A Comparison of the Molecular Mechanisms Underpinning High-Intensity, Pulsed Polychromatic Light and Low-Intensity UV-C Hormesis in Tomato Fruit

Scott[1,2*], G., Dickinson[1], M., Shama[2], G. & Rupar[1], M.

1 School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK

2 Department of Chemical Engineering, Loughborough University, Loughborough LE11 3TU, UK

*Corresponding author: G.Scott@lboro.ac.uk

Abstract

Postharvest treatment of tomato fruit with high-intensity, pulsed polychromatic light (HIPPL) has previously been shown to induce delayed ripening and disease resistance comparable to that of low-intensity UV-C (LIUV). Little, however, is known of the mechanisms underpinning postharvest HIPPL hormesis in tomato fruit. Expression of genes involved in plant hormone biosynthesis, defence, secondary metabolism and ripening were monitored 24 h post treatment (24 HPT), 10 d post treatment (10 DPT) and 12 h post inoculation with *Botrytis cinerea* (12 HPI). All genes monitored were constitutively expressed and changes in expression profiles following treatment were highly similar for both HIPPL and LIUV treatments. Expression of pathogenesis-related proteins P4, β-1,3,-Glucanase and Chitinase 9 and a jasmonate biosynthesis enzyme (OPR3), were significantly upregulated at 10 DPT and 12 HPI. Both treatments significantly downregulated the expression of polygalacturonase and flavonol synthase at 10 DPT and 12 HPI. Ethylene biosynthesis enzyme ACO1 and β-carotene hydroxylase were significantly upregulated at 24 HPT, and phenylalanine ammonia-lyase (PAL) was significantly upregulated at 12 HPI. Both HIPPL and LIUV treatments stimulate

defence responses that are mediated by salicylic acid, jasmonic acid and ethylene. This may lead to broad range resistance against both necrotrophic and biotrophic pathogens as well as abiotic stresses and herbivorous pests. Following inoculation with *B. cinerea* only *PAL* showed indication of a gene priming response for HIPPL- and LIUV-treated fruit.

Key words: Hormesis; *Solanum lycopersicum*; pulsed light; gene expression, polychromatic light.

1. Introduction

Wavelengths of light that fall between 100 and 280 nm are referred to as UV-C. The application of high-dose germicidal UV-C is extensively used in decontamination processes due to its ability to directly inactivate a range of micro-organisms. Lu *et al.,* (1987) published the first studies utilising UV-C for inducing hormesis in fresh produce. During the following three decades hormetic UV-C treatment was successfully performed on a wide range of fresh produce including climacteric and non-climacteric fruit, tubers, salads and brassicas (Ranganna *et al.,* 1997; D'Hallewin *et al.,* 1999, Costa *et al.,* 2006; Pongprasert *et al.,* 2011; Kasim & Kasim, 2012). The beneficial effects of UV-C hormesis include pathogen resistance, delayed chlorophyll degradation and improved nutritional content, all of which have been reviewed in depth by Shama & Alderson (2005), Ribeiro *et al.* (2012) and Turtoi (2013).

The majority of previous studies have been conducted with conventional low-pressure mercury sources that emit low-intensity UV-C light (LIUV) with peak emission at 254 nm. Recently, however, a number of publications have shown that high-intensity, pulsed polychromatic light (HIPPL) also induces similar hormetic benefits to that of LIUV (Oms-Oliu *et al.,* 2010; Koyyalamudi *et al.,* 2011; Rodov *et al.,* 2012; Pataro *et al.,* 2015; Scott *et al.,* 2017). In a previous study of ours, it was found that a 16-pulse treatment at 4.6 kJ/m^2/pulse of HIPPL induced both delayed ripening and increased disease resistance on tomato fruit at levels comparable levels to those achieved at a dose of 3.7

kJ/m^2 of LIUV (Scott *et al.,* 2017). The use of HIPPL reduced treatment times from 350 s to 10 s per fruit when LIUV treatments were delivered at an intensity of 20 W m^{-2}.

One of the major benefits of HIPPL and LIUV hormesis is that of induced disease resistance. Resistance is achieved through the upregulation of defence responses alongside alterations to physiology and metabolism. Such changes include phytoalexin production, delayed ripening and senescence, production of pathogenesis-related (PR) proteins and establishment of physical barriers that inhibit pathogen progression (Ben-Yehoshua *et al*., 1992; D'Hallewin *et al.,* 1999; D'Hallewin *et al.,* 2000; Mercier *et al.,* 2000; Romanazzi *et al.,* 2006; Charles *et al.,* 2008a; Charles *et al.,* 2009). PR proteins that have been shown to be induced or increase in concentration following LIUV treatment include chitinases and β-1,3-glucanases (Charles *et al.,* 2009). Such PR proteins interact directly with pathogens causing cleavage of their cell wall components leading to loss of viability (Ebrahim *et al.,* 2011).

Upon treatment with biotic and abiotic factors, defence-related genes can either be constitutively upregulated or primed locally or systemically, as reviewed by Goellner & Conrath (2008), Walters & Fountain (2009) and Walters *et al.* (2013). Priming in plants plays an important role in both induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Conrath *et al.,* 2015). The first instance of gene priming was observed following exogenous dichloroisonicotinic or salicylic acid (SA) application to parsley (*Petroselinum crispum*) cell culture (Kauss *et al.,* 1992). Priming allows the host to upregulate/downregulate defence-related genes, in response to biotic or abiotic stress, at a faster pace and to a greater extent (Conrath *et al.,* 2015). Such a response is facilitated through changes in epigenetic control including DNA methylation and histone modification; two processes involved in chromatin remodelling (Dowen *et al.,* 2012; Espinas *et al.*, 2016).

A further benefit of hormesis in tomato fruit is that of increased nutritional content through changes in secondary metabolism. Changes to secondary metabolism have been observed on a wide range of LIUV-treated fruit including tomato (*Solanum lycopersicum),* blueberries (*Vaccinium*

corymbosum), grapefruit (*Citrus paradisi*) and mango (*Mangifera indica*) (D'Hallewin *et al.,* 2000; González-Aguilar *et al.,* 2007, Perkins-Veazie *et al.,* 2008; Jagadeesh *et al*., 2011). Both HIPPL and LIUV treatments significantly increase total carotenoid and phenolic content as well as the antioxidant activities of tomato fruit (Liu *et al.,* 2009; Liu *et al.,* 2012; Pataro *et al.,* 2015). To date, however, little is known of the molecular mechanisms underpinning HIPPL hormesis in tomato fruit.

The aim of this investigation was two-fold: the first was to explore whether LIUV and HIPPL treatments induce disease resistance through similar changes in gene expression, and to identify which of the main defence signalling pathways, SA, jasmonic acid (JA) and ethylene (ET), are involved. Secondly, gene expression profiles were monitored following inoculation to determine whether genes undergo priming following treatment.

2. Materials and Methods

2.1 Plant Material

Tomato fruit, cv. Mecano, were grown in a commercial glasshouse at APS Salads (Middlewhich, UK), picked at the mature green developmental stage and delivered at ambient temperature to the University of Nottingham within 24 h of harvesting. Fruit were sorted to remove those showing surface damage or deviation from the desired developmental stage and size.

2.2 LIUV and HIPPL Treatment

Upon arrival, tomatoes were randomly assigned to treatment groups and treated at room temperature on the same day. Fruit received exposure on two sides through 180 ° axial rotation following the protocols described by Scott *et al.* (2017). LIUV treatments were carried out using a U-shaped amalgam UV source (UVI 12OU2G11 CP15/469) housed within an anodised aluminium

parabolic reflector. The source was obtained from Dr Hőnle AG, Gräfelfing, Germany. Doses of 3.7 kJ/m^2 were delivered at an intensity of 20 W m^{-2} based upon the findings of Charles *et al.,* (2008b).

HIPPL treatments were carried out with a XENON LH-840 16" ozone-free B lamp. The lamp was powered and controlled by the RT-847 cabinet and RC-802 controller, supplied by Lambda Photometrics (Harpenden, UK). The source emitted 505 J of energy per pulse with a pulse width of 360 µs at 3.2 pulses/s. Spectral emissions of the source ranged from 240 nm to 1050 nm. Fruit were placed at a distance of 10 cm from the window of the lamp housing. Using information provided by the manufacturer it is estimated that 4.6 kJ/m^2/pulse was delivered at fruit level.

After treatment, fruit were stored in the dark until sterilisation. Sterilisation was performed immediately following the completion of treatments. Tomatoes were immersed in 2 % Sodium hypochlorite (Sigma-Aldrich) for approximately 5 to 10 s. This prevented the growth of naturally occurring phytopathogens during the incubation period. Fruit were then rinsed three times in sterile distilled water (SDW), dried and immediately incubated in the dark at 13 °C at a relative humidity > 98 %. Sterilisation was performed in indirect ambient light to prevent photoreversal.

2.4 Pathogen Maintenance, Inoculum Preparation and Inoculation

A *Botrytis cinerea* culture, originally isolated from a plant of the genus *Rosa*, was supplied from the University of Nottingham's fungal collection. Cultures were grown at room temperature on potato dextrose agar (Sigma-Aldrich) supplemented with Penicillin G sodium salt (Sigma-Aldrich) at 33 mg/L and Streptomycin sulphate salt (Sigma-Aldrich) at 133 mg/L. A calibrated spore solution was made from 10 - 14 d old cultures following Scott *et al*. (2017). At 10 d post treatment (10 DPT) artificial inoculations were performed on control and treated fruit. This interval was chosen based upon Charles *et al.,* (2008b) who demonstrated near optimal disease control at 10 d following LIUV treatment. Inoculations were performed by wounding the fruit with a sterile hypodermic needle to a depth of 3 mm. A 10 µl aliquot of *B. cinerea* spores at 1x10^6 spores/ml was pipetted into the

wound. Fruit were stored at 21 °C following inoculation.

2.5 Sampling, RNA Extraction and Reverse Transcription

A No.2 cork borer (6.25 mm outer diameter) was used to take a 50 to 75 mg sample of pericarp from tissue directly facing the light sources. Samples were placed into microcentrifuge tubes and immediately frozen in liquid nitrogen. Samples were stored at - 80 °C until required. Twenty four hours before tissue homogenisation a single 4 mm steel bead (Qiagen) was cooled in liquid nitrogen and added to each microcentrifuge tube. Samples were placed into a Tissuelyser II (Qiagen) block and stored at - 80 °C overnight. Samples were homogenised using two runs of a Tissuelyser II (Qiagen) at 30 Hz for 1 min. Homogenised samples were stored at - 80 °C until RNA extraction was performed.

RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's guidelines. An on-column DNase treatment was performed with the RNASE free DNASE kit (Qiagen). A further off-column DNase step was performed with the TURBO^{TM} DNase kit (Ambion) following the manufacturer's guidelines. RNA purity and yield was assessed via NanoDrop (Thermo Scientific). All samples were then diluted to a concentration of ≤ 50 ng/µl. RNA integrity was then checked by gel electrophoresis. A 20 µl Reverse transcription reaction was then performed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems) following the manufacturer's protocol. The resulting cDNA was stored at - 20 °C until required.

2.6 qPCR

Two technical replicates were performed for each sample. Each 10 µl reaction contained 5 µl of 2x Fast SYBR® Green master mix (Applied Biosystems) and 2 µl of template cDNA. Primer concentrations and annealing temperatures were as stated in Table 1. Reactions were run on a LightCycler 480 ® (Roche) with a two-step amplification cycle. The cycle was as follows; a pre-

incubation of 10 min at 95 °C followed by 40 cycles of 95 °C for 5 s and the anneal for 45 s. Cycle threshold (Ct) values were calculated utilising the second derivative maximum method. A melting curve was run between 90 °C and 60 °C following amplification to check product specificity. Primers were optimised utilising a pooled sample and a 5-point 5-fold dilution series from which efficiency was calculated (Eq.1). Specificity of products from each primer pair was confirmed by sequencing and NCBI basic local alignment search tool (BLAST) analysis.

$$AE = D^{(\frac{-1}{\nabla})}$$

Equation 1. Amplification efficiency showing efficiency (AE), fold dilution (D) and gradient of the logarithmically plotted dilution curve (∇) (Pfaffl, 2004).

Target gene	Reference	Accession	Product Tm (°C)	Conc. (nm)	Anneal (°C)	Efficiency (%)	Sequence 5'-3'
ACT	Aimé *et al.,* 2008	U60480	75.4	100	60	81.0	F: AGGCACACAGGTGTTATGGT R: AGCAACTCGAAGCTCATTGT
ACO1	Van de Poel *et al.,* 2012	X04792	76.4	500	60	85.8	F: ACAAACAGACGGGACACGAA R: CCTCTGCCTCTTTTTCAACC
CHI9	Aimé *et al.,* 2008	Z15140	78.5	50	58	80.0	F: GAAATTGCTGCTTTCCTTGC R: CTCCAATGGCTCTTCCACAT
CRTRB	Tiecher *et al.,* 2013	SGN-U568606	77.8	500	60	101.4	F: TTGGGCGAGATGGGCACAC R: TGGCGAAAACGTCGTTCAGC
FLS	Tiecher *et al.,* 2013	GI 225321931	71.7	250	60	97.3	F: ATGGAGGCAGCTGGTGGTGAA R: CAGGCCTTGGACATGGTGGATA
GLUB	Aimé *et al.,* 2008	M80608	75.8	100	60	79.3	F: TCTTGCCCCATTTCAAGTTC R: TGCACGTGTATCCCTCAAAA
OPR3	Blanco-Ulate *et al.,* 2013	Solyc07g007870	76.8	300	60	86.0	F: TGGGTTTCCTCATGTGCCAG R: GCAGCTCCAGCAGGTTGATA
PAL	Bovy *et al.,* 2002	M83314.1	74.0	500	60	96.3	F: ATTGGGAAATGGCTGCTGATT R: TCAACATTTGCAATGGATGCA
PG	Xie *et al.,* 2014	X05656.1	74.6	250	58	78.5	F: ATACAACAGTTTTCAGCAGTTCAAGT R: GGTTTTCCACTTTCCCCTACTAA
PR1a	Aimé *et al.,* 2008	AJ011520	80.9	250	58	78.9	F: TCTTGTGAGGCCCAAAATTC R: ATAGTCTGGCCTCTCGGACA

Table 1: Details of the primers used in qPCR

2.7 Experimental Design and Data Analysis

Data was collected from two independent replicate experiments. For each experiment three fruit per treatment group per time point were analysed; n=6. Fruit were sampled before treatment (baseline expression), at 24 h post treatment (HPT), 10 d post treatment (DPT) and 12 h post inoculation (HPI). Each gene of interest was run on its own 384 well plate (Roche) along with a 5-point, 5-fold dilution series that was used to calculate the efficiency of amplification (Eq1). Following amplification qPCR samples were checked for non-specific products (melt curve analysis), Ct values ≥ 35 and technical replicate standard deviations > 0.5. Samples exhibiting these characteristics were considered unfit for further analysis and the data was re-collected. Interplate calibration was performed with a pooled sample to correct for interplate bias (Eq.2). Amplification efficiency was then used to correct Ct values following Eq. 3. Technical replicates were then averaged before further analysis.

$$Ct^{corrected} = Ct - Ct^{IPC} + \frac{1}{N}\sum_{i=1}^{N} Ct^{IPC}$$

Equation 2. Interplate calibration equation. The cycle threshold for any given sample is Ct. The Ct value of the interplate calibrator is Ct^{IPC} and N is equal to the number of plates that are being calibrated between (TATAABiocenter, 2012).

$$CtE = Ct \times \frac{Log10(AE)}{Log10(2)}$$

Equation 3. Efficiency correction of cycle threshold (Ct) values. CtE is the efficiency corrected Ct value and AE is the efficiency of amplification (Kubista & Sindelka, 2007).

Actin was used as the reference gene as in previous UV-C and *B. cinerea* inoculation studies (Liu *et al.,* 2011; Virk *et al.,* 2012; Blanco-Ulate *et al.,* 2013; Tiecher *et al.,* 2013). Following efficiency correction, actin was used to normalise the data giving ΔCt (Eq.4). Data was then normalised to baseline (pre-treatment) gene expression and fold change between treatment groups was calculated following Eq.5. For experiments utilising theoretical copy number, a copy number of 100 was assigned to the baseline gene expression levels and the further data was adjusted accordingly.

$$\Delta CtE = CtE(goi) - CtE(ref)$$

Equation 4. Normalisation of gene of interest with reference gene. CtE(goi) is the efficiency corrected Ct value for the gene of interest and CtE(ref) is the efficiency corrected Ct value for the reference gene (Pfaffl, 2004).

$$Fold\ change = 2^{-(\Delta CtET - \Delta CtEC)}$$

Equation 5. Calculating fold change. ΔCtET is the normalised and efficiency corrected mean Ct value for the treatment group and ΔCtEC is the normalised and efficiency corrected mean Ct value of the control group (Livak & Schmittgen, 2001).

Statistical analysis was performed on the efficiency corrected and normalised Ct values (ΔCt) using statistical software package SPSS 22 (IBM). One-way ANOVA with Tukey's post-hoc testing was performed. Where the homogeneity of variances assumption could not be met, Welch's robust

ANOVA was performed followed by the Games-Howell post-hoc test. Statistical significance is defined as $p \leq 0.05$.

3 Results and Discussion

Expression profiles of genes involved in plant defence, secondary mtabolism and ripening were analysed and compared for HIPPL- and LIUV-treated fruit. The comparison was made over a time course starting with 24 HPT, 10 DPT (immediately before inoculation with *B. cinerea*) and at 12 HPI. The changes in expression at each time point were calculated relative to the baseline expression before treatment.

3.1 Phytohormones and Disease Resistance

Ethylene (ET) is a plant hormone that plays a significant role in the control of ripening and ripening-related susceptibility to *B. cinerea* in tomato fruit (Cantu *et al.,* 2009). ACO (1-aminocyclopropane-1-carboxylic acid oxidase) is involved in the final oxygen-dependant step converting ACC (1-aminocyclopropane-1-carboxylic acid) to ethylene (Hamilton *et al.,* 1991 & Dong *et al.,* 1992). ACO1 is one of five identified ACO enzymes involved in ethylene biosynthesis in tomato (Hamilton *et al.,* 1991; Bouzayen *et al.,* 1993; Sell & Hehl, 2005). In our study, the expression of *ACO1* in control fruit increased during the 10 d storage by approximately 8-fold, which is consistent with *ACO1* increases during normal ripening (van de Poel *et al.,* 2012).

Expression of *ACO1* in treated fruit was shown to be significantly different from that of the control at 24 HPT. Expression levels for HIPPL- and LIUV-treated fruit were both 3.1-fold higher than that of the control (Figure 1). Conversely, at 10 DPT and 12 HPI the levels of *ACO1* in control fruit were 1.2- to 2.2-fold lower. The differences, however, were not statistically significant.

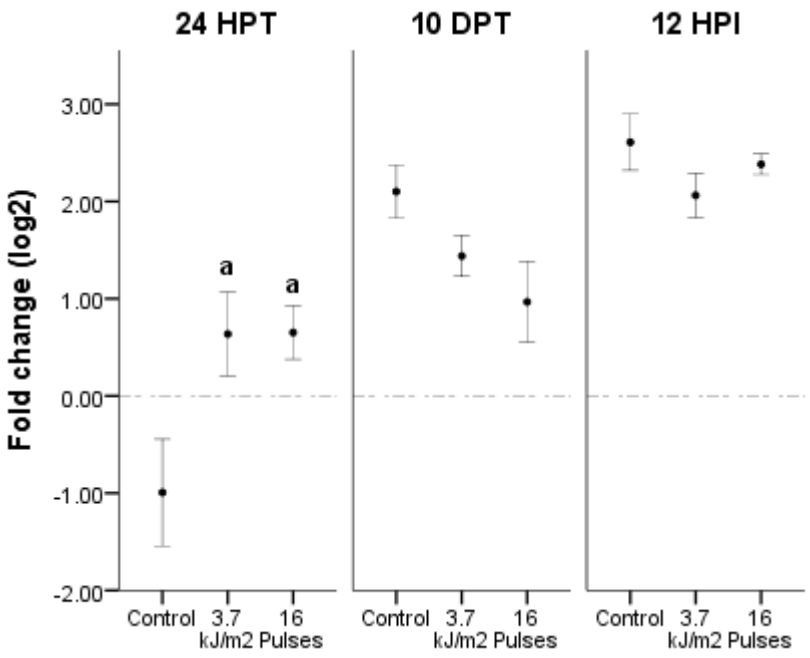


Figure 1: Relative expression of *ACO1* (1-aminocyclopropane-1-carboxylic acid oxidase 1), a bottleneck enzyme in ethylene biosynthesis, following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C source (LIUV). Samples were taken before treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical significance, within a given time point, where groups sharing labels are not significantly different at $p< 0.05$. N=6. Bars show ± 1S.E.M.

The downregulation of *ACO1* at 10 DPT and 12 HPI contributes towards the delayed ripening phenotype observed following HIPPL and LIUV treatment of tomato fruit (Liu et al., 1993; Scott et al., 2017). This is supported by two studies. Firstly, Zhefeng et al., (2008) observed that a reduction in ACO1 mRNA led to delayed ripening (colour change). Secondly, inhibition of *ACO1* was shown to lead to a reduction in ethylene biosynthesis and a prolonged shelf life (Behboodian *et al.,* 2012).

Our results are consistent with those of Maharaj *et al.* (1999) who observed a transient peak in ethylene production at 3 and 5 d after LIUV treatment followed by a lag in ethylene production and a lower maximum ethylene level from the seventh day following treatment. Similarly, Tiecher *et al*., (2013) found that *ACO* was upregulated in both the exocarp and mesocarp of tomato fruit treated with LIUV at 24 HPT while at 7 DPT, expression of *ACO* in the control was greater than that of the LIUV-treated fruit.

JA is a phytohormone whose major roles plants adaptation to herbivorous pests and necrotrophic plant pathogens (Spoel & Dong, 2012). OPR3 (12-oxophytodienoate reductase 3) is the major enzyme catalysing the penultimate enzymatic step in JA biosynthesis, where 9S, 13S-12-oxophytodienoate is reduced to a cyclopentane JA precursor (Schaller *et al.,* 2000; Breihaupt *et al.,* 2006; Bosch *et al.,* 2014).

In HIPPL-treated fruit, a slight downregulation of *OPR3* (<2-fold) at 24 HPT was detected (Figure 2). Expression in control fruit remained at the baseline levels. At 10 DPT a significant increase in *OPR3* expression was observed at 3.8- and 3.9-fold for HIPPL and LIUV treatments in comparison to the control. Following inoculation (12 HPI) *OPR3* expression increased in all groups. Expression, however, was still significantly higher in treated fruit at 2.1- and 2.2-fold for HIPPL- and LIUV-treated fruit, respectively. The initial reduction in *OPR3* expression was analogous to the results observed by Liu *et al.* (2011) which showed a 3.9-fold reduction in *OPR2* at 24 HPT following LIUV treatment; no further time points were monitored.

OPR3 upregulation following LIUV and HIPPL treatments can result in increased JA levels and activation of JA-inducible plant defences which are involved in resistance against necrotrophic pathogens (Glazebrook, 2005). This is supported by Scalschi *et al.,* (2015) who showed that *OPR3* expression determines the availability of 12-oxo phytodienoic acid (ODPA) and expression of major genes involved in JA synthesis (Scalschi *et al.,* 2015). Furthermore, silencing of *OPR3* increased susceptibility to *B. cinerea* and reduced callose deposition in tomato; a defence response against the

invading pathogen (Scalschi *et al.,* 2015). Upregulation of *OPR3,* therefore, contributes towards the control of *B. cinerea* following HIPPL and LIUV treatment; observed previously by Liu *et al.,* (1993) and Scott *et al.,* (2017).

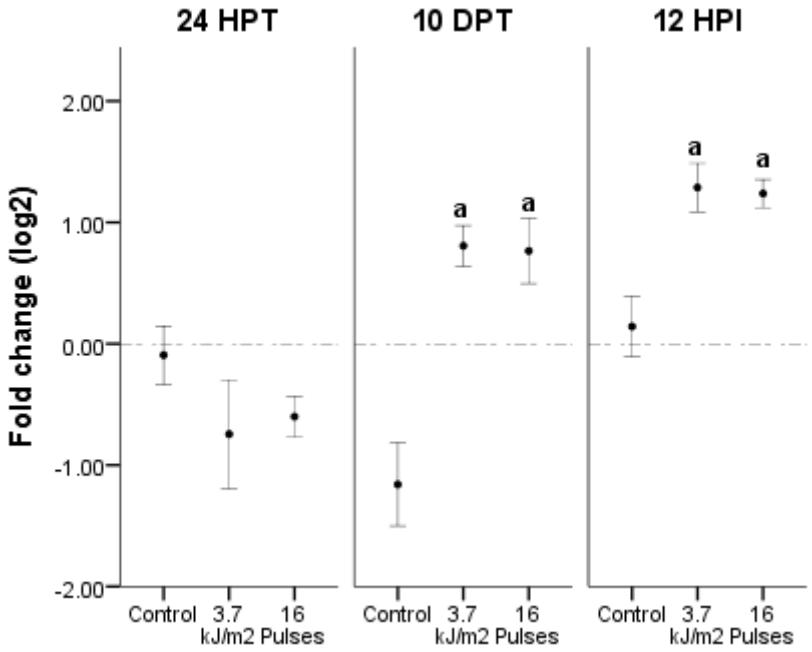


Figure 2: The relative expression of *OPR3* (12-Oxophytodienoate reductase 3), a jasmonate biosynthesis enzyme, following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical significance, within a given time point, where groups sharing labels are not significantly different at $p< 0.05$. N=6. Bars show ± 1S.E.M.

SA is a phytohormone which plays a major role in defence against biotrophic pathogens, insect pests and abiotic stress, it is also involved in DNA repair (Spoel & Dong 2012; Yan *et al.,* 2013; Song & Bent, 2014). There are at least two biosynthesis pathways for the production of SA (Lee *et al.,* 1995). It was, therefore, decided that an SA-inducible product would be monitored to infer changes in SA biosynthesis. P4 (PR1a) is a salicylic acid-inducible PR protein and marker of SAR.

P4 expression was increased in comparison to the control at each of the time-points (Figure 3). The differences, however, were only significant at 10 DPT and 12 HPI. *P4* levels in LIUV- and HIPPL-treated fruit were 50.3- and 55.5-fold and 38.0- and 35.5-fold higher than that of the control at 10 DPT and 12 HPI, respectively. Our results indicate that both HIPPL and LIUV treatments induce SA signalling following treatment.

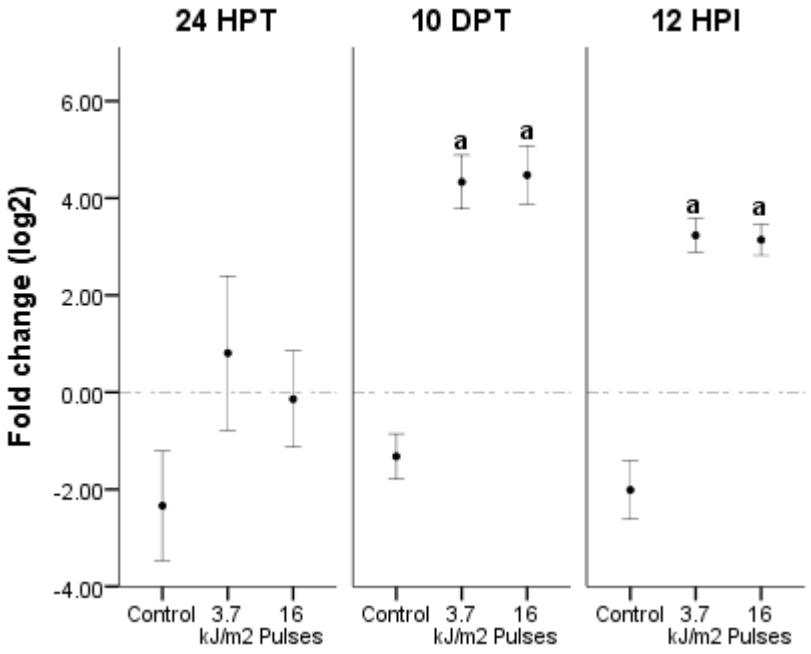


Figure 3: The relative expression of *P4* (*PR1a*), a salicylic acid-inducible pathogenesis-related protein and marker of systemic acquired resistance (SAR), following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light source (HIPPL) or 3.7 kJ/m^2 from a low-intensity UV-C source (LIUV). Samples were taken before treatment, 24 h post treatment (HPT), 10 d post

318 treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2)
319 are relative to baseline expression before treatment (dotted line). Labelling indicates statistical
320 significance, within a given time point, where groups sharing labels are not significantly different at
321 $p< 0.05$. N=6. Bars show ± 1S.E.M.

322

323 SA, however, has been shown to only play a small part in resistance against *B. cinerea*. In work
324 undertaken by Asselbergh *et al. (*2007) tomato plants expressing the bacterial gene *nahG*, which
325 cannot accumulate SA, were shown to be slightly more susceptible to *B. cinerea*. SA and P4,
326 however, play a greater role in protecting the plant against biotrophic pathogens (Glazebrook,
327 2005). The results may, therefore, indicate that HIPPL and LIUV hormesis could potentially be used
328 as a means to protect against a broad range of pathogens.

329 β-1,3-Glucanases play a number of important roles in the plant from regulating germination to
330 defence against pathogen attack. Here we observed significant upregulation in the expression of a
331 basic, intracellular, 33 kDa, ethylene-inducible and PR β-1,3,-Glucanase (*GLUB*) (van Kan *et al.,* 1992;
332 Aimé *et al.,* 2008).

333 Levels of *GLUB* were similar in all groups at 24 HPT (Figure 4). At 10 DPT, however, expression of
334 *GLUB* was increased 32.4- and 40.1-fold in HIPPL- and LIUV-treated tomato fruit, respectively. *GLUB*
335 expression increased by approximately 32-fold and 2-fold for control and treated samples following
336 inoculation (12 HPI). Expression levels in both HIPPL- and LIUV-treated fruit, however, remained
337 significantly higher than the control with 2.1- and 2.2-fold differences, respectively. A similar pattern
338 in protein expression was observed by Charles *et al.* (2009) on LIUV-treated tomato fruit. They
339 reported the induction of a basic, 33.1 kDa β-1,3,-Glucanase which increased in concentration
340 between 3 and 10 d after treatment and following inoculation with *B. cinerea*. Increased expression
341 of *GLUB* before and after the inoculation may contribute towards the increased disease resistance
342 we observed previously in HIPPL- and LIUV-treated fruit (Scott *et al.,* 2017)

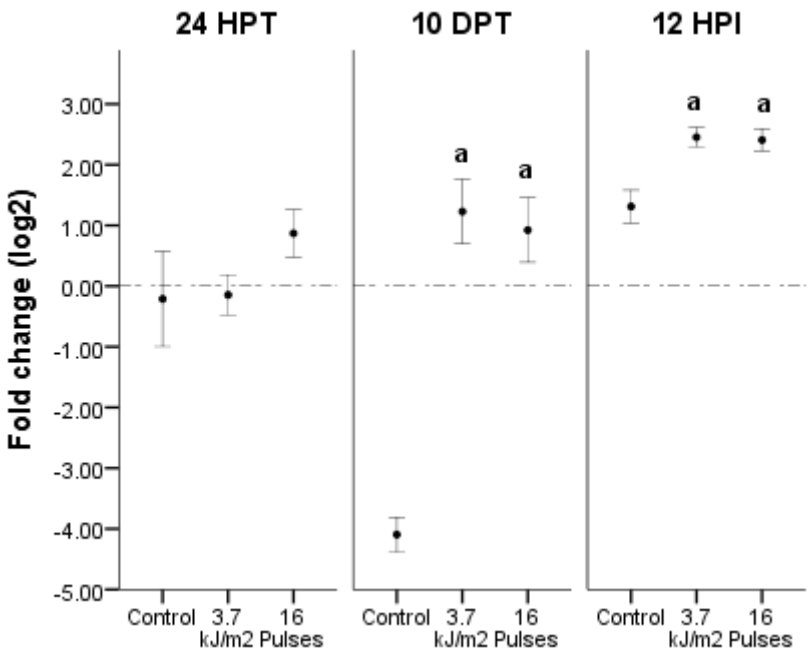


Figure 4: Relative expression of *GLUB* (β-1,3,-Glucanase), an ethylene-inducible pathogenesis related protein transcript, following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical significance, within a given time point, where groups sharing labels are not significantly different at $p< 0.05$. N=6. Bars show ± 1S.E.M.

PR chitinases are involved in the breakdown of glycosidic bonds in the cell wall of fungal pathogens. In this work we monitored the ET-, JA- and wounding-inducible chitinase CHI9 (chitinase I) (Diaz *et al.,* 2002; Wu & Bradford, 2003). CHI9 is upregulated in response to plant pests including the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* and the necrotrophic pathogen *B. cinerea* (Puthoff *et al.,* 2010; Levy *et al.,* 2015).

Expression profiles observed for *CHI9* were similar to *GLUB*. At 24 HPT a slight increase in *CHI9* expression was detected in HIPPL- and LIUV-treated fruit, while expression in the control decreased below baseline (Figure 5). At 10 DPT a statistically significant increase in expression can be seen with 10.0- and 7.3-fold differences between the control and LIUV and HIPPL treatments, respectively. This was approximately 2-fold above baseline. Following inoculation (12 HPI) expression of *CHI9* only increased in the control fruit. The expression in treated samples, however, was still significantly greater than the control at 2.9- and 3.8-fold for the HIPPL and LIUV groups. Our results indicate that disease resistance due to increased chitinase expression is a mechanism shared by both light treatments. The concentration of two chitinases observed by Charles *et al.* (2009) also showed a similar pattern of expression to those observed here. Little change in expression was reported at 3 DPT with upregulation occurring at 10 DPT and following inoculation (Charles *et al.,* 2009). Similarly, we observed an aproximately a 2-fold increase in control fruit expression following inoculation.

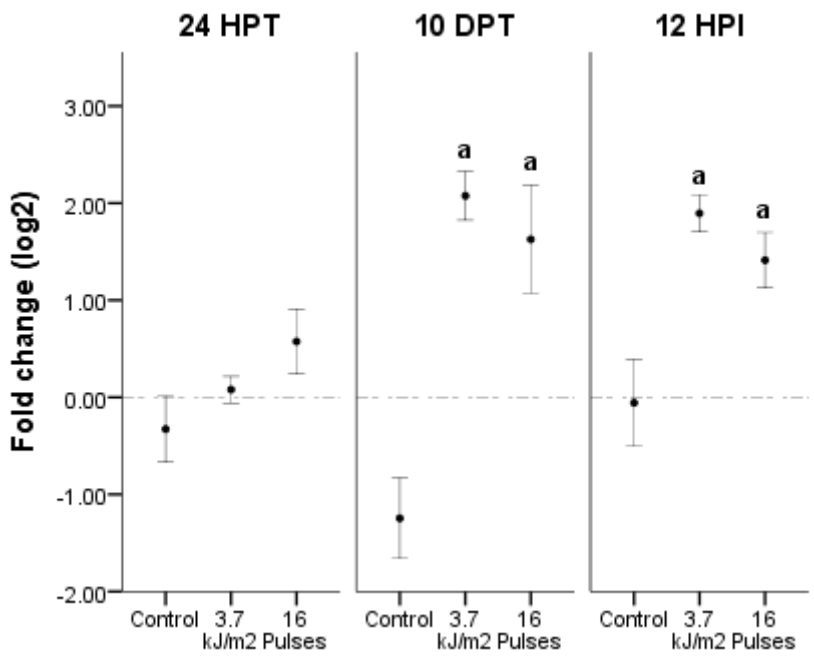


Figure 5: Relative expression of *CHI9* (Chitinase 9), a jasmonic acid-inducible pathogenesis-related protein, following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light

389 (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source. Samples were taken before
390 treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation,
391 and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before
392 treatment (dotted line). Labelling indicates statistical significance, within a given time point, where
393 groups sharing labels are not significantly different at $p< 0.05$. N=6. Bars show ± 1S.E.M.

394

395 The upregulation of JA synthesis gene *OPR3* and PR proteins *P4*, *GLUB* and *CHI9* following HIPPL and
396 LIUV treatment supports the hypothesis that the control of *B. cinerea* is achieved through induced
397 resistance mediated by SA and JA pathways (Liu *et al.,* 1993; Scott *et al.,* 2017). Furthermore, the
398 postulated broad-range resistance is further supported as all three PR proteins are also upregulated
399 in tomato's defence against both the greenhouse and silverleaf whitefly (*Bemisia tabaci* and
400 *Trialeurodes vaporariorum*) and *Fusarium oxysporum* f.sp. *lycopersici* (Puthoff *et al.,* 2010; Aime' *et*
401 *al.,* 2008). HIPPL and LIUV hormesis may, therefore, be an effective pre-harvest alternative to
402 chemical control against both pathogens and pests.

403

404 **3.2 Ripening and Secondary Metabolism**

405 A delay in ripening, through both delayed colour change and texture softening, is a further benefit of
406 LIUV hormesis which leads to extended shelf life and reduced pathogen progression (Bennett *et al.,*
407 1993; Barka *et al.,* 2000). Polygalacturonase (PG) is one of the primary hydrolases involved in the
408 breakdown of pectin in the cell wall during ripening (King & O'Donoghue, 1995). Furthermore,
409 increased polygalacturonase activity elevates tomato's susceptibility to *B. cinerea* (Bennett *et al.,*
410 1993).

411 At 24 HPT, *PG* expression was at baseline levels (Figure 6) which then increased at 10 DPT for all
412 groups. In HIPPL- and LIUV-treated fruit, however, levels of *PG* were significantly lower than the

control with 6.1- and 32.2-fold decreases, respectively. *PG* levels decreased in response to inoculation (12 HPI) with *B. cinerea* in all groups. Fruit from both treated groups, however, still showed significantly lower expression than control fruit with 15.4- and 3.0-fold less *PG* in LIUV- and HIPPL-treated fruit, respectively. Reduced expression of *PG* in HIPPL-treated fruit supports our observations that control fruit were 14.6 and 22.4 % softer than HIPPL-treated fruit at 14 and 21 DPT (unpublished data). Furthermore, Barka *et al.,* (2000) showed a reduction in PG activity following LIUV treatment. The reductions in *PG* are, therefore, likely to play a role in the delayed tissue softening observed following LIUV (Liu *et al.,* 1993) and HIPPL treatments. This is supported by Langley *et al.,* (1994) who showed that silencing of *PG* reduced tissue softening of tomato fruit.

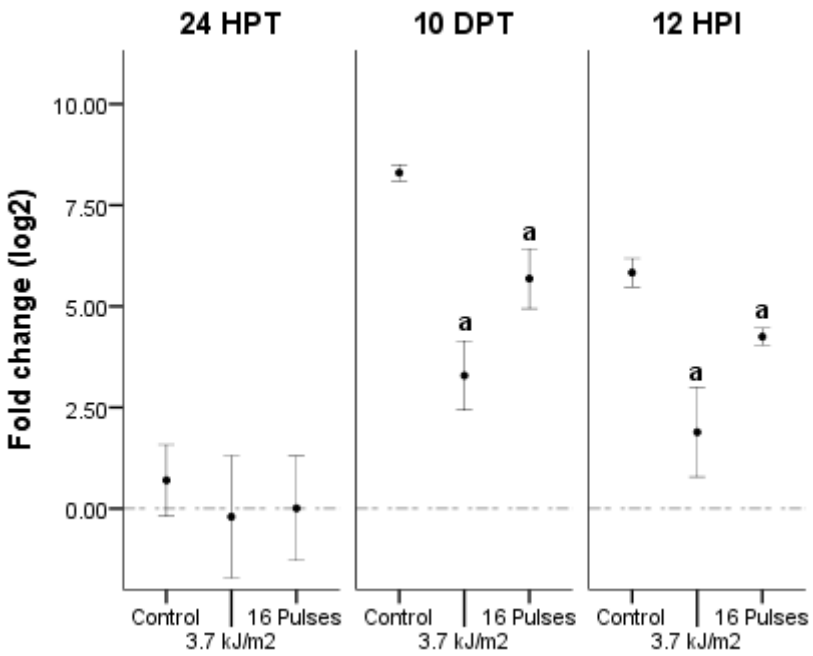
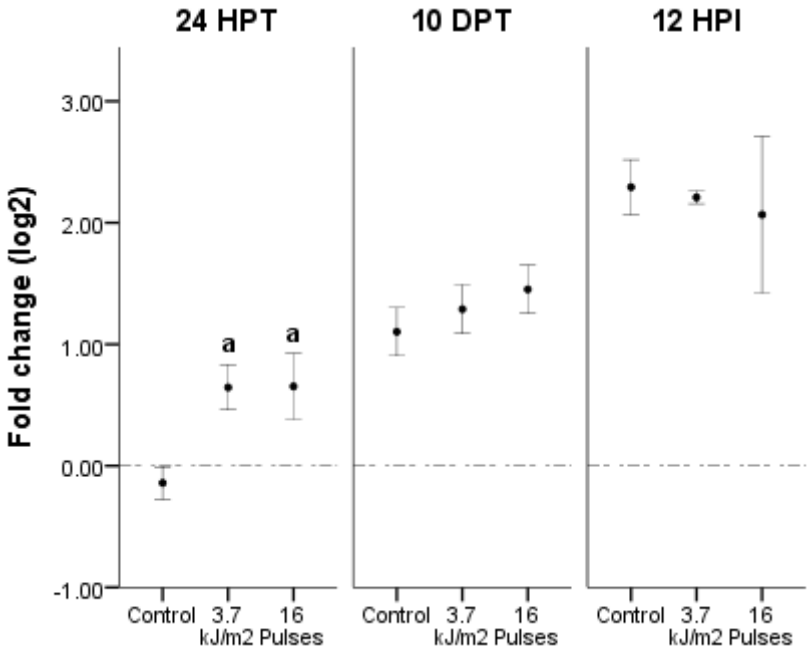


Figure 6: The relative expression of *PG* (polygalacturonase) following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical

significance, within a given time point, where groups sharing labels are not significantly different at $p < 0.05$. N=6. Bars show ± 1S.E.M.

Carotenoids are organic molecules responsible for the red, orange and yellow pigmentations found in flowers and fruits (Yuan *et al.,* 2015). The carotenoid, β-carotene, gives rise to the orange pigmentation in tomato fruit and is synthesised from the cyclisation of lycopene; the major carotenoid in tomato fruit which gives rise to their red colour (Pecker *et al.,* 1996; Tadmor *et al.,* 2005; Yuan *et al.,* 2015). Here, we monitored the expression of β-carotene hydroxylase (*CRTR-B1*) involved in β-carotene modification producing the xanthophylls zeaxanthin and lutein which impart a yellow pigmentation to plant organs (Galpaz *et al.,* 2006). These carotenoids are also found in the retina of the human eye, and their uptake through food can lower the risk of age-related macular degeneration of retina (Mares-Perlman *et al.,* 2002).

We have shown a significant 1.7-fold increase in *CRTR-B1* expression in HIPPL- and LIUV-treated fruit 24 HPT (Figure 7). At 10 DPT and 12 HPI, however, expression of *CRTR-B1* was not significantly different from that of the control. Analogous patterns of *CRTR-B1* expression along with zeaxanthin and lutein concentrations were observed by Tiecher *et al.* (2013) who reported increases in both at 1 d following LIUV treatment, and similar levels to the control at 7 DPT.



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461 **Figure 7:** Relative expression of *CRTR-B1* (β -carotene hydroxylase) following treatment with either
462 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low
463 intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT), 10 d
464 post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes
465 (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical
466 significance, within a given time point, where groups sharing labels are not significantly different at
467 $p< 0.05$. N=6. Bars show ± 1S.E.M.

468

469 The total phenolic content of tomatoes has been shown to increase following treatment with LIUV
470 (Liu *et al.,* 2009). Phenylalanine ammonia-lyase (PAL) is involved in the biosynthesis of phenolic
471 compounds. It also plays an important role in SA biosynthesis. Furthermore, phenolic compounds
472 can act as phytoalexins involved in pathogen defence, free radical absorbers and light quenchers
473 (Pietta, 2000; Sourivong *et al.,* 2007; Lev-Yadun & Gould, 2009).

474 At 24 HPT, expression of *PAL* was approximately at baseline levels in all groups (Figure 8). Following
475 10 d of storage and immediately before inoculation (10 DPT) a slight increase in *PAL* expression, in
476 comparison to the control, was observed for the treated fruit with 1.4- and 1.5-fold increases for
477 HIPPL and LIUV treatments, respectively. The differences, however, were not significant. Following
478 inoculation (12 HPT) *PAL* expression was significantly greater for both HIPPL and LIUV with a 2.0- and
479 2.1-fold increase in comparison to the control, respectively. An increase in the expression of *PAL*
480 following inoculation indicates upregulation of the phenylpropanoid pathway as PAL catalyses its
481 first step converting phenylalanine to cinnamic acid. With products including SA, flavonols and
482 anthocyanins, upregulation of the phenylpropanoid pathway following inoculation may allow

483 treated fruit to respond to pathogens faster than the control fruit resulting in effective disease
484 control as observed by Liu *et al.,* (1993) and Scott *et al.,* (2017).

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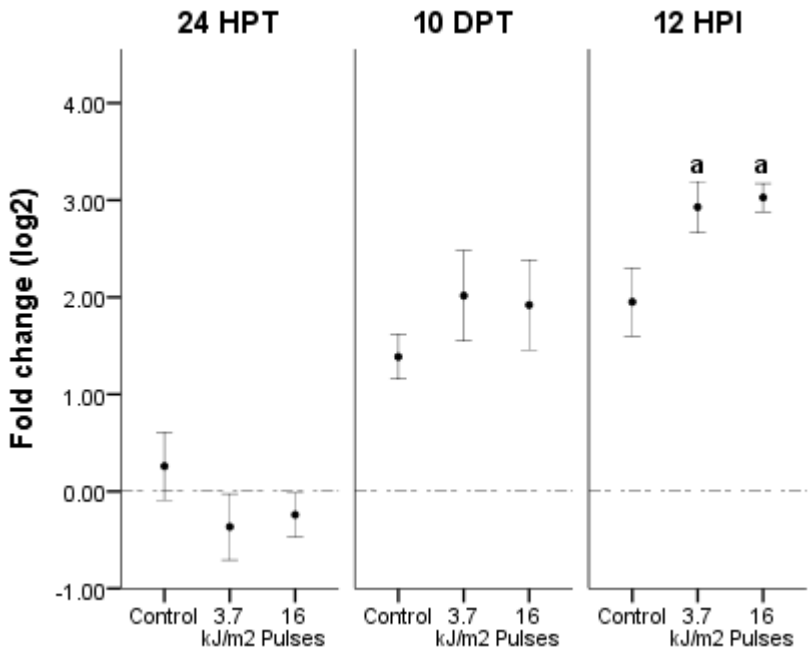


494 **Figure 8:** The relative expression of *PAL* (phenylalanine ammonia lyase) following treatment with
495 either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a
496 low-intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT),
497 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold
498 changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates
499 statistical significance, within a given time point, where groups sharing labels are not significantly
500 different at $p < 0.05$. N=6. Bars show ± 1S.E.M.

501

502 The results of this study are in agreement with Tiecher *et al.* (2013) who showed an approximately
503 2- to 3-fold increase in *PAL* in the mesocarp of tomato fruit following LIUV treatment at both 1 and 7

DPT. The exocarp, however, showed no increase in *PAL* at either 1 or 7 DPT. *PAL* expression, however, was not monitored following inoculation.

Flavonols are a group of phenolic flavonoid antioxidants which have recently been targeted for enrichment in genetically modified tomato for their health-promoting benefits (Choudhary *et al.,* 2016). Following LIUV treatment, total phenolic and flavonoid concentrations have been shown to increase. Flavonol synthase (*FLS*) is directly involved in biosynthesis of flavonols, compounds with important roles in plant-pathogen interactions due to their antioxidant properties.

FLS expression was decreased at 24 HPT with 5.8- and 2.5-fold higher concentration in the control fruit when compared to the LIUV and HIPPL treatments, respectively (Figure 9). Only the LIUV treatment was significantly different from the control. At 10 DPT, *FLS* expression further decreased with the HIPPL- and LIUV-treated fruit showing 100.3- and 109.1-fold differences when compared to the control. At 12 HPI, *FLS* expression in the control fruit decreased by approximately 4-fold to baseline levels. Expression for both treatments increased to 8.9- and 10.8-fold below the control for HIPPL- and LIUV-treated fruit, respectively. This was still significantly lower than the control.

Downregulation of *FLS* would result in decreased biosynthesis of flavonols such as myricetin, quercetin and kaempferol. A previous study by Tiecher *et al.* (2013) reported similar results in LIUV-treated tomato fruit where querecetin concentration was measured by HPLC. Decreased levels were observed in both the exocarp and mesocarp at 1 DPT and 7 DPT with an approximately 4-fold decrease in treated fruit in comparison to the control at 7 DPT. Levels of querecetin when the fruit were ripe, however, were greater in LIUV-treated fruit. In contradiction to this, however, Tiecher *et al.,* (2013) showed approximately a 2.5-fold increase at 1 DPT and a 10-fold increase in *FLS* expression at 7 DPT following treatment with LIUV.

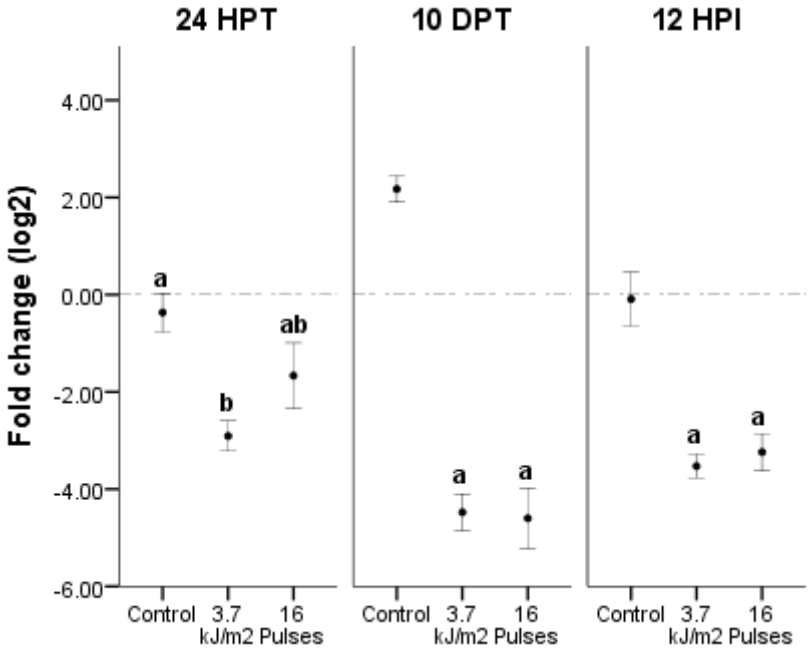


Figure 9: Relative expression of *FLS* (flavonol synthase) following treatment with either 16 pulses from a high-intensity, pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source. Samples were taken before treatment, 24 h post treatment (HPT), 10 d post treatment (DPT), immediately before inoculation, and 12 h post inoculation (HPI). Fold changes (log_2) are relative to baseline expression before treatment (dotted line). Labelling indicates statistical significance, within a given time point, where groups sharing labels are not significantly different at $p< 0.05$. N=6. Bars show ± 1S.E.M.

3.3 Gene Priming

It has been shown that both biotic and abiotic inducers of disease resistance can prime plant defences, reducing the impact of subsequent phytopathogen attack (Mur *et al.,* 1996; Latunde-Dada & Lucas, 2001; Cools & Ishii, 2002; Yang *et al.,* 2015). Defence priming is postulated to be an adaptive, low-cost defensive measure activated by a given priming stimulus, in this case HIPPL and

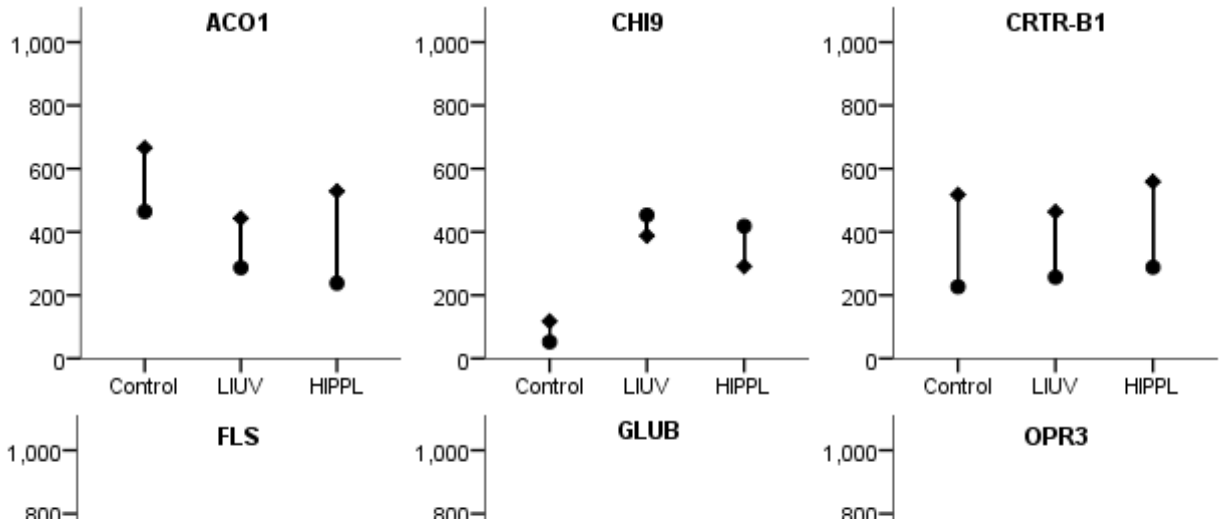
LIUV treatments. In primed plants, transcriptional responses are deployed in a faster, stronger or more sustained manner following the perception of a secondary stress (Martinez-Medina *et al.,* 2016).

Martinez-Medina *et al.,* (2016) defined a number of priming-related expression profile criteria. Firstly, a small or transient change in expression following the initial priming stimulus should be present. To identify this change, we monitored gene expression at 24 HPT. To assess whether changes were transient, samples were taken at 10 DPT, where genes exhibiting priming should show similar levels of expression to the control. Secondly, following exposure to a secondary (trigger) stimulus a faster, stronger or more sustained response should be observed. The trigger stimulus used here was inoculation with *B. cinerea*. Samples were taken at 12 HPI to assess whether a stronger response was observed. Ct values were transformed into theoretical copy number allowing the change in theoretical copy number from 10 DPT to 12 HPI to be calculated.

All of the genes in this study showed small changes in gene expression at 24 HPT; following the priming stimulus (Figures 1-9). Excluding *ACO1*, *CRTR-B1* and *PAL*, all of the genes from LIUV- and HIPPL-treated samples, however, showed an increased change in expression at 10 DPT. This indicates that the changes were not transient and may have an increased fitness cost, this is indicative of direct induction (van Hulten *et al.,* 2006). Following the triggering stimulus only *P4* and *PAL* (from HIPPL and LIUV treated samples) showed a stronger response in gene expression associated with gene priming (Figure 10). *P4*, however, also exhibited an increase in expression at 10 DPT indicating direct induction (Figure 3). Expression levels of *PAL* at 10 DPT, from LIUV and HIPPL treated fruit, is similar to that of the control and, therefore, meets the criteria of a priming-associated expression profile outlined by Martinez-Medina *et al.,* (2016) (Figure 8).

With exception of *PAL* all genes investigated in this study appear to be directly induced and fail to meet the expression profile of gene priming; a summary of the results is available in table 2. Further investigations, however, are required to provide conclusive evidence on whether or not priming is

following the secondary stimulus, analyses of histone modifications and DNA methylation and monitoring the expression of transcription factors (WRKYs and MYC2) and mitogen-activated protein kinases MPK3 and MPK6 for changes that are associated with priming (Conrath *et al.,* 2015). An involvement for priming in LIUV and HIPPL hormesis, however, is supported by further criteria outlined in Martinez-Medina *et al.,* (2016) such as a more robust defence response and broad-spectrum activity. LIUV hormesis has been shown to induce resistance against a number of pathogens on tomato fruit including *B. cinerea, Rhizopus stolonifer*, *Penicillium expansum* and *Alternaria alternata* (Liu *et al.,* 1993; Stevens *et al.,* 1997). Furthermore, HIPPL hormesis can induce resistance against *B. cinerea* and *P. expansum* on tomato fruit (Scott *et al.,* 2017; unpublished data). This is supported by previous work carried out on *Arabidopsis thaliana* in which LIUV-induced resistance to both downy mildew (*Hyaloperonospora parisitica*) and grey mould (*B. cinerea*) was observed (Kunz *et al.,* 2008; Stefanato *et al.,* 2009).



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608 **Figure 10:** Gene expression levels shown as the change theoretical copy number between samples
609 taken at 10 days post treatment (●) and 12 h post inoculation with *Botrytis cinerea* (♦). The vertical
610 line denotes the magnitude of change. Fruit were treated with either 16 pulses from a high-intensity,
611 pulsed polychromatic light (HIPPL) source or 3.7 kJ/m^2 from a low-intensity UV-C (LIUV) source and
612 compared to the untreated control. Graphs show the following genes; ***ACO1*** (1-aminocyclopropane-
613 1-carboxylic acid oxidase; a bottleneck enzyme in ethylene biosynthesis), ***GLUB*** (β-1,3,-Glucanase an
614 ethylene-inducible pathogenesis-related protein) , ***CHI9*** (chitinase 9 a jasmonic acid-inducible
615 pathogenesis-related protein) ***CRTR-B1*** (β -carotene hydroxylase), ***FLS*** (flavonol synthase), ***OPR3*** (12-
616 Oxophytodienoate reductase 3, a jasmonate acid biosynthesis protein), ***PAL*** (phenylalanine
617 ammonia lyase), ***PG*** (polygalacturonase), ***P4*** (a salicylic acid-inducible pathogenesis-related protein).

618

Table 2: Gene priming expression profile identifier summary. Criteria are defined as A) a small change following the priming stimulus B) a transient change following the priming stimulus and C) a stronger response following the triggering stimulus; as defined in Martinez-Medina *et al.,* (2016).

Gene	A	B	C	Potential priming response
ACO1	1	1	0	0
CHI9	1	0	0	0
CRTR-B1	1	1	0	0
FLS	1	0	0	0
GluB	1	0	0	0
OPR3	1	0	0	0
P4	1	0	1	0
PAL	1	1	1	1
PG	1	0	0	0

0 = No and 1 = Yes

The observed HIPPL- and LIUV-induced resistance may, therefore, be mainly due to increased expression and/or accumulation of transcripts between treatment and the day of inoculation (10 DPT). This would result in a gradual increase in resistance following light treatment, similar to that observed by Charles *et al.* (2008) following LIUV treatment of tomatoes. Priming, however, may also play a role in the induction of resistance as an expression profile analogous to that of a priming response can be seen for *PAL.* It is also possible that the priming may have occurred before or after 12 HPI was, therefore, not identified in our study. Priming responses have shown greater levels of protein activity and gene expression > 3 h following inoculation (Mur *et al.,* 1996; Latunde-Dada & Lucas, 2001; Cools & Ishii, 2002; Yang *et al.,* 2015). Further investigation is required to elucidate the full extent to which priming may play a role in LIUV- an HIPPL-induced resistance.

4. Conclusions

In our previous study (Scott *et al.,* 2017) we showed that 16 pulses of HIPPL induced similar hormetic benefits to a 3.7 kJ/m^2 LIUV treatment on both mature green and ripe tomatoes. Utilising HIPPL

638 reduced treatment times by 97.3 % to only 10 s. In this study, we have monitored the expression of
639 genes involved in ripening, secondary metabolism and defence following HIPPL and LIUV treatments.

640 On the basis of the genes monitored here, we are now able to confirm that the HIPPL and LIUV
641 sources elicit similar transcriptional changes following treatment. *GLUB*, *P4*, *CHI9* and *OPR3* were
642 significantly upregulated at 10 DPT and 12 HPI. *PG* and *FLS* were significantly downregulated at 10
643 DPT and 12 HPI. *ACO1,* and *CRTR-B1* were only significantly upregulated at 24 HPT whereas *PAL* was
644 significantly upregulated at 12 HPI. Following inoculation, only *PAL* showed an expression profile
645 analogous to that of a gene priming response. Further investigation is required to conclusively
646 confirm the presence of gene priming.

647 Importantly, we can infer that HIPPL-induced resistance, similarly to that of LIUV, is due to the
648 upregulation of PR proteins including P4, GLUB and CHI9. Moreover, a reduction in *PG* and *ACO1*
649 expression may contribute towards delayed ripening and reduced susceptibility to *B. cinerea* in
650 HIPPL- and LIUV-treated tomato fruit (Barka *et al.,* 2000; Scott *et al.,* 2017).

651 Changes in the expression of phytohormone biosynthesis genes *OPR3* and *ACO1* and SA-inducible
652 gene *P4* elucidates that both LIUV and HIPPL treatments trigger multiple defence responses
653 controlled by ET, JA and SA. The upregulation of ET and JA-inducible *GLUB* and *CHI9* further supports
654 this. This indicates that HIPPL and LIUV hormesis may provide broad range pathogen resistance
655 against biotrophic and necrotrophic pathogens and also abiotic stressors.

656

657 **Acknowledgements**

658 The study was financed by the Agriculture and Horticulture Development Board's Horticulture
659 Department (AHDB Horticulture) Project Code- PE 023 and Loughborough University. Tomato fruit
660 were provided by APS Salads (UK) to whom thanks are extended. All experimental studies were

carried out in The University of Nottingham's Plant Science department. Finally, thanks are due to Abra Millar for her grammatical input.

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