol content in

strawberry can be 36 mg/kg of fresh weight, of which 35.8 mg/kg are catechin and 0.2 mg/kg are epicatechin (Almeida et al., 2007). Other figures for catechin content in the literature are 24 mg/kg of fresh weight (Määttä-Riihinen et al., 2004), 44.7 and 49.1 mg/kg (Hannum, 2004) and 45 mg/kg of fresh weight (Aaby et al., 2012).

4.3.3 Tannins

Tannins are polyphenols with more than two aromatic rings in their structure (Robbins, 2003). They can possess 12 to 16 phenolic groups and have high molecular weight (Santos-Buelga & Scalbert, 2000). One of their most relevant features is their capacity to interact with other molecules, especially proteins. Astringency can be explained by the precipitation of proteins through their interaction with tannins (Haslam, 2007). There are two categories: the common condensed tannins or pro-anthocyanidins and the complex hydrolysable tannins (Salminen & Karonen, 2011). Condensed tannins are polymers of flavanols and leucoanthocyanidins linked by C-C and occasionally C-O-C bonds (Santos-Buelga & Scalbert, 2000; Tanner et al., 2003). Different strawberry fruit pro-anthocyanidin concentrations found in literature are 20 to 500 mg/kg (catechin equivalent) (Santos-Buelga & Scalbert, 2000); 539 to 1681 mg/kg (Buendía et al., 2010) and 30 to 188 mg/kg of fresh weight (Aaby et al., 2012). Hydrolysable tannins are heterogeneous polymers containing phenolic acids, especially gallic acid, and simple sugars, especially cyclic glucose.

In the strawberry fruit ellagitannins and gallotannins are the most common tannins (Giampieri et al., 2012): their most probable origin is gallic acid which in turn branches from an early intermediate of shikimic acid (Grundhöfer et al., 2001; Niemetz & Gross, 2005). Gallotannin hydrolysis yields gallic acid; gallotannins are not as widespread as ellagitannins (Arapitsas, 2012). Their content in strawberry can be 115 mg/kg of fresh weight (Määttä-Riihinen et al., 2004). Ellagitannin hydrolysis yields ellagic acid, which is the reason why they are called ellagitannins. Their origin is possibly a galloyl form, which is also a common intermediate for gallic acid (Clifford & Scalbert, 2000). Ellagic

acid contents reported for strawberry fruit can be 19.9 to 522 mg/kg of fresh weight (Hannum, 2004), 400 to 8700 mg/kg of dry weight (Clifford & Scalbert, 2000) after hydrolysis, and 9 to 21 mg/kg of fresh weight of free ellagic acid (Buendía et al., 2010). In turn, ellagitannins amounts can be 97 to 229 mg/kg of fresh weight (Buendía et al., 2010).

4.4 Strawberry antioxidant capacity

Antioxidants are substances that prevent or slow the oxidation of other substances. Their role is very important in plant and animal physiology, and especially so for human health (Olsson et al., 2006; Battino et al., 2009). Antioxidants protect the cell against so-called reactive species or reactive oxygen species (ROS) (free radicals). These are naturally occurring molecules that have at least one unpaired electron. They are highly reactive and can be very harmful for the plant as they are able to damage tissues, enzymes and other molecules. They are produced during respiration and photosynthesis: a difference between the availability of energy and the fixation of this energy into carbon molecules is believed to be the origin of ROS. The plant produces antioxidants in order to prevent the damage caused by ROS (Shao et al., 2008; Matkowski, 2008; Takahashi & Badger, 2011).

Antioxidants also have relevance for human health. Epidemiological studies show how the consumption of fruits and vegetables reduces the chances of chronic diseases, stroke and heart disease as well as risk of obesity, and control of diabetes. This is thought due to the presence of bioactive compounds or phytochemicals in these products (Hannum, 2004; Atkinson et al., 2005). Many of these have potential or established antioxidant properties (Carr & Frei, 1999; Matkowski, 2008; Battino et al., 2009).

Flavonoids are excellent antioxidants. These act on their own or synergistically with other antioxidants such as ascorbate or tocoferol. In addition, they seem to chelate metals therefore preventing the formation of ROS (Hannum, 2004). Organic acids,

namely oxalic, malic and citric acids, have antioxidant properties, mainly preventing metals from forming free radicals (Ma et al., 2001). Ascorbic acid is perhaps the most effective antioxidant because of its low reactivity when bound to reactive species and its high reactivity when in ion form (Carr & Frei, 1999). When measuring electrochemical responses at 400 mV to estimate antioxidant properties, ascorbic acid is the most important antioxidant (24%) followed by ellagitannins (19%) and anthocyanins (14%) (Aaby et al., 2007).

Methods to measure antioxidant capacity include hydrogen atom transfer (HAT) and single electron transfer (SAT) which in turn are the two major mechanisms by which antioxidants can deactivate free radicals. The most relevant assays are Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Power (FRAP), the Folin-Ciocalteu method and Trolox Equivalent Antioxidant Capacity (TEAC). ORAC is a HAT method whereas the other four are SAT methods (Prior et al., 2005). These methods do not reflect the whole antioxidant capacity of the sample, therefore when comparing antioxidant capacities the assay used must be carefully considered.

4.5 Environmental and developmental effects on strawberry fruit quality

The typical strawberry inflorescence is botanically classified as a cyme. As such, its flowers are ranked as primary, secondary and tertiary usually developing in a staggered manner (Hancock, 2000; Tsormpatsidis et al., 2011). The extent to which fruit quality is affected by fruit hierarchical position has been investigated by several authors. Atkinson et al. (2006), showed that there were no significant differences in ellagic acid concentrations in fruits between positions although the values tended to be higher in primary fruits. In contrast, Anttonen et al. (2006) obtained significant differences not only in ellagic acid but also anthocyanin concentration, antioxidant activity, measured by the diphenylpicrylhydrazyl (DPPH) method, and total phenolics: ellagic acid, antioxidant activity and total phenolics were higher in secondary fruits and anthocyanin concentration in primary fruits. It is important to clarify whether

phytonutrient concentration in fruit are expressed in terms of FW or DW, since under moderate or severe water deficits, fruit water content can be reduced such that phytonutrient concentrations appear to be higher (Terry et al., 2007). However, this is often due simply to less dilution, rather than to increased production per se. For instance, Terry et al. (2007) reported significantly higher total phenolic content in non-stressed primary fruits when expressed as FW, and lower contents when expressed as DW, than those of secondary fruits. These authors obtained similar results for one of the main anthocyanins, pelargonidin-3-glucoside. These findings do not concur with those of Anttonen et al (2006) for antioxidant activity, but it does for total phenolic concentrations (as FW). This is probably due to the fact that Terry et al, (2007) used the DPPH and 80% ethanol for the extraction to determine antioxidant activity whereas Anttonen et al, (2006) used the FRAP method and 70% acetone containing 0.01 M hydrochloric acid.

Time of the year can have an effect on quality of fruit of the same chronological age. Tsormpatsidis et al. (2008) studied the concentration of phenolics, flavonoids and anthocyanins in fruits at different hierarchical positions. Their conclusions did not differ significantly from those of Anttonen et al. (2011) for example, but time of harvest was important in that the primary fruit of the second pick had a significantly lower concentration of total phenolics than fruit from the first pick (June vs July). Glasshouse experiments performed by Anttonen et al. (2011) showed a decreased accumulation of total phenolics in March compared to February. It is not clear from these studies whether variations due to time are the result of environmental factors, the age of the plant or other interacting factors.

Fruit quality is also influenced greatly by the stage of fruit ripening. At the green stage, the antioxidant activity, measured by the ORAC method, and the total phenolic concentration can be highest, declining subsequently as the fruit ripens (Wang, 2000; Cheng & Breen, 1991). Special care should be taken in the interpretation of total phenolic data because it can be easily overestimated as the Folin-Ciocalteu reagent

used in the detection of the phenolics reacts with other antioxidants such as ascorbic acid (Ainsworth & Gillespie, 2007). More detailed analyses showed that anthocyanins peaked at the red stage (Fait et al., 2008; Halbwirth et al., 2006; Wang & Lin, 2000; Cheng & Breen, 1991) which has been explained as an accumulation process (Halbwirth et al., 2006). Pro-anthocyanidins, in turn, seem to be more abundant than anthocyanins at earlier stages of fruit development (Halbwirth et al., 2006). Other flavonoids accumulate at higher concentrations at earlier phases of fruit development (Halbwirth et al., 2006). At least some flavonoids are synthesized *in situ* rather than being transported from other tissues: two peaks of PAL activity can appear at 5 and 25 days after anthesis in 'Tillicum' strawberry (Cheng & Breen, 1991). Those peaks of activity may correspond to the accumulation of flavonoids and more specifically pro-anthocyanidins and anthocyanins (Cheng & Breen, 1991).

5 The plant hormone ethylene

Ethylene is the simplest alkene (C₂H₄) and the simplest unsaturated hydrocarbon after acetylene (C₂H₂). It was noted for the first time as an effect of gas and coal combustion on plants in the nineteenth century; it was first identified in 1901 by Neljubov after he realized that the abnormal growth of the peas in his lab was due to the combustion of the illuminating gas; and it was finally described as a product of plant metabolism when Gane in 1934 found that apples produce ethylene (Bleecker and Kende, 2000).

Ethylene is a gas produced especially by climacteric plants during fruit development and ripening. Nevertheless, in non-climacteric plants, such as strawberry (*Fragaria x ananassa*, Duch.), ethylene production can also be detected (Perkins-Veazie et al., 1995), the significance of which is controversial. What is commonly known as the strawberry fruit is botanically classified as a secondary fruit, where the fruits (achenes) are sitting on the receptacle (Hancock, 2000). In fact, the achenes show a response to ethylene suggesting that at least this tissue could be considered to be climacteric in its ripening behaviour (Merchante et al., 2013).

In broad terms, the physiological responses triggered by ethylene are senescence of plant organs, changes in plant growth and ripening processes. In turn, the levels of ethylene can vary at different stages of plant life and also under the effect of biotic and abiotic stresses (Morgan and Drew, 1997; Bleecker and Kende, 2000; Wang et al., 2002).

5.1 Ethylene biosynthesis

A “direct route” for ethylene biosynthesis (**Figure 0.2**) follows the sequence L-methionine, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-carboxylate (ACC). The intervening enzymes at each of the reactions are SAM synthetase, ACC synthase and ACC oxidase, respectively. ACC synthase is believed to have a central role in the rate of ethylene production (Liu & Zhang, 2004). 5'-methylthioadenosine (MAT) is a by-product of the action of ACC synthase, and the first step of a number leading to the production of L-methionine, which is known as the Methionine (Yang) cycle. In detail, MAT acts as a substrate for MAT nucleosidase producing 5'-methylthioribose (MTR) which derives to 5'-methylthioribose-1-phosphate by the action of MTR kinase; α -keto- γ -methylthiobutyric acid results spontaneously and serves as the substrate for a transaminase producing L-methionine. As has been mentioned above, ACC is the substrate of ACC oxidase, the last step for the production of ethylene. However, it can also be the substrate for ACC N-malonyl-transferase (MACC), deriving to N-malonyl-ACC (Wang et al., 2002). After some initial uncertainty, this reaction is known to be reversible (Morgan & Drew, 1997).

The expression of the enzymes involved in ethylene biosynthesis is due to multiple genes. For instance, there are 10 known genes encoding for ACC synthase in *Arabidopsis* (Wang et al., 2005). Focusing on the enzymes in the “direct route” towards the biosynthesis of ethylene, the genes encoding for SAM synthetase, ACC synthase, ACC oxidase identified in strawberry are to date (05/02/2016) the following:

- SAM synthetase: *FvSAMS1* (GenBank no.AFI38954.1) (Sun et al., 2013)

- ACC synthase: *FvACS1* (GenBank no.AAT77723), *FvACS2*, *FvACS3*, *FvACS4* (Merchante et al., 2013)
- ACC oxidase: *FvACO1* (GenBank no.CAH65482), *FvACO2* (GenBank no.CAH65483), *FvACO3* (GenBank no.AAU10090.1), *FvACO4* (Merchante et al., 2013)

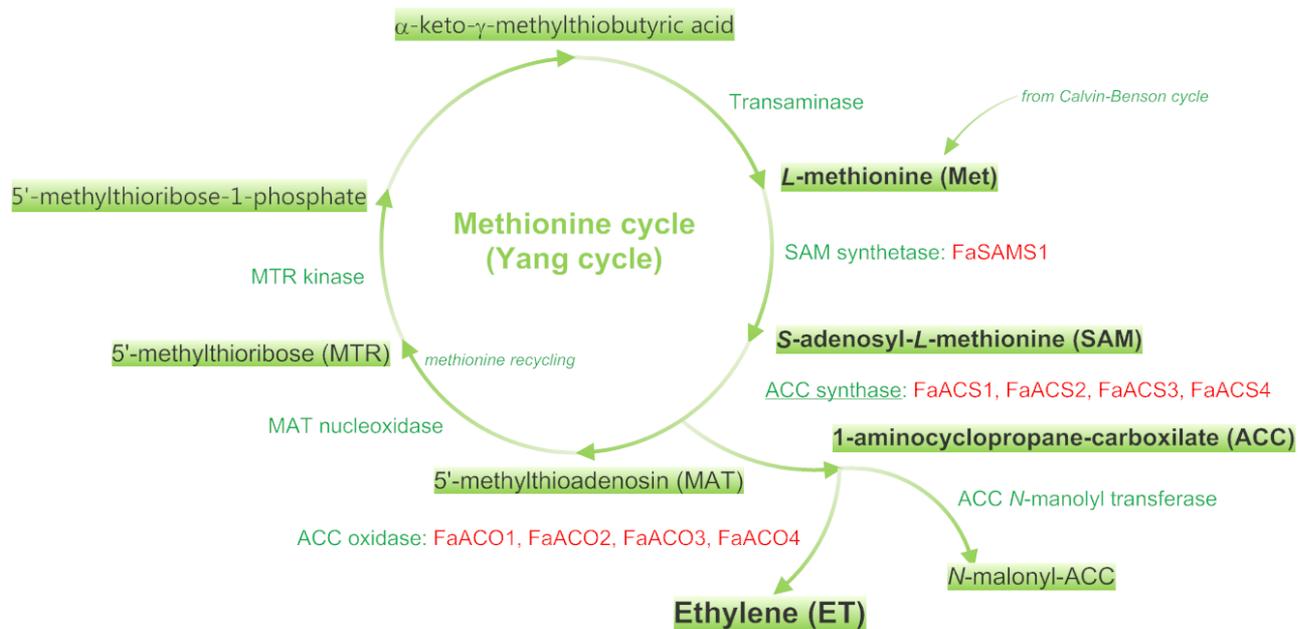


Figure 0.2. Methionine cycle described by Yang. Ethylene biosynthesis is the result of the action of mainly three enzymes: SAM synthetase, ACC synthase and ACC oxidase. It starts with L-methionine which is recycled from one of the sub-products of ACC synthase (Yang & Hoffman, 1984).

5.2 Biosynthesis regulation of ethylene biosynthesis under abiotic and biotic stress

Stress, including drought, mechanical wounding, salinity, pathogen attack, ozone and others, can increase organ ethylene production rate (Morgan and Drew, 1997; Bleecker and Kende, 2000; Wang et al., 2002). SAM synthetase is a key enzyme for ethylene biosynthesis but is not believed to be especially important for ethylene production under stress. In strawberry, the SAM pool is thought to be sufficient to allow the synthesis of ethylene while the enzyme activity is still low. When the level of the

pool decreases beyond a certain point, SAM synthetase is evoked again (Sun et al., 2013).

Both ACC synthase and ACC oxidase activities can increase under stress conditions. ACC synthase is regarded as the main player in the stress-induced production of ethylene (Liu & Zhang, 2004). This is the case because ACC oxidase is present in higher amounts than ACC synthase and it is not considered to be limiting (Liu & Zhang, 2004). However, ACC oxidase can be the most responsive enzyme under stress conditions (Kacperska, 1997). In case of wilting wheat for instance, the level of ACC oxidase can increase up to eight-fold whereas levels of ACC synthase remain similar (McKeon et al., 1995).

Regarding ethylene biosynthesis and the role of ACC synthase during stress, the plant stress-responsive mitogen activating protein kinase (MAPK) pathway is particularly interesting.. This pathway plays a central role in the communication between the cell membrane and cell nucleus, and is highly influenced by environment. The MAPK pathway has been reported to be affected by water deficit (Ripoll et al., 2014). For example, increasing levels of MPK3 and MPK6 target ACS6 and help to stabilize the enzyme, thereby increasing ethylene production rate (Liu and Zhang, 2004; Colcombet and Hirt, 2008).

5.3 Ethylene-signalling pathway

The signalling pathway is best understood in *Arabidopsis* where it is believed there are two groups of ethylene receptor proteins (Guo & Ecker, 2004): a) subfamily one (ETR1, ERS1), and b) subfamily two (EIN4, ETR2 and ERS2). There is a structural difference between these two subfamilies. Subfamily one has a histidine kinase domain whereas subfamily two has a degenerated histidine kinase domain. In the absence of ethylene, the receptors block the ethylene response pathway. When ethylene is present, it binds to the receptors and the response occurs (**Figure 0.3**). The model of this response is still incomplete but it is known that the receptor proteins can

inactivate the CTR1 protein in the presence of ethylene, which in turn unblocks the EIN2 protein and the transcriptional cascade thereafter and ultimately activates ethylene response genes. Known transcription factors involved are EIN3 (firstly) and ERF1 (secondly) (Wang et al., 2002; Guo & Ecker, 2004; Sun et al., 2013). The ethylene receptor proteins identified in strawberry so far are ETR1 and ERS1 from subfamily one and ETR2 from subfamily two. The genes encoding these proteins are *FaETR1* (GenBank no.AJ297511), *FaERS1* (GenBank no.AJ297512) and *FaETR2* (GenBank no.AJ297513) (Merchante et al., 2013).

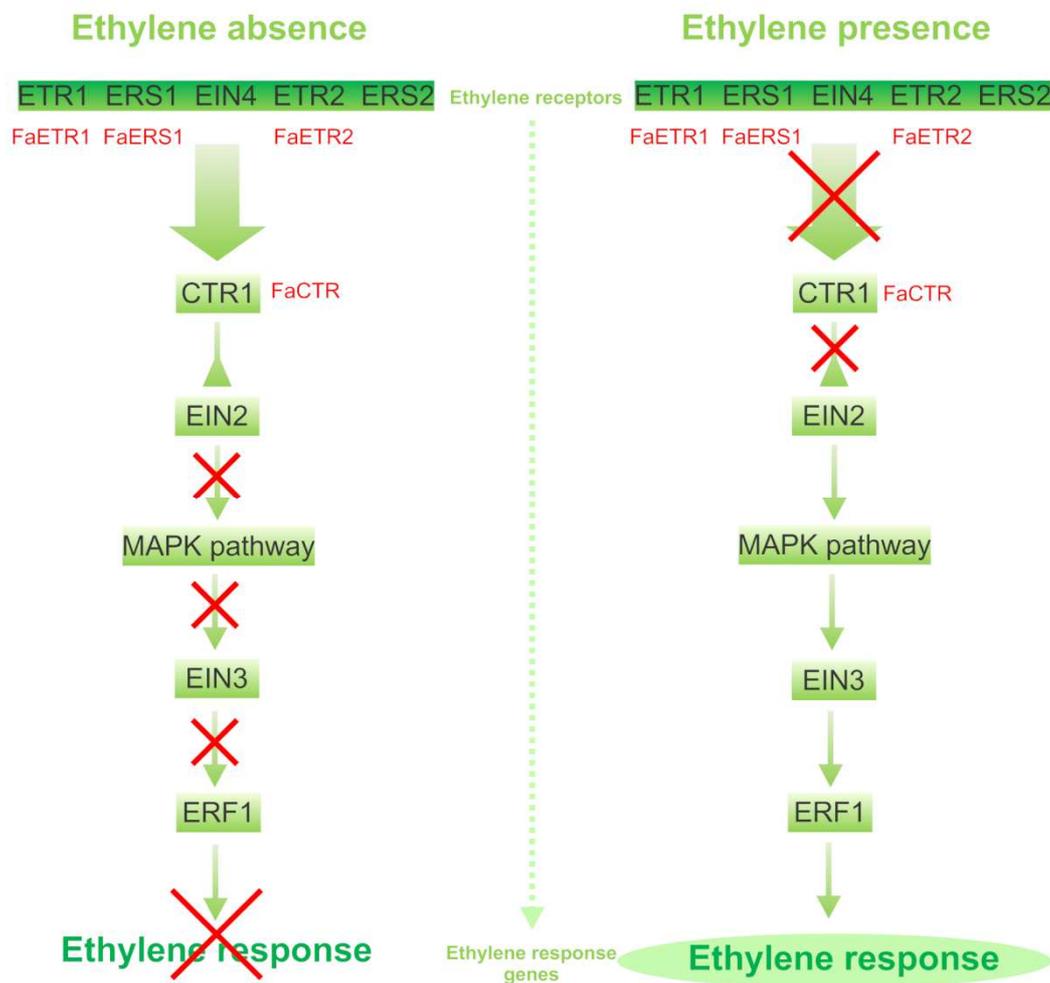


Figure 0.3. Ethylene response pathway. In the presence of ethylene, the receptors bind to the CTR1 protein preventing it from blocking the downstream signalling pathway leading to the ethylene response. In the absence of ethylene, the sequence of proteins involved in the signalling pathway are EIN2, those of MAPK pathway, EIN3 and ERF1.

5.4 Ethylene-signalling under stress

In a study investigating the expression of the proteins involved in the ethylene signal transduction, drought, salt stress, and pathogen infections were reported to trigger the overexpression of ERF1 in *Arabidopsis* (Cheng et al., 2013). It has also been shown in a study of 7,000 *Arabidopsis* genes under drought, high-salinity and/or cold stresses that a member of the ERF family (GeneBank no.AV823763) was highly induced by drought and salinity (Seki et al., 2002).

Interestingly, as well as being involved in the regulation of ethylene biosynthesis, the stress-responsive MAPK pathway seems to be involved in the ethylene response. In the presence of ethylene, the receptors are inactive so CTR1 cannot block EIN2 and the MAPK pathway. Downstream in this pathway, MPK3 and MPK6 targets EIN3 which, in turn, activates ERF1, triggering the activation of the ethylene response genes (Colcombet & Hirt, 2008). The MAPK pathway can be affected by water deficit (Ripoll et al., 2014).

5.5 Ethylene inhibitors

Given the pivotal role of ethylene in fruit ripening and post-harvest deterioration, there has been great interest in controlling either its production and/or perception. 1-Methylcyclopropene (1-MCP) commercialized as EthylBlock[®] and SmartFresh[®] is the most relevant example. It has been used since 1999 to extend the life of stored fruit and flowers. 1-MCP binds to the ethylene receptors with 10 times more affinity than ethylene and therefore effectively prevents ethylene binding and subsequent responses (Watkins, 2006). Other inhibitors of ethylene perception include silver thiosulfate (STS) which cannot be used in food because of its toxicity, some cyclic olefins such as 2,5-norbadiene and also diazo-cyclopentadiene (DACP) which permanently inactivate the receptor in the presence of fluorescent light after binding (McKeon et al., 1995; Martínez-Romero et al., 2007).

Concerning ethylene production, inhibition can potentially occur at several of the biosynthetic steps: SAM synthetase can be competitively inhibited pre- and post-harvest with polyamines. Using putrescine on peach post-harvest can increase shelf-life by 2- to 3-fold (Martínez-Romero et al., 2007). The inhibition of ACC synthase can be achieved competitively using (+) SAM or by inhibiting the cofactor pyridoxal phosphate with either aminoethoxyvinylglycine (AVG) or aminoxyacetic acid (AOA). For the inhibition of ACC oxidase, cobalt (Co²⁺) and α-aminoisobutyric acid (Aib) have been proved to act through a competitive mechanism (McKeon et al., 1995; Martínez-Romero et al., 2007). In addition, cyclopropane-1,1-dicarboxylic acid (CDA), trans-2-phenylcyclopropane-1-carboxylic acid (PCCA), 2-methyl cyclopropanecarboxylic acid (MCA) and cyclopropanecarboxylic acid (CCA) have been also tested but showed little agronomic potential (Dourtoglou & Koussissi, 2000). Inhibitors in forms other than gas have been applied to intact fruit through the peduncle (Given et al., 1988) and by means of vacuum (Tian et al., 1994; Dominguez, 1997).

Table 0.2. Compounds that can be used to inhibit ethylene biosynthesis or perception in strawberry

Target	Inhibitor	CAS number	Dosage in strawberry	Comments	References
Inhibition of ACC Synthase	AVG	49669-74-1	0.001M; incubated ¹ during 1,24 and 48 h or introduced ² through the peduncle	Spray. Competitive cofactor pyridoxal phosphate	(Basiouny, 1988) (Given & Venis, 1988)
	AOA	645-88-5	-	-	-
	(+)-AdoMet	29908-03-0	-	Spray.	-
Competitive inhibition of ACC Oxidase	Co ²⁺	-	-	Spray. Toxic	-
	Aib	62-57-7	-	Spray	-
Inhibition of ethylene perception	1-MCP	3100-04-7	2 ppm for 12 h	Gas	(Tian et al., 2000)
	STS	-	20 mM silver nitrate	Toxic food	in (Given & Venis, 1988)

AVG: aminoethoxyvinylglycine; AOA: aminoxyacetic acid; (+)-AdoMe: (+)-S-adenosyl-L-methionine; Aib: α-aminoisobutyric acid; 1-MCP: 1-methylcyclopropene; STS: silver thiosulfate.

5.6 Ethylene and fruit quality in strawberry

Normally phenolics, anthocyanin content and PAL activity increase concomitantly with increasing rates of ethylene production (Jiang & Joyce, 2003a). For example, when 1-MCP (inhibitor of ethylene action) was used in stored strawberries, PAL activity, ethylene emissions and phenolic content were reduced (Jiang et al., 2001). Other examples with transgenic plants showed that strawberry plants with diminished sensitivity to ethylene had reduced *FaPAL* expression in the achenes (Merchante et al., 2013). In contrast, when *FaSAMS1* and *FaCTR1* (at “white stage”) were down-regulated, colour development was inhibited in the whole fruit while ethylene production was increased. This was explained as a consequence of the presence of a large pool of SAM (Sun et al., 2013). However, when using ethephon (analogue of ethylene action) later in the same study, colour was partially restored (Sun et al., 2013).

5.7 Cross-talk between ethylene and other hormones

It is well known that exogenous ethylene can lead to either autocatalytic ethylene biosynthesis or an autoinhibitory ethylene biosynthesis. These are features of System II and System I signalling, respectively. System II operates during the respiratory rise in climacteric fruit whereas System I is the default situation within climacteric fruit and most tissues, and the most common system for non-climacteric fruit. Nevertheless, there seems to be a System II-like pathway in at least non-climacteric *Citrus* fruit (Katz et al., 2004). Application of ethylene to citrus peel disks can suppress ACC synthase activity and formation and reduce endogenous ethylene production (Riov & Yang, 1982a). Autoinhibitory or autocatalytic effects on endogenous ethylene production have been observed in intact citrus leaves. These effects were due to the suppression or increased synthesis, respectively, of ACC synthase and ACC oxidase (Riov & Yang, 1982b). In clover, an autocatalytic effect has been attributed to increased ACC oxidase activity (Scott et al., 2010). In tomato, a climacteric fruit, the autocatalytic effect could

be caused for a higher expression of *LsACS2* whereas the System I signalling was reported to lead to higher *LsACS1A* and *LsACS6* expression (Barry et al., 2000).

Abscisic acid (ABA) can have an antagonistic effect to that of ethylene (Sharp & LeNoble, 2001). For example, ERF1 in *Arabidopsis* under drought and salt stress can be highly induced by ethylene and jasmonic acid (JA) signalling and inhibited by ABA (Cheng et al., 2013). However, this interaction may not be antagonistic in all instances (Gazzarrini & Mccourt, 2003) nor follow a linear correlation. Ethylene production by corn leaves did not relate to water deficit unless ABA levels were low (Voisin et al., 2006) and may act additively to ABA regarding root growth (Gazzarrini & Mccourt, 2003). In addition, ABA has been reported to increase ethylene production via at least two mechanisms; exogenous application of ABA increased ACC oxidase transcripts in seeds of sugar beetroot (Hermann et al., 2007), in broccoli after harvest (Pogson et al., 1995), and ACC synthase and ACC oxidase activity in apples during ripening (Lara & Vendrell, 2000).

Some of the typical ethylene responses could, in fact, be mediated by auxin. Rungruchkanont et al., (2007) showed that application of ethylene plus either inhibitors of auxin transport or action induced abscission of stigma of *Dendrobium* flowers, whereas abscission of stigma was not induced by application of auxin (Rungruchkanont et al., 2007). Hypocotyl growth in the dark, flowering and leaf senescence were induced in *Arabidopsis* by exogenous ACC and reverted upon treatment with inhibitors of auxin transport (Vandenbussche et al., 2003). In addition, a recent paper reported that System II ethylene biosynthesis may require increasing levels of auxin (Tatsuki et al., 2013). More evidence for this cross-talk can be found at the molecular level: auxin can induce the expression of genes related to ethylene biosynthesis (*ACS* in peach, *ACO1* in rice), perception (*ETR2*) and signalling, but also ethylene was reported to induce a gene (*PIN1*) related to auxin transport (Chae et al., 2000; Trainotti et al., 2007).

There is a certain consensus in considering gibberellins (GA) and ethylene as antagonistic hormones. When looking at the activity of chlorophyllase (which breaks down chlorophyll), ethylene tends to cause an enzyme accumulation whereas GA has the opposite effect (Jacob-Wilk et al., 1999). There are at least two possible mechanisms explaining this interaction: a) a link between the CTR1 and the GA-DELLA signalling pathway, that was reported based on the finding that *ctr1-1* mutants of *Arabidopsis* had lower GA bioactive content, flowering and vegetative growth which could be rescued upon treatment with GA (Achard et al., 2007); b) GA reduction of ACC oxidase transcripts in beans (Kaneta et al., 1997).

Other hormones interact with ethylene as well, for instance cytokinins (CK) and jasmonic acid (JA). CK positively regulate ACC synthase activity and therefore ethylene production. Many of the growth effects attributed to cytokinins are actually ethylene effects (Gazzarrini & Mccourt, 2003). JA in turn seems to interact with ethylene at either the level of ERF during the ethylene response signalling (Guo & Ecker, 2004) or the level of EIN2 as well during ethylene signalling (Gazzarrini & Mccourt, 2003).

6 Background of the current project

East Malling Research has much experience in working with water deficit irrigation techniques with the aims of improving water and fertiliser use efficiencies, improving fruit quality and extending shelf-life. These can translate into benefits for the grower, retailer and consumer as that can mean producing the same yields with fewer inputs, reducing waste, and increasing health benefits for the consumer. Dr Mark Else and his team continue to explore the effects that precision and deficit irrigation have on strawberry fruit quality and the signalling involved in such effects. A former PhD student, Dr Philippa Dodds, showed in her research that organic acids and phenolics were increased by RDI and especially PRD treatments (Dodds, 2008). The transient wilting occurring during the switching of the PRD treatments was hypothesized to be

the reason behind the higher fruit quality observed with respect to RDI and the development of the TDI technique (Dr Mark Else, personal communication).

The mechanism underlying the effects of deficit irrigation on fruit quality is still to be elucidated. Among the candidate signals (ROS, pH, ions and electrical signals), hormones are of special interest because a) they are present in plants in very small amounts having large effects on the plant functioning (Weyers & Paterson, 2001); b) they are testable *in vivo* and d) there is a significant body of literature supporting the role of plant hormones in the communication of root and shoot abiotic and biotic stresses (Morgan et al., 1990; Morgan & Drew, 1997; Sharp & LeNoble, 2001; Wilkinson & Davies, 2002; Wilkinson & Davies, 2010; Peleg & Blumwald, 2011; Chen et al., 2013).

In vitro biochemical research has provided evidence of specific changes triggered by the environment, but the study *in vivo* is more challenging (Pahlic, 1993). For *in vivo* studies, Jackson (2001), inspired by Koch's postulates for pathological research (Fredericks & Relman, 1996), proposed postulates to prove the involvement of a hormone in a metabolic event (see Weyers & Paterson, 2001):

- Correlation: simultaneous changes in the timing of the hormone concentration and its potential effect
- Duplication: reproduction of the endogenous changes occurring naturally by exogenous application of the hormone
- Deletion and re-instatement: removing or decreasing the concentration of the hormone, and then re-instate the original concentration. The potential effect of the hormone should behave accordingly (i.e. It should decrease during deletion and increase during re-instatement).
- Specificity: the effects on the target cannot be mimicked by other hormones
- Relevance to higher levels of organization: in sum, the effects observed should be consistent across different environments

- Relevance to lower levels of organization: the hypothesis will be finally confirmed when the expected effects of the hormone are observed at cellular level
- Generality: the same response should appear in related taxonomic groups.

Hormones are intrinsic to metabolism, pathogens are not. Hormones are always present, triggering plant responses when their concentrations change and interacting with other signals (Wilkinson & Davies, 2002). Even more, according to “metabolic control analysis” the metabolic regulation of single events depends on numerous and interdependent effects and there are no single pacemakers in any metabolic reaction (Pahlic, 1993). There are multiple examples of this complexity in the literature.

The aim of the current project was to use the approach advocated by Jackson to elucidate the potential role of the plant hormone ethylene in the increase of fruit quality attributes during a severe water deficit and subsequent re-hydration of the strawberry cultivar ‘Elsanta’. The programme of research began by investigating and characterising plant morphological, physiological and metabolic responses to severe water deficits treatment, with a particular emphasis on fruit phenolic and organic acid accumulation and then continued by testing the potential causal role of ethylene in these responses. The hypothesis tested was that

A stress-induced increase in fruit ethylene production rate enhances berry phenolic concentrations in plants under severe water deficit

The aims of the project was to understand the role of ethylene in the regulation of stress responses and changes in fruit phytonutrient content in plants under severe water deficit

7 Objectives of this investigation

- To develop and define a severe water deficit treatment for strawberry plants cv ‘Elsanta’

- To determine whether there is a temporal correlation between the production of ethylene and the concentration of phenolics in stressed strawberry fruit
- To determine the effects of deleting the putative ethylene signal during the stress on fruit phenolic concentrations by preventing its biosynthesis a) by competition of ACC synthase (AVG) or b) by competition of ACC oxidase (Aib)
- To substitute the ethylene signal by a) ACC or b) ABA and determine effects on fruit phenolic concentrations
- To search for genetic evidence for the involvement of ethylene in the accumulation of phenolics in stressed strawberries

Chapter 2. Leaf responses to severe water deficit and other environmental factors

1 Introduction

This chapter describes the responses of leaves of strawberry plants (*Fragaria x ananassa* Duch. cv 'Elsanta') which were subjected to severe water deficit (SWD) in a controlled environment facility (Grodome) at East Malling Research during 2014.

Since most UK strawberry crops are grown under cover, they must be irrigated. Much of that water comes from areas classified by Defra as “over-abstracted” or “over licenced”. Recent legislation such as the Water Act 2014 has been issued in order to increase the regulation of water resources. In addition, the Government Abstraction Licence Reform program is generating concern in the industry because of the removal of the exemption for trickle irrigation. Inevitably the demand from the soft fruit industry is leading to an increased emphasis on the efficient use of water (Fereres et al., 2003; Knox et al., 2009; Else & Atkinson, 2010; Hanjra & Qureshi, 2010). This work on water use should help to optimize use of future fresh water and allow growers to comply with legislation.

Work at East Malling Research has shown that it is possible to achieve a 70% reduction in water volume for irrigation with respect to ADAS “best practice” (ADAS, 2003). Such reductions do not affect yield (Dr Mark Else, personal communication). Water deficit techniques not only reduce the use of water but may also enhance fruit quality, shelf-life and diminish canopy density, making fruits more visible and reducing picking costs. Plant physiological responses to drought are a valuable tool for managing water deficit treatments because they give a picture of the plant response so it can be adjusted by the irrigation (Jones, 2004). Drought treatments are known to affect gas exchange and the efficiency of photosystem II (Blanke & Cooke, 2004; Liu et al., 2007; Grant et al., 2012a,b; Snider et al., 2014) which together provide information on the effect of the treatment on the light independent and light dependent processes of photosynthesis,

as well as on stomatal conductance (Klamkowski & Treder, 2008; Razavi et al., 2008; Wilkinson & Davies, 2008; Else et al., unpublished). Leaf responses could be used to anticipate responses from other plant parts, for instance fruits. However, unless all environmental variables are tightly controlled, the responses of different plants to the same peat volumetric moisture content may not be the same. The degree to which different plant responses react to interacting environmental variables is also likely to vary (Collatz et al., 1991, 1992; Scheidegger et al., 2000; Hubbard et al., 2001; Blanke & Cooke, 2004).

Known deficit irrigation techniques include regulated deficit irrigation (RDI) and partial root zone drying (PRD), which can increase the phytonutrient content in strawberry. When equivalent volumes of irrigation water are applied, the potential of PRD to enhance ascorbic and ellagic acids concentration in strawberry fruit has been reported to be higher than that of RDI (Dodds, 2007; Terry et al., 2007a; Else & Atkinson, 2010; Giné Bordonaba & Terry, 2010). PRD involves switching irrigation from wet to dry areas of the root system, after which transient leaf wilting often occurs. Against this background, Dr Mark Else developed a novel deficit irrigation technique: transient deficit irrigation (TDI), characterized by a short period of soil drying, a 48 h period of wilting and a fast re-hydration of the soil to well-watered levels (WW). Preliminary results showed that TDI can enhance antioxidant accumulation more effectively than either RDI or PRD (Else, personal communication). Furthermore, when basil (*Ocimum basilicum*) was subjected to TDI, ascorbic acid concentration and trolox equivalent antioxidant capacity (TEAC) were higher than when subjected to RDI (Else et al., unpublished). TDI was therefore used as the starting point for the development of the SWD treatment. This treatment has yet to be fully defined but involves a period of drying, and maximum period of a 48 h wilting which may or may not be followed by re-hydration.

Monitoring of the plant response to the stress will be done by rapid and non-destructive physiological measurements: stomatal conductance, transpiration, photosynthesis,

performance of the photosystem II. These give real-time information on the status of the net photosynthesis and light dependent photosynthetic processes. Non-destructive leaf water potential measurements will not be performed because a reliable instrument was not available.

Substrate water status will be monitored by measurements of the water content with an electrical probe and sets of pot-and-plant weights. In addition, other environmental variables measured included temperature, light intensity and relative humidity. In all, the measurement of this variables gave an idea of the conditions of the plant during the experiments.

The objectives of the work described in this chapter were as follows:

- To establish the best management strategy for the SWD treatment
- To ascertain leaf plant responses and their interaction with other environmental variables during the SWD treatment

2 Materials and Methods

2.1 Overview of the experiments and data collection for this chapter

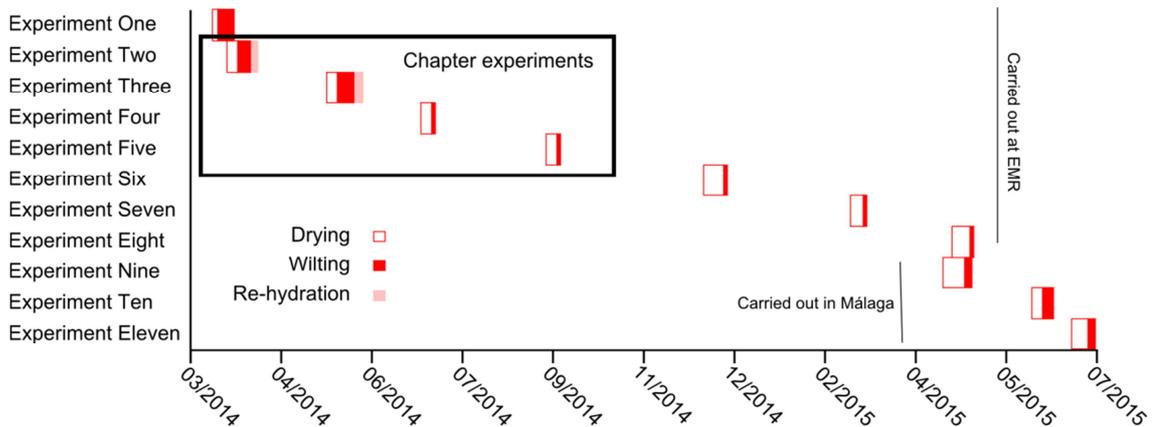


Figure 0.1. Sequence of drying, wilting and re-hydration periods for every experiment over the course of this research: experiments included in this chapter are within box “Chapter experiments” and under “Carried out at EMR”. All of them were run in the Grodome facility, chamber five at East Malling (Kent) in 2014.

The experiment one is not reported in this thesis. Leaf responses were measured during the SWD treatment and compared to control. These measurements were repeated during experiment two.

Experiment four data are included in this chapter to illustrate the variability in environment in the Grodome.

This chapter focuses on the leaf plant responses measured during the imposition of a SWD. In the experiments described here, data on fruit responses to the same stress were also collected; these are reported in Chapter 3. Fruit responses to severe water deficit and other environmental factors

2.2 Plant material

Bare-rooted strawberry plants (*Fragaria x ananassa* Duch. cv ‘Elsanta’) grade A+ (13 mm crown diameter) that had been stored at 2 °C were purchased from RW Walpole Ltd, UK. Plants were potted in 2 l polypropylene pots (Plantpak Rose container polypropylene [Ø140 x 180 mm], Desch-EPLA B.V., Netherlands) containing 1,000 to 1,200 g of peat-based compost with no additives such as fertilizer or insecticide. Four

sampling points for the WET-2 probe (Delta-T Services Ltd, UK) were made at 4 cm and 15 cm from the bottom on opposite sides of the pot. In order to stop water from evaporating from the peat surface, two covers were placed on the pot: a white polyvinyl circular cover on the top around the plant crown and a sheath-cover around the pot to minimise moisture loss from the WET sampling ports. The top cover was white in order to reflect light and to protect the root system from overheating. This cover also served to stop trusses from snapping or being damaged on the rim of the pot. The sheath-cover was made by cutting 4 cm off of the bottom of spare deep rose plant pots. Pots were placed on a 16.5 cm diameter saucer (Richard Sankey & Son Ltd, UK) to collect any run-through.

2.3 Controlled environment cultivation

Experiments were carried out in one of the six chambers of the Grodome, a secure, double-skinned, polycarbonate building, contained facility. Light control was achieved with sodium light (Osram Vialox Navcson_T) and delivered ~3,400 W per chamber at 2 m over the growing area. Light hours went from 7:00 a.m. to 7:00 p.m. over the whole growing season. Temperature control was a diurnal temperature set to 22 °C and nocturnal to 18°C.

2.4 Irrigation application, scheduling and treatments

Pots were irrigated using pressure-compensating, self-cleaning, 2 l h⁻¹ on-line TechFlow™ Junior CNL emitters fitted with 50 cm of Ø4 mm micro-tube (lace) and an on-line compact pressure compensated Angle Model (pot stake) (Netafim Ltd, Israel). Lines were polyethylene and 13 mm internal diameter attached to 19.05 mm (internal diameter) valve with a dedicated S-392 latching solenoid to control the flow (Bermad Inc., USA). Timing and duration of the irrigation events was controlled by a Galcon 6154 DC4S timer (Galcon Bakarim Agricultural Cooperative Society Ltd, Israel).

Irrigation was applied four times per day (08:00, 11:00, 14:00 and 17:00). Pots were maintained at full pot capacity by irrigating until run-off was observed and until the beginning of the treatment. When trusses started to emerge, daily irrigation event lengths were determined on the basis of accumulative evaporative demand in the form of degree hour values ($^{\circ}\text{C h}$) obtained with a SKTS 500 evaposensor and dedicated SEM 500 logger (Skye Instruments Ltd, UK). The evaposensor sensor was placed between plants at the same height as the plant canopies. The sensor had a wet and a dry thermometer. The volume of water evaporated from the sensor was correlated to the difference of temperature between the wet and dry thermometers. The relation between the evaporative demand and the length of the irrigation event was a crop factor (F_c) expressed as seconds of irrigation needed per degree hour. In order to calculate F_c , accumulated degree hours, time elapsed and gravimetric water loss were recorded once per week on at least 24 randomly distributed well-watered (WW) plants. Daily irrigation events were increased by 20% to compensate for any soil drying due to rapid vegetative growth and/or fruit production, since sudden increases in water demands would not be accounted for until F_c re-calculation.

Irrigation of SWD plants was withheld at large green fruit stage for a mean of seven days until they started to wilt. At this point, plants were supplied with an amount of water that replaced that lost by daily transpiration. Drying rate of change differed between individuals so two approaches were undertaken: a) in experiments one, two and three, plants were allowed to dry at their own rate whereas b) in experiments four, five, six and seven fast drying plants were re-hydrated daily to weight of slow drying plants. SWD was divided in three phases: 1) drying 2) sustained and transient wilting 3) re-hydration to WW soil moisture levels.

2.5 Experimental design

For each experiment, a statistically randomised experimental design was used that included six blocks, and two irrigation treatments (SWD and WW) (**Table 0.1**).

Table 0.1. Experimental design and experimental practice for experiments two, three, four and five.

	Experiment two	Experiment three	Experiment four	Experiment five
Irrigation treatment	WW SWD	WW SWD	WW SWD	WW SWD
Fruit stage	Large green White 50% red Red	Large green	Red fruit	Large green
Blocks	6	6	6	6
Relevant phases of the experiment	Drying (large green) Wilting (large green) Re-hydration (large green, white, 50% red, red)	Midday of the first day of wilting Midday of the second day of wilting One hour after re-hydration Three hours after re-hydration One day after re-hydration	"Other treatment" applied during the first night of wilting "Fruit sampling" during re-hydration phase	Eleven times: Midday of the last day of drying 10:00 a.m. on the first, second day of wilting and first day of re-hydration 13:00 on every day from last day of drying to a day after re-hydration (5 days in total) 16:00 on the first, second day of wilting and first day of re-hydration
Other treatment	No	No	Air Cabinet Cabinet + 1 ppm 1-MCP Cabinet + 0.05 ppm Ethylene Cabinet + 0.5 ppm Ethylene Cabinet + 5 ppm Ethylene	No
Number of plants	72	60	72	132
Drying approach	One block at a time, six consecutive days Plants left to dry at their own pace	All experiment at once Plants left to dry at their own pace	All experiment at once Water added to fast drying plants	All experiment at once Water added to fast drying plants
Leaf responses measurement approach	Measured at the time of fruit sampling	Measured at the time of fruit sampling	Measured at the time of fruit sampling When "Other treatment" applied	Measured at the time of fruit sampling
Fruit sampling for fruit response	When indicated in "Phase of the irrigation treatment"	When indicated in "Phase of the irrigation treatment"	One week after "Other treatment" applied	When indicated in "Phase of the irrigation treatment"

WW: well-watered; SWD: severe water deficit; large green: fruit as seven days before turning colour; 1-MCP: 1-methylcyclopropane. Primary controls indicated in **red**.

2.6 Crop protection

A major risk in all experiments was infection by powdery mildew (*Podosphaera aphanis*). Routine sprays consisting of a combination of systemic and contact

fungicides were applied regularly in rotation to avoid resistance. Routine sprays were applied every two weeks until the beginning of the treatment.

In addition, a sulphur burner (Hotbox international Ltd, UK) was installed in the experimental chamber continuously working as powdery mildew prevention measure.

2.7 Measurement of environmental factors

2.7.1 Peat volumetric moisture content

Measurements of peat volumetric moisture content (PVMC) were taken with a HH2 moisture meter and WET-2 probe (Delta-T Services Ltd, UK) at four points in the pot: (in order) top right, bottom right, top left and bottom left. In experiment two, measurements were taken right after the 11:00 a.m. and the 14:00 irrigation events on WW and SWD plants as they dried and during re-hydration; measurements on experiments three, four and five were taken every day at midday during the SWD treatment. At the time of fruit sampling measurements were also taken. Units were cubic meter of water per cubic meter of peat ($\text{m}^3 \text{m}^{-3}$).

2.7.2 Pot-and-plant weight

Weighings were done at the same time as PVMC measurements. Pots were weighed with pot cover, saucer, label and sheath cover, no water was added or tipped out between weighings. Units were given in grams per hour (g).

2.7.3 Light intensity

A data Hog 2 logger (Sky Instruments Ltd, UK) was placed among the experimental plants in each of the experiments in the Grodome (Unigro Ltd, UK). A single channel LUX sensor (Sky Instrument Ltd, UK) was attached to it. Readings were taken with a 30 min frequency. Values at sampling time were used for regression analyses, and refer to solar energy, expressed in terms of kilograms per cubic second (kg s^{-3}).

2.7.4 Temperature and relative humidity

Air temperature (T) and relative humidity (RH) were monitored in the Grodome compartment and in the air-tight cabinets used to apply 1-methylcyclopropene (1-MCP).

In the Grodome, a temperature and humidity sensor was integrated in the Data Hog 2 logger. Temperature values were given in degrees Celsius ($^{\circ}\text{C}$). Relative humidity units were expressed as the ratio of the partial pressure of water vapour to the equilibrium vapour pressure of water at the same temperature (%). Readings were taken with a 30 min frequency and values at sampling time were used for linear regression analyses.

2.8 Leaf responses: non-destructive measurements

Leaf responses were recorded twice a day in experiment two during the drying and wilting phases on control WW plants and SWD plants on fully expanded young leaves. In experiments three and five, leaf responses were recorded when fruit samples were collected for analysis. Experiment two had five fruit sampling times when fruit was at the large green stage: 1) first day of wilting, 2) second day of wilting, 3) one hour after re-hydration, 4) three hours after re-hydration and 5) 25 hours after re-hydration. Experiment five had 11 sampling times, the first being at 13:00 h of day before plants start wilting; then seven times during the two days of wilting (10:00, 13:00 and 16:00 h) and three times during re-hydration; last measurement being at 13:00 h of the second day after re-hydration.

2.8.1 Stomatal conductance

Stomatal conductance (g_s) was measured by gas exchange with a LI-6400XT Portable Photosynthesis System and a 6400-40 leaf chamber fluorimeter (LI-COR Inc., USA). Settings of the instrument were: flow at $300 \mu\text{mol s}^{-1}$, light at ambient values ($\mu\text{mol m}^2 \text{s}^{-1}$), reference CO_2 at 400 ppm and temperature at ambient values ($^{\circ}\text{C}$). Measurements were taken from the middle leaflet of a young fully expanded leaf per plant once

readings stabilised. Units were given in moles of water transpired per square meter of leaf per second ($\text{mol m}^{-2} \text{s}^{-1}$).

2.8.2 Photosynthetic rate

Readings of net photosynthesis were measured by gas exchange with a LI-6400XT Portable Photosynthesis System and its 6400-40 leaf chamber fluorimeter (LI-COR Inc., USA). Settings of the instrument were as described in 2.8.1. Measurements were taken from the middle leaflet of a young fully expanded leaf per plant once readings stabilised. Units were given in micromoles of CO_2 assimilated per square meter of leaf per second ($\mu\text{mol m}^{-2} \text{s}^{-1}$).

2.8.3 Transpiration rate

Transpiration was measured by gas exchange with a LI-6400XT Portable Photosynthesis System and its 6400-40 leaf chamber fluorimeter (LI-COR Inc., USA). Settings of the instrument were as described in 2.8.1. Measurements were taken from the middle leaflet of a young fully expanded leaf per plant once readings stabilised. Units were given in millimoles of water transpired per square meter of leaf per second ($\text{mmol m}^{-2} \text{s}^{-1}$).

2.8.4 Photosystem II efficiency

Efficiency of the photosystem II (Yield [II]) was assessed with a MiniPAM and its leaf-clip holder 2030-B (Heinz Walz GmbH., Germany). Yield (II) determination was based on the measurement of maximal fluorescence yield during the light saturation pulse (F_m') and the fluorescence yield just before the saturating pulse (F_t). The result is dimensionless:

$$\text{Yield (II)} = (F_m' - F_t)/F_m'$$

2.9 Leaf responses: light curves

Light response curves for stomatal conductance, net photosynthetic rate and transpiration rate were measured by gas exchange at midday of the second day of wilting on three WW plants and three SWD plants.

2.9.1 Photosynthetic rate, stomatal conductance and transpiration response to different light intensities

Response curves were performed with a LI-6400XT Portable Photosynthesis System and its 6400-40 leaf chamber fluorimeter (LI-COR Inc., USA). Settings of the instrument were: flow at $300 \mu\text{mol s}^{-1}$, reference CO_2 at 400 ppm and temperature at ambient values ($^{\circ}\text{C}$). Readings were taken automatically from one leaf per plant once readings had stabilized at each of the following light intensities: 1500, 100, 750, 300, 150, 100, 50, 25, 0, and $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$.

2.9.2 Efficiency of the photosystem II under different light intensities

This was assessed with a MiniPAM and its leaf-clip holder 2030-B (Heinz Walz GmbH., Germany). The leaf-clip holder was fixed at canopy height with a stand. The recording of the performance of photosystem II involved nine consecutive measurements at the following light intensities: 80, 250, 410, 580, 760, 1150, 1550, 2300 and $3300 \mu\text{mol m}^{-2} \text{s}^{-1}$. At each Yield (II) value was recorded. Yield (II) is dimensionless.

2.10 Statistical analysis

Linear regression modelling was performed on the data with response variables being leaf responses and explanatory variables being environmental variables. Models were adjusted at maximum R^2 and model assumptions were checked against normality, homoscedasticity and independence of errors. Two way analyses of variance was performed on the data of a) whole experiment and b) each irrigation phase within each experiment. Results of the latter were only presented if contradictory to those of the whole experiment. Finally, *t-tests* were carried out in order to ascertain differences

between SWD and WW at each measurement time. Statistical analyses were carried out with R version 3.1.0 (R Foundation for Statistical Computing, Austria).

3 Results

3.1 Environmental records during experiments

Environmental records were used to 1) explain the variability in leaf and fruit responses to the SWD treatment and 2) define the SWD treatment: duration of the different phases and their management.

3.1.1 Peat volumetric moisture content and pot-and-plant weights

Measurements and pot-and-plant weight (PW [g]) and peat volumetric moisture content (PVMC [$\text{m}^3 \text{m}^{-3}$]) were made to detect changes in the rate of peat drying in response to SWD and the effectiveness of subsequent re-wetting (**Figure 0.2** and **Figure 0.3**).

Experiment three and four reached full pot capacity (WW) at very similar PW and PVMC ($\sim 2,004 \text{ g}$ and $\sim 0.67 \text{ m}^3 \text{ m}^{-3}$) whereas experiment two and five reached it at lower PW and PVMC ($\sim 1,809 \text{ g}$ and $\sim 0.50 \text{ m}^3 \text{ m}^{-3}$). There was some variation in PW and PVMC within WW plants over the course of the treatment, the highest being that of experiment four ($SEM=6.25 \text{ g}$ and $0.0041 \text{ m}^3 \text{ m}^{-3}$) and the lowest in experiment three ($SEM=7.94 \text{ g}$ and $0.0046 \text{ m}^3 \text{ m}^{-3}$). Some variation in PW and PVMC was also found within WW and SWD plants: confidence intervals were largest for water stressed plants in experiment three, followed by those of experiment two, becoming wider as the treatment progressed. Standard error of the mean for WW plants was comparatively smaller, being greater in experiment two.

All experiments showed similar rates of drying ($\sim 150 \text{ g per day}$) but due to their individual full pot capacities drying phase length differed among them; it was longer for experiments three and four than for experiment five and especially experiment two. Interestingly, wilting was first recorded at similar PVMC and PW in the all experiments apart from experiment two ($\sim 0.23 \text{ m}^3 \text{ m}^{-3}$ and $\sim 1,200 \text{ g}$). Effectiveness of the re-hydration in recovering PW and PVMC to WW level varied between experiments: re-hydration for experiments four and five took hours whereas two and three took longer than a day.

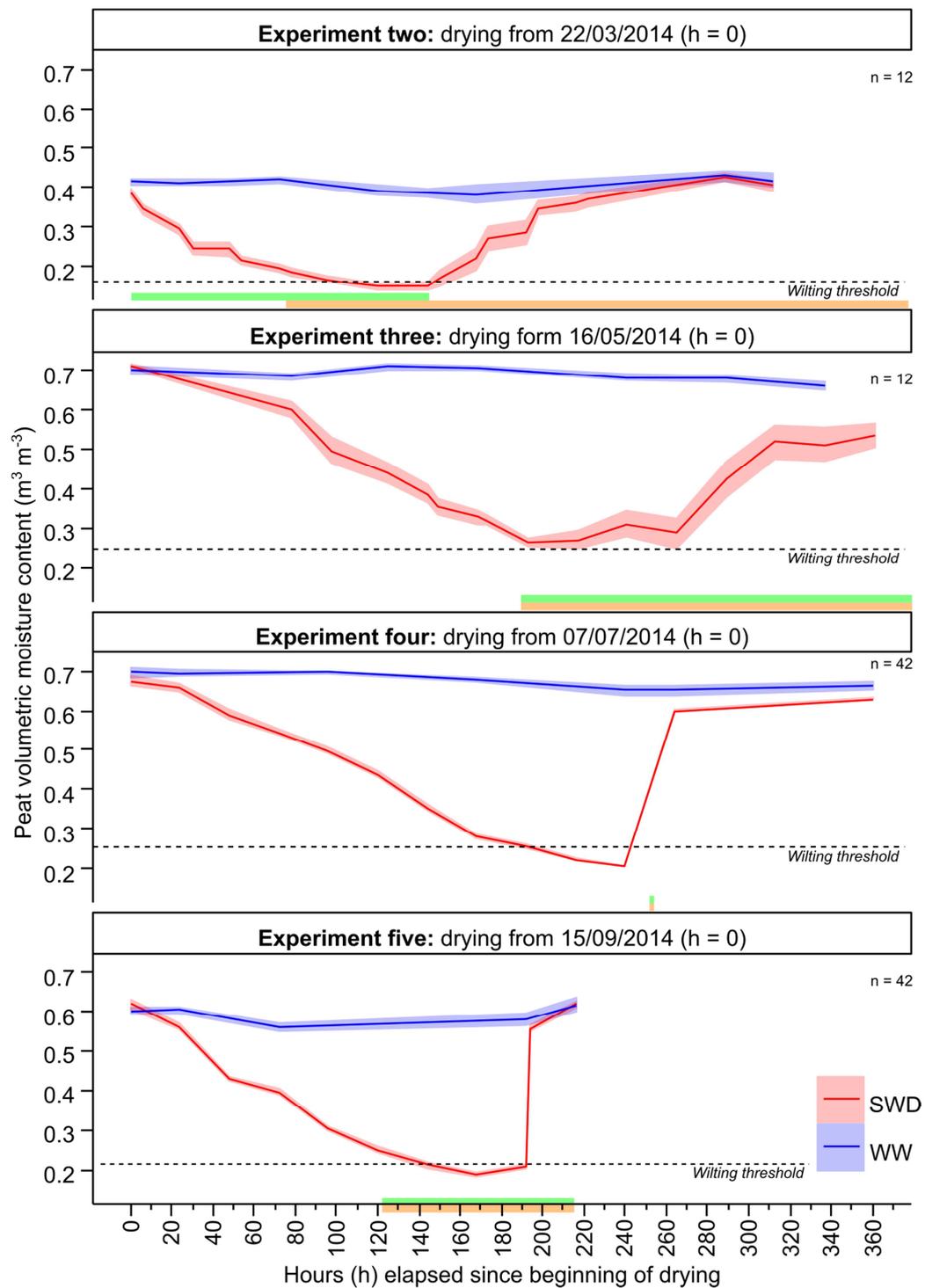


Figure 0.2. Means of peat volumetric moisture content ($\text{m}^3 \text{m}^{-3}$) for plants under severe water deficit (SWD) or well-watered conditions (WW). Readings were taken over the SWD drying period to end of experiments two, three, four and five. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014. Confidence intervals calculated as standard error of the mean are shadowed behind the mean line of each treatment and experiment. Dashed horizontal line indicates the mean of peat volumetric moisture content at the start of wilting (*wilting threshold*). Green horizontal lines represent the time length over which plant responses were measured; orange horizontal line corresponds to the time length over which fruit responses were measured.

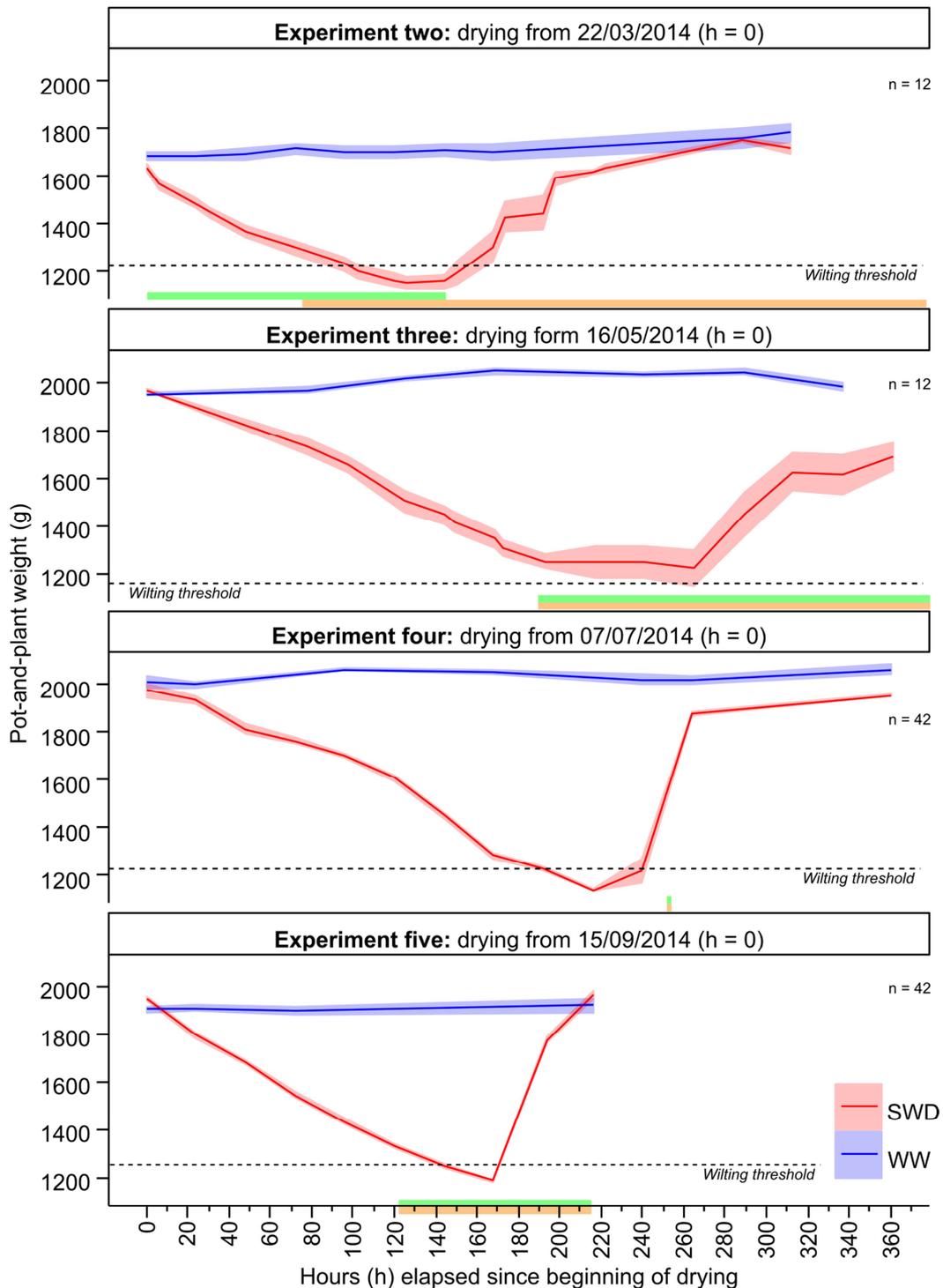


Figure 0.3. Means of pot-and-plant weight (g) for plants under severe water deficit (SWD) or well-watered conditions (WW). Readings were taken over the SWD drying period (h) to end of experiments two, three, four and five. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014. Confidence intervals calculated as standard error of the mean are shadowed behind the mean line of each treatment and experiment. Dashed horizontal line indicates the mean of pot-and-plant weight at the start of wilting (*wilting threshold*). Green horizontal lines represent the time length over which plant responses were measured; orange horizontal line corresponds to the time length over which fruit responses were measured.

3.1.2 Light intensity, ambient temperature and relative humidity

Continuous monitoring of light intensity (kg s^{-3} , same as W m^{-2}), temperature ($^{\circ}\text{C}$) and relative humidity (%) was done over the course of the experiments carried out in the Grodome in 2014. Data were used to explain the variability observed in PVMC, PW and leaf responses.

3.1.2.1 *Light intensity*

Daily mean light intensity (**Figure 0.4**) was highest during experiment four with 91.26 kg s^{-3} , followed by experiment three (57.28 kg s^{-3}), experiment five (37.53 kg s^{-3}) and experiment two (12.19 kg s^{-3}). Standard deviation was greater for experiment four data, with 126.18 kg s^{-3} , followed by experiment three (71.83 kg s^{-3}), experiment five (46.82 kg s^{-3}) and experiment two (22.60 kg s^{-3}). The peak light intensity was measured in experiment four ($\sim 500 \text{ kg s}^{-3}$) and the lowest in experiment two ($\sim 20 \text{ kg s}^{-3}$).

3.1.2.2 *Ambient temperature*

The highest mean temperature was in experiment four at 19.58°C , followed by experiment three (17.99°C), five (17.53°C) and two (15.02°C). The highest temperature was measured during experiment two, reaching almost 40°C ; the minimum temperature was also reached during this experiment (15°C). Standard deviation of temperature during experiment five (**Figure 0.5**) (6.80°C) was 32% higher than the second highest (experiment four [4.56°C]). Experiment three and experiment five followed with 3.56°C and 2.96°C .

3.1.2.3 *Relative humidity*

Mean values of relative humidity calculated from the records over the course of each experiment were in descending order: 81.43, 73.80, 72.48 and 54.48% for experiment five, three, four and two, respectively. Maximum relative humidity record was measured during experiment five, being nearly 100% and the minimum relative humidity of $\sim 12\%$ was recorded during experiment two. Relative humidity in

experiments three, four and five was lowest during wilting phase increasing during re-hydration and decreasing during drying. No such trend was observed in experiment two. The lowest relative humidity control (**Figure 0.6**) was achieved during experiment two ($SD=16.23\%$), followed by experiment four ($SD=10.40\%$) whereas experiment three and five were similar ($SD=6.71\%$ vs 6.93%).

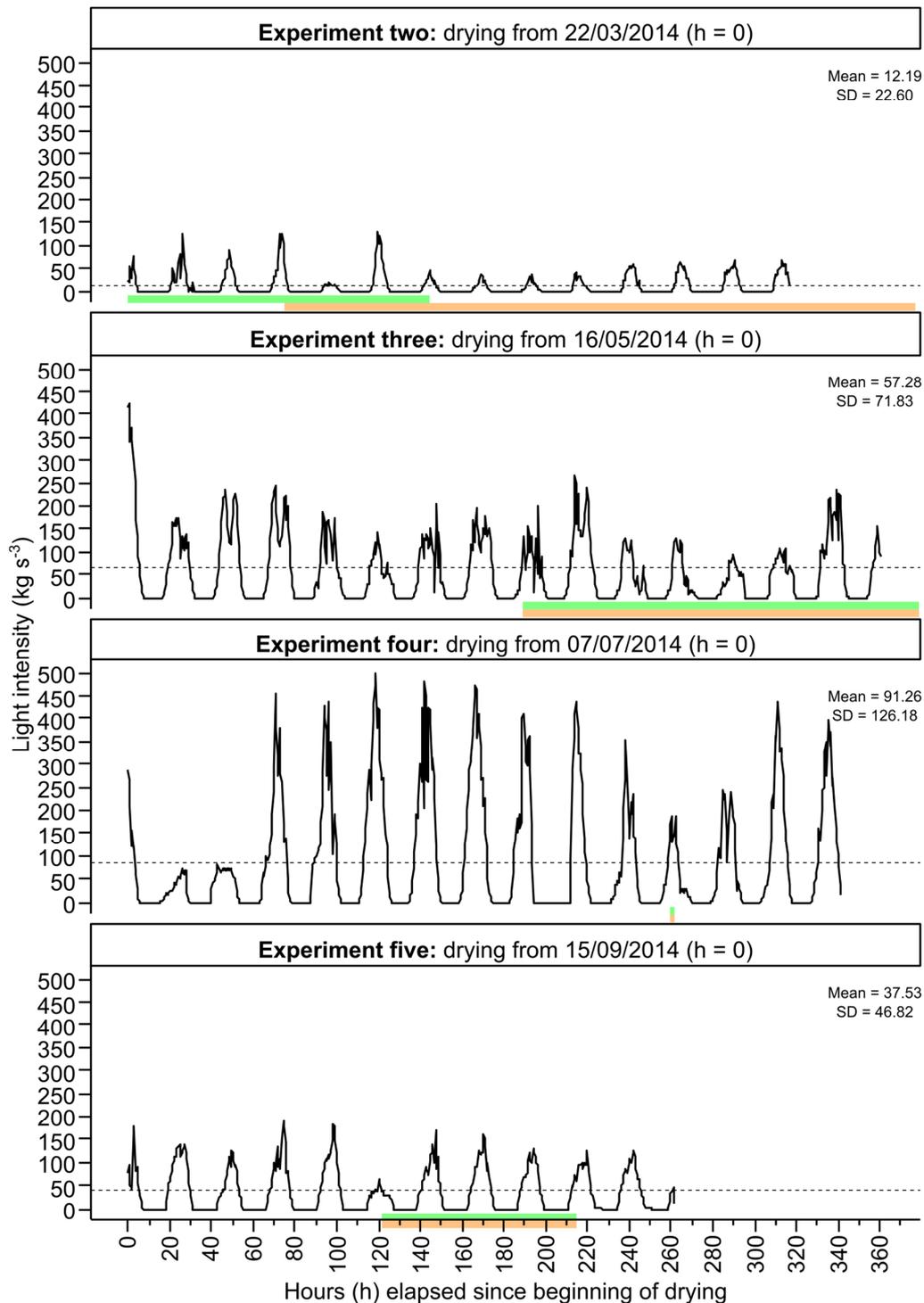


Figure 0.4. Records of light intensity (kg s^{-3}) in chamber five of the Grodome facility at East Malling Research (Kent) in 2014. Readings taken over the drying period (h) of plants severe water deficit treatment (SWD) to end of experiments two, three, four and five. Mean of the data for each experiment correspond to dashed horizontal line. Green horizontal lines represent the time length over which plant responses were measured; orange horizontal line corresponds to the time length over which fruit responses were measured.

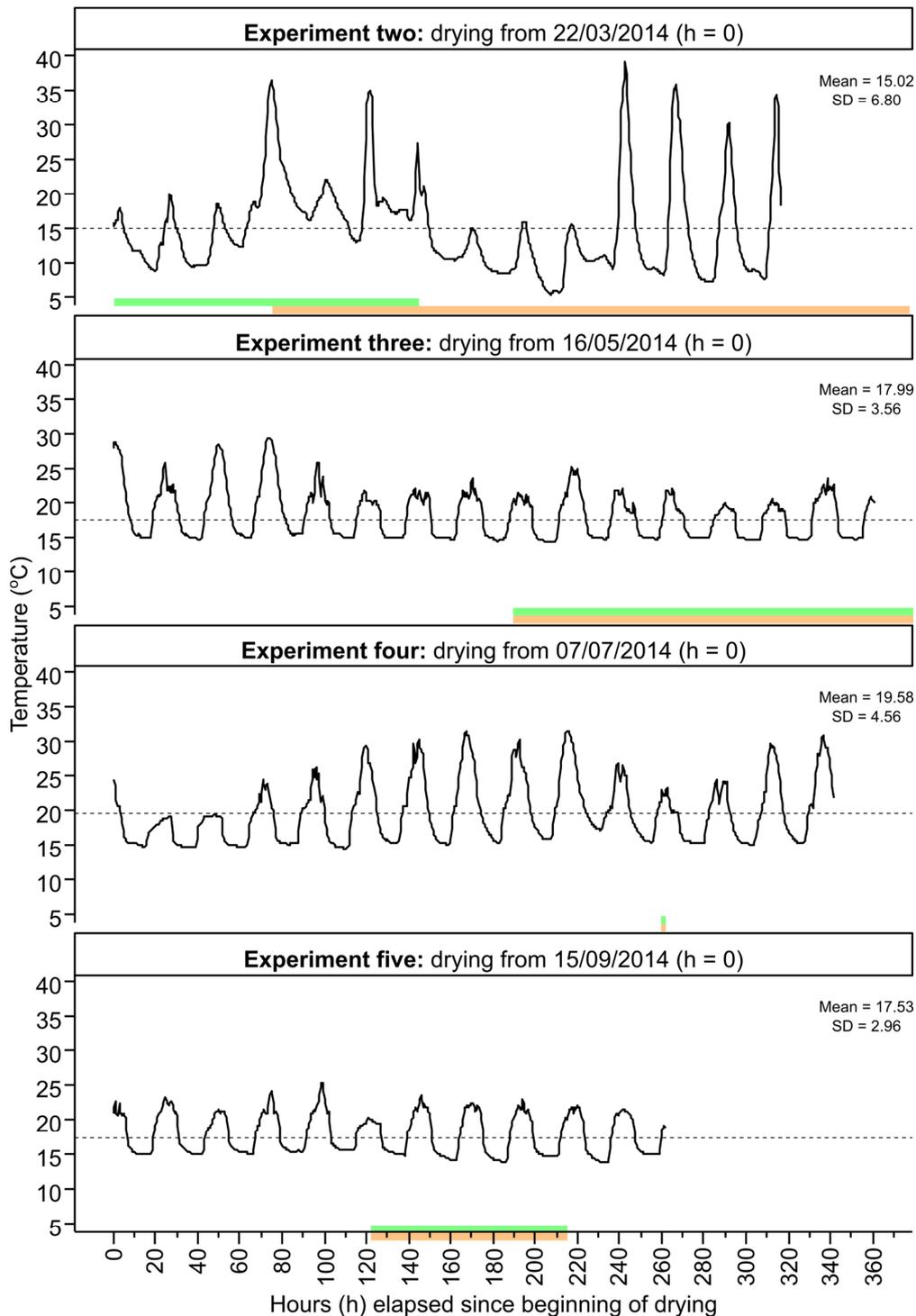


Figure 0.5. Records of temperature (°C) in chamber five of the Grodome facility at East Malling Research (Kent) in 2014. Readings taken over the drying period (h) of plants under severe water deficit treatment (SWD) to end of experiments two, three, four and five. Mean of the data for each experiment correspond to dashed horizontal line. Green horizontal lines represent the time length over which plant responses were measured; orange horizontal line corresponds to the time length over which fruit responses were measured. Pre-set temperature values were 18 °C and 22 °C for night and day temperatures respectively for all experiments.

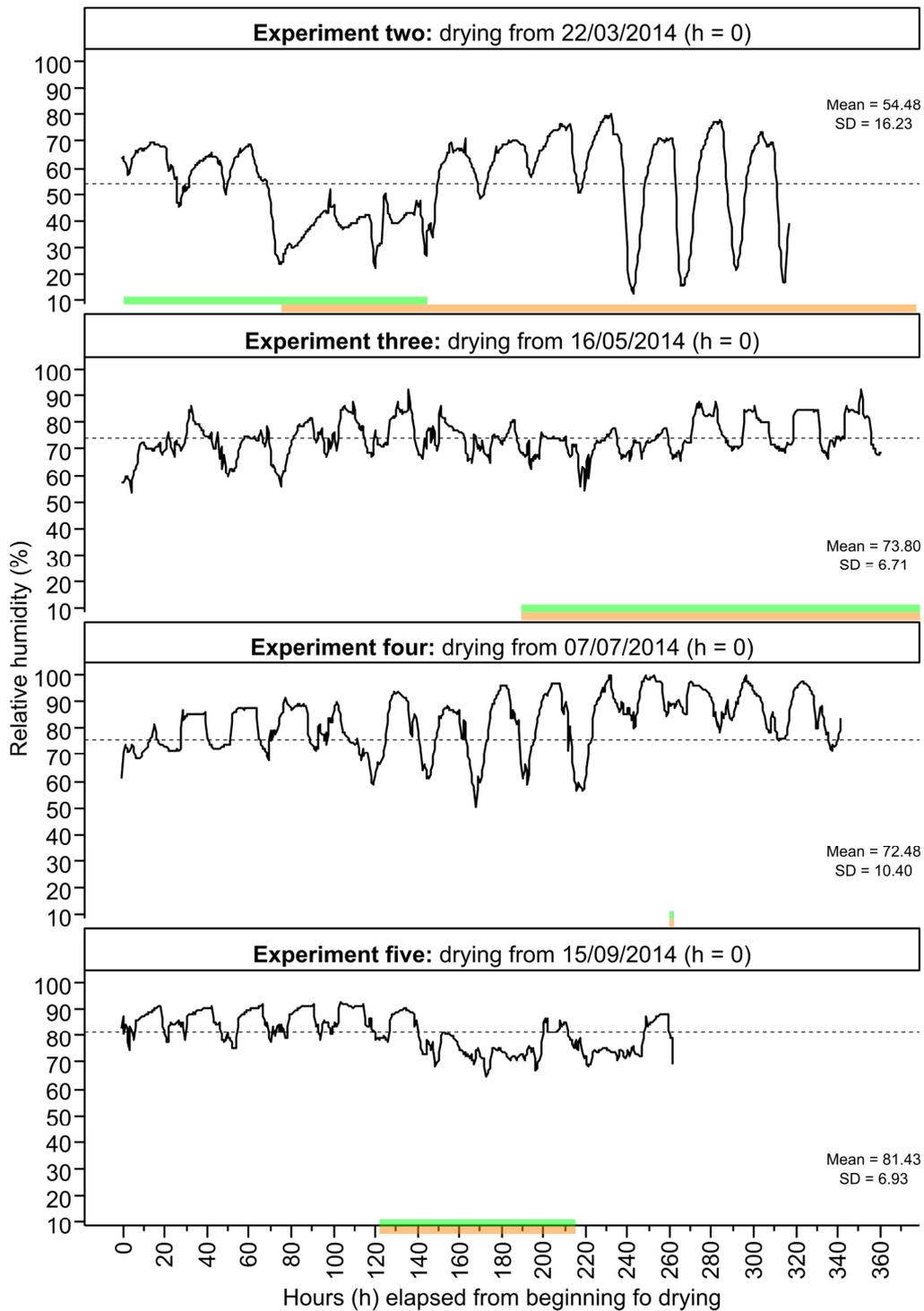


Figure 0.6. Records of relative humidity (%) in chamber five of the Grodome facility at East Malling Research (Kent) in 2014. Readings taken over the drying period (h) of plants under severe water deficit treatment (SWD) to end of experiments two, three, four and five. Mean of the data for each experiment correspond to dashed horizontal line. Green horizontal lines represent the time length over which plant responses were measured; orange horizontal line corresponds to the time length over which fruit responses were measured.

3.2 Leaf responses to SWD

3.2.1 Stomatal conductance

Stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) within experiment two was affected ($p < 0.001$) by the SWD treatment (**Figure 0.7**). Plants under control conditions (WW) had higher stomatal conductance (0.18 ± 0.008) than those under SWD (0.12 ± 0.010). Mean stomatal conductance for WW and SWD plants changed ($p < 0.001$) over the course of the experiment reflecting the different impact on stomatal conductance during drying, wilting or re-hydration phases. There was interaction between *Time* and the effect of SWD treatment, meaning that responses to the treatment were not consistent over time mainly due to the relatively long drying phase.

Stomatal conductance during the wilting phase in experiment three was affected by the SWD treatment ($p < 0.001$). SWD treatment had a negative effect on stomatal conductance (0.44 ± 0.092 vs 0.082 ± 0.021). SWD had also a negative effect on stomatal conductance during the re-hydration phase (0.30 ± 0.067 vs 0.14 ± 0.042).

Stomatal conductance in experiment five was affected ($p < 0.001$) by the SWD treatment. Plants under WW had higher stomatal conductance (0.20 ± 0.006) than those under SWD (0.09 ± 0.005). Although mean stomatal conductance of WW and SWD plants did not change over the course of the experiment (all data), it did change when only wilting was considered ($p = 0.01$) and when only re-hydration phase data were considered ($p < 0.001$) highlighting the similar impact of the SWD treatment between these two phases. There was no interaction between *Time* and the irrigation treatment meaning that the responses were consistent over the time mainly because of the long wilting phase in this experiment; that is, the long period of similar PW and PVMC in this experiment.

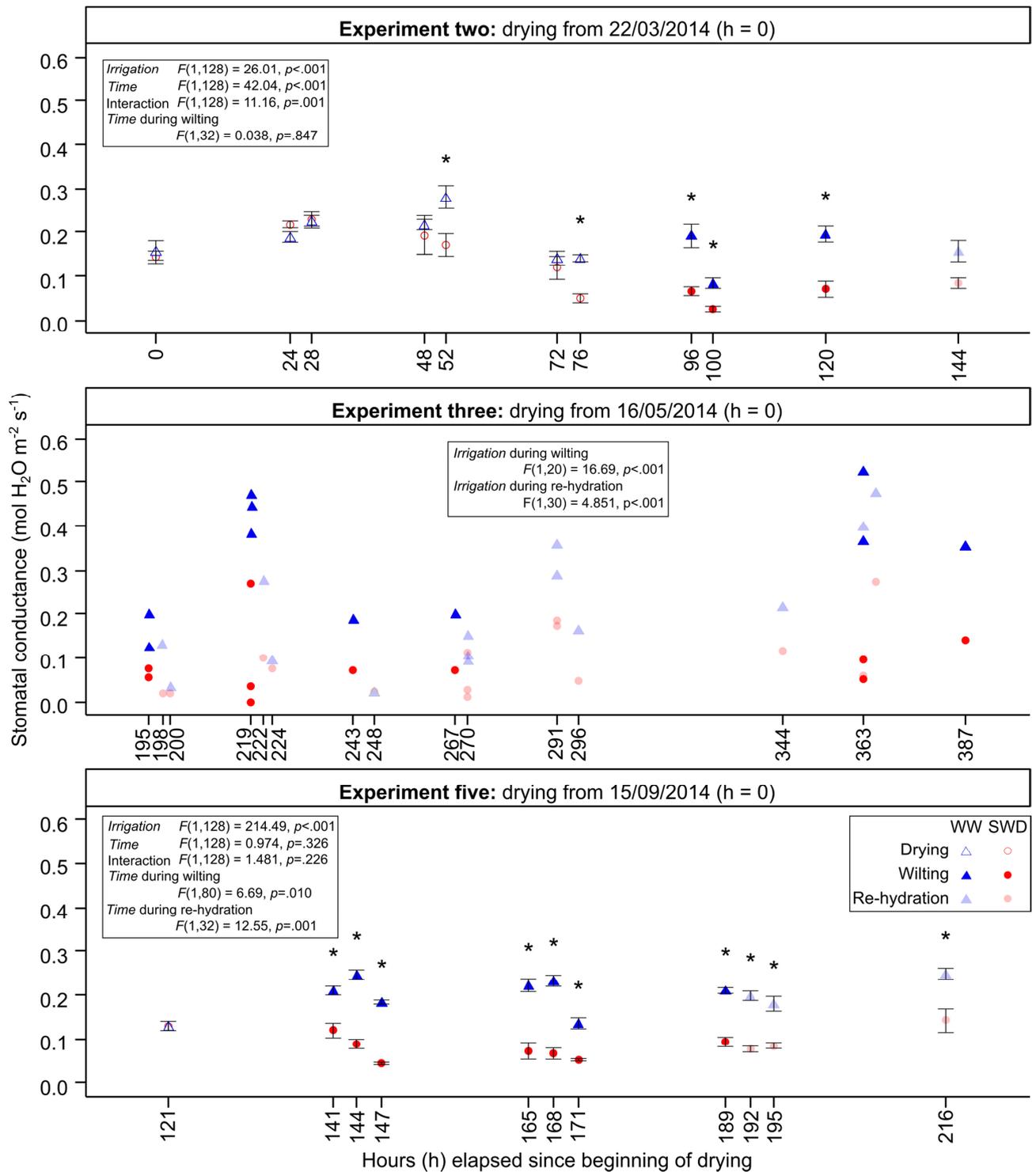


Figure 0.7 Stomatal conductance ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$) for plants under severe water deficit (SWD) or well-watered conditions (WW). Mean values \pm standard error ($n=6$) are drawn for experiments two and five. For these, readings were taken 11 successive times. In case of number two, nine during peat drying and two during plant wilting. In case of number five, one during peat drying, seven during plant wilting and three during re-hydration. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Raw data are presented for experiment three at 12:00 p.m. on the first and second day of wilting, and one, three and 24 hours after re-hydration (11:00 a.m.). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.2.2 Transpiration rate

Transpiration rate (**Figure 0.8**) and stomatal conductance were expected to be inextricably linked. That was the case in experiment two ($r(130)=0.95$, $p<.001$) and five ($r(130)=0.95$, $p<.001$) but not in experiment three ($r(56)=0.55$, $p<.001$). The SWD treatment had the expected effect on transpiration rate ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) during experiment two ($p<0.001$). Control plants had higher transpiration rates (1.85 ± 0.063) than stressed ones (1.25 ± 0.086). In addition, mean transpiration rate for WW and SWD plants changed over the course of the experiment ($p<0.001$), but there was no significant change during wilting indicating the different plant response to the three phases of the treatment. Finally, transpiration rate response to the irrigation treatment varied over the course of the experiment ($p<0.001$) probably due to the relative long period of drying.

The SWD treatment also had an effect on transpiration rate during wilting ($p<0.001$) and re-hydration phases ($p<0.001$) in experiment three. SWD reduced transpiration rate both during wilting (1.63 ± 0.130 vs 0.48 ± 0.101) and re-hydration (1.15 ± 0.095 vs 0.60 ± 0.122). Transpiration rate was negatively affected ($p<0.001$) by the SWD treatment during experiment five (2.33 ± 0.061 vs 1.16 ± 0.070). Furthermore, unlike stomatal conductance, mean transpiration rate for WW and SWD plants changed over the course of the whole experiment ($p<0.001$). There was interaction, however, between *Time* and the irrigation treatment because the responses were consistent over time mainly because of the relatively long wilting phase in this experiment (this is, the long period of similar PW and PVMC in this experiment).

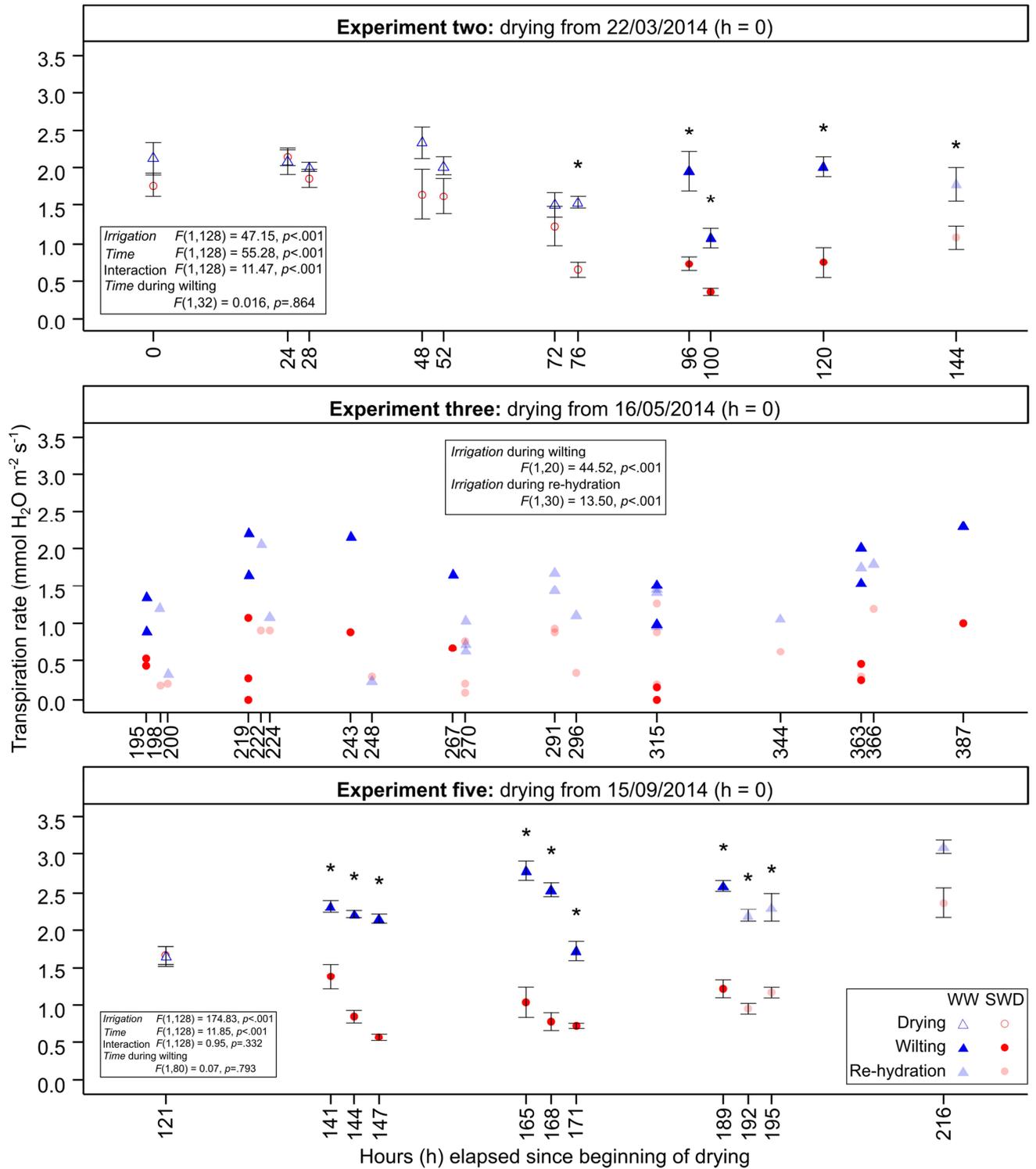


Figure 0.8. Transpiration rate (mmol H₂O m⁻² s⁻¹) for plants under severe water deficit (SWD) or well-watered conditions (WW). Mean values \pm standard error (n=6) are drawn for experiments two and five. For these, readings were taken 11 successive times. In case of number two, nine during peat drying and two during plant wilting; in case of number five, seven during plant wilting and three during re-hydration. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Raw data presented for experiment three at 12:00 p.m. on the first and second day of wilting, and one, three and 24 hours after re-hydration (11:00 a.m.). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.2.3 Photosynthetic rate

Photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) was affected by the SWD treatment ($p < 0.001$) during experiment two (**Figure 0.9**). Plants subjected to SWD had lower photosynthetic rate than controls (14.17 ± 0.881 vs 7.88 ± 0.743). In addition, mean photosynthetic rate for WW and SWD plants varied during the course of the experiment ($p < 0.001$) with the exception of the wilting period, indicating that the variation was due to the phases of the treatment. Lastly, photosynthetic rate was influenced by the interaction between *Time* and the irrigation treatment resulting, as with other plant responses, from the relative long drying period.

The SWD treatment had a negative impact ($p < 0.001$) on photosynthetic rate during the wilting (16.37 ± 0.881 vs 7.90 ± 0.743) and rehydration phases of the experiment three (12.62 ± 0.378 vs 7.86 ± 0.547). In experiment five, there was higher photosynthetic rate in control plants (14.35 ± 0.378) than in stressed ones (7.95 ± 0.547). Furthermore, since the wilting period during this experiment, where the environmental conditions were more constant, was comparatively longer than drying and re-hydration, mean photosynthetic rate for WW and SWD plants did not change over the course of the experiment although it did when only re-hydration phase was considered. The relatively long duration of the wilting period made the effect of interaction *Time* vs irrigation treatment on photosynthetic rate not significant.

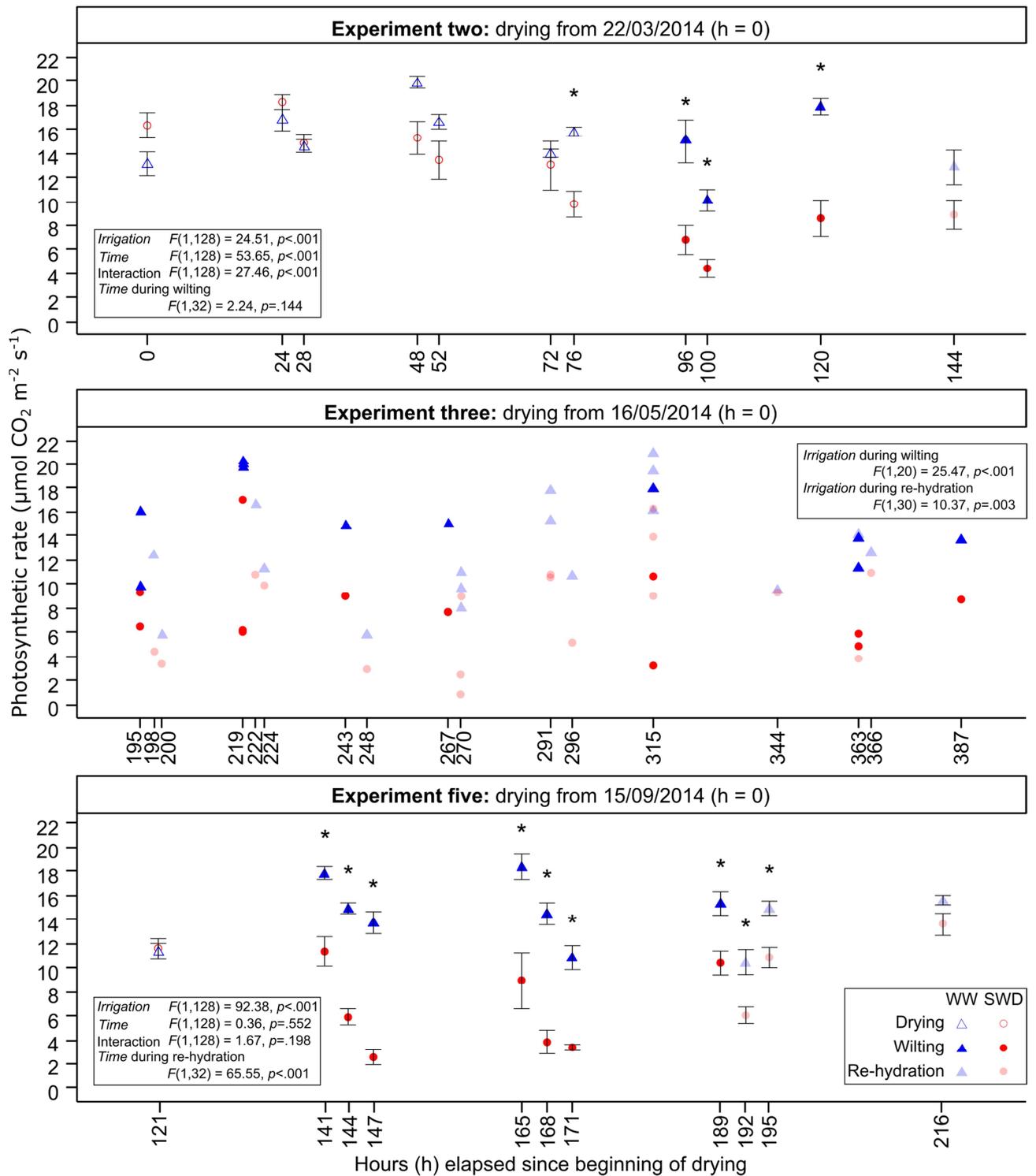


Figure 0.9. Photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) for plants under severe water deficit (SWD) or well-watered conditions (WW). Mean values \pm standard error ($n=6$) are drawn for experiments two and five. For these, readings were taken 11 successive times. In case of number two, nine during peat drying and two during plant wilting; in case of number five, one during peat drying, seven during plant wilting and three during re-hydration. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Raw data presented for experiment three at 12:00 p.m. on the first and second day of wilting, and one, three and 24 hours after re-hydration (11:00 a.m.). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.2.4 Performance of photosystem II – Yield (II)

Yield (II) data (dimensionless) were not affected by the SWD treatment during experiment two (**Figure 0.10**). Further analyses revealed that the Yield (II) measurements were affected by the treatment at midday of the first and second day of wilting, and the day before the wilting phase started. In addition, mean Yield (II) for WW and SWD plants changed during the course of the whole experiment ($p < 0.001$). Finally, the effect of the interaction between *Time* and the irrigation treatment was not significant suggesting that environmental variables could have not exerted a major influence on the performance of the photosystem II during this experiment.

In experiment three, although mean Yield (II) values were lower for SWD plants the effect of the SWD treatment on Yield (II) during wilting or re-hydration phases was not significant. SWD affected the performance of the photosystem II during experiment five ($p < 0.001$). The treatment reduced Yield (II) with respect to WW plants (0.71 ± 0.007 vs 0.64 ± 0.016). At midday of the second day of wilting the SWD reduced the performance of the photosystem II. In addition, mean Yield (II) for WW and SWD plants did not vary over the course of the whole experiment or when only considered during a treatment phase, indicating that in this experiment Yield (II) could have had greater degree of independence from the environment. This reduced environmental effect on Yield (II) was further supported by the no-significant interaction between *Time* and the irrigation treatment from the ANOVA output.

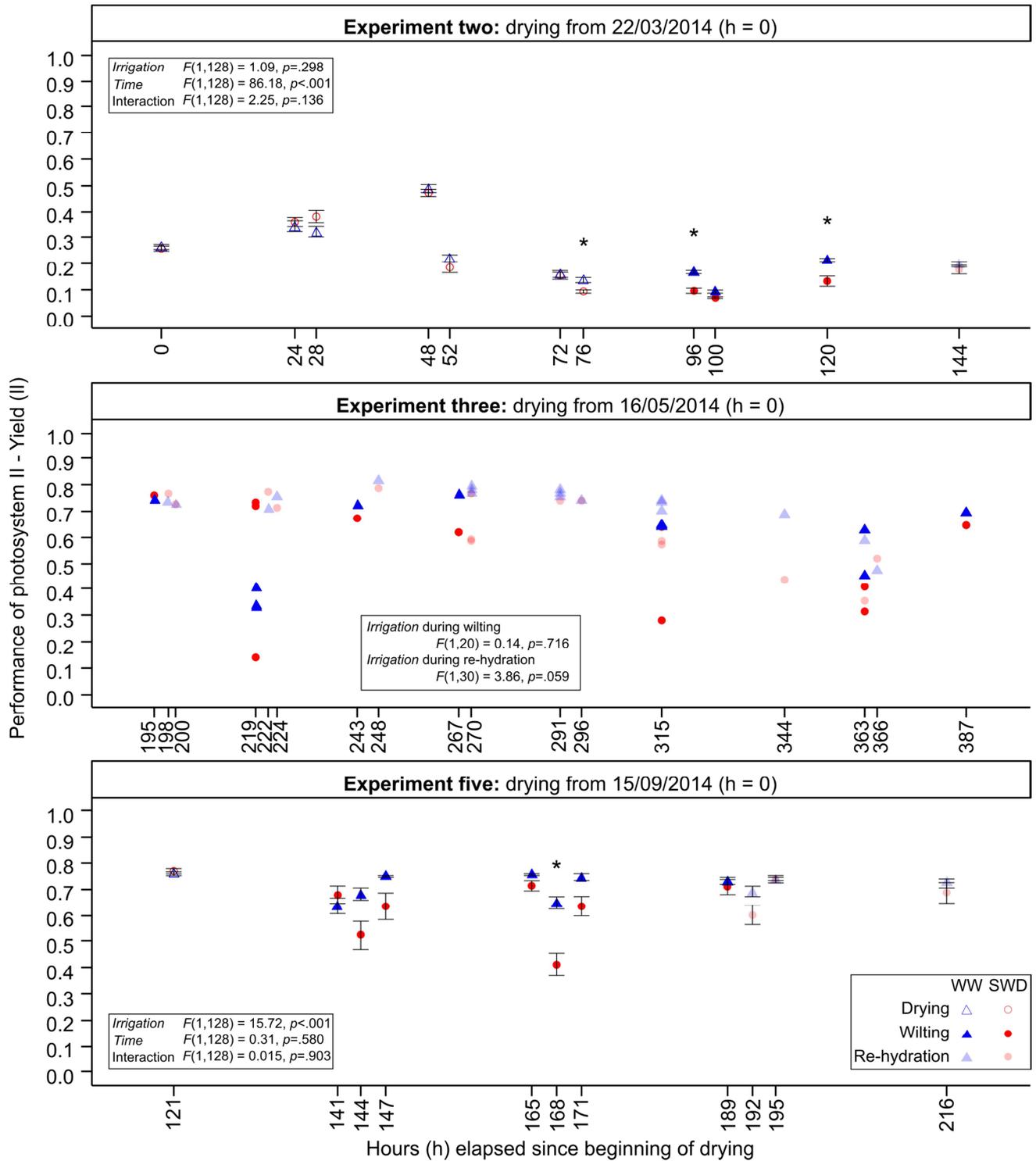


Figure 0.10. Performance of the photosystem II (dimensionless) for plants under severe water deficit (SWD) or well-watered conditions (WW). Mean values \pm standard error ($n=6$) are drawn for experiments two and five. For these, readings were taken 11 successive times. In case of number two, readings were taken after one minute of actinic light exposure, nine times during peat drying and two during plant wilting; in case of number five, one time during peat drying, seven during plant wilting and three during re-hydration. Statistical differences detected with t-test are indicated with an asterisk ($p<0.01$). Raw data presented for experiment three at 12:00 p.m. on the first and second day of wilting, and one, three and 24 hours after re-hydration (11:00 a.m.). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.3 Interaction between environmental factors and leaf responses

This analysis was done to obtain evidence on the influence of environment on leaf responses and potentially to find which of the leaf responses is more specific for the SWD treatment. All predictors initially considered for linear regression analyses (**Table 0.2**) were finally included in the models for photosynthetic rate, stomatal conductance and transpiration rate but radiation and experiment two effect were not included in the Yield (II) model. Models were adjusted to maximise overall fit (R^2), so although most predictors were highly significant for the model in which they were included, those related to experiment and phase in the transpiration rate model had higher p -values and therefore lower significance. R^2 of these models ranged from 0.41 for the stomatal conductance model to 0.81 for the Yield (II) model, all being highly significant. The Yield (II) model supported and expanded the findings reported in **Section B.2.8.4**, showing a greater independence from environmental factors such as PW/PVMC and light intensity/temperature.

Table 0.2. Multiple regression analysis results for leaf responses to environment

	Photosynthetic rate	Stomatal conductance	Transpiration rate	Yield (II)
Intercept	-16.59 ($p < .001$)	-6.51×10^{-1} ($p < .001$)	-1.69 ($p < .001$)	1.32×10^{-5} ($p < .001$)
Weight	0.01 ($p < .001$)	1.67×10^{-4} ($p < .001$)	2.00×10^{-3} ($p < .001$)	8.92×10^{-5} ($p < .001$)
Radiation	0.04 ($p < .001$)	1.79×10^{-3} ($p < .001$)	4.95×10^{-3} ($p = .001$)	
Experiment two	12.74 ($p < .001$)	4.04×10^{-1} ($p < .001$)	-0.18 ($p = .583$)	
Experiment three	10.73 ($p < .001$)	4.77×10^{-1} ($p < .001$)	-0.92 ($p = .004$)	4.56×10^{-1} ($p < .001$)
Experiment five	9.72 ($p < .001$)	3.89×10^{-1} ($p < .001$)	-0.36 ($p = .281$)	4.87×10^{-1} ($p < .001$)
Re-hydration	-5.39 ($p < .001$)	-9.66×10^{-2} ($p < .001$)	-0.64 ($p < .001$)	-8.43×10^{-2} ($p < .001$)
Wilting	-3.60 ($p < .001$)	-7.14×10^{-2} ($p < .001$)	-0.31 ($p = .023$)	-1.26×10^{-1} ($p < .001$)
	$R^2 = 0.51$; $F(7,375) =$ 59.06; $p < .001$	$R^2 = 0.41$; $F(7,375) = 39.68$; $p < .001$	$R^2 = 0.55$; $F(7,375) = 67.3$; $p < .001$	$R^2 = 0.81$; $F(5,316) = 271.2$; $p < .001$

Results of four analyses, one for each leaf response. Models were adjusted to maximise R^2 . Weight and peat moisture, and temperature and radiation correlate so peat volumetric moisture content and temperature were excluded from the analysis. Response variables are those in the first row and predictors those in the first column. Significance levels (p) are presented for the coefficients (β) of each model. In addition, R^2 , F statistic and significance level of the model are presented at the bottom.

Further study of the effect of light on stomatal conductance, transpiration rate, photosynthetic rate and Yield (II) of plants under WW or SWD treatments revealed that

stomatal conductance (**Figure 0.11 [A]**) and transpiration rate (**Figure 0.11 [B]**) were little affected by decreasing light intensity (in this case photosynthetically active radiation [PAR]) whereas photosynthetic rate (**Figure 0.11 [C]**) and the efficiency of photosystem II (**Figure 0.11 [D]**) responded to increasing PAR. Apart from photosynthetic rate at low light intensity and Yield (II) at lowest and highest light intensities, SWD had a significant and negative impact on all leaf responses. Values of leaf responses increased with increasing light intensity with the exception of performance of the photosystem II, where increasing PAR lead to lower Yield (II) measurements.

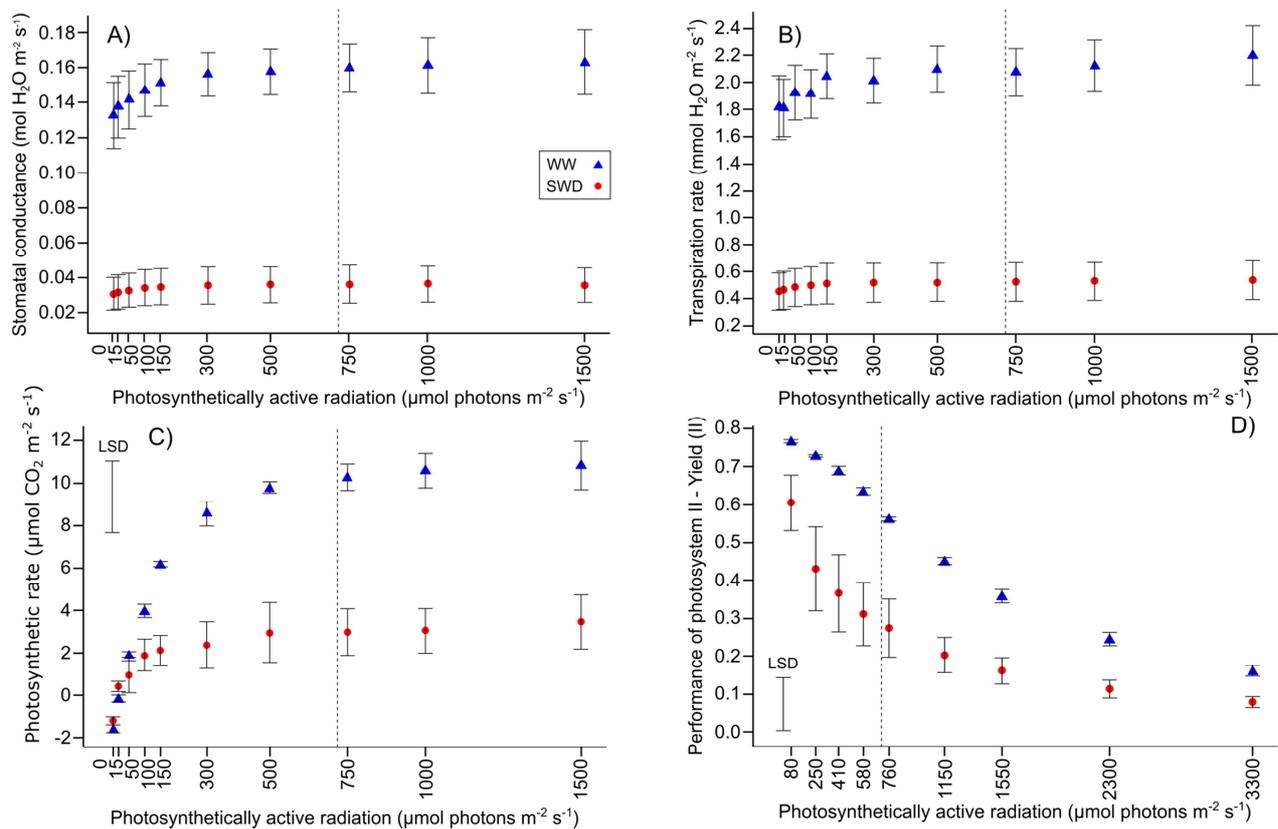


Figure 0.11. Response curves for (A) stomatal conductance (mol $\text{H}_2\text{O m}^{-2} \text{s}^{-1}$), (B) transpiration rate (mmol $\text{H}_2\text{O m}^{-2} \text{s}^{-1}$), (C) photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$) and (D) performance of the photosystem II (dimensionless) to decreasing photosynthetic active radiation (PAR [$\mu\text{mol photons m}^{-2} \text{s}^{-1}$]) for plants under severe water deficit (SWD) or well-watered conditions (WW). Mean values \pm standard error ($n=3$) are presented. Measurements were taken at 12:00 p.m. on the second day of wilting in December 2014. Vertical dashed line indicates the maximum measured ambient PAR in any of the experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

4 Discussion

The experiments discussed in this chapter were designed to describe the leaf responses from plants under SWD and their interaction with environmental factors such as light intensity, temperature and relative humidity in order to a) explain potential variability in plant responses in future experiments and b) identify leaf responses which would further improve the management of the SWD treatment because of their specificity to SWD rather than to the interacting environmental factors.

4.1 Establishment of the management strategy for the SWD treatment

Curves for PVMC and pot-and-plant weight (PW) from plants subjected to SWD showed the three distinctive phases for the SWD treatment: a) drying phase equivalent to the initial downward slope, b) a 48 h period in between drying and re-hydration in which plants were in wilting phase and c) re-hydration phase corresponding to the final upward slope (**Figure 0.2** and **Figure 0.3**).

4.1.1 Drying phase

Plants within the same block in experiments two and three, although were grouped according to their evaporative demands and started drying simultaneously, reached wilting at different times. Evaporative demands within groups were still variable. In contrast in experiments four and five, water was added to plants with higher demand for irrigation, and consequently faster drying pace, to match PVMC/PW of plants with slower drying pace, resulting in a uniform drying and narrower confidence intervals. Managing the drying phase as in experiments two and three left plant responses exposed to the effects of changing temperatures, light intensity and relative humidity besides those of the SWD treatment. However, the irrigation regime was the same for all of the plants. In contrast, managing the drying as in experiments four and five eliminated the variability in plant responses at the time of sampling due to environmental factors other than PVMC/PW.

4.1.2 Wilting and re-hydration phases

The drying phase finished once plants were seen wilting. PVMC/PW of SWD plants at the end of the drying phase was annotated and from then water was added to keep plants at that PVMC/PW. The wilting phase was maintained for 48 h because longer wilting periods were shown to affect fruit expansion rates previously (Mark Else, unpublished results). Peat re-hydration of SWD plants to WW PVMC/PW was not achieved in experiments three and four. This was likely to be due to hysteresis: according to Hogart et al. (1988) successive re-hydrations of a substrate lead to eventual lower water holding capacity. In turn, peat re-hydration could be achieved during experiment two because of the lower peat density. In consequence these pots held less water which made them less susceptible to hysteresis (Kaboos, 2014). Finally, WW water content of experiment five was never maintained at full pot capacity so hysteresis could be avoided, allowing rapid and full re-hydration to WW PVMC/PW levels. In order to achieve successful re-hydration experimental plants had to be potted with 1,200 g of peat in 2 l pots and re-hydrated to $1,900 \text{ g}/0.600 \text{ m}^3 \text{ m}^{-3}$.

4.1.3 Reliability of the Grodome facility on environmental control

Experiments were carried out in the Grodome, a contained facility with temperature control and artificial lighting. During 2014, on-going engineering and building-management software issues meant that the Grodome had to be serviced a number of times to try to improve temperature control (**Figure 0.5**). The facility had good temperature control under low external temperature and its limitations became evident in case of high external temperature because of the limited cooling performance. In addition, the chamber that had to be used for these experiments did not have humidity control (**Figure 0.6**). Relative humidity was solely dependent on the accumulated water input and the ambient temperature. The facility was not fitted with shading cover to protect the experiment against high external light intensities. Light control under low external intensities was limited to less than 100 kg s^{-3} (**Figure 0.4**). Despite these

limitations, the Grodome has proved the best controlled environment facility at East Malling Research and so it was used throughout the second and third years of this PhD programme.

4.2 Leaf plant responses and their interaction with other environmental variables during the SWD treatment

4.2.1 Stomatal conductance

The SWD had a significant effect on stomatal conductance during the experiments run in 2014 (**Figure 0.7**). There were a number of exceptions appearing during drying in experiments two and five which may have been due to the fact that a) plants were experiencing only mild peat drying that was not sufficient to trigger physiological responses, and b) there were low temperatures and lower evaporative demands. When comparing mean values during the wilting phase, the highest occurred in experiment three (WW=0.44 vs SWD=0.08 mol H₂O m⁻² s⁻¹) which was run throughout summer; whereas in experiments two and five, completed in colder and less sunny months, had lower mean value. This finding was in agreement with the data found in the literature for stomatal conductance for WW 'Elsanta' plants, which during the summer months ranges from ~0.19 to ~0.52 mol H₂O m⁻² s⁻¹ (Liu et al., 2007; Grant et al., 2012a,b). There appears not to be published data on stomatal conductance for wilting strawberry plants; however under 65% regulated deficit irrigation (which is a severe stress) values range from ~0.02 to ~0.39 mol H₂O m⁻² s⁻¹ (Liu et al., 2007; Grant et al., 2012a,b). Stomata closure could be due to root-sourced or shoot-sourced ABA (Davies et al., 2005). Presumably, stomatal conductance of plants under SWD would have been influenced by changes in the apoplastic pH which in turn could have had an effect on ABA distribution on the leaf. In this instance no need for root-source ABA would have been needed for stomatal closure (Dodd et al., 1996; Wilkinson & Davies, 1997; Davies et al., 2002; Davies et al., 2005).

4.2.2 Transpiration rate

Transpiration rate data (**Figure 0.8**) followed a very similar pattern to that of stomatal conductance which was not unexpected given the fact that, besides temperature, relative humidity, wind and radiation, stomatal opening determines plant evapotranspiration (Farquhar & Sharkey, 1982). Consequently a correlation was found between these two variables in experiment two and five. Uncoupling between stomatal conductance and transpiration rate can occur, however, when stomata are fully open (Jarvis & McNaughton, 1986); experiment three did not show the expected correlation which may have been because plant evapotranspiration was compromised by limited root water uptake. This may have been because of the high temperatures during this experiment. An example of this can be found at h=219 when temperature was $\sim 26^{\circ}\text{C}$, stomatal conductance the highest of all experiments ($\sim 0.4 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and transpiration a modest $\sim 2 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (see **Figure 0.7** and **Figure 0.8**).

4.2.3 Photosynthetic rate

Although there were a number of exceptions during drying and re-hydration of experiment two and five, plants subjected to the SWD treatment had a significantly lower photosynthetic rate than control plants (**Figure 0.9**). The highest mean photosynthetic rate was measured in experiment three during summer because CO_2 assimilation was highest (as stomatal conductance was highest) and light most intense; whereas experiments two and five, run during colder and less sunny months, had lower mean values. Comparable data in the literature on photosynthetic rate for WW 'Elsanta' plants ranged from 4.7 to $10.2 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (Keutgen et al., 1997, 2005) which was lower than the mean values measured during the course of this investigation. This could be because of the low PAR value ($450 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) used by those authors for the gas exchange (Keutgen et al., 1997, 2005). On the other hand, withholding irrigation to 'Cambridge favourite' and 'Florika' plants for four days reduced their photosynthetic rate to $\sim 8 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (Blanke & Cooke, 2004) in

contrast to $\sim 13 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ measured here. This could be because the different cultivars used. Reduction of the photosynthetic rate in the experiments reported in this thesis could be explained by Blanke & Cooke (2004) findings who explained the reduction in photosynthetic rate by a decrease in internal CO_2 concentration presumably caused by stomata closure. During re-hydration phase, however, stomatal conductance remained affected whereas photosynthesis did not pointing to other causes; perhaps the supply of ATP from the photosystems which stopped being limiting once these recover from the stress (Suzuki et al., 2012) (see **Figure 0.10**).

4.2.4 Performance of the photosystem II – Yield (II)

SWD had a significant effect on the performance of Photosystem II (**Figure 0.10**). Reactive oxygen species originating from Mehler reactions in the chloroplasts are counteracted by antioxidants and when damage occurs to the electron transport chain, it can normally be repaired unless water deficit stress is too severe or light intensities too high (Nishiyama & Murata, 2014). ROS are regarded as signalling molecules involved in many plant metabolic processes, including ethylene metabolism (Wang et al., 2002; Shao et al., 2008; Mittler et al., 2011; Suzuki et al., 2012). Consequently, Yield (II) measurements may reflect when ethylene is expected to be produced under SWD.

The effect of water deficit on the Yield (II) is controversial (Snider et al., 2014). For instance, Razavi et al. (2008) reported no significant changes in MPQ or Yield (II) of strawberry 'Elsanta' plants under water deficit stressed conditions (minimum peat-and-perlite volumetric moisture content of $\sim 0.2 \text{ m}^3 \text{ m}^{-3}$); a finding that disagrees with the findings of the present research. Yet the authors provided no detail of other interacting environmental variables which could be relevant to the absence of an effect of water deficit (Nishiyama & Murata, 2014).

4.2.5 Interaction between environmental factors and leaf responses

Changes in temperature, relative humidity and peat moisture affected stomatal conductance and transpiration; stomatal conductance, in turn, influenced photosynthetic rate which also can be directly affected by environmental factors (Collatz et al., 1991, 1992; Scheidegger et al., 2000; Hubbard et al., 2001; Blanke & Cooke, 2004).

The most important result from the four regression analyses carried out to study these relationships (**Table 0.2**) was that, unlike the rest of leaf responses, the variation in Yield (II) data was best explained if light intensity was not included in the model, leaving pot-and-plant weight as the single environmental variable explaining the Yield (II) response measured. Hence Yield (II) and the environmental treatment applied to the plants were related. This finding is further supported by the highest R^2 achieved for Yield (II) model in comparison to the other models. The study of Razavi et al. (2008), however, contradicts this finding. These authors obtained no-significant differences in Yield (II) measurements on strawberry plants under water deficit conditions but they did not consider a putative effect of light intensity in their measurements.

Light intensity and pot-and-plant weight were the most important variables in explaining the variability of leaf responses with the exception of Yield (II), which was only affected by pot-and-plant weight. Their interaction was studied through four light curves measured on leaves of SWD and WW plants on the second day of wilting, one for each of stomatal conductance (**Figure 0.11 [A]**), photosynthetic rate (**Figure 0.11 [C]**), transpiration rate (**Figure 0.11 [B]**) and performance of the photosystem II (**Figure 0.11 [D]**).

Light intensity is mostly relevant for photosynthesis because light supplies the required energy for the fixation of atmospheric CO_2 but at the same time excessive light intensities can inhibit photosynthesis by increasing the rate of ROS formation (Nishiyama & Murata, 2014). In addition, it has long been known that stomata respond to light intensity and the blue, red and green light wavelengths (Farquhar & Sharkey, 1982).

According to these light response curves, stomatal conductance and transpiration increased only slightly with increasing light intensity. According to the regression analyses, however, light intensity was highly relevant in explaining the variability of those two leaf responses. Since light intensity and temperature correlated in the experiments of this research, temperature was not incorporated in the regression analyses. Hence during this investigation temperature may have had a bigger effect on stomatal conductance and transpiration than light intensity.

Photosynthetic responses at low light intensities were not affected by the SWD treatment; however, at higher intensities it was clearly evident that WW plants were able to use that extra supply of energy in a significantly more efficient way than SWD plants. The shape of these curves matched those found in the literature (Collatz et al., 1992; Hdidier & Desjardins, 1994; Chabot, 2008).

The performance of photosystem II decreased at similar rates in WW and SWD plants with increasing light intensity levels; the different response observed was due to a pot-and-plant weight effect. Regression showed that, unlike the rest of leaf responses, the performance of photosystem II was affected by pot-and-plant weight and not by light intensity. Borkowska (2006) showed an analogous pattern for the F_o component (used in Yield [II] determination). This seems to be the first work studying the effect on Yield (II) of water deficit irrigation in strawberry at increasing light intensities. Because of the specificity of Yield (II) responses to PVMC/PW, this leaf response could be the best option to manage the irrigation treatment under study.

5 Conclusions

5.1 Establishment of the management strategy for the SWD treatment

The SWD treatment imposed on the strawberry plants (*Fragaria x ananassa* Duch. cv 'Elsanta') had three distinctive phases, namely drying, wilting and re-hydration. Plant drying occurred at different rates. In order to circumvent this complication in subsequent experiments, water was added to plants with higher demand for irrigation, and consequently faster drying pace, to match PVMC/PW of plants with slower drying pace. Experience gained during the experiments reported in this chapter showed that this approach exposed plants to the same environmental factors during the course of drying. Wilting was reached at similar PW/PVMC (no in experiment two) and was maintained for 48 h. Yield (II) was identified as the main plant response to wilting.

In order to achieve rapid re-hydration, experimental plants must be potted with 1,200 g of peat in 2 l pots and re-hydrated to 1,900 g/0.600 m³ m⁻³. Duration of drying and re-hydrating the plants will be finally decided in the next chapter because depended on fruit responses.

5.2 Leaf plant responses and their interaction with other environmental variables during the SWD treatment

Light curves and regression analyses on leaf responses indicated that stomatal conductance and transpiration responded mainly to temperature and SWD whereas photosynthesis responded to light intensity and SWD. The regression analyses showed that, unlike the rest of leaf responses, the performance of photosystem II was affected by pot-and-plant weight and not by light intensity. This suggested Yield (II) as the most specific leaf response to the SWD treatment. In addition, the light curves showed that Yield (II) decreased with increasing light intensities, and that the rate of Yield (II) reduction was similar between SWD and WW plants; the different response observed was due to a pot-and-plant weight effect. Because of the specificity of Yield (II) responses to PVMC/PW, this leaf response could be the best option to manage the

irrigation treatment under study. Results presented in the next chapter will determine whether Yield (II) measurements on leaves could also be used to estimate fruit responses such as ethylene production.

Chapter 3. Fruit responses to severe water deficit and other environmental factors

1 Introduction

In this chapter the responses of fruits of strawberry plants (*Fragaria x ananassa* Duch. cv 'Elsanta') during severe water deficit (SWD) are reported. The experiments were carried out in the Grodome facility at East Malling Research in 2014. Treatments such as transient deficit irrigation (TDI) can increase the concentration of phenolics more than other treatments, such as regulated deficit irrigation (RDI) or partial root zone drying (PRD) (Else et al., unpublished). The mechanisms underlying the effects of deficit irrigation on fruit quality are yet to be elucidated but may be related to a transient wilting of the plant (Dr Mark Else, personal communication). The main hypothesis of the research described in this thesis was that an ethylene-mediated increase in phenolics in the fruit is triggered by SWD and the resultant wilting. The strategy adopted was to test a number of Jackson's postulates (Weyers & Paterson, 2001) of which, in this chapter, only the first is included: correlation. This requires the study of the timings of changes in phenolic concentration level and ethylene production rate in the fruit of strawberry cv 'Elsanta' subjected to SWD.

There are a number of reports of ethylene production under water deficit by other crops such as wheat, bean, cotton, miniature rose, *Arabidopsis*, tomato, kaki, maize and apple (El-Beltagy & Hall, 1974; Apelbaum & Yang, 1981; Wang et al., 1987; Morgan et al., 1990; Narayana et al., 1991; Ouvrard et al., 1996; Nakano et al., 2003; Sobeih et al., 2004; Voisin et al., 2006; Liu et al., 2008; Cheng et al., 2013). Although evidence for increased ethylene production under water deficit by direct measurement of the gas concentration is abundant (El-Beltagy & Hall, 1974; Wang et al., 1987; Nakano et al., 2003; Sobeih et al., 2004; Voisin et al., 2006), there is still some controversy because an effect of water deficit stress in promoting ethylene production rate has not always been found (Morgan et al., 1990; Narayana et al., 1991).

There are published examples where phenolic concentration increased with increasing ethylene production (Jiang & Joyce, 2003). For example, when an inhibitor of ethylene perception (MCP-1) was used in stored strawberries, the activity of a phenolic production rate limiting enzyme (PAL), ethylene emissions and phenolic content were reduced (Jiang et al., 2001). Merchante et al. (2013) and Sun et al. (2013) provided the first molecular evidence that ethylene is required for strawberry ripening. Merchante et al. (2013) used strawberry plants with diminished sensitivity to ethylene and unknown ethylene production rate to show reduced *FaPAL* expression in the achenes. Sun et al. down-regulated *FaSAMS1* and *FaCTR1* at the “white stage” via tobacco rattle virus-induced gene-silencing technique, inhibiting colour development in the whole fruit while increasing ethylene production rate (Sun *et al.*, 2013). In these studies, either perception or the subsequent signalling was hindered and phenolic content was reduced. Ethylene production, however, could have been high in both cases but they did not measure it. Although ethylene production seems very important for phenolic production, an increase in ethylene production may not mean an increase in phenolics; which is a good reminder that correlation does not necessarily imply causation.

Wilkinson & Davies (2010) hypothesized that ethylene and ozone can up-regulate H_2O_2 production, leading to stomatal closure. It is not clear, however, whether ethylene triggers the ROS increase rather than ozone, but other authors sequence the event as first, production of ethylene and second, concentration of ROS (Wang et al., 2002; Nishiyama & Murata, 2014). As causation has not yet been clearly established, it could be that ROS generated under stress triggers ethylene production. Li et al. (2004) point to the fact that ethylene had no effect on ROS production in leaves of spring wheat seedlings and more interestingly, whatever promoted ethylene production in stressed leaves also promoted ROS production (Li et al., 2004). A recent review proposes that light can directly damage photosystem II whereas other abiotic stresses inhibit the repair of the damage (Nishiyama & Murata, 2014). ROS originated from Mehler reactions in the chloroplasts are counteracted by antioxidants and when there is

damage to the electron transport chain, the harm can normally be repaired unless water deficit stress is quite severe or light intensities are too high. Incidentally, ROS are regarded as signalling molecules involved in, among other things, ethylene metabolism (Wang et al., 2002; Shao et al., 2008; Mittler et al., 2011). Yield (II), this is the efficiency of the photosystem II in driving energy towards photosynthesis (values between 0 and 1: 0 all energy is lost, and 1 all energy goes to photosynthesis), can be a valuable instrument to determine when ROS levels overcome plant antioxidant mechanisms and, as Li et al. (2004) point out, an indication as to when ethylene biochemistry may be affected.

The objectives of the work described in this chapter were as follows:

- Determination of the optimum duration of the duration of drying and re-hydration phases
- Develop a sampling method for ethylene production by strawberry fruit, and decide which ripening stage to sample during the severe water deficit (SWD)
- Determine whether there is temporal correlation between the production of ethylene and the concentration of phenolics in the fruit of plants (*Fragaria x ananassa* Duch. cv 'Elsanta') subjected to SWD

2 Materials and Methods

2.1 Experiments included in this chapter

Experiments included in this chapter are those described in the previous chapter: experiment two, three, four and five. Unless otherwise stated, plant materials, controlled environment conditions, irrigation, fertigation, crop protection, experimental design and measurement of environmental factors were as in Chapter 2.

2.2 Fruit sampling

Three fruits from each plant were used for ethylene measurements and total phenolic analyses (more information in **Section C.2.3**). Once harvested, fruit were immersed immediately in liquid nitrogen and transferred to -8 °C until total phenolic analysis. In experiment four, two lots of three fruits for each experimental unit were kept separate, one for the quantification of ethylene and one for total phenolics.

Fruit samples were taken at six times during the course of the SWD treatment in experiment two (**Figure 0.1 [c]**):

Large green fruit (one week before fruit turned white) were taken on the day the SWD treatment was applied, then again at midday of the second day of wilting and one hour after re-hydration.

During the re-hydration period, fruits were taken as they reached de-greening (white), reddening (50% red) and fully ripe stages.

In each block, the drying phase was imposed one day apart from the previous one and within each block one well-watered [WW] and one SWD plant were paired and allocated to a targeted harvesting time (experimental unit comprised one plant). Additionally, of large green, white, 50% red and red fruit was harvested block by block for further analyses (experimental unit comprised seven plants). A final pick of fully ripe fruit was done just before the end of the experiment (431 h).

In experiment three, fruits were taken at large green stage over the course of the SWD treatment (**Figure 0.1 [b]**). The first samples were taken at midday of the first day of

wilting; the second at midday of the second day of wilting; the third one hour after re-hydration; which took place at 11:00 a.m. of the same day; the fourth three hours after re-hydration and the last one was taken 25 hours after re-hydration. In this experiment, all plants were left to dry, independently of the block they were in and at their own pace so each pair (SWD and WW) reached wilting phase on different day and consequently was sampled on different days.

In experiment five, fruit were taken at eleven time points during the course of the SWD treatment, starting from midday of the day before wilting commenced (**Figure 0.1 [a]**). Then sampling took place three times a day over three consecutive days, at 10:00, 13:00 and 16:00; a final sample was taken at midday on the fourth day. All plants of the experiment started the drying phase simultaneously.

In experiment four (not in the figure), samples for measurement of ethylene production were taken at midday of the second day of wilting. Samples of red fruit for total phenolic analyses were taken seven days later.

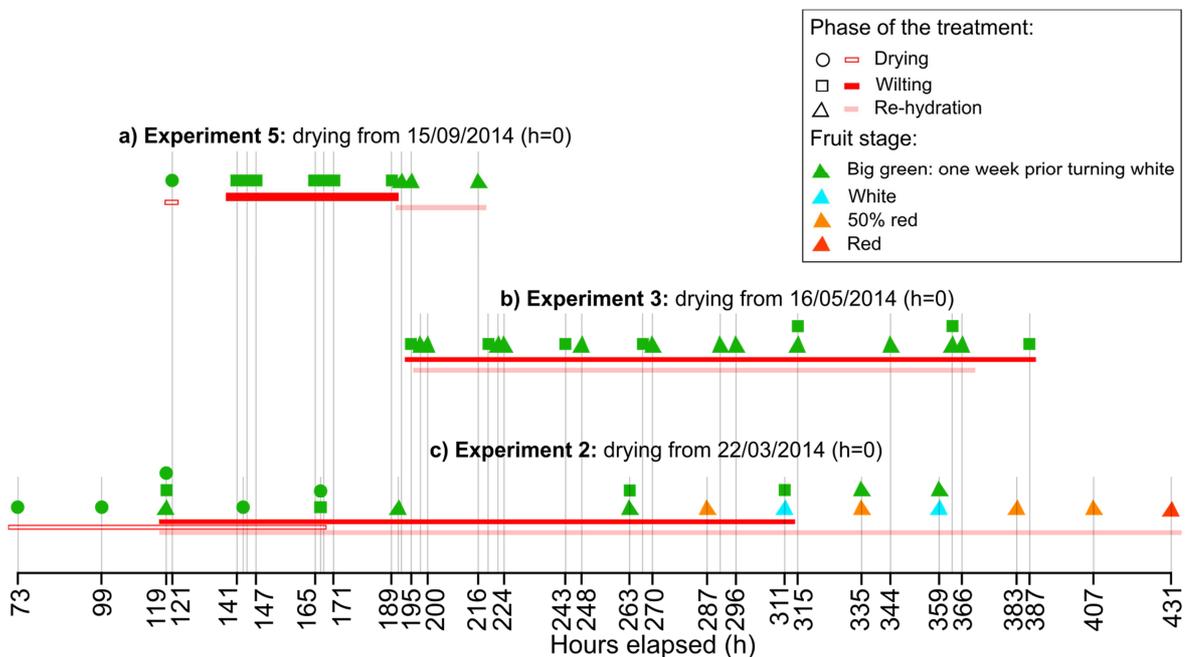


Figure 0.1. Schematic representation of fruit sampling for ethylene production rate and total phenolic concentration analyses over the course of the SWD treatment in experiments two (c), three (b) and five (a).

2.3 Chemical Analyses

2.3.1 Sample preparation for phytochemicals analyses

Once fruit was harvested and after ethylene production rate had been determined, the calyx was removed, the fruit placed in a Whirl-Pak[®] sampling bag (Nasco Ltd., USA) and immediately immersed in liquid nitrogen. Samples were placed in a freeze drier (Telstar LyoQuest, Telstar, Spain) where they were subjected to cool plus vacuum treatment that removed all water from them. Shelves were at 30 °C, the condenser was at -52.6 °C and pressure was set at 0.2 mBar.

2.3.2 Total phenolics by Folin-Ciocalteu method

Quantification of total phenolics was carried out according to the protocol of Ainsworth & Gillespie's (2007) as follows. All chemicals were obtained from Sigma-Aldrich Inc. (USA) unless otherwise stated.

2.3.2.1 *Extraction*

Finely ground freeze dried material (0.1 g) was introduced in a two ml Sure Lock[®] vial (Eppendorf GA., Germany) containing 1.5 ml of 0.05 mol l⁻¹ potassium phosphate buffer, pH 6.5 (phosphoric acid). It was homogenized for 1 min by vortexing; extraction was left to complete in the dark at 4 °C while shaking at 500 mot min⁻¹ for 24 hours. Samples were centrifuged (Sigma 4-16KS, Sigma GmbH., Germany) at 4,700 rev min⁻¹ and 4 °C for 20 min.

2.3.2.2 *Quantification by spectrophotometry*

Supernatants were diluted (1:4) in 0.05 mol l⁻¹ potassium phosphate buffer pH 6.5 (phosphoric acid). A 0.36 ml aliquot was pipetted into an HPLC tube with 0.73 ml of 10% (vol/vol) Folin-Ciocalteu reagent and 2.91 ml of 0.7 mol l⁻¹ of sodium carbonate. The absorbance of samples, calibration solutions and blanks was immediately quantified using an Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology Inc, USA) at 760 nm and re-quantified after two hours of incubation in the dark at 35°C.

The calibration curve was calculated using gallic acid as standard (**Table 0.1**). The stock solution was made of 1 mg per ml gallic acid in 95% (vol/vol) methanol.

Table 0.1. Calibration solutions for total phenolic quantification

Cuvette	Gallic Acid	95% MeOH
1	0	3600
2	100	3500
3	400	3200
4	700	2900
5	1000	2600
6	1400	2220

Total phenolic concentration was expressed as milligrams gallic acid equivalents per gram of dried weight of sample (mg GAE g⁻¹ DW) and calculated using the absorbance of the sample converted using the calibration curve.

2.3.3 Ethylene quantification by GC-FID

Ethylene was quantified using gas chromatography (GC) (more information follows).

The sampling procedure was developed for the current investigation. An overview of the quantification method can be found in Colgan et al. (2010).

2.3.3.1 *Head space sampling*

Three fruit were introduced into a 50 ml volumetric flask. The air space volume was reduced with wax beads (2 mm diameter) in order to increase ethylene concentration in headspace sample. A rubber stopper/septum was placed on top of the flask to produce an air-tight seal. After exactly 1 h, a 1 ml head space was taken and injected into the GC-FID for ethylene quantification.

2.3.3.2 *Quantification by GC-FID*

The GC used (6890N, Agilent technologies UK Ltd, UK) was fitted with a flame ionization detector and an activated alumina column. The conditions of the run were: temperature of the oven 250 °C, the carrier gas (N₂) flow rate was 10 ml min⁻¹, the hydrogen (H₂) and compressed air were set at flow rates of 35 and 250 ml min⁻¹ respectively. Each run lasted 60 s and the concentration of the sample was determined

by calibration with a 1 part per million ethylene standard (Sigma-Aldrich Inc., USA).

The injections were 1 ml volume.

Whole fruit were then weighed and head space measured in order to give results as nanolitres of ethylene per kilogram of fruit per hour ($\text{nl kg}^{-1} \text{FW h}^{-1}$).

2.3.4 Organic Acid quantification by HLPC in green fruit

The following quantification by high performance liquid chromatography (HLPC) was adapted from an in-house method at EMR. The extraction method from the EUberry manual (2010) was optimised for green strawberry fruit.

2.3.4.1 *Extraction of organic acids*

Chemicals used for extractions were purchased from Sigma-Aldrich Inc. (USA) unless stated otherwise. Finely ground freeze-dried material (0.1 g) was introduced into a 2 ml Sure Lock® vial along with 1.5 ml of the extraction solution. The extraction solution was 0.02 mol l^{-1} potassium phosphate buffer at pH 2.7 (phosphoric acid) alongside 0.005 mol l^{-1} of *tris*-(2-carboxyethyl)phosphine (TCEP) in order to protect samples from oxidation. Homogenization for 1 min by vortexing followed; extraction was left to complete in the dark at $4 \text{ }^{\circ}\text{C}$ while shaking at 500 mot min^{-1} for 15 min. The samples were centrifuged at $4,700 \text{ rev min}^{-1}$ and $4 \text{ }^{\circ}\text{C}$ for 20 min, then $300 \text{ }\mu\text{l}$ of the supernatant was diluted in 1.5 ml of phosphate buffer. A 0.45 ml aliquot of each dilution was pipetted and filtered into a Single Step® filter vial. At this point samples were ready for injection and could be kept at $-20 \text{ }^{\circ}\text{C}$ in the freezer.

2.3.4.2 *Calibration*

Stock solutions were 1 mg per ml for oxalic acid and ascorbic acid, and 10 mg ml^{-1} for citric acid and malic acid in 0.1 mol l^{-1} potassium phosphate buffer at pH 2.7 in 5 ml volumetric flasks. Most standards were purchased from Sigma-Aldrich Inc., but citric acid was purchased from Fisher Scientific UK Ltd. Stock solutions were kept at minus $4 \text{ }^{\circ}\text{C}$.

A four point calibration was carried out for each of the standards. Amounts of stocks and buffer for each of the vials were as shown in **Table 0.2**.

Table 0.2. Calibration solutions for organic acid quantification by HPLC

Vial	Oxalic	Ascorbic	Malic	Citric	Buffer
1	200	200	100	200	100
2	100	100	50	100	550
3	50	50	30	50	770
4	25	25	20	25	880

Units given in microliters (μl)

2.3.4.3 Quantification by HPLC

Organic acids were quantified with a Walters Alliance 2690 HPLC system with a Walters 410 Differential Refractometer (Walters Inc. USA). HPLC conditions were as follows: 10 μl injection volume, Phenomenex Synergi Hydro RD 250 x 4.60 mm and four microns HPLC column fitted with an C18 cartridge, column at ambient temperature, and flow rate of 1 ml per min. The mobile phase was 0.01 mol l^{-1} potassium phosphate buffer at pH 2.5 running for 12 min with no delay between injections. For quantification, calibration curves were compared against the sample data using Millennium 32 4.0 software (Walters Inc. USA). Results were expressed as milligrams per gram of dry weight of sample (mg g^{-1} DW).

2.3.5 Polyphenol quantification by HPLC in green fruit

Initially, standards of eight phenolic compounds, ascorbic acid and shikimic acid were run to establish retention times and to enable their quantification in the samples. Only ellagic acid and ascorbic acid in green fruit were detected with the following method. The rest of the phenolics were catechin, p-coumaric acid, epicatechin, kaempferol, pelargonidin-3-glucoside, caffeic acid and ferulic acid. All chemicals used here were purchased from Sigma-Aldrich Inc. (USA) unless otherwise stated. The following method is based on the protocol of Atkinson et al. (2006).

2.3.5.1 *Extraction*

A sample of finely ground freeze-dried material (0.1 g) was introduced into a 2 ml Sure Lock[®] vial along with 2 ml of 80% (vol/vol) methanol. Homogenization for 1 min by vortexing followed; extraction was left to complete in the dark at 4 °C while shaking at 500 mot min⁻¹ for 15 min. Samples were centrifuged (Sigma 4-16KS, Sigma GmbH., Germany) at 4,700 rev min⁻¹ and 4 °C for 20 min.

2.3.5.2 *Hydrolysis and purification*

After centrifuging the samples, two 300 µl aliquots of the supernatant were used to measure the total ellagic acid, free ellagic acid and ascorbic acid. For hydrolysis, each aliquot was mixed with 180 µl of 3.9 mol l⁻¹ hydrochloric acid and heated to 100 °C for 2 h. The hydrolysates were purified by passage through a reverse phase column, Discovery[®] DSC-18 (Sigma-Aldrich Inc, USA). The column was washed with 1 ml of pure methanol and the pre-equilibrated with 1 ml of ultra-pure water. Then 300 µl of the hydrolysate was applied to the column, followed by 200 µl of ultra-pure water to remove impurities. Finally, the analytes retained in the C-18 were eluted in to a Single Step[®] filter vial by application of 450 µl of 80% (vol/vol) methanol.

For purification of free ellagic acid (no hydrolysis was performed), a reverse phase column Discovery[®] DSC-18 was used as a filter, retaining the impurities instead of the analytes. The C-18 was washed with 1 ml of pure methanol followed by pre-equilibration with 1 ml of 80% (vol/vol) methanol. The supernatant was added with 100 µl of 80% (vol/vol) methanol. The eluate from the column was collected into a Single Step[®] filter vial, evaporated to dryness and re-dissolved with 450 µl of methanol (20:10 v/v).

2.3.5.3 *Calibration*

Calibration solutions were made of stock solutions of 1 mg per ml ascorbic acid in water and 1 mg ml ellagic acid in dimethyl sulfoxide (DMSO). Standards were

purchased from Sigma-Aldrich Inc. The calibration solutions for each of the standards are shown in **Table 0.3**.

Table 0.3. Calibration solutions for ascorbic and ellagic acid quantification by HPLC

Vial	Ascorbic	Ellagic
1	200	160
2	150	120
3	100	80
4	50	40
5	25	4

Units are microliters (μl)

2.3.5.4 Quantification by HPLC

Free and total ellagic acid, and ascorbic acid were quantified using a Walters Alliance 2690 HPLC system with a Walters 410 Differential Refractometer. HPLC conditions were as follows: 10 μl injection volume, Phenomenex Luna C-18 150 x 2 mm and three microns HPLC column fitted with a C-18 cartridge, column at ambient temperature, and constant flow rate of 1 ml per min. The mobile phases were (B) acetonitrile and (D) 0.8% trifluoroacetic acid (TFA). The following gradient was used: zero to two minutes, 90% D and 10% A; two to 20 min, linear increase to 40% B (and 60% D); 20 to 25 min, isocratic conditions and then 25 to 27 min, linear decrease to initial conditions; 27 to 30 min, kept at the same conditions; finally the column was left to equilibrate for 10 min before the next injection. For quantification of the acids, the calibration curves were compared against the sample data using Millenium 32 4.0 software.

Total ellagic acid and free ellagic acid was expressed as nanograms per gram of dry weight of sample (ng g^{-1} DW). Ascorbic acid was expressed as milligram per gram of dry weight.

2.4 Statistical analysis

Linear regression modelling was performed on the data with response variables being fruit responses (ethylene production rate and total phenolic concentration) and

explanatory variables being environmental variables and leaf responses. An additional regression analysis was performed to study the relation between ethylene production rate and total phenolic concentration. Models were adjusted at maximum R^2 and model checked against normality, homoscedasticity and independence of errors. Two way analyses of variance were performed on the data of a) whole experiment and b) each irrigation phase. Results of the latter were only presented if contradictory to those of whole experiment. *T-tests* were carried out to ascertain differences between SWD and WW at each hour or sampling time. Finally, in order to study the relation between ethylene production rate and total phenolic concentration, a cross-correlation plot was calculated for each of the experiments in this chapter. Statistical analyses were carried out using R version 3.1.0 (R Foundation for Statistical Computing, Austria).

3 Results

3.1 Responses of ripening strawberry fruit to SWD

Ethylene production rate ($\text{nl kg}^{-1} \text{h}^{-1}$) was highest from fruits at large green stage and declined during ripening. Ethylene production in experiment two was positively affected ($p < 0.05$) by the severe water deficit (SWD) treatment (**Figure 0.2**). Plants subjected to SWD produced more ethylene (165.40 ± 17.68) than control well-watered (WW) plants (129.99 ± 11.78). Further exploration of the data revealed that at “Day 2 of wilting” the SWD produced the most significant difference of all sampling times during the experiment ($t[7.93] = 2.90$, $p = 0.02$; 288.50 ± 36.96 vs 165.12 ± 21.02).

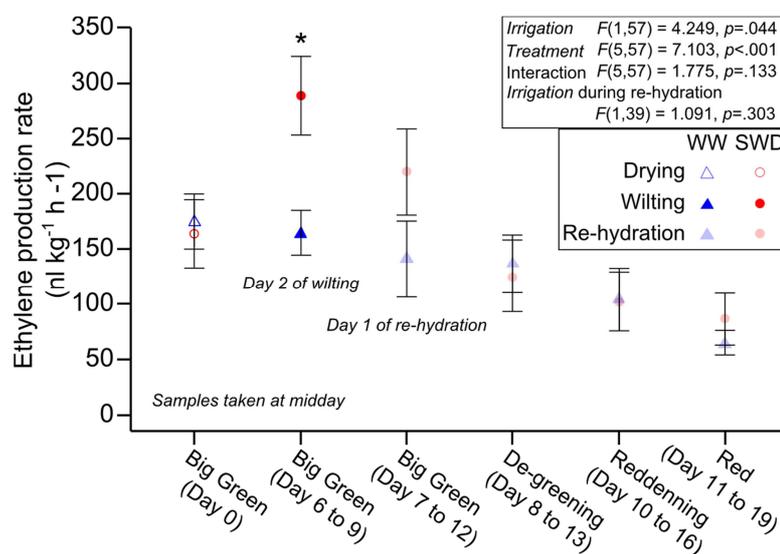


Figure 0.2. Ethylene production rate ($\text{nl kg}^{-1} \text{h}^{-1}$) of fruits at different ripening stages under severe water deficit (SWD) or well-watered (WW) conditions. Data were obtained during experiment two. Plants were re-hydrated at 11:00 a.m. Mean values \pm standard error ($n=6$) are presented. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Measurements were made at 12:00 p.m., when plants were in the targeted harvesting time. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Total phenolic concentration ($\text{mg GAE g}^{-1} \text{DW}$) varied across sampling times (**Figure 0.3**). This variation depended on ripening stage and time of sampling. In addition, concentration of total phenolics was shown to be affected by the SWD treatment ($p < 0.05$) only when all data were included in the same analysis. Fruit of plants subjected to SWD accumulated more phenolics than control (WW) plants (12.02 ± 0.37

compared to 11.39 ± 0.41). Further analyses revealed that this difference was highest when fruit was at the red stage (from Day 13 to 18) ($t[7.49]=2.71$, $p=0.03$; 9.26 ± 0.61 vs 7.40 ± 0.31).

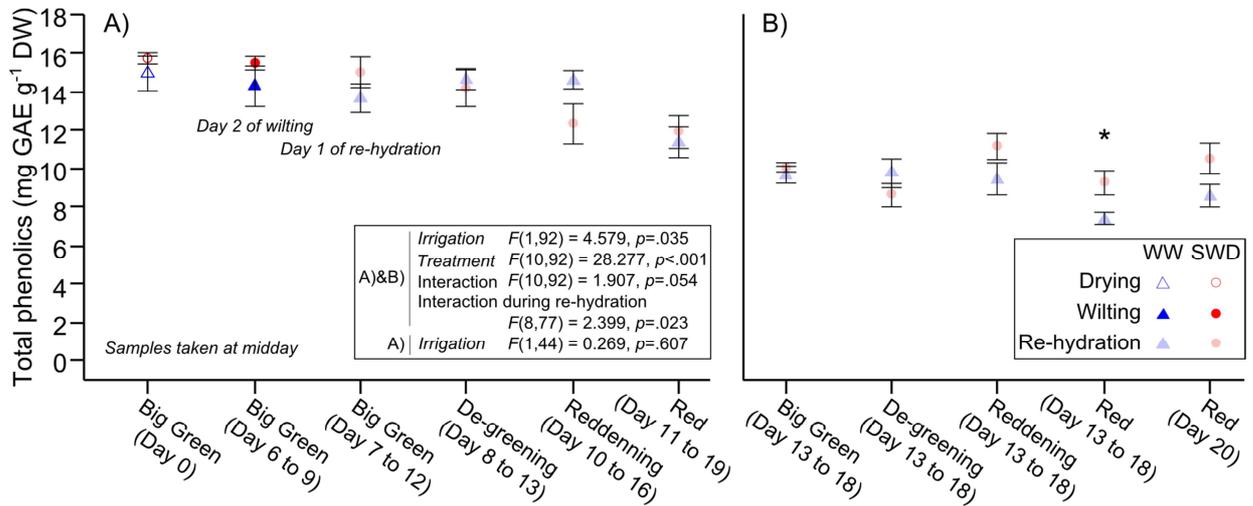


Figure 0.3. Total phenolic concentration (mg GAE g⁻¹ DW) in fruits at different ripening stages and under severe water deficit or well-watered conditions. Data were obtained during experiment two: A) samples were harvested with ethylene production rate samples; B) samples were taken block by block, all fruit stages at once; and red (Day 20) were taken just before the end of the experiment. Plants were re-hydrated at 11:00 a.m. Mean values \pm standard error ($n=6$) are presented. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Samples were taken at 12:00 p.m., when plants were in the target phase of the irrigation treatment and fruit were in the target ripening stage. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.2 Large green fruit responses during the SWD phases

Figure 0.4 shows ethylene production rate (nl kg⁻¹ h⁻¹) of green fruit in experiments three (A) and four (B). The SWD treatment affected positively ethylene production in comparison to WW ($p=0.006$) when all the data were included in the same analysis. Fruit subjected to SWD had on average a higher ethylene production rate than controls (552.30 ± 71.43 vs 317.46 ± 40.11). Although the time at which fruit were harvested after the imposition of the stress did not have a significant effect on the average ethylene production rate, further analyses revealed statistical differences between SWD and WW averages at midday of the second day of wilting in experiments three ($F[1,6]=12.19$, $p=0.013$; 599.69 ± 232.94 vs 258.73 ± 65.62) and four ($f[6.3]=3.48$, $p=0.012$; 508.18 ± 69.53 vs 230.15 ± 39.15), and 1 h after re-hydration in experiment

three ($t[8.8]=2.89$, $p=0.019$; 392.46 ± 33.05 vs 235.53 ± 43.15). SWD treated fruit produced more ethylene than controls in all these cases.

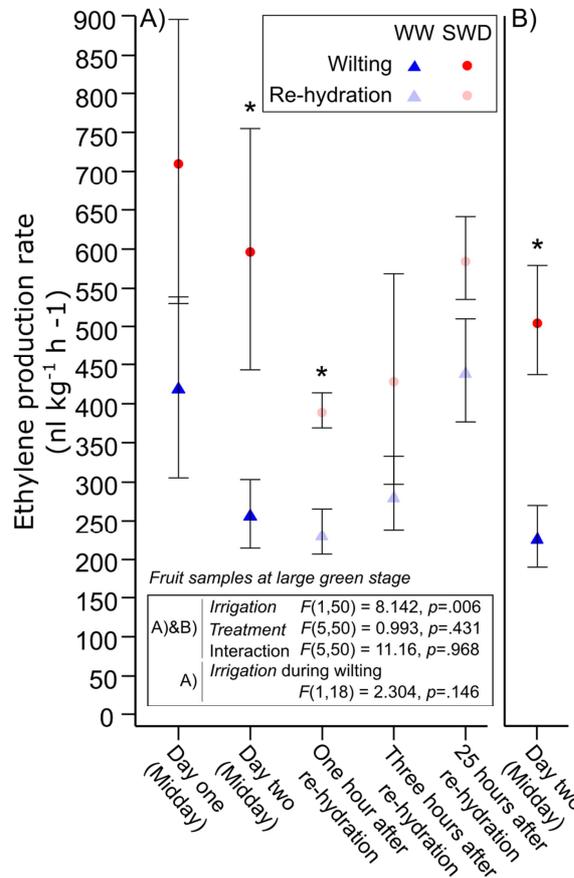


Figure 0.4. Ethylene production rate (nl kg⁻¹ h⁻¹) of green fruits at different times during wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n = 6$) are presented for experiment three (A) and four (B). Plants were re-hydrated at 11:00 a.m. Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Average ethylene production rate (nl kg⁻¹ h⁻¹) for green SWD fruits was significantly higher ($p=0.02$) than for WW fruit (244.12 ± 15.66 vs 203.61 ± 7.83), and also changed during the experimental period ($p=0.032$) (Figure 0.5). The SWD treatment did not affect ethylene production during re-hydration, however. Consistent with previous results, there was a significant effect of SWD at hour 168, at midday of the second day of wilting ($t[10]=2.84$, $p=0.032$; 362.76 ± 67.61 vs 165.11 ± 16.87).

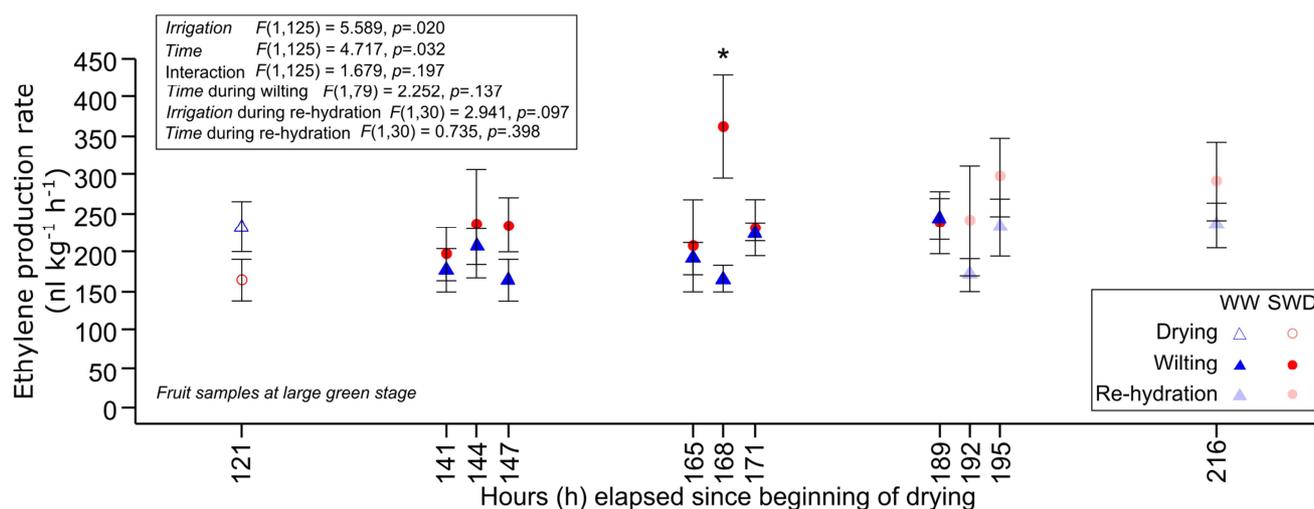


Figure 0.5 Ethylene production rate ($\text{nl kg}^{-1} \text{h}^{-1}$) of green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected with t-tests are indicated with an asterisk ($p<0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Total phenolic concentration ($\text{mg GAE g}^{-1} \text{DW}$) in fruit harvested during experiment three (A) and four (B) (Figure 0.6) was significantly affected by the SWD treatment ($p<0.001$). On average, SWD fruit had higher phenolic concentration than WW fruit (12.70 ± 0.45 vs 10.73 ± 0.40). The average of total phenolic concentration (WW and SWD) from experiment four was significantly lower than that for experiment three. In addition, the results of the analysis of the whole set of data (in Figure 0.6 [A] and [B]) and only experiment three (Figure 0.6 [A]) showed that the effect of the SWD treatment changed between experiments. Further analysis revealed significant differences between average phenolic concentration in SWD and WW at midday of day two of wilting ($t[5.22]=2.84$, $p=0.034$; 13.06 ± 0.48 vs 9.30 ± 1.23), and also three hours after re-hydration ($t[6.04]=3.79$, $p<0.001$; 15.20 ± 0.57 vs 10.69 ± 1.04).

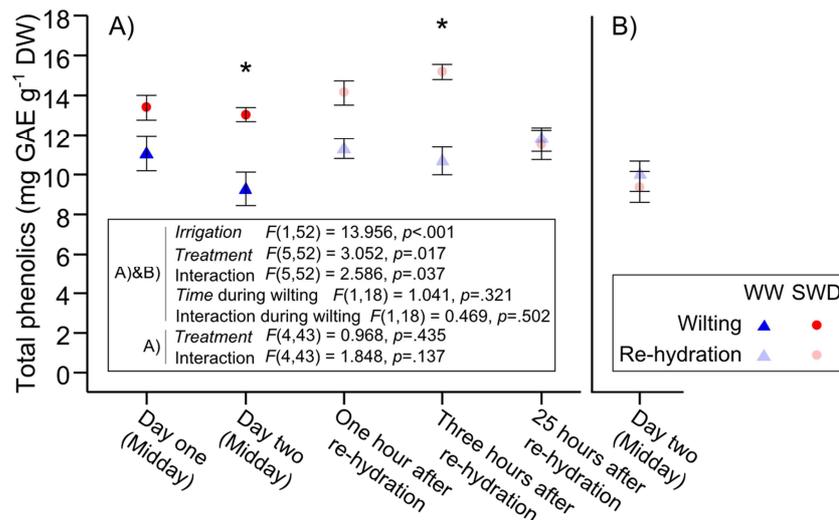


Figure 0.6. Total phenolic concentration (mg GAE g⁻¹ DW) in fruit at different times during wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error (n=6) are presented for large green fruit in experiment three (A) and red fruit in experiment four (B). Statistical differences detected with t-test are indicated with an asterisk ($p < 0.01$). Plants were re-hydrated at 11:00 a.m. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

The concentration of phenolics during experiment five (**Figure 0.7**) was also positively affected by the SWD treatment ($p=0.002$; 244.12 ± 15.66 vs 203.61 ± 7.83). Neither effects of *Time* nor the *Interaction* could be found, indicating that neither the average total phenolic concentration, nor the SWD treatment effect changed over time. The SWD effect was especially prominent three hours after midday on the second day of wilting ($t[10]=3.05, p=0.015$; 231.16 ± 35.54 vs 225.90 ± 10.35).

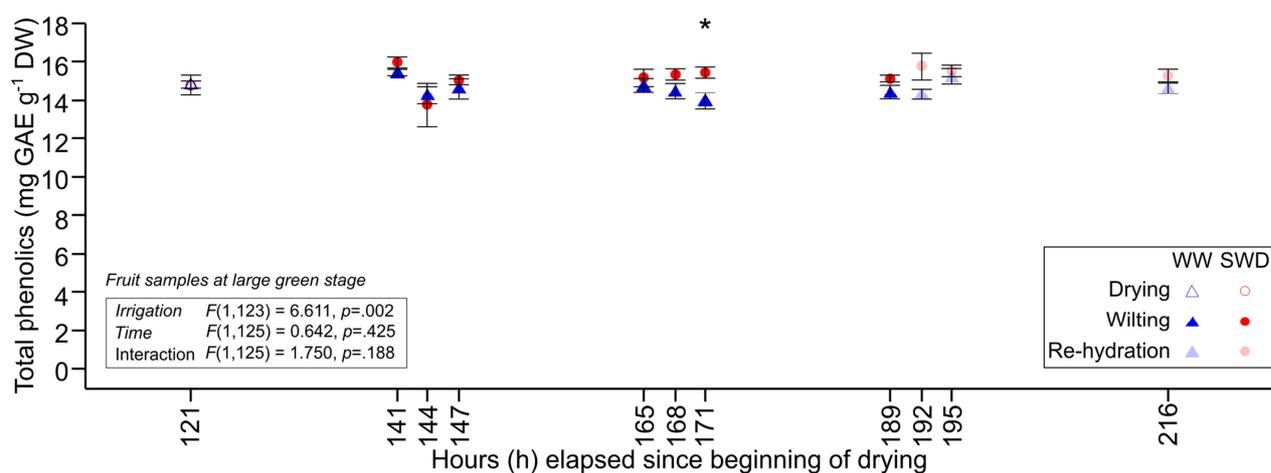


Figure 0.7. Total phenolic concentration (mg GAE g⁻¹ DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error (n=6) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p < 0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.3 Total phenolic concentration and ethylene production rate

All predictors initially considered for linear regression analysis (**Table 0.4**) were included in the model for total phenolic concentration. The model was adjusted to maximum R^2 and peat moisture, temperature, stomatal conductance and transpiration were excluded from the analyses because correlations were found between these and pot-and-plant weight (with PVMC), temperature (with radiation) and photosynthesis (with transpiration and stomatal conductance). Total phenolic concentration in the fruit was explained by radiation, ethylene production, pot-and-plant weight, experiment five, phase of the SWD treatment and the rate of photosynthesis. Of these, only the intercept, radiation, weight and experiment five effects were significant; ethylene production, however, was close to significant levels ($p=.064$). In all, the model explained 48% of the variability of the total phenolic concentration.

Table 0.4. Relation between phenolic concentration and selected leaf responses, environmental variables and fruit ethylene production rate

	(Total phenolics) ²
Intercept	193.130 ($p < .001$)
Radiation	-0.160 ($p = .047$)
Ethylene production	0.031 ($p = .064$)
Weight	-0.025 ($p = .016$)
Experiment five	86.378 ($p < .001$)
Re-hydration	23.256 ($p = .198$)
Wilting	7.282 ($p = .678$)
Photosynthesis	-1.048 ($p = .145$)
$R^2 = 0.48$;	
$F(7,216) = 30.19$;	
$p < .001$	

Regression analyses for the effect of ethylene production rate and other variables on total phenolic accumulation by the fruit from drying to re-hydration and during wilting. Response variable was transformed and models were adjusted to maximum R^2 . Response variables are those in the first row and predictors those in the first column. p -values (p) are presented for the coefficients (β) of each model. In addition, R^2 , F statistic and significance level of the model are presented at the bottom.

Cross-correlation between ethylene production rate and total phenolic accumulation was calculated in order to find support for a putative temporal correlation between these two variables (**Figure 0.8**). Significant correlation at negative *Lag* values would indicate that ethylene production rate changed before phenolic accumulation took place and conversely, positive *Lag* values would indicate that ethylene production rate changed after phenolics accumulated in the fruit. The results of the autocorrelation function (ACF) were not significant in any case. However, in each of the experiments there tended to be positive correlation at negative *Lag* values in the proximity of 0, and negative correlation at positive *Lag* values, suggesting that ethylene production changed before total phenolics accumulated in the fruit.

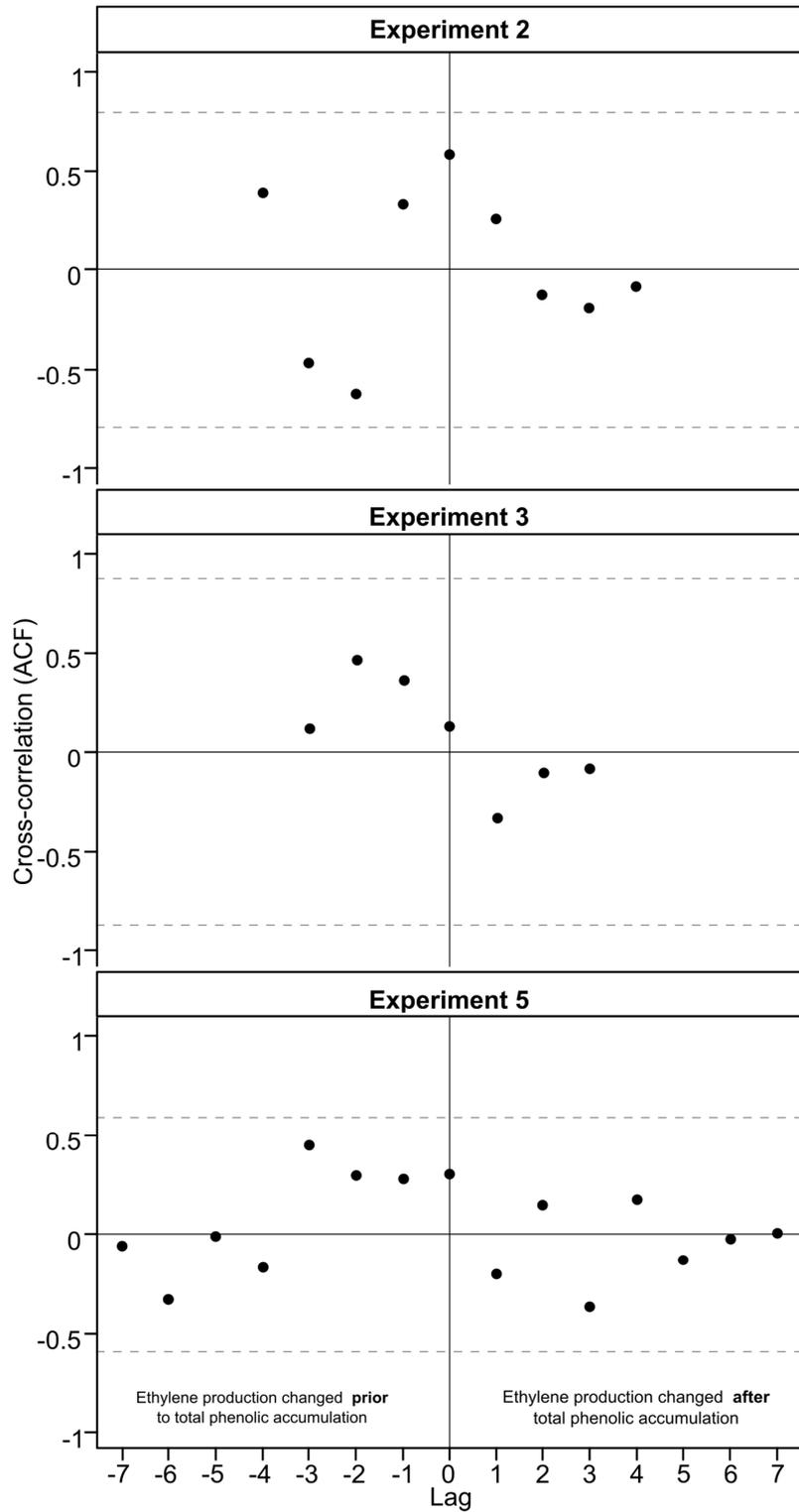


Figure 0.8. Cross-correlation plots between ethylene production rate and total phenolic accumulation for experiments two, three and five. Each point denotes the result of autocorrelation function (ACF) between ethylene production rate at time t and total phenolic concentration at time $t+Lag$. Confidence intervals (95%) for each experiment are represented by the two dashed lines; if a point is not within the confidence interval the cross-correlation is not significant.

3.4 Free, total ellagic and organic acids concentration in large green fruit over the course of the SWD treatment

The two-way ANOVA on the ascorbic acid concentration (mg g^{-1} DW) from experiment five did not reveal an effect of the SWD treatment (**Figure 0.9**). Yet the t-test indicated a significant and positive effect of the SWD treatment in comparison to WW at midday of the first day of wilting (hour 144) ($t[10]=3.73$, $p<0.01$; 3.70 ± 0.08 vs 2.85 ± 0.21) and an opposite effect at midday of the second day of wilting (hour 168) ($t[10]=-3.19$, $p=0.01$; 3.39 ± 0.14 vs 3.95 ± 0.10). This latter effect of the SWD occurred again during the re-hydration phase (hour 216) ($t[10]=-2.80$, $p=0.02$; 3.04 ± 0.28 vs 3.96 ± 0.16).

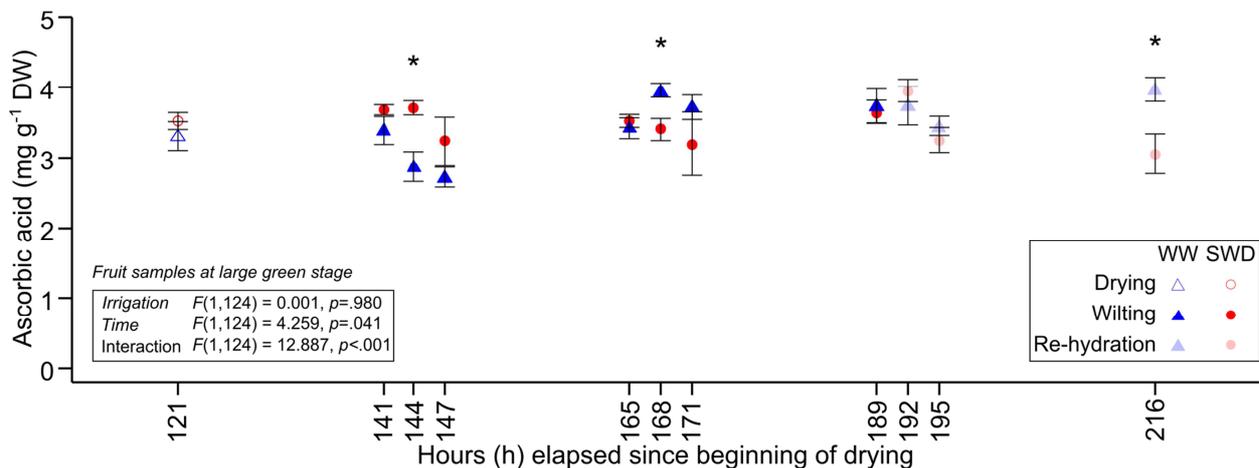


Figure 0.9. Ascorbic acid concentration (mg g^{-1} DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p<0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Analysis of the citric acid data from experiment five (mg g^{-1} DW) showed no significant main effects (**Figure 0.10**). During re-hydration, average citric acid concentration changed significantly ($p=0.037$) and the SWD treatment reduced significantly the fruit concentration of citric acid compared to the WW control ($t[10]=-2.80$, $p=0.02$; 47.10 ± 3.63 vs 62.22 ± 4.45).

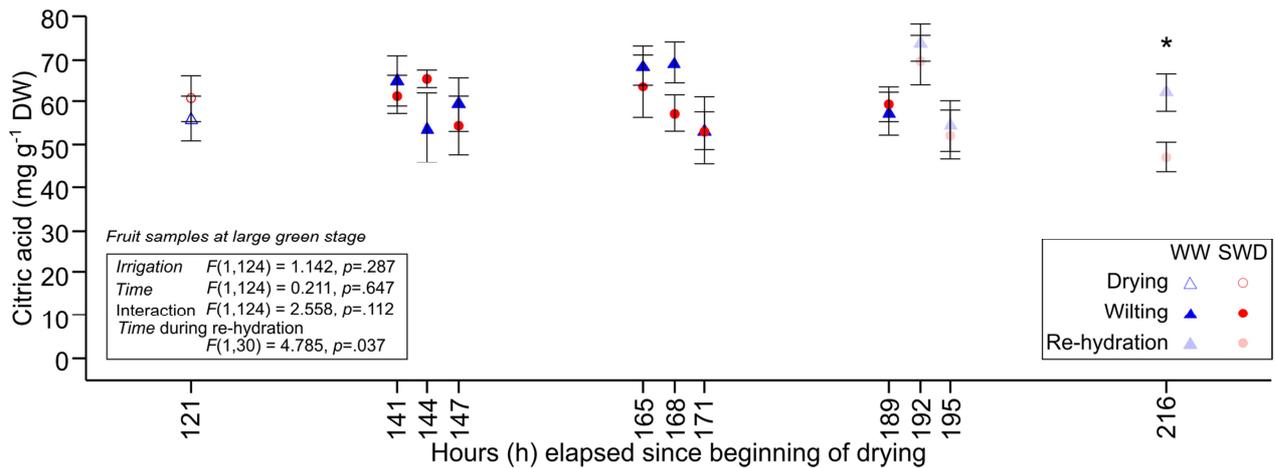


Figure 0.10. Citric acid concentration (mg g^{-1} DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p < 0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Malic acid concentration (mg g^{-1} DW) was reduced significantly ($p < 0.001$) by the SWD treatment in experiment five (**Figure 0.11**) (11.46 ± 0.57 vs 14.94 ± 0.51). This effect was especially clear during the second day of wilting and during re-hydration: t-tests revealed significant differences at midday of the second day of wilting and at every sampling occasion during the third day.

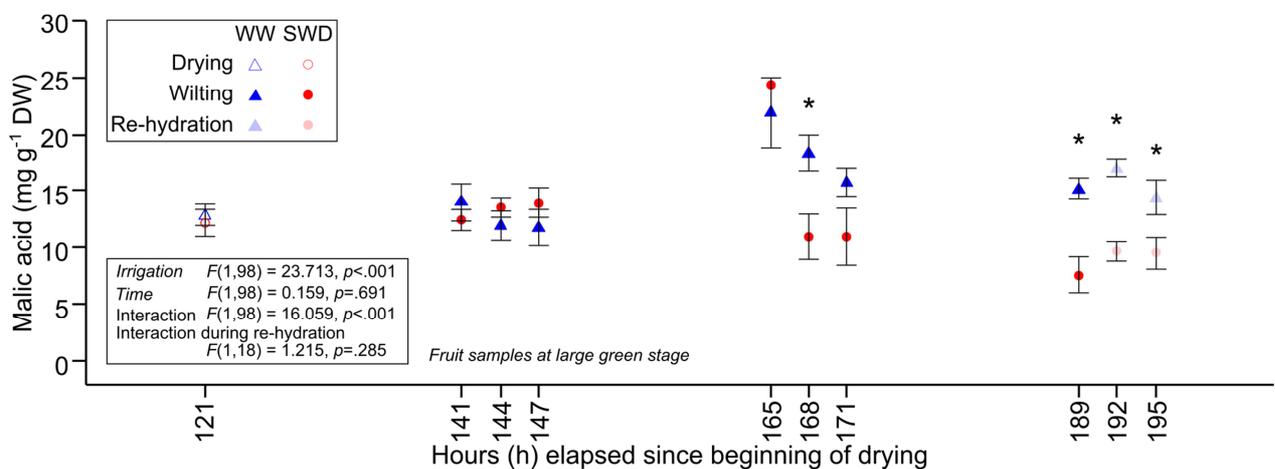


Figure 0.11. Malic acid concentration (mg g^{-1} DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p<0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Neither the two-way ANOVA performed on the free ellagic acid data ($\mu\text{g g}^{-1}$ DW) from experiment five (**Figure 0.12**) nor the t-tests showed any significant differences. During the wilting phase, however, average free ellagic acid concentration changed significantly ($p=0.037$) and seemed to decrease over time.

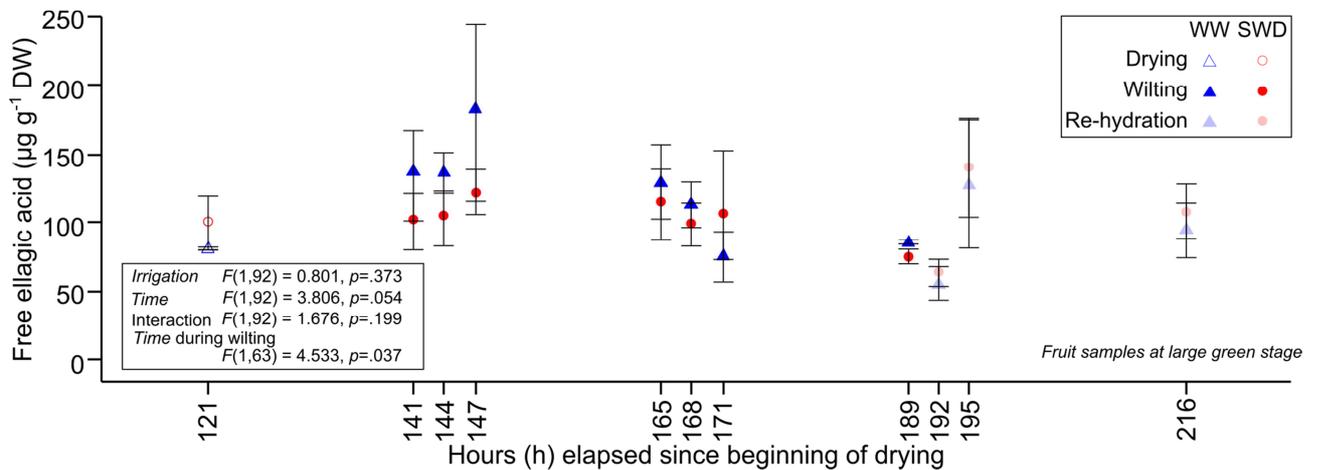


Figure 0.12. Free Ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p<0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

Total ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) from experiment five (**Figure 0.13**) was affected by the SWD treatment ($p=0.006$). The concentration was higher in fruit treated with SWD than in the WW controls (1579.83 ± 69.63 vs 1329.35 ± 52.11).

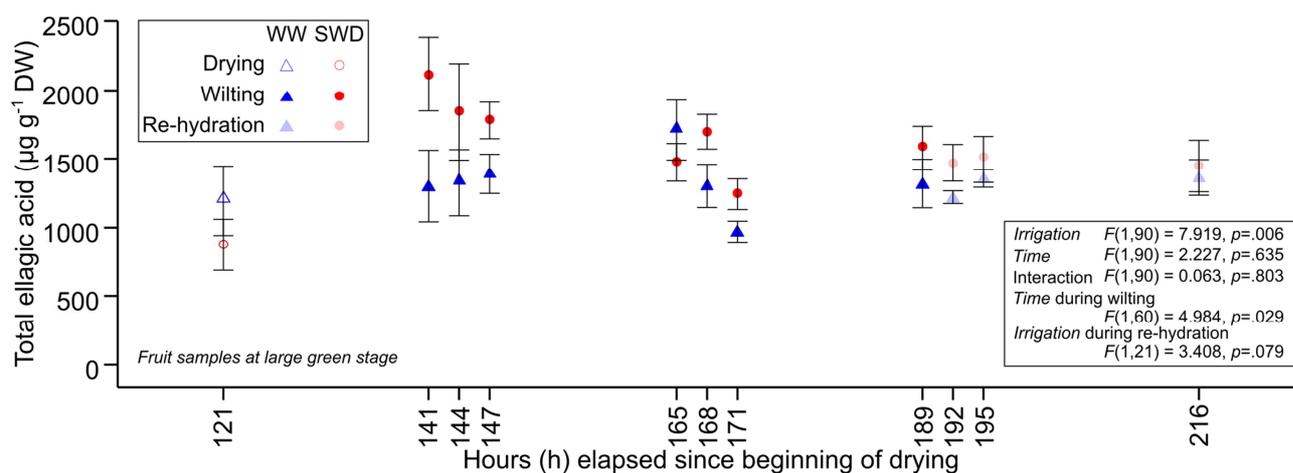


Figure 0.13. Total Ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) in green fruits at different times during drying, wilting or re-hydration, under severe water deficit (SWD) or well-watered (WW) conditions. Mean values \pm standard error ($n=6$) are presented for experiment five. Sampling was done 11 successive times, once during drying, seven times during wilting and three times during re-hydration. Statistical differences detected by t-tests are indicated with an asterisk ($p<0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2014.

3.5 Most relevant environmental factors and leaf responses explaining the variations in total phenolic concentration and ethylene production rate

Multiple regression analyses were carried out on data from experiments two, three and five to test whether pot weight, light intensity (radiation), the experiments, the treatment phase, photosynthetic rate or Yield (II) data predicted the variability in ethylene production rate or total phenolics during drying, wilting and re-hydration, or only during wilting (**Table 0.5**). Models were adjusted to maximum R^2 . Pot weight and peat moisture correlated as did temperature and light intensity, stomatal conductance and photosynthetic rate, and finally transpiration rate and photosynthetic rate, so peat moisture, temperature, stomatal conductance and photosynthetic rate were excluded from the analyses.

Table 0.5. Relation between phenolic concentration or ethylene production rate and selected leaf responses and environmental variables

	From drying to full re-hydration		During wilting	
	log(Ethylene production rate)	(Total phenolics) ²	Ethylene production rate	Total phenolics
Intercept	6.15 ($p<.001$)	213.89 ($p<.001$)	-759.35 ($p=.027$)	11.92 ($p<.001$)
Radiation	-3.01×10^{-4} ($p=.001$)	-0.16 ($p=.035$)	10.29 ($p<.001$)	-0.013 ($p=.047$)
Weight	2.97×10^{-3} ($p=.011$)	-3.64×10^{-2} ($p<.001$)	-0.38 ($p=.032$)	0.002 ($p=.003$)

Experiment five	-0.35 ($p < .001$)	78.98 ($p < .001$)		1.25 ($p = .003$)
Re-hydration	4.73×10^{-2} ($p = .782$)	27.47 ($p = .135$)		
Wilting	-0.11 ($p = .486$)	9.67 ($p = .594$)		
Yield (II)			774.28 ($p = .001$)	
Photosynthesis	-1.37×10^{-2} ($p = .095$)			
	$R^2 = 0.27$; $F(6,224) = 14.93$; $p < .001$	$R^2 = 0.43$; $F(4,227) = 36.00$; $p < .001$	$R^2 = 0.52$; $F(3,60) = 24.26$; $p < .001$	$R^2 = 0.41$; $F(3,60) = 15.47$; $p < .001$

Results of four analyses, for ethylene production rate and total phenolic concentration by the fruit from drying to re-hydration and during wilting. Response variables were transformed if needed, models were adjusted to maximum R^2 . Pot weight and peat moisture, and temperature and radiation correlated so peat moisture content and temperature were excluded from the analysis. Response variables are those in the second row and predictors those in the first column. p -values (p) are presented for the coefficients (β) of each model. In addition, R^2 , F statistic and significance level of the model are presented.

When all data were included in the analyses, the variability in ethylene production rate was explained by radiation, pot-and-plant weight, experiment five, the re-hydration and wilting phases and the photosynthesis data. Total phenolic data variability was explained by the same predictors apart from the photosynthetic rate, and again the irrigation phase effects were not significant. Response variables required transformation: log transformation for ethylene production rate and square transformation for total phenolics.

Focussing on the analysis of data from the wilting phase, the variability in ethylene production rate was explained only by environmental factors and the performance of the photosystem II, all of which were significant; total phenolics was explained by environmental variables and the effect of experiment five, all of which were also significant.

The overall fit of the models ranged from 0.27 (ethylene production rate model, for whole data set) to 0.52 (data from wilting phase), all of which were highly significant.

4 Discussion

4.1 Ethylene production rate of strawberry fruit subjected to SWD

During plant wilting, the ethylene production rate of green strawberry fruit increased significantly in SWD plants, compared to that of WW plants. The difference in ethylene production rate between WW and SWD plants persisted one hour after re-hydration in experiment five but disappeared three hours after re-hydration. Whatever was causing the increase in ethylene production seemed to be inherent to wilting. More importantly, increased ethylene production by green fruit subjected to SWD was consistently observed on the second day of wilting throughout the work presented in this thesis.

There was great variation in ethylene production in green fruit within and between experiments. Strawberry fruit produce a small amount of ethylene (~ 100 to ~ 500 $\text{nl kg}^{-1} \text{h}^{-1}$) in comparison to climacteric fruits such as apples and tomatoes (~ 20 to ~ 150 $\mu\text{l kg}^{-1} \text{h}^{-1}$) (O'Donnell et al., 1996; Song & Bangerth, 1996) which means that small errors during sampling or measuring procedures would result into large error bars. This variability could also be partly explained by the sharp and transient increase in ethylene production when fruit is about to turn white, as reported by Iannetta et al. (2006).

Red fruit tended to produce less ethylene compared to other developmental stages, ranging from $65 \text{ nl kg}^{-1} \text{h}^{-1}$ to $230.1 \text{ nl kg}^{-1} \text{h}^{-1}$. According to some authors, whole red fruit produce more ethylene than fruit at any other developmental stage (Iannetta et al., 2006). Other studies suggest that when results are expressed as production of ethylene per gram fresh weight, then it is green fruit which produce more ethylene (Abeles & Takeda, 1990; Perkins-Veazie et al., 1995). Green fruit from WW plants produced on average $220.6 \text{ nl kg}^{-1} \text{h}^{-1}$; in experiment four though, production reached $299 \text{ nl kg}^{-1} \text{h}^{-1}$ whereas in experiment two production was only $130 \text{ nl kg}^{-1} \text{h}^{-1}$. The reported changes in temperature and radiation between experiments may help to explain this variability. These values were well within the limits found in the literature for ethylene production rates from WW strawberry green fruit which range from 50 to

546 nl kg⁻¹ h⁻¹ (Abeles & Takeda, 1990; Perkins-Veazie et al., 1995; Iannetta et al., 2006). Such a wide range can be partly explained by the fact that the data from these studies come from different varieties (including 'Elsanta') and from sampling attached and detached fruit (Morgan et al., 1990; Narayana et al., 1991).

Green fruit subjected to SWD produced a significantly higher amount of ethylene than WW controls, ranging from 362.8 nl kg⁻¹ h⁻¹ to 713.2 nl kg⁻¹ h⁻¹ which is consistent with other reports of higher ethylene production in tissues of water stressed plants (El-Beltagy & Hall, 1974; Wang et al., 1987; Nakano et al., 2003; Sobeih et al., 2004; Voisin et al., 2006). However, this evidence is questioned by some authors, who report that attached tissue does not show such an increase whereas detached tissue does (Morgan et al., 1990; Narayana et al., 1991). However, light intensities used by Morgan et al. (1990) and Narayana et al. (1991) in their growing cabinets (70 to 260 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were low in comparison to usual ambient levels ($\sim 400 \mu\text{mol m}^{-2} \text{s}^{-1}$). Since ROS are signalling molecules for ethylene metabolism (Wang et al., 2002; Shao et al., 2008; Mittler et al., 2011) the increase observed could be due to the presumably high ROS production inferred by the Yield (II) measurements during wilting.

Regression analyses on the data collected during experiments two, three, four and five were performed to gain a better understanding of the influence of environmental responses other than soil moisture content. During the SWD treatment, ethylene production rate had a significant response to radiation and pot-and-plant weight. The response of ethylene metabolism to light has been reported before: Hogan (2008) measured ethylene production from leaves as well as recording ambient temperature and light intensity, finding that in some cases ethylene production and light/temperature positively correlated both in plants subjected to water stress and in well-watered controls. Munné-Bosch et al. (2002) measured ACC concentration in drying rosemary and found that it was positively correlated to light intensity and relatively independent of the drought itself. These reports reinforce the idea of the important role that light may play on ethylene production during the SWD treatment.

When considering ethylene production rate during wilting, the model explained 52% of the variability in ethylene production rate. Moreover, as well as radiation and pot-and-plant weight, ethylene production rate responded to the performance of photosystem II. The relationship between the performance of photosystem II (Yield [II]) and reactive oxygen species ROS was explained in the previous chapter but there may be a relation as well between ethylene metabolism and ROS (Wang et al., 2002; Shao et al., 2008; Mittler et al., 2011)., which suggests that Yield (II) could be used to estimate when the ethylene production rate is likely to be high in response to water deficit stress and consequently to inform decisions on when to sample the fruit.

The concentration of ROS was not measured during this work. Further exploration of the correlation over time of ROS accumulation with ethylene production rate and performance of photosystem II under different light intensities and water deficit interactions could be a first step towards finding whether ROS are behind the ethylene increase measured.

4.2 Phenolic concentration in fruit of strawberries subjected to SWD

According to the literature, total phenolics in WW red 'Elsanta' fruit range from 22.46 to 23.63 mg GAE g⁻¹ DW (Terry et al., 2007). In turn, green fruit always has more phenolics, when expressed as total phenolic concentration per gram of fruit, than red fruit; and phenylalanine ammonia-lyase (PAL - a rate limiting enzyme for biosynthesis of phenolic compounds) has decreasing activity as the fruit develops (Cheng & Breen, 1991; Wang & Lin, 2000; Halbwirth et al., 2006). Data from experiment two agree with the trend that these authors describe: green fruit phenolics accumulated to 14.95, decreasing to 11.32 mg GAE g⁻¹ DW at the red fruit stage. However, the highest value measured during the 2014 experiments on red fruit was substantially lower (14.4 mg GAE g⁻¹ DW) than the ones reported previously which may be explained by the fact that aqueous solvents such as the potassium phosphate buffer used here extract 40%

less phenolics than the 80% methanol solvent used by Terry et al. (2007) (Turkmen et al., 2006).

During the present research, significant differences in total phenolic concentration in red fruit were only detected six days after re-hydration whereas red fruit harvested four or eight days after re-hydration did not show such an concentration. For green fruit, however, statistical differences arose at midday of the second day of wilting or three hours after. In green fruit, this difference was sometimes detected during re-hydration, but not always. This inconsistent result suggests that despite the evidence provided by Terry et al. (2007) and Dodds (2008) of an increase in phenolic concentration in strawberry fruit under water deficit a) the concentration of phenolics may not depend solely upon the SWD treatment; b) SWD may cause too severe a stress for the concentration of phenolics. Yield (II) during the wilting phase was the lowest during the SWD treatment indicating that the photosystems may have been compromised (Nishiyama & Murata, 2014). Although ROS generation may be a key signal for the biosynthesis of phenolics (Jacobo-Velázquez et al., 2011), if ROS generation were exacerbated by the stress, phenolic reduction could be explained by their role as antioxidants (Shao et al., 2008; Matkowski, 2008); and c) sampling of red fruit during experiment two and four took place too long after the imposition of the stress and therefore there was a period while plant phenolics were recovering to control levels. A detailed time-course of phenolic concentration during the re-hydration phase while recording environmental factors would help to understand the reasons for this.

4.3 Relation between total phenolic concentration and ethylene production rate

The literature gives examples where phenolic contents vary with different ethylene production rates in strawberry fruit (Jiang *et al.*, 2001; Jiang & Joyce, 2003). There is some evidence of this relationship at a molecular level too based on plants whose ethylene biosynthesis or perception has been silenced (Merchante *et al.*, 2013, Sun *et al.*, 2013). Cross-correlation plots on the data collected during the course of the 2014

experiments showed a positive but non-significant correlation at high negative *Lags*, suggesting that increases in fruit ethylene production rate were followed by an concentration of total phenolics in fruit.

Regression analysis did not show that ethylene production rate explained the variation on total phenolics during the experiments, but it was not far from significance ($p=0.064$). Alongside other variables including radiation, pot-and-plant weight, the experiment and the phase of the treatment, the model explained nearly 50% of the variability in fruit total phenolic concentration ($R^2=0.48$) which was a higher prediction power than when ethylene was excluded in the model ($R^2=0.27$). Therefore, this results suggested that although weak, there may be a relation between ethylene production rate and total phenolic biosynthesis. Whether this is only a temporal correlation or there is a cause-effect relation is something that requires more testing.

4.4 Total, free ellagic acid and organic acid concentration in fruit of strawberries subjected to SWD

Green strawberry fruit have a higher concentration of the health-related compounds total ellagic acid or free ellagic acid than red fruit (Hannum, 2004; Atkinson et al., 2005; Vatterm & Shetty, 2005; Giampieri et al., 2012). Total ellagic acid contents in green achenes ranged from 1,370 to 20,730 $\mu\text{g g}^{-1}$ DW and in the receptacle from 1,320 to 8,430 $\mu\text{g g}^{-1}$ DW (Maas et al., 1991). Concentrations during experiment five ranged from 553 to 2,901 $\mu\text{g g}^{-1}$ DW for whole fruit, which are at the lower end of the ranges reported. Reported free ellagic acid contents for whole green fruit ranged from 347 to 1,042 $\mu\text{g g}^{-1}$ DW (Olsson et al., 2004) and from 54 to 220 $\mu\text{g g}^{-1}$ DW (Kosar et al., 2004), ranges that are remarkably different, possibly because of the extraction and quantification methods used: Olsson et al. performed a triple extraction whereas Kosar et al. (2004) used a single extraction. Concentrations during experiment five were more similar to those reported by Kosar et al. (2004), ranging from 23 to 288 $\mu\text{g g}^{-1}$ DW; here a single extraction was also performed. Regarding the potential effect of water deficit

on free ellagic acid, Dodds et al. (2007) reported increases of up to 270% free ellagic acid in red fruit of plants subjected to Partial Rootzone Drying and supplied with 60% of their evaporative demands (PRD60). SWD had no significant effect on free ellagic acid concentration in green fruit but it had an effect on the total ellagic acid content. Although overall the SWD treatment increased total ellagic acid accumulation, there were no significant differences in either total or free ellagic acid during experiment five at any sampling time. Nevertheless total phenolic concentration was significantly increased on the second day of wilting. The Folin-Ciocalteu reagent, used for total phenolics determination, is believed to be specific for phenolics, but some other compounds do react with it, for instance ascorbic acid (Ainsworth & Gillespie, 2007; Everette et al., 2010). Ascorbic acid concentration during experiment five, however, showed a significant decrease at midday of the first day of wilting and a significant increase at midday of the second day of wilting that did not relate to changes in total phenolic accumulation. The first significant increase in total phenolics was detected when total and free ellagic acids increased simultaneously. That total and free ellagic acid did not show a significant change whereas total phenolic was significantly increased was perhaps due to a) the fact that the HPLC method used to detect these individual compounds was based on its chromatographic properties rather than the antioxidant capacity of the Folin-Ciocalteu reagent; b) the presence of other interfering agents than ascorbic acid, or d) the different extraction methods used for the ellagic acid and the total phenolics assays. Ellagic acid should have been extracted using the same solvent used for the total phenolic determination in order to obtain better justification for this discrepancy.

According to Kafkas et al. (2007), concentrations of citric, malic and ascorbic acids for WW green strawberry fruit ranged from 6.73 to 17.26, 2.55 to 5.29 and 0.01 to 0.60 g kg⁻¹ FW, respectively. Experiment five data showed ranges for the same organic acids of 27.33 to 88.89, 4.65 to 25.2 and 1.32 to 4.8 mg g⁻¹ DW, respectively. Differences may have arisen from the use of different extraction methods, experimental conditions

or due to the fact that different strawberry varieties were studied. There was no clear effect of water deficit on organic acid concentration in red fruit. Two reports agree that plants subjected to 50 ml irrigation volume per day accumulate significantly less ascorbate, citrate and malate than plants under 200 ml irrigation (Terry et al., 2007; Bordonaba & Terry, 2010). On the other hand, a 60% PDR treatment was reported to increase the concentration of ascorbic acid in strawberry fruit by 55% (Dodds et al., 2007). No increases in ascorbic acid were detected during experiment five; the transient decrease observed at midday of the second day of wilting may have been due to a) the role of ascorbic acid as cofactor for ACC oxidase (Van de Poel & Van Der Straeten, 2014), or perhaps to b) its role as antioxidant (Valpuesta & Botella, 2004). Water deficit did not induce significant changes in citric acid concentration but concentrations of malic acid were, in general, lowered in SWD-treated plants and particularly from midday on the second day of wilting onwards. Malic acid is less energetically effective to recycle CO₂ molecules for photosynthesis under drought conditions than citric acid. Franco et al. (1992) explained that under these conditions, malic acid would deplete whereas citric acid concentration would increase. However, it is likely that the SWD treatment was contributing to the increased production of ROS for which organic acids could be scavengers because of their role as antioxidants (Shao et al., 2008; Matkowski, 2008). Although the plant may have been producing these compounds to counteract ROS induced by the effects of SWD on the photosystem II efficiency, its capacity to do so may have been limited by the extent and intensity of the stress (Shao et al., 2008).

4.5 Relationship between the performance of the photosystem II and fruit quality

Finally, on the hypothesis that Yield (II) could be used to inform fruit sampling time, it has been shown that Yield (II) measurements on leaves could be a good indicator of when to expect an increased ethylene production in the fruit. However, regression

analyses showed that variability in total phenolics was not explained by Yield (II) measurements, accounting for neither whole set of data nor just data during wilting phase. Three things should be noted regarding this finding: a) total phenolics responded to light intensity and Yield (II) did so too, b) the R^2 for both models is under 45% so caution should apply when conclusions are based on these models and c) Atkinson et al., (2006) noted that whereas 'Elsanta' plants did not accumulate more ellagic acid under conditions where photosynthetically active radiation was reflected, 'Flamenco' did, so this finding may have been different if other cultivars were used.

5 Conclusions

5.1 Fruit ripening stage, sampling time and ethylene sampling method

Low Yield (II) values were related to high ethylene production by regression analysis. This information, alongside the results from the previous chapter, suggests that Yield (II) could be used to determine when ethylene responses in the fruit are more likely to occur. The highest ethylene production rate was measured in large green fruit and the difference between SWD and WW control plants was maximal at this fruit stage as well. Therefore, the optimum time to sample fruit was at large green fruit stage at the midday of the second day of wilting; defining midday as the brightest part of the day, and large green stage as the stage seven days before fruit turns colour.

5.2 Cross-correlation between ethylene production and phenolic concentration

Ethylene production rate clearly increased in fruit of stressed plants on the second day of wilting, but an associated increase in total phenolics was not always detected. It has been suggested that the SWD may produce a too severe stress. Yield (II) during the wilting phase was the lowest during the SWD treatment indicating that the photosystems may have been compromised by excessive ROS production (Nishiyama & Murata, 2014). The phenolics reduction sometimes observed could be explained by their role as scavengers of an exacerbated production of ROS by the severity of the stress (Shao et al., 2008; Matkowski, 2008).

Cross-correlation analyses revealed concentration of phenolics in the strawberry fruit at the time of an increased ethylene production rate or up to *Lag* minus three (in experiment 5). In addition, regression analyses pointed to an effect of light on both total phenolic and ethylene production, especially during the wilting phase. The effect of light on phenolic concentration, particularly ellagic acid, has been reported before (Atkinson et al., 2006), and ROS may be key signalling molecules for phenolic biosynthesis under stress (Jacobo-Velázquez et al., 2011). The effect of light on ethylene production rate increase, in turn, could be due to the fact that expression of

genes encoding for ACC synthase is mainly upregulated by light (Munné-Bosch et al., 2002) or the role of ROS as signal for ethylene metabolism (Wang et al., 2002; Shao et al., 2008; Mittler et al., 2011). During wilting phase the light effect on the physiology of the plant was more evident.

Chapter 4. Pharmacological and genetic evidence of ethylene mediated phenolic accumulation during SWD

1 Introduction

Given & Venis (1988) concluded that ethylene did not play an essential role in the ripening of the strawberry fruit. Silencing of genes related to ethylene biosynthesis and signalling (Sun et al., 2013) or partial silencing of ethylene perception (Merchante et al., 2013) resulted into inhibited or delayed fruit ripening. This chapter provides pharmacological and genetic evidence for ethylene mediation of phenolic accumulation in fruit and leaves of strawberry plants subjected to severe water deficit (SWD).

There are two main strategies that can be used to prove the involvement of a hormone in a metabolic event: a pharmacological approach (Weyers & Paterson, 2001) and the use of transgenic plants (Merchante et al., 2013). The first approach comprises testing for correlation between the hormone and the changes it potentially triggers, deletion and re-instatement of the hormone and specificity. This strategy is based on Jackson's postulates (Weyers & Paterson, 2001). The chemicals will be applied to SWD plants to try to ensure that, as far as possible when using non-specific inhibitors, other processes affected by SWD that may interact with the ethylene/phenolic response are still triggered. The second approach involves using transformed plants with down-regulated key genes for ethylene biosynthesis, metabolism or perception. Merchante et al. (2013) produced the *etr1-1* plants used for experiments described in this chapter. These plants have a limited sensitivity to ethylene.

The temporal correlation between the ethylene signal and its potential effect has been tested in previous chapters: an increased ethylene production rate has been identified at midday of the second day of wilting both in fruit and leaves of strawberry. In addition, phenolics accumulated in fruit at the same time as the peak in ethylene production, or not long after. Results of the autocorrelation plot and the regression analysis confirmed that ethylene production rate increased prior to total phenolic accumulation. Ellagic

acid and ellagitannins were identified as the most important phenolics in the strawberry fruit in terms of concentration.

ACC synthase (1-aminocyclopropane-1-carboxylic acid synthase) and ACC oxidase are the main enzymes involved in ethylene biosynthesis (Yang & Hoffman, 1984). Inhibition of their activity can be achieved pharmacologically by using aminoethoxyvinylglycine (AVG) a competitive inhibitor of ACC synthase activity (Basiouny, 1988; Given & Venis, 1988) or aminoisobutyric acid (Aib) a competitive inhibitor of ACC oxidase activity (Satoh & Esashi, 1980). If phenolic accumulation in fruit of plants subjected to water stress is an ethylene-mediated response, the effects of this inhibition should result in a reduced concentration of phenolics with respect to the control.

Once the signal had been inhibited, ethylene production could be re-instated with ethephon (Liu et al., 2008), ethylene (Riov & Yang, 1982b) or perhaps ACC (Basiouny, 1988; Perkins-Veazie et al., 1995; Perkins-Veazie et al., 1996), assuming that ACC oxidase activity was not limiting. If ethylene is indeed the causal signal, then its re-instatement should increase phenolic concentration to SWD levels.

However, increased phenolic accumulation in fruit from water stressed plants might be mediated by other hormones. Although abscisic acid (ABA) has different roles to ethylene, it is a well-known stress signal in drought response (Slovik et al., 1995; Wilkinson & Davies, 1997; Bahrin et al., 2002; Dodd et al., 2008b; Dodd et al., 2008a), and it plays an important role in the regulation of strawberry fruit ripening. It has been reported, for example, that inhibiting strawberry ABA receptors prevents fruit ripening (Chai et al., 2011; Jia et al., 2011). In this research reported in this chapter, a specific ethylene-mediated response was hypothesized so ABA application should not trigger phenolic accumulation.

Pharmacological studies have proved very useful in physiological research; however, changes provoked by application of chemicals can produce pH and redox potentials shifts (Gehring et al., 1990) and consequently lead to unpredictable metabolic

reactions (Chance, 1952; Alberty & Bloomfield, 1963; Pahlic, 1993; Xu, 1997). In order to minimize this effect, the concentrations of chemicals used in these experiments should be as low as possible. An alternative approach is to use genetically modified plants designed to study the role of the particular chemical of interest (Weyers & Paterson, 2001). Merchante et al. (2013) developed a group of transgenic plants (*etr1-1*) with a reduced sensitivity to ethylene, in which they measured relative expression of genes related to strawberry ripening. In the same year, Sun et al. (2013) published a report of their work on silencing genes related to ethylene signalling and biosynthesis in strawberries observing inhibition of ripening. These genetically modified plants would be an ideal tool to use in the investigation of the ethylene-mediated accumulation of phenolics in fruit of strawberry plants exposed to SWD. Prof. Victoriano Valpuesta from the University of Málaga provided the *etr1-1* plants on which the SWD was applied and in which the expression of genes related to ethylene and phenolic biosynthesis was measured by RT-qPCR.

Objectives covered in this chapter were:

- To test the effect of the inhibition, re-instatement or replacement of the ethylene signal during SWD on phenolic accumulation
- To obtain genetic evidence for the involvement of ethylene in the accumulation of phenolics during SWD in transformed plants with lower sensitivity to ethylene

2 Materials and Methods

2.1 Experiments included in this chapter

Results of experiment four have been included in chapters 2 (leaf responses) and 3 (fruit responses of air control). In this chapter, results of the application of ethylene and 1-MCP on ethylene are included and discussed (experiment four). With the exception of **Section 4-2.2.1** in this chapter, materials and methods for this experiment have been already described in previous chapters. The rest of the materials of methods in this chapter describe the experiments carried out in 2015: seven, eight, nine and ten. Experiments seven (inhibitors) and eight (ACC and ABA) were focussed on application of chemicals. Experiment nine studied the effect of the SWD on the leaves of *etr1-1* plants whereas experiment ten looked at the effect of the treatment on the fruit of three commercial cultivars. This experiment was carried out in order to obtain information of the expression levels of genes related to ethylene and phenolic biosynthesis in fruit.

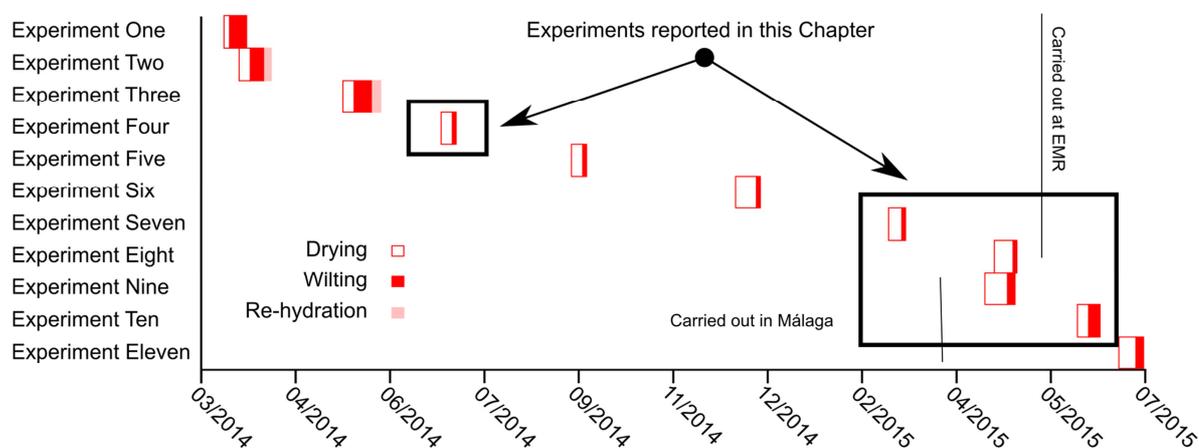


Figure 0.1. Sequence of drying, wilting and re-hydration periods for every experiment over the course of this research: experiments included in this chapter are within boxes. Experiment four, seven and eight were run in the Grodome facility, chamber five at East Malling (Kent) in 2014 and 2015. Experiments nine and ten were run in a contained facility for transgenic material at the IFAPA centre in Churriana in 2015.

2.2 Experiment four

2.2.1 Ethylene and 1-MCP treatment

Plants were placed in 360 l polypropylene air-tight containers and exposed to either 0.05, 0.5 or 5 ppm of ethylene, or 1 ppm of 1-methylcyclopropene (1-MCP) (SmartFresh™, AgroFresh Hayward, USA). These concentrations were lowest effective for strawberries found in the current literature (Jiang et al., 2001; Bower et al., 2003) and the known fruit ethylene production from previous experiments (see **Sections 3-3.1** and **3-3.2**). Fans were used to circulate and homogenize the internal atmosphere of the container. Treatments were carried out from 17:00 h on the first day of wilting till 08:00 h of the morning of the second day of wilting.

2.2.2 Leaf sampling

Three young leaves with leaflets still closed of each six SWD plants and six WW plants were excised at midday of the second day of wilting and immediately introduced, along with 3 mm diameter wax beads in order to reduce the head space, in to a 25 ml glass vial which was immediately sealed with a cap containing a septum insert. Ethylene production rate and ellagic acid concentration analyses were performed using the same protocol as described for fruit (see **Sections 3-2.3.3** and **3-2.3.5**).

2.3 Experiments seven and eight

Experiments seven and eight were run at East Malling Research. Unless otherwise stated, plant materials, controlled environment conditions, measurement of environmental factors, leaf responses and chemical analyses were the same as those described in Chapters 2 and 3.

2.3.1 Fruit sampling

Yield (II) measurements were taken around midday of the second day of wilting; when SWD plants had a significantly lower Yield (II) compared to WW controls. Based on these data, three fruits at large green stage were taken from each plant. Fruit were placed in a 50 ml volumetric flask in order to quantify ethylene production rate.

Immediately after determination fruit was immersed in liquid nitrogen and stored for further chemical analyses.

2.3.2 Management of the drying and wilting phases of SWD plants

Prior to the start of substrate drying (day 0), plants were irrigated to pot capacity each day, and then irrigation was withheld for 10 days until wilting was first observed. In order to synchronize drying, larger plants that dried the substrate more rapidly were topped up to the weight of smaller, more slowly drying plants. Plants were kept on the verge of wilting by re-hydrating plants to PVMC/PW when signs of wilting were first detected.

2.3.3 Experimental design

The design for experiments seven and eight included four blocks and 12 and 10 treatments, respectively. Each treatment was applied to 12 plants.

Decision on the concentrations for the aminoethoxyvinylglycine (AVG) treatments were based on Basiouny (1988) and Given & Venis (1988) who reported the use of 1 mM AVG. The range of concentrations chosen aimed to identify the lowest AVG concentration able to reduce SWD ethylene production rate to WW levels. A 5 mM concentration was also used in case a higher concentration was needed.

Since no reports could be found for aminoisobutyric acid (Aib) use on strawberry at the time of this experimental design a wider range of concentrations was chosen for it.

1-aminocyclopropane-1-carboxylic acid (ACC) were chosen on the basis of the expected volume of the fruit at the time of the injection and the concentration of ACC in green strawberry fruit reported by Perkins-Veazie et al. (1995). A five times lower and a five times higher concentration completed the range of concentrations of AVG tested. Finally, abscisic acid (ABA) concentrations were chosen according to the concentration in green fruit reported by Symons et al. (2012) and the volume of the green fruit at the

time of the injection. A five times higher and a five times lower concentration completed the range used (**Table 0.1**).

Table 0.1. Treatments applied in experiments seven and eight

Experiment seven	Experiment eight
WW	WW
SWD	SWD
SWD + H ₂ O	SWD + H ₂ O
SWD + H ₂ O + 40 µM AVG	SWD + H ₂ O + 1 mM AVG
SWD + H ₂ O + 200 µM AVG	SWD + H ₂ O + 1 mM AVG + 200 µM ACC
SWD + H ₂ O + 1 mM AVG	SWD + H ₂ O + 1 mM AVG + 1 mM ACC
SWD + H ₂ O + 5 mM AVG	SWD + H ₂ O + 1 mM AVG + 5 mM ACC
SWD + H ₂ O + 80 µM Aib	SWD + H ₂ O + 1 mM AVG + 1 mM ABA
SWD + H ₂ O + 400 µM Aib	SWD + H ₂ O + 1 mM AVG + 5 mM ABA
SWD + H ₂ O + 2 mM Aib	SWD + H ₂ O + 1 mM AVG + 25 mM ABA
SWD + H ₂ O + 10 mM Aib	
SWD + H ₂ O + 50 mM Aib	

AVG (aminoethoxyvinylglycine), Aib (aminoisobutyric acid), ACC (1-aminocyclopropane-1-carboxylic acid) and ABA (abscisic acid)

2.3.4 Application of chemical treatments to the fruit

Three large green fruit from each experimental unit were selected to be injected and sprayed with a solution of the chemical. Twenty microliters of those solutions were injected with a 100 µl syringe (Hamilton Co., USA) at a 50 mm depth, aiming for the fruit core by passing through the calyx and parallel to the peduncle. In addition, 1 ml of the solution was evenly sprayed on to the fruits. Chemicals were applied to fruit at 17:00 h on the first day of wilting.

2.3.5 Leaf responses: non-destructive/survey measurements

Leaf responses were measured at the time of fruit sampling. Yield (II) measurements were taken around midday of the second day of wilting in order to estimate when fruit ethylene production was likely to be at its highest (see **Section 4-2.3.1**), and at the time of fruit sampling.

2.3.6 Chemical analyses

Ethylene production rate, ascorbic acid, ellagic acid and total ellagic acid were determined in fruit from experiments seven and eight.

2.3.7 Statistical analysis

Fisher's least significant difference test was carried out after ANOVA. Results from any pair of treatments were statistically different if their difference was higher than LSD. Statistical analyses were performed using R version 3.1.0 (R Foundation for Statistical Computing, Austria).

2.4 Experiments nine and ten

Experiments nine and ten were run in the Instituto de investigación y formación agraria y pesquera (IFAPA) centre in Churriana, Málaga (Spain). Chemical analyses for experiment nine were carried out at Málaga University whereas for experiment ten they were carried out at the IFAPA.

2.4.1 Plant material

Strawberry plants (*Fragaria x ananassa* Duch. cv 'Chandler') used in experiment nine were developed by Prof Victoriano Valpuesta's team at Málaga University and comprised eight non-transformed 'Chandler' controls and four of each of the *etr1-1* lines 10 and 12 (lowest sensitivity to ethylene of all *etr1-1* lines) transformed by Merchante et al. (2013). Plants were propagated vegetatively.

The success of the transformation was evaluated by phenotyping the different transformed lines (Merchante et al., 2013). Those lines with lower expression of the *ert1* gene were used in this work. In order to check whether the transformation persisted in the generation of the lines under study, the expression of the *ert1* gene was re-evaluated.

The plant materials used in experiment ten were supplied by Dr Elsa Martínez Ferry and consisted of six plants of each of the cultivars 'Liberty', 'Sahara' and 'Safari'. Plants were propagated by stolon in 2014 and planted in the autumn.

2.4.2 Experimental design

Experiment nine comprised 16 plants, eight were 'Chandler' non-transformed, and four of each transformed line. Four 'Chandler' were under WW conditions whereas the remaining 12 plants were subjected to SWD. Experimental plants were randomized and guard plants were placed along each side of the experiment.

Experiment ten comprised 18 plants, three cultivars 'Liberty', 'Sahara' and 'Safari', six plants of each. Each cultivar had half of the plants under SWD and the rest were kept as WW controls.

2.4.3 Husbandry

Because of the size of the plants, in experiment nine they were kept in 1 l pots whereas plants in experiment ten they were planted in 6 l pots. In both cases compost was a mixture of organic compost and sand (10:1 [v/v]). Continuous monitoring of plant health was carried out throughout plant development. Fertilizer used was 15-10-15 2MgO plus calcium nitrate. The pH and EC of the irrigation water were uncontrolled. Integrated pest and disease management was followed in both experiments. Experiment nine was run in a greenhouse facility dedicated to work with transgenic material in the IFAPA centre in Churriana, Málaga (Spain). Experiment ten was conducted in a contained facility dedicated to transgenic material.

2.4.4 Irrigation treatment and sampling

Prior the start of drying (day 0), plants were irrigated to pot capacity, then irrigation was withheld for 10 days until wilting was first observed. In order to synchronize drying, larger plants were irrigated with small volumes of water to the weight of smaller, more slowly drying plants as described earlier.

In experiment nine, at midday on the second day of wilting, the youngest leaf of each experimental plant was sampled and immediately immersed in liquid nitrogen for further ethylene and phenolic analyses.

In experiment ten, at midday on the second day of wilting, three fruits at large green stage were picked from each experimental plant and immediately immersed in liquid nitrogen for further analyses.

2.4.5 Environmental records

A Data Hog 2 logger (Sky Instruments Ltd, UK) was placed among the experimental plants in each of the experiments to monitor ambient temperature, relative humidity and light intensity throughout each experiment.

2.4.6 Physiological responses to drying of substrate

Pots were weighed to monitor the rate of substrate drying every day at midday. In addition, PVMC substrate moisture and pore EC measurements were made every day at midday with a HH2 and WET probe (Delta-T Devices Ltd, UK) through two sampling ports made in opposite sides of the pot. Plant responses were monitored every two days from day 0. Net photosynthetic rate, stomatal conductance and drying were obtained by leaf gas exchange (LI-COR Inc., USA) and the performance of the photosystem II was assessed using chlorophyll fluorescence (Heinz Walz GmbH., Germany). Each of these measurements was repeated at the time of harvest.

2.4.7 Molecular analysis of the relative expression genes related to ethylene and phenolic biosynthesis (RT-qPCR)

2.4.7.1 *RNA extraction*

The protocol used for RNA extraction was adapted from Gambino et al., (2008). Approximately 100 mg of frozen tissue from each sample were lysed (Tissuelyser, QIAGEN B.V., Netherlands) and weighed in a 2 ml tube. Then, 900 µl of the extraction buffer (2% CTAB, 2.5% PVP-40, 2.0 M NaCl , 100 mM Tris-HCl pH 8.0, 25 mM EDTA

pH8.0, 2% β -mercaptoethanol) at 65 °C were pipetted, vortexed and mixed rapidly to avoid the sample thawing and RNA degradation. First extraction occurred after 10 min incubation at 65 °C (Water bath; Memmert GmbH, Germany), when an equal volume of chloroform isoamyl alcohol (24:1 v/v) was added followed by vortexing and centrifugation at 14,000 g for 10min at 4 °C (Micro 22R; Andreas Hettich GmbH, Germany). The supernatant was recovered in a new 2 ml tube and extracted a second time. The supernatant of this second extraction was transferred to a new 2 ml tube and LiCl added to a final concentration of 3 M and vortexed. The solution was left to precipitate on ice for 30 min and subsequently centrifuged at 14,000 g for 20 min. All liquid was removed, and the resulting pellet dissolved in 500 μ l of SSTE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.5% SDS, 1 M NaCl). The final extraction was done with 500 μ l of chloroform isoamyl alcohol (24:1 v/v) vortexed and centrifuged as in each of the extractions. The supernatant was transferred to a 1.5 ml tube and 0.7 volume of cold isopropanol added and immediately vortexed and centrifuged at 14,000 g for 15 min at 4 °C. All supernatant was removed and the remaining pellet washed with 75% ethanol, vortexed, briefly centrifuged and left to dry. The pellet was then dissolved in 30 μ l of deionised water. RNA concentration was checked using a spectrophotometer (Thermo Fisher Scientific Inc., USA. Software: ND-100 3.1.0) and quality was assessed by running a solution of 0.7 μ l sample and 9.3 μ l of deionised water in a gel (1% agarose, 1% *tert*-butyl alcohol, Red Safe™).

2.4.7.2 RNA purification

Turbo™ DNase (Thermo Fisher Scientific Inc., USA) was used to degrade DNA present in the samples according to the instructions detailed by the manufacturer with some modifications: a volume corresponding to 8 μ g of RNA was pipetted into a 1.5 ml tube along with 6 μ l of the DNase:buffer mix (1:5 v/v). Deionised water was added to make 50 μ l final volume. The solution was incubated for 30 min at 37 °C (TS-100 Thermoshaker, Boeckel GmbH, Germany). After incubation, volume was increased to

100 μl with water, and an equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1 v/v) was added. The mixture was thoroughly vortexed and centrifuged for 5 min at 14,000 g and 4 °C. The supernatant was transferred to a new 1.5 ml tube along with an equal volume of chloroform followed by vortexing and centrifugation as described above. The supernatant was transferred to a 1.5 ml tube to which 1/10 volume of 3 M NaOAc pH 5 and 2.5 volumes of 100% ethanol were added. The mixture was left to precipitate on ice for 30 min, then centrifuged for 30 min at 14,000 g and 4 °C. The supernatant was removed and the pellet washed with 70% ethanol, vortexed and centrifuged for 5 min, at 14,000 g and 4 °C. After being left to dry, the pellet was dissolved in 15 μl of deionised water. RNA concentration was quantified using spectrophotometry. Five μl of each sample was diluted to 100 ng μl^{-1} and the quality of the latter checked on a gel. Finally, PCR with primers for the actin gene was carried out to check that DNA was fully degraded using an Ambition[®] RNA Kit (Thermo Fisher Scientific Inc., USA). Each well contained 14.86 μl of water, 5 μl of buffer, 2 μl of 25 mM MgCl, 0.5 μl of 10 mM nucleotides, 0.5 μl of each of the primers (10 μM), 0.64 μl of 1:40 TAQ (enzyme) and 1 μl of sample. The incubation ran as follows (Thermocycler PTC-200; Mic Group Inc., USA): 5 min at 95 °C and then 33 cycles of 30 sec at 95 °C, 25 sec at 60 °C, 1 min at 72 °C, followed by 5 min at 72 °C, and then held at 12 °C. The resulting DNA amplification, if any, was checked on 1.5% agarose gel (no bands should be visible since all DNA should have been degraded). There were wells for positive, negative controls and 1 with 5 μl molecular weight marker.

2.4.7.3 *cDNA synthesis (Retrotranscription)*

Retrotranscription was carried out with iScript[™] cDNA Synthesis Kit (Bio-Rad Laboratories Inc., USA): the mix was formed of the corresponding volume for 1 μg of RNA, 4 μl of the kit mix, 1 μl of transcriptase (enzyme) and water to make a 20 μl final volume. The incubation ran (Thermocycler MJ Mini[™], Bio-Rad Laboratories Inc., USA) for 5 min at 25 °C, 30 min at 42 °C, 5 min at 85 °C and held at 4 °C indefinitely, if

desired. Finally, PCR with primers for the actin gene was carried out in order to check cDNA quality. The mix included 13.86 μ l of water, 5 μ l of buffer, 2 μ l of 25 mM MgCl, 0.5 μ l of 10 mM nucleotides, 0.5 μ l forward and reverse primers for the of actin, GAPDH genes (10 μ M), 0.64 μ l of 1:40 TAQ (enzyme) and 2 μ l of cDNA. The incubation ran as follows: 5 min at 95 °C and then 33 cycles of 30 sec at 95 °C, 25 sec at 60 °C, 1 min at 72 °C, followed by 5 min at 72 °C and then held at 12 °C. The resulting cDNA quality was checked on 1.5% agarose gel. There were wells for positive, negative controls and two with 5 μ l of molecular weight marker.

2.4.7.4 *Quantitative PCR*

For the quantification of the expression of the genes of interest SSoFast™ EvaGreen™ Supermix (Bio-Rad Laboratories Inc., USA) was used: each well had 10 μ l of EvaGreen, 1 μ l of each 10 μ M forward and reverse primers of interest, 1 μ l of sample and water to make a final volume of 20 μ l. Quantification was done in a real-time PCR detection system (MyIQ™. Bio-Rad Laboratories Inc., USA). Threshold value was set at 50 Ct. Relative expression was calculated according to The Livak method (Livak et al., 2003).

2.4.8 Statistical analyses

For experiment nine data Fisher's least significant difference test was carried out after ANOVA. Results from any pair of treatments were statistically different if their difference was higher than the LSD. For experiment ten, a two-way analysis of variance with effects of irrigation and cultivar was performed on the data. Statistical analyses were done with R version 3.1.0 (R Foundation for Statistical Computing, Austria).

3 Results

3.1 Effect of the inhibition of the ethylene signal on fruit responses

3.1.1 Effect of Aib and AVG on ethylene production rate

In experiment seven, fruits responded to the severe water deficit (SWD) treatment (4.51 ± 0.15 ; $LSD=0.49$), as expected, by increasing ethylene production rate ($\ln[nl\ kg^{-1}\ h^{-1}+1]$) with respect to well-watered fruit (WW) (3.61 ± 0.34) (**Figure 0.2**). Only one out of the nine treatments tested, injection and spray of 1 mM aminoethoxyvinylglycine (AVG) (4.39 ± 0.22) effectively reduced the production of ethylene; in contrast, 400 μ M aminoisobutyric acid (Aib) (5.48 ± 0.17) significantly enhanced ethylene production rate when compared to values from plants in which only water was injected (4.80 ± 0.18).

A clear dose response to AVG or Aib was not observed in these experiments. However, the inhibition may have worked better at higher concentration of AVG or Aib whereas at lower concentrations, ethylene production rates were higher than Control but not significantly.

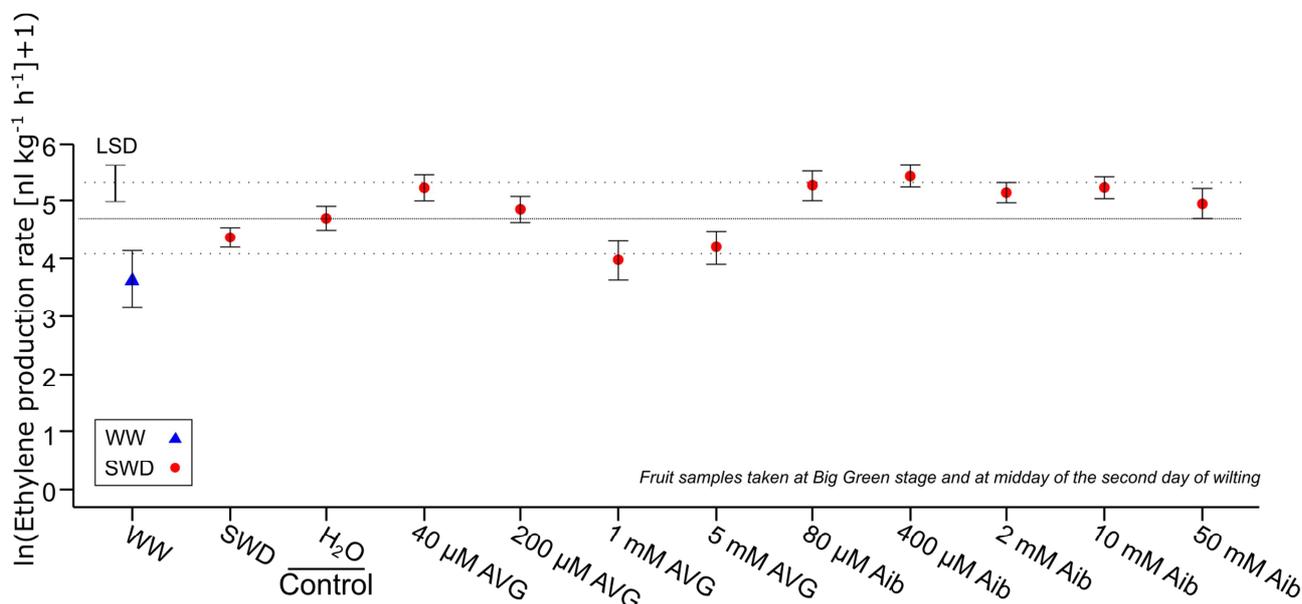


Figure 0.2. Effect of the inhibition of ACC synthase activity by aminoethoxyvinylglycine (AVG) or ACC oxidase activity by aminoisobutyric acid (Aib) on ethylene production rate ($nl\ kg^{-1}\ h^{-1}$) from wilting plants. Each point corresponds to mean value \pm standard error ($n=12$). Inhibitors were injected at 17:00 h of the first day of wilting and ethylene production measured at 13:00 h of the second day of wilting. Fisher's least significant difference test was carried out after ANOVA [$F(11,127)=4.047$, $p<.001$]. Any pair of treatments were statistically different if their difference was higher than LSD. Treatments having statistical effect fell beyond Control mean \pm LSD (represented by dotted lines). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.1.2 Effect of Aib and AVG on ascorbic, free and total ellagic acid concentration

Analysis of variance did not reveal any effect of the treatments (see x axis factors) on the ascorbic acid concentration (mg g^{-1} DW) in the fruit ($F[11,131]=1.761$, $p=0.067$) in experiment seven (**Figure 0.3**). Nevertheless, ascorbic acid concentration was negatively affected in SWD-treated fruit with respect to fruit from WW plants, which was expected from previous work (4.67 ± 0.25 vs 5.64 ± 0.38). Further exploration of the data showed that this effect was significant ($t[18.98]=-2.10$, $p=0.049$). No other treatment effects were detected.

The potential relationship between ethylene production rate and ascorbic acid concentration was explored using regression analysis. The model included the effect of leaf responses, treatments with inhibitors, of this experiment and peat volumetric moisture content (PVMC). Firstly, optimised fit of the model was very low ($R^2=0.20$) and the p -value for the estimate of ascorbic acid was not significant ($p=0.064$). In summary, there was no evidence of a relationship between changes in ascorbic acid concentration and ethylene production rate.

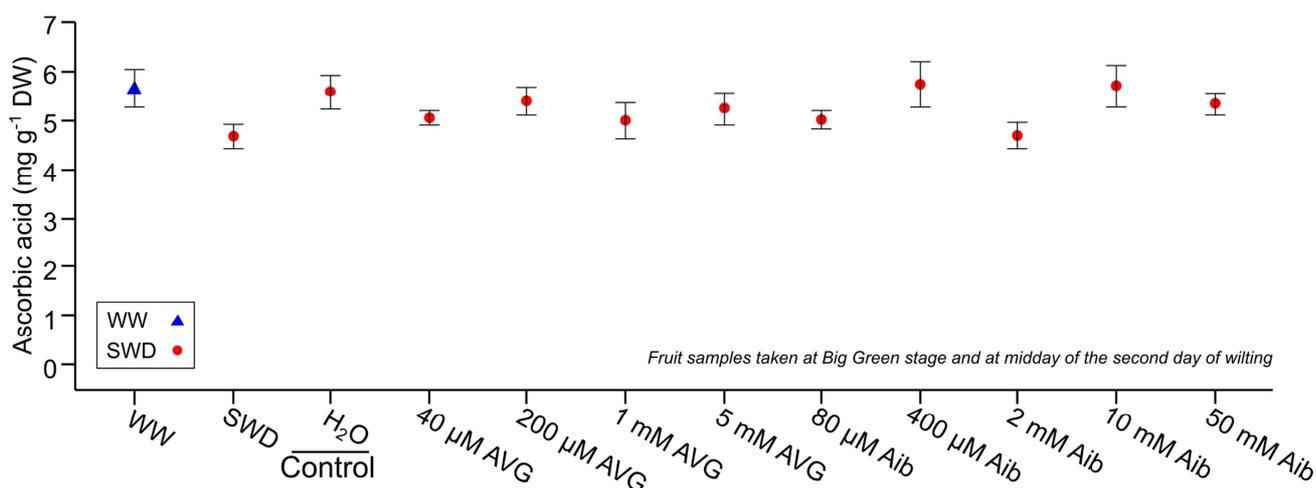


Figure 0.3. Effect of inhibitors of ACC synthase activity aminoethoxyvinylglycine (AVG) or ACC oxidase activity aminoisobutyric acid (Aib) on ascorbic acid concentration (mg g^{-1} DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n=12$). Inhibitors were injected at 17:00 h of the first day of wilting and samples for analysis taken at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(11,131)=1.761$, $p=0.067$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

Analysis of variance with effect of the treatments (see x axis factors) on free ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) (**Figure 0.4**) did not show differences between treatments ($F[11,130]=1.49$, $p=0.142$). Further exploration with t-tests revealed that free ellagic acid concentration was significantly increased by the SWD treatment with respect to WW (103.27 ± 10.10 vs 72.10 ± 6.24 ; $t[18.33]=2.62$, $p=0.017$).

Regression analysis was used to explore the relationship between ethylene production rate and free ellagic acid concentration. The model included the effect of leaf responses, treatments of this experiment and PVMC. The fit of the model was only $R^2=0.20$, and the p -value for the estimate of free ellagic acid was not significant ($p=0.37$). However, the free ellagic acid concentration in SWD treated fruit is significantly lower than in fruit in which ethylene production was inhibited. In all, although the t test seemed to indicate otherwise, the regression analysis suggested that there was no evidence from the data for a relationship between ellagic acid and ethylene production rate.

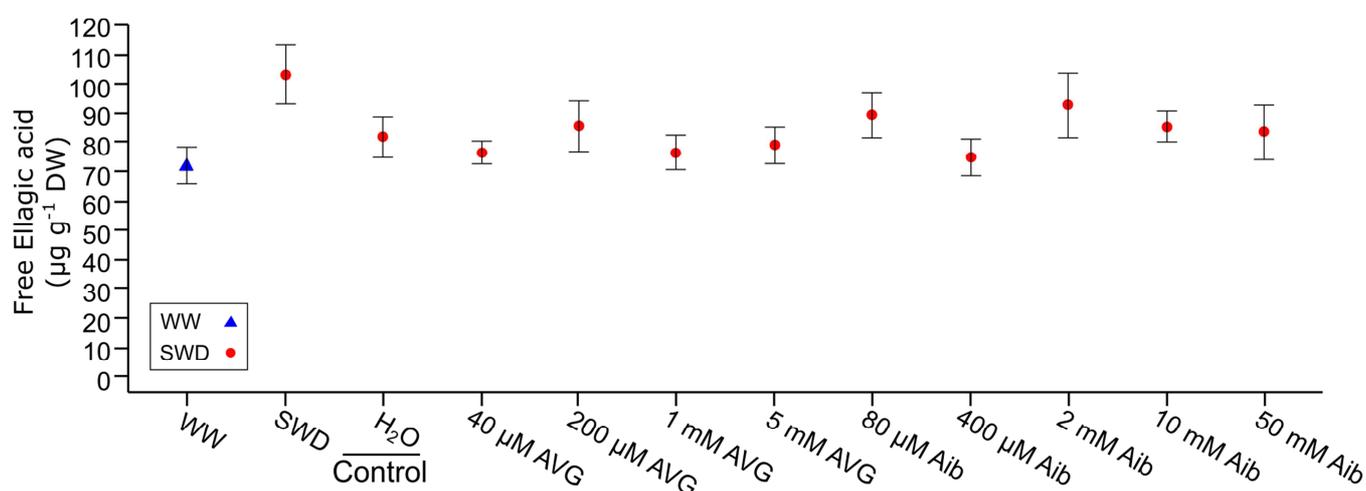


Figure 0.4. Effect of inhibitors of ACC synthase activity by aminoethoxyvinylglycine (AVG) or ACC oxidase activity by aminoisobutyric acid (Aib) on free ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n=12$). Inhibitors were injected at 16:00 h of the first day of wilting and samples for analysis taken at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(11,130)=1.49$, $p=.142$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

Analysis of variance did not reveal an effect of the treatments (see x axis factors) on the total ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) in the fruit ($F[11,127]=0.897$, $p=0.54$) in experiment seven (**Figure 0.5**). Further analyses with t tests did not show differences between any of the treatments and the Control. However, fruit from SWD-treated plants accumulated higher ellagic and ellagitannins in comparison to those from WW plants (885.90 ± 38.99 vs 778.05 ± 68.39).

The relationship between the ethylene production rate and total ellagic acid concentration was explored with regression analyses. The model included the effect of leaf responses, treatments of this experiment and PVMC. The fit of the model was only $R^2=0.27$, however the p -value for the estimate of total ellagic acid was highly significant ($p<0.01$). Therefore, there could be a relationship between total ellagic acid and ethylene production rate.

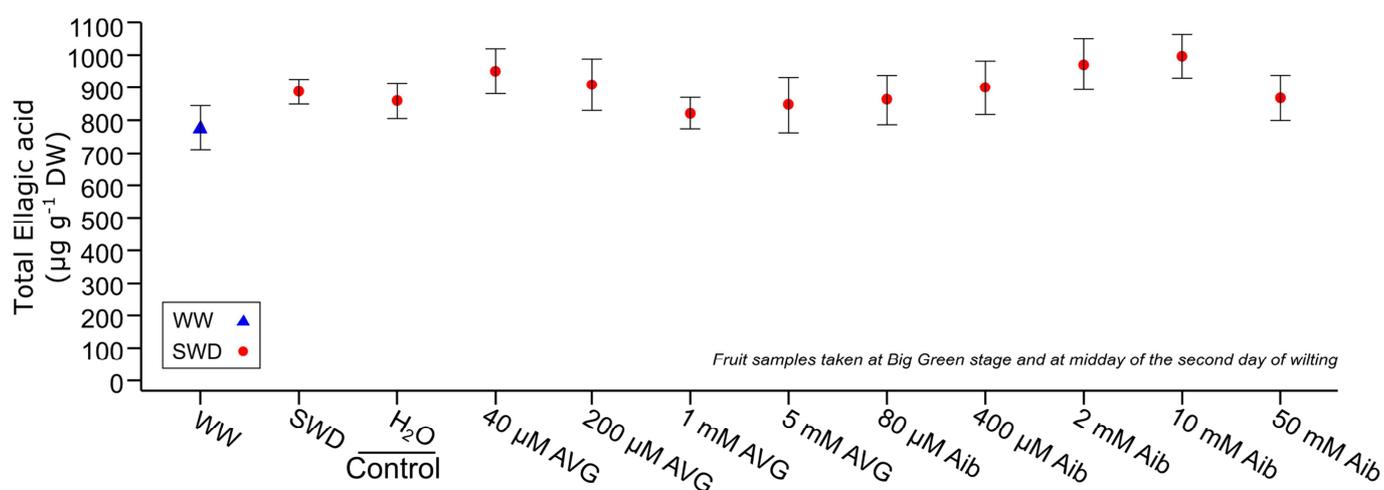


Figure 0.5. Effect of the inhibition of ACC synthase activity by aminoethoxyvinylglycine (AVG) or ACC oxidase activity by aminoisobutyric acid (Aib) on total ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n=12$). Inhibitors were injected at 16:00 h of the first day of wilting and samples for analysis taken at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(11,127)=0.897$, $p=.546$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.2 Effect of exogenous ethylene on ethylene production rate from wilting and well-watered plants

The SWD treatment significantly increased (LSD=0.49) ethylene production rate of green fruit ($\ln[\text{nl kg}^{-1} \text{ h}^{-1}]$) compared to fruit from WW plants in the Grodome and not moved to the cabinet (Control - CH5) (6.57 ± 0.27 vs 5.69 ± 0.14) and after over-night air incubation in the cabinet (Control-Cabinet) (6.19 ± 0.13 vs 5.38 ± 0.18) (**Figure 0.6**). Fruit from plants incubated in the air-tight cabinet with 1-methylcyclopropene (1-MCP) did not significantly change their ethylene production rate with respect to their Control counterparts (6.15 ± 0.14 vs 5.71 ± 0.12).

Ethylene production rate decreased in response to increasing exogenous ethylene concentrations in plants subjected to SWD, being significantly lower than SWD Control-Cabinet plants at 5 ppm ethylene concentrations (6.19 ± 0.13 vs 5.56 ± 0.30). Hence plants subjected to SWD may show auto-inhibitory ethylene production. Interestingly, plants under WW conditions showed auto-catalytic ethylene production at 0.05 ppm exogenous ethylene (5.38 ± 0.18 vs 5.87 ± 0.15) but not at higher exogenous ethylene concentrations.

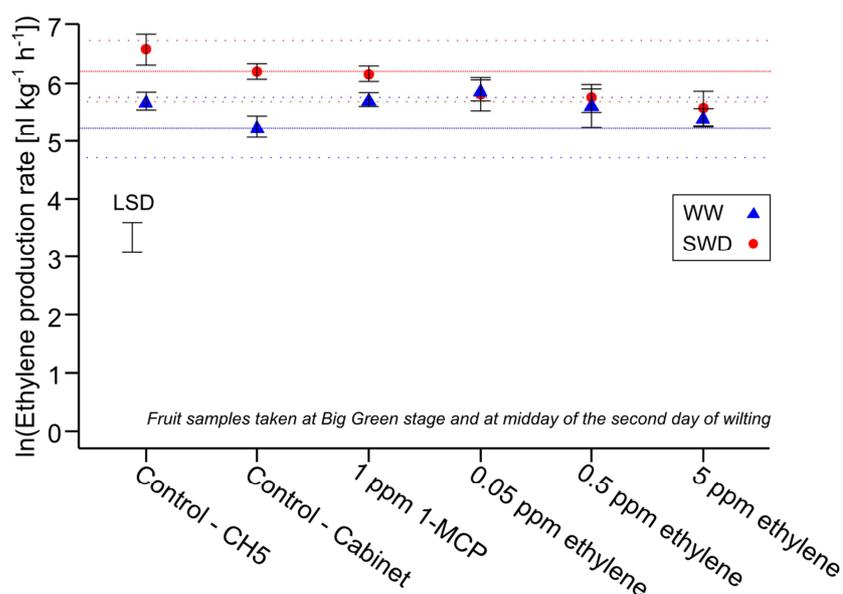


Figure 0.6. Effect of the inhibition of ethylene perception by 1-methylcyclopropene (1-MCP) and exogenous application of ethylene on ethylene production rate ($\ln[\text{nl kg}^{-1} \text{h}^{-1}]$). Each point corresponds to mean value \pm standard error ($n=6$). 1-MCP and ethylene was applied overnight from 16:00 h of the first day of wilting and samples for analysis taken at 13:00 h of the second day of wilting. Fisher's least significant difference test was carried out after ANOVA [$F(11,54)=2.27$, $p=.023$]. Any pair of treatments were statistically different if their difference was higher than LSD. Treatments having statistical effect fell beyond Control-cabinet mean \pm LSD (represented by dotted lines: blue for WW and red for SWD). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.3 Re-instatement and replacement of the ethylene signal

3.3.1 Effect of ACC and ABA on ethylene production rate

In experiment eight, the SWD treatment significantly increased ($\text{LSD}=0.48$) ethylene production rate ($\ln[\text{nl kg}^{-1} \text{h}^{-1}]$) from fruits with respect to WW (5.67 ± 0.12 vs 4.87 ± 0.26) (**Figure 0.7**). The attempted inhibition of endogenous ethylene production rate carried out here with 1 mM AVG succeeded in reducing endogenous ethylene production rate in green fruit with respect to the Control (5.51 ± 0.29 vs 6.26 ± 0.19).

Re-instatement of the endogenous ethylene production rate in fruit treated with AVG was achieved with 200 μM , 1 mM and 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC). However, endogenous ethylene production rates did not reach Control levels, especially when 5 mM ACC was applied. No clear dose-response in terms of ethylene production rate to the applied ACC was detected. Replacement of endogenous ethylene production rate in fruit treated with AVG was achieved with 5 mM and 25 mM abscisic acid (ABA) but not with 1 mM ABA (5.39 ± 0.21 vs [Control] 6.26 ± 0.19).

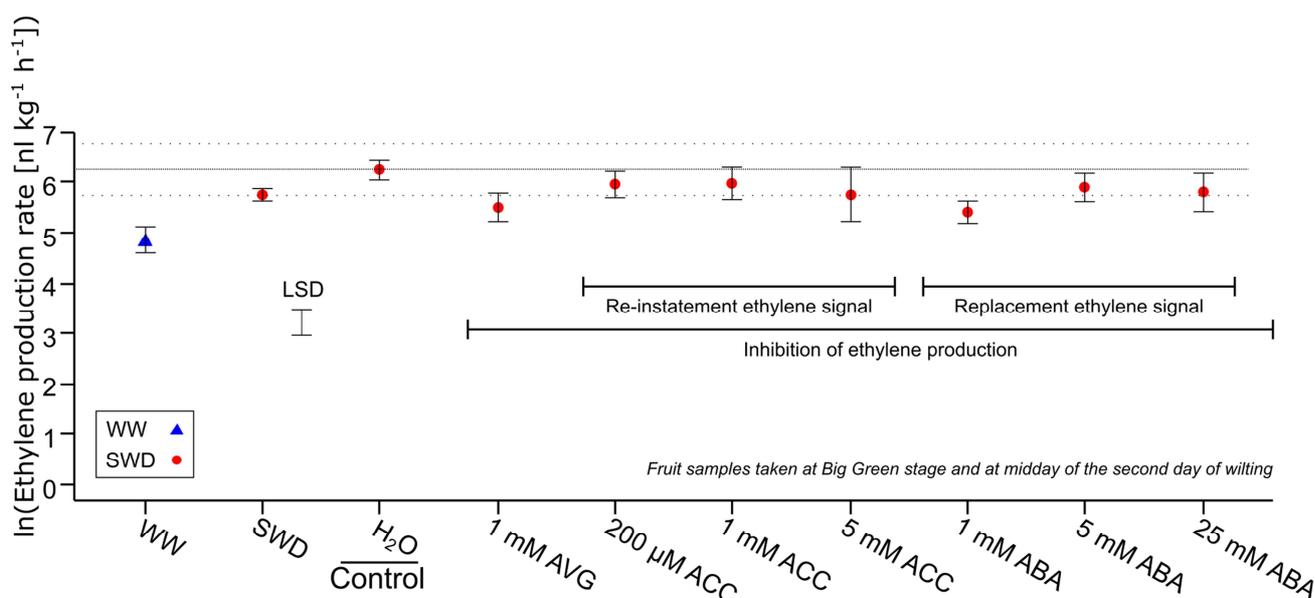


Figure 0.7. Effect of the inhibition and re-instatement of the ethylene signal with aminoethoxyvinylglycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) or replacement of the ethylene signal with abscisic acid (ABA) on ethylene production rate ($\text{nl kg}^{-1} \text{h}^{-1}$) from wilting plants. Each point corresponds to mean value \pm standard error ($n=9$). Inhibitors alongside ACC or ABA were injected at 16:00 h of the first day of wilting and ethylene production measured at 13:00 h of the second day of wilting. Fisher's least significant difference test was carried out after ANOVA [$F(9,127)=4.469$, $p=0.041$]. Any pair of treatments were statistically different if their difference was higher than LSD. Treatments having statistical effect fell beyond Control mean \pm LSD (represented by dotted lines). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.3.2 Effect of ACC and ABA on ascorbic acid, and free and total ellagic acid concentration

Analysis of variance did not reveal an effect of the treatments (see x axis factors) on the ascorbic acid concentration ($\text{mg g}^{-1} \text{DW}$) in the fruit ($F[9,79]=0.702$, $p=0.70$) in experiment eight (**Figure 0.8**). The inhibited endogenous ethylene production rate did not affect ascorbic acid concentration in green fruit with respect to the Control (6.62 ± 0.49 vs 7.06 ± 0.39); consequently, although the use of ACC or ABA did not produce any significant effect with respect to Control fruits, the failure of the AVG treatment to inhibit ethylene production meant that it was not possible to obtain evidence for a restored ascorbic acid concentration to Control levels by using ACC or ABA.

The relationship between ethylene production rate and ascorbic acid concentration was explored with regression analyses. The model included the effect of leaf

responses, treatments of this experiment and PVMC. However, the fit of the model was only $R^2=0.023$; similarly the p -value for the estimate of ascorbic acid was non-significant ($p=0.58$). Therefore, according to the analysis of the data from experiment eight there was no evidence for a relationship between ascorbic acid and ethylene production rate.

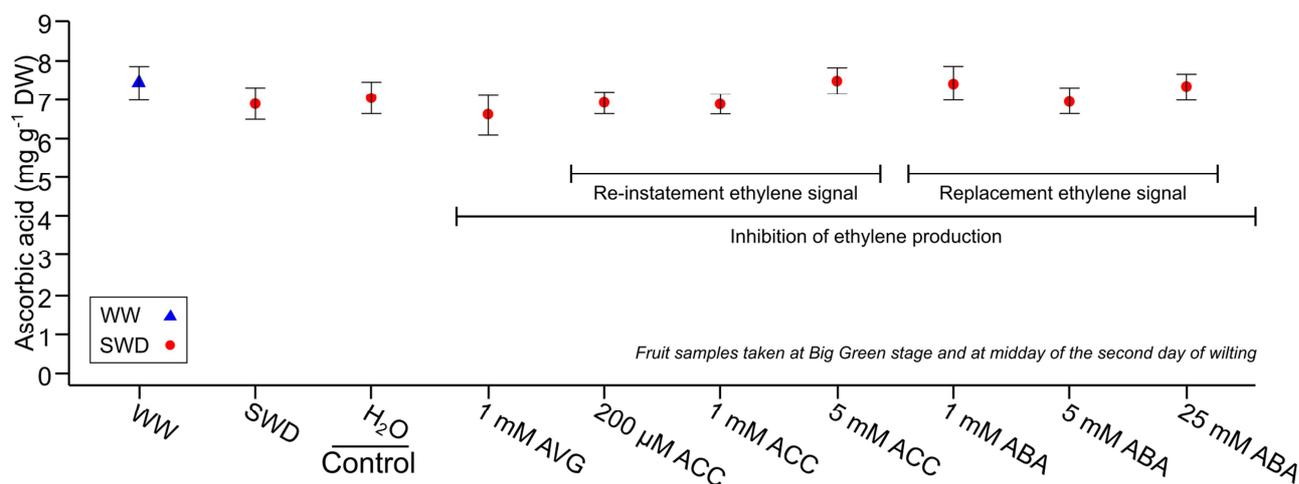


Figure 0.8. Effect of the inhibition and re-instatement of the ethylene signal with aminoethoxyvinylglycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) or replacement of the ethylene signal with abscisic acid (ABA) on ascorbic acid concentration (mg g^{-1} DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n = 9$). Inhibitors alongside ACC or ABA were injected at 16:00 h of the first day of wilting and ethylene production measured at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(9,71)=0.908$, $p=0.523$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

Analysis of variance did not reveal an effect of the treatments (see x axis factors) on the free ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) in the fruit ($F[9,79]=0.364$, $p=0.95$) in experiment eight (**Figure 0.9**). The inhibited endogenous ethylene production rate did not seem to affect free ellagic acid concentration in green fruit with respect to the Control (104.16 ± 21.19 vs 95.24 ± 20.23); thus, although the use of ACC or ABA did not produce any significant effect with respect to Control fruits, the failure of the AVG treatment in inhibiting endogenous ethylene production rate meant that it would not be possible to obtain evidence for a potential restoration of free ellagic acid concentration to Control levels by using ACC or ABA.

The relationship between the ethylene production rate and ellagic acid concentration was explored with regression analyses. The model included the effect of leaf responses, treatments of this experiment and PVMC. However, the fit of the model was only $R^2=0.023$; similarly the p -value for the estimate of ellagic acid was non-significant ($p=0.58$). Therefore, according to the analysis of the data of experiment eight there was no evidence for a relationship between ellagic acid and ethylene production rate.

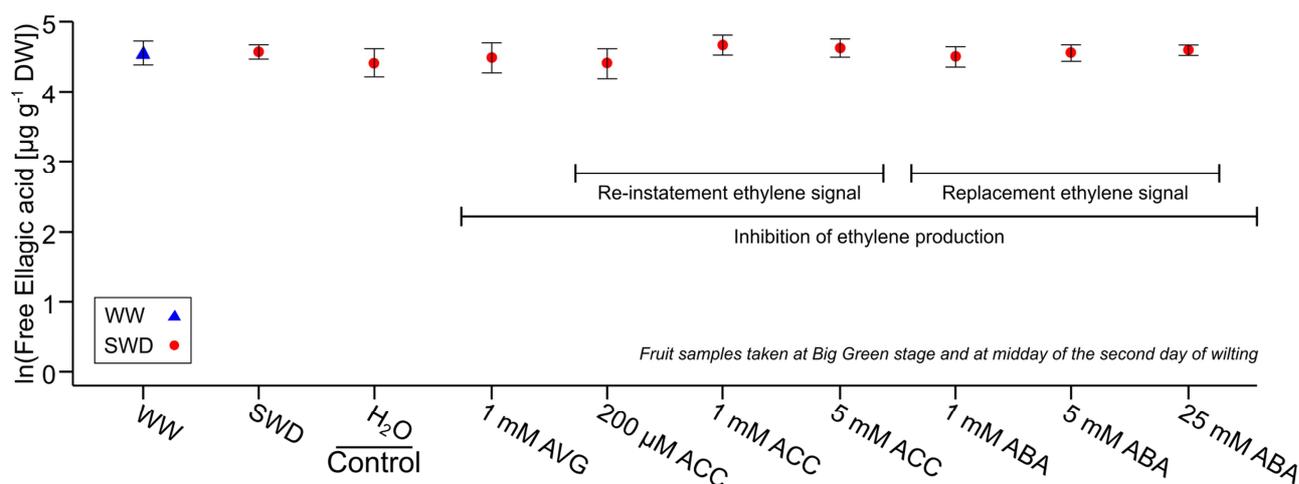


Figure 0.9. Effect of the inhibition and re-instatement of the ethylene signal with aminoethoxyvinylglycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) or replacement of the ethylene signal with abscisic acid (ABA) on free ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n = 9$). Inhibitors alongside ACC or ABA were injected at 16:00 h of the first day of wilting and ethylene production measured at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(9,79)=0.364$, $p=.949$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

Analysis of variance did not reveal an effect of the treatments (see x axis factors) on the concentration of free ellagic acid and ellagitannins (mg g^{-1} DW) in the fruit ($F[9,71]=0.908$, $p=0.52$) in experiment eight (**Figure 0.10**). The inhibited endogenous ethylene production rate did not seem to affect free ellagic acid and ellagitannin concentration in green fruit with respect to the Control (425.15 ± 49.95 vs 380.31 ± 19.54); thus, although the use of ACC or ABA did not produce any significant effect with respect to Control fruits, the failure of the AVG treatment in inhibiting endogenous ethylene production rate meant that it would not be possible evidence for

a potential restoration of free ellagic acid and ellagitannin concentration to Control levels by using ACC or ABA.

The relationship between the ethylene production rate and ellagic acid and ellagitannins concentration was explored with regression analyses. The model included effect of leaf responses, treatments of this experiment and PVMC. However, the fit of the model was only $R^2=-0.03$; similarly the p -value for the estimate of ellagic acid and ellagitannins was non-significant ($p=0.94$). Therefore, according to the analysis of the data of experiment eight there was no evidence for a relationship between ellagic acid and ellagitannins, and ethylene production rate.

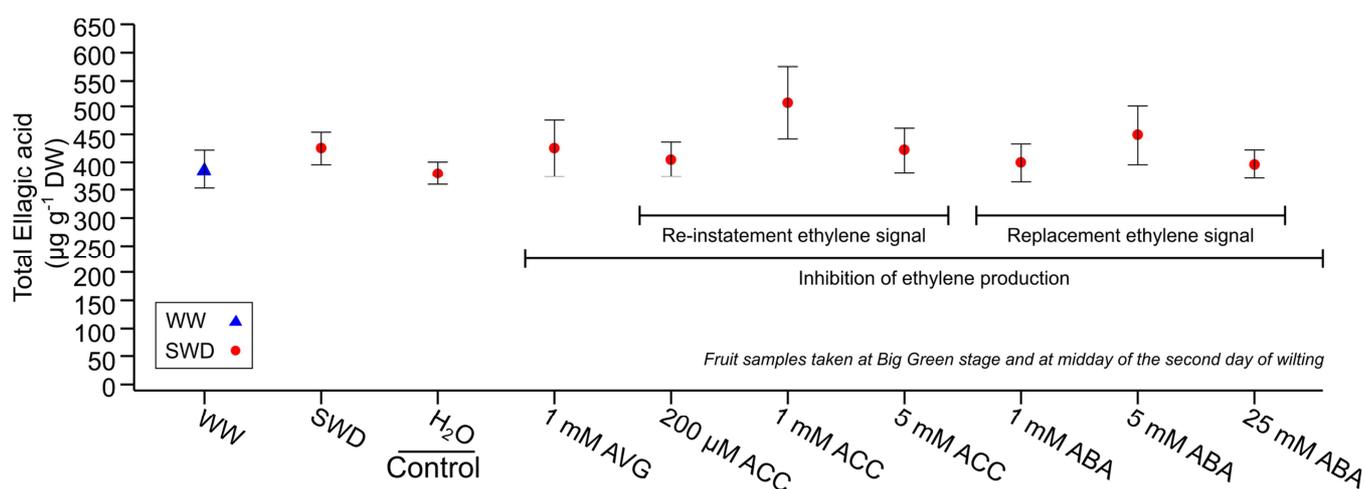


Figure 0.10. Effect of the inhibition and re-instatement of the ethylene signal with aminoethoxyvinylglycine (AVG) and 1-aminocyclopropane-1-carboxylic acid (ACC) or replacement of the ethylene signal with abscisic acid (ABA) on total ellagic acid concentration ($\mu\text{g g}^{-1}$ DW) from wilting plants. Each point corresponds to mean value \pm standard error ($n=9$). Inhibitors alongside ACC or ABA were injected at 16:00 h of the first day of wilting and ethylene production measured at 13:00 h of the second day of wilting. ANOVA did not show treatment effect [$F(9,71)=0.908$, $p=0.523$]. Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.4 Effect of the SWD treatment on leaf ethylene production rate and total ellagic acid concentration

Determination and subsequent statistical analysis of the ethylene production rate from strawberry leaves at midday of the second day of wilting during the SWD treatment (Figure 0.11 [A]) showed that leaves of plants subjected to the stress treatment

produced ethylene significantly faster than in control WW plants ($2,119.44 \pm 184.16$ vs 1329.03 ± 200.27 ; $t[15.89]=2.90$, $p=0.01$). The concentration of ellagitannins (**Figure 0.11 [B]**) was higher in leaves from SWD plants than from WW plants (306.42 ± 57.41 vs 229.64 ± 33.93); however, this increased concentration was not significant ($t[12.98]=1.15$, $p=0.27$).

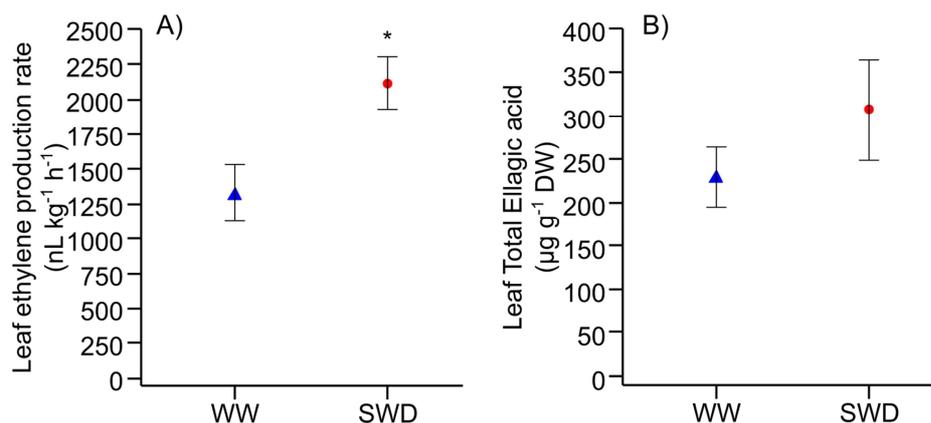


Figure 0.11. Effect of the SWD treatment on (A) leaf ethylene production rate and (B) total ellagic acid concentration on leaves ($\text{nL kg}^{-1} \text{h}^{-1}$). Each point corresponds to mean value \pm standard error ($n=9$). Samples were taken at 13:00 h on the second day of the wilting phase. Statistical differences detected with t-test with respect to WW are indicated with an asterisk ($p < 0.01$). Experiments ran in the Grodome facility (chamber five) at East Malling Research (Kent) in 2015.

3.5 Effect of the SWD treatment on the expression of selected genes in strawberry leaves of transformed plants with low sensitivity to ethylene

The *etr1-1* plants showed an abnormally slow growth due to the cold winter of 2014 in Málaga. Consequently, the number of fruits was insufficient for RNA extraction. Strawberry leaves produce ethylene and accumulate total ellagic acid in response to SWD (**Section 4-3.4**), so in the absence of any fruit, the study of the expression of the genes was carried out on leaves.

The imposition of the SWD treatment to the wild type ‘Chandler’ plants affected the expression levels of genes related to leaf ethylene biosynthesis (**Figure 0.12 [A], [B] or [C]**) and to phenolic biosynthesis in leaves (**Figure 0.12 [D]**). *FaACS1* was significantly down-regulated whereas *FaACO1* and *FaACO4* were significantly up-regulated by the SWD treatment. *FaPAL* expression under the SWD treatment was

between 20 and 40% less than the WW, but was not-significant in any case. Interestingly, patterns of gene expression in the two *etr1-1* lines (L10 and L12), with a decreased sensitivity to ethylene, showed a different response to the SWD treatment. L10 had lower expression of *FaACS1*, *FaACO1* and *FaACO4* than did the L12 line, but these expression levels were not significantly different to those of the WW plants. In turn, the expression of *FaACO1* and *FaACO4* was similar to the SWD ‘Chandler’. The SWD treatment seemed to have a similar effect on the expression of these genes in all lines.

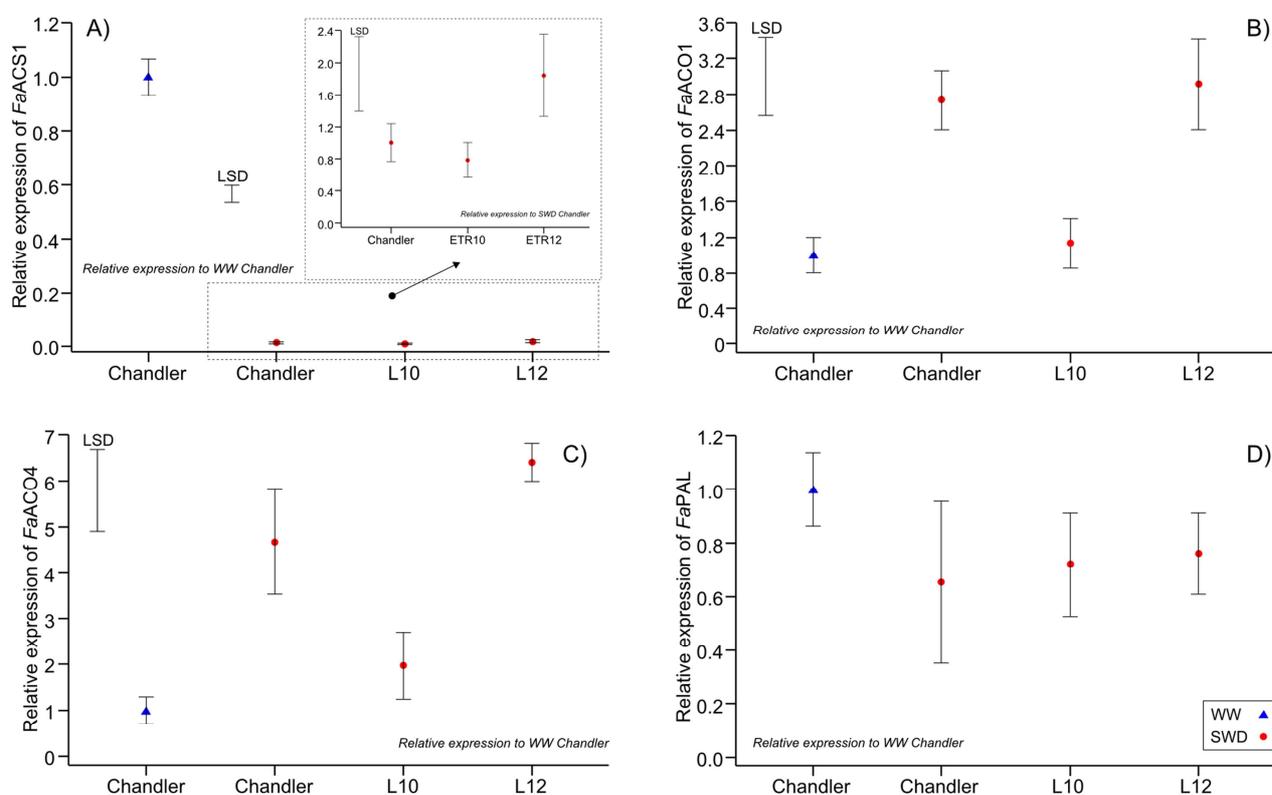


Figure 0.12. Effect of the SWD treatment on the relative expression of (A) *FaACS1*, (B) *FaACO1*, (C) *FaACO4* and (D) *FaPAL* in leaves of the transformed lines L10 and L12 (lower sensitivity to ethylene), and their wild type cv ‘Chandler’. Each point corresponds to mean value \pm standard error (n=4) of the relative expression of the gene with respect to a control. Samples for analyses were taken at midday of the second day of wilting. Fisher's least significant difference test was carried out after ANOVA and any pair of treatments were statistically different if their difference was higher than LSD. Experiment ran at the IFAPA research centre in Churriana, Málaga (Spain) in 2015.

3.6 Effect of the SWD treatment on the expression of selected genes in fruit of three strawberry cultivars

The imposition of the SWD treatment on 'Liberty', 'Safari' and 'Sahara' strawberry cultivars did not affect the expression of genes related to fruit ethylene biosynthesis (**Figure 0.13 [A]**) or phenolic biosynthesis in fruit (**Figure 0.13 [B] or [C]**). There was a clear effect of cultivar on levels of gene expression: 'Sahara' had the lowest expression of all genes under study (always lower than 30% of the expression of 'Liberty') and 'Liberty' the highest.

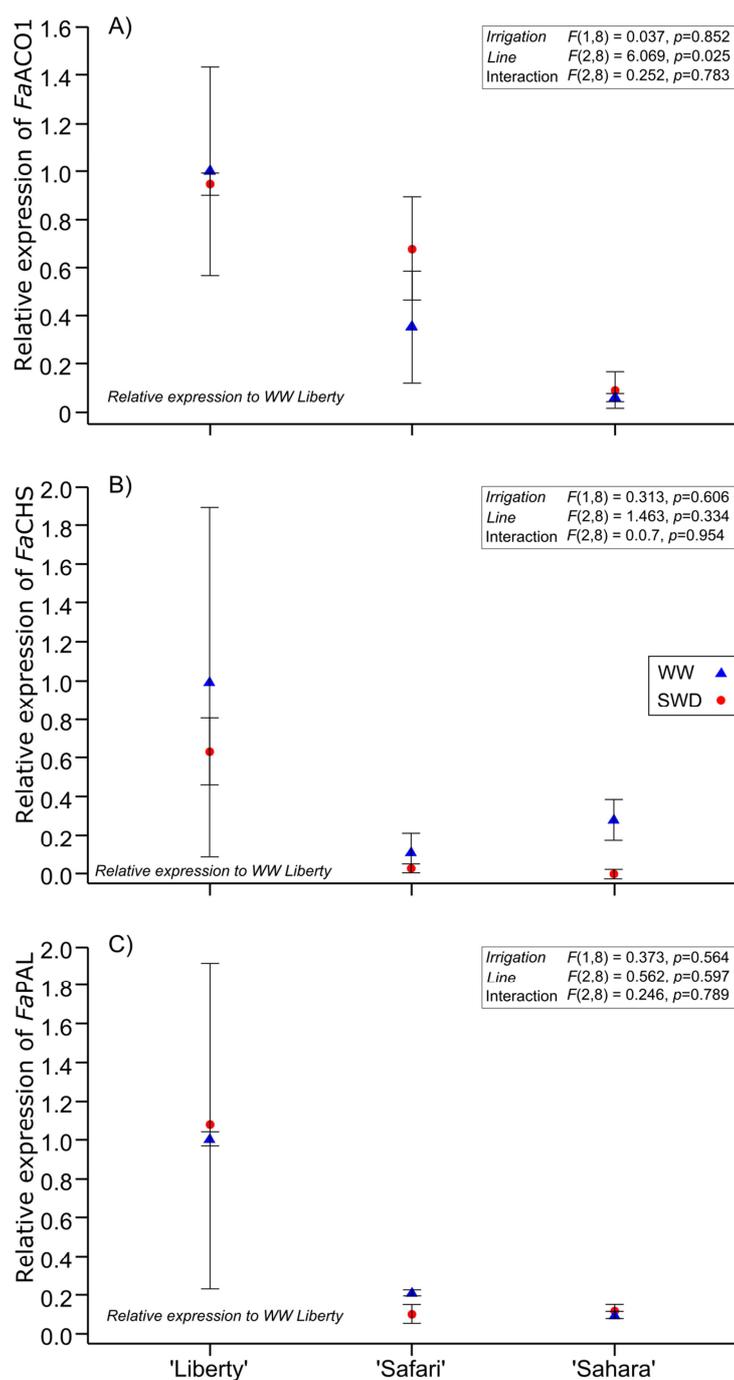


Figure 0.13. Effect of the SWD treatment on the relative expression of (A) *FaACO1*, (B) *FaCHS* and (C) *FaPAL* in fruits of 'Liberty', 'Safari' and 'Sahara' strawberry cultivars. Each point corresponds to mean value \pm standard error ($n=3$) of the relative expression of the gene with respect to a control (WW Liberty). Samples for analyses were taken at midday of the second day of wilting. Experiment ran at the IFAPA research centre in Churriana

4 Discussion

4.1 Effect of AVG and Aib on ethylene production rate by fruits subjected to SWD

4.1.1 ACC synthase inhibition by AVG

Inhibition of ACC synthase activity was attempted with the injection and spray of 40, 200, 1,000 and 5,000 μM of aminoethoxyvinylglycine (AVG), and of ACC oxidase activity with 80, 400, 2,000 and 10,000 and 50,000 μM of aminoisobutyric acid (Aib) (see **Section 4-2.4.2**); from those, only 1 mM AVG managed to significantly inhibit ethylene biosynthesis. Given & Venis (1988) provided the only published example in which AVG is introduced to attached strawberry fruit. At a 1 mM concentration they concluded that a) AVG did not have an effect on PAL activity nor anthocyanin concentration in ripening fruit, and b) ethylene did not play an essential role in the ripening of the strawberry. Later reports questioned the latter conclusion and recent molecular work on transgenic strawberry plants with down-regulated genes involved in ethylene biosynthesis or perception indicates that ethylene plays an essential role in strawberry ripening (Sun et al., 2013; Merchante et al., 2013). Basiouny, (1988) applied a 1 mM AVG concentration to detached white fruit and reported a significant decrease in ethylene production rate 48 hours after application. A more recent report showed that application of 0.5 mM AVG decreased significantly ACC oxidase activity in green, white and red strawberry fruit which was presumably due to a reduced ACC production (Atta-Aly et al., 2000). Therefore, it was expected that 1 mM AVG injections and topical applications reduced fruit ethylene production. In this thesis, lower AVG concentrations seemed to increase ethylene production in fruit from SWD-treated plants perhaps because a) the competitive inhibition depends upon concentrations of both SAM and AVG (Boller et al., 1979; Satoh & Yang, 1988) and b) AVG could have increased levels of ACC synthase in the tissue (Yoshii & Imaseki, 1981).

4.1.2 ACC oxidase inhibition by Aib

To compensate for the lack of information in the literature on the use of Aib in strawberry, the number of concentrations of Aib tested was higher than those of AVG. No inhibition was detected here but ethylene production was significantly increased at 400 μ M Aib. This greater ethylene production upon treatment with Aib has already been reported (Satoh & Esashi, 1980) and because of the competitive nature of Aib, inhibition of ACC oxidase would depend on relative concentrations of its substrate (ACC) and the competitive inhibitor (Aib) (Satoh & Esashi, 1980). A second hypothesis is that the effects observed were due to Aib inhibiting ACC synthase instead. This non-specific effect has been observed in cut carnation flowers sprayed with 10 mM Aib (Kosugi et al., 2014), and suggests that Aib may not be as specific to ACC oxidase as previously thought.

4.2 Effect of AVG and Aib on total, free ellagic and ascorbic acids in fruits subjected to SWD

4.2.1 ACC synthase inhibition by AVG

A relationship between ethylene production rate and ellagic acid concentration in the fruit was found by regression analysis. Ellagic acid is the main phenolic when extracted from achenes and receptacle of strawberry together (Häkkinen & Heinonen, 1999). Evidence for a temporal correlation between ethylene and phenolics has been reported before for stored strawberries (Jiang *et al.*, 2001; Jiang & Joyce, 2003). Although ascorbic acid and free ellagic acid concentration were similar during experiment five and in experiment nine, total ellagic acid concentration in this experiment was approximately half than during experiment five. This kind of variation was reported by Atkinson et al. (2006) for 'Elsanta' plants between two consecutive years (2006). In the work reported here, environmental conditions varied between experiments, despite the use of a controlled environment facility. The largest variation was in radiation suggesting that higher light intensities may reduce total ellagic acid accumulation

perhaps because of their role as antioxidants (Shao et al., 2008; Matkowski, 2008), scavenging ROS produced in the chloroplasts during the water deficit (Nishiyama & Murata, 2014).

The average concentrations for ascorbic acid, free and total ellagic acids in the 1 mM AVG treatment were between the SWD control and the WW control suggesting that inhibition of the ethylene production rate in SWD-treated plants during the second day of wilting may have had an effect on phytonutrient accumulation which might have become significant in a experiment with larger number of replicates.

Application of AVG to fruits translates into improved storability, sometimes measured as firmness, colour, chlorophyll content or phytonutrient content (Martínez-Romero et al., 2007). In plums (non-climacteric), AVG application had a positive effect on total phenolic and antioxidant activity during storage (Ozturk et al., 2012), and in cherries (non-climacteric) increased total phenolics, total antioxidant capacity but not total anthocyanin at the time of harvest (Kucuker & Ozturk, 2011). Given and Venis (1988) reported an increased anthocyanin concentration in strawberry fruit treated with AVG. PAL activity was lower, however, so the treatment might have inhibited the overall production of phenolics, but not anthocyanin concentration. No ethylene measurements were reported in those studies, so it is possible that ethylene production rate might have been promoted rather than inhibited (Boller et al., 1979; Satoh & Yang, 1988). In addition, the relevance of these reports must be considered carefully because the experiment discussed here focused on green fruit subjected to SWD where anthocyanin content was not detected when measured.

4.2.2 ACC oxidase inhibition by Aib

Although 400 μ M Aib managed to significantly increase ethylene production rate, that rise did not translate into a significant increase in the concentration of ascorbic, free or total ellagic acids. The free ellagic acid concentrated in fruit treated with 400 μ M Aib was lower than the control, but total ellagic acid and ascorbic acid were higher but not

significant. Wang et al., (2015) applied Aib at a concentration of 100 mM to longan fruit at the time of harvest and reported several effects during postharvest, including a negative impact on the activity of peroxidase (POD), polyphenoloxidase (PPO) (believed to oxidise phenolics) and gallic acid concentration but higher concentration of catechins, epicatechin, flavonoids and total phenolics (Wang et al., 2015). Perhaps a reduction of gallic acid and consequently ellagic acid biosynthesis could have been achieved here by expanding the range of Aib concentrations to 100 mM (Clifford & Scalbert, 2000). Aib may have also inhibited ellagitannin hydrolysis and increased the synthesis of ellagitannins from existing gallotannins so free ellagic acid concentration was reduced whereas total ellagic acid increased (Clifford & Scalbert, 2000).

4.3 Effect of exogenous ethylene on ethylene production rate from wilting and well-watered plants

Exposing strawberry plants to 5 ppm of exogenous ethylene reduced endogenous ethylene production rate in green fruits from plants exposed to SWD, but not in fruit from WW plants. Inhibition of the ethylene perception by 1-methylcyclopropene (1-MCP) did not affect ethylene production rates. Together these findings suggest that exogenous ethylene had an autoinhibitory effect on its own production in SWD-treated plants. According to Atta-Aly et al. (2000), exposing strawberry fruits for 3 days to 0.13 ppm concentration of exogenous ethylene had a positive effect on ethylene production rate from white and half coloured fruit but not from green fruit. This feedback mechanism has been long known to be dependent on the developmental stage of the fruit (Bufler, 1986). Also, higher exogenous ethylene concentration could have had a different effect on green fruit. Perkins-Veazie et al., (1995) inhibited ethylene perception of detached strawberry green fruit with 0.5 mM silver thiosulfate (STS) but did not observe any changes in ethylene production rate. When STS was combined with 1 mM ACC, ethylene production was increased 11-fold. However, when only 1mM ACC was applied, the increase was 3-fold. These findings suggest that at the green

stage, strawberry fruit ethylene production may follow a System 1 (autoinhibitory mechanism) which can be suppressed by inhibiting ethylene perception.

The autoinhibitory effect could be due to a reduction in the synthesis of ethylene at the ACC synthase or ACC oxidase level, or diversion of ACC to Malonyl-ACC. Examples of the autoinhibitory effect in other non-climacteric fruits include the application of ethylene to citrus peel disks, which resulted in lowered endogenous ethylene production due to a suppression of ACC synthase activity and formation (Riov & Yang, 1982a). The exogenous ethylene effect on ethylene production by intact citrus leaves showed an autoinhibitory and also autocatalytic effect which was due to suppression and increased synthesis, respectively, of ACC synthase and ACC oxidase (Riov & Yang, 1982b). This autocatalytic effect was observed in a later report on citrus where the expression of *CsACS1* was induced upon ethylene treatment (Katz et al., 2004).

4.4 Effect of the re-instatement or replacement of ethylene production on ethylene production rate

4.4.1 Re-instatement of the ethylene signal by ACC

Inhibition and re-instatement of ethylene production rate to control levels (from fruit injected and sprayed with water) was attempted with the injection and spray of 1 mM AVG plus 200 μ M, 1 mM or 5 mM 1-aminocyclopropane-1-carboxylic acid (ACC) (see **Section 4-2.4.2**) Addition of ACC to strawberry fruit aimed to by-pass ACC synthase activity so that ethylene production rate was unaffected by AVG. Partial inhibition of ethylene production was achieved by 1mM AVG and production was successfully re-instated with all concentrations of ACC tested. Basiouny (1988) reported the effect of ACC on fruit at different developmental stages: in the case of white fruit, control produced 0.16 μ l fruit⁻¹ h⁻¹ and increased to 2.57 μ l fruit⁻¹ h⁻¹ in treated fruit; in case of red fruit ethylene production went from 0.23 to 6.12 μ l fruit⁻¹ h⁻¹. For green fruit, Perkins-Veazie et al. (1995, 1996) reported an increase in ethylene production rate upon treatment with the same ACC concentration as used here, and a higher ACC

oxidase activity. Application of 0.5 mM ACC to water deficit stressed wheat leaves also yielded a higher ethylene production which was possibly due to an increased ACC oxidase activity triggered by the water deficit (Apelbaum & Yang, 1981).

4.4.2 Replacement of the ethylene signal by ABA

Replacement of the ethylene signal was attempted with injection and spray of 1 mM AVG plus 1 mM, 5 mM or 25 mM abscisic acid (ABA) (see **Section 4-2.4.2**). The lowest concentration failed to restore ethylene production rate but the highest concentrations did. A review on plant hormone cross-talk by Gazzarrini & Mccourt (2003) highlighted how complex the relation between ethylene and ABA can be: antagonistic at the level of germination, synergistic with respect to root growth and appearing not to interact in other processes such as stomatal closure. The literature is full of examples of this complexity (Voisin et al., 2006, Wilkinson & Davies, 2010). For instance, ABA concentration increases as the strawberry fruit develops (Symons et al., 2012) whereas ethylene production rate decreases (Perkins-Veazie et al., 1995), suggesting an antagonistic effect during ripening which has been reported in at least one study on root elongation from fruit and seed of *Beta vulgaris* (Hermann et al., 2007). On the other hand, Sun et al. (2010) showed that at the beginning of grape ripening there was synergism between these two hormones. Synergism seems to be more common, based on the numerous examples reported for various species (Vendrell & Busea, 1989; Riov et al., 1990; Gomez-Cardenas et al., 1996; Tari & Nagy, 1996). ABA could increase ethylene production due to higher expression of genes encoding for ACC oxidase or ACC synthase. This interaction has been reported previously when applied ABA increased ACC oxidase transcripts in seeds of sugar beetroot (*Beta vulgaris*) (Hermann et al., 2007), broccoli (*Brassica oleracea*) after harvest (Pogson et al., 1995), and ACC synthase and ACC oxidase activity in apples (*Malus domestica*) during ripening (Lara & Vendrell, 2000). The study of Ouvrard et al. (1996) provides the most solid support for results reported here as ACC oxidase

transcripts were reported to increase upon ABA treatment in water stressed sunflower leaves (*Helianthus annuus*).

4.5 Effect of the re-instatement or replacement of the ethylene production on total, free ellagic and ascorbic acids in fruits subjected to SWD

4.5.1 Reinstatement of the ethylene signal by ACC

Total phenolic accumulation and antioxidant activity can be enhanced in strawberries by treatment with ~36 mM ethylene (Heredia & Cisneros-Zevallos, 2009b). This effect can be extended to other crops such as carrots, lettuce, celery, white onions and red onions under 1,000ppm of ethylene (Heredia & Cisneros-Zevallos, 2009a; 2009b). The potential ethylene production from the ACC used during this research was lower than that described in the Heredia and Cisneros-Zevallos reports so it is not surprising that a similar effect on phenolics was not observed. Exogenous ethylene could trigger phenolic biosynthesis at least at three points during the phenolic biosynthetic pathway by increasing levels of chalcone synthase (CHS), flavanone 3-dioxygenase (F3H) and flavonol 3-O-glucosyltransferase (El-Kereamy et al., 2003).

ACC oxidase requires ascorbic acid as a cofactor for the conversion of ACC to ethylene (Ververidis & John, 1991). ACC oxidase seems to be located in the apoplast of the tissues whereas most of the ascorbic acid is located in the symplast (John, 1997). This point supports the idea that a more likely source of this reduction in ascorbic acid could be the oxidation by ROS (Valpuesta & Botella, 2004). Ethylene can increase the ROS production to which the plant responds by producing ascorbic acid (Valpuesta & Botella, 2004; Shao et al., 2008; Wilkinson & Davies, 2010). Hence, in this case, it might be that the accumulation of ascorbic acid depended on the rate of synthesis versus the rate of oxidation. Under SWD the rate of oxidation must have been greater than the rate of synthesis.

4.5.2 Replacement of the ethylene signal by ABA

ABA seemed a good candidate to replace the ethylene signal because it is a well-known stress signal for drought (Slovik et al., 1995; Wilkinson & Davies, 1997; Bahrn et al., 2002; Dodd et al., 2008b; Dodd et al., 2008a), and it plays an important role in the regulation of strawberry fruit ripening. Molecular evidence for the importance of ABA in fruit ripening was found when inhibition of genes regulating the expression of the ABA receptors CHLH/ABAR, PYR1 or the key biosynthesis element NCED1 caused inhibition of strawberry ripening, and anthocyanin and sugar concentration in strawberry fruit (Chai et al., 2011; Jia et al., 2011). Exogenous ABA application to strawberries post-harvest also enhanced anthocyanin accumulation and increased PAL activity and phenolic concentration (Jiang & Joyce, 2003b). Since most anthocyanins are glucosides of anthocyanidins (Clifford, 2000; Lopes-da-Silva et al., 2007; Giampieri et al., 2012), higher sugar concentration might have a direct impact on anthocyanin concentration and so in phenolic concentration. Ascorbic acid concentration upon treatment with ABA was not significantly affected here, but this could be due to the high concentrations of ABA used. Jiang & Zhang, (2001) managed to increase ascorbic acid concentration in leaf segments of maize seedlings (*Zea mays*) vacuum infiltrated with 10 or 100 μM ABA solutions. However, 1 mM concentration did not modify ascorbic acid concentration.

4.6 Effect of the SWD treatment on leaf ethylene production rate and total ellagic acid concentration

Leaf ethylene production has been used as an indicator of stress conditions in strawberries (Hogan et al., 2006). Analyses of ethylene production rate from leaves of strawberry plants under SWD revealed a higher production from stressed plants, and a higher concentration of total ellagic acid whereas ascorbic and ellagic acids concentration was not detected. Ethylene production was higher than those normally found in fruit, therefore leaf-sourced ethylene could have an effect on fruit phenolic

concentration. However, the air in Grodome chamber was continuously stirred and renewed; moreover ethylene in the air from the Grodome chamber was always undetectable.

4.7 Effect of the SWD treatment on the expression of selected genes in leaves of transformed strawberry plants with low sensitivity to ethylene

The study of the expression of selected genes related to ethylene and phenolic biosynthesis from leaves of transformed lines included the relative expression of *FaACS1*, *FaACO1*, *FaACO4* and *FaPAL*. Preliminary results from experiments at East Malling Research showed a) an increased ethylene production and b) increased total ellagic concentration in leaves (see **Section 4-4.6**). Therefore, the enhanced relative expression of some of the genes involved in the biosynthesis or metabolism of ethylene and ellagic acid/phenolics was anticipated. However, since these are only a few of all the genes involved in the regulation of ACC synthase, ACC oxidase and PAL (Prof Victoriano Valpuesta, unpublished results), gene expression results could not rule out an effect of the SWD on the enzyme activity regulation and consequently on ethylene and phenolic biosynthesis. Other genes involved in the regulation of these enzymes include *FvACS2*, *FvACS3*, *FvACS4*, *FvACO2* and *FvACO3* (Merchante et al., 2013), and at least another gene encoding for PAL (Prof Victoriano Valpuesta, unpublished results). Nevertheless, this work provides valuable information on the expression of genes related to ethylene and phenolic biosynthesis in strawberry plants under SWD.

Relative expressions of *FaACO1* and *FaACO4* were increased by the SWD treatment in 'Chandler' and the *etr1-1* line L12 (reduced sensitivity to ethylene). In contrast, *FaACS1* was down-regulated by the SWD treatment. Consequently, and assuming that enzyme activity was affected in the same way, ethylene production could only be explained by the presence of a pool of ACC whose origin could either be pre-treatment

leaf-sourced ACC or root-sourced ACC. The latter instance has been reported before for *Citrus reshni* seedlings (Gomez-Cadenas et al., 1996).

The relative expression of *FaPAL* was not affected by the SWD treatment. According to the RNAseq information provided by Prof Victoriano Valpuesta (unpublished results), there may be another gene encoding for PAL. This does not necessarily mean eight copies of the gene. Being the cultivated strawberry a polyploid organism some of the homeologs could have been missing (Koh et al. 2010). The expression of *FaPAL* reported here did not support the synthesis of phenolics under SWD. PAL activity, however, could be irrelevant for ellagic acid concentration because ellagic acid synthesis begins with an early intermediate of phenylalanine in the shikimate pathway, the 5-dehydroshikimate (Clifford & Scalbert, 2000; Niemetz & Gross, 2005).

Interestingly, regarding *FaACO1* and *FaACO4*, the two transformed lines responded differently to the SWD treatment. Whereas expression of these genes was clearly up-regulated in the wild type and in L12, in L10 there was no effect of the SWD treatment. Genetic transformation required a selection based on the lower relative expression of the combined effect of all *FaETR* genes (Merchante et al., 2013). However, in experiment nine, the transformation triggered changes in the expression of *FaACO1* and *FaACO4* in at least one of the lines selected.

4.8 Effect of the SWD treatment on the expression of selected genes in strawberry fruits of 'Liberty', 'Safari' and 'Sahara' strawberry cultivars

In view of the lack of fruit from the *etr1-1* plants for RT-qPCR analyses, 'Liberty', 'Safari' and 'Sahara' strawberry plants were subjected to SWD order to provide evidence for the main hypothesis of this research. Relative expression of the genes *FaACO1*, *FaPAL* and *FaCHS* was determined and there was no observed significant effect of SWD on the expression of these genes in any of the cultivars. However, as discussed above, these were only few of all genes involved in enzyme activity (Prof. Victoriano Valpuesta, unpublished results) so results could not rule out an effect of the

SWD on the enzyme activity regulation and consequently on ethylene and phenolic biosynthesis. In addition, the large error bars were due to the low number of replicates which was limited by the available material.

5 Conclusions

5.1 Effect of the inhibition, re-instatement or replacement of the ethylene signal during SWD on phenolic concentration

One mM AVG significantly reduced ethylene biosynthesis. Ethylene production was significantly increased by the application of 400 μ M Aib. Because of the competitive nature of the inhibitors used, they work best when the natural substrates for these enzymes are at lower concentrations (Boller et al., 1979; Satoh & Esashi, 1980; Satoh & Yang, 1988).

No effect of inhibitors was observed on concentrations of free ellagic acid and ascorbic acid. According to the regression analyses, total ellagic acid concentration in the fruit, however, responded to the altered ethylene production rate. Although ascorbic acid and free ellagic acid concentration were similar during experiment five and experiment nine, total ellagic acid concentration in experiment ten was approximately half than during experiment five, which could be the result of the different light, temperature and relative humidity during these experiments (Atkinson et al., 2006). This highlights the importance of carrying out research of this nature in a precisely controlled environment, which, at the time the Grodome did not provide.

An AVG-induced reduction in ethylene production was partially restored by ACC or ABA, at all concentrations tested. Fruit concentrations of phytonutrients, however, were not affected suggesting that although pharmacological studies have proved very useful in physiological research, unintended changes provoked by application of chemicals such as shifts in pH and redox potentials (Gehring et al., 1990) may lead to unpredictable metabolic reactions (Chance, 1952; Alberty & Bloomfield, 1963; Pahlic, 1993; Xu, 1997).

5.2 Genetic evidence of the involvement of ethylene in the concentration of phenolics during SWD in transformed plants with lower sensitivity to ethylene

Preliminary results showed increased ethylene production and total ellagic concentration in leaves of plants subjected to SWD. The relative expression of genes related to ACC oxidase activity (*FaACO1* and *FaACO4*) was significantly up-regulated under the SWD in one of the transgenic lines but not in the other. This was an undesired result produced by the genetic manipulation.

Genes related to ACC synthase and PAL activities were not up-regulated by the SWD treatment. However, not all the genes involved in their regulation were studied (Prof. Victoriano Valpuesta, personal communication). No enhanced expression of the genes under study was found in fruit of plants subjected to SWD.

Chapter 5. Concluding remarks and future work

1 The Severe Water Deficit treatment and its effect on the performance of the photosystem II, ethylene production rate and concentration of phenolics

Ethylene is a stress hormone whose production rate in plant tissues is often increased by water deficit (El-Beltagy & Hall, 1974; Wang et al., 1987; Nakano et al., 2003; Sobeih et al., 2004; Voisin et al., 2006; Ripoll et al., 2014). However, some authors report that ethylene production was not increased under water deficit (Morgan et al., 1990; Narayana et al., 1991). The concentration of phenolics in the fruit of strawberry exposed to root water deficit has been reported by Terry et al. (2007) and Dodds (2008). This accumulation is well known to be affected by water deficit; probably by an redox mechanism which has been shown to increase PAL activity in carrot (Jacobovelázquez et al., 2011). This evidence, combined with the known effect of ethylene on fruit quality in strawberry (Jiang et al., 2001; Jiang & Joyce, 2003; Merchante et al., 2013; Sun et al., 2013), particularly the concentration of phenolics, make ethylene an interesting candidate for the study of the regulation plant responses to water deficit in strawberry fruit.

Dodds et al. (2008) compared the effect of three RDI treatments and three PRD treatments with WW control on the concentration of phenolics and reported that 'Elsanta' strawberry plants irrigated with 80% or 60% of their evaporative demands had higher concentration of phenolics in fruit under PRD than in WW controls, whereas in plants watered with 60% of their evaporative demand under RDI, phenolic concentrations were unchanged. TDI was developed from the observation that a transient wilting occurred in PRD-treated plants during the switching of the irrigation between the dry and the wet zones, and this was hypothesised to be the trigger for the enhanced fruit phenolic concentrations (Dr Mark Else, personal communication). Plants under TDI are subjected to a gradual drying treatment until the first signs of wilting are observed, then maintained at the point of wilting for 48 h by frequent but

short irrigation events, then re-hydrated to WW conditions. The severe SWD used here was adapted from the TDI treatment, and was fully characterised in experiment five in 2014. Similarly to TDI, SWD involved a period of drying and a period of wilting. Initially, irrigation was withheld so that gradual substrate drying was imposed; plants with greater canopy areas and therefore higher water demands reached the wilting point earlier so fruit had to be sampled on different days. The potential impact of this sampling procedure on the consistency of results was addressed in experiment four by re-hydrating fast-drying plants to PVMC of slow-drying plants. Since the focus of the research was established once it was confirmed that the highest ethylene production rate in large green fruit (seven days before turning colour) took place at midday (when light intensity was at its greatest) of the second day of the wilting phase, the re-hydration phase became irrelevant for the SWD treatment in subsequent experiments. It was necessary to develop a rapid method of quantifying the plant responses to the SWD to effectively manage the drying phase of the treatment and the fruit sampling strategy. In order to achieve this, leaf responses to limited substrate water availability were used; these included photosynthetic rate, performance of the photosystem II [Yield (II)], stomatal conductance and transpiration rates. The environmental factors affecting evapotranspiration (pot-and-plant weight, PVMC, temperature, light intensity and relative humidity) were also measured and used to explain the variability of the responses to the treatment, including fruit responses.

Some authors have questioned whether Yield (II) responds to water deficit (Razavi et al. 2008, Snider et al., 2014). Here, Yield (II) was measured in WW and SWD plants at decreasing light intensities, and results suggested that photosystem II of WW plants introduced more electrons in the electron transport chain than in SWD plants. In addition, according to the regression analyses Yield (II) was the most specific response to the treatment because Yield (II) responded to pot-and-plant weight while other leaf responses such as photosynthesis, stomatal conductance, and transpiration responded to pot-and-plant weight and light intensity or temperature. This, and the fact

that regression analyses suggested that Yield (II) could be used to indicate when ethylene production from SWD plants was highest with respect to WW plants, meant that measurements of Yield (II) were adopted as the main tool for deciding the time of fruit sampling and managing the drying phase of the SWD treatment.

In WW conditions, ethylene production rate and phenolic concentration in large green fruit was the highest of all the ripening stages studied. During the wilting phase, fruit ethylene production was higher than in fruit from WW plants. However, fruit concentrations of total phenolics were not always higher than control values, being greater in experiment three and five, but not during experiment two. This inconsistency has been attributed to environmental factors other than the substrate drying (Ripoll et al., 2014) itself caused in the experiments discussed in this thesis by poor environmental control. This effect has been observed in strawberry by Atkinson et al. (2006) who reported two-fold variation in total ellagic acid in 'Elsanta' strawberry fruit between two consecutive years. However, the SWD treatment did have a positive significant effect on the concentration of fruit phenolics in the three experiments. Concentration of these phytonutrients under water deficit has been reported previously by Terry et al. (2007) when investigating the potential role of ABA as signal for this accumulation of phenolics and Dodds (2008).

Average ethylene production during experiment two was $130 \text{ nl kg}^{-1} \text{ h}^{-1}$ whereas in experiment three, production was $299 \text{ nl kg}^{-1} \text{ h}^{-1}$. Both values were within the limits reported for strawberry (Abeles & Takeda, 1990; Perkins-Veazie et al., 1995; Iannetta et al., 2006). The large variation could be partly explained by the interaction of substrate drying with other environmental factors (Ripoll et al., 2014). The regression analyses showed that light intensity was a major predictor for ethylene production rate and total phenolic accumulation. Light can affect phenolic accumulation as reported by Atkinson et al. (2006) who noted that 'Flamenco' strawberry plants accumulated ellagic acid in response to reflected photosynthetically active radiation; and can have an effect on ethylene production rate as described by Hogan (2008) and Munné-Bosch et al.

(2002), presumably via ACC accumulation. Although experiments two and three were carried out in a semi-controlled environment, light intensity was very different between the two experiments due to fluctuations in natural light intensity. The regression analyses also revealed that changes in Yield (II) coincided consistently with the rise in ethylene production and was highly significant to explain the variation on total phenolic data which reinforced the idea of using Yield (II) as tool to decide when to sample the fruit.

Experiment five provided more details of the changes in ethylene production and phenolic accumulation in large green fruit over the course of the SWD treatment. Concentrations of catechin, p-coumaric acid, epicatechin, kaempferol, pelargonidin-3-glucoside, caffeic acid and ferulic acid were not identified in any of fruit samples analysed. The only phenolic found in the samples was ellagic acid, which is the most important phenolic in terms of concentration according to Häkkinen & Heinonen (1999). Total phenolics accumulation was significantly higher than control values even though total ellagic acid or free ellagic acid concentrations were not significantly different. This was perhaps due to the different extraction methods used, the fact that the total phenolics assay was based on capacity of the Folin-Ciocalteu reagent to oxidize phenolics (reducing power of the phenolics) whereas the quantification of ellagic acid was based on its chromatographic properties (absorption of light of different wavelengths), or perhaps to the presence of interfering agents with the Folin-Ciocalteu reagent other than ascorbic acid that also increase under SWD. Ellagitannins were proposed to be the main single phenolic affected by the SWD because the relative difference between total ellagic acid concentration in fruit under SWD and fruit under WW was greater than the difference in free ellagic acid.

Results obtained during 2014 revealed that large green fruit of strawberry plants subjected to SWD produced more ethylene than WW fruits. However, it was not clear whether the fruit accumulated ellagitannins in response to this increased ethylene production. The cross-correlation analyses indicated that under SWD, the fruit

produced ethylene and then followed an concentration of phenolics. Despite the fact that the correlations were not statistically significant, the trend concurred with earlier reports of ethylene-mediated production of phenolics in strawberry fruit (Jiang et al., 2001; Merchante et al., 2013; Sun et al., 2013). This hypothesis was further explored using a pharmacological approach based on Jackson's postulates (Fredericks & Relman, 1996), and a molecular approach utilising the transgenic (*etr1-1*) and commercial plant material (cv 'Liberty', 'Sahara' and 'Safari').

2 Pharmacological approach to test whether ethylene mediates phenolic concentration in strawberry fruit of plants under SWD

Ethylene production was significantly inhibited by injection and spray of 1 mM AVG which agrees with the reports by Basiouny (1988) and Atta-Aly et al. (2000). Ethylene production was significantly increased by 400 μ M Aib which has also been reported previously (Satoh & Esashi, 1980). AVG and Aib are competitive inhibitors that are most effective when their natural substrates, ACC synthase and ACC oxidase, respectively, are at low concentrations. AVG and Aib effectiveness is also dependent on their concentrations. However, when the natural substrate concentration increases beyond a threshold level, inhibition fails and the enzyme is free to act on the accumulated substrate, consequently ethylene production increases greatly (Boller et al., 1979; Satoh & Yang, 1988). None of the concentrations of the inhibitors tested significantly reduced or increased total or free ellagic concentration in the fruit. However, regression analysis revealed a significant effect of ethylene production rate on total ellagic acid concentration in the fruit. Taken together with the fact that in experiments three and five, the phenolic concentration occurred at the same time or shortly after the ethylene production rate increased, the results of the regression analysis provide evidence for an ethylene-mediated accumulation of phenolics, possibly ellagitannins, in fruit of strawberry plants under SWD.

Lowered ethylene production induced by AVG was restored by ACC or abscisic acid (ABA) applied to fruit by all concentrations tested apart from 1 mM ABA. Applications of ACC by-passed the inhibition of ACC synthase caused by AVG but ABA presumably increased ethylene production rate by increasing ACC oxidase or ACC synthase activity (Pogson et al., 1995, Ouvrard et al. 1996; Lara & Vendrell, 2000; Hermann et al., 2007). A reduction in total fruit ellagic acid concentration was expected to be produced by the inhibition; however, it was not affected by the 1 mM AVG treatment. No effect of the re-instatement by ACC or the replacement by ABA on total ellagic acid concentration was observed. In addition, no relationship between total ellagic acid and ethylene production rate was found. This result may be due to a) the fact that despite previous results, the concentration of phenolics in fruit of plants subjected to SWD is not mediated by ethylene, or b) an undesired effect of the ABA and ACC treatments which, in turn, was the main limitation of the pharmacological approach: exogenous application of chemicals to the plant produce changes in the chemistry of the tissue, particularly pH and redox potential, which in turn can affect metabolic processes other than those originally targeted (Chance, 1952; Alberty & Bloomfield, 1963; Gehring et al., 1990; Pahlic, 1993; Xu, 1997). For example, the synthesis of phenolics seems to be under redox control (Jacobo-Velázquez et al., 2011). That is why the dose-response experiments reported here aimed to find the lowest effective concentration of AVG, Aib, ACC and ABA.

3 Molecular approach to test whether ethylene mediates phenolic concentration in fruit and leaves of strawberry plants under SWD

The molecular approach involved the use of genetically modified plants designed to study the role of the ethylene signal in the ripening of strawberry fruit (Weyers & Paterson, 2001). The *ert1-1* plants used were developed by Merchante et al (2013); these had a reduced sensitivity to ethylene. This approach was complemented by

studying the relative expression of key genes related to ethylene and phenolic metabolism in fruit and leaves.

The success of the transformation was evaluated by phenotyping the different transformed lines (Merchante et al., 2013). Those lines with lower expression of the *ert1* gene were used in this work. In order to check whether the transformation persisted in the generation of the lines under study, the expression of the *ert1* gene was re-evaluated.

Due to an the unusually cold winter in Málaga in 2014-2015 plants did not develop full size and the number and size of fruit on the transgenic plants at the time of the experiments was small and insufficient for RNA extraction. The experiment was repeated with a new batch of plants but these again failed to produce enough fruit. Plants were propagated and grown in a contained facility for transgenic material with no heating in place from which removing material was highly restricted. Therefore, plants could not be moved to a warmer location. Both batches were planted in the autumn of 2013 and used during the spring of 2014. Preliminary analysis during experiment four of the ethylene production rate and total ellagic acid in leaves of plants subjected to SWD showed that leaves produced around 8-fold more ethylene than fruit. In addition, leaves under SWD not only produced more ethylene but also accumulated more total ellagic acid than WW plants. There was not instrumentation available to measure ethylene production rate in Málaga. In view of this information the decision was made to use leaves instead of fruit in experiment nine. Experiment ten provided the material for the RT-qPCR on fruit of the strawberry cultivars 'Liberty', 'Sahara' and 'Safari' strawberry.

The primers under study were obtained using the available sequence of the *Fragaria vesca* genome. Rousseau-Guentin et al. (2009) showed that the genome of the diploid *F. vesca* contributes to the octoploid genome of *F. ananassa* with extremely high sequence identity (Bombarely et al., 2010). These primers would have revealed a combined expression of all homeologs of the gene of interest.

Expression of the *FaACO1* and *FaACO4* genes was promoted by the SWD treatment in leaves. Interestingly, the expression of these genes was not affected by the treatment in L10 but it was affected in L12 and 'Chandler'.

In turn, expression of *FaACS1* was down-regulated. There are other known genes expressing for ACC synthase and ACC oxidase (Merchante et al., 2013; Sun et al., 2013), but if enzyme activity was directly related to the genes studied here, these results suggest that there must have been ACC available from other sources. Despite the fact that transpiration rate was low during the wilting phase, the ACC that fuelled the higher ethylene production may have been root-sourced (Else et al., 1994; Else et al., 1995; Gomez-Cadenas et al., 1996; Tiekstra et al., 2000). However, ethylene production rate in fruit during the re-hydration phase was lower than in the wilting phase. Hence the most likely source is a pool of ACC that was present before the imposition of the SWD treatment that was sufficient to support the increased rate of foliar ethylene production. Else & Jackson (1998) showed that foliar concentrations of ACC in well-watered tomato plants were sufficient to support a basal rate of ethylene production.

FaPAL was not affected by the SWD treatment. For the most likely route for ellagic acid biosynthesis PAL could be irrelevant because ellagic acid most probable origin is gallic acid whose biosynthesis in turn branches from an early intermediate of shikimic acid (Grundhöfer et al., 2001; Niemetz & Gross, 2005).

RT-qPCR performed on fruit material from 'Liberty', 'Sahara' and 'Safari' strawberry varieties revealed no effect of the SWD on the relative expressions of *FaACO1*, *FaPAL* and *FaCHS* genes. This information suggests that the combined expression of each these genes cannot explain the enhanced ethylene production rate and phenolic concentration in fruit of plants under SWD. Although the treatment was applied and fruit sampling done according to the protocols developed in the experiments in the UK, ethylene production could not be measured (the instrument was not available in

Málaga) so the SWD may not have produced the same effects with 'Liberty', 'Sahara' and 'Safari' in Málaga than it did with 'Elsanta' in East Malling.

4 Future work

4.1 Investigation of the interaction between light intensity and substrate moisture deficits

Experiments at East Malling Research were carried out in a semi-controlled environment facility where light intensity, temperature and relative humidity varied from experiment to experiment. This was due to problems with the environmental control within the facility due to problems with contractors during the time of the experiments which led to variations between experiments and lowered the reproducibility of the results. Nevertheless, since all environmental metrics were recorded, it was possible to study their effect on the plant responses to SWD, including the variation in ethylene production rate and total phenolic concentration in the fruit. A recent review by Nishiyama & Murata (2014) highlighted the fact that water deficit itself may not be the primary reason for the production of antioxidants, which include phenolics, but rather an interaction between water deficit and light intensity is involved presumably due to a redox mechanism (Jacobo-Velázquez et al., 2011; Ripoll et al., 2014).

Regression analyses on the measurements taken during the work in this thesis revealed light intensity as a significant environmental factor affecting plant responses to SWD. Although this is valuable information, randomized experiments in a completely controlled environment such as growth cabinets would help to gain a better understanding of the interaction between light intensity and substrate water deficits. Substrate moisture thresholds and light thresholds from such research would help in the application of a consistent stress to a given cultivar of strawberry in geographic areas exposed to different average light intensities.

Use of severe water deficit is highly influenced by environmental factors, particularly light. In a commercial context the light intensities should be monitored and used to schedule irrigation in order to achieve the desired effects (i.e. increase in fruit quality)

4.2 Further the testing of Jackson's postulates

Here, unequivocal evidence for a stress-induced increase in ethylene production rate in fruit of plants subjected to SWD is provided. Conclusive evidence on the concentration of phenolics, including free and total ellagic acid, in the fruit in response to the enhanced ethylene production was not obtained: experiments two and four did not show the concentration of total phenolics whereas three and five did. In those cases where total phenolics were increased, it remains to be seen whether ellagitannins are the main contributory factor. Extraction solutions for both assays (total phenolics and ellagic acid) should be the same in order to obtain comparable results in future experiments. Variation in total ellagic acid concentrations during experiment seven (inhibition) was explained by the ethylene production rate data, but this was not the case in experiment eight (restoration or replacement). In order to gain insight in the ethylene-mediated phenolic concentration in the fruit, the remaining postulates proposed by Jackson (Weyers & Paterson, 2001) should continue to be tested. Ideally, these experiments would be carried out in a fully controlled environment facility and with a larger number of plants. First, future experiments should include a wider range of these chemicals targeting both ACC synthase and ACC oxidase, and perhaps use different inhibitors to AVG and Aib (**Table 0.2**). A larger number of ACC and ABA concentrations should also be tested, and the responses of inhibited fruit to other hormones such as auxins would also help. New experiments should test the reproduction of the endogenous changes occurring naturally by application of ethylene, check that the effects observed are consistent across different environments, and observe whether the same response appears in related taxonomic groups. Results from these experiments along with the results reported in this thesis may help to

strengthen the evidence for ethylene-mediated total ellagic concentration in the fruit of the strawberry. However, the limitations of the pharmacological approach should be considered carefully (see **Section 5-2**), and should be a priority to find the lowest 5-effective concentrations of the chemicals.

4.3 Study the expression of greater number of genes under the SWD treatment

The molecular work carried out here investigated the expression of a limited number of genes related to ethylene biosynthesis (*FaACO1*, *FaACO4* and *FaACS1*) and phenolic biosynthesis (*FaPAL* and *FaCHS*) in strawberry fruit or leaves. There are other genes involved in the regulation of ACC synthase and ACC oxidase in strawberry such as *FvACS2*, *FvACS3*, *FvACS4*, *FvACO2* and *FvACO3* (Merchante et al. 2013). By comparison with the genome of *Arabidopsis*, some tentative roles for some of the genes with no known function have now been assigned. That is the case for at least one other *FaPAL* gene (Victoriano Valpuesta, personal communication). The limited availability of transgenic plant material did not allow a more complicated experiment with the *etr1-1* plants, but the molecular work completed here could be expanded and improved by studying the regulation of a larger number of genes related to ethylene biosynthesis and phenolic concentration by the SWD treatment in fruit of a larger number of *etr1-1* lines in a time-course experiment. Ideally, the work should focus on fruit in order to a) confirm that the SWD treatment increases ethylene production, b) find whether there are genes yet to report by testing primers of candidate genes revealed by RNAseq, c) complemented by determination of the activities of ACC synthase, ACC oxidase, PAL and CHS. Since, as discussed earlier in the text, the biosynthesis of some phenolics may not be limited by PAL activity, concentrations of total and free ellagic acid in the fruit of transgenic plants should be also measured.

Alternatively, development of transformed lines in which a complete down-regulation of ACC synthase or ACC oxidase was achieved would provide material to continue the testing. Such plants under SWD would indicate which of the enzymes is affected by the

treatment, and whether there is an effect on the phenolic concentration. If there was an effect, exogenous ethylene should restore the concentration of phenolics. However, a large number of lines of each transformation should be tested in order to strengthen the evidence for an ethylene-mediated accumulation of phenolics in fruit of strawberry.

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