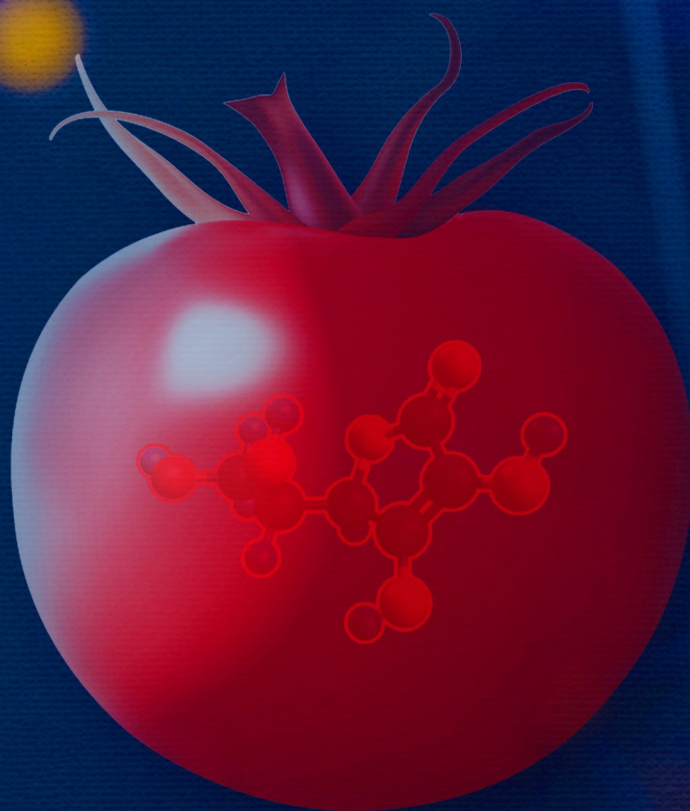# Lighting the way for increased

vitamin C

in tomatoes

Nikolaos Ntagkas

Propositions

1. Illuminating tomato fruits improves their vitamin C content.
   (this thesis)

2. Light regulation of vitamin C in tomato fruits is mediated through photosynthesis.
   (this thesis)

3. The limited attention currently given to the exploration of space will ultimately lead to a plateau in scientific and technological development.

4. The emergence of a technological singularity will bring vast alterations to the current lifestyle that humans ought to prepare for.
   (B. Goertzel 2007 Artif. Intell. 171, 1161–1173)

5. Simplifying scientific messages in order to reach the average person does not undermine science but inspires a science-centred society.

6. Scientists cannot perform good science if they are driven only by a sense of duty or personal benefit.

Propositions belonging to the thesis, entitled
Lighting the way for increased vitamin C in tomatoes

Nikolaos Ntagkas
Wageningen, 3 July 2020

Lighting the way for increased vitamin C in tomatoes

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Lighting the way for increased vitamin C in tomatoes

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To Shuang.
We are earthlings looking for answers,
and in the process we found love.

Contents

Chapter 1

General Introduction

1.1 L-ascorbate, a metabolite essential for human health

Vitamin C (ASC; L-ascorbate; CID 54670067; originally called hexuronic acid) is an organic molecule with the formula $C_6H_8O_6$. ASC was discovered in 1912 and firstly synthesized in the lab in 1933. It is found in two enantiomer forms the L-ascorbic acid and the D-ascorbic acid. Out of the two, the L- form is more common in plant tissue. The D- form is easily synthesized in the lab but has no significant function in biological systems. ASC is a white solid in its pure form. It is solvable in water which results in solutions of mild acidity and it is a mild reducing agent. Glucose is a precursor of ASC and these two compounds are structurally related to each other.

ASC deficiency is a pathological condition known as scurvy. It has been observed and documented since the ancient years (Stone, 1966). ASC regulates the synthesis of several proteins including collagen (Sunada et al., 1988). Furthermore, presence of ASC in cell culture of human cells results in stabilisation of collagen (Davidson et al., 1997). Consequently, scurvy has a wide range of symptoms manifested mostly through events that relate to collagen instability and thus loss in the integrity of membranes. Scurvy had already been observed early in the modern era (13th century) mostly in groups of people that experienced poor diet such as voyagers and sailors. ASC had paramount importance in exploration and development of civilisation. A diet rich in citrus fruits (and other fruits and vegetables containing considerable amounts of ASC) has been observed since then to have a reversible effect on scurvy.

Even though scurvy is currently rare (Agarwal et al., 2015), ASC remains in the spotlight of research as it has been related to very important physiological disorders of the human body. ASC is very well known for its antioxidant function as it is a direct scavenger of reactive oxygen species (ROS). Its protective function over cell membranes is also indirect by regenerating the antioxidant form of vitamin E (Beyer, 1994). In certain cases ASC acts as a pro-oxidant (Chen et al., 2005, 2007). Through in vitro studies ASC has been proposed to have high potential for cancer prevention and treatment in humans (Chen et al., 2005, 2007; Du et al., 2012). ASC deficiency has also been related to other diseases such as diabetes mellitus and the coronary heart disease (Boekholdt et al., 2006; Li and Schellhorn, 2007; Mandl et al., 2009). ASC plays key roles in the development of the human body by regulating gene expression (Chung et al., 2010; Minor et al., 2013) and as co-factor for enzymatic activity (Hutton et al., 1967). ASC is one of the most well studied phytochemicals and is one of the few phytochemicals for which health claims are officially allowed. As illustrated above it has been related to a wide range of health benefits. However, the allowed health claims are restricted to its beneficial effects on the immune system (EFSA Panel on Dietetic Products, 2010).

Animals including certain primates maintain the ability to synthesize ASC. However, humans are not able to synthesize ASC because the activity of L-gulono-γ-lactone oxidase (the enzyme catalysing the terminal step in L-ascorbate biosynthesis) is

insufficient due to several mutations that occurred during evolution (Chatterjee, 1973; Nishikimi et al., 1994; Asensi-Fabado and Munné-Bosch, 2010; Lachapelle and Drouin, 2011). Therefore, humans rely on external uptake of ASC. The recommended dietary allowance for ASC is 75 to 90 mg/day, depending on various factors such as age and sex (Monsen, 2000). Dietary supplements enriched with high amounts of ASC (up to 1000 mg) have been compared with plant sources to cover the recommended dietary allowance for ASC (Bjelakovic et al., 2004). Uptake of ASC from plant sources is more effective in reducing redox stress in vivo in humans compared to the same uptake from artificial supplements (Inoue et al., 2008). This is potentially due to the presence of other substances that amplify the beneficial effects of ASC: (1) ASC becomes a more efficient ROS scavenger in the presence of flavonoids (2) plants contain elements such as potassium that increase ASC availability in vivo affecting excretion or absorption (Thiel, 2000; Inoue et al., 2008; Fitzpatrick et al., 2012). Plants remain to date the primary natural source of ASC in the human diet and as such there is great scientific interest in increasing the ASC levels of plant tissues.

ASC is an important metabolite for plants as it plays multiple roles for various developmental processes. Higher stress tolerance to the abiotic environment has been attributed to increased ASC levels by genetic modification (Lim et al., 2012). ASC is related to flowering. A delay in flowering of arabidopsis plants was the result of an artificial increase of ASC (Kotchoni et al., 2009). ASC could play a critical role in regulating the wall stiffening process during cell differentiation (de Pinto and De Gara, 2004). ASC is involved in the physiology of seeds. ASC is a regulatory factor of seed germination (Behairy et al., 2012). Furthermore, manipulation of ASC levels through artificial feeding of the immediate precursor of the main biosynthetic pathway (L-galactono-1,4-lactone) resulted in a delay of the programmed cell death in wheat kernels while it improved the kernel quality (Paradiso et al., 2012).

ASC is a highly important metabolite for human health while it has a regulatory function in several processes in plants. Therefore, the ability to manipulate ASC in plants provides the possibility to (1) positively influence human health by improving ASC levels of crops and (2) regulate growth and development of plants and seeds. The potential of breeding for higher ASC in tomato has been illustrated by the identification of plant populations that have the required variability (Markovic et al., 2000; Leiva-Brondo et al., 2012). Nevertheless, breeding for higher ASC would yield results only after a considerable amount of time, as a breeding program typically takes between 7 and 15 years depending on the crop. Current trends in horticulture allow a better control through the abiotic environment (e.g. high technology greenhouses and indoor farming). Lighting technologies for such application are of particular interest not only because of the fact that light has a regulatory effect on ASC (Chapter 2) but also because LED lighting systems are being introduced into production systems. Some of the first descriptions of the effects of light on ASC in plants dates back to the 1940s (Hansen and Waldo, 1944) while the underlying physiological regulation of ASC levels in plants by the abiotic environment is under investigation since the 1960s (Menser,

1964). By expanding our understanding on the physiology behind light regulation of ASC we will be able to utilise the current horticultural systems to improve ASC levels in plants by light applications.

1.2 Light affects the levels of L-ascorbate and other metabolites in plants

Light is an environmental factor that affects ASC levels in plants. In a number of dicot and monocot plants species higher irradiances resulted in higher ASC levels in the leaves compared to those exposed to lower irradiances (Abraham et al., 1970; Bartoli et al., 2006; Li et al., 2009, 2010; Fukunaga et al., 2010; Massot et al., 2012). ASC levels in the fruits also increase when plants are exposed to higher irradiances (Li et al., 2009, 2010). Tomato is not only a preferred crop in the study of light effects on ASC, but also a model plant for fruit bearing crops (Kimura and Sinha, 2008). In a similar fashion to other species and tissues, tomato fruits achieve higher ASC levels when they are exposed to higher irradiances compared to fruits exposed to lower levels (Labrie and Verkerke, 2012; Massot et al., 2012). Reducing the irradiance around the fruits (by local shading) while leaving unaffected the irradiance on the rest of the plant resulted in lower fruit ASC levels (Gautier et al., 2008), indicating that the effects of light on fruits are local and that local biosynthesis determines ASC levels in the fruits rather than import of ASC from the leaves. Furthermore, ASC levels in tomato fruits correlate with irradiance fluctuations through the year (Massot et al., 2010). Characteristics of the spectrum of visible light like the red to far-red ratio (Bartoli et al., 2009) and the intensity of the blue light (Lester, 2006; Ohashi-Kaneko et al., 2007) can affect the ASC levels in leaves. However, spectral effects on ASC levels of tomato fruits are not well documented.

The metabolome of the tomato fruit changes drastically with the progress of development. Tomatoes accumulate lycopene, beta-carotene, lutein, violaxanthin, auroxanthin, neoxanthin, chlorophylls a and b with the progress of growth on the plant. By the appearance of the climacteric respiratory peak, the levels of chlorophylls decrease while lycopene accumulates (Laval-Martin et al., 1975). The climacteric peak is a critical point in development as disruption of the ethylene signal leads to reduced carotenoid accumulation (Alba et al., 2005). These changes in pigments reflect the colour transitions of the tomato fruits from green to yellow (breaker stage) and then red. Accumulation of carotenoids, lycopene and flavonoids is linked to light signalling in apples and grapes (Solovchenko et al., 2006; Takos et al., 2006; Ristic et al., 2007). In tomato fruits, red light induced carotenoids accumulation, a process reversible by far-red light (Alba et al., 2000). Phytochrome signalling is therefore involved in the regulation of the levels of carotenoids. Overexpression of cryptochrome (blue light photoreceptor) via the CRY2 gene leads to accumulation of flavonoids and

lycopene in fruits (Giliberto et al., 2005). Silencing of a positive (LeHY5) and a negative (LeCOP1LIKE) regulator of photomorphogenic responses lead to lower and higher levels of carotenoids (Liu et al., 2004). These facts support the idea that changes in certain metabolites (predominantly pigments) in tomatoes are regulated by light.

1.3 The physiology behind light regulation of L-ascorbate

ASC being an effective ROS scavenger, is an integral part of the plant's natural defence mechanism against redox stress. Physiological processes that result in production of ROS often result in ASC biosynthesis. Electron transport processes like photosynthesis and respiration regulate ASC levels in plants at different biochemical control points. Gene expression of ascorbate peroxidase is regulated by the rate of photosynthetic electron transport. The signal for this regulation has been proposed to be mediated through the redox state of plastoquinone (Karpinski et al., 1997). Indeed photosynthetic inhibitors retard ASC accumulation when arabidopsis leaves are exposed to light (Yabuta et al., 2007). The respiratory electron transport has also been linked to ASC levels. GLDH (the last enzyme in the main biosynthetic pathway for ASC (D-man/L-gal) is located in the inner membrane of mitochondria (Bartoli et al., 2000). Furthermore, GLDH activity is directly coupled with the respiratory transport rate at the level of cytochrome C (Schimmeyer et al., 2016). Application of respiratory inhibitors resulted in a dramatic reduction of ASC levels (Millar et al., 2003; Bartoli et al., 2006). Furthermore, the alternative oxidase respiratory pathway is also linked to ASC levels as AOX overexpressors accumulated more ASC compared to the wild types (Bartoli et al., 2006). Evidence so far proves a close relationship between respiration, photosynthesis and ASC levels in leaves. However, there is less evidence for the involvement of respiration and photosynthesis in regulation of ASC in fruits. Furthermore, it is not yet known whether photosynthesis and respiration are part of the physiological mechanism of light regulation of ASC.

1.4 The biochemistry behind light regulation of L-ascorbate

The levels of ASC in plants are determined by a dynamic equilibrium between the processes of biosynthesis, recycling and turnover. When the rate of the input processes (biosynthesis and recycling) exceed that of turnover, there is ASC accumulation in the tissue. Several biochemical pathways have been identified to exist in plants. The primary ASC biosynthetic pathway (D-man/L-gal) synthesizes ASC from D-glucose through an 11-step pathway (Wheeler et al., 1998). Other biosynthetic pathways have also been proposed to operate in plants. The galacturonate pathway has been characterized in strawberry, as a salvage pathway since it utilises the carbon released from cell wall breakdown (in the form of galacturonate). It synthesizes ASC through a brief pathway

1

which is a bypass of the D-man/L-gal pathway (Agius et al., 2003). As it is related to cell wall breakdown, this pathway is expected to become more active with the progress of development in fruits. Genes encoding steps of two other pathways, the gulose and myo-inositol pathways have also been identified in plants (Wolucka and Van Montagu, 2003; Lorence et al., 2004). Furthermore, ASC recycling is directly linked to glutathione regeneration. The biochemical pathways for ASC turnover (breakdown) have also been described (Nakano and Asada, 1981; Chen et al., 2003). The D-man/L-gal pathway is the described as the main biosynthetic pathway for ASC biosynthesis (Wheeler et al., 1998). The contribution of other biosynthetic pathways is often neglected. Furthermore, the response and contribution of individual biosynthetic pathways to the total ASC pool when ASC is manipulated by light, has never been investigated.

As the main biosynthetic pathway for ASC starts with a simple hexose (D-glucose), it has been proposed that soluble carbohydrate content directly regulates the size of the ASC pool (Smirnoff and Pallanca, 1996; Nishikawa et al., 2005; Badejo et al., 2012). Investigating whether this is indeed the case, is relevant for light regulation of ASC as light will influence soluble carbohydrate content indirectly through affecting the rate of photosynthesis. This hypothesis has been previously tested in a variety of species and tissues with variable results. Sucrose feeding of broccoli inflorescences resulted in a retardation of ASC depletion (Nishikawa et al., 2005) while sucrose or glucose feeding of barley seedlings did not affect ASC levels (Pallanca and Smirnoff, 1999). Besides their substrate function, sugars have also been proposed to regulate ASC levels via signalling effects on gene expression. In tomato fruits the expression of biosynthetic (Badejo et al., 2012), recycling and turnover genes (Nishikawa et al., 2005) are modulated from artificial feeding of sucrose. It has been also proposed that the effects of soluble carbohydrates on ASC are species specific (Massot et al., 2010).

1.5 Light affects fruit quality beyond vitamin C

Apart from ASC also other metabolites beneficial for human health are found in tomato fruits. Lycopene, the pigment responsible for the red colour of the tomato fruits has been proposed to be related to the prevention of cardiovascular diseases (Müller et al., 2016) and certain types of cancer (Ford and Erdman, 2012). Carotenoids, a family of metabolites known for the their characteristic yellow and orange colours, have also been proposed to have beneficial effects on human health (Krinsky and Johnson, 2005) due to their antioxidant function and also due to the fact that certain of these compounds are precursor for vitamin A. Tomato as a typical climacteric fruit undergoes a dramatic change in the rate of respiration during ripening which is accompanied by changes in the sugar content (thus taste), the pigmentation of the pericarp (Liu et al., 2009) as well as the structure of cell walls and therefore the texture of the fruits (Carrari et al., 2006). The above mentioned traits might be affected by the ambient light conditions (irradiance and spectral distribution) during ripening (Zhou and Singh, 2002; Giliberto et al., 2005).

1.6 Aim and content of this thesis

The light environment to which the plant is subjected to, affects aspects of its metabolic profile. The aim of this thesis is to investigate the physiological processes that mediate the effects of light on ASC levels in tomato fruits. Furthermore, the effects of light over the broader metabolome of the tomato fruit are investigated.

The biochemical network for ASC regulation in plants is extensive. Numerous physiological processes are involved in regulation of ASC. In **Chapter 2** a literature review was carried out with the aim to create a universal map of the biochemical pathways for ASC biosynthesis, recycling and turnover and discuss their contribution to the ASC pool and how they are regulated by light. Interactions between biochemical pathways are presented and the underlying homeostatic mechanism is discussed. In the second part of this review, the physiological processes linked to ASC regulation are presented in a network of possible interactions and their possible importance in light regulation of ASC is discussed.

Based on the findings of the literature review, a series of experiments was designed and carried out (**Chapter 3**). A variety of light treatments was applied (irradiance dose-response, time-series in light and darkness, and light quality) with the aim to test the involvement of the proposed physiological processes in light regulation of ASC. All the experiments were carried out with tomato fruits that were detached from the plant. This was done for two reasons: (1) to avoid a potential influence of the rest of the plant on ASC levels of the fruits as it has been previously found that ASC may be transported from the leaves to the fruits (Hancock et al., 2003) and (2) to be able to apply the light treatments under a uniform and controlled environment while maintaining the rest of the environmental factors at constant levels. The light effects were tested over a range of genotypes in order to confirm the universality of the physiological mechanism for light regulation of ASC. Mitochondria and chloroplasts are both essential components of the ASC biosynthetic machinery. In this series of experiments, both the respiratory and photosynthetic traits were quantified and discussed in respect to ASC upregulation by light.

The main biosynthetic pathway for ASC biosynthesis begins from a simple hexose (D-glucose). In **Chapter 4** the physiological relationship between soluble carbohydrates and ASC during light regulation was investigated. The levels of soluble carbohydrates in tomato fruits have been modified via different approaches (fruits load manipulation and artificial feeding) and the response of ASC levels was monitored. It was also studied whether the soluble carbohydrate content of tomato fruits correlated with the ASC levels when the latter was manipulated by light treatments. Furthermore, the precursors of biosynthetic pathways other than the D-man/L-gal have been measured in an effort to analyse the involvement of alternative biochemical pathways that might mediate the regulation of ASC by light.

In **Chapter 5**, the research aim was to identify which ripening aspects of the tomato fruits are affected by the ambient light environment. A broad image of the tomato metabolome as affected by light was produced. Tomato fruits ripened under light and darkness or different spectral quality treatments and the respective changes in the metabolome were monitored through means of metabolomics. A variety of metabolites related to health, taste and visual quality were studied.

Finally, **Chapter 6** consists of a general discussion of the presented research.

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Chapter 2

Light regulates vitamin C in plants:
an integrated view on
physiology and biochemistry

2

L-ascorbate (vitamin C, ASC) is an antioxidant that is essential for the proper function not only of plants but also animals. Light is a major regulatory factor for ASC levels in plants. In this paper, we review the regulation of ASC by light and the involved biochemical and physiological processes. Several biochemical pathways for ASC biosynthesis have been proposed to exist in plants. We aim to determine the contribution of these biochemical pathways on ASC levels and, locate the steps of them that are affected by light. From biochemical and genetic studies only evidence for ASC biosynthesis occurring via the D-mannose/L-galactose biosynthetic pathway was found. Alternative pathways might account for ASC biosynthesis only in transgenic plants. Apart from biosynthesis, recycling and turnover of ASC might affect the size of the ASC pool. Light regulation of ASC levels in plants occurs primarily via effects on biosynthesis. In addition, light affects ASC homeostasis and translocation within the plant. Light regulation of ASC has been studied for individual physiological processes without taking into account possible interactions. By establishing the physiological network behind light regulation of ASC for both leaves and fruits, we developed a novel hypothesis on interactions between the physiological processes that regulate ASC. We conclude that respiration and photosynthesis interact in light regulation of ASC biosynthesis via carbohydrate availability.

Abstract

List of abbreviations

ASC	L-ascorbate
D-man/L-gal	D-mannose/L-galactose
HXK	hexokinase
PGI	phosphoglucose isomerase
PMI	phosphomannose isomerase
PMM	phosphomannomutase
GMP	GDP-D-mannose pyrophosphorylase
GME	GDP-D-mannose 30,50-epimerase
GGP	GDP-L-galactose phosphorylase
GPP	L-galactose-1-P phosphatase
GalDH	L-galactose dehydrogenase
GLDH	L-galactono-1,4-lactone dehydrogenase
GalUR	D-galacturonate reductase
APX	ascorbate peroxidase
MDHAR	monodehydroascorbate reductase
AO	ascorbate oxidase
GR	glutathione reductase
DHAR	dehydroascorbate reductase
MDHA	monodehydroascorbate
DHA	dehydroascorbate
GSH	glutathione
GSSG	oxidized glutathione
UV	ultraviolet radiation
VTC2	GDP-L-galactose phosphorylase gene
AMR1	ascorbate D-man/L-gal pathway regulator 1
GalUR	D-galacturonate reductase
AOX	alternative oxidase
ATP	adenosine triphosphate
PCD	programmed cell death

2.1 Plants are an essential source of ascorbate for humans

L-ascorbate (ASC; vitamin C; CID 54670067) is a multirole chemical compound, essential for proper functioning of the human body. Official health claims for ASC are allowed due to its beneficial effects on the immune system (EFSA Panel on Dietetic Products, 2010). ASC plays multiple roles in the functioning of the human body. As a direct reactive oxygen species (ROS) scavenger it is considered an important antioxidant. ASC protects membrane and other hydrophobic compartments from oxidative damage also indirectly by regenerating the antioxidant form of vitamin E (Beyer, 1994). In some cases

ASC also acts as a pro-oxidant (Chen et al., 2007, Chen et al., 2005). ASC promotes both cell growth and protein synthesis including that of collagen (Sunada et al., 1988). Furthermore, presence of ASC in human cells culture results in collagen stabilization (Davidson et al., 1997). ASC acts as an enzymatic co-factor in numerous cases (e.g. hydroxylation of peptidyl-proline to peptidyl-hydroxyproline; Hutton et al., 1967). Finally, ASC has been reported to positively affect health by epigenetic control of the genome activity (Minor et al., 2013, Chung et al., 2010).

Maintaining ASC above the critical levels is of pivotal importance for human health. Biosynthesis of ASC does not occur in the human body because the activity of L-gulono-y-lactone oxidase (the enzyme catalysing the terminal step in L-ascorbate biosynthesis) is deficient. The respective genes accumulated deleterious mutations with the progress of evolution (Chatterjee, 1973, Asensi-Fabado & Munné-Bosch, 2010, Nishikimi et al., 1994, Lachapelle & Drouin, 2011). Therefore, humans rely solely on external sources of ASC. The beneficial effects of artificial ASC supplements have been questioned (Bjelakovic et al., 2004). Additionally, supply of ASC from plant tissues leads to higher reduction of ROS, than when it is supplied by artificial supplements (Inoue et al., 2008). It was found in guinea pigs that the beneficial function of ASC becomes more efficient in the presence of flavonoids (Cotereau et al., 1948). Specific plant tissues contain substances that increase ASC availability in vivo by affecting absorption or excretion (e.g. potassium; Fitzpatrick et al., 2012, Inoue et al., 2008, Thiel, 2000). As plants are considered the primary source of ASC in human diet, enhancing ASC content of plant tissues is of high importance for sustaining and promoting human health.

ASC also plays a pivotal role for the proper functioning of plant organisms. It has been associated with a variety of physiological processes in plants. ASC is involved in the control of flowering. ASC-deficient arabidopsis mutants (vtc1-1, vtc2-1, vtc3-1, and vtc4-1) flowered and senesced earlier than wild type plants irrespective of the photoperiod. This has been associated with upregulation of the expression of circadian clock and photoperiodic pathway genes. Artificial increase of ASC delayed flowering (Kotchoni et al., 2009). ASC also regulates the development of seeds. Higher ASC induced by L-galactono-1,4-lactone (the immediate precursor of ASC) feeding resulted in delayed ASC depletion as naturally observed with seed maturation. This delay in ASC depletion resulted in delayed programmed cell death in durum wheat (Triticum durum) kernels' storage tissues, delayed kernel dehydration as well as improved kerned filling (Paradiso et al., 2012). ASC has also proved to be involved in seed germination (Behairy et al., 2012). Cell differentiation is another process regulated by ASC. With the transition from meristematic to differentiated cells, ASC levels as well as ASC peroxidase genes change significantly. It has been proposed that ASC in cell walls interferes with secretory peroxidases in the regulation of cell wall stiffening, a processes that occurs during differentiation (de Pinto & De Gara, 2004). Finally, ASC has also been proposed to play a pivotal role in stress tolerance. Transgenic plants with higher ASC levels have typically improved tolerance to abiotic stress (Lim et al., 2012). In fungi an analogue form of ASC (D-erythroascorbate) is found (Baroja-Mazo et al., 2005).

2.2 The necessity for an integrated view

Plants in protected cultivation have lower levels of ASC compared to field crops. This is usually attributed to reduced irradiance levels plants experience in greenhouses (Massot et al., 2010). ASC in plants follows the seasonal variations in irradiance (Massot et al., 2010). Extensive literature confirms that irradiance is an abiotic factor with pronounced effects on ASC levels in plants (section 3). Light quality also affects ASC levels in plants (section 3). Modern protected cultivation provides the opportunity to optimise lighting conditions for plants as supplemental light is a common strategy in high technology greenhouses. Modern technologies (e.g. LEDs) enable the easier, more accurate and more energy efficient regulation of the light environment in the greenhouse. Along with plant breeding it will eventually lead to plant products with improved nutritional properties such as increased ASC levels.

Light regulates ascorbate through different ways. Respiration, carbohydrates and photosynthesis are physiological components involved in the mechanism for light regulation of ASC. Respiration affects the enzymatic activity of a major biosynthetic gene. Carbohydrates are the substrate for ASC biosynthesis. Photosynthesis regulates expression of biosynthetic, recycling and turnover genes. Enhancing ASC in plants by light treatments remains a challenging task. Besides direct effects of light on gene expression, knowledge on the regulation of ASC from respiration, carbohydrates and photosynthesis is essential. Furthermore, a homeostatic mechanism for ASC has been proven to exist in plants. Our knowledge on the physiology of light regulation of ASC levels remains ambiguous. Significant interactions between the underlying physiological processes and how these affect ASC levels have not been studied. There is little doubt that genetic engineering can be the means to enhance ASC. But, achieving higher ASC levels in plants requires also optimisation of the physiological processes involved accounting for any possible interactions among them.

An integrated view on the network of underlying physiological processes (respiration, photosynthesis, carbohydrate availability) is of high relevance for both physiologists and geneticists. The regulatory effects of these physiological processes on ASC are known. However, little is known about how possible interactions between respiration and photosynthesis affect ASC. In this review, we present an overview of the biochemical and physiological network for light regulation of ASC: (1) Through a brief review of the biosynthesis, recycling and turnover pathways we aim to identify control points for ASC and how they are regulated by light. We discuss the contribution of individual pathways to ASC pool. (2) In the second part of the review we aim to describe a novel hypothesis on the interaction between respiration and photosynthesis in light regulation of ASC.

2.3 Light is an important regulatory factor of ASC levels in plants

Presence of light is essential for ASC accumulation (Yabuta et al., 2007). ASC was higher in leaves grown under high irradiance than in leaves grown under shade for both monocots (rice: Fukunaga et al., 2010 and wheat: Abraham et al., 1970) and dicots (arabidopsis; Bartoli et al., 2006b, Dowdle et al., 2007, Gatzek et al., 2002a, kiwi; Li et al., 2010c, tomato; Massot et al., 2012, apple; Li et al., 2009, cowpea; Reid, 1938, grapefruit; Cakmak et al., 1995 and other evergreen species; Grace & Logan, 1996). Similarly, ASC of fruits increases with an increase in ambient irradiance in apple (Li et al., 2009), tomato (Massot et al., 2012, Labrie & Verkerke, 2012) and small berries (Hansen & Waldo, 1944). ASC of tomato fruits also correlates with fluctuations of irradiance throughout the year (Massot et al., 2010). Irradiance is therefore a control factor for ASC in plant tissues.

Higher red – far red ratios enhanced ASC levels in phaseolus vulgaris leaves (Bartoli et al., 2009). Blue light also promotes ASC in a variety of leafy vegetables (Ohashi-Kaneko et al., 2007, Lester, 2006). Consequently the spectral distribution of light (red:far red ratio of 1.1 or pronounced blue fraction) result in the enhancement of ASC. Ultraviolet radiation (UV) enhances ASC levels in soybean sprouts (Xu et al., 2005). Narrower spectral bands of UV-B and UV-C reduce ASC in tomato fruits (Giuntini et al., 2005, Maharaj et al., 2014). Even though UV as a stress factor results in the production of ROS (Mittler, 2002), it does not always result in enhancement of ASC.

2.4 The biochemistry behind ASC regulation and how it is affected by light

2.4.1 Biosynthesis

The existence of four biosynthetic pathways for ASC has been proposed in plants: D-mannose/L-galactose, galacturonate, myo-inositol and gulose pathways.

The D-mannose/L-galactose pathway (D-man/L-gal) was initially proposed by the identification of D-mannose and L-galactose as ASC precursors and GDP-D-mannose-3,5-epimerase (GME; EC 5.1.3.18) as the enzyme that catalyses this interconversion. It is an 11-step pathway that uses D-glucose as initial precursor (Wheeler et al., 1998). The commonality of the precursors as well as studies with several plant species, suggest a common pathway among plants. The D-man/L-gal pathway accounts for the vast majority of ASC found in plants. The genes encoding the enzymes that catalyse all steps have been identified (Conklin et al., 1999, Wolucka & Van Montagu, 2003, Dowdle et al., 2007, Laing et al., 2007, Laing et al., 2004, Gatzek et al., 2002b, Imai et

al., 1998). The first 6 steps of the D-man/L-gal pathway lead to the biosynthesis of cell wall precursors (GDP-D-mannose and GDP-L-galactose).

Gene expression levels from the later steps of the D-man/L-gal correlates with ASC levels: GMP (Badejo et al., 2007, Badejo et al., 2008, Cronje et al., 2012) and VTC4/GPP (Conklin et al., 2006). However, a causal relationship between the expression of these genes and ASC levels via means of genetic modification has been proved only for GME (Gilbert et al., 2009) and VTC2/GGP (Bulley et al., 2012, Laing et al., 2007, Laing et al., 2017). In kiwi co-expression of GME with GGP results in a synergistic increase of ASC, which is 2-fold the increase from overexpression of GGP alone (Bulley et al., 2009). In this case, overexpression of GME alone was found to result in little or no increase of ASC. GalDH overexpression did not correlate with higher ASC levels (Gatzek et al., 2002b). GLDH has also been broadly discussed as a possible control point (Linster & Clarke, 2008, Alhagdow et al., 2007, Tokunaga et al., 2005). The work of Yoshimura (2014) is notable as they monitored gene expression rates of many genes from the D-man/L-gal pathway. In this case, no significant difference was observed in ASC levels between arabidopsis plants transiently expressing PMI1, GMP/VTC1, GME, and GPP/VTC4. ASC levels in the plants transiently expressing VTC2 were 2.5-fold higher than those in non-genetically modified plants. The proposed control points are located at the later steps of the pathway (dedicated part). Control points on the first steps are rarely mentioned. Even though carbohydrates often correlate with ASC levels (section 5.1), a causal relationship has not yet been proven.

Light can alter gene expression patterns, affecting some of the proposed ASC biosynthesis control points of the D-man/L-gal pathway. ASC increased in continuous light and decreased in darkness (Yoshimura et al., 2014). This effect of light was enhanced in overexpressors of several steps of the D-man/L-gal pathway. Seven ASC related genes in leaves and two in ripe fruits (including genes for GMP, GPP, GME and GGP) were downregulated by shading (Massot et al., 2012, Dowdle et al., 2007, Yabuta et al., 2007, Reuhs et al., 2004). Apart from genes involved in different steps of ASC biosynthesis, a number of light-regulated transcription factors have been identified that are part of the upstream signal transduction pathway. AMR1 (ascorbate D-man/L-gal pathway regulator 1) is a transcription factor that negatively affects the last six steps of the D-man/L-gal biosynthetic pathway (GMP, GME, GGP, GPP, GalDH and GLDH). High light intensity induced a decrease in AMR1 transcripts with an accompanied increase in ASC (Zhang et al., 2009).

GGP is able to catalyse the conversions of D-mannose-1-P and GDP-L-galactose to GDP-D-mannose and L-galactose-1-P respectively. The activity of GGP as a GDP-galactose phosphorylase is 10-fold higher compared to its activity as transferase (Linster et al., 2008). Therefore, in vivo GGP most likely acts as a GDP-galactose phosphorylase. This information gave rise to a hypothetical pathway for the synthesis of ASC (VTC2 cycle; Laing et al., 2007). As there is insufficient evidence of its function along with the D-man/L-gal pathway in vivo (Linster & Clarke, 2008), it will not be further discussed in this review.

Degradation of the cell wall in strawberry fruits results in production of Me-D-galacturonate. In a 3-step pathway (galacturonate pathway), Me-D-galacturonate is converted to L-galactonate and thereafter to L-galactono-1,4-lactone, the immediate precursor of ASC. The potential for the in vivo function of this salvage pathway was proposed in strawberry with the identification of the gene encoding D-galacturonate reductase (GalUR), the enzyme catalysing conversion of D-galacturonate to L-galactonate (Agius et al., 2003, Badejo et al., 2012).

GalUR was suggested to be a possible control point (Upadhyaya et al., 2009, Agius et al., 2003). Agius (2003) illustrated a correlation between ASC and GalUR activity. There was also an increase in ASC when GalUR is overexpressed. Feeding galacturonate in ester form results in improvement of ASC in tomato (Badejo et al., 2012) and in arabidopsis cell cultures (Davey et al., 1999). The work of Loewus (1999) contradicts the above. Via critical labelling experiments Loewus proved that a very small proportion of ASC results from uronic acids in strawberries. It is therefore uncertain whether the galacturonate pathway contributes considerably to the ASC pool in plants. The activity of the galacturonate pathway was also found to depend on the developmental stage of the tissue (Badejo et al., 2012). The galacturonate pathway utilises carbon coming out of cell wall breakdown (salvage pathway). Therefore, it could be contributing to the ASC pool only in fruits and it could become active only at later stages of development. This remains still hypothetical. Future work for the determination of the contribution of the galacturonate pathway has to be done across different developmental stages especially in fruits. To date there is no convincing evidence for light effects on the galacturonate pathway.

The myo-inositol pathway has also been proposed to exist in plants. In this pathway myo-inositol is converted to ASC in four steps with immediate precursor L-gulono-1,4-lactone (Lorence et al., 2004). All enzymes that catalyse these reactions have been identified in plants (Lorence et al., 2004, Torabinejad et al., 2009). Enzymatic activity of L-gulono-1,4-lactone dehydrogenase/oxidase (EC 1.1.3.8), the enzyme that catalyses the conversion of L-gulono-1,4-lactone to ASC was detected in potato (Jain & Nessler, 2000). Overexpression of this enzyme in transgenic plants leads to higher levels of ASC (Radzio et al., 2003, Maruta et al., 2010). Overexpression of MIOX4 (the gene encoding myo-inositol oxygenase) in arabidopsis plants resulted in contradicting results. Zhang et al. (2008) observed an increase whereas, ASC did not increase in the work of Endres and Tenhaken (2009). Another study revealed upregulation of ASC with overexpression of MIOX4 only under the external supply of myo-inositol (Zhang, 2012) . This suggests that the endogenous amount of the precursor could be insufficient. The myo-inositol pathway does not account for considerable amounts of ASC in non-genetically modified plants. Its potential has been illustrated only in GMO plants. Further genomic studies are required for a better understanding of the function of the gulose and myo-inositol pathways in plants. Effects of light on these pathways have not been documented.

The last pathway to be discussed is the gulose pathway. GME also catalyses a second epimerisation reaction of GDP-D-mannose that produces GDP-L-gulose, an ASC precursor found in animals. As this reaction is reversible, GDP-L-gulose can be recovered back to GDP-D-mannose. The state of this chemical equilibrium is potentially affected by the activity of the later steps in the gulose pathway (GDP-L-gulose, L-gulose 1-P and L-gulose). The L-gulose pathway was proposed to exist in plants where, GDP-L-gulose is converted to ASC with immediate precursor L-gulono-1,4-lactone (Wolucka & Van Montagu, 2003). However, the existence of the gulose pathway is based on the in vitro assays. Enzymatic activity of L-gulono-1,4-lactone dehydrogenase/oxidase (EC 1.1.3.8), the enzyme that catalyses the conversion of L-gulono-1,4-lactone to ASC was detected in potato (Jain & Nessler, 2000). No possible control points or light effects have been mentioned regarding the gulose pathway.

In conclusion, the D-man/L-gal pathway which is the only that accounts for ASC in non-genetically modified plants, is found to be regulated by light. The contribution of myo-inositol and gulose pathways is considered very minor as it is only described partially or found in transgenic plants. Improving ASC levels via the gulose and myo-inositol pathways in plants has up to now only been achieved through genetic modification. Light regulation of ASC has been related to the expression of genes located on the D-man/L-gal pathway.

2.4.2 Turnover and recycling

Turnover of ASC includes ROS scavenging and non-enzymatic degradation. ASC acts as a ROS scavenger due to its ability to donate electrons. During this catabolic process, ASC is converted to monodehydroascorbate (MDHA). This oxidation is catalysed by ascorbate oxidase (AO; EC 1.10.3.3) and ascorbate peroxidase (APX; EC 1.11.1.11; Nakano & Asada, 1981). Monovalent oxidation of ASC to MDHA is catalysed by iron ions and prolyl-hydroxylase (Borsook et al., 1937, Myllylä et al., 1978, Weissberger et al., 1943). MDHA may be converted back to ASC by the catalytic action of monodehydroascorbate reductase (MDHAR; EC 1.6.5.4; Hossain et al., 1984). The next step of ASC catabolism is the conversion of MDHA to dehydroascorbate (DHA) which is a non-enzymatic reaction. Then it breaks down to oxalate, L-threonate, L-tartrate and other compounds through independent pathways. The L-threonate is enzymatically catalysed to 4-O-oxalyl-L-threonate. This turnover pathway might also operate non-enzymatically. Specific steps in this pathway might generate peroxide which contributes to the role of ASC as pro-oxidant (Green & Fry, 2005). In certain species (e.g. Vitaceae) the catabolism proceeds by the conversion of L-idonate to L-tartrate (Williams and Loewus, 1978). The process of ASC turnover was found to be stimulated by darkness (Truffault et al., 2016).

Recycling of MDHA and DHA back to ASC takes place through two pathways. MDHA is converted back to ASC in a reaction catalysed by MDHAR. Reduction of the relatively unstable DHA is catalysed by a more complex system. DHA is reduced to ASC by

dehydroascorbate reductase (DHAR; EC 1.8.5.1) which also catalyses the conversion of glutathione (GSH) to its oxidised form (GSSG). The oxidation status of GSH is important for ASC recycling as GSH is the reducer of ASC. Therefore, the relevant enzyme glutathione reductase (GR; EC 1.8.1.7) is also important for determining ASC levels. The ASC recycling mechanism in plants has been reported to be operational in many sub-cellular compartments such as mitochondria, chloroplasts, peroxisomes and cytosol (Jimenez et al., 1997, Dalton et al., 1993, Koshiba, 1993, Yamaguchi et al., 1995) with the exception of the apoplast. A summary of all biochemical pathways for ASC is presented in Figure 1.

Recycling and turnover pathways are potential control points for ASC (Chen et al., 2003, Zhang et al., 2011a). APX downregulation and upregulation of DHAR and MDHAR result in ASC accumulation (Zhang et al., 2011a, Chen et al., 2003, Eltayeb et al., 2006, Eltayeb et al., 2007). Gest et al. (2013) illustrated the complexity for regulation of ASC via recycling at the level of MDHAR (reduction of MDHA to ASC). Unexpectedly, tomato overexpressors of MDHAR showed a decrease in ASC in leaves. Lines where MDHAR was silenced had increased ASC levels in both leaves and fruits. No light effects have been reported on the recycling and turnover of ASC. The need to investigate further the potential of genes involved in recycling and ASC turnover has been highlighted by several authors (Li et al., 2010a, Li et al., 2011, Zhang et al., 2011b, Aragüez et al., 2013, Chen et al., 2003, Qin et al., 2011, Eltayeb et al., 2007).

2.4.3 Gaps in understanding the light regulation of ASC

Up to date there is no solid proof that any of the biosynthetic pathways except the D-man/L-gal operate in non-genetically modified plants. Light specifically, affects the later steps in this pathway. The galacturonate and myo-inositol pathways illustrate several possible control points. However, the effects of light on these pathways remain largely unknown. Evidence for the operation of the galacturonate, gulose and myo-inositol pathways is based on correlations between gene expression of biosynthetic genes and ASC levels.

Work to date mostly focuses on biosynthesis. Recycling and turnover are two processes with possible control points. Recycling and turnover could contribute to different extend depending on the species, tissue or developmental stage. However, the activity of the recycling and turnover pathways in different tissues and species has not been investigated by modifying the expression of relevant genes. Light effects on recycling and turnover of ASC are largely unknown. Expanding our knowledge here is essential in better understanding further light regulation of ASC. Studies with genetically modified plants and isotope feeding reveal important biochemical pathways and define specific genes as possible control points.

Upregulation of a gene in ASC biosynthesis does not always result in more ASC possibly due to low availability of other enzymes or precursors in the same pathway.
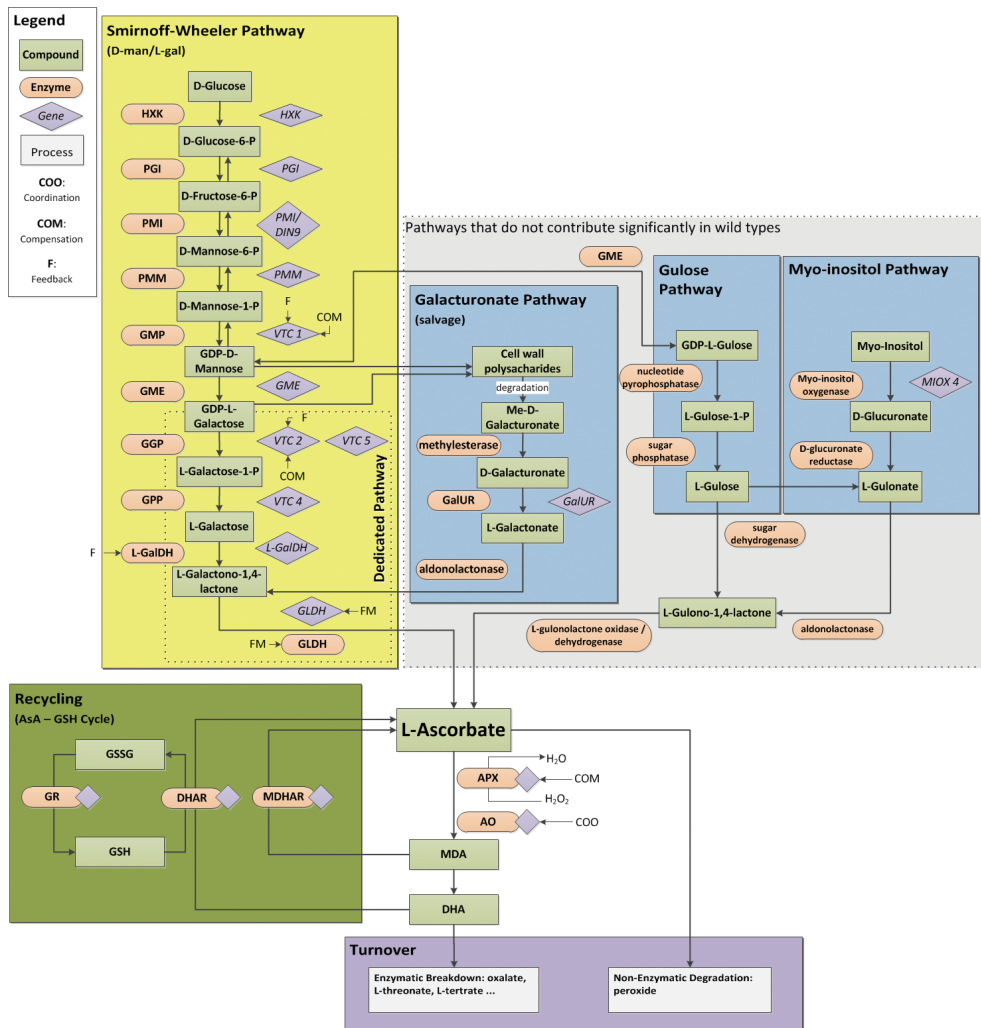
FIGURE 1 | Map of the biochemical pathways for biosynthesis, recycling and turnover of L-ascorbate acid in plants. The biosynthetic pathway that accounts for ASC found in plants is highlighted in yellow while alternative ones are highlighted in blue. Turnover pathway is highlighted in purple and recycling pathway is highlighted in green. Enzymes presented are: HXK, hexokinase; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose 30,50-epimerase; GGP, GDP-L-galactose phosphorylase; GPP, L-galactose-1-P phosphatase; GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; GalUR, D-galacturonate reductase; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; AO, ascorbate oxidase; GR, glutathione reductase; DHAR dehydroascorbate reductase. Chemical compounds are illustrated as such: MDHA, monodehydroascorbate; DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione.

The tight homeostatic control of ASC levels in specific species as well as epistatic effects (e.g. overexpression of GalUR occasionally coincides with upregulation of GLDH expression) reveal that biochemical pathways for biosynthesis, recycling and

catabolism interact at the genome level. By gene expression rate measurements, post-transcriptional and epistatic effects are not considered. Due to the complexity of the underlying gene network an empirical model will not be able to provide an in depth understanding of these processes. Further genotypic investigations including effects of other abiotic factors will allow the construction of an analytical model for accurate prediction of ASC in commercial crops. Part of such an analytical model should be a gene network analysis, as it will be able to account for any kind of interactions between biochemical pathways at the gene level (Hammer et al., 2006).

2.4.4 Translocation

A network for long-distance transport of ASC exists in higher plants (Franceschi & Tarlyn, 2002, Tedone et al., 2004). ASC produced in mature leaves is loaded into the phloem sieve tube elements and transferred to shoots, roots, and generative organs (Hancock et al., 2003). ASC is transported through the plasma membrane via facilitated diffusion (Horemans et al., 1996), proton driven transport (Takahama, 1996) and ASC-DHA exchange mechanisms (Horemans et al., 1998). ASC was also found to conjugate with glycosides in the phloem and transported to the sink organs also in this form (Li et al., 2010b). Mature leaves maintain high ASC levels and no ASC translocation is observed to them therefore, defined as ASC sources. Translocation of ASC from mature leaves to tomato fruits was observed only for green/unripe fruits (Badejo et al., 2012). This suggests that translocation of ASC in interaction with the developmental stage, are factors that can affect ASC content of plant tissues. Even though there is sufficient evidence for ASC transportation in plants, the broader scheme of the ASC transport is not yet established (Horemans et al., 2000). Irradiance has a positive effect on ASC at plant level. However, it is unknown whether translocation patterns of ASC within the plant change in respects to light.

2.4.5 Balancing input and output

ASC levels in plants are determined by the balance between the rates of biosynthesis, recycling, translocation (input) and turnover (output) within the range the homeostatic mechanism allows. Even though biosynthesis is mostly in the attention of research, ASC turnover and recycling are processes that account for major ASC changes. ASC turnover was found to be up to 63% of the ASC pool per day and partially affected by recycling (Truffault et al., 2016). Furthermore, genetically modified plants over expressing recycling genes, had significantly higher ASC levels (Wang et al., 2010). Upregulation of the input and/or downregulation of the output processes do not necessarily result in improvement of ASC levels. The ascorbate homeostatic mechanism is defined as the sum of processes that tend to maintain ASC at a stable equilibrium. The homeostatic mechanism for ascorbate includes feedback, coordination and compensation effects. Biosynthesis and turnover are subjected to feedback control from the ASC pool size.

Exogenous ASC application resulted in a dramatic reduction of ASC biosynthesis rate (Pallanca & Smirnoff, 2000, Wolucka & Van Montagu, 2003). This drop in the rate of biosynthesis is attributed to reduced activity of enzymes of the D-man/L-gal pathway (GalDH, GLDH and GMP; Mieda et al., 2004, Tabata et al., 2002). Additionally, the rate of turnover increased linearly with the ASC pool size (Pallanca & Smirnoff, 2000).

The feedback mechanism of ASC pool to its biosynthesis involves the regulation of GGP. This regulation requires a cis-acting upstream open reading frame (uORF; Laing et al., 2015). High levels of ASC induce translation of uORF which inhibits GGP translation. This results in reduction of ASC biosynthesis through the D-man/L-gal biosynthetic pathway. The direct linkage of ASC concentration to GGP transcription via uORF allows a rapid feedback control of the D-man/L-gal biosynthetic pathway. This has been related to stress conditions such high irradiance (Bulley & Laing, 2016). Yabuta et al. (2010) found GGP to be regulated by irradiance with a concomitant increase in ASC. What remains unknown is whether uORF is involved in light regulation of GGP and ASC. It could be that light dampens the uORF feedback response allowing ASC upregulation. Light might also have direct effects on downstream enzymes of the D-man/L-gal biosynthetic pathway that lead to ASC accumulation at higher irradiances. Information on how uORF translation is affected by irradiance is not yet available.

Genetically modified plants overexpressing or supressing an enzyme for biosynthesis or turnover did not have altered levels of ASC, as other genes will react in a compensating way (compensation). Suppression of GMP in tomato resulted in an increase of the expression rates of other D-man/L-gal genes (Zhang, 2012). On the contrary, genetically modified plants that overexpress biosynthesis genes might show genetic responses in the same direction (coordination). Transgenic tomato plants overexpressing GME were found to have decreased expression of AO which was associated with an observed ASC accumulation (Zhang, 2012). The extent of homeostatic control is species specific: ASC homeostasis in Ribes nigrum was proposed to tightly control of the ASC pool (Hancock et al., 2007). Other species are more responsive to irradiance (e.g. Solanum lycopersicum). Identifying the points of homeostatic control will allow the increase of ASC by focusing breeding at the limiting steps of the D-man/L-gal pathway. Several of the expression rates of the involved genes increase at higher irradiances (section 4.1). Light is potentially the abiotic factor with the most pronounced effects on ASC levels in plants, by lifting the constraints of the homeostatic mechanism.

2.5 The physiological network behind light regulation of ASC levels

2.5.1 Photosynthesis and soluble carbohydrates

Light regulation of ASC involves several physiological processes. ASC is a scavenger of ROS that are vastly produced during the process of photosynthesis. This suggests a direct link between ASC biosynthesis and photosynthesis (Figure 2). The rate of photosynthetic electron transfer defines the plastoquinone pool redox state, which is a regulator of antioxidant enzymes (Karpinski et al., 1997, Karpinski et al., 1999, Madhusudhan et al., 2003). Photosynthetic inhibitors (DCMU and ATZ) arrested ASC accumulation in arabidopsis leaves by reducing expression of genes related to ASC biosynthesis: GMP, GGP, GPP and GLDH (Yabuta et al., 2007). In tomato fruits DCMU reduced ASC only in green/unripe fruits (Badejo et al., 2012). Red/ripe tomatoes were not affected as the photosynthetic apparatus is highly dismantled in this developmental stage. The rate of photosynthesis regulates ASC biosynthesis and therefore ASC levels in plants, via the plastoquinone redox state (Figure 2). High irradiance might have an additional positive effect on ASC by increasing the number of chloroplasts (Enfissi et al., 2010, Noguchi et al., 2005). There is some evidence for photosynthetic electron transfer effects on ASC turnover. DCMU affected the expression levels of APX in arabidopsis (Karpinski et al., 1997) and tobacco (Yabuta et al., 2004) leaves, however with limited effects on ASC levels. Improved rated of photosynthesis at higher irradiances will lead to accumulation of ASC. Therefore, maintaining a highly efficient photosynthetic apparatus will result in achieving higher ASC levels. ASC potentially helps in such direction by being an electron donor to photosystem I in light induced transport of electron in chloroplasts (Ivanov et al., 2001). Similarly the water-water cycle is worth mentioning as it protects PSI from photo-oxidative stress (Asada, 1999) and therefore is potentially important for accumulation of ASC by maintaining optimal photosynthetic rates.

The rate of photosynthesis is an important factor for sucrose and consequently glucose levels of the plant tissue. In the primary ASC biosynthetic pathway (D-man/L-gal) glucose is the initial precursor. This might imply that soluble carbohydrates have a regulatory effect on ASC levels in plants (Figure 2). Indeed, exogenous sucrose feeding in detached broccoli inflorescences delayed ASC depletion (Nishikawa et al., 2005) and in tomato fruits it increased ASC (Badejo et al., 2012). On the contrary, exogenous application of sucrose or glucose in barley (Smirnoff & Pallanca, 1996) and pea embryonic axes (Pallanca & Smirnoff, 1999) did not affect ASC levels. Carbohydrates regulate ASC levels via effects on ASC-related gene expression. A positive effect of sucrose feeding was observed on the expression rates of biosynthetic genes (VTC1, VTC2, and L-GalLDH) in tomato fruits (Badejo et al., 2012) as well as on recycling and turnover genes (APX, MDHAR, DHAR, GLDH and GR; Nishikawa et al., 2005). Xiang et. al. (2011a) observed that regulation of APX by ROS is strongly affected by exogenous

application of carbohydrates. In a low light environment it is possible that ASC is limited due to substrate deficiency or lack of carbohydrate effects (section 5.3). Massot et al. (2010) suggested that the effects of carbohydrates on ASC are genotype specific. In summary, soluble carbohydrates have potentially regulatory action on ASC in plants via regulation of expression of ASC biosynthetic, recycling and turnover genes.
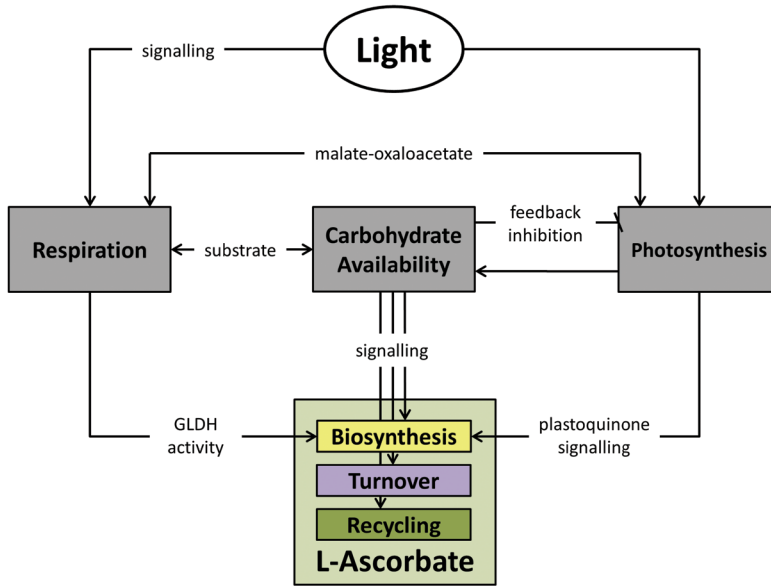
FIGURE 2 | The physiological network behind light regulation of L-ascorbate in plants. Interactions between underlying physiological processes (grey boxes) in affecting ASC regulatory processes (colour boxes) are illustrated with arrows. Colours correspond to Figure 1. (GLDH, L-galactono-1,4-lactone dehydrogenase).

2.5.2 Respiration

The link between ASC biosynthesis and respiration is located at the last step of the biosynthetic pathway (Figure 2). The enzyme involved in this last step (GLDH) is located in the inner membrane of mitochondria (Bartoli et al., 2000), where also complex I of the respiratory electron transport chain (ETC) is located (Millar et al., 2003). GLDH is supposedly part of complex I of the respiratory ETC (Schimmeyer et al., 2016). It has an integral role in the respiratory ETC as it is essential for the accumulation of complex I as proved in arabidopsis leaves (Pineau et al., 2008). GLDH is not present in chloroplasts. Mitochondria isolated from potato leaves were able to synthesize ASC from L-galactono-1,4-lactone (Bartoli et al., 2000). These observations suggest that the mitochondrion is an essential part of the ASC biosynthetic apparatus. Experiments with respiratory inhibitors (KCN and rotenone) revealed that respiratory ETC through complex I increases GLDH activity in arabidopsis and is essential for maximal ASC

biosynthesis rates (Millar et al., 2003, Bartoli et al., 2006b). Biosynthesis of ASC is linked to cytC which is part of the ETC (cytC; link between complexes III and IV in respiratory ETC). CytC is an electron acceptor from GLDH and therefore, a substrate for this enzyme (Leferink et al., 2008). Consequently, availability of oxidized cytC increases ASC biosynthesis as proved for intact potato mitochondria (Bartoli et al., 2000). The link between respiration and ASC biosynthesis has also been illustrated in studies of stress-induced alteration of mitochondrial proteins. During heat-stress induced programmed cell death (PCD) a decrease in GLDH activity and in the levels of steady state ASC pool were observed (Valenti et al., 2007). Heat stressed induced PCD causes the alteration of mitochondrial proteins that provide adenosine triphosphate (ATP) and nucleoside triphosphates. However, this research cannot exclude the possibility that it is complex I that inhibits GLDH. Increasing light levels resulted in higher respiration rates in arabidopsis (Bartoli et al., 2006b) and tomato (Poorter et al., 2013). In the case of Bartoli et. al., (2006b) in arabidopsis, the increase of ASC under high irradiance was attributed to both the higher content of oxidized cytC and the increased activity of complex I of the respiratory ETC. High irradiance might increase ASC levels in plants not only by increasing the biosynthetic capacity of individual organelles, but also by increasing the number of mitochondria (Noguchi et al., 2005). Alternative oxidase (AOX; enzyme that provides an alternative route for respiratory ETC bypassing cytC) is also linked with ASC biosynthesis. Higher AOX capacity is negatively correlated with ROS in arabidopsis in vivo (Umbach et al., 2005). Leaves of AOX-overexpressing arabidopsis plants accumulated more ASC compared to wild-type and antisense leaves. This effect was pronounced at higher light intensities (Bartoli et al., 2006a). The importance of AOX in regulating light effects on ASC biosynthesis of leaves and fruits in different developmental stages is a topic for further investigation. An increased AOX pathway capacity might be beneficial especially under high irradiances by maintaining cytC in a more oxidized state, preventing over reduction of mitochondrial transporters (Millar & Day, 1997). AOX silenced mutants had more cytC implying the presence of a regulatory homeostatic mechanism (Bartoli et al., 2006b). Furthermore, there is no research on the possible link between respiration and ASC recycling and turnover. Respiration was found to replenish NAD(P)H which is needed for MDHAR and GR (Millar et al., 2003). However, how NAD(P)H regulates ASC via turnover and recycling remains largely unknown. In summary, both the cytC and the AOX respiratory pathways affect ASC levels via regulation of the activity of GLDH.

2.5.3 The link between photosynthesis and respiration in light regulation of ASC

As previously discussed, respiration and photosynthesis are two key processes in light regulation of ASC. A positive relation between respiratory or photosynthetic rates and ASC level is expected. However, this correlation is not always observed in literature, a fact that suggests a possible interaction between respiration and photosynthesis in

light regulation of ASC. This complex relationship does not allow the evaluation of the importance of the two processes in light regulation of ASC. Research up to date has never studied how these two physiological processes interact in regulating ASC levels in plants. In each case, research either focused only on photosynthesis or on respiration. Photosynthesis has effects on biosynthetic genes while respiration affects the enzymatic activity at the D-man/L-gal biosynthetic pathway (sections 5.1 and 5.2 respectively). Expression of the ASC - GSH cycle genes has been found in both chloroplasts and mitochondria (Chew et al., 2003). Consequently, both organelles are integral parts of the ASC biosynthetic and recycling apparatus. Mitochondria and chloroplasts have been proposed to interact in the biosynthesis of a variety of secondary metabolites some of them associated with plant responses to stress (Mackenzie & McIntosh, 1999). When tomato leaf discs were incubated in ASC and consecutively illuminated, carbon assimilation increased with a coupled increase in starch (Nunes-Nesi et al., 2005). This suggests that ASC levels in plant tissue are linked to respiration and photosynthesis.

Carbohydrates are not only an important component of light regulation of ASC (section 5.2) but also an interaction point between respiration and photosynthesis. Carbohydrate availability might have an effect on ASC biosynthesis by affecting respiration as it is the substrate for respiratory activity in plant cells (Azcón-Bieto & Osmond, 1983, Monteiro et al., 2002). The rate of respiration as affected by light, might affect the levels of soluble carbohydrates (Ögren et al., 1997). Furthermore, exogenous glucose was found to be a cue for increasing the number of mitochondria and regulate respiratory ETR via regulating the activity of hexokinase (Xiang et al., 2011b). Respiration via the alternative pathway is affected by various abiotic factors including irradiance (Mackenzie & McIntosh, 1999). Respiratory activity in the alternative pathway requires availability of carbohydrates (Lambers, 1982). Therefore, carbohydrate availability is a cue that may regulate ASC via both cytC and the alternative respiratory pathway. Furthermore, carbohydrate availability is strongly dependent on the photosynthetic rate and thus modulated by irradiance and light quality. High levels of carbohydrates confer feedback inhibition effect on photosynthesis (Paul & Foyer, 2001). This has been observed in a variety of species including some with considerable concentrations of ASC (Goldschmidt & Huber, 1992). Another interaction between respiration and photosynthesis is the dissipation of energy excess of the photosynthetic ETC. In illuminated leaves reductants are transferred to the cytosol via the malate-oxaloacetate shuttle affecting the respiratory ETC (Noguchi & Yoshida, 2008). These observations suggest that both photosynthetic and respiratory ETCs (cytC) as well as the alternative respiratory ETC (AOX) are required for maximal ASC levels. Respiration and photosynthesis as affected by light regulate ASC levels. Even though the links between respiration, photosynthesis and ASC biosynthesis have been proven to exist, their importance in light regulation of ASC has not been recognised.

In summary, respiration is a regulator of the activity of GLDH and photosynthesis is a cue for ASC biosynthetic genes. Respiration and photosynthesis may interact via carbohydrate availability in light regulation of ASC. Furthermore, carbohydrates have

effects on ASC biosynthesis, recycling and turnover genes. Effects of respiration and photosynthesis on ASC recycling or turnover have not been thoroughly investigated. In addition, direct effects of light have only been reported for biosynthesis (section 4).

2.6 Conclusions

Light has a regulatory function for ASC levels in plants. ASC in plant tissues increases with increasing irradiance due to stimulation of the D-man/L-gal biosynthetic pathway. The physiological network for light regulation of ASC in plants involves respiration and photosynthesis. Both processes regulate ASC via effects on the D-man/L-gal biosynthetic pathway. Furthermore, these two physiological processes interact in light regulation of ASC via soluble carbohydrates. For both leaves and fruits respiration, photosynthesis and carbohydrate availability play a role in ASC light regulation but their relative importance may vary.

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Chapter 3

Light regulation of vitamin C in tomato fruit is mediated through photosynthesis

3

Higher levels of irradiance result in higher accumulation of ascorbate in leaves and fruits. Photosynthesis and respiration are an integral part of the physiological mechanism of light regulation of ascorbate in leaves, but little is known about the light regulation of ascorbate in fruit. The aim of this study was to investigate whether fruit illumination alone is sufficient for ascorbate increase in tomato fruit and whether this light signal is mediated by respiration and photosynthesis. First the changes of ascorbate with the progress of fruit development were investigated and subsequently detached fruit of different tomato genotypes were exposed to different irradiances and spectra. Measurements were performed on ascorbate, respiration, photosynthesis and chlorophyll content of the fruit. When attached to the plant, there was no effect of development on ascorbate from the mature green to the red stage. Detached fruit stored in darkness did not accumulate ascorbate. However, when exposed to 300-600 μmol m^{-2} s^{-1} light detached mature green fruit (photosynthetically active) substantially accumulated ascorbate, while mature red fruit (non-photosynthetically active) did not respond to light. Photosynthesis correlated with this increase of ascorbate while no correlation between respiration and ascorbate was found. Spectral effects on ascorbate in detached tomato fruit were limited. These results indicate that the signal for light regulation of ascorbate is perceived locally in the fruit and that fruit illumination alone is sufficient for a considerable increase in ascorbate levels for as long as the fruit contains chlorophyll. It is shown that photosynthetic activity of the fruit is an integral part of the response of ascorbate to light in tomato fruit. The light induced increase in ascorbate levels occurred in a range of genotypes, indicating a universal effect of light to ascorbate in tomato fruit.

Abstract

3.1 Introduction

L-ascorbate (ASC; vitamin C; CID 54670067) is an antioxidant compound found in considerable amounts in plant tissue. In addition to its antioxidant activity, ASC has pro-oxidant effects. ASC is a compound with high potential for cancer prevention and treatment in humans (Chen et al., 2007, 2005; Du et al., 2012). ASC deficiency in the human body has been related to the coronary heart disease and diabetes mellitus (Boekholdt et al., 2006; Li and Schellhorn, 2007; Mandl et al., 2009; Paolisso et al., 1994). Due to its widely proclaimed beneficial effects on the human immune system, ASC has been characterized as essential for human health (EFSA Panel on Dietetic Products, 2010). The human organism through the progress of evolution lost the ability to synthesize ASC (Asensi-Fabado and Munné-Bosch, 2010; Chatterjee, 1973; Nishikimi et al., 1994). Plants are considered the most important source of ASC for humans as the bioavailability of ASC from plants is higher than that from artificial supplements (Bjelakovic et al., 2004; Fitzpatrick et al., 2012; Inoue et al., 2008).

One of the most widely discussed abiotic factors affecting ASC in plants is light. Increasing the irradiance level, increases ASC levels of leaves (Bartoli et al., 2006; Dowdle et al., 2007; Fukunaga et al., 2010). A response of ASC to light has also been reported for fruits like kiwi (Li et al., 2010), tomato (Massot et al., 2012; Ntagkas et al., 2016), apple (Li et al., 2009) and grapefruit (Cakmak et al., 1995). Shading of tomato fruit while still growing attached to the plant resulted in lower ASC levels (Gautier et al., 2008). When tomato fruit were illuminated with LEDs while still on the plant, they achieved higher ASC levels than in non-illuminated fruit (Labrie and Verkerke, 2012). Therefore, it is possible that the light signal for increase of ASC levels of the fruit is perceived locally from the fruit.

The spectrum of light may also affect ASC in plants. Increasing the red:far red ratio increased the ASC concentration in bean seedlings (Bartoli et al., 2009). Blue light also increased ASC levels in lettuce, spinach and other leafy vegetables (Lester, 2006; Ohashi-Kaneko et al., 2007). The situation seems to be complicated as UV light enhanced ASC levels in soybean seedlings (Xu et al., 2005) while UV-B and UV-C light reduced ASC in tomato fruit (Giuntini et al., 2005; Maharaj et al., 2014).

The photosynthetic electron transport rate regulates gene expression of ascorbate peroxidase which affects the ability of the tissue to scavenge H_2O_2. This signal is mediated via changes in the redox state of the plastoquinone pool (Karpinski et al., 1997). In arabidopsis leaves, photosynthetic inhibitors such as DCMU and ATZ, arrested the accumulation of ASC under light, by reducing the activity of the D-Man/L-Gal biosynthetic pathway (Yabuta et al., 2007). Inhibition of photosynthesis in green tomato fruit also resulted in arrested ASC levels in the fruit (Badejo et al., 2012). The rate of photosynthetic electron transport is also linked to ASC turnover with limited effects however on ASC levels (Karpinski et al., 1997).

Respiration is another physiological process mediating light regulation of ASC. The last enzyme in the D-Man/L-Gal biosynthetic pathway (GLDH) is located in the inner membrane of mitochondria (Bartoli et al., 2000). GLDH is a functional part of complex I of the respiratory electron transport chain (Schimmeyer et al., 2016) and an electron donor to cytochrome C. Oxidized cytochrome C results in higher ASC levels as proven in isolated potato mitochondria (Bartoli et al., 2000). Inhibition of respiration at the level of complex I (via application of KCN and rotenone) result in a considerable reduction in ASC levels (Bartoli et al., 2006; Millar et al., 2003). The alternative oxidase (AOX) respiratory pathway has also been associated to ASC biosynthesis. ASC accumulated in leaves of AOX overexpressing arabidopsis mutants while it did not in the wild type (Bartoli et al., 2006). It remains elusive whether respiration and photosynthesis mediate the signal for increase of ASC when tomato fruit are treated with higher irradiances. Respiration and photosynthesis possibly interact in light regulation of ASC (Chapter 2).

It is known that light regulates ASC levels in leaves and that respiration and photosynthesis are an integral part of this regulatory mechanism. The aim of the present study was to investigate whether the signal of light for increase of ASC levels in tomato fruit is perceived locally in the fruit and whether this light signal is mediated by respiration and photosynthesis. It was hypothesized that light may increase both respiratory and photosynthetic activities and hence may stimulate ASC accumulation. To test these hypotheses first the changes of ASC with the progress of development were investigated and subsequently detached tomato fruit of different genotypes were exposed to different irradiances and spectra.

3.2 Materials and methods

3.2.1 Plant material

Tomato fruit (*Solanum lycopersicum*) were harvested from the glasshouse of a commercial grower (Royal Pride Holland) in Middenmeer (N 52° 46' 58'', E5° 03' 42''), the Netherlands. Fruit were transported to Wageningen University and Research facilities in Wageningen, the Netherlands. In all experiments fruit were selected from the 3rd and 4th positions of the truss (counting acropetally) when they were within a specific range of colour, firmness and weight (Table 1). Only trusses at the same position on the plant with 8 fruit per truss were used. The developmental stage of the fruit was characterised based on lycopene (NAI) and chlorophyll (NDVI) content related indices (table 1). Five experiments were performed. In Experiments (Exp.) 1, 2, 3 and 5 the commercial cultivar Vimoso (40 g/fruit) was used. In Exp. 4, five commercial cultivars bearing mature fruit of different size (20 to 160 g/fruit; Table 1) were tested. For experiments 2 to 5 the light treatments begun approximately 5 hours after harvest. As difference experiments took place in different parts of the year, fruit for all experiments

were picked from a greenhouse equipped with artificial light. This way the difference in growth irradiance was minimized to the best possible extend so that the fruit would have comparable initial ASC levels.

3.2.2 Set-up of experiments

In Exp. 1 the ASC levels of fruit of different developmental stages that matured in the greenhouse was measured. Four developmental stages were selected (mature green, breaker, red, advanced red). The fruit matured to the designated developmental stage while attached on the plant. The ambient irradiance in the greenhouse at fruit level was measured with a handheld quantum sensor (LI-250; Li-Cor Inc., Lincoln, Nebraska, USA). The measurement took place at noon with clear sky where the irradiance is expected to be at its highest. Irradiance was measured at fruit level at a total of 50 points of the row the fruit were harvested from. Irradiance at fruit level on June 16th (harvest point of Exp. 1) was found to be 98 $\pm$6.8 µmol m^{-2} s^{-1}.

In Exp. 2, in order to characterise ASC levels during ripening of detached fruit in light and darkness, mature green tomato fruit were placed for 15 days in 500 µmol m^{-2} s^{-1} of white light and darkness. In Exp. 3, the response of ASC levels to irradiance was studied by keeping mature green and red tomato fruit under a range of irradiance levels (0, 8, 144, 306 and 616 µmol m^{-2} s^{-1}) for 7 days. In Exp. 4, four tomato cultivars with different fruit size were exposed to 300 µmol m^{-2} s^{-1}, an irradiance level causing considerable accumulation of ASC (Exp. 3). In Exp. 2, 3 and 4 a broad spectrum (white light) was used to avoid lack of spectra potentially essential for increasing ASC. In Exp. 5 the effect of spectrum on ASC was tested. Tomatoes were placed at a combination of 100 µmol m^{-2} s^{-1} white background irradiance supplemented with 250 µmol m^{-2} s^{-1} of monochromatic light (red, blue and far-red). The green light treatment was an exception due to the output limitations of the LEDs. It was a combination of 150 µmol m^{-2} s^{-1} green light and 200 µmol m^{-2} s^{-1} of white light, such that total irradiance was kept constant.

In Exp. 2 to 5 after the transfer from the greenhouse, fruit were placed in a climate-controlled room which contained 5 compartments with one light treatment per compartment. The calyx was removed and the fruit were placed with the calyx scar pointing downwards to the table in order to minimize water loss. Fresh weight (FW) measurements confirmed uniform water loss for all treatments. In Exp. 3, 4 and 5 fruit lost 0.62% of the initial weight after 7 days. In Exp. 2 fruit lost 2.4% of their initial weight after 15 days in the treatments. LED light was applied continuously (24 hours per day) in all experiments. The broad/white spectrum was supplied by blue phosphorous coated LEDs (GreenPower LED, Philips, The Netherlands). Far red was supplied by LED production modules (Green Power LED, Philips, The Netherlands). Blue and red light was supplied by LEDs with dominant wavelengths of 450 nm and 638 nm, respectively (types Royal Blue and Red Luxeon K2, Lumileds Lighting Company, San Jose, CA, USA). Green was supplied by custom made LED modules with dominant wavelength

at 520 nm. The LEDs were suspended 80 cm from the surface of the bench. The sides and bottom of the compartments were covered with neutrally reflective MC-PET sheets (SRF-A032T, Sekisui Plastics Co., LTD, Osaka, Japan) in order to improve irradiance levels and light distribution. These reflective properties were verified with a spectrophotometer (USB-4000, Ocean Optics, Dunedin, FL, USA) with the use of an integrated sphere in an obscure box. Spatial distribution of irradiance and spectrum was measured in a 5 by 5 cm grid with a spectroradiometer (USB2000, Ocean Optics, Duiven, The Netherlands; calibrated against a standard light source).

The total illuminated area was 80 x 50 cm out of which an area of 60 x 30 cm was selected for the placement of samples based on the light distribution measurements. Light distribution within the selected area was within 10 µmol m^{-2} s^{-1} for all light treatments of all experiments and 0.5 µmol m^{-2} s^{-1} for the 8 µmol m^{-2} s^{-1} irradiance treatment of Exp. 3. To further ensure uniform exposure of all fruit to the light treatments, fruit were rotated daily within the selected area. Phytochrome stationary state (PSS) was calculated according to equation 1 (Sager et al., 1988).

$$PSS = \left(\sum\nolimits_{300}^{800} N_\lambda \sigma_{r_\lambda} \right) / \left(\sum\nolimits_{300}^{800} N_\lambda \sigma_{r_\lambda} + \sum\nolimits_{300}^{800} N_\lambda \sigma_{fr_\lambda} \right) \qquad \text{(eq.1)}$$

Where N: photon flux at wavelength (nm), $r\lambda$: photochemical cross-section of red absorbing phytochrome state, $fr\lambda$: photochemical cross-section of far-red absorbing phytochrome state. Air temperature was 18 °C, relative humidity was 70% and CO_2 concentration was between 352ppm and 428 ppm for all experiments. Fruit temperature was monitored with k-type thermocouples attached to the lower side of the fruit on TC-08 data loggers (Picotechnology LTD., Cambridge, UK; table 1). Thermocouples were calibrated in distilled water at freezing and boiling point. Fans were placed in the openings below and above the reflective material on the sides, to avoid temperature deviations. Eventually, the fruit temperature was approximately 0.5 °C higher than the air temperature with maximum temperature difference between the treatments of 0.4 °C (table 1).

3.2.3 Fruit colour and firmness

Changes in fruit colour were measured by a hand-held photodiode array spectroradiometer (PA1101, CP, Germany). Measurements were taken at three spots on the equatorial region of the fruit. This spectroradiometer provides the normalized anthocyanin index (NAI) calculated according to remittance spectra at 570 and 780 nm (R_{570} and R_{780} respectively) and the normalized difference vegetation index (NDVI) calculated according to remittance spectra at 660 and 780 nm (R_{660} and R_{780} respectively; Equations 2 and 3). NAI and NDVI values correlate with lycopene and chlorophyll contents respectively (Kuckenberg et al., 2008).

$$NAI = \frac{R_{780} - R_{570}}{R_{780} + R_{570}}$$

<div align="right">(eq.2)</div>

$$NDVI = \frac{R_{780} - R_{660}}{R_{780} + R_{660}}$$

<div align="right">(eq.3)</div>

Firmness of the fruit was measured by an acoustic detector (AFS, AWETA, Nootdorp, The Netherlands). The system measures the fruit weight (m, g/fruit) and the resonant frequency (f, Hz) of the fruit after it is hit by a small plastic piston. The firmness index (Fi) is calculated according to equation 4.

$$Fi = \frac{f^2 m^{2/3}}{10^6}$$

<div align="right">(eq.4)</div>

3.2.4 Ascorbate and dehydroascorbate

L-ascorbate (ASC) and dehydroascorbate (DHA) were measured at the beginning of the treatments and after 7 days in Exp. 3, 4 and 5 or every 3 days in Exp. 2. For each treatment 10 fruit were combined in pairs into 5 replicates. Each replicate consisted of a pool of 6 pericarp discs of 1 cm diameter from two fruit. In tomato fruit ASC is mostly localized in the pericarp (Badejo et al., 2012). The discs were taken from the fruit side which was directly illuminated by the LEDs. The pericarp discs were then frozen in liquid nitrogen and grinded to fine powder.

0.2 g of fresh frozen powder was thawed on ice in dark with the addition of 0.5 ml ice cold 3.3% meta-phosphoric acid. Samples were placed in an ultrasonic bath for 10 minutes and consecutively centrifuged at 25000 rcf at 4°C for 10 minutes. 100μl of each sample was transferred to another HPLC vial where DHA was reduced to L-ASC with 50μl DTT 5mM and 400mM Tris base (Davey et al., 2003). The reduction of L-ASC to DHA took place in darkness for 15 minutes and was stopped with the addition of 50μl o-phosphoric acid 8.5%. Extracts were measured in a high-performance liquid chromatography system (P580 pump, UVD 340S detector, Dionex Corporation, Sunnyvale, USA). Calibration of the HPLC was performed with authentic ASC solutions of known concentration.

TABLE 1 | Overview of set-up of experiments investigating the effects of light on L-ASC in detached tomato fruit. Irradiance and PSS measurements represent the mean of 40 measurements equally distributed over the illuminated area. Data on fruit weight, NAI (lycopene index) and NDVI (chlorophyll index) are based on 10 replicate fruit and fruit temperature is based on 8 replicate fruit.

Experiment	Scope	Treatment Duration (days)	Spectrum	Irradiance (μmol m^{-2} s^{-1})
1	ASC time course of fruit on plant	-	-	-
2	Time course in light and darkness	15	Darkness	0
			White	500 ±10
3	Response to irradiance	7	Darkness	0
			White	8 ±0.1
			White	144 ±2
			White	306 ±5
			White	616 ±12
4	Cultivar differences	7	Darkness	0
			White	306 ±12
5	Response to light spectrum	7	Red and white	253 and 122 respectively (375 ±10)
			Blue and white	255 and 118 respectively (373 ±11)
			Far Red and white	258 and 119 respectively (377 ±12)
			Green and white	155 and 209 respectively (364 ±12)
			White	378 ±9
			Darkness	0

3

PSS	Tomato Cultivar	Average Fruit Temperature (°C)	Fruit Developmental Stage at Harvest		
				Average NAI	Average NDVI	Average Fresh Weight (g)
-	Vimoso	-	Green Mature	-0.6 ±0.02	0.07 ±0.02	43 ±3
			Breaker	-0.3 ±0.04	-0.2 ±0.05	
			Red	0.2 ±0.01	-0.6 ±0.02	
			Advanced Red	0.6 ±0.01	-0.6 ±0.01	
-	Vimoso	18 ±0.20	Green mature	-0.6 ±0.01	0.07 ±0.02	43 ±4
0.83		18.4 ±0.13				
-	Vimoso	18.1 ±0.12	Green mature	-0.6 ±0.02	0.07 ±0.01	43 ±3
0.83		18 ±0.18	Red	0.2 ±0.02	-0.6 ±0.02	
0.83		18.3 ±0.21				
0.83		18.4 ±0.17				
0.83		18.5 ±0.17				
-	Robinio	18.4 ±0.13	Green mature	-0.6 ±0.01	0.07 ±0.02	21 ±2
0.83	Vimoso					43 ±2
	Axiradius					101 ±11
	Roterno					98 ±9
	Komeet					160 ±17
0.88	Vimoso	18.2 ±0.12	Green mature	-0.6 ±0.03	0.07 ±0.02	43 ±3
0.7		18.5 ±0.18				
0.67		18.4 ±0.18				
0.83						
0.83		18.3 ±0.22				
-		18.1 ±0.17				

3.2.5 Measurements of respiration and photosynthesis

Respiration of individual tomato fruit was measured with a portable infra-red gas exchange system (LI-6400; Li-Cor Inc., Lincoln, Nebraska, USA). A single tomato fruit was placed in a transparent, hollow PVC sphere which was integrated at the sampling circuit of the LI-6400 with the cuvette bypassed (Savvides et al., 2013). The sphere reduced irradiance by 10% without affecting the spectrum. Gas exchange rates were measured both under light and darkness (dark respiration) by covering the sphere with a non-transparent hood. For fruit in darkness, the CO_2 rate was logged for 15 minutes after a stabilization period of 15 minutes. A chlorophyll fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic) was used to measure photosynthetic electron transport efficiency in photosystem II (PSII) under 300 µmol m^{-2} s^{-1} with a saturating pulse of 3500 µmol m^{-2} s^{-1}. Maximum quantum efficiency of photosystem II (Fv/Fm) was measured after 30 minutes dark adaptation. Fv/Fm was measured under a saturating pulse of 3000 µmol m^{-2} s^{-1}. FluorCam v.5.0 software was used to operate the measurement protocol in FluorCam 700MF.

3.2.6 Statistical analysis

One-way ANOVA was used to test the effects of the developmental stage on L-ASC (Exp. 1). Two-way analysis of variance (ANOVA) was used to test the effects of two factors on L-ASC (light treatment and time in Exp. 2, light treatments and developmental stage in Exp. 3, light treatments and cultivar in Exp. 4 and spectrum and time in Exp. 5). Individual plants were treated as independent replicates. This may have underestimated the random variance hence, we conducted our tests at P=0.01 instead of the commonly used P=0.05 with post hoc Tukey's honestly significant difference (HSD) multiple comparison tests (P≤0.01). Statistical analyses were carried out with the R software (R 3.0.1; R Project for Statistical Computing, Vienna, Austria).

3.3 Results

3.3.1 ASC in tomatoes of different developmental stages

Tomato fruit of four different developmental stages were harvested and their ASC content was analysed (Exp. 1). During fruit maturation on the plant from mature green, breaker to red fruit lycopene index increased and chlorophyll index decreased, but ASC did not significantly change (Figure 1). Light red is the stage that fruit are typically harvested and fully red refers to over-ripe fruit. In fully red fruit ASC was 5-7 mg/100 FW higher than in the other developmental stages (Figure 1).
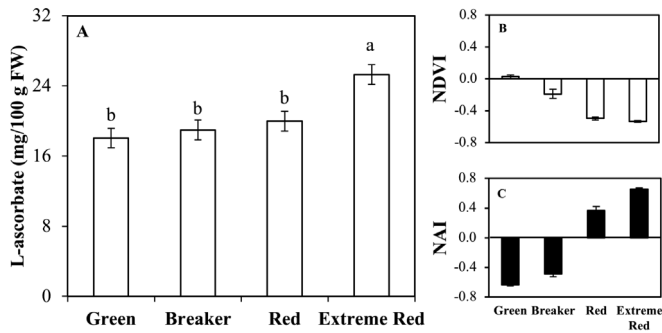
FIGURE 1 | L-ascorbate concentration (A), chlorophyll index (NDVI, B) and lycopene index (NAI, C) of greenhouse grown tomato fruit harvested at 4 different developmental stages. Measurements were performed immediately upon harvest. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 1).

3.3.2 Effects of light on ASC during ripening

Detached mature green tomato fruit were placed under white LED (500 µmol m^{-2} s^{-1}) light and darkness for 15 days (Exp. 2). During fruit ripening in darkness for 15 days ASC did not show any significant changes (Figure 2). During fruit ripening under white light, ASC levels in the pericarp were increased 4.8 times from day 0 (mature green) compared to day 9 (breaker stage) with no further change thereafter. By the end of the experiment the fruit of both treatments had ripened to the red stage as indicated by the similar NAI and NDVI values (Figure 2). No differences in firmness between the two treatments were observed at the end of the experiment (data not shown).
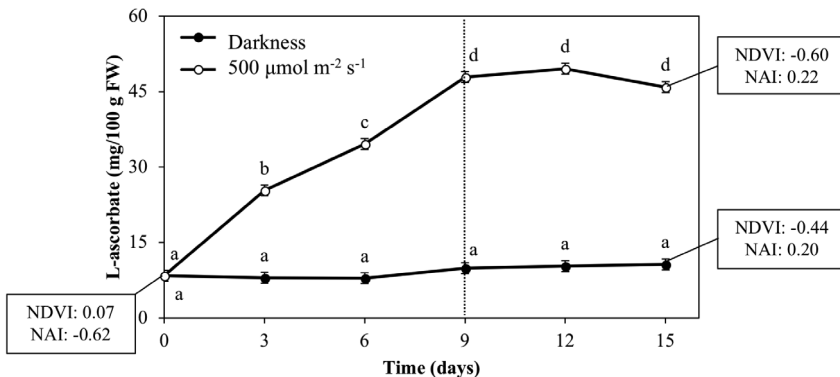
FIGURE 2 | L-ascorbate concentration of tomato fruit kept under 500 µmol m^{-2} s^{-1} of white light (open symbols) and darkness (closed symbols) for 15 days. At the beginning of the treatments the fruit were in mature green stage. Broken line indicates the time point fruit entered the breaker stage. NAI and NDVI indices are presented for the initial and final time points. Error bars (when larger than symbol size) represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

Efficiency of photosystem II (PSII), maximum photosynthetic efficiency of photosystem II (Fv/Fm) and respiration were measured at day 2 and day 14 after treatments with white LED light (500 µmol m^{-2} s^{-1}) and in darkness (Exp. 2). PSII was found to be significantly higher in fruit that were kept in light (500 µmol m^{-2} s^{-1}) compared to fruit in darkness (2 and 14 days; Figure 3A). Fv/Fm was not different between fruit held in light and darkness at 2 days. At day 14 however, fruit in the light treatment had significantly lower Fv/Fm in comparison to fruit in the dark treatment (Figure 3B). The light treatment might have a small effect on ripening as NDVI was slightly lower in the light treatment that results in the observed reduction in the Fv/Fm. However, this is most likely within a range that is not expected to affect ASC levels as observed in Exp. 1.
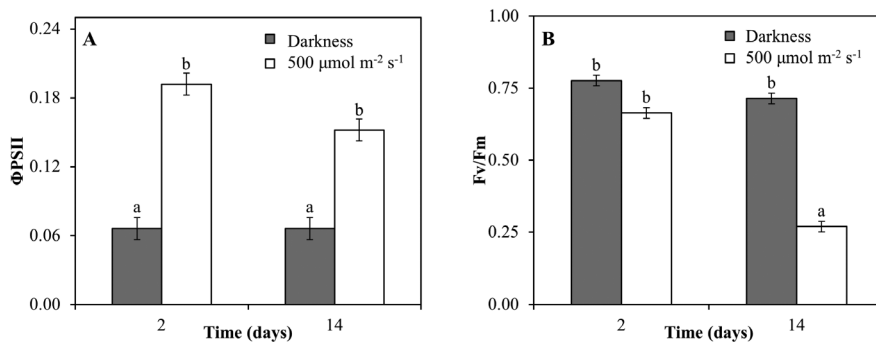
FIGURE 3 | (A) Efficiency of photosystem II (PSII) and (B) maximum photosynthetic efficiency of photosystem II of tomato fruit kept under 500 µmol m^{-2} s^{-1} (white bars) and darkness (black bars). Measurements took place after 2 days and after 14 days in the treatment. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

At the second day of the treatment dark respiration rates were significantly lower in the light treatment compared to the dark treatment, while at day 14 there were no significant differences anymore in dark respiration (Figure 4). There was no difference between light and dark respiration in the light treatment (Figure 4).
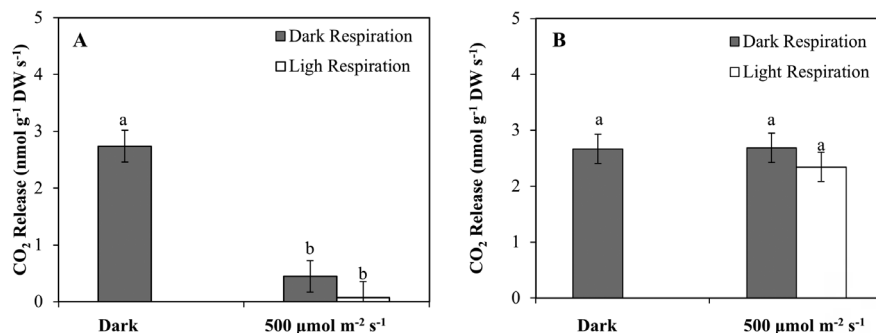
FIGURE 4 | Rate of CO_2 release from tomato fruit kept in darkness or 500 µmol m^{-2} s^{-1} white light. Measurements took place at day 2 (A) and day 14 (B) of the treatment. Dark bars indicate respiration in darkness (CO_2 release in darkness) while white bars represent respiration in light (CO_2 release in light).

Respiration under light was not measured for fruit kept in dark. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 2).

### 3.3.3 Effects of irradiance on ASC in green and red fruit

At start the ASC levels were similar in red and green fruit (Figure 5). Applying different irradiances in the range of 0 to 600 µmol m^{-2} s^{-1} to mature green fruit, showed that the treatment of 300 µmol m^{-2} s^{-1} yielded the highest observed ASC levels. Irradiances below 150 µmol m^{-2} s^{-1} did not significantly affect the ASC concentration. The ASC concentration in red fruit was not significantly affected by irradiance. Initial ASC levels of green tomatoes did not differ significantly from that of red tomatoes (15 and 17 mg 100 g^{-1} of fresh weight, respectively).
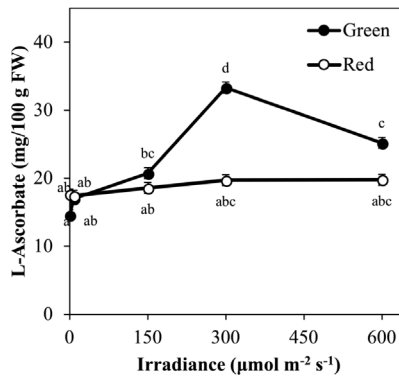
FIGURE 5 | Effect of irradiance on L-ascorbate concentration in tomato fruit after 7 days light treatment on detached fruit. At the beginning of the experiment (t=0) mature green and red fruit were picked from the plant and irradiance treatments were applied. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 3).

3.3.4 ASC increases with higher irradiance in several tomato cultivars

The effect of 7 days of white LED light (300 µmol m^{-2} s^{-1}) on ASC was investigated in detached mature green fruit from five different cultivars grown under comparable conditions in a commercial greenhouse (Exp. 4). In darkness ASC levels did not increase in any of the cultivars (Figure 6). In the light, ASC levels of all five tomato cultivars increased during ripening of mature green fruit compared to fruit that ripened in darkness (Figure 6). Depending on the cultivar the increase varied from 1.8 to 2.4 times (Figure 6).
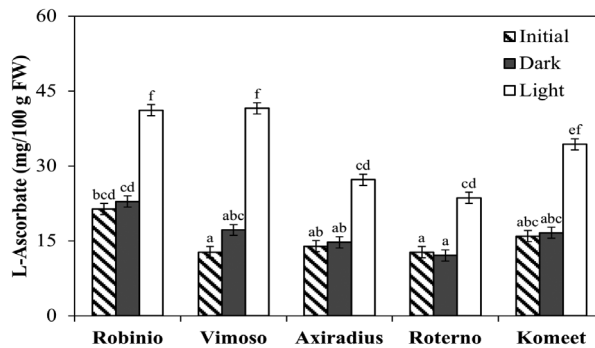
FIGURE 6 | L-ascorbate concentration of fruit of five commercial cultivars (robino, vimoso, axiradius, roterno and komeett) kept under 300 μmol m^{-2} s^{-1} white light (white bars) and darkness (black bars) for 7 days. Fruit from all cultivars at the beginning of the treatment (striped bars) were at the mature green stage. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 4).

3.3.5 Spectral effects on ASC

To study the effect of spectrum on ASC levels (Exp. 5) detached tomato fruit were kept for 7 days under background white LED light (100 μmol m^{-2} s^{-1}) while supplemented with monochromatic LED light (250 μmol m^{-2} s^{-1}). ASC levels in all light treatments were significantly higher compared to the darkness and the initial ASC levels. The highest levels of ASC were achieved in the blue treatment (Figure 7), which was about 10% higher than fruit under white light. The ASC level of fruit under red and green light did not significantly differ from those under white light (Figure 7). Far-red resulted in significantly lower ASC levels compared to all other light treatments (Figure 7).
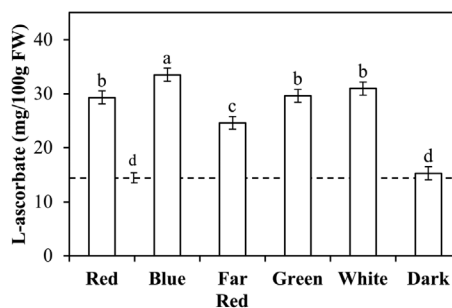
FIGURE 7 | Effect of light spectrum on L-ascorbate concentration of tomato fruit. Detached fruit were kept for 7 days under 250 μmol m^{-2} s^{-1} of monochromatic (red, blue and far-red) light combined with 100 μmol m^{-2} s^{-1} of white light; total irradiance was 350 μmol m^{-2} s^{-1} in all treatments. However, the green light treatment was a combination of 150 μmol m^{-2} s^{-1} green light and 200 μmol m^{-2} s^{-1} of white light. There was also a darkness treatment. Broken line indicates L-ascorbate levels at the beginning of the light treatments (t=0). All light treatments had the same light sum. Error bars represent standard errors of mean, letters indicate statistical difference at P≤0.01 with n=5 (Exp. 5).

3.4 Discussion

3.4.1 ASC in tomato fruit does not increase substantially with the progress of development

For detached tomato fruit, ASC does not increase considerably with the progress of development (from mature green to red) under irradiances below 150 µmol m^{-2} s^{-1}.

This is in line with the absence of developmental effects on ASC content of tomato fruit when tomatoes grown in a greenhouse at the lower part of the canopy where average light intensities were lower than 150 µmol m^{-2} s^{-1}. The light conditions in the greenhouse during this time of the year may have only minor effects on ASC levels. It is only during over-ripening from red to extreme red that ASC increased which is in line with other studies (Ioannidi et al., 2009; Yahia et al., 2001). This response is not related to irradiance. The limited increase of ASC with prolonged development is attributed to the activation of the galacturonate pathway (Agius et al., 2003). The galacturonate pathway converts carbon released from cell wall breakdown to ASC. Its contribution in red ripe tomato fruit is limited while the pathway is presumably inactive in green fruit (Badejo et al., 2012). As in the current experiment there was an increase of approximately 20%, it is hypothesized that this is the result of both the primary (D-Man/L-Gal) and galacturonate pathways.

3.4.2 Light improves ASC levels of tomato fruit by local biosynthesis

Broadband visible light can increase ASC levels in several species. This effect has been proven for both leaves (Bartoli et al., 2006; Fukunaga et al., 2010; Massot et al., 2012) and fruit (Labrie and Verkerke, 2012; Li et al., 2010, 2009; Ntagkas et al., 2016). In line with previous work, ASC levels are higher in fruit kept in light compared to darkness. Leaf irradiation may also regulate ASC in fruit as ASC is synthesized in mature leaves and transported through phloem sieve tube elements to the fruit (Hancock et al., 2003). However, regulation of ASC in tomato fruit is more dependent on fruit irradiance compared to leaf irradiance (Gautier et al., 2008). In the current work it has been shown that fruit irradiation increases ASC levels by up to approximately 500%. Translocation of ASC from the leaves to the fruit was excluded as the fruit were placed in light after detachment from the plant and removal of the calyx. This indicates that increase of ASC by fruit illumination is due to light effects on biosynthesis, recycling and/or turnover locally in the fruit. Various genotypes of different fruit sizes, ranging from 20g to 160g average fruit weight, respond similarly. This suggests a universal effect of light on ASC in tomato fruit.

Tomato fruit from all experiments have been harvested from the same greenhouse compartment at different seasons of the year. Fruit for Exp. 2 have been harvested

in spring while fruit for all other experiments have been harvested in summer. The temporal variation of irradiance (higher daily quantum integral in summer compared to spring) is the reason for the difference in starting levels (t=0) of ASC between Exp. 2 and the rest of the experiments.

In experiment 3, the maximum ASC levels after 7 days were achieved at 300 μmol m^{-2} s^{-1} (about 35 mg/100g of fresh weight). In experiment 2, ASC levels after 7 days exposure to 500 μmol m^{-2} s^{-1} were slightly higher (about 40 mg/100g of fresh weight). In experiment 3, ASC levels at 600 μmol m^{-2} s^{-1} were lower than 300 μmol m^{-2} s^{-1}. This indicates that at a 7-day treatment the optimal irradiance for stimulating ASC in tomato fruit is around 500 μmol m^{-2} s^{-1}, with irradiances above 600 μmol m^{-2} s^{-1} being supra-optimal. A possible explanation for this reduction is that ASC may be utilized in encountering the high oxidative load observed at high irradiances.

Specific spectra of the visible part of the spectrum have an effect on ASC in green tissue. *Phaseolus vulgaris* leaves grown under low red:far-red had lower ASC levels compared to higher red:far-red (Bartoli et al., 2009). In accordance, ASC was higher in tomato fruit kept in additional red light than when kept under additional far-red with the same irradiance. This might be associated with either signalling through phytochrome or the extra photosynthetically active radiation of the red-light treatment. ASC increased also in leaves under blue light (Lester, 2006; Ohashi-Kaneko et al., 2007). The results of the current work are in line with this, as ASC increased (146%) when fruit were kept in broadband light with additional blue. When broadband light was compared to light with high proportion of either red or green, no additional effect of the latter was observed. In all light treatments, the white background light was supplied at 100 μmol m^{-2} s^{-1} which is not expected to increase ASC. Signal limitation of ASC increase due to lack of specific spectra is also not expected. It can be concluded that spectral effects are limited that cryptochromes and phototropins do not play an important role. ASC levels in the supplementary far-red treatment were higher than initial levels and fruit stored in darkness. This is not likely explained by the white background light, nor the additional PAR form the far-red lamps (5 μmol m^{-2} s^{-1}) and might be a phytochrome related effect. Ascorbate peroxidase synthesis has been proposed to be regulated by phytochrome (Thomsen et al., 1992).

3.4.3 Light increases ASC levels in harvested tomato fruit via effects on photosynthesis

ASC in irradiated fruit ceased to increase when the fruit lost their green colour by entering the breaker stage. Similarly, red fruit did not increase in ASC in response to light, whereas green fruit achieved higher ASC levels when kept in more than 150 μmol m^{-2} s^{-1}. This suggests that photosynthetic activity is essential for ASC accumulation. The involvement of photosynthesis in light regulation of ASC is also supported by the spectral treatments. Light spectra that are expected to result in higher photosynthetic

rates (white, blue and red) resulted in higher ASC level in tomato fruit compared to darkness and spectra that result in lower photosynthesis (far-red). No considerable differences in photosynthesis are expected between the red, blue and green light treatments (Paradiso et al., 2011) which is in line with no differences observed in ASC between these treatments. Green light has beneficial effects on ASC potentially due to light absorption from the tissues below the pericarp as it penetrates deeper in the fruit. Expected photosynthetic rates for the spectral treatments correlate with ASC levels for all spectral treatments. It can be concluded that a minimum amount of chlorophyll in the tissue of tomato fruit is essential for light regulation of ASC.

Involvement of photosynthesis in light regulation of ASC has been previously proven in leaves but not in fruit. In leaves, high irradiances result in higher photosynthetic electron transport. The latter regulates the plastoquinone redox state which affects the gene expression of ASC related enzymes (Karpinski et al., 1997). Arabidopsis leaves treated with ATZ and DCMU (photosynthetic inhibitors) did not achieve higher ASC levels when placed under light, compared to non-treated plants. This is attributed to reduced activity of the D-Man/L-Gal pathway (Yabuta et al., 2007). In the time course experiment, photosynthetic rates were higher when fruit kept in light compared to darkness (Figure 3A). Inhibition of photosynthesis with DCMU also reduced ASC levels in mature green tomato fruit but not in red fruit (Badejo et al., 2012). It can be concluded that a minimum photosynthetic rate in tomato fruit is essential for light induced increase of ASC.

Respiration is also related to ASC. The last enzyme of the main biosynthetic pathway (GLDH) is located in mitochondria (Bartoli et al., 2000). GLDH is part of complex I of the respiratory electron transport chain (Schimmeyer et al., 2016). CytC is an electron acceptor from GLDH (Leferink et al., 2008). When cytC is oxidized, the enzymatic activity of GLDH increases resulting in ASC accumulation, given that the substrate for this reaction is sufficient (Bartoli et al., 2000). In the time course experiment, respiratory rates and ASC levels in different light treatments did not correlate. Respiratory CO_2 emissions were lower in fruit at 500 µmol m^{-2} s^{-1} compared to darkness at day 2. Respiration rates were similar in dark and light treated fruit at the end of the treatment. Light suppression of dark respiration in darkness might be a potential explanation of such a response (Sharp et al., 1984). The involvement of respiration in light regulation of ASC in tomato fruit cannot be dismissed in its entirety. Treatment of arabidopsis leaves with respiratory inhibitors (KCN and rotenone) proved that respiration is essential for the achievement of maximal ASC biosynthetic rates (Bartoli et al., 2006; Millar et al., 2003).

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3.5 Conclusions

ASC levels increased in detached mature green fruit when they were exposed to higher irradiances. ASC levels of red tomato fruit did not respond to irradiance treatments. Spectral effects on ASC were limited. Furthermore, the ASC levels increase when the fruit still contain considerable amounts of chlorophyll and is manifested across a range of different sized cultivars. Therefore, it can be concluded that the light signal for increase of ASC is perceived only by chlorophyll containing fruit and fruit illumination is sufficient for considerable ASC upregulation in the fruit pericarp. This effect is mostly independent of the light spectrum. The rate of fruit photosynthesis correlated with the light induced increase in ASC. There was no correlation between the respiratory rate of the fruit and ASC levels. Therefore, it can be concluded that the positive effect of light on ASC levels of tomato fruit is mediated primarily through fruit photosynthesis.

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3

Chapter 4

Light-induced vitamin C accumulation in tomato fruits is independent of carbohydrate availability

4

Abstract

L-ascorbate (ASC) is essential for human health. Therefore, there is interest in increasing the ASC content of crops like tomato. High irradiance induces accumulation of ASC in green tomato fruits. The D-mannose/L-galactose biosynthetic pathway accounts for the most ASC in plants. The myo-inositol and galacturonate pathways have been proposed to exist but never identified in plants. The D-mannose/L-galactose starts from D-glucose. In a series of experiments, we tested the hypothesis that ASC levels depend on soluble carbohydrate content when tomato fruits ripen under irradiances that stimulate ASC biosynthesis. We show that ASC levels considerably increased when fruits ripened under light, but carbohydrate levels did not show a parallel increase. When carbohydrate levels in fruits were altered by flower pruning, no effects on ASC levels were observed at harvest or after ripening under irradiances that induce ASC accumulation. Artificial feeding of trusses with sucrose increased carbohydrate levels, but did not affect the light-induced ASC levels. We conclude that light-induced accumulation of ASC is independent of the carbohydrate content in tomato fruits. In tomato fruit treated with light, the increase in ASC was preceded by a concomitant increase in myo-inositol.

4.1 Introduction

L-ascorbate (ASC; vitamin C) is a phytochemical known for its antioxidant properties and positive effect on nutritional iron availability. It is essential for a healthy human body (EFSA Panel on Dietetic Products, 2010). Humans are unable to synthesize ASC (Chatterjee, 1973; Nishikimi et al., 1994; Asensi-Fabado and Munné-Bosch, 2010). Therefore, they rely on a fruit- and vegetable-rich diet for sufficient amounts of ASC. ASC bioavailability in the human body is higher when ASC originates from plant products compared to artificial sources (Bjelakovic et al., 2004; Inoue et al., 2008; Fitzpatrick et al., 2012).

Improving the ASC content of edible plant parts is an interesting option to contribute to public health. This requires thorough understanding of the biochemical and physiological processes involved in ASC regulation in plants. When plants are exposed to higher light intensities, their ASC contents increase (Bartoli et al., 2006; Dowdle et al., 2007; Yabuta et al., 2007; Li et al., 2010). Tomato appears very responsive to light in terms of ASC levels: ASC content of tomato fruits increased when the fruit trusses were exposed to higher irradiances during cultivation (Gautier et al., 2008; Labrie and Verkerke, 2012; Massot et al., 2012). Detached green mature tomato fruits that ripened under light, achieved up to 5 times higher ASC levels in their pericarp than fruits that ripened in the dark (Chapter 3). This response has been related to a light-induced increase in photosynthetic activity (Chapter 3; Madhusudhan et al., 2003) and a direct signalling effect of light on ASC biosynthesis-related genes (Massot et al., 2012). In order to optimize the light environment for ASC accumulation, a better understanding of the underlying physiological network is essential.

There are several proposed pathways of ASC biosynthesis in plants. The vast majority of the ASC pool comes from the D-mannose/L-galactose (D-man/L-gal) biosynthetic pathway, with D-glucose as the initial substrate and mannose and galactose as the intermediate products (Wheeler et al., 1998). In view of this substrate–product relationship, the general hypothesis that the content of soluble carbohydrates (glucose, fructose, and sucrose) regulates the content of ASC emerged (Smirnoff and Pallanca, 1996; Nishikawa et al., 2005; Badejo et al., 2012). Besides the D-man/L-gal pathway, the galacturonate pathway has been identified in ripening strawberry fruits; this pathway synthesizes ASC from galacturonate, which is a product of cell wall breakdown (Agius et al., 2003). Even though the galacturonate biosynthetic pathway has been thoroughly described, to date there is limited evidence for its contribution to the ASC pool in tomato (Chapter 2). Two more pathways for ASC biosynthesis, namely the myo-inositol pathway and the gulose pathway, have also been proposed to exist in plants (Wolucka and Van Montagu, 2003; Lorence et al., 2004). Genes encoding different steps in the latter pathway have been identified and transgenic plants overexpressing the gene encoding one enzyme of the myo-inositol pathway (myo-inositol oxygenase) achieved higher ASC levels (Zhang et al., 2008). There is yet limited evidence for a

major contribution of these alternative pathways to the ASC pool (Chapter 2). Thus, in non-genetically modified plants the D-man/L-gal biosynthetic pathway is the main route determining the size of the ASC pool and its activity is potentially affected by soluble carbohydrate availability.

Apart from being a substrate for ASC biosynthesis, carbohydrates play a signalling role in a variety of processes including the development of antioxidant networks (BolouriMoghaddam et al., 2010). In tomato fruits and broccoli florets, carbohydrate feeding resulted in an increase in the expression rates of certain genes involved in ASC biosynthesis, such as GDP-D-mannose pyrophosphorylase (VTC1), GDP-D-mannose 3,5-epimerase (VTC2), and L-galactose-1-P phosphatase (L-GalLDH), as well as recycling and turnover genes, such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Nishikawa et al., 2005; Badejo et al., 2012). Therefore, soluble carbohydrates appear to regulate ASC levels by acting not only as a substrate but also as signalling cues for ASC biosynthetic, recycling and turnover genes.

The hypothesis that the pool of soluble carbohydrates regulates the ASC levels has been tested in a variety of different plant species and tissues, though with variable results. Artificial carbohydrate feeding did not increase the ASC content of photosynthesizing tissue in both pea seedlings (Pallanca and Smirnoff, 1999) and in arabidopsis leaves (Yabuta et al., 2007). However, soluble carbohydrate feeding of harvested broccoli inflorescences delayed ASC depletion during storage (Nishikawa et al., 2005). Whether availability of carbohydrates limits ASC biosynthesis in tomato fruits remains to be elucidated.

Soluble carbohydrates are the substrate for ASC biosynthesis via the D-man/L-gal biosynthetic pathway. Higher light intensities stimulate the biosynthesis of ASC (Chapter 3). We hypothesize that this stimulation of ASC accumulation by light might at least be partially caused by increased production of soluble carbohydrates. The aim of this research is to investigate whether ASC accumulation in tomato fruits is limited by soluble carbohydrate availability. To test this hypothesis, a number of experiments were conducted in order to obtain different levels of soluble carbohydrates in tomato fruits: (1) exposing detached fruits to different light intensities, (2) cultivation of plants with different fruit loads, and (3) artificial feeding of detached fruits with carbohydrates. In these experiments, ASC contents were quantified and related to the carbohydrate contents.

4.2 Materials and methods

4.2.1 Experiment 1—Detached mature green fruits stored in light and darkness

In Experiment 1 mature green tomato fruits (Solanum lycopersicum cv. Vimoso) were harvested in spring from a commercial glasshouse (Royal Pride Holland) in Middenmeer, the Netherlands. The fruits were taken from the 3rd and 4th positions of the truss (counting acropetally, starting from the oldest fruit present on the truss) from trusses of totally 8 fruits. After harvest, uniform fruits were selected according to colour (-0.6 ± 0.03 NAI and 0.07 ± 0.02 NDVI colour indices), size (6.2 ± 0.3 cm) and weight (43 ± 3 g). Light treatments took place in a climate-controlled room (Wageningen University and Research facilities) which contained 2 compartments: one with a light treatment (500 µmol m^{-2} s^{-1} of white light at fruit level and blue phosphorous-coated LEDs (GreenPower LED, Philips, The Netherlands) and the other in darkness. The LEDs were placed at a height of 80 cm above the fruits. All sides of the compartments were covered with neutrally reflective MC-PET sheets (SRF-A032T, Sekisui Plastics Co., LTD, Osaka, Japan) in order to improve light distribution. Irradiance difference between the brightest and darkest points of the area where the fruits were placed was 10 µmol m^{-2} s^{-1}. In order to expose fruits to light uniformly, the fruits were placed in all spots of the illuminated area by daily rotation. The spectrum of the LED light treatment was measured with a spectroradiometer (USB2000, Ocean Optics, Duiven, The Netherlands; calibrated against a standard light source) and had a phytochrome stationary state (Sager et al., 1988) of 0.83. Air temperature was 18°C in all compartments. The temperature of the epidermis of the fruits in the light treatment was approximately 0.1°C higher than air temperature and the difference between the light and darkness treatments was less than 0.4°C. Air and fruit temperature was monitored with k-type thermocouples (shielded from direct radiation) on TC-08 data loggers (Picotechnology LTD., Cambridge, UK) calibrated in distilled water at freezing and boiling point. Relative humidity in the room was 70%. After removal of the calyx, the fruits were placed with the scar downwards in order to minimize fruit water loss. In these conditions the fruits in both treatments had lost 2.4% of their initial weight after 15 days. ASC was analyzed in fruit pericarp as in tomatoes the vast majority of ASC is found in the pericarp (Moco et al., 2007; Badejo et al., 2012). ASC was analysed every 3 days until the end of the 15-day light and darkness treatments. The duration of this experiment was 15 days as ASC levels were observed to increase from day 0 to day 9 and then remained unchanged after the fruit entered the breaker stage (day 9 to day 15). The initially green-mature fruit reached the red-mature stage by the end of the 15 day treatment. From each treatment 10 fruits were paired into 5 replicates. Each replicate consisted of 6 pericarp discs (2 fruits, 3 discs per fruits, 1 cm diameter).

4.2.2 Experiment 2—Soluble carbohydrate content varied by fruit load

In Experiment 2 tomato seeds (Solanum lycopersicum cv. Komeett) were sown on August 6, 2015, shoots were grafted on a Maxifort rootstock and then topped, resulting in two stems per plant. On October 8 they were planted on rockwool slabs at a density of 2.5 stems per m2 in a glasshouse compartment at the research facilities of Wageningen University and Research, Bleiswijk. Average air temperature was 20 °C, ambient CO_2 concentration was 600–800 ppm during daytime and relative humidity was 80%. In addition to the daily outside global radiation, 185 µmol m^{-2} s+ of supplementary red and blue LED (95% red and 5% blue, Philips Greenpower) light was applied from midnight until sunset. Nutrient supply and pest and disease control followed commercial practices. Flowers were pollinated by bumble bees. In order to produce fruits with different carbohydrate content, 3 fruit load treatments were applied: trusses were pruned to either 1, 3, or 5 flowers per truss. This pruning was applied to all trusses of an individual plant. Fruits were harvested in March, at the red mature stage. Ten fruits per pruning treatment were paired into 5 replicates with each replicate consisting of 6 pericarp discs from two fruits. All fruits were harvested at the same time point. Each replicate originated from a different plant from a truss at the same height on the plant. The fruit load treatments were randomized over all 30 plants of the line.

4.2.3 Experiment 3—Detached fruits with variable soluble carbohydrate content due to fruit load, are exposed to light and darkness

In Experiment 3 tomato plants (Solanum lycopersicum cv. Komeett) were grown in a glasshouse compartment next to the compartment of Experiment 2. All conditions and fruit load treatments were similar as in Experiment 2 unless otherwise stated. Sowing and transplanting dates were identical to Experiment 2. In this glasshouse compartment, far-red LEDs (research module far-red, Philips Greenpower) were additionally installed with an intensity of 40 µmol m^{-2} s^{-1}. Fruits from different fruit load treatments were harvested at the mature green stage and stored for 7 days under darkness or at 300 µmol m^{-2} s^{-1} of white light (blue phosphorous-coated LEDs; GreenPower LED, Philips, The Netherlands) until ripening (red mature stage). Storage conditions were identical to Experiment 1. For the ASC and carbohydrate measurements, 10 fruits were paired into 5 replicates, and each replicate consisted of 6 pericarps from two fruits. Fruit load treatments were randomized over all 30 plants of the line.

4.2.4 Experiment 4—Truss feeding experiment

In Experiment 4 tomato trusses (Solanum lycopersicum cv. Vimoso) were taken from the same glasshouse as those of Experiment 1. The trusses were harvested when fruits were at the immature green stage (slightly smaller than mature green),

in order to facilitate the sucrose uptake from the feeding. Intact trusses bearing 8 fruits per truss were harvested early in the morning. The cut stem end of the truss was submerged in tap water during transportation from the greenhouse to the lab in order to prevent air embolism of the xylem. Upon arrival at the lab of Wageningen University and Research, where the trial took place, 1 cm from the cut end of the truss stem was excised under water in order to remove possible cavitation that might have taken place during transportation. On the freshly cut end, 5 cm flexible silicon tube was attached. The tube was connected to falcon tubes (50 mL) containing sucrose solutions of different concentrations (20, 10, 5, 1, and 0 g/100mL H_2O). To achieve the same osmotic potential in all solutions 0, 1.77, 2.52, 3.03, and 5.32 g of mannitol was added respectively to the 20, 10, 5, 1, and 0 g/100mL sucrose solutions. The cut end was recut by 0.5 cm every three days to remove any wound induced blockages. The tomato trusses attached to the feeding system were stored for 10 days in temperature-controlled cabinets. Irradiance was 280 µmol m^{-2} s^{-1} applied continuously, using the same white LEDs as in Experiment 1. This irradiance level should be sufficient to increase the ASC content considerably (Chapter 3). Temperature and relative humidity were identical to Experiment 1. After 10 days of sugar feeding the fruits had reached the red mature stage. At that time, fruits in positions 1 and 2 (counting acropetally, starting from the oldest fruit present on the truss) were analysed for ASC. Ten fruits were paired into 5 replicates, each replicate consisting of half a tomato coming from two fruits.

4.2.5 ASC and total soluble carbohydrates determination

In Experiment 1, contents of ASC, soluble carbohydrates (sucrose, glucose, and fructose) and the ASC precursors myo-inositol and galacturonic acid were measured in the pericarp, using gas chromatography coupled to mass spectrometry (GC-MS) of derivatized polar compounds (Mokochinski et al., 2018). Methanol (0.7 mL) containing ribitol as an internal standard was added to 100 mg of frozen fruit powder, vortexed for 10 s, and then mixed for 10 min at 950 r.p.m. After 15 min sonication and 10 min centrifugation, 250 µL of the supernatant was re-extracted with 185.5 µL of chloroform and 375 µL of ultrapure water. Aliquots (50 µL) of the upper (polar) phase were dried in a speedvac overnight. Prior to GC-MS analysis, these dried extracts were derivatized online using a CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and both methoxyamine and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as derivatization agents. An alkane mixture (C10–C30) was automatically added to each sample. The derivatized polar extracts were then analysed by a GC-MS system comprising an Agilent 6890 gas chromatographer (Agilent Technologies, Santa Clara, USA) coupled to a Pegasus III time-of-flight MS (Leco Instruments, Saint Joseph, USA). Target compounds were annotated by matching both the observed electron impact (EI) mass spectra and retention indexes with the Golm EI-spectral database (http://gmd. mpimp-golm.mpg.de/). Their relative levels were calculated from the corresponding GC-MS peak heights with correction for the internal standard.

In Experiments 2, 3, and 4, ASC and soluble carbohydrates were measured. The pericarp samples were frozen in liquid nitrogen immediately after dissection and mechanically grinded to fine powder under liquid nitrogen. Approximately 0.2 g of the powder was mixed with 0.5 mL 3.3% metaphosphoric acid and thawed on ice in darkness. After 10-minute sonication (Branson 2200; Branson Equipment Co., Shelton, CT, USA), samples were centrifuged at 25,000 rcf at 4°C for 10 minutes. The supernatant was used for L-ASC determination. In 100 µL of each of these extracts L-DHA was reduced to ASC in a separate tube by the addition of 50 µL DTT 5mM and 400mM Tris base (Davey et al., 2003). In this second extract, total ASC was determined allowing the calculation of DHA by subtracting the amount of reduced ASC. However, DHA was below the detection threshold and therefore not considered in our further analysis. Extracts were measured in a high-performance liquid chromatography system (P580 pump, UVD 340S detector, Dionex Corporation, Sunnyvale, USA). From the initial pulverized tomato sample, 0.3 g was taken for the determination of soluble carbohydrates. After 5 mL of 85 % ethanol was added, the samples thawed on ice and consecutively placed in thermal bath at 80 °C for 20 minutes and centrifuged at 8500 rpm at 4 °C for 5 minutes. The supernatants were transferred to Eppendorf tubes and dried in a SpeedVac (SPD 2010; Thermo Fisher Scientific Inc., Asheville, NY, USA) for 2 hours. Carbohydrates were resuspended in 1 mL Milli-Q water and samples were sonicated for 10 min. After diluting the extract 10 times, soluble carbohydrates were quantified using high-pressure anion exchange chromatography (HPAEC; ICS5000; Dionex, Sunnyvale, CA, USA) by the use of an anion exchange column (250x-2 mm; CarboPac PA1; Dionex) at 25 °C. Chromatograms were analyzed in Chromeleon 7.0 software (Dionex) and glucose, fructose and sucrose were quantified using calibration curves of authentic standards.

4.2.6 Measurements of respiration and photosynthesis

The photosynthetic electron transport rate in photosystem II (PSII) of the tomato fruits was measured under 300 µmol m^{-2} s^{-1} with a chlorophyll fluorescence imaging system (FluorCam 700MF, Photon System Instruments, Brno, Czech Republic). With the same system maximum quantum efficiency of photosystem II (Fv/Fm) after 30 minutes dark adaptation was also measured. Fv/Fm was measured under a saturating pulse of 3500 µmol m^{-2} s^{-1}. FluorCam v.5.0 software was used to operate the measurement protocol in FluorCam 700MF. Ten replications (individual fruits) were done per treatment.

Fruit respiration was assessed by measuring CO_2 exchange of the fruits. A portable infrared gas exchange system (LI-6400; Li-Cor Inc., Lincoln, Nebraska, USA) was used. A single tomato was placed in a transparent, plastic sphere, which was bypassed in the sampling circuit of the LI-6400. The leaf cuvette of the device was isolated from the sampling circuit (Savvides et al., 2013). The sphere reduced irradiance by 10% with no effects on the spectrum. Temperature in the sphere was 18,5 °C and CO_2 concentration of the reference was set to 450ppm. The CO_2 emission rate was logged

for 15 min after a stabilization period of 15 min. Ten replications (individual fruit) were done per treatment.

4.2.7 Statistical analysis

Two-way analysis of variance (ANOVA) was used to test the effects of two factors on ASC, ASC precursors, carbohydrates, respiration, and photosynthesis data: light treatment and time on L-ASC in Experiment 1, fruit load and light treatment in Experiment 3, and sucrose feeding and light in Experiment 4. One-way ANOVA was used to test the effects of fruit load on L-ASC (Experiment 2). In all experiments, the significance of carbohydrate content explaining variability in ASC was tested by linear regression analysis. The pool of material from two fruits was considered an independent replicate. This may have underestimated random variance. Therefore, the tests have been conducted at =0.01 instead of the commonly used =0.05 with post hoc Tukey's honestly significant difference (HSD) multiple comparison tests (=0.01). Statistical analyses were carried out with GenStat 18th edition (VSN, International, Hempstead, UK).

4.3 Results

4.3.1 Detached fruits in light and darkness

The aim of Experiment 1 was to investigate whether there is a correlation between carbohydrate content and ASC in detached fruits that matured under LED light or in darkness. In light, the ASC steadily increased during the experiment; in darkness ASC did not change (Chapter 3). Both in fruits stored in light and darkness the relation between the levels of ASC and individual carbohydrates (sucrose, fructose, and glucose) was not statistically significant (Figure 1A–C, respectively).
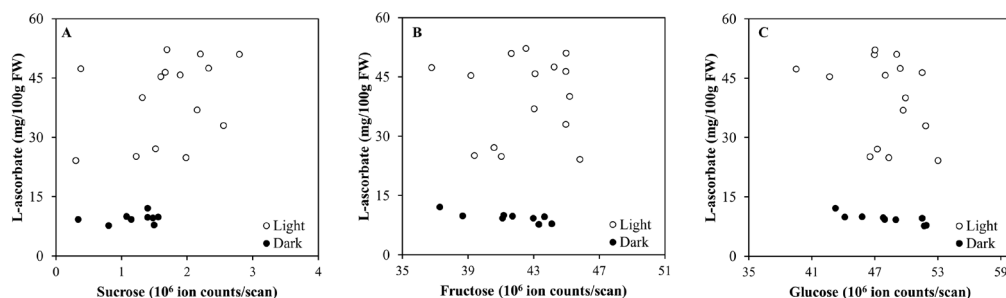
FIGURE 1 | Correlation between L-ascorbate (ASC) and sucrose (A), fructose (B), and glucose (C) for detached tomato fruits that ripened in light (500 µmol m^{-2} s^{-1}) or darkness for 15 days (Experiment 1). Each data point is based on pooled sample of pericarp discs of 2 fruits.

The relative content of the ASC precursor myo-inositol in the fruits increased about 2-fold within six days of storage in the light (Figure 2A). From day 6 until the end of the experiment (day 15) myo-inositol remained stable. For fruits stored in darkness, no significant changes in myo-inositol were observed over time (Figure 2A). Galacturonate was relatively low in the fruits until day 9 in the light, thereafter its content increased rapidly (Figure 2B). ASC content in light showed a significant positive correlation with myo-inositol (r=0.59, Figure 2C). The linear fitting however between ASC and myo-inositol was not significant for the dark treatment (r=0.48). ASC significantly correlated with galacturonate for both the light (r=0.63) and dark (r=0.98) treatments.
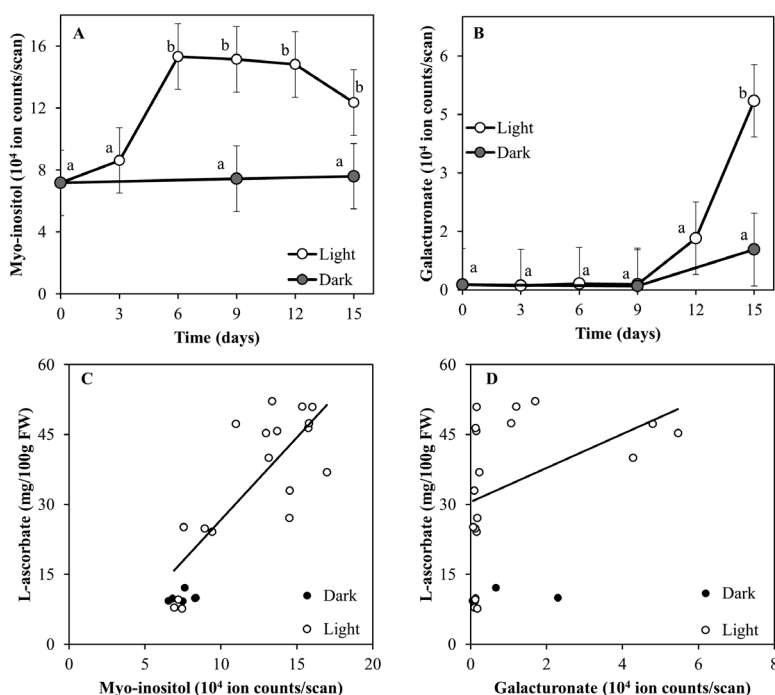
FIGURE 2 | Relative level of ASC precursors myo-inositol (A) and galacturonate (B) in detached mature green tomato fruits stored in 500 µmol m^{-2} s^{-1} light (open symbols) and darkness (closed symbols) for 15 days. C and D show the relation between ASC and ASC precursors (myo-inositol and galacturonate, respectively) for the light and darkness treatments. Each data point is based on pooled sample of pericarp discs of 2 fruits. Solid lines in C and D represent a linear fitting on data points of the light treatment. Error bars represent standard errors of means, letters indicate statistical difference at =0.01; n=5; Experiment 1).

ASC levels of tomato fruit stored for 15 days in light and darkness were measured every three days. ASC in the light treatment increased approximately five-fold until day 9 when fruit entered the breaker stage (from 8 to 48 mg/100 g FW), and remained constant from day 9 until day 15. In the dark treatment, ASC did not show any significant changes from the beginning of the experiment until day 15 (8–11 mg/100g FW; Chapter 3).

4.3.2 Soluble carbohydrate content varied by fruit load

The aim of Experiment 2 was to investigate whether carbohydrate content in red fruits, as varied by different fruit load levels, correlates with ASC content of tomato fruits that ripened on the vine. Reducing the number of fruits per truss resulted in significantly higher total carbohydrate levels when fruits had reached the red mature stage (Figure 3A). There were no significant effects of fruit load treatments on ASC content (Figure 3B). Hence, ASC did not correlate with carbohydrate content (Figure 3C).
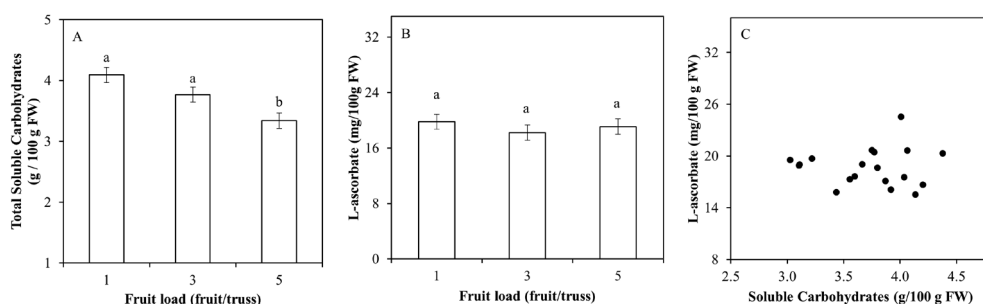
FIGURE 3 | Relation between fruit carbohydrate and ASC levels in mature red tomato fruits grown at 3 different fruit load levels (1, 3, or 5 fruits per truss). Total soluble carbohydrate content (sum of sucrose, fructose, and glucose) (A) and ASC content (B) . Correlation between ASC and carbohydrate content of individual samples (C). Each data point is based on pooled sample of pericarp discs of 2 fruits. Error bars represent standard errors of means, letters indicate statistical difference at =0.01; n=5; Experiment 2).

The aim of Experiment 3 was to investigate whether variable carbohydrate content in mature green fruits, as induced by different fruit load treatments, correlates with ASC in tomato fruits that were ripened under light (until red mature stage). Total soluble carbohydrate level in fruits increased significantly with lower fruit load but was not affected by light conditions during storage (Figure 4A). There were no significant differences in initial fruit ASC level among the three fruit load treatments (Figure 4B). Subsequent storage in light resulted in a significant increase of ASC of about 300% as compared to the initial content. Storage in darkness also resulted in an increase in fruit ASC but to a lesser extend (~70%) than storage in light. There was no significant effect of fruit load on ASC level of stored fruits at the end of the light treatment (Figure 4B). Linear regression analysis showed that there was no correlation between ASC and soluble carbohydrates both for the initial time point as well as at the end of the light and darkness treatments (Figure 4C).
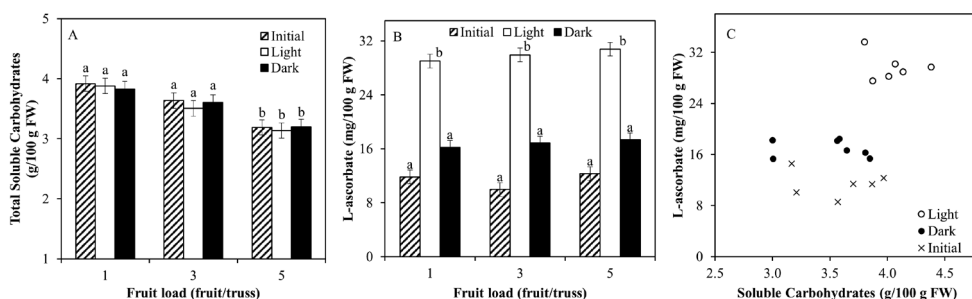
FIGURE 4 | Relation between fruit carbohydrate and ASC levels in mature green and light or dark ripened tomato fruits that were grown at 3 different fruit load levels (1, 3, or 5 fruits per truss). Total soluble carbohydrate content (sum of sucrose, fructose, and glucose (A) and ASC levels (B)) before and after ripening in light 300 µmol m^{-2} s^{-1} or darkness for 7 days. Correlation between ASC and carbohydrate content of individual samples (C). Each data point is based on pooled sample of pericarp discs of 2 fruits. Error bars represent standard errors of means, letters indicate statistical difference at =0.01; n=5; Experiment 3).

4.3.3 Soluble carbohydrate content varied by truss feeding

The aim of Experiment 4 was to investigate whether different carbohydrate content as induced by sucrose feeding of the truss, correlates with ASC content in tomato fruits that ripened in light during the sucrose feeding. Tomato trusses were fed with sucrose solutions of different concentrations under light and dark conditions for seven days. Soluble carbohydrates of fruits fed with 20 % (w/v) sucrose solution and kept in light, were significantly higher than their initial content (data not shown) and the lower sucrose feeding levels at either light or darkness (Figure 5A). ASC content was significantly higher at the light treatment compared to the dark treatment. However, sucrose feeding did not affect the ASC content of the fruits in the light nor in darkness (Figure 5B). A linear regression showed only a significant correlation between ASC and soluble sugar content in the light (r = 0.53; Figure 5C).

Sucrose feeding did not result in higher respiration rates of the fruits: the average CO_2 release rate was 18 mL kg^{-1} h^{-1} (± 0.2) for both the light and dark treatments. There were no significant differences in the rate of photosynthesis of fruits from different feeding treatments, with an average PSII of 0.39 for both treatments. Fv/Fm was lower in the light compared to darkness; averaged over all sucrose feeding treatments Fv/Fm was 0.55 and 0.78 for light and darkness, respectively. This difference in Fv/Fm was most likely related to a slightly accelerated development of the fruit in light.
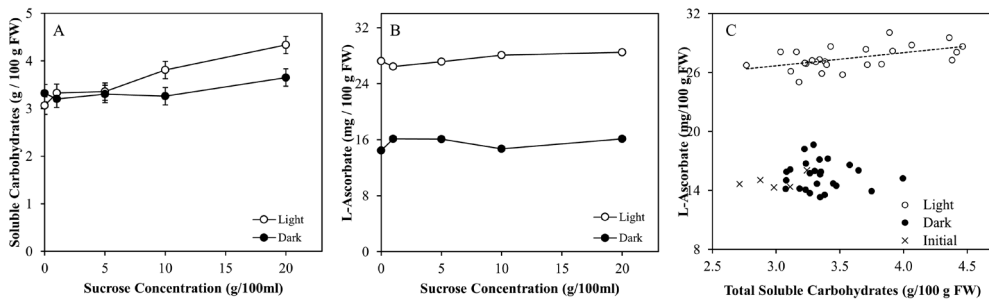
FIGURE 5 | Effect of sucrose feeding on fruit total soluble carbohydrate content (sum of sucrose, fructose, and glucose (A) and ASC content (B)) in tomato when detached immature green fruits were fed with 5 different sucrose concentrations during 7 days of ripening until red in light and darkness. Correlation between ASC and soluble carbohydrates content for fruits in light and darkness (C). Line in C represents a linear fitting of data points of the light treatment. Each data point is based on pooled sample of pericarp discs of 2 fruits. Error bars represent standard errors of means, =0.01; n=5; Experiment 4). Error bars in Figure B are smaller than symbol size.

4.4 Discussion

4.4.1 Light regulation of ASC is not via soluble carbohydrates

The D-man/L-gal ASC biosynthetic pathway is considered to contribute the most to the ASC pool in plants, unless genetically modified (Chapter 2). The D-man/L-gal pathway utilizes glucose in producing ASC in eleven steps with intermediate products such as D-mannose and L-galactose (Wheeler et al., 1998). As glucose is the product of sucrose breakdown and the precursor of ASC in the D-man/L-gal pathway, we tested the hypothesis that soluble carbohydrate availability correlates with ASC content. High light intensity positively affects the ASC levels in treated mature green tomato fruits (Chapter 3). Here we showed in detached tomato fruits that when ASC accumulation was induced by light, there was no significant parallel change in soluble carbohydrates. When soluble carbohydrate content was varied, either by different fruit loads on the plant or by feeding sucrose to detached trusses, ASC levels did not change in parallel to the carbohydrate content. That was also the case for fruits with different carbohydrate content that were ripened under irradiance levels that stimulate the ASC biosynthesis. Since no correlation between the levels of soluble carbohydrates and ASC was found, carbohydrates are apparently not the causal the causal factor for differences in ASC levels in harvested fruit and are not determinate for the accumulation of ASC under light.

In the current work, we show the absence of a correlation between carbohydrates and ASC levels. This is in line with research in some other species and tissues. Exogenous

application of sucrose or glucose in barley (Smirnoff and Pallanca, 1996) and pea (Pallanca and Smirnoff, 1999) embryonic axes did not cause ASC accumulation. ASC content of broccoli typically decreases with storage. Feeding of detached broccoli inflorescences did not result in ASC increase but in delayed ASC depletion (Nishikawa et al., 2005). In the same study, it was also found that sucrose feeding increased the expression rates of turnover (APX), recycling (MDHAR, DHAR, and GR), and biosynthetic (L-galactono-1,4-lactone dehydrogenase: GLDH) genes suggesting that carbohydrates regulate ASC through direct signalling. These signalling effects require further research using a variety of species as it has been suggested that carbohydrate regulation of ASC is species specific (Massot et al., 2010). Feeding arabidopsis plants with sucrose reduced ASC in the leaves due to inhibitory effects on PSII (Yabuta et al., 2007). This may suggest a regulatory role of photosynthesis for ASC in green tissue. The rate of photosynthetic electron transfer regulates the redox state of plastoquinone and that affects the ASC content through signalling on ASC enzymes (Madhusudhan et al., 2003). In conclusion, it is most likely that the beneficial effect of light on ASC is mediated through photosynthesis (Chapter 3) and signalling effects on expression of genes of the D-man/L-gal pathway (Massot et al., 2012), rather than by an increase in the substrate of ASC. Our research does not disprove that carbohydrate regulation of ASC in tomato could be induced after carbohydrate levels exceed a certain threshold. However, if there would be such a threshold, the commonly found carbohydrate concentrations in tomato fruits are above this threshold.

Incubation of micro-tom fruits in 5 g/100mL sucrose solution (Badejo et al., 2012) led to an increase in ASC. This increase has been suggested to be due to a possible rise of the respiration rates due to carbohydrate feeding. The rate of respiration regulates the ASC content by regulation of the enzymatic activity of the last step (GLDH) of the D-man/L-gal biosynthetic pathway (Bartoli et al., 2000; Millar et al., 2003). The effect of carbohydrate feeding on respiration rates differs between our research (no changes in respiration rates) and the previously mentioned case, potentially due to differences in plant material and feeding methods in between the two cases. This puts forward the idea that carbohydrates might affect the ASC levels only in the cases they regulate the rate of respiration but most likely not through direct signalling.

4.4.2 ASC accumulation coincides with increased availability of myo-inositol and galacturonate

Besides the D-man/L-gal pathway, other biosynthetic pathways for ASC have been proposed for plants. The galacturonate pathway produces ASC from the D-mannose released as a product of cell wall breakdown (Agius et al., 2003). As D-mannose is an intermediate product of the D-man/L-gal pathway, ASC production through the galacturonate pathway might also be dependent on the D-mal/L-gal pathway and consequently on soluble carbohydrate availability. When mature green fruits were stored in light, an increase in ASC was observed. Galacturonate—a precursor in the

secondary biosynthetic pathway—started to increase only after ASC had reached its highest level. This late increase of galacturonate (from six days after turning red) was most likely due to the progress of development (ripening) of the fruits and was not the consequence of increased activity of the D-mal/L-gal pathway as there was not an increase of carbohydrates. The rate of conversion of exogenously fed galacturonate to ASC is higher in mature red tomato fruits and the pathway is in vivo induced by ripening (Badejo et al., 2012). Even though the galacturonate pathway has been proposed to be a possible control point for ASC (Upadhyaya et al., 2009), there is to date no evidence that this pathway may contribute considerably to the ASC pool in non-genetically modified plants.

A four-step biosynthetic pathway that synthesizes ASC from myo-inositol with an intermediate precursor—L-gulono-1,4-lactone—has been proposed but not identified yet in plants (Lorence et al., 2004; Torabinejad et al., 2009). The light-induced increase of ASC in mature green fruits that ripened under light was preceded by an increase in myo-inositol by three days. The fact that the substrate for ASC biosynthesis through the myo-inositol pathway was being built up ahead of the ASC response puts into perspective the potential contribution of this pathway to the ASC pool when tomato fruit are stored in the light. So far, ASC biosynthesis of considerable amounts of ASC has been observed only in overexpressors of steps of the myo-inositol pathway (Radzio et al., 2003; Zhang et al., 2008; Maruta et al., 2010). Overexpression of strawberry myo-inositol oxygenase (MIOX) in transgenic tomato plants lead to higher ASC content in the leaves only when myo-inositol is artificially fed (Zhang, 2012). This suggests that myo-inositol content is likely insufficient for ASC biosynthesis in leaves. However, as seen in Experiment 1, myo-inositol content might not be a restriction for ASC accumulation in tomato fruits. The myo-inositol pathway is probably unlikely in non-genetically modified plants (Endres and Tenhaken, 2009). Current research does not provide sufficient evidence for the contribution of the myo-inositol pathway in plants. However, as the levels of the precursor correlate with ASC levels in tomato, it would be worth investigating if the activity of this pathway in tomato fruit would be affected by myo-inositol feeding (e.g., radioactive isotopes).

4.5 Conclusions

Soluble carbohydrates are the precursor for ASC biosynthesis. Mature green tomato fruits when allowed to ripen under higher light levels achieve higher ASC content. As light may stimulate photosynthesis and consequently availability of sugars in the plant, we hypothesized that the positive effects of light on ASC content could be due to an increase in soluble carbohydrates. In a series of experiments, it has been shown that mature green tomato fruits achieve higher content of ASC when kept in light, but no correlation with the soluble carbohydrate content has been observed. When carbohydrate content was altered either by fruit pruning treatments or artificial truss

feeding, no effects on ASC content was observed either. It is concluded that ASC accumulation by light is not due to an increase in soluble carbohydrates and that soluble carbohydrates do not limit ASC biosynthesis as increasing the carbohydrate concentration does not result in ASC increase. The increase of ASC in fruit stored in light was preceded by an increase in myo-inositol. However, further research is essential to determine if the myo-inositol biosynthetic pathway produces considerable amounts of ASC.

4

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Chapter 5

Modulation of the tomato fruit metabolome by light

Tomato is a commonly used crop for studying the biochemical processes underlying the ripening of climacteric fruits. A wide range of metabolites such as carotenoids, alkaloids and phenolic compounds change during ripening. It is known that ambient light can modulate the activity of certain biochemical pathways in plants. In the current work we investigated the effects of light directly supplied to the fruits, on the metabolome of the fruit pericarp during ripening. To enable specifically studying these light effects, fruits were picked from greenhouse-grown plants at their mature green stage and then exposed to well-controlled conditions with light, supplied by LEDs, as the only varying factor; control fruits were kept in darkness. Two independent experiments were performed: in Experiment 1 the fruits were exposed to either white LED light or darkness for 15 days, while in Experiment 2 fruits were exposed to different light spectra (blue, green, red, far-red, white) added to a background of white light for 7 days. Changes in the global metabolome of the fruit pericarp were monitored using both targeted and untargeted metabolomics approaches. Both carotenoids and flavonoids, as well as other potentially health-beneficial compounds like tocopherols and phenolic acids, accumulated faster under white light compared to darkness. Alkaloids and chlorophylls decreased faster, in fruits exposed to white light compared to darkness. Furthermore, flavour-related metabolites also changed in the presence of light. The light quality treatments affected fewer compounds compared to Experiment 1, with blue being the most effective treatment altering the metabolome. The light environment during ripening of tomatoes has a wide range of effects on the metabolome by regulating health and taste related metabolites.

Abstract

5.1 Introduction

Tomato is an important crop widely produced around the world. Due to its nutritional content, tomato plays an important role in human health (Giovannucci, 1999). An adequate understanding of the environmental factors and the underlying physiological processes that regulate the nutritional content of the tomato fruits is essential for the production of fruits with enhanced nutritional value. Furthermore, due to its well-studied genome and suitability for genetic manipulation, tomato is a model crop for the study of a variety of metabolic processes (Faurobert et al., 2007).

Tomato fruits contain a wide range of metabolites with some of the most important being carotenoids, flavonoids and other phenolic compounds as well as flavour and aroma related compounds. Tomato contains considerable amounts of metabolites that act as antioxidants (phenolic compounds and carotenoids) and vitamins (e.g. vitamins C and E). It is most known as a crop rich in the carotenoid all-trans lycopene. Consumption of foods rich in lycopene has been proposed to be related to the prevention of multiple types of cancer (Giovannucci, 1999; Ford and Erdman, 2012) and cardiovascular diseases (Müller et al., 2016). Certain claimed health benefits of lycopene such as prostate cancer prevention remain debatable to date (Basu and Imrhan, 2007; Jatoi et al., 2007). Apart from all-trans lycopene, tomatoes contain carotenes which contribute to the specific colour of orange-coloured varieties and are also known to have beneficial effects on the human body by acting as antioxidants and being the main vitamin A precursors (Krinsky and Johnson, 2005). Furthermore, carotenoids are the precursors of a variety of volatiles in fruits, generated through the action of carotenoid-cleavage enzymes (Vogel et al., 2010).

Tomato is a climacteric fruit that is able to ripen after harvest. The climacteric peak in internal ethylene concentration, typically observed during the colour transition from green to red, stimulates a variety of developmental processes resulting in drastic changes in the metabolome. When the fruits enter the breaker stage, chlorophylls are broken down while flavonoids and carotenoids increase dramatically (Liu et al., 2009). Besides the visible pigment changes other phenomena also occur during ripening of the tomato fruits. Typically starch is broken down to hexoses and the production of aromatic volatiles is upregulated. Furthermore, modifications of the cell walls occur resulting in the loss of firmness (Fraser et al., 1994; Giovannoni, 2001; Carrari et al., 2006). These ripening-related changes determine to a large extent the typical sensory characteristics of tomatoes.

Light is the primary source of energy in plants and as such it affects the metabolome not only of the leaves but also of the fruits. Besides being present in leaves, phytochromes (Alba et al., 2000) and cryptochromes (Giliberto et al., 2005) have been found in the pericarp of tomato fruits. Therefore, not only light intensity but also light spectrum may potentially influence the metabolome of tomato. Higher irradiance levels on

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tomato fruits can stimulate the accumulation of ascorbic acid in the pericarp (Chapter 3) through an overall increased photosynthetic activity in the fruit but not related to increased carbohydrate availability (Chapter 4). The light spectrum had small effects on ascorbate levels of tomato fruits (Chapter 3). Next to upregulating ascorbate, the light spectrum may influence other fruit metabolic pathways like those involved in pigmentation. For instance, in cranberries red light increased the anthocyanin content as compared to white and far-red light (Zhou and Singh, 2002). To better characterize how light regulates the quality and shelf life of fruits, research has to be expanded to both nutritional health-related as well as flavour and aroma-related metabolites. This knowledge becomes highly relevant with the increasing integration of LEDs in horticultural lighting systems (Morrow, 2008; Urrestarazu et al., 2016), as LED usage potentially opens up a wide range of applications including the regulation and fine-tuning of the crop metabolome. LEDs have not only been related to a high energy efficiency but they also allow application of light with specific wavelengths (Massa et al., 2008).

The abiotic environment and especially light during the pre- or postharvest part of the tomato supply chain plays an important role in the ripening process as it may affect the metabolome of the fruits. The aim of this research was to study the effect of light on the metabolic profiles of tomato fruits during ripening. We tested the hypothesis that the presence of light and its spectrum can modulate the fruit composition. Detached tomato fruits were exposed to treatments with and without light and different light spectra. Both targeted and untargeted metabolomics analyses were performed, which provided us the ability to study multiple changes in the metabolome simultaneously. We discuss the observed changes in metabolite profiles in relation to the potential impact on visual quality (pigment), nutritional quality (vitamins and antioxidants) as well as organoleptic characteristics (flavour-related compounds, sugars, acids).

5.2 Materials and methods

5.2.1 Plant material

Tomato fruits (Solanum lycopersicum cv. Vimoso) from a commercial glasshouse (Royal Pride Holland) in Middenmeer, the Netherlands were used. Trusses of 8 mature green fruits that were hanging on the same position on the plants were harvested and transported to Wageningen University and Research facilities in Wageningen, the Netherlands within 3 hours of harvest. To ensure a similar developmental stage, only fruits from positions 3 and 4 in the truss counting acropetally (from the oldest to the youngest fruits) were used. The selection of fruits was further narrowed down by measuring and selecting fruits of similar fresh weight (43 ±3 g) and colour (pigment analyser spectroradiometer, PA1101, CP, Germany).

5.2.2 Light treatments and abiotic environment

We performed two experiments to determine the effects of light intensity and light quality on the metabolite composition of tomato. In experiment 1 (Exp. 1) the overall effect of light was investigated by treating detached fruits with either white light or complete darkness for 15 days. In experiment 2 (Exp. 2) fruits were treated with different light spectra for 7 days. Both experiments have been previously described (Chapter 3). In both experiments, small compartments with an illuminated area of 80 X 50 cm were used for the application of the light treatments (2 compartments in Exp.1 and 6 compartments in Exp.2). Each compartment consisted of an aluminium frame that supported the LED module on top and was covered on the sides by highly reflective MC-PET sheets (SRF-A032T, Sekisui Plastics Co., LTD, Osaka, Japan) which significantly improved light distribution. This material was chosen for its ability to reflect light without affecting the spectrum, a property that was further verified by a spectrophotometer with the use of an integrated sphere in a dark box (USB-4000, Ocean Optics, Dunedin, FL, USA). The compartments were placed on the top of tables inside a climate cell and each compartment represented an individual light treatment. The distance between the LED modules and the surface of the table was 80 cm. The fruits were placed in the middle of the compartment (in an area of 60 x 30 cm) where the light was most evenly distributed (less than 10 μmol m^{-2} s^{-1} difference between the brightest and darkest spots). In order to expose the fruits equally to the light, fruits were daily rotated within the illuminated area. Irradiance and spectral distribution were measured in all treatments on a 5 by 5 cm grid with a spectroradiometer (USB2000, Ocean Optics, Duiven, The Netherlands; calibrated against a standard light source). Light was continuously applied (24 hours per day) in both experiments.

In Exp. 1 the fruits were ripened for 15 days in complete darkness or under white light (broad spectrum) of 500 μmol m^{-2} s^{-1}. White light was supplied with blue phosphorous coated LEDs (GreenPower LED research module, Philips, The Netherlands). In Exp. 2 the fruits were treated with 4 different monochromatic spectra (blue, red, far-red, and green) for 7 days. The irradiance levels of the monochromatic spectra were set at 250 μmol m^{-2} s^{-1}. In all monochromatic treatments there was a white background light of 100 μmol m^{-2} s^{-1} supplied by the same blue phosphorous coated LEDs as used in Exp. 1. Thus, the total irradiance in each treatment of Exp. 2 was 350 μmol m^{-2} s^{-1}. Red and blue monochromatic light had dominant wavelengths at 638 nm and 450 nm, respectively, and were supplied by Royal Blue and Red Luxeon K2 LEDs (Lumileds Lighting Company, San Jose, CA, USA). Far-red monochromatic light had a dominant wavelength at 740 nm and was supplied by GreenPower LED research module (Philips, The Netherlands), while the green light was provided by custom made modules with a dominant wavelength at 520 nm (Philips prototype). Due to energy output limitations, the green light treatment consisted of only 150 μmol m^{-2} s^{-1} green light supplemented with 200 μmol m^{-2} s^{-1} of white light, yielding a total irradiance identical to the other treatments.

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To ensure an even temperature distribution between and within the compartments, we used fans producing a turbulent air flow. The temperature in each compartment was logged with TC-08 data loggers (Picotechnology LTD., Cambridge, UK). During all treatments the relative air humidity was 70% and the CO_2 concentration was between 352 and 428 ppm. The average temperature of the fruits was 18.5°C (±0.15°C) in all treatments, as measured with k-type thermocouples (calibrated in freezing and boiling distilled water) attached on the epidermis at the lower side of the fruits to avoid direct shortwave radiation on the thermocouple. At the start of treatments, the calyx of the fruits was removed and the fruits were placed with their calyx scar on the table plate, in order to minimize the rate of water loss. Water loss rates were similar between all treatments of the same experiment, as was established by frequently weighing the fruits (data not shown). In Exp.1 the fruits lost 2.4% of their initial weight, while in Exp.2 the fruits lost 0.62% of their initial weight. No significant differences in weight loss was observed between treatments within each experiment (data not shown). After the light treatments, 3 pericarp discs per fruit were removed from the equator of the fruits with a cork borer of 1cm diameter. Only the outer pericarp and epidermis were included (no columella tissue). One sample. i.e. one biological replicate, consisted of a pool of 2 fruits (6 pericarp discs in total per pool). Upon dissection, the sample was flash frozen in liquid nitrogen. Each sample was then ground into a fine powder using liquid nitrogen. Samples were stored at -80°C before metabolomics analyses.

5.2.3 Metabolomics platforms – extraction, analysis and data processing

Metabolite extractions and analyses were all performed as recently described (Mokochinski et al., 2018), using 100 mg fresh weight of frozen powder per extraction. In short, lipid-soluble compounds including carotenoids, tocopherols and chlorophylls were extracted using chloroform/methanol with 0.1% butylhydroxytoluene as antioxidant, and analysed in a targeted manner by C_{30}-reversed phase HPLC (Waters) coupled to photodiode array (PDA) detection for both carotenoids and chlorophylls, and fluorescence (Fl) detection for tocopherols (in short: HPLC-PDA-Fl). Semi-polar compounds, including flavonoids, phenylpropanoids, alkaloids and various volatile-glycosides, were extracted in 75% methanol containing 0.1% formic acid and analysed in a comprehensive manner by C_{18}-reversed phase HPLC (Waters) coupled to both PDA detection and a LTQ-Orbitrap FTMS hybrid system (Thermo) with electrospray ionisation (ESI) in negative mode (in short: LC-MS). Polar compounds, including sugars, organic acids and amino acids, were extracted using water-methanol-chloroform and the polar phase was dried, derivatized with methoxyamine and N-methyl-N-(trimethylsilyl) trifluoroacetamide and mixed with 1 μl of an alkane series (C10-C30) using an online derivatization/injection robot (CTC Analytics) and analysed in a comprehensive manner by an Agilent 6890 GC coupled to Pegasus III TOF MS with electron impact (EI) ionisation (in short: GC-MS).

Samples from both Exp.1 and Exp.2 were all extracted and analysed in a single series in a random order. The processing of data from the targeted HPLC-PDA-Fl analysis of lipid-soluble compounds (isoprenoids) was performed using Empower software (Waters); these lipid-soluble compounds were annotated and quantified using authentic standards. Both LC-MS and GC-MS data were processed in an untargeted manner, using Metalign software (Lommen, 2009) for automatic noise estimation, unbiased peak picking and alignment. The resulting datasets were then further processed by grouping mass features derived from the same compound (mass clusters), in order to remove mass signal redundancy and retain a single representative total ion value per metabolite, using MSClust software (Tikunov et al., 2012). In addition, the resulting mass clusters actually represent low energy-ESI (pseudo) mass spectra in the case of LC-MS and high energy-EI mass spectra in the case of GC-MS; these mass spectra were used for annotation of selected compounds. For LC-MS, the molecular ion was manually checked in the pseudo spectrum and annotated based on comparisons of retention time, accurate mass, isotopic pattern and (pseudo)mass spectrum information, and the corresponding PDA spectrum if available, with in-house databases (Moco et al., 2006) and on-line available metabolite databases such as KNApSAcK, HMDB and MassBank. For GC-MS, the mass spectra from MSClust and calculated retention indices, based on the retention of the added alkane series, was compared with available EI-spectral libraries such as the NIST2014 and the Golm spectral database (http://gmd.mpimp-golm.mpg.de/), as well as an in-house library of derivatized standards.

5.2.4 Statistical analysis

Unsupervised multivariate analysis (Principal Component Analysis, or PCA; GeneMaths XT, Applied Maths, Belgium) was performed to visualize global differences between samples, based on log2-transformed and mean-centred metabolite data. Analysis of variance (ANOVA) was used to test either the effect of the light treatment or to compare the metabolite initial and final levels of metabolites. In cases when metabolite intensities did not exceed the detection threshold, their levels were assumed as zero. The ANOVA then took place with the rest of the treatments, the LSD was calculated and used to test if the treatments differ significantly from zero. The pool of six pericarp discs, from two fruits (three discs per fruit), was considered an independent replicate. Three replicates (three pools of two fruits each) per treatment were measured. Considering each sample as independent replicate may have underestimated random variance. Therefore, the multiple comparison tests have been conducted at =0.01 instead of the more commonly used =0.05, using post hoc Tukey's significant difference (HSD) tests.

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5.3 Results

5.3.1 Time course of metabolites in light compared to darkness (Exp. 1)

As was judged by the colour and firmness changes (Chapter 3), the ripening process of the detached tomato fruits proceeded more or less similarly in both the light (500 μmol m^{-2} s^{-1}) and dark treatments. In both treatments, the green colour as assessed visually had disappeared by day 9 at which time the fruits entered the breaker stage (transitionary stage between green and red coloration). From day 9 onwards both light and dark exposed fruits gradually developed in terms of colour though with slightly higher green and slightly lower red coloration in the dark-ripened fruits (Chapter 3).

In the metabolomics profiling of samples from both the irradiance and light quality experiments the targeted analysis by HPLC-PDA-Fl resulted in absolute levels of 9 well-known lipid-soluble tomato isoprenoids. The untargeted LC-MS platform yielded relative abundance levels of 437 mainly semi-polar metabolites of which 108 were putatively identified, while the untargeted GC-MS platform yielded relative levels of 109 polar compounds of which 22 were putatively identified.

The targeted analyses of the lipid-soluble isoprenoids (Figure 1) indicated that the chlorophyll b content (Figure 1C) significantly declined after 9 days, and this decline was faster in light than in darkness. In the light, all other isoprenoids except the relatively low abundant delta-tocopherol (Figure 1I), showed an increase during the first 6-12 days, some after an initial lag phase. Finally the contents stabilized for lutein, alpha- and beta-carotene and alpha-tocopherol. Zeaxanthin decreased again after 12 days (Figure 1B), while the pattern of lycopene indicates that it did not reach a maximum yet (Figure 1F). The time courses for most metabolites (lutein, alpha-tocopherol, alpha- and beta-carotene) might be considered to be similar but with minor shifts in time. Except for chlorophyll (Figure 1C), there were no significant changes over time for isoprenoids in fruits placed in darkness. Gamma-tocopherol increased over time in light but not in darkness. However, at 15 days there was no significant difference between the light and dark treatments (Figure 1H).
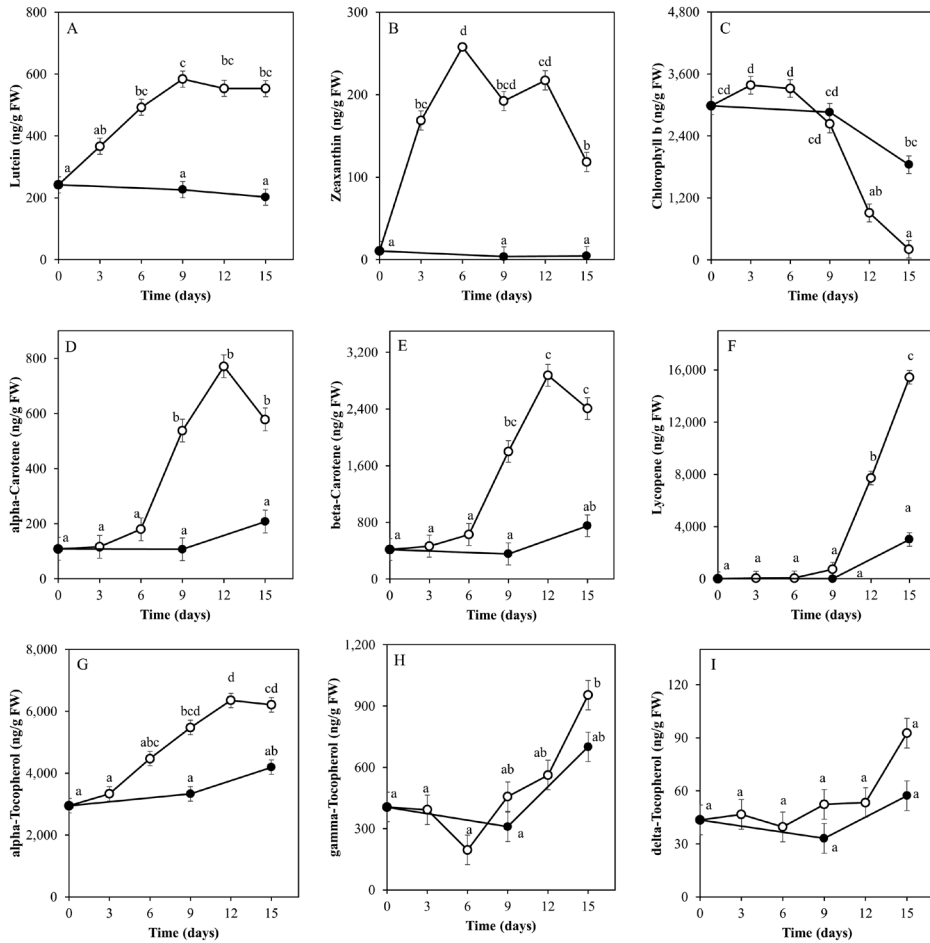
FIGURE 1 | Levels of lipid-soluble metabolites (isoprenoids) in tomato pericarp from Exp. 1, measured by LC-PDA-Fl. The fruits were treated with either white light (500 µmol m^{-2} s^{-1}; open symbols) or darkness (closed symbols) for 15 days and sampled at indicated days. Error bars indicate standard errors; letters indicate significant differences between treatments or days at =0.01, n=3 (3 pools of 2 fruits each).

In order to determine the overall effect of light or darkness on the tomato pericarp metabolites detected by LC-MS, a principal component analysis (PCA) was carried out based on the variation in the relative intensity values of all 437 compounds detected (Figure 2). The main effect observed (1st PC) obviously corresponds to the time, i.e. fruit ripening. At both day 9 and day 15 the light-treated fruits were on average further away from day 0 (mature green fruits) then the fruits that were placed in darkness, suggesting faster fruit ripening. The 2nd PC coincides with light versus dark, suggesting differential biochemistry during ripening.
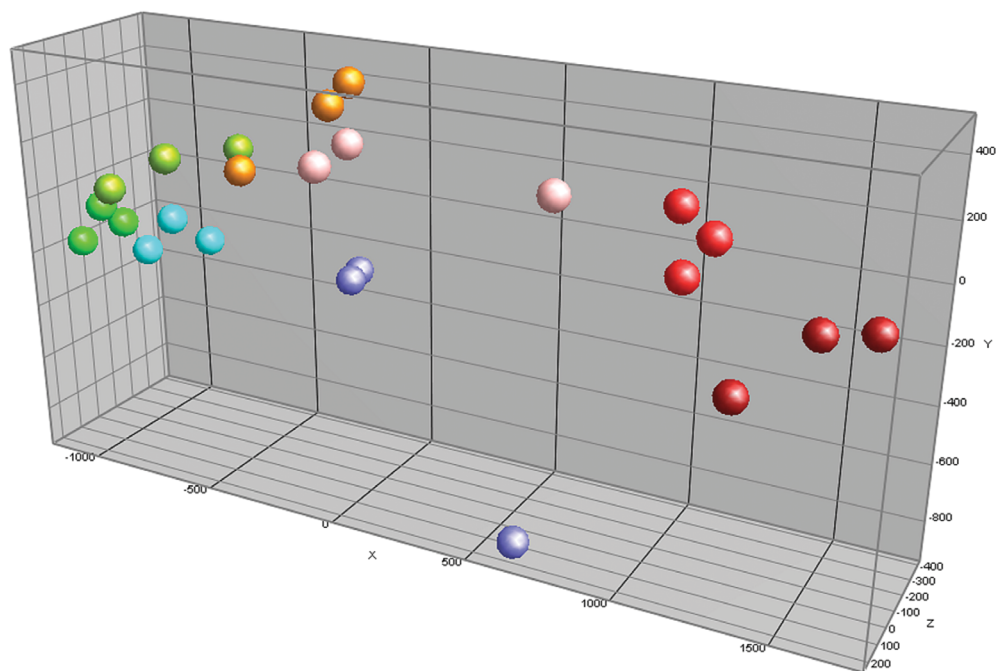
FIGURE 2 | Principal component analysis (PCA) of tomato samples from Exp. 1, based on their variation in relative abundance of 437 metabolites detected by LC-MS based untargeted metabolomics. The fruits were treated with either light (500 µmol m^{-2} s^{-1}) or darkness during 15 days. Samples of the light treatment were measured every 3 days and of the dark treatment at 9 and 15 days after beginning of the experiment (t=0 days). PC1=X-axis=42.7% explained variation; PC2=Y-axis=13.7% explained variation; PC3=Z-axis=7.3% explained variation (Light treatment: t=0 days light green points, t=3 days dark green points, t=6 days orange points, t=9 days pink points, t=12 days light red points, t=15 days dark red points. Dark treatment: t=0 days light green points, t=9 days blue points, t=15 days purple points).

From the 437 metabolites detected by LC-MS, we selected a limited number of compounds for investigating their specific patterns upon treatment with light and darkness (Figure 3). At day 15 the phenolic compounds naringenin-chalcone, naringenin-C-diglycoside, sinapic acid, caffeoylquinic acid and phloretin-C-diglycoside, as well as the umami-flavour related L-glutamic acid, were significantly more abundant in light-exposed fruits compared to fruits kept in darkness (Figure 2). Naringenin-C-diglycoside and sinapic acid were not detectable in dark-treated fruits at all. A variety of other naringenin and caffeoylquinic acid-conjugates as well as L-threonate and the flavonol-glycoside rutin were also higher in light compared to darkness (Supplementary Table 1). In total, at day 15 of treatment the level of 79 compounds (18%) were higher in light compared to darkness while only 4 metabolites (0.9%) were lower (Supplementary Table 1). The final level of caffeoylquinic acid, L-glutamic acid, naringenin-C-diglycoside, sinapic acid and phloretin-C-diglycoside were significantly

higher compared to their initial levels when fruit were treated with light (Figure 2). Likewise, the umami-flavour compound L-glutamic acid increased in time and more in light than in darkness (Fig 3C). In light, naringenin-chalcone was higher than the initial at 12 days while there was no significant difference at 15 days. In the light, in total 156, (35.7%), of all LC-MS compounds detected significantly increased over time while 31 compounds (6.8%), decreased (Supplementary Table 2). For fruits stored in darkness, only 31 metabolites changed significantly with time. Only a few of these metabolites were the same with the metabolites that changed over time under light.
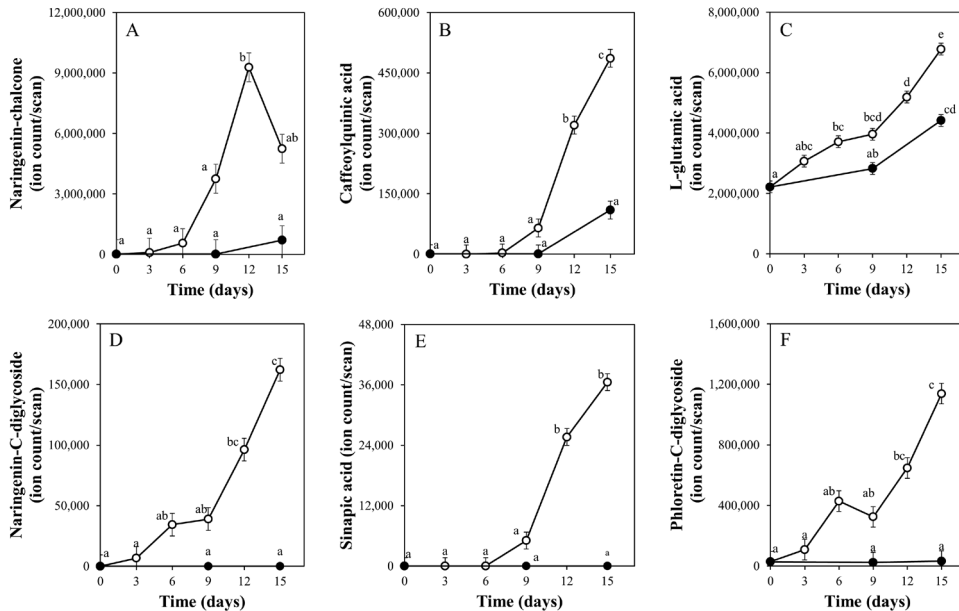
FIGURE 3 | Relative levels of selected metabolites in tomato pericarp from Exp.1, measured by LC-MS. The fruits were treated with white light (500 μmol m^{-2} s^{-1}; open symbols) or darkness (closed symbols) for 15 days and sampled at indicated days. Error bars indicate standard errors, letters indicate significant differences between treatments or days at =0.01, n=3 (3 pools of 2 fruits each).

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Next to influencing the global composition of secondary metabolites, light also exerted a clear effect on primary metabolites as detected by GCMS (Figure 4) . Again, the main effect (1st PC; X-axis) coincided with time (fruit ripening), while light-treated fruits showed faster ripening compared to the dark treatment. In contrast to the secondary metabolites (Figures 1 and 2), for primary metabolites we did not observe a separate grouping of dark versus light-treated fruits.
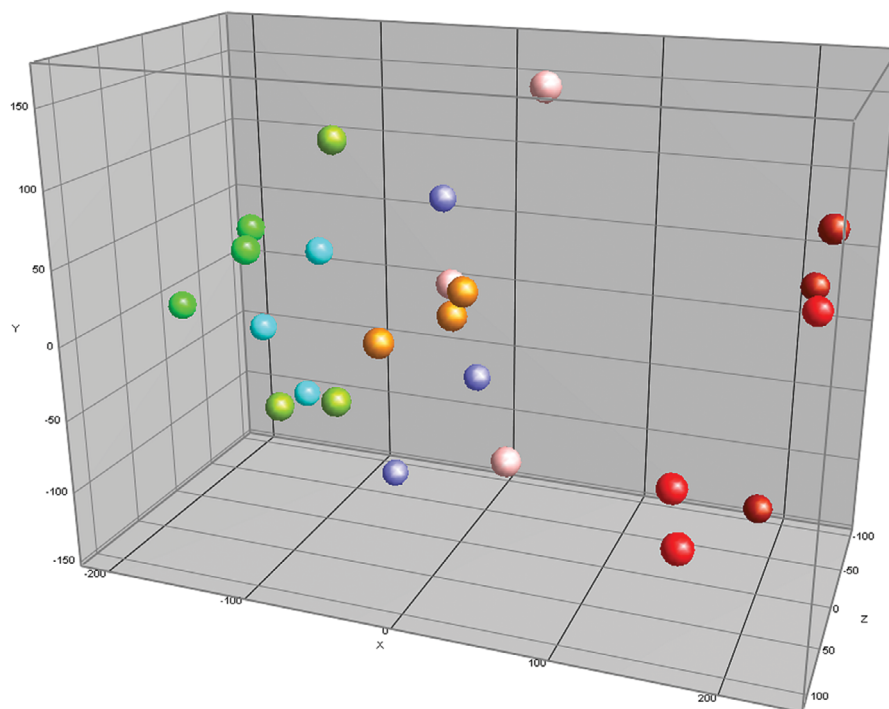

FIGURE 4 | Principal component analysis (PCA) of tomato samples from Exp. 1, based on their variation in relative abundance of 109 metabolites detected by GC-MS based untargeted metabolomics. The fruits were treated with either light (500 µmol m^{-2} s^{-1}) or darkness during 15 days. Samples of the light treatment were measured every 3 days and of the dark treatment at 9 and 15 days after beginning of the experiment (t=0 days). PC1=X-axis=26.1% explained variation; PC2=Y-axis=14.3% explained variation; PC3=Z-axis=10.6% explained variation (Light treatment: t=0 days-light green points, t=3 days-dark green points, t=6 days-orange points, t=9 days-pink points, t=12 days-light red points, t=15 days-dark red points. Dark treatment: t=0 days-light green points, t=9 days-blue points, t=15 days-purple points).

Figure 5 shows some selected differentially accumulating primary metabolites. At the end of the experiment glutamic acid, xylose, galacturonic acid and myo-inositol were significantly higher in the light than in the dark (Figures 5C to 5F). Malic acid at 15 days was lower in the light treated fruits (Figures 5A) while gamma-aminobutyric acid (GABA) was significantly lower in light at 12 days with no significant difference

at 15 days. At the end of the light treatment the levels of 19 compounds (17.4% of total metabolites detected) were significantly higher, while only 2 (1.8%) were lower as compared to the dark treatment (Supplementary Table 3). In the light glutamic acid, xylose, galacturonic acid and myo-inositol (Figures 5C to 5F) gradually increased over the 15 days treatment, while malic acid and gamma-aminobutyric acid decreased (Figures 5A and 3B); in the light, in total 16 (14.7%) compounds increased and only 6 (5.5%) decreased over the 15 days of light exposure (Supplementary Table 4).
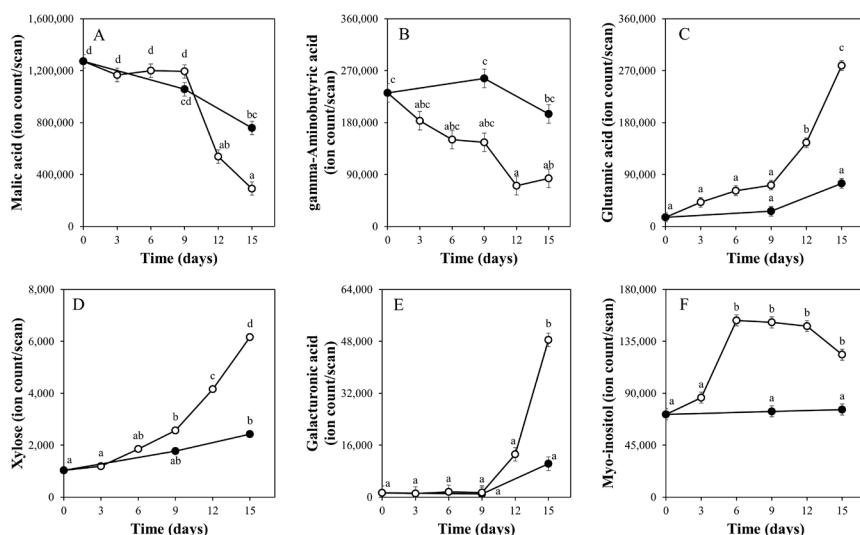
FIGURE 5 | Relative levels of selected metabolites in tomato pericarp from Exp.1, measured by GC-MS. The fruits were treated with white light (500 μmol m^{-2} s^{-1}; open symbols) or darkness (closed symbols) for 15 days and sampled at indicated days. Error bars indicate standard errors, letters indicate significant differences between treatments or days at =0.01, n=3 (3 pools of 2 fruits each).

5.3.2 Light Spectrum (Exp. 2)

We subsequently investigated the effect of different light spectra on the tomato metabolite composition, as compared to white light (see above) and no light at all (darkness). Here the fruits were analysed after 7 days of treatments. In the group of carotenoids, lutein (Figure 6A) was largely unaffected by the light quality treatments or darkness. Compared to the initial level, lutein increased only in the blue light treatment. On the contrary, zeaxanthin (Figure 6B) levels of the red and blue light spectrum treatments increased significantly compared to both the start of the light treatments and the dark treatment (initially and in dark zeaxanthin was not detectable). Alpha-carotene increased under blue and green light (Figures 6D) while beta-carotene increased only in blue light (Figures 6E) with the rest of the light treatments and darkness having no influence compared to the initial levels. The three tocopherols (Figures 6G,

6H, and 6I) also showed variable responses to the light spectrum treatments. Alpha-tocopherol was positively affected only by blue light, gamma-tocopherol was highest in darkness and delta-tocopherol increased in fruits under green light compared to the initial. Chlorophyll (Figure 5C) decreased equally in all light spectrum treatments and dark treatment without a particular wavelength effect. Lycopene levels (Figure 6F) increased in fruits at far-red, green and darkness compared to the initial (Figure 6F).
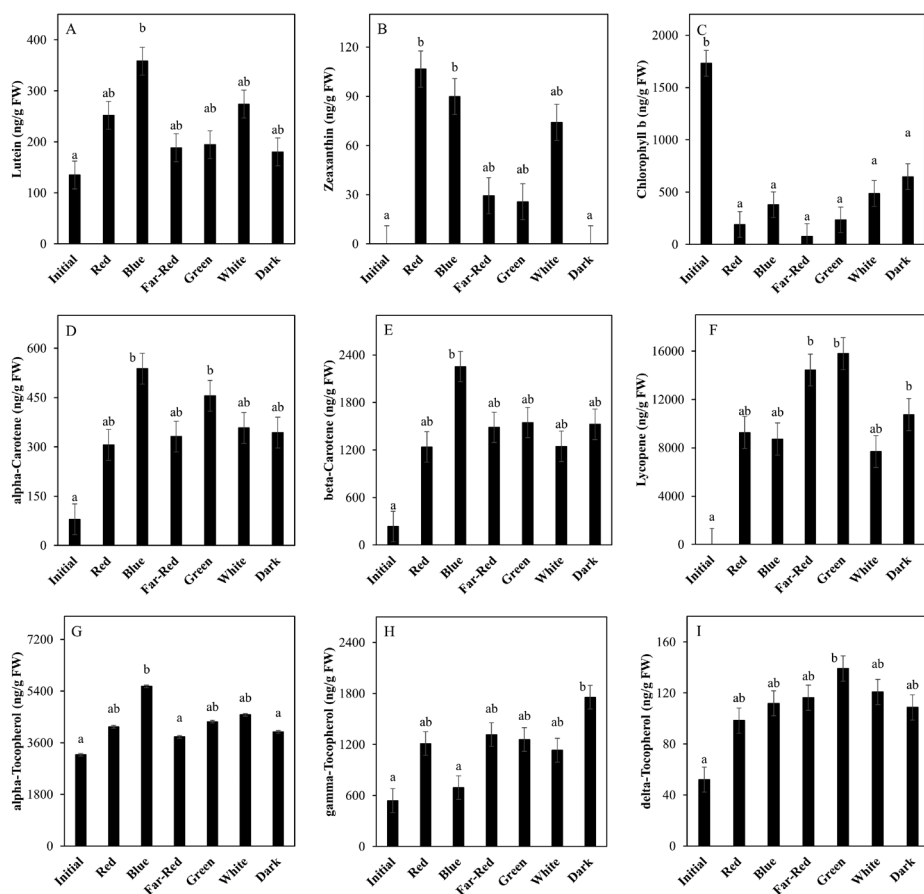
FIGURE 6 | Levels of lipid-soluble metabolites (isoprenoids) in tomato pericarp from Exp. 2, measured by LC-PDA-Fl. The fruits were exposed to white light (background) supplemented with monochromatic red, blue, far-red and green light, as well as to broad-spectrum white light (white) or no light at all (dark) for 7 days. Initial values represent fruits at start of experiment. Error bars indicate standard errors, letters indicate significant differences between light treatments at =0.01, n=3 (3 pools of 2 fruits each).

In order to establish the effects of these contrasting light spectra on semi-polar and polar metabolites, the same fruits were also analysed using untargeted LCMS and GCMS based metabolomics approaches, respectively. A principle component

analysis (PCA) based on the LCMS data of all samples including the fruits at the start of experiment (Supplementary Figure 1) clearly indicates that the 1st PC (X-axis) corresponds to differences between day 0 and day 7) of experiment, i.e. ripening time effect (cf. Figure 2). Thus, in order to zoom in into possible light quality effects, we performed a local PCA based on the 7 days-treated fruits only (Figure 7). In this local PCA, all light-treated fruit samples were separated from the fruits kept in darkness, with blue light exerting the largest effect (largest difference with dark-treated fruits on the X-axis, PC1) followed by white light, while all far-red, green and red lights had intermediate effects.
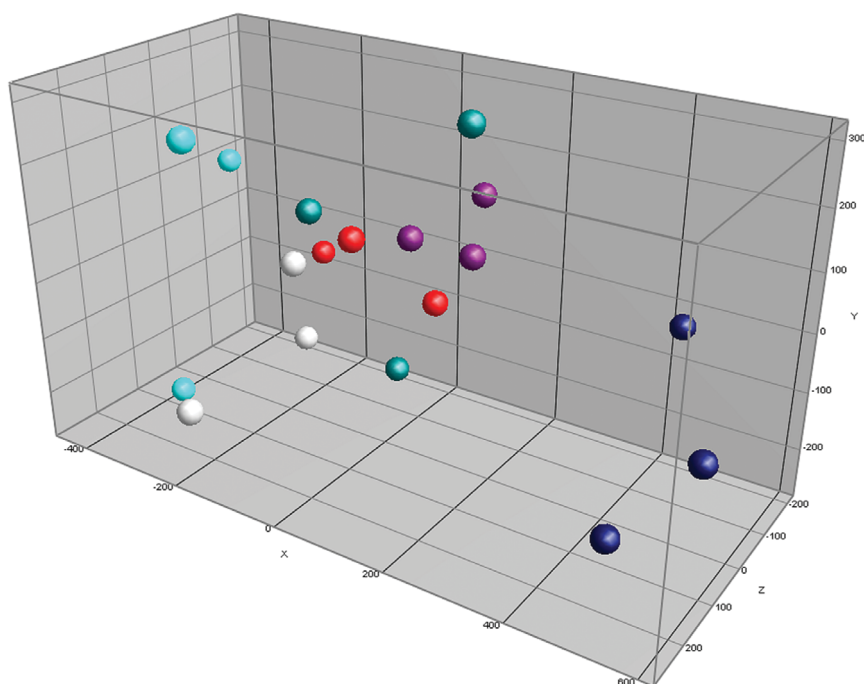
FIGURE 7 | Principle component analysis (PCA) of tomatoes treated with different light qualities, based on 437 tomato pericarp metabolites detected by LC-MS based untargeted metabolomics. The fruits were treated with different light spectrum treatments (350 µmol m^{-2} s^{-1}) and darkness for 7 days (Exp. 2). PC1=X-axis=21.7% explained variation; PC2=Y-axis=12.6% explained variation; PC3=Z-axis=10.6% explained variation (Red treatment-red points, blue treatment-light blue points, far-red treatment-purple points, green treatment-green points, white treatment-white points, dark treatment-dark blue points).

From the 437 compounds detected by LCMS, we again selected some secondary metabolites to show their individual responses to the various light quality treatments (Figure 8). The naringenin mono- (Figure 8D) and dihexoside (Figure 8A) conjugates, resulting from the flavonoid biosynthetic pathway, were low at the start of the trial and remained largely unaffected in the dark treatment. The phenylpropanoid

conjugates ferulic acid-hexose and sinapic acid-hexose were higher in the blue and white treatments compared to initial levels (Figures 8B and 8F), while another phenylpropanoid conjugate, caffeic acid-hexose (Figure 8C), was relatively low in the beginning of the experiment and increased by all treatments similarly. Quercetin 3-O-glucoside was significantly higher in the blue treatment compared to the other treatments and initial levels (Figure 8E). In the overall comparison of the various light spectra, 29 LCMS compounds were found to be significantly higher and 4 lower in blue light compared to far-red light, 5 were higher and 1 lower in red versus far-red treatments, 11 higher and 1 lower in blue light compared to red light, and finally 2 higher and 9 lower when comparing green to white light (Supplementary Table 5).

With regard to the primary metabolites detected by untargeted GCMS, a PCA based on all samples indicated a clear effect of the 7 days treatment time, as well as an effect of light versus dark exposure (Supplementary Figure 2). However, these metabolites are rather limited in all comparisons (blue vs far-red, 8 metabolites significantly different; blue vs red, 3 metabolites significantly different; green vs white, 5 metabolites significantly different) and none of them has been positively annotated with high confidence.
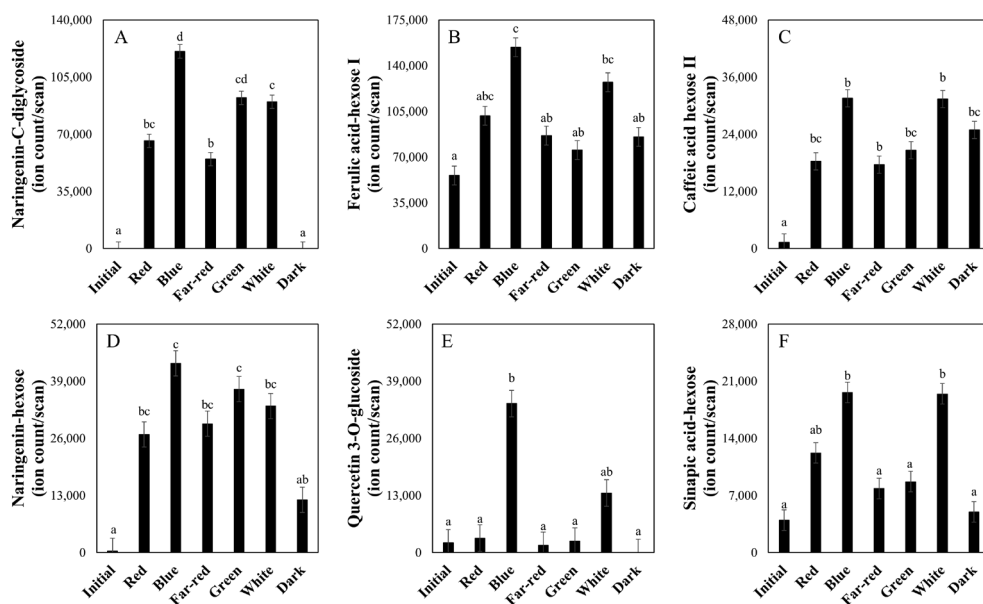
FIGURE 8 | Relative levels of selected metabolites in tomato pericarp from Exp. 2, measured by LC-MS. The fruits were treated with white light (background) supplemented with monochromatic red, blue, far-red and green light, as well as to broad-spectrum white light (white) or no light at all (dark) for 7 days. Error bars indicate standard errors, letters indicate significant differences between light treatments at =0.01, n=3 (3 pools of 2 fruits each).

5.4 Discussion

5.4.1 Light affects ripening-related processes

In previous chapters, we showed that light intensity and spectral quality can affect the levels of ascorbic acid (vitamin C) and investigated the possible underlying physiological and biochemical mechanisms (Chapters 2 and 3). In the current work we expanded our research towards the effect of light on the global metabolome of tomato fruit pericarp, via means of comprehensive metabolomics technologies. We focused on metabolites related to visual quality (pigment), nutritional quality (vitamins and antioxidants) as well as organoleptic characteristics (flavour-related compounds, sugars, acids).

The level of pigments, specifically lycopene, in the pericarp is one of the key aspects in overall visual quality of red tomato fruits. Lipid-soluble pigments including lycopene accumulated over time with exposure to light. Furthermore, exposure of mature green tomatoes to white LED-light for 15 days resulted in both an increase in all carotenoids and a faster decrease in chlorophyll as compared to exposing fruits to darkness. Even though fruit in darkness also turned red (but to a lower extent than in light), the increase in lycopene during the dark treatment was not statistically significant. This only suggests that ripening in terms of lycopene accumulation proceeds with a faster pace under light than in darkness. In contrast, fruit firmness progressed in a similar manner in both the light and dark treatments (Chapter 3). Furthermore, significant differences between these light and dark treatments in sucrose levels have not been observed (Chapter 4). These results indicate that not all aspects of fruit ripening are influenced by light. Fruit exposed to light do not simply ripen faster than fruit in darkness. Although there were no statistically significant differences in levels of individual pigments, the different light spectra only showed a tendency for minor effects on pigments in the fruits, with blue and far-red light being the most contrasting treatments. However, as in Exp. 2 the light sum was lower than in Exp.1 (lower intensity and shorter duration) any effects of specific wavelengths may be more visible when using higher light intensities. It can be concluded that the presence of light accelerates specific ripening-related processes in pigmentation.

Typically, with the progress of ripening and concomitantly to the colour changes, a decrease in fruit firmness is usually observed, as a result of the hydrolysis of cell walls (Huber, 1983). In our experiments, chlorogenic acid, one of the side products in lignin biosynthesis (Boerjan et al., 2003), was similar in light and dark exposed fruits. However, other phenolic compounds directly related to the lignin pathway such as sinapic acid were higher in the light-exposed fruits. As there were no macroscopic differences in fruit firmness between light and darkness (Chapter 3), it is concluded that the light effects on the lignin pathway are insufficiently strong to affect the firmness of the fruit.

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5.4.2 The nutritional value of tomato fruits is regulated by light

Tomato is a good source for a variety of metabolites with beneficial or potentially beneficial effects on human health. The fruits contain vitamin E (tocopherols) as well as carotenoids such as lycopene, alpha- and beta-carotene (provitamin A) and xanthophylls such as lutein and zeaxanthin. A common function of the above mentioned metabolites is that they potentially act as antioxidants in the human body, which have been related to the prevention of certain diseases (Ristow, 2014). Moreover, the antioxidant function of these molecules protects also the plant tissue from oxidative damage (Young, 1991; Munné-Bosch and Alegre, 2002). Reactive oxygen species (ROS) are mainly produced during photosynthesis and mitochondrial respiration. A higher light intensity usually results in a higher photosynthetic rate and thus potentially in a higher production rate of ROS. In the current research, treatment with light resulted in an increase of the above mentioned antioxidants, as compared to keeping fruits in darkness (Figure 1). This response was generally observed up to day 9, at which time point the fruits entered the breaker stage and started breaking down chlorophyll, thus fruit photosynthesis is expected to be reduced dramatically upon further fruit ripening (Chapter 3). This orchestrated response of antioxidants to light is potentially a defence mechanism of the tissue against photosynthesis-related oxidative stress.

Due to the absorbance spectrum of chlorophylls, blue and red LED resulted in higher photosynthetic rates compared to both green and far-red light (McCree, 1971), with a likely concomitant effect on the ROS production in the fruit tissue. However, identifying specific light-signalling effects appeared more complex. Red and blue light (compared to far-red and green) each increased the content of a limited number of antioxidants (e.g. zeaxanthin and alpha-tocopherol) potentially due to upregulation of the photosynthetic rate. Furthermore, antioxidants such as flavonoids (e.g. naringenin-hexoses), phenylpropanoids (e.g. caffeic acids and dicaffeoyl-quinic acids) and other phenolic compounds were also upregulated by blue light relative to other LED colours. These results suggest wavelength-specific light signalling effects. Therefore, specific photoreceptors are potentially involved in the observed differential effects of led qualities on the composition of antioxidants and other fruit metabolites.

Other health related metabolites typically found in tomato fruits increased when fruits were exposed to light. Phenols such as naringenin, caffeic acid derivatives and rutin potentially have a beneficial effect on human health through their antioxidant function. Specifically, naringenin is a flavonoid which has been related to the prevention and treatment of Alzheimer's disease and cancer (Yang et al., 2017), while phloretin is a naturally occurring phenol with antioxidant function (Rezk et al., 2002). Light stimulated the accumulation of all above mentioned phenols in tomato pericarp. Another major antioxidant, ascorbic acid (vitamin C) was also higher in the light treatment (Chapter 3). Other metabolites for which various health effects have been suggested, such as L-threonate increased in tomatoes when exposed to light. Moreover, as glycoalkaloids significantly decreased upon exposure to light (Supplementary Table 1), a potential

negative effect of tomato consumption on the human intestine i.e. aggravation of inflammatory bowel disease (Patel et al., 2002) is less likely in fruits treated with light compared to fruits kept in darkness. It is thus concluded that light may enhance the potential nutritional quality of tomato fruit.

5.4.3 Taste related metabolites of tomato fruits can be manipulated by light

Studies employing both consumer and trained sensory panels as well as metabolic measurements define those taste aspects that are the main drivers for consumer liking. Consumer liking of tomato fruits is predominantly driven by soluble carbohydrate content and to a lesser extent by the content of organic acids and texture (Verkerke et al., 1997). To achieve a better prediction power in future modelling of taste and liking, a higher level of complexity has to be integrated. For example, the various sugars found in tomatoes have a different impact on sweetness as sensed by the human tongue (Mahawanich and Schmidt, 2004). In the current research no significant changes in taste related sugars (sucrose) between the irradiance or light spectrum treatments have been observed. From the two most abundant organic acids and key to sour taste -malic acid and citric acid- (Baldwin et al., 1998), only malic acid was slightly increased in the light treatment compared to darkness. As malic acid was not quantified, the actual effect of its light-induced increase is unknown yet but could potentially affect taste. In parallel, a slight decrease of gamma-aminobutyric acid (GABA) was observed when fruits were treated with light. GABA is an inhibitory transmitter that can affect taste buds associated with the perception of sweet, bitter and umami (Dvoryanchikov et al., 2011). GABA has been proposed to be an inhibitor of sweetness and thus light may have a beneficial effect on sweetness by reducing the level of this sweetness inhibitor. Further research is essential in order to decipher the exact impact of this light-induced GABA alteration on tomato taste perception. In addition, glutamic acid increased substantially upon exposure to light, a result that potentially leads to the enhancement of the umami taste perception (Halpern, 2000). Naringenin also increased by light, which may have a potential effect on the perception of bitterness (Ortuño et al., 1995). Guaiacol and methyl-salicylate (phenolic volatiles), present in tomato as non-volatile glycosides can be released via de-glycosylation upon eating/cell disruption due to endogenous glycosidase activity, giving a characteristic smoky aroma (Buttery et al., 1990). Guaiacol-xylosyl-glucoside and methyl-salicylate-xylosyl-glucoside (xylosyl-glucose conjugates of guaiacol and methyl-salicylate, respectively) are well-known substrates for glycosidase-induced release of smoky aroma (Tikunov et al., 2013). Both compounds were detected in the fruits, but their relative levels were not different between light and dark exposed fruits, nor between fruits exposed to different light qualities (data not shown). It is thus likely that the light environment did not influence the smoky aspect of the tomato aromatic profile.

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5.5 Conclusions

Irradiance treatments significantly influenced the metabolic profiles of tomato pericarps while light spectrum had a more narrow range of effects. Exposure to light accelerated ripening in terms of pigment and flavonoid accumulation and alkaloids reduction in the pericarp. Light positively influenced the level of a variety of health related compounds, such as tocopherols, carotenoids and phenolic compounds, thus potentially increasing the nutritional value of these fruits as compared to darkness. Effects of light spectrum on the metabolome were less clear yet, exerting diverse effects with blue being the most effective. Light also significantly influenced the levels of some well-known flavour-related compounds such as malic acid, glutamate and GABA, but not the levels of glycosides of volatile compounds that are typically released upon eating tomatoes. The results of the present study provides a detailed insight in the effects of LED light on tomato fruits and how light can improve the quality of tomato fruits.

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Supplementary material

SUPPLEMENTARY TABLE 1 | Responsive annotated metabolites detected in tomato pericarp by liquid chromatography mass spectroscopy (LC-MS), when fruits were treated with light (500 μmol m^{-2} s^{-1}) vs darkness for 15 days (Exp.1; =0.01, n=3).

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between light and dark at 15 days)	Level of annotation	T-test
13.42	595.17	Naringenin-C-diglycoside	349.63	2	0.001
30.52	479.12	unknown	165.80		0.008
31.96	459.09	unknown	100.29		<0.001
15.63	223.06	Sinapic acid	77.91	2	<0.001
21.36	203.06	unknown	70.37		<0.001
25.74	587.20	unknown	65.61		<0.001
32.48	819.27	unknown	58.70		<0.001
31.62	445.08	unknown	58.68		0.001
32.70	425.14	unknown	57.87		<0.001
19.24	433.11	unknown	50.66		0.009
13.78	351.13	unknown	48.94		0.001
20.89	577.25	N307	44.88	4	<0.001
25.47	415.20	unknown	43.52		0.009
33.58	287.06	unknown	41.71		<0.001
21.34	333.06	unknown	41.12		<0.001
22.60	577.25	C26H42O14	39.30		0.002
24.95	283.06	unknown	38.48		0.004
25.92	449.11	unknown	36.38		0.004
22.91	597.18	Phloretin-C-diglycoside	34.68	2	0.003
13.04	309.12	unknown	29.58		<0.001
28.96	855.25	unknown	28.45		0.002
29.71	429.14	unknown	27.62		0.002
29.57	283.06	unknown	26.45		<0.001
22.37	245.09	unknown	25.42		<0.001
44.11	855.31	unknown	24.80		<0.001
38.86	542.15	unknown	23.67		<0.001
23.46	335.17	unknown	23.23		<0.001
17.28	759.23	unknown	22.22		0.009
29.28	511.18	unknown	19.66		0.003
26.82	433.11	Naringenin-hexose	19.24		0.003
35.70	623.18	unknown	18.49		0.001

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Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between light and dark at 15 days)	Level of annotation	T-test
35.75	775.28	unknown	17.71		0.002
34.19	623.18	unknown	17.17		<0.001
39.90	271.06	naringenin	15.83	1	<0.001
25.58	283.06	unknown	15.72		0.004
35.79	923.28	unknown	15.14		<0.001
39.94	431.90	unknown	14.99		<0.001
27.02	1120.52	unknown	14.03		0.006
30.58	221.07	unknown	13.94		0.003
20.25	367.10	3-O-Feruloylquinic acid	12.22	2	<0.001
23.39	577.25	C26H42O14	12.01	4	<0.001
7.81	371.06	unknown	11.87		0.003
2.30	351.06	unknown	11.83		0.009
12.59	351.07	unknown	11.81		<0.001
36.94	677.15	3,4,5-Tricaffeoylquinic acid	11.19	1	0.002
24.29	433.11	Naringenin-hexose	10.71	2	0.004
31.89	221.07	unknown	10.19		0.004
32.03	677.28	N713	10.10	4	0.007
24.83	415.20	unknown	9.38		0.001
14.57	351.07	unknown	8.53		0.001
28.10	515.12	dicaffeoylquinic acid IV	7.49		0.003
40.39	271.06	naringenin-chalcone	7.43	1	0.009
22.06	405.18	unknown	7.41		0.006
39.04	677.15	unknown	6.92		<0.001
26.39	415.20	unknown	6.84		0.002
25.62	515.12	dicaffeoylquinic acid II	6.75	2	0.004
21.04	662.18	unknown	6.42		0.008
2.16	175.02	Ascorbic acid	6.15	2	0.003
4.81	345.08	unknown	5.85		0.007
38.30	677.15	Tricaffeoylquinic acid II	5.72	2	<0.001
1.96	326.11	unknown	5.17		<0.001
2.03	535.15	unknown	5.11		0.003
20.32	391.16	unknown	4.58		0.008
12.66	353.09	caffeoylquinic acid	4.44	3	0.004
21.77	525.52	unknown	4.44		0.002
32.25	515.12	dicaffeoylquinic acid V	4.34	2	<0.001

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between light and dark at 15 days)	Level of annotation	T-test
21.70	609.15	quercetin-3-O-rutinoside (rutin)	4.34	1	0.007
21.65	345.15	unknown	4.27		<0.001
17.44	1272.58	unknown	4.24		0.006
26.68	515.12	dicaffeoylquinic acid III	3.99		0.001
32.30	411.20	unknown	3.44		0.010
27.18	415.20	unknown	3.10		0.004
2.09	135.03	L-Threonate	2.75	3	0.003
3.73	286.97	unknown	2.59		0.003
34.82	1180.54	unknown	2.42		0.004
32.55	845.21	unknown	2.40		0.007
1.86	331.05	unknown	2.15		0.007
2.61	333.06	unknown	2.07		0.007
2.01	195.05	unknown	1.53		0.008
2.63	317.05	unknown	0.53		0.001
40.57	539.25	unknown	0.45		0.003
1.90	214.05	unknown	0.31		0.001
30.99	917.23	unknown	0.13		<0.001

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SUPPLEMENTARY TABLE 2 | Responsive annotated metabolites detected in tomato pericarp by liquid chromatography mass spectroscopy (LC-MS), when fruits were treated with light (500 µmol m^{-2} s^{-1}) for 15 days compared to initial levels (Exp.1; =0.01, n=3).

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
28.10	515.12	dicaffeoylquinic acid IV	1518.54		0.002
32.03	677.28	N713	1130.41	4	0.003
39.90	271.06	naringenin	619.00	1	<0.001
36.94	677.15	3,4,5-Tricaffeoylquinic acid	596.26	1	0.001
22.37	245.09	unknown	557.21		<0.001
12.66	353.09	caffeoylquinic acid	478.72	3	<0.001
20.89	577.25	N307	454.79	4	<0.001
22.60	577.25	C26H42O14	380.62		0.002
13.42	595.17	Naringenin-C-diglycoside	364.57	2	0.001
25.62	515.12	dicaffeoylquinic acid II	351.29	2	0.002
26.75	839.33	unknown	331.50		0.002
40.39	271.06	naringenin-chalcone	331.43	1	0.003
32.25	515.12	dicaffeoylquinic acid V	165.62	2	<0.001
31.96	459.09	unknown	158.12		<0.001
24.29	433.11	Naringenin-hexose	157.07	2	0.002
20.19	667.80	unknown	155.39		<0.001
15.23	491.18	N231	150.30	4	0.002
23.39	577.25	C26H42O14	144.52	4	<0.001
22.98	760.82	double charged: >1500 D	137.72	4	<0.001
27.02	1120.52	unknown	131.74		0.004
24.95	283.06	unknown	127.38		0.003
38.30	677.15	Tricaffeoylquinic acid II	127.21	2	<0.001
23.77	328.65	double charged: >1500 D	126.49	4	<0.001
22.19	760.82	unknown	120.10		<0.001
26.82	433.11	Naringenin-hexose	115.21		0.002
24.23	291.14	unknown	109.14		0.002
13.04	309.12	unknown	103.31		<0.001
40.45	334.06	unknown	99.49		<0.001
21.00	328.65	unknown	95.82		<0.001
29.05	855.33	unknown	95.80		0.003
25.92	449.11	unknown	93.35		0.004
26.68	515.12	dicaffeoylquinic acid III	89.05		<0.001
30.52	479.12	unknown	87.67		0.008
32.03	694.28	unknown	84.63		<0.001

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
39.94	431.90	unknown	84.57		<0.001
15.63	223.06	Sinapic acid	83.29	2	<0.001
39.04	677.15	unknown	82.61		<0.001
9.90	341.09	caffeic acid hexose III	81.76	2	<0.001
18.72	189.08	unknown	80.98		0.004
16.13	295.14	unknown	74.05		<0.001
33.58	287.06	unknown	72.58		<0.001
11.42	175.06	unknown	72.17		<0.001
39.20	821.32	unknown	72.05		0.002
34.08	691.26	unknown	70.51		0.006
25.74	587.20	unknown	69.66		<0.001
21.36	203.06	unknown	66.73		<0.001
30.70	693.28	unknown	65.18		0.006
26.59	505.13	unknown	62.44		<0.001
31.62	445.08	unknown	60.58		0.001
13.78	351.13	unknown	60.49		0.001
32.48	819.27	unknown	59.49		<0.001
2.08	193.04	galacturonate/glucuronate	57.74	3	<0.001
32.70	425.14	unknown	57.51		<0.001
40.30	431.90	unknown	57.28		<0.001
17.28	759.23	unknown	56.71		0.008
24.97	503.12	unknown	52.37		<0.001
25.80	581.15	unknown	51.63		0.001
2.30	351.06	unknown	50.42		0.006
13.17	593.24	unknown	49.71		<0.001
12.59	351.07	unknown	49.19		<0.001
34.82	1180.54	unknown	48.05		<0.001
30.58	221.07	unknown	47.87		0.002
17.44	1272.58	unknown	47.28		<0.001
20.32	391.16	unknown	47.04		<0.001
19.24	433.11	unknown	45.71		0.009
39.51	867.33	unknown	42.96		0.001
25.47	415.20	unknown	42.60		0.009
22.91	597.18	Phloretin-C-diglycoside	40.25	2	0.003
10.24	327.11	unknown	40.23		0.002
12.75	817.20	unknown	40.00		0.006

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Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
21.34	333.06	unknown	39.62		<0.001
21.04	662.18	unknown	38.48		0.001
11.35	353.09	5-Caffeoyl-quinic acid	36.32	2	<0.001
23.46	335.17	unknown	34.11		<0.001
29.28	511.18	unknown	33.99		0.003
17.07	295.05	unknown	33.90		<0.001
34.60	1150.53	unknown	33.68		0.005
9.03	341.09	caffeic acid hexose II	32.75	2	<0.001
28.96	855.25	unknown	30.19		0.002
29.57	283.06	unknown	29.45		<0.001
5.24	378.13	unknown	28.97		<0.001
29.71	429.14	unknown	28.77		0.002
19.94	433.11	Naringenin-hexose	28.19		0.009
4.81	345.08	unknown	27.95		0.003
23.05	471.19	unknown	26.87		0.002
44.11	855.31	unknown	25.92		<0.001
2.03	535.15	unknown	23.95		<0.001
11.05	341.09	caffeic acid hexose IV	23.69	2	<0.001
38.86	542.15	unknown	23.09		<0.001
40.14	805.33	unknown	22.27		0.009
43.02	1009.45	unknown	21.94		<0.001
35.70	623.18	unknown	19.51		0.001
35.75	775.28	unknown	18.98		0.002
11.12	343.10	unknown	18.49		0.002
24.83	415.20	unknown	17.87		<0.001
24.18	371.11	unknown	17.29		0.002
23.21	336.15	unknown	17.22		0.002
2.16	175.02	Ascorbic acid	16.84	2	<0.001
15.45	797.31	N178	16.33	4	0.002
19.37	1272.58	Esculeoside B + FA	16.32	2	<0.001
39.56	857.30	unknown	16.31		0.002
25.17	903.22	unknown	16.22		<0.001
35.79	923.28	unknown	16.19		<0.001
17.03	569.21	unknown	16.13		0.004
31.89	221.07	unknown	16.09		0.004
34.19	623.18	unknown	15.43		<0.001

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
25.58	283.06	unknown	15.19		0.004
30.65	871.23	unknown	14.92		0.003
24.52	1017.38	unknown	14.46		0.008
20.07	748.82	unknown	14.26		<0.001
25.04	686.79	unknown	13.45		0.008
1.96	326.11	unknown	12.44		<0.001
30.49	688.29	unknown	12.43		<0.001
7.81	371.06	unknown	12.27		0.003
20.66	760.82	unknown	11.94		0.002
28.51	887.22	unknown	11.40		<0.001
22.06	405.18	unknown	11.26		0.005
28.71	1152.54	unknown	10.64		0.008
35.41	688.29	unknown	9.95		0.001
14.57	351.07	unknown	9.95		<0.001
25.29	1150.53	unknown	9.58		0.004
8.60	371.10	unknown	9.30		0.004
26.39	415.20	unknown	8.44		0.002
15.02	355.10	Ferulic acid-hexose II	7.82	2	0.004
3.52	284.01	unknown	7.49		<0.001
16.04	427.18	unknown	7.42		0.008
21.77	525.52	unknown	7.32		0.002
21.65	345.15	unknown	6.90		<0.001
1.86	331.05	unknown	6.47		<0.001
14.53	385.11	Sinapic acid-hexose	5.93		0.003
32.30	411.20	unknown	5.13		0.006
20.25	367.10	3-O-Feruloylquinic acid	5.13	2	<0.001
21.97	471.19	unknown	5.07		0.007
14.01	355.10	Ferulic acid-hexose I	5.06	2	<0.001
14.39	335.13	unknown	4.96		<0.001
15.92	567.19	dimethyl-dihydroxyphenyl hexoside	4.74	3	0.002
24.65	1110.53	unknown	4.71		0.002
29.80	965.29	unknown	3.89		0.005
32.55	845.21	unknown	3.82		0.003
32.34	921.26	unknown	3.65		0.006
2.95	515.06	unknown	3.42		0.005

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Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
13.96	329.09	4-Hydroxy-2-(hydroxymethyl) benzoic acid-hexoside	3.37	3	<0.001
14.12	341.09	caffeic acid-hexose V	3.27	2	<0.001
21.70	609.15	quercetin-3-O-rutinoside (rutin)	3.21	1	0.007
19.08	403.16	unknown	3.08		0.002
1.92	146.05	L-Glutamic acid	3.06	1	<0.001
2.61	333.06	unknown	3.05		<0.001
27.18	415.20	unknown	2.99		0.003
21.22	359.13	C10H14O4-hexoside	2.96	3	0.002
3.73	286.97	unknown	2.59		0.003
2.54	221.03	unknown	2.37		<0.001
2.16	259.02	unknown	2.31		0.002
20.41	471.19	N317	2.20	4	0.005
15.79	431.19	N238	2.17	4	0.003
2.01	195.05	unknown	1.40		0.005
2.21	451.05	unknown	1.38		0.005
3.77	565.05	UDP-glucose	0.73	1	0.002
23.66	1094.54	Hydroxytomatine III + FA	0.66	2	0.008
22.33	1094.54	Hydroxytomatine II + FA	0.56	2	<0.001
2.41	455.10	unknown	0.51		0.006
3.82	606.07	UDP-N-acetyl-hexose amine II	0.46	3	0.003
2.28	133.01	malic acid	0.45	1	0.002
1.82	145.06	L-Glutamine	0.43	1	0.003
2.65	184.99	unknown	0.42		0.001
2.63	317.05	unknown	0.41		<0.001
2.01	341.11	sucrose	0.31	1	0.008
31.33	1136.55	Acetoxy-tomatine III + FA	0.23	2	0.001
26.86	1096.55	N514_C50H85NO22 + FA	0.22		0.001
1.95	325.12	unknown	0.17		<0.001
29.64	1134.53	unknown	0.17		0.004
30.47	1078.54	alpha-Tomatin + FA	0.12	1	<0.001
29.32	1076.53	dehydrotomatin + FA	0.12	2	0.001
2.03	549.17	unknown	0.11		0.005
1.90	214.05	unknown	0.11		<0.001
30.63	1136.55	Acetoxy-tomatine II + FA	0.10		<0.001
29.84	1108.55	unknown	0.08		<0.001
29.98	1078.54	alpha-Tomatin isomer + FA	0.08	2	0.001

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Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	Level of annotation	t-test
30.43	1069.52	unknown	0.08		<0.001
30.34	1128.52	unknown	0.07		<0.001
31.10	1076.53	unknown	0.05		<0.001
29.91	1109.56	unknown	0.05		<0.001
30.38	1136.55	Acetoxy-tomatine I + FA	0.05		<0.001
21.16	1094.54	Hydroxytomatine I + FA	0.05	2	<0.001
33.94	1150.56	unknown	0.04		0.001
31.55	1048.53	unknown	0.03		0.002
43.50	407.26	unknown	0.02		<0.001
2.47	269.05	unknown	0.01		<0.001

SUPPLEMENTARY TABLE 3 | Responsive metabolites detected in tomato pericarp by GC-MS, after fruits were treated with light (500 µmol m^{-2} s^{-1}) compared to treatment with darkness for 15 days (Exp.2; =0.01, n=3).

Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between light and darkness at 15 days)	T-test
19.24	192	Unknown	528.56	<0.001
19.18	163	Unknown	309.17	<0.001
19.20	82	Unknown	224.53	<0.001
19.45	73	Unknown	224.00	<0.001
20.34	103	Unknown	155.50	0.003
21.97	189	Unknown	74.87	<0.001
16.52	131	Unknown	30.93	<0.001
21.96	333	Unknown	24.71	0.002
13.20	74	Unknown	13.78	0.008
16.50	173	Unknown	8.53	<0.001
10.28	61	Unknown	8.39	<0.001
17.60	333	Unknown	6.90	0.002
17.39	115	Galacturonic acid	4.69	0.007
6.20	166	Unknown	3.94	<0.001
13.98	363	Glutamic acid	3.73	0.001
14.32	73	Xylose	2.54	<0.001
17.90	147	Unknown	1.99	0.012
14.40	73	Unknown	1.77	0.010
18.83	221	myo-inositol	1.63	0.005
12.92	129	GABA	0.43	0.004
12.37	350	Malic acid	0.38	<0.001

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SUPPLEMENTARY TABLE 4 | Responsive metabolites detected in tomato pericarp by GC-MS, after fruits were treated with light (500 µmol m^{-2} s^{-1}) for 15 days compared to initial levels (Exp.2; =0.01, n=3).

| Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between 15 and 0 days in light)	T-test
17.60	333	Unknown	716.23	<0.001
21.96	333	Unknown	421.25	0.002
21.97	189	Unknown	80.21	<0.001
10.28	61	Unknown	37.25	<0.001
17.39	115	Galacturonic acid	36.80	<0.001
16.52	131	Unknown	28.08	<0.001
13.98	363	Glutamic acid	17.45	<0.001
12.98	84	Glutamic acid	9.62	<0.001
14.32	73	Xylose	5.98	<0.001
16.50	173	Unknown	5.71	<0.001
6.20	166	Unknown	5.48	<0.001
14.40	73	Unknown	4.18	0.001
15.32	86	Unknown	2.65	0.003
12.77	148	Asparagine	2.57	0.006
17.90	147	Unknown	1.95	0.009
15.52	147	Unknown	1.49	0.006
7.66	59	Unknown	0.68	<0.001
12.92	129	GABA	0.36	0.005
8.72	144	Unknown	0.26	0.007
12.37	350	Malic acid	0.23	<0.001
11.63	86	Unknown	0.22	0.004
23.77	133	Unknown	0.10	<0.001

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SUPPLEMENTARY TABLE 5 | Effect of light spectrum during treatment of tomato fruits on metabolites detected by LC-MS. Fruits were treated for 7 days with a combination of monochromatic light (250 µmol m^{-2} s^{-1} for blue, red and far-red and 200 µmol m^{-2} s^{-1} for green treatments) and background broadband light (100 µmol m^{-2} s^{-1} for blue, red and far red and 150 µmol m^{-2} s^{-1} for green treatments; Exp. 2; =0.01, n=3).

Treatments Compared	Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between the first and second light treatment at 15 days)	Level of annotation	T-test
Blue VS Far Red	13.49	179.03	unknown	29.01		0.002
Blue VS Far Red	21.34	333.06	unknown	15.68		<0.001
Blue VS Far Red	31.78	435.13	unknown	10.82		0.003
Blue VS Far Red	28.98	481.13	unknown	9.18		0.003
Blue VS Far Red	26.46	451.12	unknown	7.88		0.007
Blue VS Far Red	12.63	171.03	unknown	7.72		0.003
Blue VS Far Red	15.34	351.07	unknown	7.53		0.002
Blue VS Far Red	14.57	351.07	unknown	7.45		<0.001
Blue VS Far Red	16.53	351.07	unknown	7.42		0.002
Blue VS Far Red	12.59	351.07	unknown	6.97		0.001
Blue VS Far Red	26.37	433.11	Naringenin-hexose	6.66	2	0.003
Blue VS Far Red	27.13	495.11	unknown	5.32		0.010
Blue VS Far Red	19.24	433.11	unknown	5.00		0.001
Blue VS Far Red	19.94	433.11	Naringenin-hexose	4.86		0.003
Blue VS Far Red	4.81	345.08	unknown	3.34		<0.001
Blue VS Far Red	22.04	449.11	unknown	2.92		0.003
Blue VS Far Red	14.53	385.11	Sinapic acid-hexose	2.50		0.002
Blue VS Far Red	25.92	449.11	unknown	2.48		0.002
Blue VS Far Red	22.60	577.25	C26H42O14	2.45		0.005
Blue VS Far Red	28.96	855.25	unknown	2.44		0.001
Blue VS Far Red	13.42	595.17	Naringenin-C-diglycoside	2.20	2	<0.001
Blue VS Far Red	9.03	341.09	caffeic acid hexose II	1.79	2	0.002
Blue VS Far Red	14.01	355.10	Ferulic acid-hexose I	1.78	2	0.005
Blue VS Far Red	31.62	445.08	unknown	1.72		0.008
Blue VS Far Red	15.02	355.10	Ferulic acid-hexose II	1.58	2	0.005
Blue VS Far Red	1.96	326.11	unknown	1.52		0.004
Blue VS Far Red	24.29	433.11	Naringenin-hexose	1.47	2	0.008
Blue VS Far Red	28.10	515.12	dicaffeoylquinic acid IV	1.38		0.009
Blue VS Far Red	3.77	565.05	UDP-glucose	1.33	1	0.004
Blue VS Far Red	22.98	760.82	double charged: >1500 D	0.74	4	0.002
Blue VS Far Red	1.90	214.05	unknown	0.59		0.008
Blue VS Far Red	30.49	688.29	unknown	0.44		0.002
Blue VS Far Red	25.04	686.79	unknown	0.16		0.004

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Treatments Compared	Retention Time (min)	Mass	Metabolite Name	Response to treatment (times increase between the first and second light treatment at 15 days)	Level of annotation	T-test
Red VS Far Red	29.89	312.12	unknown	6.23		0.009
Red VS Far Red	25.92	449.11	unknown	2.48		0.002
Red VS Far Red	30.70	693.28	unknown	2.44		0.010
Red VS Far Red	32.03	677.28	N713	2.05	4	0.005
Red VS Far Red	2.11	526.12	unknown	1.49		0.003
Red VS Far Red	32.48	819.27	unknown	0.18		0.003
Blue VS Red	28.98	481.13	unknown	4.08		0.007
Blue VS Red	19.24	433.11	unknown	3.85		0.002
Blue VS Red	29.71	429.14	unknown	3.76		<0.001
Blue VS Red	21.34	333.06	unknown	2.96		0.005
Blue VS Red	28.96	855.25	unknown	2.55		0.001
Blue VS Red	14.57	351.07	unknown	2.34		0.009
Blue VS Red	4.81	345.08	unknown	2.21		0.003
Blue VS Red	22.04	449.11	unknown	1.91		0.004
Blue VS Red	13.42	595.17	Naringenin-C-diglycoside	1.83	2	<0.001
Blue VS Red	28.10	515.12	dicaffeoylquinic acid IV	1.34		0.008
Blue VS Red	29.61	221.07	unknown	1.10		0.004
Blue VS Red	32.48	819.27	unknown	0.06		0.001
Green VS White	30.70	693.28	unknown	3.84		0.006
Green VS White	2.59	259.02	unknown	1.19		<0.001
Green VS White	3.43	565.05	unknown	0.93		0.003
Green VS White	24.97	503.12	unknown	0.65		0.003
Green VS White	10.24	327.11	unknown	0.46		0.003
Green VS White	14.53	385.11	Sinapic acid-hexose	0.45		0.006
Green VS White	14.57	351.07	unknown	0.29		0.004
Green VS White	28.71	1152.54	unknown	0.29		<0.001
Green VS White	15.34	351.07	unknown	0.26		<0.001
Green VS White	16.53	351.07	unknown	0.23		0.009
Green VS White	22.73	463.09	Quercetin 3-O-glucoside	0.19	1	0.004

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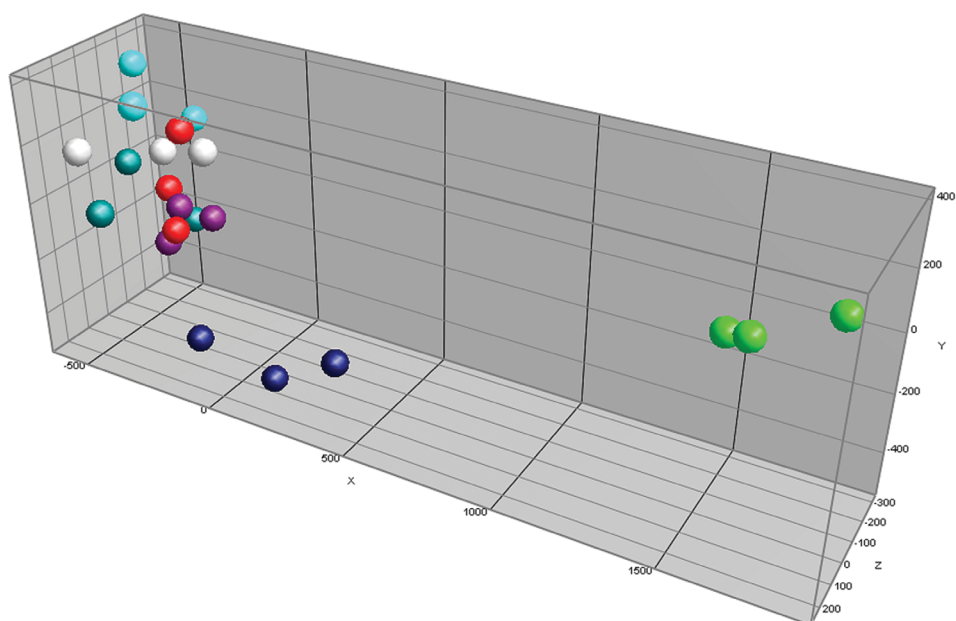
SUPPLEMENTARY FIGURE 1 | Principle component analysis (PCA) of tomatoes treated with different light qualities, based on 437 tomato pericarp metabolites detected by LC-MS based untargeted metabolomics. The fruits were treated with different light spectrum treatments (350 µmol m^{-2} s^{-1}) and darkness for 7 days (Exp. 2). PC1=X-axis=37% explained variation; PC2=Y-axis=12.4% explained variation; PC3=Z-axis=7.3% explained variation (Red treatment-red points, blue treatment-light blue points, far-red treatment-purple points, green treatment-green points, white treatment-white points, dark treatment-dark blue points, starting material-light green points).

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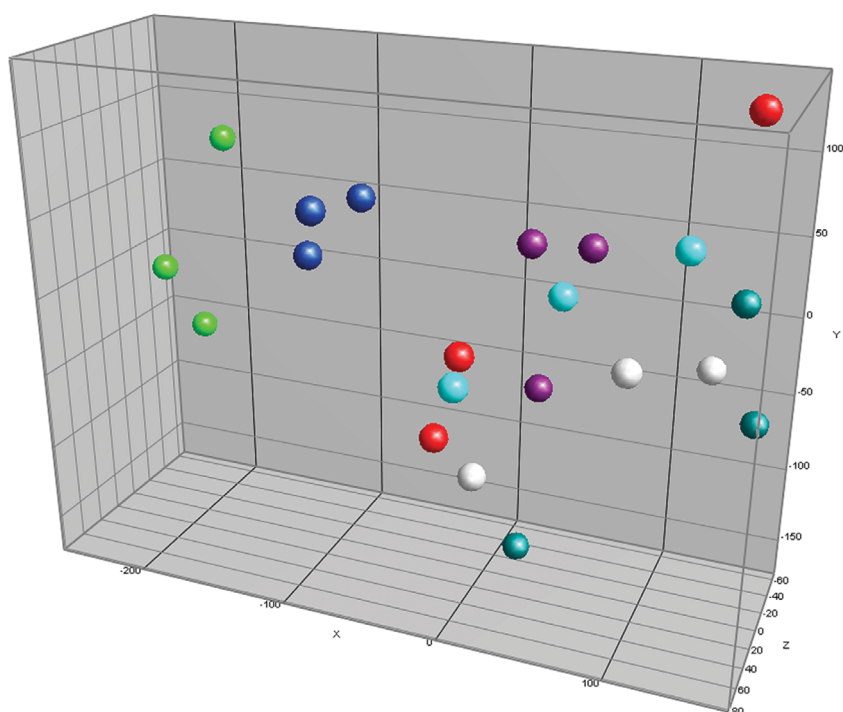
SUPPLEMENTARY FIGURE 2 | Principle component analysis (PCA) of tomatoes treated with different light qualities, based on 109 tomato pericarp metabolites detected by GC-MS based untargeted metabolomics. The fruits were treated with different light spectrum treatments (350 µmol m^{-2} s^{-1}) and darkness for 7 days (Exp. 2). PC1=X-axis=24.9% explained variation; PC2=Y-axis=13.9% explained variation; PC3=Z-axis=8.3% explained variation (Red treatment-red points, blue treatment-light blue points, far-red treatment-purple points, green treatment-light green points, white treatment-white points, dark treatment-dark blue points, starting material-light green points).

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Chapter 6

General Discussion

The positive effects of light on ASC levels in leaves have been manifested over a wide range of species. Effects of light on ASC in fruits are not as extensively studied (Chapter 2). My work investigates the physiological mechanism behind light regulation of ASC in tomato fruits. Latest developments in LED technology expand even further the possibility to manipulate the quality of horticultural products. Therefore, producing knowledge around the underlying physiological mechanisms is timely. This chapter of the thesis has the following structure. First, the elements of the physiological pathway for light regulation are discussed. Initially I focus on irradiance responses and then on responses to light quality. Then the involved biochemical pathways that potentially mediate the light response are discussed. Finally, an overview of the practical implications of the commercial application of such technology either in the pre- or postharvest part of the supply chain is presented.

6.1 The physiological pathways for light regulation of ASC

It is by now well proven that light regulates ASC levels in plant tissue. It is quite common to monitor the ASC changes in parallel to the gene expression of several biosynthetic, recycling and turnover genes during light treatments. While this approach provides with valuable information on which are the limiting steps on the main biochemical pathways, it does not provide any information on the physiological processes involved in light regulation of ASC. In my research, only green fruits which were photosynthetically active were found to respond to the light treatments. Once fruits enter the breaker stage and chlorophyll breaks down, the fruits' ASC concentration ceases to respond to the light treatments (Chapter 3). Exposure to high irradiances results in an increase of the photosynthetic rate of the fruit. The rate of the photosynthetic electron transport determines the redox state of the plastoquinone pool which has a signalling effect on the biosynthesis of ASC (Karpinski et al., 1997, 1999; Madhusudhan et al., 2003). The involvement of photosynthesis in the regulation of the ASC pool was further confirmed by the use of photosynthetic inhibitors. The photosynthetic inhibitors atrazine and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) resulted in retardation of light-induced ASC accumulation in Arabidopsis leaves (Yabuta et al., 2007). A by-product of photosynthesis is the formation of reactive oxygen species in the chloroplasts, where ASC is transported via the PHT4;4 transport protein (Miyaji et al., 2015). Even though the oxidative stress of fruit tissue has not been measured in my research, it is fair to assume that reactive oxygen species are being produced as there is distinct rate of photosynthesis in green fruit (Chapter 3). It is therefore hypothesized that the coupling between ASC levels and photosynthesis could have developed through evolution as a natural defence mechanism against reactive oxygen species. Even though it is shown in my work (Chapter 3) that many antioxidants increase under conditions that stimulate fruit photosynthesis, a coupling between photosynthesis (or respiration) and other antioxidant compounds has not yet been described.

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The respiration process has also been proposed to be involved in light regulation of ASC. GLDH the last enzyme in the main respiratory pathway is located in the inner membrane of mitochondria (Bartoli et al., 2000) and is an integral part of complex I of the respiratory electron transport chain (Schimmeyer et al., 2016). Consequently, the rate of respiratory electron transfer across the inner membrane of mitochondria correlates positively with the enzymatic activity of GLDH and, therefore, ASC levels. Biosynthesis of ASC from L-galactono-1,4-lactone was possible in isolated mitochondria from potato cells (Bartoli et al., 2000). In my research, higher irradiances induced an increase in ASC but a concomitant increase in the rate of respiration was not observed. Furthermore, when the fruits entered the breaker stage (when the climacteric peak in respiration is expected) there was not an increase in ASC (Chapter 2). This suggests that in tomato fruits the mechanism behind ASC regulation is largely independent of the respiratory rate of the fruit.

The main biosynthetic pathway for ASC (D-man/L-gal) synthesizes ASC with D-glucose as initial precursor. Indeed, a soluble carbohydrate (sucrose) has been found to affect the expression rates of ASC related genes (Xiang et al., 2011). Effects on biosynthetic genes such as VTC1, VTC2, and L-GalDH have been observed in tomato fruit (Badejo et al., 2012). Carbohydrate feeding affects also the expression of recycling and turnover genes such as APX, MDHAR, DHAR, GLDH and GR (Nishikawa et al., 2005). Therefore, it has been hypothesized that soluble carbohydrate availability regulates the rate of ASC biosynthesis and consequently ASC levels. Indeed exogenous sucrose feeding of broccoli inflorescences delayed ASC depletion typically observed during postharvest. However, this might not be the case for all species as exogenous application of sucrose or glucose to barley (Smirnoff and Pallanca, 1996) and pea seedlings (Pallanca and Smirnoff, 1999) had no effect on ASC levels. In my research (Chapter 4) the effects of carbohydrates have been tested in a variety of experimental setups where soluble carbohydrates have been manipulated by exogenous application or fruit pruning. In all cases and in accordance to literature (Smirnoff and Pallanca, 1996; Pallanca and Smirnoff, 1999), there was no correlation between the soluble carbohydrate content and the ASC levels. In an approach where ASC levels were manipulated by light, there was no correlation with soluble carbohydrates.

Fruits being sink organs contain considerably higher levels of soluble carbohydrates compared to source organs such as leaves. Therefore, it may be that in tomato fruits the soluble carbohydrate concentration is never a limitation for the biosynthesis of ASC. ASC was shown to be independent of carbohydrate levels also in source organs (Smirnoff and Pallanca, 1996). The hypothesis that soluble carbohydrate levels are not a limitation for ASC biosynthesis, is yet to be tested as the relationship between ASC and soluble carbohydrates in fruits has been tested only by increasing the content of the latter. To expand our knowledge on how carbohydrates might regulate ASC levels (either as substrate or signalling molecules) genetically manipulated plants with low carbohydrate content can be used (e.g. starch-less or near starch-less mutants). A way of quantifying the flux of carbohydrates into ASC in future experiments, is to feed the tissue with 14C-labelled ASC precursors (Loewus, 1999).

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In summary, in tomato fruit it is most likely that the regulatory effects of light on the size of the ASC pool are mediated through photosynthetic signalling rather than respiration or substrate availability. However, respiration, photosynthesis and carbohydrates might interact in light regulation of ASC (Chapter 2). Soluble carbohydrate feeding of Arabidopsis plants has inhibitory effects on photosynthesis with consequent negative effects on ASC levels (Paul and Foyer, 2001). This potential indirect effect of carbohydrates on ASC via photosynthesis, is often not taken into account in feeding experiments. In my work it was shown that the rate of respiration does not affect ASC in tomato fruits (Chapter 3). In leaves where respiration has a regulatory function on the ASC pool size (Bartoli et al., 2006), carbohydrate feeding might have an indirect effect on ASC levels by increasing the rate of respiration. While respiration regulates the enzymatic activity of GLDH (the last enzyme in the main biosynthetic pathway for ASC), photosynthesis regulates in particular the expression of biosynthetic genes. Genes of the ASC-glutathione cycle are present both in mitochondria and chloroplasts (Chew et al., 2003). Therefore, both mitochondria and chloroplasts are integral parts of the biosynthetic and recycling machinery for ASC. The two organelles have been previously found to interact in the biosynthesis of a variety of secondary metabolites (Mackenzie and McIntosh, 1999). Another level of interaction between the two organelles is the malate-oxaloacetate shuttle where excessive energy during photosynthesis is dissipated (Noguchi and Yoshida, 2008).

6.2 Light quality effects on ASC and the tomato metabolome

The spectral distribution of light has limited effects on ASC levels in the pericarp of tomato fruit (Chapter 3). Certain light spectra were found to be more effective than others but differences between light quality treatments were of a small magnitude. When enhancing the red and blue part of the spectrum ASC levels increased compared to enhancing the far-red part of the spectrum. Light absorption of chlorophyll is pronounced in the red and blue part of the spectrum. There is minimum chlorophyll absorption of far-red radiation and thus only an extremely small part of photosynthesis is driven by far-red light. Therefore, higher photosynthetic rates are expected in the red and blue treatments compared to the far-red. The fact that higher ASC levels were detected in the red and blue treatments also supports the idea that light regulation of ASC in tomato fruit is mediated through photosynthesis.

As light spectrum had some effects on ASC, certain photoreceptors may be involved in light regulation of ASC. The photostationary state of phytochrome (PSS) is largely affected by red and far-red radiation and less by blue (Sager et al., 1988). In my research additional red induced considerable ASC accumulation compared to additional far-red. In accordance, red LED light was effective in delaying ASC depletion in broccoli florets (Ma et al., 2014). The beneficial effects of red light in the latter case were related to biosynthetic (VTC2 and GLDH) and recycling (MDAR) genes. Blue light is mostly

absorbed by cryptochrome and phototropins photoreceptors (Lin, 2000). In accordance to our results, blue LED light increased ASC in the fruit of three citrus varieties while red LED irradiation had no effects on ASC content of these fruit (Mastropasqua et al., 2012). This suggests the involvement of blue light photoreceptors besides phytochrome, such as cryptochrome and phototropins. Specifically blue light affected biosynthetic (VTC1, VTC2, VTC4, and GLDH) and recycling (GR, MDAR, DHAR) genes. Photoreceptors for red and blue in plants seem to be involved in light regulation of ASC with variable effects. Even though it has been shown that phytochrome is involved in regulation of ASC levels, only indications for the involvement of other photoreceptors exist to date. Further understanding how photoreceptors are involved in light regulation of ASC will provide with information for fine tuning the light spectrum to achieve maximum ASC accumulation.

Light quality effects on ASC are extended beyond the visible spectrum (480-700nm). UV-C radiation delays the ASC loss during postharvest that is typically observed in broccoli (Lemoine et al., 2010). Effects of ultraviolet irradiation may vary depending on the specific spectrum. UV-B (280-315nm) radiation carries more energy compared to UV-A (315-400nm) and less than UV-C (100-280nm). All UV spectra might induce damage to the cells when supplied in substantial amounts with UV-B and UV-C having particularly detrimental effects on living cells through lipid peroxidation, DNA strand breaks and ROS production. ASC has been identified as a key molecule of the antioxidant mechanism of plants against UV-B radiation (Conklin et al., 1996). UV-B irradiation induced increases in ASC in a variety of cases (Jansen et al., 2008). However, certain species or tissues might defend against UV induced redox stress by other antioxidants instead of ASC. In sunflower cotyledons UV-B radiation influenced the ratio of reduced to oxidized glutathione but not the ratio of ASC to dehydroascorbate (Costa et al., 2002). UVR8, a specific photoreceptor for UV-B is found in plants (Jenkins, 2014). UVR8 knockout mutants were deficient in ASC (Singh et al., 2014). Therefore, UV regulation of ASC is not only indirectly through induction of redox stress but also directly through UVR8. This research with mutants has been to its entirety conducted in arabidopsis leaves and it is yet unknown whether the same physiological system exists in fruit and whether tomato fruit would be responsive to UV irradiation in terms of ASC content and redox state.

Apart from ASC, other metabolites are also affected when tomato fruits are subjected to different light quality treatments. This response is potentially a defence mechanism in encountering the increased redox load that is expected from the light induced upregulation of photosynthesis and respiration. In my research, the antioxidant zeaxanthin accumulated upon exposure to blue and red light compared to far-red, while lycopene and carotenoids did not differ between the light quality treatments. However, these patterns were not always consistent with literature where red light was found to have a beneficial effect for the lycopene and carotenoid content of tomato fruit (Schofield and Paliyath, 2005; Liu et al., 2009). Red LED light positively affects -cryptoxanthin, while blue light did not affect carotenoids in citrus fruits (Ma

et al., 2012). Individual metabolites might respond differently to spectral treatments. The effects of light quality on the metabolome are probably executed via different photoreceptors. Furthermore, effects of light quality on the metabolome might depend on the developmental stage and/or the genotype.

6.3 The biochemistry of the regulation of ASC by light

The plant genome encodes biosynthetic enzymes for ASC that belong to distinct biosynthetic pathways. Although ASC recycling and turnover take place through only one established mechanism, several biosynthetic pathways for ASC biosynthesis have been proposed to exist in plants. The D-mannose/L-galactose (D-man/L-gal) biosynthetic pathway synthesizes ASC through 11-steps with initial precursor D-glucose. The dedicated part of the pathway consists of 4 steps with GDP-L-galactose as initial precursor and L-galactono-1,4-lactone as last precursor of ASC (Wheeler et al., 1998). The galacturonate pathway (also named secondary or salvage pathway) utilises carbon released from cell wall breakdown in the form of me-D-galacturonate to synthesize ASC (Agius et al., 2003). The myo-inositol pathway synthesizes ASC in 4 steps with initial precursor L-gulono-1,4-lactone (Lorence et al., 2004). Finally, the gulose pathway synthesizes ASC from GDP-L-gulose. This pathway is typically found in animal organisms. However, genes encoding certain steps of the gulose pathway have also been identified in plants (Wolucka and Van Montagu, 2003). As explained in Chapter 6.2 the main precursor for ASC biosynthesis is glucose without it being a limitation or considerable boost for ASC biosynthesis in the range typically found in tomato fruits (Chapter 6.2).

Isotope feeding studies confirm that the vast majority of the ASC pool in non-genetically modified plants is attributed to the D-man/L-gal pathway (Chapter 2). There are no conclusive quantification studies of the ASC output of the galacturonate, gulose and myo-inositol biosynthetic pathways. Furthermore, evidence for the contribution of the galacturonate, myo-inositol and gulose pathways to the ASC pool, comes mostly from correlations between expression of biosynthetic genes and ASC levels (Chapter 2). As cell walls break down with the progress of fruit ripening, the contribution of the galacturonate pathway is expected to be increased at later stages of development. For the same reasoning, the galacturonate pathway might account for greater fraction of the ASC pool in fruit than in leaves, a hypothesis that has not yet been tested. However, in my work it is shown that there is no correlation between galacturonate and ASC for fruit that ripened in both light and darkness. Furthermore, the activity of the galacturonate pathway is not affected by the light treatments. It may be that only in over-ripe fruits, the galacturonate pathway accounts for a considerable part of the ASC pool.

Light was found to induce ASC accumulation because of upregulation of gene expression of several biosynthetic genes that are predominantly located in the D-man/L-gal pathway. There was to date no evidence for the contribution of the myo-inositol biosynthetic pathway to the ASC pool in plants (Chapter 2). When tomato fruit were exposed to light in my research, a significant correlation was observed for the first time between myo-inositol and ASC (Chapter 4). This proposes that during exposure to light, apart from the activity of the D-man/L-gal pathway, the myo-inositol pathway might also be activated. Nevertheless, it remains unknown whether it contributes considerably to the ASC pool. Overexpressors of L-gulono-1,4-lactone dehydrogenase/oxidase (the enzyme that catalyses the conversion of L-gulono-1,4-lactone to ASC) in higher plants lead to accumulation of ASC. This indicates the myo-inositol pathway in plants can produce considerable amounts of ASC. However, for this hypothesis to be confirmed, gene expression of the relevant steps of this pathway or isotope feeding studies have to be carried out.

6.4 Light applications can improve the overall quality of tomato fruits

A wide range of health related metabolites are found in tomato fruits (e.g. vitamins C, E and K1, lycopene, beta-carotene, chlorogenic acid and naringenin). Several of these metabolites have been found to increase in the presence of light. Out of all the tested light environments, mature green tomato fruit achieved a maximum amount of ASC (~48 mg/100g of fresh weight) when kept at 500 µmol m^{-2} s^{-1} for 9 days. Therefore, the daily required intake for humans can be covered with approximately 200 g of such a fruit. Tomato fruit treated with these LED light treatments can easily be characterised as "source of" (15% of daily required intake) or "high in" (30% of daily required intake) ASC. However, the light treatment is effective only when applied to green fruit. When fruits are already ripe there is no opportunity to increase vitamin C by light. Therefore, if application would be done post-harvest, this is relevant only for supply chains in which fruits are harvested green. Antioxidants such as lutein, zeaxanthin, lycopene, beta-carotene and tocopherols increase in light compared to darkness. These metabolites have also been indicated to be related to the proper function of the human body. Even though health effects for certain of these metabolites are debatable, most of these molecules have been associated with promoting human health due to their antioxidant function (EFSA Panel on Dietetic Products, 2011). Many of them are indispensable (tocopherol and beta-carotene characterised respectively as vitamin E and provitamin A) for the proper function of the human body. LED illumination of mature green tomato fruit has a generalised beneficial effect to antioxidants.

As many antioxidants increase with exposure of tomatoes to light, the total antioxidant capacity of the tissue is expected to increase. After harvest the maintenance respiration provides the energy essential for the tissue to stay alive. A by-product of this process is reactive oxygen species (ROS). An enhanced ROS content might have a negative

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effect on shelf life as they negatively influence the integrity of the cell membranes and consequently decrease cell turgor and cellular functions. Increased antioxidant capacity of the tissue will counteract ROS and therefore minimize the harmful effect of ROS. It is therefore hypothesized that enhanced antioxidant content due to illumination of the fruit might extend shelf life in terms of firmness and water loss, as it has been observed in tomatoes enriched with anthocyanins (Zhang et al., 2013). Indeed, in my research fruit with enhanced ASC content (illumination with 300 µmol m^{-2} s^{-1} for 7 days) had slower firmness degradation when stored in darkness compared to non-upregulated fruit (data not presented).

The visual appearance, firmness, taste and nutritional value of tomato fruit change with the progress of ripening. With respect to the appearance, after the climacteric peak the concentration of the pigments mostly in the pericarp undergo drastic changes. Typically, chlorophyll breaks down while lycopene and carotenes increase (Choi et al., 2010). This explains the colour transition of the fruit from green to red. The presence of light during ripening considerably increases the concentration of lycopene and carotenes while it induces an earlier chlorophyll breakdown. Firmness of tomato fruits typically decreases during the postharvest part of the chain (Oms-Oliu et al., 2011). For climacteric fruit like tomato, a peak in polygalacturonase content is observed (Brady et al., 1985) leading to increased cell wall metabolism and a reduction in firmness. In my research there was an increase in products of cell wall metabolism such as naringenin (Chapter 5). However, as in my research no considerable changes in firmness have been observed in response to light, the mentioned effects of light on cell wall metabolism are limited to a range that does not considerably affect the firmness of the fruit (estimated based on acoustic measurements).

Taste characteristics of the fruit also change during ripening. Typically starch breaks down to glucose. This change in the primary metabolome results in an increase in perceived sweetness. Soluble carbohydrates of detached fruit were not affected by the light environment after harvest. Sweetness is the main driver for consumer liking. However, taking into account in models not only soluble carbohydrates (°brix) but also texture and organic acids, does increase the predictability of consumer liking. A variety of organic acids has been found to be affected by the postharvest light treatments. However, out of the organic acids found in high concentrations in tomato only malic acid was found to increase in response to light, while citric acid was not affected. Therefore, no considerable changes in perceived sourness are expected with the light treatments. Regarding texture, no differences in the firmness of the fruit have been detected between fruit stored in light and darkness. However, certain products of cell wall metabolism have been found to be influenced by the light treatments. A more elaborate texture analysis would provide a better understanding on the potential effects of light on sensory related texture traits (juiciness, gumminess, crunchiness, toughness etc.).

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In summary, since postharvest illumination of mature green tomato fruit does not affect the main drivers of consumer liking (sweetness, sourness and texture), it is not expected that the postharvest lighting treatments produce neither inferior, nor superior fruit in terms of consumer liking. However, the main drawback of the application of postharvest fruit remains the fact that the fruit have to be harvested at the mature green stage. Harvesting mature green tomato fruit is more common in long supply chains and these fruits are typically treated as fruits of inferior quality compared to the ones that ripen normally on the plant. When tomatoes are harvested mature green they usually lack the soluble carbohydrates that they would have accumulated if they would have ripened naturally on the plant. Such a strategy is therefore expected to negatively influence consumer liking. LED light potentially has similar beneficial effects on green mature tomato fruit irrespectively of whether they are still attached to the plant or detached (as in my research). In a potential preharvest application, several concerns have to be taken into account for the development of an effective system. The preharvest application of LEDs for the upregulation of ASC in tomato fruits has been previously tested (Labrie and Verkerke, 2012) with similar effects on ASC. According to the findings of my work, to have considerable effects on ASC approximately 300 µmol $m^{-2} s^{-1}$ at fruit level are required. This means a very high intensity, which seems not very practical. This intensity might be realised currently only with intense intercrop lighting which produces heat that leads to a disturbance in the thermal balance of the plant. As the plants transpired more in this case, more frequent irrigation was essential which required adaptation of the composition of the nutrient solution. The above mentioned have been confirmed in a greenhouse experiment.

Many modern greenhouses can facilitate the application of LED lighting. Some growers are already using LEDs in the greenhouse. The advantage of these modules is that due to small size and low radiative heat, they can be installed very close to the fruit (such as intercrop lighting) as opposed to HPS lighting that can be installed only on the top of the canopy (Kaukoranta et al., 2015). However, the way LED lighting is currently applied is unlikely to cause significant upregulation of ASC. Light from both top mounted modules and between plants are placed close to the photosynthetically active leaves and thus away from the fruit. Therefore, fruits produced in current systems with intercrop lighting are not expected to have higher ASC levels. In the case specific modules will be placed at fruit height aiming to improve ASC, light use for photosynthesis will be probably inefficient as this part of the plant is typically defoliated and the light is mostly absorbed by the stems which have low photosynthetic efficiency.

LED illumination of mature green harvested tomato fruits led to improvements of the nutritional quality, without negative effects on taste. After fruits and vegetables are harvested and in the supply chain, the produce is typically subjected to conditions optimal for prolonging shelf life in terms of texture and visual quality (transport and storage in darkness and reduced temperature). However, these conditions have negative effects on taste and nutritional quality. This consolidated approach in the industry has led to a general opinion that the quality of the produce at harvest point

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is the maximum possible quality and that this generally decreases in the postharvest supply chain. In my research I showed that postharvest lighting treatments of unripe fruits are capable of increasing certain quality traits of the tomato fruit as improved quality is achieved compared to that at harvest point. Such an application might be of industrial significance in the future (Nicole et al., 2018). Further work on the topic will pave the way for the development of industrial postharvest lighting strategies with the aim not only to maintain but also to improve the quality of the produce.

Developing these postharvest lighting strategies is now timely due to the vigorous technological development in the field of LEDs. Compared to more traditional light sources used for horticultural applications such as HPS lamps, LEDs have multiple advantages: compact size, modular designs, relatively low heat emissions, able to steer the spectral distribution of light, long operating time and output linear to electrical input (Folta et al., 2005; Bourget, 2008). Furthermore, the latest LED technological advancements achieved light production efficiencies (mol PAR per J electricity) that exceeded this of HPS lamps and keep increasing (Pattison et al., 2018). Higher light production efficiency means a lower heat output relative to light output compared to HPS lamps and therefore, a smaller effect on the temperature of the crop and the produce. Furthermore, the compact design of LEDs allows them to be placed closer to the produce. Due to the above facts LEDs can be characterized as more environmentally friendly and suitable for application in confined environments such as storage climate rooms, indoor growth systems, and greenhouses (Nhut and Nam, 2010; Shimada and Taniguchi, 2011).

My research proposes the application of LEDs for the improvement of certain quality traits by illumination of tomato fruit during the postharvest part of the supply chain (Nicole et al., 2018). This application is relevant only for certain supply chains in which tomato fruits are harvested mature green and ripened later (sometimes by means of ethylene) in designated ripening rooms. LEDs can easily be suspended on shelves and placed in storage rooms or transportation units due to their small size and low radiative heat (Nicole et al., 2018). In this system, 10 shelves would require 1Kw per m^2 which would probably require some cooling. In order to make the postharvest application of light financially sustainable, the treatment has to be short and efficient. In this work it is shown that continuous illumination for 7 days at 300 µmol m^{-2} s^{-1} is sufficient for causing approximately 2 times increase in pericarp ASC levels. In a separate experiment where fruit were treated with 412 µmol m^{-2} s^{-1} for 7 days approximately a 3-fold increase in ASC was achieved. These light treatments result in approximately 40 mg ASC/100g of fresh weight and therefore these fruits are "high in vitamin C" as they would contain more than 30% of the daily required intake for humans (conventional tomatoes in the best case are only a "source of vitamin C" containing no more than 15% of the daily required intake). Daily needs in ASC may be covered by the consumption of a couple medium sized light treated tomatoes. Furthermore, the increased levels of ASC as a result of light treatment, were maintained for 14 days post illumination where the fruit were stored in common postharvest conditions (19 °C and 19 µmol m^{-2} s^{-1}; indicative

conditions measured in supermarkets in the Netherlands). In tomato fruits where ASC has been upregulated by prior light exposure the increase is maintained even after illumination. Another practical question is the storage temperature. If the treatments are applied during storage, research has to confirm the beneficial effects of LED light are also evident in temperatures lower than in my study (18°C), at which storage of fruit during transportation in longer supply chains may occur. In commercial practice, tomatoes are seldom stored below 12°C due to chilling injury sensitivity. Therefore, illumination at average storage and transportation temperatures (15°-19°C) is not expected to have very different effects on ASC compared to what has been observed in my study.

Environmental factors other than light affect ASC metabolism in plants. Abiotic stress is a strong stimulus for ASC regulation. Salinity stress induced ascorbate accumulation in bean (Telesiñski et al., 2008). Drought stress in sorghum decreased the activity of turnover (APX) and recycling (MDHAR) enzymes while it increased the activity of another recycling enzyme (DHAR). Sunflower plants experiencing drought, had increased activity of turnover (APX) and recycling (DHAR) enzymes (Zhang and Kirkham, 1996). The activity of AO (recycling enzyme) in pumpkin seedlings positively correlated with oxygen concentration ranging from higher than atmospheric to anoxic conditions (De Tullio et al., 2007). Both macro- and micronutrients affect ASC levels in plants. Plants experiencing limited nitrogen availability had lower levels of ASC compared to plants with sufficient nitrogen availability (Robinson, 1997). The levels of Mn, Zn or Cu affect the activity of APX (recycling enzyme) in tobacco plats (Yu et al., 1998). Finally, the biome around the plant such as pathogenic bacteria might also affect ASC metabolism e.g. through production of exopolysaccharides (de Pinto et al., 2003). Studying further the effects and the interactions of both abiotic and biotic factors on ASC in plants, will facilitate even further increases in ASC levels.

6.5 Practical concerns for increasing ASC levels in tomato fruit by LED light application

Below are listed some recommendations for the pre- or postharvest application of light, with the aim to increase the nutritional quality of tomato fruits.

1. Developmental stage at harvest (for postharvest application): Only green fruit respond to light in terms of ASC increase and only mature green (full sized but still green) are able to ripen normally postharvest. Therefore, tomato fruits have to be harvested when mature green (full sized but still green). If the fruits are harvested at a time-point where they can stay green for at least a week, then the light treatments will induce considerable upregulation of ASC (up to 60 mg/100g of fresh weight has been achieved in my work, compared to 20 mg/100g when

the same fruit ripens on the plant). However, fruit that ripen off the plant achieve typically lower °brix which results in inferior taste.

2. Light intensity: Intensities between 300 and 500 µmol m^{-2} s^{-1} at fruit level, induce considerable accumulation of ASC. Very high light intensities (>600 m^{-2} s^{-1}) might bring opposite results. If the application of light is done in the greenhouse while the fruits are still attached on the plant (and given the current heat emissions of LEDs), such light intensities are expected to change the heat balance of the plant. Irrigation frequency has to be readjusted and consequently the composition of the nutrient solution has to be readjusted.

3. Kind of lamps to be used: Any broadband light source that contains considerable fractions of red and blue light will induce accumulation of ASC. Light sources that have pronounced far-red are to be avoided, as they will not be as efficient in increasing ASC. Avoid light sources with considerable heat output as they will heat the fruits and the plant. In postharvest applications, light modules can be placed on top of shelves during storage or transportation. Fruits have to be laid on a single layer so that all fruits are uniformly exposed to light. When light is applied in the greenhouse, intercropping light modules placed close to the fruit should be the most effective. Placement should be such that the light that does not reach the fruit, will be intercepted by leaves which can promote photosynthesis and thereby yield. In some greenhouses, UV light is being used for purposes of disinfection of the crop. When applied at the lower part of the plant where the fruit are hanging, it might have a beneficial effect on ASC levels (by evoking the mechanism for protection from oxidative stress). In such case, relatively low dosages would be sufficient but due to safety issue this should only be applied when personnel is not in the greenhouse. This would first need to be investigated.

4. Storage temperature (for postharvest application): the effect of temperature on ASC during storage is not yet well understood. With tomato being sensitive to chilling injury fruit, storage temperature lower than 12°C for more than 3 days is not recommended. The mentioned temperature is indicative and might differ between cultivars. When the storage temperature is measured it has to refer to the temperature of the fruits and not the air temperature as the two might differ considerably when the fruits are illuminated (due to the thermal input of radiation). The selected intensity and lamps used have to be placed in a way that does not affect the temperature of the fruits.

5. Packaging (for postharvest application): typically macro-perforate packaging is used for packing tomato fruit in order to avoid accumulation of ethylene around the fruit. Plastic films that do not alter significantly the quality of light have to be used.

6.6 How breeding can take the next step in increasing ASC in tomatoes

1. Maintain chlorophyll in the fruits for longer: Typically with the progress of ripening of tomato fruits, chlorophylls break down while lycopene and carotenoids accumulate in the pericarp of the fruit. In my work the importance of photosynthesis in light regulation of ASC is highlighted. Breeding for tomato fruit that maintain a minimum chlorophyll level and consequently a minimum photosynthetic rate would potentially lead to even higher than the documented ASC levels when the fruit are kept under light. LeSGR1 (stay green protein) gene silencing in tomato inhibits chlorophyll degradation (Hu et al., 2011). However, these fruits are typically darker. Such an approach could interfere with consumer liking as it potentially alters the visual quality of the fruits (darker fruit).

2. The next step in genetic manipulation: There are several critical control points known in all biosynthetic pathways for ASC. It is projected that in the future novel and safer approaches for genetic manipulation will be allowed for plant breeding. When CRISPR-Cas9 would be allowed in the future for bringing new cultivars to the market, silencing negative regulators or increasing copy number variation of critical control points can be applied. This may lead to higher levels of ASC given that the homeostatic mechanism allows it.

6

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Summary

L-ascorbate is a phytochemical essential for human health. Light regulates L-ascorbate (vitamin C; ASC) levels in plants with the vast majority of the evidence referring to leaves. In the present study the focus was on the effects of light on ASC levels in tomato fruits. The aim of this work as explained in **Chapter 1**, was to investigate which physiological processes and how do they mediate the effects of light on ASC levels in tomato fruits. Furthermore, the effects of light over the broader metabolome of the tomato fruit are investigated.

In **Chapter 2**, a literature review is presented with the aim to highlight the physiological and biochemical network mediating the regulation of ASC by light. Respiration, photosynthesis and soluble carbohydrates are proposed to control ASC levels in plants. Possible interactions between these physiological components and their importance in light regulation of ASC in both leaves and fruits were discussed. Furthermore, a broad biochemical map for ASC biosynthesis, recycling and turnover was presented and the contribution of specific pathways to the ASC pool is discussed. It is concluded that the main biosynthetic pathway (D-man/L-gal) for ASC accounts for the vast majority of the ASC pool in leaves as well as in fruits.

In **Chapter 3**, the effects of light intensity and spectrum on ASC levels in detached tomato fruits were investigated. ASC levels of fruits increased under light compared to darkness, as long as the fruits were green. Red fruits did not respond to the light treatments. Accumulation of ACS under light was not much affected by the light spectrum. The effect of light on ASC is proposed to be universal for tomato as several tested genotypes were found to be similarly responsive to the same light treatments. As only mature green fruits respond to the light treatments and the rate of photosynthesis correlated with ASC levels, it is put forward that the regulatory effect of light on ASC in tomato fruits is mediated through photosynthesis. No correlation between ASC levels and respiration rates was found.

The precursor for ASC biosynthesis via the D-man/L-gal biosynthetic pathway is glucose. The hypothesis that the availability of soluble carbohydrates regulates ASC levels in tomato fruits was tested in a series of experiments presented in **Chapter 4**. The correlation between ASC and carbohydrates was tested in different settings: fruits with different ASC levels due to light intensity treatments, fruits with different carbohydrate levels due to fruit pruning treatments and fruits with different carbohydrate levels due to artificial feeding with carbohydrates. In all of the cases no correlation between the ASC levels and soluble carbohydrates was found. This suggests that soluble carbohydrates are not a limitation for ASC accumulation. Galacturonate, the precursor for the secondary/salvage biosynthetic pathway did not increase when ASC was upregulated by light. However, myo-inositol –a precursor of a pathway that was previously thought not to contribute in the ASC pool in non-genetically modified plants- increased when ASC was upregulated by light treatments.

Apart from ASC, a variety of other fruit metabolites respond to the light environment. In two experiments presented in **Chapter 5**, light versus darkness and spectrum effects on the broad metabolic profile of tomato fruits were tested. Exposure of mature green fruits to light lead to acceleration of ripening as reflected in a more pronounced pigment and flavonoid accumulation and alkaloids reduction. Furthermore, presence of light positively influenced the levels of carotenoids, tocopherols and phenolic compounds. As these metabolites have been proposed to be beneficial for human health, the light treatment compared to darkness produces potentially fruits with improved nutritional value. The light treatment also affected flavour-related compounds such as malic acid, glutamate and GABA. Hence, light may improve the taste of fruits. These results should further be confirmed with sensory studies. Above mentioned results indicate that light treatments can be used to improve and/or fine tune certain quality traits of the tomato fruits.

Chapter 6 summarizes and discusses the findings of Chapters 2 to 5 with the aim to present an overview of the physiological mechanism that mediates the regulation of ASC by light in tomato fruits. Practical concerns for the application of light aiming to improve tomato fruit quality are discussed. Furthermore, suggestions on approaches plant breeding may implement in improving ASC in plants are presented.

Samenvatting

Vitamine C, ofwel L-ascorbaat (ASC) is een stof die door planten wordt aangemaakt en essentieel is voor de menselijke gezondheid. Licht reguleert het niveau van L-ascorbaat in planten, en het grootste deel van het bewijs hiervoor is afkomstig van onderzoek aan bladeren. In de huidige studie ligt de nadruk op de effecten van licht op ASC niveaus in tomatenvruchten. Het doel van dit werk, zoals uitgelegd in **Hoofdstuk 1**, was om te onderzoeken welke fysiologische processen hierbij betrokken zijn en hoe deze de effecten van licht op ASC niveaus in tomaten tot stand brengen. Verder zijn de effecten van licht op het bredere metaboloom van de tomaten onderzocht.

In **Hoofdstuk 2** wordt een literatuuroverzicht gepresenteerd. Het doel was het fysiologische en biochemische netwerk van de lichtregulatie van ASC te begrijpen. Ademhaling, fotosynthese en oplosbare koolhydraten worden voorgesteld als regulatoren van de ASC niveaus in planten. Mogelijke interacties tussen deze fysiologische componenten en hun belang bij de lichtregulatie van ASC in bladeren en vruchten worden besproken. Verder wordt een brede biochemische kaart voor ASC biosynthese, recycling en afbraak gepresenteerd en wordt de bijdrage van specifieke biosynthese routes aan de ASC pool besproken. Er wordt geconcludeerd dat het grootste deel van de ASC pool in bladeren en in vruchten door de D-man/L-gal biosynthese route wordt bepaald.

In **Hoofdstuk 3** werden de effecten onderzocht van lichtintensiteit en spectrum op ASC niveaus in geoogste tomatenvruchten. ASC niveaus van vruchten namen in het licht toe in vergelijking met vruchten in het donker, zolang de vruchten groen waren. Rode vruchten reageerden niet op de lichtbehandelingen. De toename van ASC onder licht werd niet sterk beïnvloed door het lichtspectrum. Verschillende tomatengenotypen reageerden op dezelfde manier op de lichtbehandelingen. Op basis daarvan wordt voorgesteld dat het effect van licht op ASC universeel is voor tomaten. Omdat alleen groene vruchten op de lichtbehandelingen reageren en de snelheid van fotosynthese met de ASC niveaus correleert, wordt het regulerende effect van licht op ASC in tomaten waarschijnlijk gemedieerd door fotosynthese. Er was geen correlatie tussen ASC niveaus en ademhalingsactiviteit van de vruchten.

De precursor van ASC via de D-man / L-gal biosynthese route is glucose. De hypothese dat de beschikbaarheid van oplosbare koolhydraten de ASC niveaus in tomaten regelt, werd onderzocht en beschreven in **Hoofdstuk 4**. In een reeks experimenten werd de correlatie tussen ASC en koolhydraten getest: vruchten met verschillende ASC niveaus als gevolg van behandelingen met verschillende lichtintensiteiten, vruchten met verschillende koolhydraatniveaus als gevolg van vruchtdunningsbehandelingen en vruchten met verschillende koolhydraatniveaus als gevolg van kunstmatige voeding met koolhydraten. In alle gevallen was er geen correlatie tussen de ASC niveaus en oplosbare koolhydraten. Dit suggereert dat oplosbare koolhydraten geen beperking

vormen voor ASC accumulatie. Galacturonaat, de precursor van de secundaire biosynthese route, nam niet toe wanneer ASC door licht werd gereguleerd. Echter, myo-inositol nam toe, gelijktijdig met ASC als gevolg van de lichtbehandelingen. Myo-inositol is de precursor van een biosynthese route waarvan eerder werd gedacht dat deze niet bijdraagt aan de ASC pool in niet-genetisch gemodificeerde planten.

Naast ASC reageren ook verschillende andere vruchtmetabolieten op licht. In **Hoofdstuk 5** werden in twee experimenten licht versus donker en spectrumeffecten op het brede metabole profiel van tomaten getest. Blootstelling van volwassen groene vruchten aan licht leidde tot versnelling van de rijping, hetgeen tot uiting kwam in een meer uitgesproken accumulatie van pigmenten en flavonoïden en vermindering van alkaloïden. Bovendien verhoogde de aanwezigheid van licht de niveaus van carotenoïden, tocoferolen en fenolverbindingen. Gezien de vermeende gunstige effecten van deze metabolieten op de menselijke gezondheid, levert de lichtbehandeling in vergelijking met donker mogelijk vruchten op met een verbeterde voedingswaarde. De lichtbehandelingen hadden ook invloed op smaakgerelateerde verbindingen zoals appelzuur, glutamaat en GABA. Daarom zou licht de smaak van vruchten kunnen verbeteren. Deze resultaten moeten verder worden bevestigd met sensorische onderzoeken. Bovenstaande resultaten geven aan dat lichtbehandelingen kunnen worden gebruikt om bepaalde kwaliteitskenmerken van de tomatenvruchten te verbeteren.

Hoofdstuk 6 vat de bevindingen van de Hoofdstukken 2 tot 5 samen en bespreekt deze met als doel een overzicht te geven van het fysiologische mechanisme dat de regulering van ASC door licht in tomaten tot stand brengt. Mogelijkheden voor praktische toepassing van licht om de kwaliteit van tomaten te verbeteren, worden besproken. Verder worden suggesties gegeven voor een veredelingsaanpak om ASC in planten te verbeteren.

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Orion (Ntagkas-Fan), my awesome little hunter. Your consciousness has been around only during the last part of this work. You haven't seen me being all obsessed, frustrated, determined, anxious, troubled, single-minded … (the list is endless) to the extent I was during my PhD. Honestly, I am really happy for this. Nevertheless, by the skills I have acquired during the PhD, I feel now a tiny bit more equipped to guide you in life. One very important thing. I will never expect you to follow the same path as me. I only hope that you find what makes you happy in life, and become really good at it. Both your mother and I will always be there for you. Thank you for being the greatest inspiration to finish this.

Finally, to my magnificent wife **Shuang Fan**. You were such a big part of this. You kept me on my toes reminding me that I can't win always but, I can lose every day. No matter the hardships you didn't allow me to lose my fundamental interest in science, this magical alliance of reason and result. When under the domes of anguish I dwelled and when I was breeding thorns, you were there to eliminate any space for self-pity and misery. You taught me self-respect. You always reminded me that no matter what the impact of our work is, we rise from ashes and stone and that we should be grateful for the opportunities we were given. You never cease to amaze me. There is so much strength and courage in you. We did it αγάπη μου, once more. And this is not even the greatest thing we'll ever do.

About the Author

Nikos Ntagkas was born in Corfu, Greece, in March 1987. At the age of 6 he moved with his family to Paramythia. Being raised very close to the natural environment he was a keen observer of the plant and animal species found typically in the Mediterranean ecosystem. In 2004 Nikos was admitted to the School of Agriculture of the Aristotle University of Thessaloniki for his BSc studies in the specialisation of horticulture and viticulture. He carried out his BSc thesis in the lab of apiculture studying Nosema apis, a microsporidian that causes dysentery in the common bee. During his internship he studied how different pruning strategies affect the harvest time of roses. In 2010 he moved to Wageningen, the Netherlands for an MSc in Plant Sciences, with specialisation in Greenhouse Horticulture in Wageningen University. He did his MSc thesis in Horticulture Supply Chains (currently Horticulture and product Physiology) where he investigated the effects of irradiance on leaf initiation rate of cucumber and tomato under the supervision of Andreas Savvides and Wim van Ieperen. For his internship, Nikos worked in Forschungszentrum Jülich, Germany under the guidance of Hendrik Poorter. Through means of meta-analysis (metaphenomics) he summarized the effects of multiple environmental factors on growth and developmental traits over large groups of plant species. These two experiences made the image clear for Nikos that he would be pursuing a career in research. Consequently he carried out his PhD which is presented in this book, from July 2013 to June 2017. Nikos currently resides in Maasdijk, the Netherlands. He is married to Shuang Fan and together they raise their son Orion Ntagkas-Fan. He works in the team biochemistry of the R&D department of Rijk Zwaan. In his free time Nikos composes and records contemporary death metal music.

List of Publications

Scientific Publications

Ntagkas N, Min Q, Woltering EJ, Labrie C, Nicole CCS, Marcelis LFM. 2016. Illuminating tomato fruit enhances fruit Vitamin C content. Acta Horticulturae 1134, 351–356.

Ntagkas N, Woltering EJ, Marcelis LFM. 2018. Light regulates ascorbate in plants: An integrated view on physiology and biochemistry. Environmental and Experimental Botany 147, 271–280.

Ntagkas N, Woltering E, Nicole C, Labrie C, Marcelis LFM. 2018. Light regulation of vitamin C in tomato fruit is mediated through photosynthesis. Environmental and Experimental Botany 158, 180–188.

Ntagkas N, Woltering E, Bouras S, de Vos RCH, Dieleman JA, Nicole C, Labrie C, Marcelis LFM. 2019. Light-induced vitamin C accumulation in tomato fruits is independent of carbohydrate availability. Plants 8, 86.

Patent

Nicole CCS, Marcelis L, Woltering E, Ntagkas N. 2018. System and method for post-harvest treatment of vegetables and fruits.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)
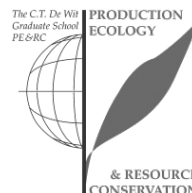
Review of literature (6 ECTS)
- Light regulates vitamin C: an integrated view on physiology and biochemistry

Writing of project proposal (1.5 ECTS)
- Healthy fruits by localised LED light on fruits

Post-graduate courses (3 ECTS)
- Applied methods in crop physiology; Aarhus University (2016)

Competence strengthening / skills courses (2.2 ECTS)
- Peer consultation (2014)
- Competence assessment (2014)
- Entrepreneurship in and outside science (2016)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.4 ECTS)
- PE&RC Day (2013, 2014, 2015)
- PE&RC Weekend (2013, 2015)

Discussion groups / local seminars / other scientific meetings (15 ECTS)
- FLOP (2013-2017)
- SEMINAR (2013-2017)
- STAIR (2013-2017)

International symposia, workshops and conferences (7.9 ECTS)
- V International conference postharvest unlimited; oral presentation (2014)
- BeNeLuxSHS symposium: plant control by LED light; oral presentation (2015)
- VII International symposium on light in horticulture LightSym; oral presentation (2016)

Lecturing / supervision of practicals / tutorials (4.5 ECTS)
- Concepts in environmental plant physiology (2013, 2015)
- Postharvest physiology (2015)
- Advanced methods for plant-climate research in controlled (2016)
- Physiology and development of plants in horticulture (2016)

Supervision of MSc students
- Healthier tomato fruits: the effects of light on vitamin C
- Soluble carbohydrates are not a limitation in L-ascorbate upregulation by LED in tomato
- Effect of micronutrients on cucumber postharvest quality