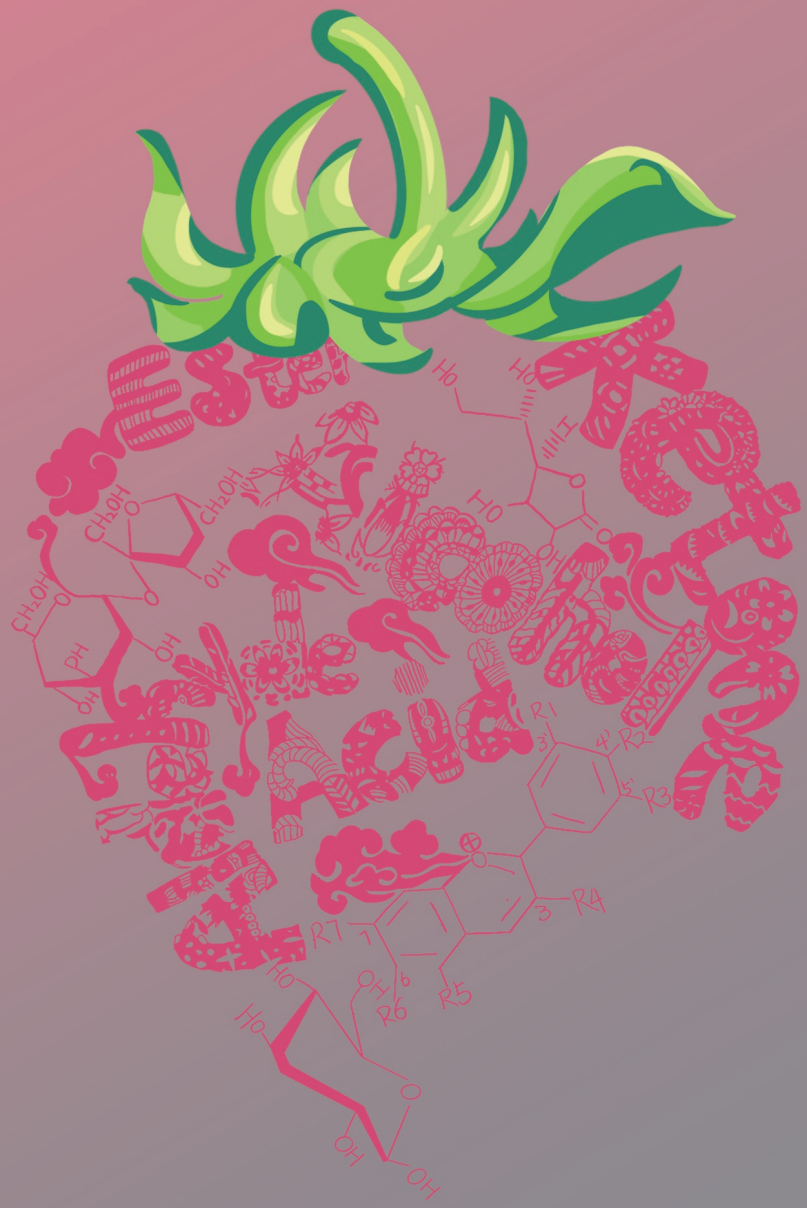
The association between metabolic status and susceptibility to ***Botrytis cinerea*** in strawberry fruit



HUA LI

Propositions

1. Additional far-red light during cultivation improves the overall quality of strawberry fruit.
   (this thesis)

2. Applying high concentrations of CO_2 to strawberry fruit during postharvest delays spoilage by *Botrytis cinerea*.
   (this thesis)

3. Emphasis on novelty by the majority of journals disrupts the scientific enterprise.

4. The brain's function to forget shapes us as individuals.

5. The growing popularity of vegetarianism advances human civilization.

6. Trees outperform humans when it comes to sharing 'nutrients' with their neighbors that are facing adversity.

Propositions belonging to the thesis, entitled

The association between metabolic status and susceptibility to *Botrytis cinerea* in strawberry fruit

Hua Li
Wageningen, 30 March 2022

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Hua Li

Thesis

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Chapter 1

General Introduction

1.1 Breeding priorities of strawberry and its primary cause of spoilage

The cultivated strawberry (*Fragaria* × *ananassa*) is an allo-octoploid species (2n = 8x = 56) that is consumed worldwide as fresh or processed fruit (Shirasawa et al., 2021). *F.* × *ananassa* is a hybrid generated by interspecific crosses between two distantly related species: *Fragaria virginiana* (a forest-floor strawberry from North America) and *Fragaria chiloensis* (a wild strawberry from South America) (Pillet and Folta, 2015; Folta and Barbey, 2019). Botanically, a strawberry is a false fruit composed of tiny individual true fruits (achenes, average 200 on each strawberry) embedded in a fleshy receptacle (Azam et al., 2019). Hereafter, the term 'strawberry fruit' refers to the receptacle with achenes.

Strawberry fruit are appreciated for their unique organoleptic properties (e.g., shiny appearance, delicacy), nutritional value (e.g., vitamins, minerals) and antioxidants (Seeram, 2008; Giampieri et al., 2014). As the most popular summer berry in Europe, the greenhouse cultivation and the use of diverse cultivars have extended the production season of strawberry fruit, resulting in a total annual production of 1.3 million tonnes (CBI, 2021).

1.1.1 Breeding priorities

In general, strawberry breeders focus on two aspects. The first aspect relates to fruit characteristics such as color, shininess, size, aroma, taste and shelf life; the secondary aspect relates to agronomic traits such as yield, seasonality and disease resistance. The supplier-priority selection focuses on disease resistance, shelf life, yield, appearance and uniformity. The consumer-assisted selection focuses on taste, aroma, nutrition and minimal pesticide usage (Klee and Tieman, 2018; Mezzetti et al., 2018).

Strawberry fruit are affected by several pathogens - including fungi, bacteria, viruses and nematodes. Pesticide applications are the most common practice to control pathogen infection and reduce economic loss in modern strawberry production (Petrasch et al., 2019a). Due to the extensive use of pesticides on strawberry plants, strawberry leads the list of the 2021 Environmental Working Group's 'dirty dozen' (EWG's 2021 Shopper's Guide to Pesticides in Produce | Dirty Dozen) as the most contaminated fruit. Biological control is gradually being developed as a sustainable alternative for protecting strawberry from spoilage caused by pathogens (Petrasch et al., 2019a).

1.1.2 Fruit spoilage caused by fungi

Strawberry fruit, particularly red ripe fruit that are detached from plants, are prone to spoilage mainly caused by a range of fungi, including *Botrytis cinerea*, *Rhizopus stolonifer*, *Mucor* spp., *Podosphaera aphanis*, *Colletotrichum* spp. and *Penicillium* spp. (Feliziani and Romanazzi, 2016). *B. cinerea* is regarded as the primary postharvest pathogen contaminating strawberry fruit. Botrytis fruit rot also occurs during cultivation on strawberry stems, leaves, flowers, unripe and ripe fruit (Petrasch et al., 2019a). The visible symptom mostly begins with a brownish circular area where the infected tissue becomes softer than the surrounding 'healthy' tissue. Afterwards, the infection site is covered by fluffy grey mycelia, on which the sporulation occurs (Feliziani and Romanazzi, 2016). The rotten areas are soft and mushy, becoming leathery and dry in the absence of high humidity.

Some other destructive fungi, *R. stolonifer* and *Mucor* spp., causing Rhizopus and Mucor fruit rots, closely resemble each other in the field but visibly do not look like Botrytis fruit rot. The sporangia of *Rhizopus* appear dry, whereas the sporangia of *Mucor* appear wet or sticky due to a viscous liquid film. The infection manifests as water-soaked soft rot in fruit that are covered with thin, cotton-like mycelia with black sporangia (Feliziani and Romanazzi, 2016). *P. aphanis* is the cause of powdery mildew in strawberry plants and fruit. Infected flowers do not produce fruit or produce deformed fruit. Infected immature fruit are firm and become dehydrated. Infected mature fruit look seedy with colonies that look powdery and white (Koike et al., 2018). Powdery mildew is particularly severe in protected cultivation systems.

1.2 Pathogenicity factors released by *B. cinerea*

Pathogens, according to lifestyles, are classified as biotrophic (obtaining nutrition from living plant cells), necrotrophic (obtaining nutrition from dead or dying cells) or hemibiotrophic (an early biotrophic phase followed by a necrotrophic phase) (Van Kan et al., 2014). *B. cinerea* is generally considered as a necrotrophic fungus, however, a recent review of Veloso and Van Kan (2018) proposed that its lifestyle should be reconsidered as hemibiotrophic as it has a short biotrophic phase before triggering the cell death of hosts. *B. cinerea* is one of the most well-studied necrotrophic fungi (Veloso and Van Kan, 2018), causing grey mold disease in many plant species, including strawberry. *B. cinerea* promotes necrotrophic infection by releasing pathogenicity factors in different phases of fungal infection (Van Kan, 2006; Kars and Van Kan, 2007; Tian et al., 2016; Zhang et al., 2021).

A global gene expression profile of *B. cinerea* during infection of unripe and ripe tomato fruit revealed that the fungus alters the repertoire of pathogenicity factors that it releases in the host tissue during the course of fruit ripening (Petrasch et al., 2019b). In this study, the common strategies of infecting both unripe and ripe fruit included 1) reactive oxygen species (ROS) production via glucose oxidases and laccases; 2) enzymatic cell wall degradation by polygalacturonases or cellulases; 3) accumulation of phytotoxic metabolites (the sesquiterpene botrydial and the polyketide botcinic acid). When attacking unripe tomato fruit, *B. cinerea* is capable of detoxifying ROS by superoxide dismutases (SOD) and catalases; the fungus is also able to alter the pH by oxalic acid production (Petrasch et al., 2019b). Interestingly, Müller et al. (2018) reported that the host tissue acidification plays a role in forming lesions on apple fruit, noting that the role of citric acid was more prominent than that of oxalic acid. While attacking ripe tomato fruit, the fungus releases multiple Carbohydrate-Active Enzymes to further decompose the host cell wall, which releases sugars supporting the fungal growth (Petrasch et al., 2019b).

The flexibility of *B. cinerea* in exploiting different types of pathogenicity factors, as well as the functional redundancy between pathogenicity factors themselves can explain why so many host plants are at least to some extent susceptible to fungal infection. A study by Leisen et al. (2021) demonstrated that a deletion mutant of *B. cinerea* that lacks 10 phytotoxic protein-encoding genes and genes required for production of two phytotoxic metabolites still remained pathogenic in several plant species, including apple fruit.

1.3 The infection modes in strawberry tissues caused by *B. cinerea*

Infections of strawberry fruit by *B. cinerea* are characterized as either primary infection (via floral organs) or secondary infection (via receptacle tissues) (Bristow et al., 1986).

Upon infection of strawberry floral organs, Powelson (1960) observed hyphae growing from infected stamens into the calyx and then to the receptacle and he suggested that senescent flower parts (i.e., infected senescent petals, stamens and sepals) are important sources of inoculum for fruit infection. The conidia especially germinate on stigmas, facilitated by the stigmatic fluid that provides sufficient moisture and nutrition (Bristow et al., 1986). After entering the stigma, hyphae grow slowly within the transmitting tissues of styles, following the pathway to the ovules used by pollen tubes. The hyphae rarely reach the achenes or receptacles and appear to remain largely

quiescent for up to 4-6 weeks (Bristow et al., 1986; Williamson et al., 2007). During quiescence, the fungus remains inactive before invading fruit tissues rapidly at the ripe stage. The occurrence of quiescence in strawberry fruit is presumably due to 1) shortage of accessible nutrient sources (e.g., carbohydrates) for *B. cinerea* growth and reproduction; 2) considerable levels of preformed or induced antifungal compounds (e.g., polyphenols and pathogenesis-related proteins) (Prusky and Lichter, 2007; Prusky et al., 2013).

The most important inoculum source for secondary infection on strawberry receptacles derives from diseased flowers and fruit (Droby and Lichter, 2007). The fungus activates the necrotrophic phase during secondary infection without quiescence (Holz et al., 2007). Generally, when fruit are incubated at room temperature (22 to 25 °C), grey mold lesions become visible between 48 to 72 hours post inoculation, depending on genotype (Xiong et al., 2018; Haile et al., 2019; Lee et al., 2021).

1.4 Defense responses of strawberry associated with ripening

During fruit development and ripening, strawberries undergo physical changes in size, color, firmness and chemical changes in primary and secondary metabolism, as well as changes in the extent and types of defense responses against pathogenic infections. Petrasch et al. (2019a) summarized the sequential occurrences of ripening events in strawberry fruit that are essential for *B. cinerea* to break through the quiescence phase of infection. In turn, B*. cinerea* releases pathogenicity factors that may promote fruit ripening.

To cope with *B. cinerea* infection, strawberry has evolved different layers of defense systems, including preformed defenses and induced defenses. Strawberry preformed defenses play a critical role when fruit are confronted by *B. cinerea* infection (Amil-Ruiz et al., 2011), leading to the quiescence phase following the primary infection. Both physical and chemical factors contribute to protecting strawberries from *B. cinerea*. Strawberry fruit of different genotypes perform differently upon either natural spoilage (Chandler et al., 2006; Seijo et al., 2008; Lewers et al., 2012, 2013) or artificial infection (Bestfleisch et al., 2015b; Petrasch et al., 2021) by *B. cinerea*, which is attributed to variations in defense mechanisms.

1.4.1 Firmness

Fruit firmness is a complex trait that is determined by several factors, including cell shape, turgor of parenchyma cells, the extension and strength of adhesion areas between adjacent cells, and the presence of non-parenchyma cells (Harker et al., 1997). During ripening, the loss of turgor pressure along with the disassembly of the parenchyma cell walls and middle lamella reduce cell adhesion and lead to fruit softening (Harker et al., 1997; Toivonen and Brummell, 2008). The host cell wall acts as a structural barrier against fungal infection. The disassembly of the cell wall provides nutrients (e.g., sugars) for the fungus to grow and reproduce (Blanco-Ulate et al., 2016a).

Amil-Ruiz et al. (2011) listed several studies that described the associations between mRNA expression levels or enzyme activities of cell wall degrading enzymes and fruit firmness in different strawberry cultivars. Few studies have elucidated the role of strawberry cell wall degrading enzymes as a defense response to pathogens. For instance, overexpression of *F. x ananassa* pectin methylesterase 1 (*FaPE1*) in *Fragaria vesca* reduced the methyl esterification of soluble and chelated pectins (Osorio et al., 2008). Consequently, a pathogenesis-related gene involved in the salicylic acid pathway was continuously expressed, which reduced the susceptibility of transgenic strawberry fruit to *B. cinerea* (Osorio et al., 2008). A recent transcriptome analysis of *B. cinerea* inoculated *F. vesca* strawberry fruit revealed that genes encoding endogenous cell wall modifying or degrading proteins were differentially expressed with a larger number of genes in unripe than ripe fruit (Haile et al., 2019). In summary, cell wall related enzymes of strawberry fruit play decisive roles in facilitating *B. cinerea* infection.

1.4.2 Flavor-related metabolites

Soluble sugars and non-volatile organic acids

When strawberry fruit ripen on the plant, the levels of soluble sugars such as glucose, fructose and sucrose rise (Jia et al., 2011, 2013; Jiu et al., 2018). Sucrose functions as a signal, accelerating pigmentation in strawberry fruit (Jia et al., 2013; Luo et al., 2019, 2020). There are several conflicting reports about the changes in major organic acids (i.e., citric- and malic acid) of strawberry fruit during ripening. Montero et al.(1996) found that fruit citric acid content gradually increased with ripening, while malic acid showed a transient peak before reaching the fully ripe stage. Mahmood et al. (2012) reported that contents of these acids continuously increased throughout ripening, whereas Van de Poel et al. (2014) did not notice dramatic changes in the contents of

the two acids as fruit ripened. Finally, Min et al. (2020) reported that citric acid levels decreased whereas malic acid levels increased in fruit between the green and the fully red stages. These contradictory observations may be related to the different cultivars and cultivation conditions. It is impossible to make a general statement about the development of organic acid levels during ripening of strawberry fruit due to the fact that the studies have been performed by different researchers using different cultivars and protocols.

Information about fruit susceptibility to *B. cinerea* and the association with soluble sugars or non-volatile organic acids is limited. Such an association was suggested in the observations of Mundy and Beresford (2007) that the spoilage of grape berries by *B. cinerea* increased significantly with increasing sugar concentrations. However, transgenic tomato fruit containing low levels of malic acid in the green stage showed increased soluble sugar contents in the red ripe stage (at harvest) and decreased water loss and wrinkling during postharvest, which did not affect the susceptibility to *B. cinerea* (Centeno et al., 2011). Studies on tomato *Cnr* mutant fruit that do not accumulate high content of sugars showed that the mutant is hyper-susceptible to *B. cinerea* when compared with the wild type, even at the unripe stage (Blanco-Ulate et al., 2016b). The above studies do not suggest a clear role of either sugars or non-volatile acids in strawberry susceptibility to *B. cinerea*.

Volatile organic compounds (VOCs)

More than 900 VOCs have been identified in strawberry fruit so far, primarily esters, acids, lactones, aldehydes, furans, alcohols, ketones, and terpenoids (Ulrich et al., 2018). Many of these volatile metabolites are important for taste and aroma perception. Changes in volatile compounds are used as indicators of strawberry ripening (Forney et al., 2000; Ménager et al., 2004). During strawberry fruit ripening, the levels of volatile esters, terpenes, furanones, and acids generally increase whereas aldehyde levels decrease (Du et al., 2011; Van de Poel et al., 2014).

The biological implications of volatile compounds present in ripe strawberry fruit on interactions with *B. cinerea* have been investigated. For instance, *B. cinerea* infection induced the production of linalool, which showed direct antifungal activity against *B. cinerea* by inducing oxidative stress and damaging fungal cell membrane integrity (Xu et al., 2019). γ-Decalactone upregulated the expression of strawberry genes contributing to defense responses against fungi (Sánchez-Sevilla et al., 2014). Besides having an impact on strawberry fruit defense responses against *B. cinerea*, volatile compounds may also benefit the fungus. Neri et al. (2015) investigated the effects of a

variety of volatile compounds on *B. cinerea* growth *in vitro* and found that volatile esters (e.g., ethyl butanoate, butanoate, methyl butanoate, hexyl butanoate) and furanones (e.g., furaneol and mesifurane) stimulated *B. cinerea* growth.

Green leaf volatiles (GLVs) play a role in triggering plant defense, especially in 'priming' vegetative plant tissues against fungal attack (Scala et al., 2013). GLVs are mainly fatty acid-derived C6 compounds, including aldehydes, alcohols and esters, which are synthesized via the hydroperoxide lyase branch of the oxylipin pathway (Matsui, 2006). Myung et al. (2007) proposed that C6 aldehydes may chemically modify secreted proteins of *B. cinerea* during the interaction on the surface of fungal tissues, which could interfere with the fungal pathogenicity. (E)-2-Hexenal has been suggested to be useful as an antifungal agent (Archbold et al., 1997; Arroyo et al., 2007; Wakai et al., 2019), however, the compound may also stimulate *B cinerea* infection in strawberry fruit by triggering sulfate assimilation of the fungus (Xu et al., 2021). From the above, it can be derived that ripening related volatiles may play a role in susceptibility.

1.4.3 Antioxidants

Ascorbic acid

Ascorbic acid, also named ascorbate or vitamin C, is a well-known but surprisingly poorly understood primary metabolite. Ascorbic acid has antioxidant properties, which result from its non-enzymatic reduction of superoxide ($O_2^{\bullet-}$), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and other radicals (Njus et al., 2020). These radicals take a single H atom from ascorbic acid, oxidizing it to monodehydroascorbate (Buettner and Jurkiewicz, 1996; Carr and Frei, 1999). Monodehydroascorbate does not react with oxygen or other molecules to generate more reactive radicals due to the resonance stabilization; therefore, it is an effective radical scavenger (Smirnoff, 2018).

The changes in ascorbic acid levels during fruit ripening are species dependent. In kiwifruit, the ascorbic acid content increases to a peak level at the immature green stage and decreases until fully ripe. In peach fruit, the content gradually decreases during ripening. By contrast, in apple (Lemmens et al., 2020), tomato (Dumas et al., 2003; Gautier et al., 2008; Ioannidi et al., 2009; Badejo et al., 2012), grape (Cruz-Rus et al., 2010) and strawberry fruits (Cruz-Rus et al., 2011), the ascorbic acid level increases as fruit ripen. The high level of ascorbic acid in ripe fruit could play a role in response to the fungal attack.

The involvement of ascorbic acid in fruit-*B. cinerea* interactions is poorly studied. A higher content of ascorbic acid in apple fruit was accompanied by less severe *B. cinerea*

infection (Davey et al., 2007). Furthermore, increases in enzymatic activities of SOD and ascorbate peroxidase (APX), as well as a decrease in the ascorbic acid level were observed in apple fruit infected by *B. cinerea* (Bui et al., 2019). The increase in SOD activity, which resulted from the fruit and/or the fungus, was to detoxify the excessive ROS that accumulated during the fruit-*B. cinerea* interaction (Rolke et al., 2004; López-Cruz et al., 2017). With the progression of *B. cinerea* infection, the APX activity increased, which caused a more rapid consumption of ascorbic acid (Bui et al., 2019). It is very likely that ascorbic acid in ripe strawberry fruit serves as an important antioxidant to delay fruit spoilage by *B. cinerea*.

Flavonoids

Flavonoids are an important group of antioxidants in strawberry fruit with a basic structure of C6–C3–C6 (Giampieri et al., 2014). Flavonoids are classified into different subgroups based on the carbon of the C ring to which the B ring is attached and the degree to which the C ring is unsaturated and oxidized (Panche et al., 2016). In red ripe strawberry fruit, the main subgroups of flavonoids include flavonols, proanthocyanidins, anthocyanins (Almeida et al., 2007; Aaby et al., 2012). Flavonoids exist in the form of aglycones, glycosides, and methylated derivatives. In nature, the majority of flavonoids occur in the form of glycoside derivatives. The modifications of flavonoids often alter their solubility, reactivity and stability (Mierziak et al., 2014).

The antioxidant activity of flavonoids is largely determined by the configuration, substitution, and the total number of hydroxyl groups (Cao et al., 1997; Haenen et al., 1997; Burda and Oleszek, 2001; Sekher Pannala et al., 2001). The most significant determinant of scavenging ROS is the configuration of the B ring hydroxyl as it donates hydrogen and an electron to hydroxyl, peroxyl, and peroxynitrite radicals, producing a relatively stable flavonoid radical (Burda and Oleszek, 2001; Sekher Pannala et al., 2001). In addition, polymerization of flavonoid monomers could enhance their antioxidant activity (Heim et al., 2002). For instance, proanthocyanidins are effective antioxidants due to the high number of hydroxyl groups in their molecules. The antioxidant activity of proanthocyanidins is determined by the length of their oligomer chain and the type of ROS with which they react (Lotito et al., 2000). Glycosylation of flavonoids reduces their antioxidant activity as compared to the corresponding aglycones (Kumar and Pandey, 2013). For instance, the more sugar units occur at C3 and C5 positions of anthocyanins, the lower the antioxidant activity (Sadilova et al., 2006).

Proanthocyanidins and anthocyanins are end products of the phenylpropanoid pathway and they require the same metabolic intermediates, the colorless

leucoanthocyanidins. Proanthocyanidins are known as condensed tannins, conferring astringency to fruit (Dixon et al., 2005; Dixon and Sarnala, 2020). In strawberry fruit, the level of proanthocyanidins is high in the small green stage and gradually decreases until fruit are fully ripe (Almeida et al., 2007; Schaart et al., 2013). By contrast, anthocyanins start to accumulate at the turning stage when fruit approach full expansion (Almeida et al., 2007; Carbone et al., 2009). Anthocyanins are the primary red pigments in commercial strawberry cultivars (Da Silva et al., 2007; Castillejo et al., 2020). Strawberry genotypes vary in the external red coloration, ranging from orange (e.g., cv. Elsanta) to dark red (e.g., cv. Purple Wonder). The different types of anthocyanins in red strawberry fruit are mainly derived from pelargonidin and cyanidin aglycones, namely pelargonidin-3-glucoside, pelargonidin-3-malonylglucoside, pelargonidin-3-rutinoside, cyanidin-3-glucoside (Almeida et al., 2007; Aaby et al., 2012; Giampieri et al., 2014).

The role of proanthocyanidins from strawberry fruit in remaining the quiescent phase of *B. cinerea* infection was proposed by Jersch et al. (1989). They found that in inoculated unripe strawberry fruit, the total content of proanthocyanidins was negatively correlated with the growth of *B. cinerea* mycelia and the inhibitory effect of proanthocyanidins extracted from unripe fruit was greater than that of red ripe fruit. It was later found by Hébert et al. (2002) that cultivars with higher levels of proanthocyanidins were less susceptible to *B. cinerea* infection. Furthermore, Terry et al. (2004) reported that extracts of strawberry flowers at the post-anthesis showed greater antifungal activity than the white bud and full bloom stages. Given the previous finding that the susceptibility of strawberry flowers to *B. cinerea* increases with age until petal-fall (Hennebert and Gilles, 1958), Terry et al. (2004) suggested that antifungal compounds in strawberry flowers may contribute to initiating the quiescence phase of *B. cinerea* infection. Compared to flower tissues or fruit tissues in other stages, fruit in the green stage (approximately 7 days after anthesis) exhibited the greatest antifungal activity, possibly related to levels of catechin, epicatechin and gallate (Terry et al., 2004). More recently, accumulation of proanthocyanidins, ellagitanins, and catechins was shown to be induced in unripe strawberry fruit inoculated with *B. cinerea* (Puhl and Treutter, 2008; Nagpala et al., 2016). Besides their protective effect against *B. cinerea*, proanthocyanidins impair the taste of strawberry fruit due to the astringency (Dixon et al., 2005; Dixon and Sarnala, 2020), which may result in an unpleasant perception of fresh strawberries to consumers.

Cyanidin-3-glucoside and pelargonidin-3-glucoside displayed inhibitory effects on *B. cinerea* growth *in vitro* (Tao et al., 2010). Transgenic tomato fruit that contained higher

anthocyanin levels in the fruit skin (resulting in purple skin), showed lower susceptibility to *B. cinerea* and longer shelf life which might be due to the antioxidant properties (Bassolino et al., 2013; Zhang et al., 2013). Red ripe strawberry fruit are abundant in anthocyanins and due to their strong antioxidant activity, it is logical to hypothesize that anthocyanins participate in defense against fungal infection. This beneficial effect of anthocyanins has, however, not yet been systematically investigated in strawberry fruit.

1.5 Reducing susceptibility to *B. cinerea* by altering metabolic profiles of fruit via pre- & postharvest regimes

Entirely resistant strawberry cultivars do not exist (Bristow et al., 1986; Schestibratov and Dolgov, 2005; Bestfleisch et al., 2015). Manipulation of pre- and/or postharvest conditions are the current alternatives implemented to improve or retain strawberry fruit physical and chemical defense responses to *B. cinerea.* These pre- and postharvest factors may include light, temperature, air humidity, atmosphere composition). Here I focus on two factors, light spectrum during preharvest and atmospheric composition during postharvest.

1.5.1 Light spectrum

Effects of light on plant growth result from light duration, direction, intensity and spectrum. Light-emitting diodes (LEDs), highly efficient light sources that can provide monochromatic and broadband light, are increasingly used to adjust the light conditions in greenhouses. LED lighting allows for controlling primary and secondary metabolism to improve nutraceutical, organoleptic, and dietary value of horticultural products by manipulating light characteristics (SharathKumar et al., 2020). Below I will focus on effects of light spectrum on defense and metabolism of plants and fruit.

Plants perceive visible light spectra (400-700 nm), UV (below 400 nm) and far-red (700-800 nm) light using a cassette of photoreceptor proteins such as phytochromes, cryptochromes, phototropins and UVR8 (Thoma et al., 2020). Light spectrum affects photosynthesis, morphology and metabolism (Demotes-Mainard et al., 2016; Huché-Thélier et al., 2016), thereby regulating plant growth and defense (Lazzarin et al., 2021).

Plant defense responses against necrotrophic pathogens are influenced by light spectrum via endogenous phytohormonal signaling, which is primarily orchestrated by

jasmonic acid (JA) (Glazebrook, 2005; Moreno and Ballaré, 2014; AbuQamar et al., 2017). The well-studied low red: far-red ratio increases susceptibility of vegetative tissues to necrotrophic fungi by inactivation of phytochromes, particularly phytochrome B (phyB). Inactivation of phyB reduces JA-dependent defense responses by promoting DELLA degradation and increasing JASMONATE ZIM-DOMAIN (JAZ) stability (Ballaré, 2014; Leone et al., 2014). By contrast, UVB light enhances defense responses through JA-dependent and JA-independent signaling mechanisms. UVB light elevates levels of phenolic compounds mediated by UVR8 and these chemicals serve as direct defense responses against pathogens (Ballaré, 2014).

Many studies have focused on promotion of important secondary metabolites of strawberry fruit. Short wavelengths, such as UV and blue light increase flavonoid production in fruit, often by increasing the expression of genes in the flavonoid biosynthetic pathway (Zoratti et al., 2014). The contents of anthocyanins and proanthocyanidins were higher in strawberry fruit grown under light with a 1:1 ratio of red to blue light compared to fruit grown under white light or monochromatic red or blue light (Zhang et al., 2018a). Light conditions can also affect strawberry volatile production. For instance, the production of methyl butyrate increased in response to far-red light; blue light decreased hexyl butyrate accumulation approximately 5-fold (Colquhoun et al., 2013).

Notably, the application of different light regimes always affects the behavior of both the host and *B. cinerea* (Schumacher, 2017). For instance, Janisiewicz et al. (2016) reported that a four-hour dark period following UV-C illumination (~60 seconds) treatment effectively killed *B. cinerea* conidia and controlled grey mold of strawberries. Additional research about how light affects *B. cinerea* growth and pathogenicity has been reviewed by Schumacher (2017).

1.5.2 Controlled atmosphere

Postharvest shelf life of horticultural products depends on their respiration rate (Kader, 1992; Brash et al., 1995) which in turn, is determined by storage temperatures and the composition of the surrounding atmosphere (Wang et al., 2019). Appropriate controlled or modified atmosphere (lowered O_2 and increased CO_2) storage prolongs shelf life and retains flavor and nutrients in many horticultural products. The physiological responses to alterations in atmosphere vary among crops, growth conditions, maturity at harvest and organ parameters (Rama and Narasimham, 2003). In strawberry fruit, for instance, the postharvest condition of 5 to 10 kPa O_2 and 15 to 20 kPa CO_2 at 0 to 5 °C is recommended to extend its shelf life, with limited loss of taste

and nutrients (Yahia, 2009). The recommended O_2 and CO_2 concentrations slow down fruit respiration and prevent the occurrence of fermentation and senescence (Kanellis et al., 2009; Li et al., 2019). However, the recommended CO_2 concentration may not be sufficiently fungistatic to *B. cinerea* as García-Gimeno et al. (2002) found that the fungus stopped growing only when the concentration of CO_2 was higher than 30 kPa. Attempts have been made to further prolong strawberry shelf life by elevating CO_2 concentrations to 30 or 40 kPa, however, side-effects occurred such as discoloration, and emission of fermentation-associated volatiles (Wszelaki and Mitcham, 2000; Almenar et al., 2006; Nakata and Izumi, 2020). Stepwise increases of CO_2 and decreases of O_2 in few days to reach the final concentrations efficiently extended the shelf life of blueberries without damaging fruit nutritional quality such as sugars, acids and ascorbic acid (Falagán et al., 2020). This stepwise CO_2 increment may also be applicable to improve strawberry fruit tolerance to high CO_2 levels such that the taste and nutritional compounds can be retained and growth of *B. cinerea* can be effectively reduced.

1.6 Consumer liking

'Consumer liking' is used to describe the chemistry of consumer preference (Klee and Tieman, 2018). In strawberry fruit, sweetness and flavor intensity are two sensory attributes highly associated with consumer liking, which is largely determined by the sugar content (Schwieterman et al., 2014; Fan et al., 2021a). Fan et al. (2021a, 2021b) characterized that glucose, fructose, sucrose and certain volatile compounds are important for predicting the consumers' perception of sweetness based on data originated from ~50 genotypes and several years. The characterized volatiles largely overlapped in two studies, including butanoic acid esters, (E)-2-pentenal, nonanal. Hexyl acetate and isopropyl acetate are characterized to negatively affect sweetness (Fan et al., 2021b). γ-Decalactone (peach-like aroma), occurring in all strawberry genotypes, contributed to sweetness and fermented flavor in Fan et al. (2021a, 2021b) respectively. Fan et al. (2021b) further characterized volatiles that could predict sourness (e.g., (Z)-linalool oxide, butyl butyrate), green (e.g., α-terpineol), astringent (e.g., (Z)-linalool oxide, pentanal), woody (e.g., butyl butyrate, methyl acetate) flavors.

It is important to consider the contribution of these metabolites to consumer liking of strawberry fruit as reducing strawberry susceptibility to *B. cinerea* may be at the expense of important quality aspects.

1.7 Thesis outline

Until ~2015, many studies have focused on individual quality traits of strawberry fruit and its susceptibility to *B. cinerea.* Only a limited number of studies have performed a systematic analysis of multiple physical and chemical features of the fruit that contribute to improved nutrition, flavor and shelf life. This knowledge gap has hampered the breeding process, as it is unclear to breeders which traits can contribute to improving fruit resistance to *B. cinerea* and thereby increasing fruit shelf life and marketability. In recent years, insights into strawberry fruit defense mechanisms are increasing especially thanks to the use of 'omics' technologies (Nagpala et al., 2016; Xiong et al., 2018; Haile et al., 2019; Xu et al., 2019, 2021; Badmi, 2020; Lee et al., 2021; Li et al., 2021). However, information about which metabolites in strawberry fruit contribute to their defense is still limited.

The objectives of this thesis were:

- To disentangle genetic, physiological and metabolic factors that affect the susceptibility of red ripe strawberry fruit to *B. cinerea.*
- To evaluate how susceptibility of red ripe strawberry fruit to *B. cinerea* changes as a result of preharvest (i.e., light spectrum) and postharvest (i.e., controlled atmosphere) treatments. Furthermore, to elucidate physiological mechanisms of the observed changes (if any) in fruit susceptibility.
- To evaluate the contribution of sugars, acids and volatile organic compounds to consumer liking.
- To list genetic, physiological and metabolic characteristics of strawberry that may be beneficial in reducing fruit susceptibility to *B. cinerea* without impairing consumer liking and nutritional quality.

As spoilage due to *B. cinerea* is the main reason for quality loss and end of shelf life in strawberry, we evaluated the effectiveness of treatments mainly with respect to spoilage caused by *B. cinerea.*

This thesis consists of six chapters. **Chapter 1** serves as a general introduction of the thesis; it introduces the research background and research objectives. **Chapters 2 to 5** (Figure 1) describe the experimental work and **Chapter 6** is a general discussion.

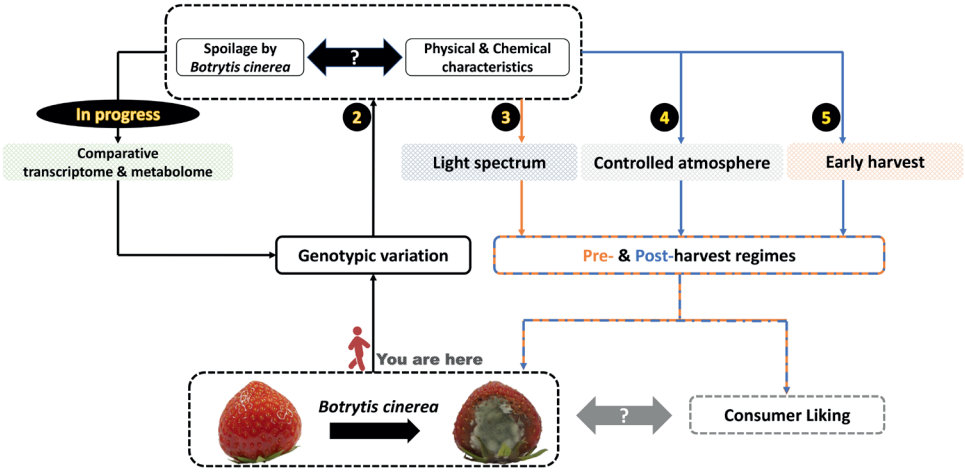

Figure 1 Research route of the thesis. The research described in this thesis started with characterizing the genotypic variation of susceptibility to *Botrytis cinerea* (grey mold disease) in red ripe strawberry fruit and the association between strawberry physical and chemical characteristics and natural spoilage caused by *B. cinerea* (Chapter 2). Pre- and postharvest regimes including light spectrum (Chapter 3), controlled atmosphere (Chapter 4) and harvest stage (Chapter 5) were applied to strawberry fruit and the metabolite contents and susceptibility to *B. cinerea* were determined. Effects of ripeness on consumer liking were further investigated and presented in Chapter 5. A comparative transcriptome and metabolome study was also conducted to investigate the differential responses to *B. cinerea* infection of two strawberry genotypes with contrasting susceptibility derived from the results demonstrated in Chapter 2. The transcriptome combined with metabolome study is still in progress and will be published separately. The corresponding chapters are indicated by the numbers in the black dots.

Chapter 2 describes the association between the shelf life and the physical and chemical characteristics in strawberry fruit. A standardized protocol to evaluate shelf life of strawberry fruit was developed in a controlled environment (low temperature with high humidity). Seventeen genotypes were selected based on contrasting flavor, color, firmness, and shelf life. Physical characteristics (e.g., weight, color, firmness) and selected primary (e.g., soluble sugars, organic acids) and secondary metabolites (e.g., proanthocyanins, anthocyanins, volatiles) of red ripe strawberries at harvest were determined and spoilage during the storage was assessed. Correlation analyses were conducted between spoilage rates and measured fruit characteristics across all genotypes.

As light is a pivotal environmental factor for plant growth and defense, we investigated the effects of light spectra on strawberry fruit susceptibility to *B. cinerea* and the results are presented in **Chapter 3**. We further studied the underlying physiological and metabolic mechanisms. LED light (red, blue and additional far-red) was applied to strawberry plants during cultivation. Fruit at different ripening stages were harvested;

the physical and chemical characteristics were determined as well as the inoculation with *B. cinerea* conidia was conducted.

Controlled atmosphere techniques are commonly used as a commercial practice to extend shelf life of fresh products. Strawberry is generally tolerant to the environment with high concentrations of CO_2 (15 to 20 kPa), in which spoilage by *B. cinerea* can be notably reduced. We tested if the stepwise increment of high CO_2 levels is beneficial to fruit nutritional quality and shelf life, which is demonstrated in **Chapter 4**. Stepwise increments of CO_2 levels to 20 or 30 kPa were applied during postharvest storage to strawberry fruit. The *B. cinerea* spoilage incidence was evaluated and specific metabolites (e.g., sugars, acids) were quantified at the moment of harvest and after postharvest storage.

To prolong the shelf life, strawberry fruit are generally harvested before they are fully ripe. We investigated the effects of maturity at harvest and subsequent cold storage on consumer liking, which is largely dependent on fruit sweetness, aroma attributes, and volatile compositions (**Chapter 5**). Strawberries were harvested either at the ¾ red stage or fully red stage and stored at 4 °C for few days and subjected to sensory profiling, color, firmness, GC-MS and PTR-ToF-MS measurements.

Finally, a general discussion is presented in **Chapter 6**.

Chapter 2

The association between the susceptibility to *Botrytis cinerea* and the levels of volatile and non-volatile metabolites in red ripe strawberry genotypes

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(submitted)

Abstract

Primary and secondary metabolites of strawberry fruit may play roles in stimulating or reducing the susceptibility to *Botrytis cinerea*. The relations between physical and chemical characteristics (e.g., color, firmness, volatile and non-volatile metabolites) of red ripe strawberries and the natural spoilage caused by *B. cinerea* were investigated. The spoilage rates differed between genotypes, and this was consistent over two successive years. Among seventeen genotypes, a more intense red coloration of the fruit skin was associated with a lower spoilage rate. Additionally, weakly negative correlations were found between the levels of anthocyanins, ascorbic acid, malic acid and spoilage rates. No clear correlations were found between spoilage rates and soluble sugars, most volatiles, firmness and dry weight percentage. High levels of two volatile compounds, ethyl butanoate and 1-hexanol, were correlated to high spoilage rates. These characteristics may assist strawberry breeders in selecting for genotypes with reduced susceptibility to *B. cinerea.*

Keywords: *Fragaria × ananassa*, strawberry genotypes, *Botrytis cinerea*, color, antioxidants, flavor

2.1 Introduction

Strawberry (*Fragaria × ananassa*) fruit have a distinct flavor, delicate texture and high nutritional value highly appreciated by consumers. Strawberries are prone to fungal infection, primarily due to grey mold caused by *Botrytis cinerea* (Petrasch et al., 2019a). Grey mold disease causes substantial losses during cultivation and postharvest storage. The spoilage of red ripe fruit results from either infection of open flowers (primary infection) or penetration through receptacles (secondary infection). The spoilage of strawberry fruit to *B. cinerea* is determined by the density of airborne conidia, pre- and postharvest conditions (e.g., temperature, humidity, light) and genotype-dependent fruit characteristics. Entirely resistant strawberry genotypes do not exist. Genetically determined resistance to *Botrytis* has been documented and quantitative resistance loci (QRLs) have been identified in e.g., tomato (Finkers et al., 2007) and gerbera (Fu et al., 2017b). However, the QRLs identified in these studies represent large chromosomal regions with hundreds of genes. Furthermore, it is challenging to identify strawberry QRLs due to its octoploid genome structure (Amil-Ruiz et al., 2011).

Few studies have attempted to systematically disentangle the genetic and physiological factors that affect the susceptibility of strawberry fruit to *B. cinerea* (Petrasch et al., 2019a, 2021). Amil-Ruiz et al. (2011) proposed a schematic overview of strawberry defense mechanisms. In short, when responding to pathogenic fungi, strawberry first relies on preformed defense responses at physical (e.g., cuticle, cell wall) and chemical (i.e., phytoanticipins) levels. After penetration by the fungus, active defense responses are induced, such as cell wall reinforcement, phytoalexin accumulation, oxidative burst and pathogenesis-related (PR) protein accumulation. Efforts have been made to understand the molecular and cellular interplay between strawberry fruit and *B. cinerea* (Haile et al., 2019; Lee et al., 2021). Information on how several important fruit metabolites influence the susceptibility to *B. cinerea*, however, remains limited.

Changes in fruit metabolites (e.g., composition and levels) and cell wall structure occur as fruit ripen, which has been demonstrated to increase the susceptibility to *B. cinerea* of strawberry fruit (Haile et al., 2019; Lee et al., 2021). Soluble sugars (e.g., glucose, fructose and sucrose) as primary metabolites serve as a carbon source for *B. cinerea* and/or participate in ripening signaling processes (Durán-Soria et al., 2020) to promote fungal infection. By contrast, transgenic tomato fruit with a higher level of malic acid in the green stage led to a decreased soluble sugar level in the red ripe stage (at harvest) and increased wrinkling during postharvest. These features resulted in increased

susceptibility to *B. cinerea* (Centeno et al., 2011). The role of sugars in fruit-*B. cinerea* is complex, presumably depending on concentrations.

Another type of important, highly abundant strawberry fruit metabolites are proanthocyanidins and anthocyanins, compounds with antioxidant activities and derived from the phenylpropanoid biosynthetic pathway. Proanthocyanidins provide an astringent flavor in immature fruit and the high level is negatively correlated to the susceptibility of strawberries to *B. cinerea* (Hébert et al., 2002). Anthocyanins are pigments conferring the red color to strawberry fruit. Transgenic tomato fruit with higher levels of anthocyanins were less susceptible to *B. cinerea* infection (Zhang et al., 2013), presumably due to the antioxidant activity of anthocyanins. Another antioxidant in strawberry fruit is ascorbic acid. The involvement of ascorbic acid in fruit susceptibility to *B. cinerea* was only studied in apples. Higher ascorbic acid levels in the sun-exposed side of apple fruit reduced the lesion expansion compared to the shaded side (Bui et al., 2019).

In addition to non-volatile metabolites, research has also been performed on the relation between strawberry volatile composition and *B. cinerea* infection. The primary importance of volatile compounds is that they may provide a pleasant aroma to consumers. Volatile compounds with both appealing odor and antifungal activity properties are therefore of interest. For instance, *B.cinerea* infection in 'Akihime' strawberries induced production of linalool, a terpene conferring a sweet and citrus-like note to red ripe strawberry fruit. Linalool possesses antifungal activity towards *B. cinerea* by causing oxidative stress and impairing the membrane integrity of the fungal cells (Xu et al., 2019). Green leaf volatiles (GLVs) are proposed to contribute to defense responses to fungal infection. GLVs include aldehydes, alcohols of C_6 compounds and some esters. (E)-2-hexenal is the most intensely investigated GLV and is suggested to be useful as an antifungal agent (Archbold et al., 1997; Wakai et al., 2019). However, Xu et al. (2021) proposed that (E)-2-hexenal may also facilitate *B cinerea* infection of strawberry fruit by inducing sulfate assimilation in the fungus.

The above examples illustrate that strawberry fruit characteristics may contribute to determining the susceptibility to *B. cinerea*, although these studies involved only a limited number of genotypes. There are also numerous reports showing variation in fruit susceptibility to *B. cinerea* among strawberry cultivars (Chandler et al., 2006; Seijo et al., 2008; Lewers et al., 2013), however, these studies did not describe the genotypic characteristics or physiological traits that may mediate the increased resistance. Recently, Petrasch et al. (2021) attempted to bridge this knowledge gap using genomic

prediction. These authors developed and investigated training populations segregating for fruit quality traits predicted to affect susceptibility.

This study aimed to determine the association between fruit characteristics and their propensity for natural spoilage by grey mold (i.e., without artificial inoculation with *B. cinerea* conidia) in 17 strawberry genotypes. Physical and chemical characteristics of red ripe fruit were measured at harvest and fruit spoilage development caused by *B. cinerea* was evaluated during postharvest storage. We discuss the implications of our observations for selecting cultivars with reduced susceptibility to *B. cinerea* and list strawberry characteristics that may be correlated to improved fruit shelf life without impairing flavor.

2.2 Materials and methods

2.2.1 Strawberry batches, cultivation, storage & measurements

Strawberry plants (*Fragaria × ananassa* Duch.) were cultivated in a greenhouse at Eck en Wiel, the Netherlands. Fruit were harvested in the red ripe stage in June, 2018 and April, 2019 and immediately transported to the laboratory. Fruit without mechanical damage and of uniform size and color were selected. Fruit from each cultivar within the same harvest were placed individually in weighing boats (46 × 46 × 8 mm, L × W × H).

In 2018, fruit from five genotypes (cultivars Elsanta, Jive, Verdi and breeding selections E2012-1494, E2012-1648) were harvested up to three times with a one-week interval with approximately ninety fruit per genotype and harvest. Fruit from each harvest were randomly assigned to three sub-batches per genotype and harvest. Each sub-batch was placed in a crate and stored at either 5, 10 or 15 °C, always at ~95% relative humidity in darkness for up to 20 d to monitor the spoilage incidence.

In 2019, fruit from 17 genotypes were harvested with approximately sixty fruit per genotype. The first harvest contained fruit from 11 genotypes (cultivars Elsanta, Figaro, Korona, Lambada, Malling Centenary, Rumba, Sonata, Vivaldi and breeding selections E2010-286, E2014-0692). The second harvest contained fruit from six genotypes (cultivars Florence, Jive, Opera and breeding selections E2008-321, E2012-1494, E2012-1648). The two harvests were carried out with a one-week interval. Fruit were randomly assigned to three sub-batches per genotype, placed in a crate and stored overnight at 5 °C in darkness and ~95% relative humidity at three positions within the cold room. The next day four fruit per sub-batch were randomly selected for fresh weight, dry weight, color and firmness measurements. In addition, antioxidants (total anthocyanins,

proanthocyanidins and ascorbic acid), soluble sugars (glucose, fructose and sucrose), non-volatile organic acids (malic-, citric- and oxalic acid) and volatile composition were measured. All other fruit were thereafter stored in darkness at 12 °C and ~95% relative humidity and used for quantifying the spoilage incidence every two days for up to seven days.

2.2.2 Spoilage rate, overall visual quality and spoilage modelling

For the 2018 harvests, spoilage was expressed as the cumulative percentage of *B. cinerea*-infected fruit over time. For the 2019 harvests, a more elaborated overall visual quality (OVQ) scoring system was used that scored strawberries over time according to the rating system depicted in Figure 1.

Figure 1 Progression of natural spoilage occurring in strawberry fruit caused by grey mold disease. Fruit are stored in darkness at 12 °C and ~95% relative humidity. Score 5 = healthy fruit; score 4 = fruit with dry bruises; score 3 = fruit with wet bruises; score 2 = infected fruit showing brown spots without visible mycelia; score 1 = infected fruit with visible mycelia. Note: not every strawberry showed all infection phases according to our observation. Fruit infected by other fungi are excluded when occurred.

Spoilage modelling, either recorded as the cumulative percentage of affected fruit (2018) or by the OVQ scoring system (2019), was carried out as proposed by Hertog et al. (1999) and applied by Schouten et al. (2002). In short, the spoilage development over time was described by the following differential equations that differ only in the sign, positive for the increasing cumulative spoilage data recorded in 2018 (Eq. (1)) and negative for the decreasing OVQ scores in 2018 (Eq. (2)).

$$\frac{\mathrm{d}N}{\mathrm{d}t} = k \cdot N \cdot \frac{N_{max} - N}{N_{max}} \; with \; N(t=0) = N_0 \qquad (1)$$

$$\frac{\mathrm{d}N}{\mathrm{d}t} = -k \cdot N \cdot \frac{N_{max} - N}{N_{max}} \; with \; N(t=0) = N_0 \qquad (2)$$

with *N* the spoilage in % for the 2018 dataset or the OVQ score for the 2019 dataset, N_0 the initial spoilage in % (2018) or the initial OVQ score (2019), *k* the spoilage rate (in d^{-}

[1]) and N_{max}, the maximum spoilage, set to 100% in 2018 or 1 (Figure 1) in 2019. The spoilage rate is assumed to depend on temperature according to Arrhenius' law (Eq. (3))

$$k = k_{ref} \cdot e^{\frac{E}{\mathrm{R_{gas}}}\left(\frac{1}{\mathrm{T_{ref}}}-\frac{1}{\mathrm{T}}\right)} \quad (3)$$

with R_{gas} is the gas constant (8.314 J mol^{-1} K^{-1}), k_{ref} the spoilage rate at reference temperature T_{ref} (in K) and E, the energy of activation (in KJ mol^{-1}) that expresses the dependence of the spoilage rate on temperature T (in K). The shelf life, the time (in d) it takes until strawberries show brown spots (OVQ score 2) for the 2019 dataset was calculated using the analytical solution of Eq. 2 according to Eq. 4.

$$\text{Shelf life} = -\frac{ln\left(-\frac{N_0(N_{max}-2)}{2\,(N_0-N_{max})}\right)}{k} \quad (4)$$

2.2.3 Color measurements

Strawberry color was assessed using image analysis. Images were acquired using a LED cabinet (IPSS Engineering, Wageningen, the Netherlands) containing a RGB camera (MAKO G-192C POE, Allied Vision, Stadtroda, Germany). The RGB images were calibrated using a 24-patch color checker card (Colour checker classic, X-rite Europe GmbH, Regensdorf, Switzerland). Image analysis was carried out using multi-threshold color image segmentation in the RGB color space to remove the strawberry background. Strawberry color was expressed as (1/R*100) (Schouten et al., 2002).

2.2.4 Fruit weight, firmness and percentage dry weight

Fruit fresh weight was measured using a MS6002TS balance (Mettler-Toledo GmbH, Giessen, Germany). Firmness was measured using the whole fruit by a commercial acoustic firmness tester (AFS, AWETA, Nootdorp, the Netherlands) with the tick power of the plunger set to 12. The AFS combines a resonant frequency (f in Hz) and mass (m, in kg), measured by an inbuild balance, into a FI (firmness index) according to Eq. 5.

$$FI = \frac{f^2 m^{\frac{2}{3}}}{10^4} \quad (5)$$

Following the fresh weight and firmness measurements, whole strawberry fruit were chopped into pieces ground into powder using an analytical mill (IKA A11, IKA, Staufen, Germany), and stored at -80 °C until further analysis. Part of the frozen powder was

freeze-dried for sugar extraction and to determine dry weight percentage, allowing to express all nutrients (except for volatile composition, see below) per g dry weight (DW).

2.2.5 Total anthocyanin and proanthocyanin extraction and determination

For extraction of anthocyanins, 0.5 g frozen powder was dissolved in 1.5 mL 50% methanol containing 1% HCl extraction. The supernatant was filtered through a 0.45 µm cellulose filter with the absorbance measured at 520 nm using a UV-Vis spectrophotometer (Varian Cary 4000, Canada). Pelargonidin-3-glucoside (Extrasynthase, Genay, France) was used as the standard.

Proanthocyanidins were measured according to Prior et al. (2010) with slight modifications. Twenty mg freeze-dried powder was dissolved in 10 mL extraction solution (75% acetone, 24.5% distilled water and 0.5% acetic acid). The mixture was vortexed for 30 s and placed into an ultrasonic bath for 30 min at room temperature. The mixture was subsequently shaken for an hour at room temperature, followed by centrifugation for 20 min at 8.500 × *g* at 12 °C. An aliquot of 70 µL was then mixed with 210 µL acidified ethanol (12.5% HCl, 12.5% deionized water and 75% ethanol containing 0.1% 4-dimethylaminocinnamaldehyde). Analysis was conducted using a plate reader (CLARIOstar Plus, BMG Labtech, Germany) by reading the absorbance at 640 nm per min for 30 min. Varying concentrations (20, 50 and 100 µg mL^{-1}) of procyanidin A2 (Extrasynthese, Genay, France) were used to quantify the total proanthocyanidin level.

2.2.6 Ascorbic acid extraction and determination

Frozen powder (0.2 g) was thawed on ice in darkness with the addition of 1 mL ice cold 3.3% meta-phosphoric acid (MPA). Samples were sonicated for 10 min and then centrifuged at 21.000 × *g* at 4 °C for 10 min. The supernatant was filtered through a 0.45 µm cellulose filter and injected into a HPLC system consisting of a GS50 pump (Dionex), a 340S UV-VIS detector (Dionex) and a MIDAS autosampler (Spark Holland) equipped with a ProntoSIL 120-3 C18 AQ, 250 × 3mm column (Knauer). The column was eluted with 400 µL L^{-1} H_3PO_4 + 2.5 mL L^{-1} MeOH + 0.1 mM EDTA in distilled water followed by a wash step with 30% acetonitrile in distilled water at a flow rate of 0.35 mL min^{-1} at 35 °C. Ascorbic acid was detected at 243 nm. The quantification was carried out using ascorbic acid standard (Acros Organics) prepared in 3.3% MPA.

2.2.7 Sugar and organic acid extraction and determination

Fifteen mg of freeze-dried powder was mixed with 5 mL 75% ethanol followed by shaking and incubating at 80 °C in a water bath for 20 min, then centrifuged at 8.500 × *g* for 5 min at 4 °C. One mL supernatant was dried using a vacuum centrifuge at 55 °C for 2.5 h. The pellet was resuspended with 1 mL distilled water and placed into an ultrasonic bath for 10 min, followed by centrifugation for 10 min at 4 °C at 14.800 × *g*. Before analysis, the supernatant was diluted 50 times and 5 times with distilled water for sugars and organic acids, respectively.

The sugar samples were loaded onto a High Performance Anion Exchange Chromatograph with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Scientific), equipped with a CarboPac1 (250 × 2 mm) column (Dionex) eluted with 100 mM NaOH at a flow rate of 0.25 mL min^{-1} at 25 °C. Calibration of the HPLC was performed with glucose, fructose and sucrose from Sigma-Aldrich.

Organic acids in the extracts were analyzed using an IC system equipped with a GS50 pump (Dionex), ED50A detector (Dionex), Triathlon autosampler (Spark Holland) operating in the conductivity mode and an ASRS ultra II 2 mm suppressor (Dionex). Anions were separated at 30 °C on an IonPac AS11HC (250 × 2 mm) column (Dionex), using the following multi-step gradients: 1 mM NaOH, 0 min; 1 mM NaOH, 1 min; 14 mM NaOH, 23 min; 30 mM NaOH, 31 min; 60 mM NaOH, 41 min at a flow rate of 0.38 mL min^{-1}. The quantification was carried out using oxalic acid, DL-malic acid and citric acid from Sigma-Aldrich.

2.2.8 Volatile emission detection by SPME/GC-MS and data processing

Extraction and detection of volatile metabolites were performed according to Tikunov et al. (2005) with slight modifications. Frozen powder (0.5 g) was added into vials with 0.5 mL distilled water. The vials were closed and incubated at 30 °C for 10 min. After adding 1 mL 100 mM, pH 7.5 EDTA/NaOH, 2.2 g solid $CaCl_2$ powder was mixed thoroughly to inhibit enzyme activity. One mL of the extracts was transferred into a 10 mL crimp cap vial for headspace SPME/GC–MS detection. Individual vials were randomized to avoid systematic memory effects and placed into a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). A 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre (Supelco, Bellefonte, USA) was exposed for 20 min to the vial headspace under continuous agitation and heating at 50 °C. The trapped compounds by SPME were desorbed into a Trace GC Ultra gas

chromatograph (Thermofisher, Waltham, USA) injector for 1 min at 250 °C. Chromatographic separation was achieved on an Zebron ZB-5 (50 m × 0.32 mm × 1.05 µm) column (Phenomenex) with helium as the carrier gas (at constant flow 2 mL min^{-1}). The GC interface and MS source temperatures were 260 and 250 °C, respectively. The GC temperature program started at 45 °C for 2 min, was then increased to 250 °C at a rate of 5 °C min^{-1} and finally held at 250 °C for 5 min. Including oven cooling, the total run time was 60 min. Mass spectra in the 35 to 400 m/z range were recorded by an DSQII electron impact MS (Thermofisher) at a scanning speed of 2.8 scans s^{-1} and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using Xcalibur software (http://www.thermo.com). The raw data generated by SPME/GC-MS were processed by MetAlign™ software package (http:// www.metalign.nl) for baseline correction, noise estimation and ion-wise mass spectral alignment. The VOCs mass spectra and quantitative ion fragments were extracted using MSClust software (http:// www.metalign.nl) (Tikunov et al., 2012). VOCs were identified by matching mass spectra and the retention indices of the compounds extracted to the NIST mass spectral library using NIST MS Search software (http://www.nist.gov). Peak height of these compounds was used for further statistical analysis.

2.2.9 Statistical analysis

The spoilage model was assumed to directly relate to the spoilage in percentage fruit affected (in the 2018 harvests) or the OVQ score (in the 2019 harvests) with the differential equations (Eq. 1 or Eq. 2) applied in OptiPa (Hertog et al., 2007), a dedicated tool for data analysis using differential equations. In the 2018 harvests, all spoilage data were analyzed simultaneously with the initial spoilage (N_0) estimated per sub-batch and the reference spoilage rate (k_{ref}) estimated per cultivar and the energy of activation (E) estimated in common for all batches and T_{ref} set to 12 °C (Eq. 3). Similarly, all OVQ scoring data were analyzed simultaneously with N_0 estimated per sub-batch and the spoilage rate (k) estimated per genotype.

Fresh weight, dry weight, color and firmness, antioxidants, soluble sugars and titratable acid data per cultivar were subjected to one-way ANOVA using GENSTAT (19th Edition, VSN International Ltd., Hemel Hempstead, UK). Statistical treatment effects were calculated at $\alpha = 0.05$. Homogeneity and normality of residuals in the ANOVA were tested using Bartlett's test and the Shapiro-Wilk test, respectively and all accepted ($P > 0.05$). Fisher's protected least significant difference (LSD) test was used as post-hoc test for the parameters with significant treatment effects in ANOVA. All other analyses were carried out using R3.5.0 (R Core Team, 2021). Principal component analysis (PCA) and

hierarchical clustering analysis of genotypes were carried out using the factoextra package. The Ward's minimum variance method (ward. D2) was used for hierarchical clustering analysis. The correlation matrix was conducted using the corrplot package.

2.3 Results

2.3.1 Natural spoilage rates over two consecutive years are highly correlated

Spoilage data of strawberry batches from five genotypes (Elsanta, Jive, Verdi, E2012-1494, E2012-1648), recorded in both years, were described using either Eq. 1 and Eq. 3 (2018) or Eq. 2 (2019). The reference spoilage rate (k_{ref}) was estimated per genotype with the initial spoilage (N_0) estimated per harvest for the data gathered in 2018 in one joint estimation. The spoilage rate (k) was estimated in common for three sub-batches per genotype with N_0 estimated per sub-batch for the data gathered in 2019 in one joint estimation. Estimated parameters are presented in Table S1, including the variance explained, either 93.4% (2018) or 98.4% (2019). Representative spoilage data and the corresponding simulation for the 2018 dataset are shown for three sub-batches of one harvest, stored either at either 5, 10 or 15 °C for two genotypes (E2012-1494 and Jive) (Figure 2A). Similarly, representative spoilage data and the corresponding simulation for the 2019 dataset are shown for three sub-batches of 'E2012-1494' and 'Jive' (Figure 2B). The reference spoilage rate k_{ref} was estimated at 12 °C for the 2018 dataset. The spoilage rate for the 2019 dataset was estimated for strawberries stored at 12 °C. Therefore, the reference spoilage rates from the 2018 dataset and the spoilage rate from the 2019 dataset can be compared. Spoilage rates of the five genotypes across both years were highly correlated (Figure 2C).

In 2019, the spoilage rates of 17 genotypes were estimated by applying Eq. 2. 'Lambada' and 'E2012-1494' strawberries had the lowest and highest spoilage rates, respectively (Figure 2D). The shelf life was calculated by using the estimated values from Table S1 and applying Eq. 4. The shelf life ranged between 3.6 ('E2012-1494') and 9.0 d ('Vivaldi') (Figure 2E).

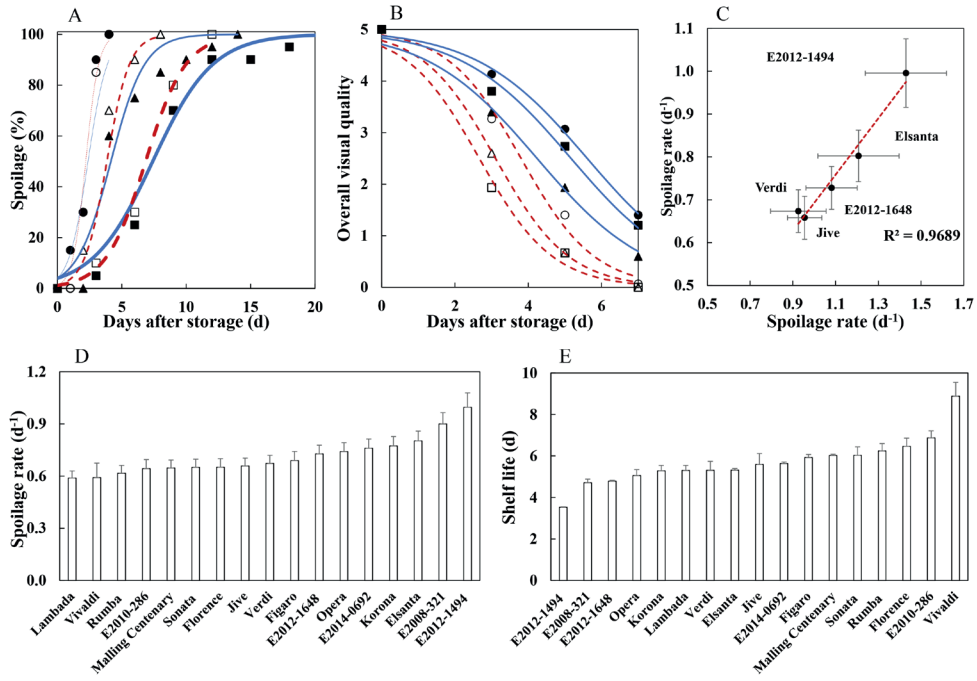

Figure 2 Spoilage rates and shelf life of different genotypes. (A) Spoilage over time for strawberries from one harvest of the 2018 dataset for 'E2012-1494' (open symbols, red lines) and 'Jive' (solid symbols, blue lines) of three sub-batches, stored at either 15 (○, ●), 10 (Δ, ▲) and 5 °C (□, ■) with simulated model behaviors. (B) Overall visual quality (OVQ) scores for three sub-batches at 12 °C (circles, triangles and squares) of the 2019 dataset for 'E2012-1494' (open symbols) and 'Jive' (closed symbols) with simulated model behaviors. (C) Relation between the estimated spoilage rates at 12 °C of the five cultivars cultivated in 2018 and 2019. (D) The estimated spoilage rates and (E) calculated shelf life at 12 °C for all strawberry batches from the 2019 dataset.

2.3.2 Relations between non-volatile characteristics and spoilage rates

Physical and chemical (fresh weight, dry weight percentage, firmness, color, soluble sugars, non-volatile acids, proanthocyanidins and anthocyanins) characteristics in 17 strawberry genotypes at the red ripe stage were determined immediately after harvest for the 2019 harvests. The genotypes varied in appearance, such as color, size and shape (Figure 3A). The measured values of individual fruit characteristics are presented in Figure S1. Color analysis indicated that 'E2012-1494' and 'Rumba' strawberries were the lightest- and darkest red colored strawberries, respectively (Figure S1A). Fresh weight varied between 12.1 ('Lambada') and 32.8 g ('2012-1648') (Figure S1B). 'Korona' and '2012-1648' fruit showed the lowest and highest firmness index values, about a 2-fold difference (Figure S1D). 'E2012-1494' fruit showing the shortest shelf life (Figure

1E) contained low contents of ascorbic acid, proanthocyanidins and anthocyanins (Figure S1E, F, G). In contrast, 'Florence' strawberries had a long shelf life with higher contents of above mentioned three compounds as well as citric acid and malic acid (Figure S1H). The glucose and fructose levels were close among genotypes ranged between 180 and 270 mg g^{-1} DW (Figure S1I). Sucrose levels ranged between ~60 and ~250 mg g^{-1} DW. The ratio of total sugar to total acid ranged from 4.13 ('Florence') to 9.18 ('Rumba' and 'Lambada'). All these three genotypes had relatively low spoilage rates.

PCA revealed that PC1 and PC2 described 58.6% of the total variance in 17 genotypes with respect to all phenotypic characteristics, non-volatile compounds and the spoilage rate (*k*) (Figure 3B). The largest positive loadings encountered in PC1 were glucose and fructose and largest negative loadings were oxalic and ascorbic acid. In PC2 fresh weight and dry weight percentage showed the largest positive and negative loadings, respectively. Genotypes displayed as red squares were characterized by high dry weight percentage and sucrose levels; genotypes displayed as blue circles were characterized by abundant acids (except for malic acid), anthocyanins and proanthocyanidins; genotypes displayed as purple triangles were characterized by high fresh weight and firmness; genotypes displayed as yellow diamonds were characterized by high contents of fructose and glucose. The spoilage rate (*k*) was negatively correlated with the red coloration (r = -0.63). Notably, in the PCA plot, the vector of the spoilage rate (*k*) was close to the vectors representing glucose and fructose (but perpendicular to sucrose) and opposite to acids (except for malic acid), anthocyanins and proanthocyanidins. The spoilage rate (*k*) was weakly positively correlated to fructose (r = 0.38), glucose (r = 0.26) and malic acid (r = 0.43) whereas it was weakly negatively correlated to anthocyanins (r = -0.32) and ascorbic acid (r = -0.35). No correlation between the spoilage rate (*k*) and sucrose or firmness was observed (Figure 3C, S2A).

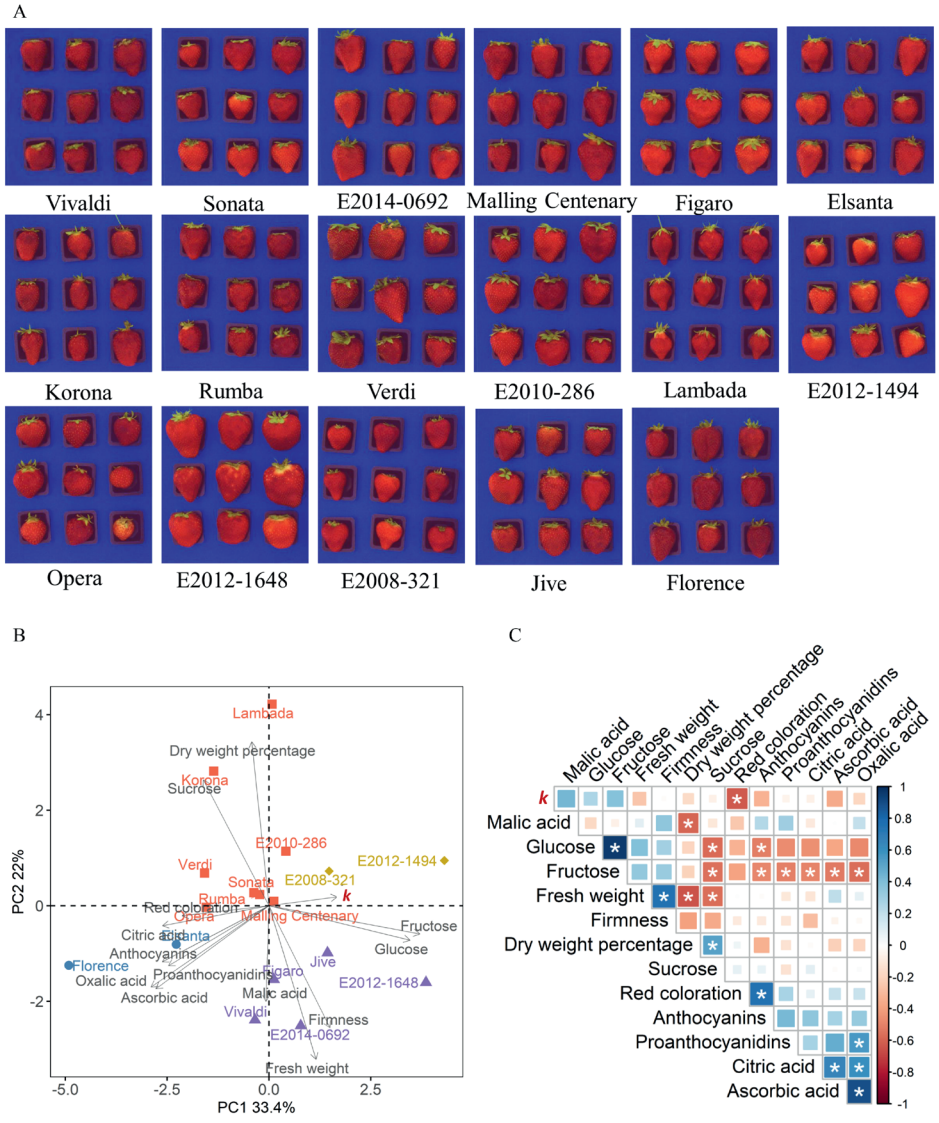

Figure 3 Correlation analysis of non-volatile characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage. (A) Representative images of all genotypes at harvest. (B) PCA biplot showing the score of each genotype (points) and the loading of fruit characteristics (arrows in grey). (C) Correlation matrix. Positive correlations are displayed in blue and negative correlations in red. The areas of squares and color intensity (light to dark) are proportional to the Pearson correlation coefficients. Asterisks denotes significant correlation coefficients ($P < 0.05$).

2.3.3 Relations between volatile characteristics and spoilage rates

Fifty-two volatiles were tentatively identified when measuring the volatile compounds of strawberries from 17 genotypes harvested in 2019. PCA revealed that PC1 and PC2 described 36.9% of the total variance with respect to volatile characteristics and the spoilage rate (*k*) (Figure 4A, B). Along PC1, genotypes displayed as purple triangles were mainly characterized by the production of high levels of esters such as ethyl hexanoate, methyl hexanoate and hexyl acetate. Genotypes displayed as blue circles were mostly characterized by aldehydes, including (E)-2-hexenal and (E)-2-octenal. Genotype E2014-0692 was characterized by a low abundance of mesifurane and high abundance of hexyl acetate. Along PC2, genotypes displayed as red squares were mainly characterized by esters (e.g., methyl 3-methylbutanoate, methyl butanoate) and volatile acids (e.g., octanoic acid, 2-methylbutanoic acid and hexanoic acid). Interestingly, the vector of the spoilage rate (*k*) was near esters and alcohols but opposite to aldehydes vectors. Figures 4C and 4D describe the correlation between the spoilage rate (*k*) and the production of different volatiles. For 23 volatile compounds, there was a negative correlation, none of which was significant (Figure 4C). For 29 volatile compounds, there was a positive correlation, and the correlations between the spoilage rate (*k*) and the contents of methyl anthranilate, ethyl hexanoate and 1-hexanol were statistically significant (Figure 4D).

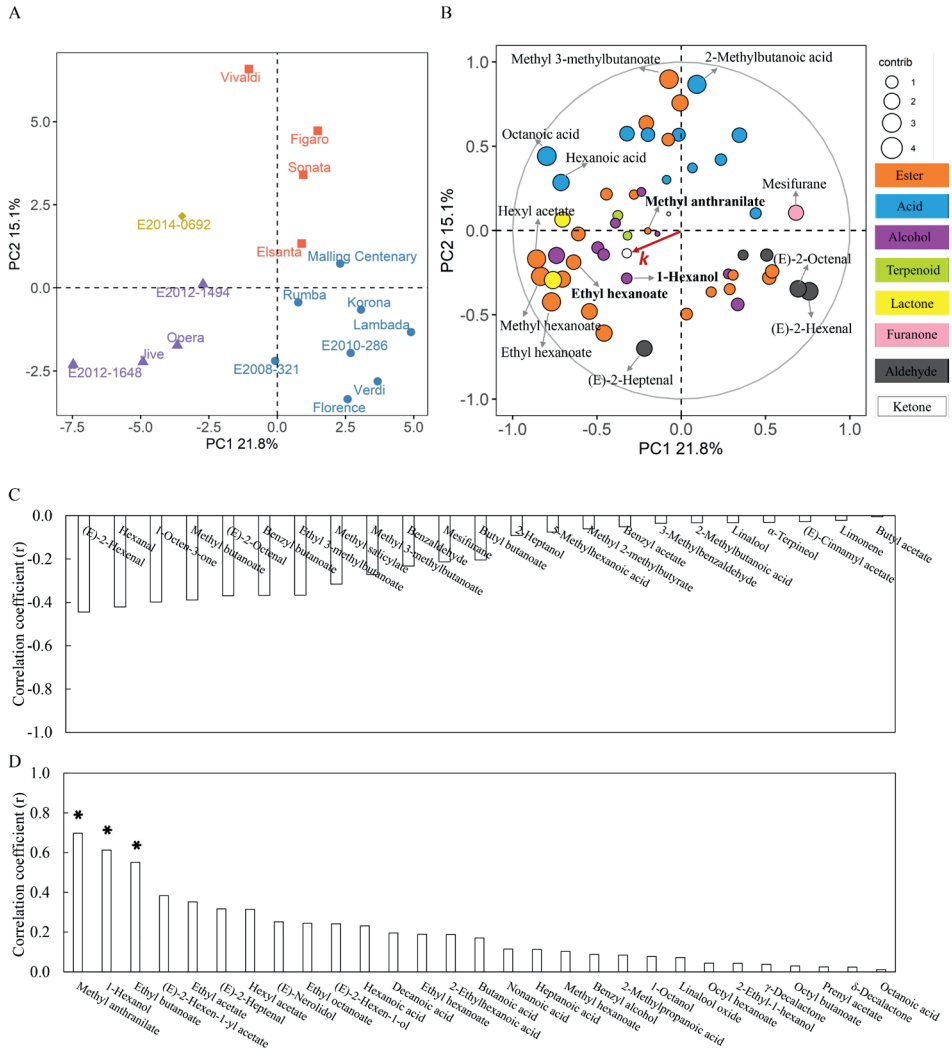

Figure 4 Correlation analysis of volatile characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage. (A) PCA score plot showing the score of each genotype (points). (B) PCA loading plot showing the loading of fruit characteristics. Negative correlations (C) and positive correlations (D) between volatile characteristics and the spoilage rate. The date is originated from the correlation matrix (Figure S2.3A). Asterisks denotes significant correlation coefficients ($P < 0.05$).

2.4 Discussion

2.4.1 Spoilage rates of strawberry fruit are genotype-dependent

Previous studies on *B. cinerea* spoilage in strawberry fruit have reported the effects of year, cultivar and the cultivar × year interaction on fruit spoilage (Hegyi-Kaló et al.,

2019). Some of these studies reported that the variation of *B. cinerea* spoilage between years was more profound than genotypic differences within years (Chandler et al., 2006; Seijo et al., 2008). In our research, the initial spoilage (N_0), the estimated initial spoilage of each batch, varied among harvests, cultivars and years (Table S1). In 2019, the initial spoilage rates (N_0) were less variable as compared to 2018, probably because the disease pressure on different cultivars was more similar in 2019. This lower variation of N_0 in 2019 was possibly achieved because all genotypes were grown in the same greenhouse and harvested at an interval of only two weeks.

The spoilage rate (*k*), estimated per genotype, showed consistent rankings in five genotypes across two years (Figure 2C), indicating that the spoilage rate is a characteristic that is associated with the genotype. In other words, the approach to separate the initial spoilage (dependent on year and harvest), from the genotype-dependent spoilage rate, allows screening for the characteristics linked to the intrinsic, genetically determined susceptibility to *B. cinerea*. In addition, the spoilage performance of fruit is also affected by ripeness at harvest to some extent. External color as an indication of ripeness, is often used as a harvest criterion. However, ripeness is also determined by sugar and non-volatile acid levels and aroma composition. Our data showed that fruit containing higher levels of glucose and fructose as well as volatile compounds with a fruity, sweet and berry-like notes (e.g., methyl hexanoate and ethyl hexanoate) but low levels of non-volatile acids and volatile compounds with a green note tend to spoil rapidly (Figure 3B, 4B).

2.4.2 Towards reducing strawberry susceptibility without impairing consumer liking

Strawberry sugar levels and the susceptibility to *B. cinerea* increase with ripening. Soluble sugars as the major carbon source for the fungal growth and reproduction may promote the infection processes. However, the hypothesis that sugars act as a signal of the host cell at the infection site to activate its defense responses has also been discussed. For instance, the “Sugars Will Eventually be Exported Transporters” (SWEET) help to translocate sugars to infection sites such that the host defense metabolism can be activated (Tadege et al., 1998). Hu et al. (2019) found an increase in sucrose content of strawberry leaves infected by *B. cinerea* and proposed that the increase in sucrose content could act as an early indicator for grey mold infection in strawberry leaves before the occurrence of visible symptoms. However, the contents of glucose, fructose and sucrose of strawberry fruit are about 12, 22, and 66 folds higher than in leaves (Akšić et al., 2019), which may suggest that the high sugar availability in fruit could

interfere with the detection of any subtle changes in sugars upon fungal infection. Indeed, several research groups have attempted to unveil the dynamic interplay between strawberry fruit and *B. cinerea* using transcriptome analysis, and none of these studies have provided information about the roles of sugars or sugar transporters in response to *B. cinerea* infection (Haile et al., 2019; Lee et al., 2021). In these investigations, fungal conidia were applied to strawberries and the lesion expansion was monitored and inoculated fruit tissue was sampled.

In our research, we quantified the fruit metabolites at harvest and evaluated the subsequent natural spoilage caused by *B. cinerea*. We noticed the tendency that a higher spoilage rate was correlated to higher levels of fructose and glucose but not to the level of sucrose or total sugar content (Figure 3B). However, sucrose is the main factor contributing to variations in total sugar content and *B. cinerea* may not be experiencing a lack of sugars upon infection of ripe fruit. Hence, we agree with the statement by Petrasch et al. (2019a) that in the strawberry fruit-*Botrytis* interaction, the high sugar availability might not play a significant role in fruit spoilage by grey mold.

Amongst the correlations between volatile composition and spoilage rates, we observed that the levels of ethyl butanoate, 1-hexanol and methyl anthranilate were significantly and positively corelated to the spoilage rate (Figure 4C). Ethyl butanoate is among the most universally detected esters in strawberries with a fruity, sweet and 'pineapple-like' note and is synthesized via esterification of butyric acids with ethanol. In line with our observation, Daugaard and Lindhard (2000) reported that 'Senga Sengana' and 'Korona' strawberries were more susceptible to *B. cinerea* than 'Honeoye' strawberries, which corresponded to a higher emission of ethyl butanoate in 'Senga Sengana' and 'Korona' strawberries compared to 'Honeoye' strawberries (Hakala et al., 2002). In addition, ethyl butanoate was shown to stimulate *B. cinerea* conidial germination *in vitro* over a wide range of concentrations (from 0.062 to 12.3 µl L^{-1}) (Neri et al., 2015).

As a primary alcohol, 1-hexanol is synthesized from the lipoxygenase (LOX) pathway, where hexanal is catalyzed by alcohol dehydrogenases (ADH) into 1-hexanol (Dudareva et al., 2013), that is consumed to metabolize downstream esters. Both hexanal (8 µl L^{-1}) and 1-hexanol (48 µl L^{-1}) exhibited potential as a postharvest fumigant for controlling grey mold spoilage in strawberries (Archbold et al., 1997). Vandendriessche et al. (2012) reported a negative correlation between the 1-hexanol content and the infection severity of strawberries caused by *B. cinerea*. By contrast, we found that a higher level of 1-hexanol was associated with a more rapid spoilage across 17 strawberry genotypes.

The phytotoxic effect of 1-hexanol to overall fruit quality (e.g., discoloration, loss of turgidity) was also reported in some strawberry cultivars (Archbold et al., 1997).

Methyl anthranilate reduced *B. cinerea* growth *in vitro* (Chambers et al., 2013). Methyl anthranilate provides a distinct 'grape-like' odor to the diploid strawberry *Fragaria vesca* and several *Fragaria × ananassa* cultivars, however, it is not universally present in cultivated strawberry cultivars (Ulrich and Olbricht, 2014). Of the strawberry genotypes tested in the current study, 'E2012-1494' fruit showed a high content of methyl anthranilate, accompanied by the highest spoilage rate among all 17 genotypes. As the other 16 genotypes produced only negligible levels of methyl anthranilate, the role of this compound in the strawberry-*B. cinerea* pathosystem remains unclear.

Sugar levels (particularly sucrose content) and volatile compounds, in particular esters, terpenes, and furans are important determinants of strawberry sweetness and flavor (Schwieterman et al., 2014). Our data did not reveal significant correlations between *B. cinerea* spoilage and sugar contents, esters (with the exception of ethyl butanoate), terpenes and furans. This suggests that it may be feasible to breed strawberry genotypes that are less susceptible to *B. cinerea* without impairing the metabolite profiles that are important for consumer liking.

2.4.3 Increased red coloration is associated with lower spoilage rates but increased antioxidant levels are not

Increasing the anthocyanin content by genetic modification reduced susceptibility to *B. cinerea* of tomato, presumably due to the antioxidant activity (Zhang et al., 2013). Our results are partly in agreement with these findings, as strawberry genotypes displaying lower spoilage rates had more intense red color (Figure 3C). The red color of strawberry was positively correlated to the total level of anthocyanins. There was, however, no significant correlation between the total anthocyanin levels and the spoilage rate. We also tested the correlation between the spoilage rate and the peak areas of pelargonidin-3-glucoside or pelargonidin-3-malonyl-glucoside (main anthocyanins detected among tested genotypes). The correlation coefficients were not significant but close to the value originated between the spoilage rate and the total anthocyanin content (data not shown). The lack of the direct correlation between spoilage rates and anthocyanin levels may be explained by the observations that the external and internal coloration and anthocyanin accumulation of strawberries are not necessarily synchronized. Furthermore, although anthocyanins are the main source of red color in strawberry fruit, the more intense red coloration of certain genotypes might be

associated with a higher abundance of other phenolic compounds that reduce the susceptibility. Hegyi-Kaló et al. (2019) observed that the inhibition of *B. cinerea* growth was more profound on grape berries with dark-skin color than on white-skinned berries that contain lower concentrations of phenolic compounds.

The total proanthocyanin content was not correlated to the spoilage rates according to our results (Figure 3C). Others have reported that less susceptible cultivars of grapes and strawberries had higher quantities of proanthocyanidins (Hébert et al., 2002). Jersch et al. (1989) observed that aqueous extracts of immature strawberry fruit could suppress *B. cinerea* conidial germination and mycelial growth. They suggested that proanthocyanidins delay the spoilage by *B. cinerea* through inhibiting the enzymatic activity of fungal polygalacturonases, one of the key enzyme activities involved in fruit rotting processes. The observation that proanthocyanidin contents at harvest were not correlated to postharvest spoilage by *B. cinerea* might be due to 1) the minor differences in the proanthocyanidin contents of red ripe strawberry fruit compared to unripe strawberries (Schaart et al., 2013); 2) that the different types of proanthocyanidins (Schaart et al., 2013) may matter instead of total proanthocyanidins. Notably, higher proanthocyanidin levels may correspond to higher anthocyanin levels in red ripe strawberries in 17 strawberry genotypes (Figure S2A). Zhang et al. (2013) reported that anthocyanins in transgenic tomato fruit do not reduce *B. cinerea* growth directly but rather improve the antioxidant capacity, thereby delaying senescence processes. However, it is unclear whether the inhibition of *B. cinerea* growth is caused by an accumulation of anthocyanins or proanthocyanidins, or both, because the dihydroflavonol 4-reductase (*SlDFR*) produces the precursors of both classes of compounds. Anthocyanins may still play a more important role in strawberry-*B. cinerea* pathosystem compared to proanthocyanidins in red ripe strawberries. Notably, attempts to modify proanthocyanidin levels may result in a negative impact on fruit taste given the astringent flavor caused by proanthocyanidins. Hence, further research is needed on the genetic modification of anthocyanin biosynthesis and the measurement of fruit susceptibility to *B. cinerea.*

The ascorbic acid content was weakly negatively correlated to the spoilage rate (Figure 3C). Likewise, Davey et al. (2007) reported that a higher content of ascorbic acid in apples was correlated with less severe *B. cinerea* infection. Bui et al. (2019) observed increased enzymatic activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX), accompanied by a decrease in the ascorbic acid level in apple fruit during the infection by *B. cinerea*. They proposed that the increase in SOD activity could be originated from apple fruit or *B. cinerea* in response to the oxidative stress as SOD is

important for detoxification of reactive oxygen species either the fruit- and/or fungus-produced (López-Cruz et al., 2017). Ascorbic acid is no longer recycled but consumed by APX in apples with increasing severity of *B. cinerea* infection, presumably due to a disruption in redox equilibrium.

2.5 Conclusions

This study investigated the association between physical and chemical characteristics (e.g., firmness, color, volatiles and antioxidants) of strawberry fruit and their natural spoilage by *B. cinerea*. The spoilage rate is genotype-dependent as it consistently differed between genotypes over two years. Within the same year among seventeen genotypes, a higher intensity of red coloration was associated with a lower spoilage rate whereas a high production of ethyl butanoate and 1-hexanol was associated with a higher spoilage rate. High sugar contents might not play a significant role in fruit spoilage by grey mold. These characteristics may serve as markers for strawberry breeders to select for genotypes with reduced susceptibility and favorable consumer liking.

Author contribution

Hua Li: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Dorthe H. Larsen**: Methodology, Writing - review & editing. **Ruimin Cao**: Investigation, Formal analysis. **Arjen C. Peppel**: Methodology, Resources. **Yury M. Tikunov**: Methodology, Resources, Writing - Review. **Leo F.M. Marcelis**: Writing - Review & Editing. **Ernst J. Woltering**: Conceptualization, Writing - Review & Editing. **Jan A.L. van Kan**: Writing - Review & Editing. **Rob E. Schouten**: Conceptualization, Formal analysis, Writing - Review & Editing.

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Supplementary materials

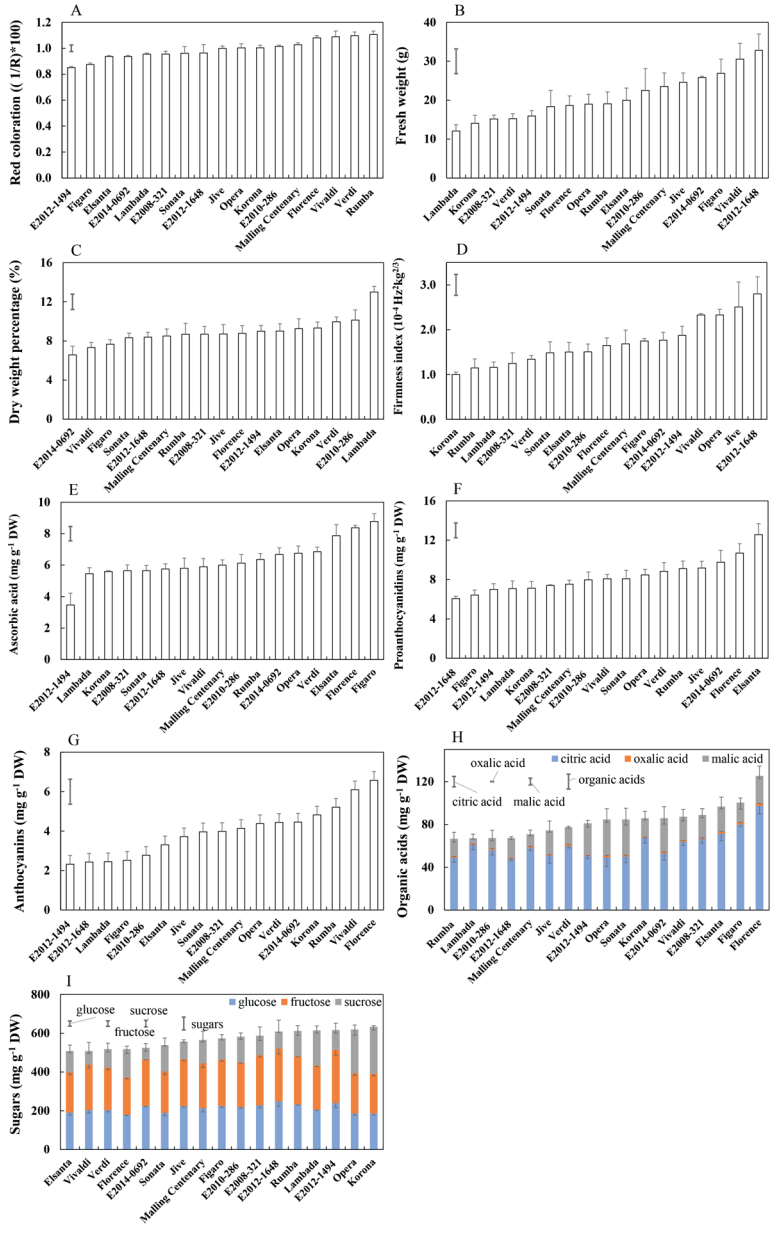

Figure S1 Non-volatile quality characteristics in 17 strawberry cultivars harvested at the red ripe stage in 2019. Bar graphs of (A) red coloration, (B) fresh weight, (C) dry weight percentage, (D) firmness index, (E) ascorbic acid, (F) proanthocyanidins, (G) anthocyanins, (H) organic acids and (I) soluble sugars. One-way analysis of variance test (ANOVA) at α=0.05 is applied due to accepted assumptions for normality and homogeneity ($P > 0.05$). Data represent means (n = 3) and error bars represent standard deviation.

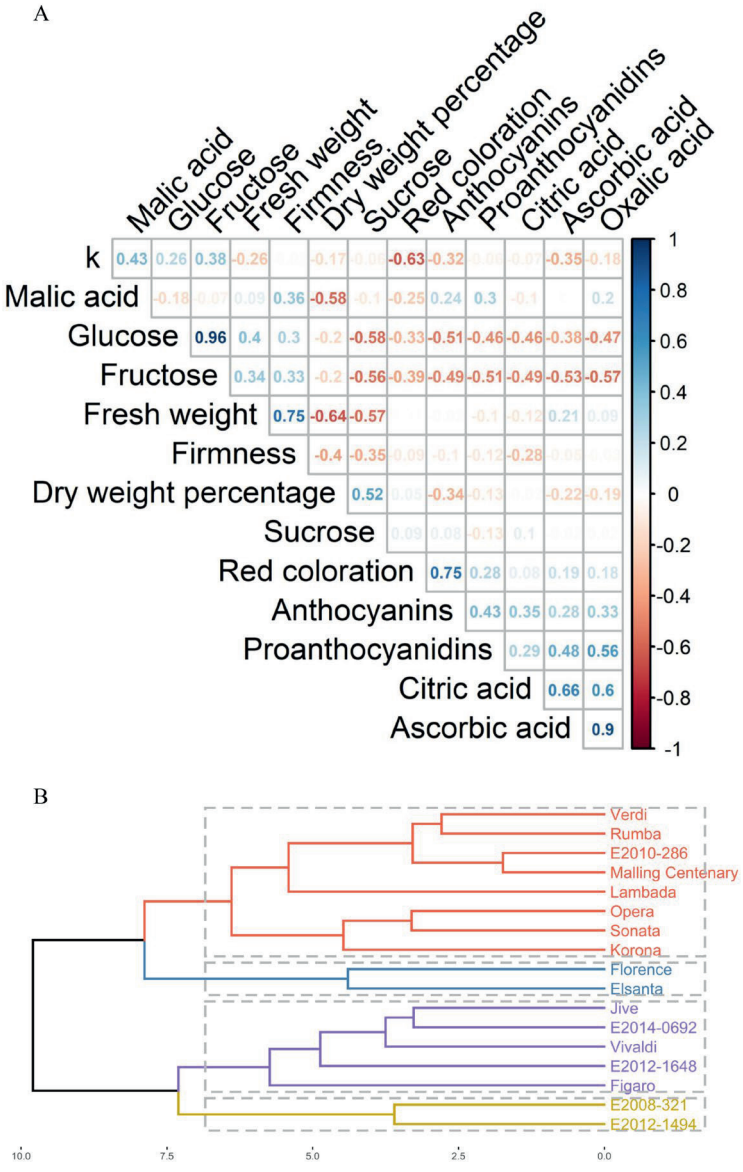

Figure S2 Correlation analysis of non-volatile characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage and hierarchical clustering analysis of genotypes based on volatile characteristics. (A) Correlation matrix of non-volatile fruit characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage in 2019. (B) Hierarchical clustering analysis is computed by the Ward's minimum variance method (ward. D2).

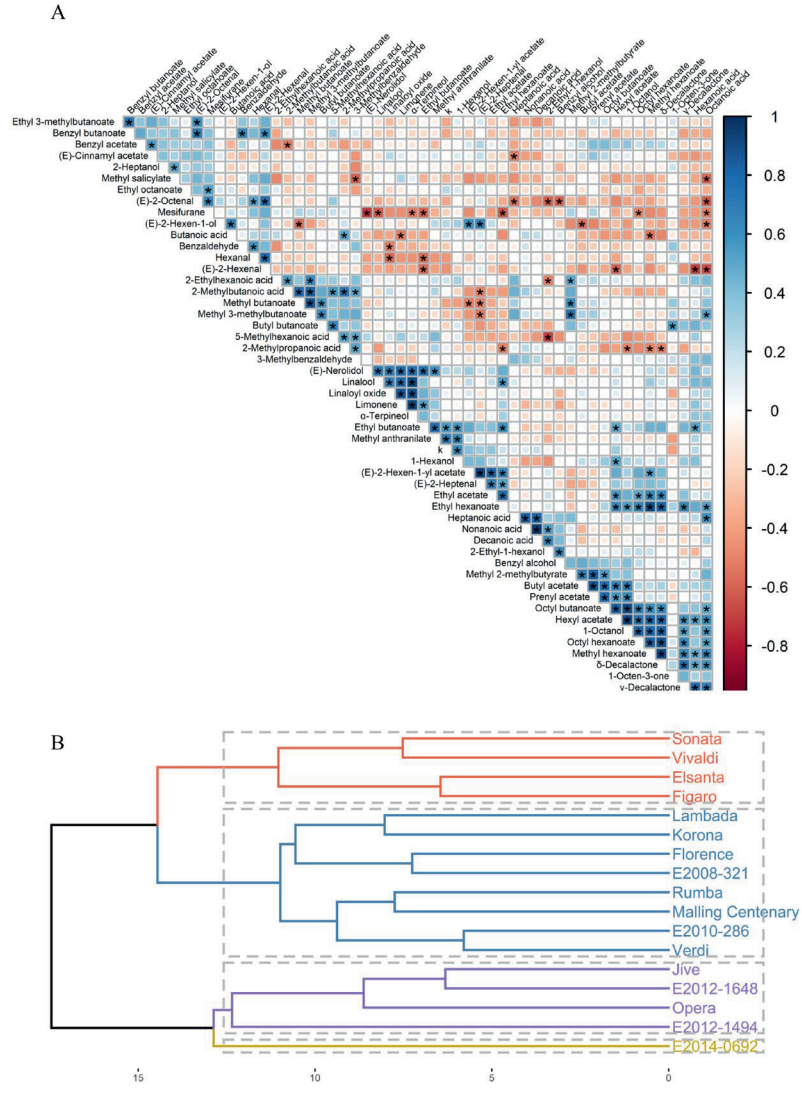

Figure S3 Correlation analysis of volatile characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage and hierarchical clustering analysis of genotypes based on volatile characteristics. (A) Correlation matrix of volatile characteristics and spoilage rates using 17 strawberry genotypes harvested at the red ripe stage in 2019. Asterisks denotes significant correlation coefficients ($P < 0.05$). (B) Hierarchical clustering analysis is computed by the Ward's minimum variance method (ward. D2).

Table S1 Parameter estimates for the analysis of the spoilage data for the 2018 and 2019 datasets for five cultivars with indicated standard deviation.

	2018					**2019**				
Cultivar	Harvest	k_{ref} (day^{-1})		N_0 (-)		sub-batch	k (day^{-1})		N_0 (-)	
		Mean	SD	Mean	SD		Mean	SD	Mean	SD
Elsanta	1	1.21	0.19	3.2	1.8	1			4.89	0.03
						2	0.80	0.06	4.89	0.03
						3			4.91	0.03
Verdi	1	0.93	0.13	6.2	2.4	1			4.88	0.03
						2	0.67	0.05	4.72	0.06
						3			4.79	0.05
E2012-1494	1	1.43	0.19	1.2	0.7	1			4.89	0.04
	2			6.4	2.3	2	1.00	0.08	4.68	0.09
						3			4.79	0.06
E2014-1648	1	1.08	0.12	2.2	0.9	1			4.77	0.05
	2			17.2	3.5	2	0.73	0.05	4.79	0.05
						3			4.78	0.05
Jive	1	0.96	0.08	4.0	1.2	1			4.88	0.03
	2			7.5	1.9	2	0.66	0.05	4.72	0.06
	3			9.4	2.1	3			4.84	0.04
E (kJ mol^{-1})		75.7	3.5							
Adminstrative information										
N_{max}		100					5			
T_{ref} (°C)		12					-			
R^2_{adj} (%)		93.4					98.4			

SD indicates standard deviation.

Chapter 3

Red, blue and far-red light do not affect fruit susceptibility to *Botrytis cinerea* despite altering sugar and secondary metabolite contents in strawberry

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Abstract

Primary and secondary metabolites of strawberry fruit may be involved in the interaction between the host and the ubiquitous fungal pathogen *Botrytis cinerea*. Light is an essential environmental factor regulating both plant growth and defense through its impact on primary and secondary metabolism. This study tested the hypothesis that light spectrum affects the fruit susceptibility to *B. cinerea* through its impact on primary and secondary metabolites of strawberry fruit. Strawberry plants (*Fragaria × ananassa,* 'Furore') were grown in a climate chamber under different red to blue light ratios (90% red : 10% blue, 50% red : 50% blue, 10% red : 90% blue). In addition, the response to additional far-red (FR) (~ 50 µmol m^{-2} s^{-1}) was studied on top of a 50% red : 50% blue light background. In all experiments a total photosynthetic photon flux density (PPFD) of ~180 µmol m^{-2} s^{-1} was applied with a 16 h day length. Fruit at the turning and red ripe stages were harvested for analyses of physical and chemical characteristics (fresh weight, dry weight, firmness, brix, sugars, antioxidants and volatile composition). Harvested red ripe fruit were inoculated with *B. cinerea* conidia and the lesion development was monitored at 12 °C in darkness. Low red to blue light ratios resulted in improved ascorbic acid and proanthocyanidin levels but lower levels of sucrose, higher (E)-2-hexenal, (Z)-3-hexenol. Additional FR light resulted in faster red coloration of fruit on the plant, indicating a faster ripening process. Meanwhile, FR light lowered the levels of ascorbic acid and anthocyanins but stimulated the accumulation of soluble sugars and esters that may improve fruit flavor quality. Despite effects of different light regimes (red to blue ratios with and without additional FR) on fruit ripening and metabolite contents, fruit susceptibility to *B. cinerea* was not affected.

Keywords: *Fragaria × ananassa,* carbohydrates, antioxidants, grey mold, LEDs

3.1 Introduction

Plants rely on the cell wall thickness, cuticle, phytoanticipins (i.e., constitutive phytochemicals) and phytoalexins (i.e., inducible phytochemicals) to cope with pathogen infections (Underwood, 2012; Newman et al., 2013). Efforts have been made to strengthen basal and induced defenses in multiple plant species via genetic or environmental modification. A specific light spectrum during cultivation affects photosynthesis and secondary metabolism (Demotes-Mainard et al., 2016; Huché-Thélier et al., 2016), thereby regulating plant growth and defenses (Lazzarin et al., 2021). Strawberry is an important fruit crop worldwide and the production and shelf life is primarily impeded by the fungal pathogen *Botrytis cinerea* during cultivation and postharvest. In the strawberry fruit-*B. cinerea* pathosystem, the effects of light spectrum during cultivation on the fruit's physical properties, metabolite contents and on their potential to defend themselves against *B. cinerea* have not been studied yet.

Plants use light spectra between 400 nm and 700 nm for photosynthetic activity (Davis and Burns, 2016) and perceive this via a set of photoreceptor proteins such as phytochromes, cryptochromes, phototropins and UVR8 (Thoma et al., 2020). Light spectrum modulates plant defenses against necrotrophic pathogens via endogenous phytohormonal signal-transduction predominantly orchestrated by jasmonic acid (JA) (Glazebrook, 2005; Moreno and Ballaré, 2014; AbuQamar et al., 2017). Amongst the spectra, red (600-700 nm), blue (400-500 nm) and far-red (700-800 nm, FR) light are mostly studied. A lower R:FR ratio causes a photoconversion of phytochromes from the active FR-absorbing form (Pfr) to inactive R-absorbing form (Pr) (Franklin, 2008; Legris et al., 2019). Inactivation of phytochrome B (phyB) reduces JA-dependent defense responses by promoting DELLA (repressors of gibberellic acid (GA)-responsive plant growth) degradation and increasing JASMONATE ZIM-DOMAIN (JAZ) (inhibitor of JA responsive plant defense) stability (Ballaré, 2014; Leone et al., 2014). This results in shifting the balance away from JA-mediated defense and toward gibberellic acid (GA)-mediated shade-avoidance syndrome responses (Ballaré, 2014). Intrinsic JA levels could be reduced by FR-induced upregulation of a sulfotransferase (ST2a) (Fernández-Milmanda et al., 2020). Moreover, the combination of red (600-700 nm) and blue light (400-500 nm) increased intrinsic JA levels compared to white light with FR (380-760 nm) in *Arabidopsis thaliana* (Mirzahosseini et al., 2020). However, it needs further elaboration on how the change in basal JA levels influences downstream metabolism and defense responses in plants.

Soluble sugars produced by plants are carbon sources for fungal growth and reproduction. Tomato leaves were more susceptible to *B. cinerea* infections when plants were grown under additional FR light (Ji et al., 2019; Courbier et al., 2020). Courbier et al. (2020) proposed that additional FR light increased the leaf susceptibility to *B. cinerea* by elevating glucose and fructose contents and reducing JA-mediated defense responses, which may also hold true for fruits. There is also evidence in favor of sugar-mediated enhancement of plant defense (Bolouri Moghaddam and Van den Ende, 2012; Lemonnier et al., 2014; Bezrutczyk et al., 2018). It remains unclear whether or not light spectrum, especially additional FR light would affect fruit sugar contents and the susceptibility to *B. cinerea*.

An 'oxidative burst' is one of the early plant defense responses to pathogens, which refers to the production of reactive oxygen species (ROS) (Chadha, 2019). Antioxidants in strawberry fruit, such as ascorbic acid, anthocyanins and proanthocyanidins may play roles in neutralizing excessive produced ROS thereby reducing the oxidative stress. Davey et al. (2007) reported that a higher ascorbic acid content in apple fruit was associated with less severe *B. cinerea* infection and enzymes that are known for scavenging ROS might be involved (Bui et al., 2019). Ascorbic acid content in tomato fruit can be influenced by light spectra (Ntagkas et al., 2019; Zushi et al., 2020). Light spectrum may also affect the levels of anthocyanin and proanthocyanidins, both are flavonoids synthesized from the phenylpropanoid pathway. Strawberry fruit grown under 50% : 50% red : blue light contained increased levels of anthocyanins and proanthocyanidins in fruit compared to fruit grown with either white light or monochromatic red or blue light (Zhang et al., 2018a). Fruit crops abundant in proanthocyanidins or anthocyanins show reduced susceptibility to *B. cinerea* (Jersch et al., 1989; Saks et al., 1996; Schaefer et al., 2008; Bassolino et al., 2013; Zhang et al., 2013) and flavonoids are considered as direct defense-related metabolites (Lazzarin et al., 2021).

Volatile compounds are considered to be involved in plant-pathogen interactions in an indirect manner (Lazzarin et al., 2021). Green leaf volatiles (GLVs), mainly fatty acid-derived C6 compounds are capable of triggering plant defense responses, particularly in 'priming' vegetative plant tissues against fungal attack (Scala et al., 2013). For instance, Myung et al. (2007) proposed that C6 aldehydes may chemically modify *B. cinerea* secreted proteins during interaction on the surface of fungi, potentially interfering with fungal virulence. The antifungal activity (E)-2-hexenal might result from its role in altering the conidial structures of the cell wall and plasma membrane, causing cell death (Arroyo et al., 2007). Strawberry has a distinct and complex volatile profile,

of which some esters and GLVs might act as early indicators to aid penetration by *B. cinerea* (Neri et al., 2015; Xu et al., 2021). Low R:FR partially reduced production of constitutive and methyl jasmonate (MeJA)-induced GLVs and terpenoids emitted in *Arabidopsis* (Kegge et al., 2013).

In the current study, we investigated how light spectra (i.e., red, blue and FR) during cultivation affect strawberry fruit susceptibility to *B. cinerea*. Different red to blue light ratios with and without FR light were applied to strawberry plants. Fruit at the turning and red ripe stages were harvested for analysis of their physical and chemical characteristics (fresh weight, dry weight percentage, firmness, brix, sugars, antioxidants, volatile composition). Red ripe fruit were inoculated with *B. cinerea* spores and the progress of infection was monitored. We hypothesized that light spectrum affects the susceptibility through its impact on primary and secondary metabolites of fruit. More specifically, low red to blue light ratios might reduce the susceptibility to *B. cinerea* infection through increased accumulation of antioxidants such as proanthocyanidin, anthocyanin and ascorbic acid levels. Adding FR to a red and blue light background may stimulate *B. cinerea* infection by elevating soluble sugar contents.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Two batches of everbearing strawberry (*Fragaria × ananassa,* 'Furore') plants from the cold storage were grown. The first batch in October 2020 was obtained from Eck en Wiel, the Netherlands; the second batch in January 2021 was obtained from Luttelgeest, the Netherlands. Plants were transplanted in 3 L plastic pots with two plants per pot and transferred to a climate chamber. The potting soil contained horticultural clay, Swedish sphagnum peat, Baltic peat, garden peat, dolokal extra potting soil (bulk), PG-mix. Treatments started at the moment of transplanting. The climate chamber was divided into 8 compartments of W 0. 6 × L 1.4 × H 1.5 m and the plant density was 19 plants m^{-2}. The compartments were separated by white reflective white plastic. Day and night temperatures were maintained at 22 °C and 15 °C and the relative humidity was set at 70% and 50%, respectively. Relative humidity (RH) and temperature in each compartment were recorded with keytag dataloggers (KTL-508, Keytag, The Netherlands). Measured values were within 10% RH and 1 °C relative to the setpoints during the course of the experiment. Fertigation (electrical conductivity 1.6 dSm^{-1}, pH 5.7-6.0; containing 1.0 mM NH_4^+, 5.0 mM K^+, 3.0 mM Ca^{2+}, 1.2 mM Mg^{2+}, 10.8 mM NO_3^-,

1.4 mM SO_4^{2-}, 0.9 mM PO_4^{2-}, 35 µM Fe^{3+}, 15.0 µM Mn^{2+}, 10.0 µM Zn^{2+}, 15 µM B, 0.9 µM Cu^{2+}, 0.8 µM MoO_4^{2-}) was applied together with water four times per week. Spical and Swirski Ulti-Mite (Koppert biological systems, The Netherlands) were introduced every two weeks for biological control of aphids. Brown leaves caused by *B. cinerea* infections were removed weekly. After 4-week cultivation, small plastic cages were placed in the pots to support the plants during growth.

3.2.2 Light treatments

Each compartment was equipped with LED lights (Green Power LED Research Module, Philips, Eindhoven, The Netherlands) with blue (400-500 nm), red (600-700 nm) and far-red (700-800 nm) emitting diodes. Spectral distribution and photon flux density (PFD) were measured with a spectroradiometer (Specbos 1211UV, Jeti, Jena, Germany). The treatments were 10% red : 90% blue (1:9 R:B), 90% red : 10% blue (9:1 R:B), 50% red : 50% blue (1:1 R:B), 50% red : 50% blue + ~ 50 µmol m^{-2} s^{-1} far-red (1:1 R:B+FR) (Table 1). A photoperiod of 16 h and a photosynthetic photon flux density (PPFD) of ~ 180 µmol m^{-2} s^{-1} at the top of the plant was provided from transplanting until end of end of experiment (approximately 70 days). Plants were randomized twice a week within each compartment to minimize variations in the light distribution. The height of the LED modules was adjusted weekly to maintain a constant light intensity at the top of the plants throughout the experiment.

Table 1 Light levels measured at the top of plant.

Light spectra	PPFD[a]	Blue[b]	Green[c]	Red[d]	Far-red[e]	PSS[f]
	µmol m^{-2} s^{-1}					
1:1 R:B+FR[g]	178.1 ± 2.9	89.0 ± 2.2	0.9 ± 0.1	87.9 ± 0.8	50.3 ± 0.4	0.76
1:1 R:B	174.0 ± 1.1	83.6 ± 2.5	1.1 ± 0.1	89.3 ± 0.9	0.8 ± 0.1	0.87
1:9 R:B	183.0 ± 2.8	163 ± 2.6	1.5 ± 0.1	18 ± 0.1	0.2 ± 0.0	0.74
9:1 R:B	180.0 ± 0.1	17.3 ± 0.3	0.4 ± 0.0	162.2 ± 0.2	0.8 ± 0.0	0.88

[a]PPFD: photosynthetic photon flux density (400-700 nm) measured at the top of plants. Values are the means ± standard error of means (n = 4) of four blocks of each treatment. The light intensity of each light compartment was based on 12 measurements equally distributed over the illuminated area.
[b-e] Blue (400-500 nm), green (500-600 nm), red (600-700 nm), and far-red (700-800 nm).
[f] PSS: Phytochrome photostationary state, calculated according to Sager et al. (1988).
[g] R:B, red : blue; FR, far-red.

3.2.3 Fruit color monitoring and fruit sampling

Three times per week manual pollination with a paint brush was applied to fully open flowers and individual flowers were labelled following pollination. During fruit

formation starting at the green white stage, fruit color was recorded 3 times per week by using a color scale (score 1 for green-white; score 2 for white; score 3 for turning; score 4 for ½ pink-red; score 5 for ¾ pink-red; score 6 for fully pink-red; score 7 for fully light-red; score 8 for fully red ripe) (Figure 1) (Schaart et al., 2013; Neri et al., 2015).

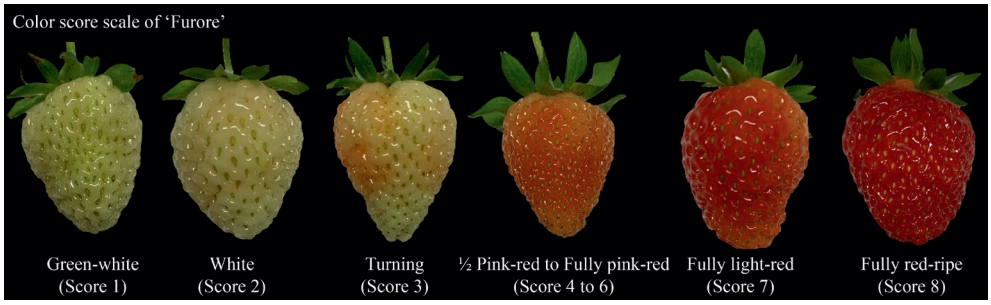

Figure 1 Color scales of ripening stages in 'Furore' strawberry fruit.

From each compartment, fruit were harvested at two color stages (turning and fully red ripe) for 2 to 6 times during the cultivation period, depending on the treatment. For each harvest time, 5 fruit at the turning stage and 5 at the fully red ripe stage were used for physical and chemical analyses at harvest; 5 fruit in fully red ripe stage were stored at 12 °C and ~85% RH for 3 days, followed by physical and chemical analyses; 10 fruit in fully red ripe stage were inoculated with *B. cinerea* spore suspension and stored at 12 °C and ~85% RH for 5 days to quantify lesion expansion. The fruit physical and chemical analyses included fresh weight, firmness, brix, dry weight percentage, soluble sugars, ascorbic acid, anthocyanins, proanthocyanidins, volatile composition.

Following initial fruit quality measurements (weight, firmness, brix), fruit of the same stage and harvest were then pooled and frozen with liquid nitrogen, ground into powder using an analytical grinder (IKA A11, IKA, Staufen, Germany) and stored at -80 °C until further analysis. Part of the frozen powder was freeze-dried for sugar extraction and to determine dry weight percentage, allowing to express all nutrients per g dry weight (except for volatile composition, see below).

3.2.4 *Botrytis cinerea* growth and inoculation

B. cinerea strain B05.10 was cultured on Malt Extract Agar (MEA) Medium in petri dishes and grown for approximately 1 week in darkness, followed by 24-h UV light treatment to induce sporulation (Hayashi et al., 2001). Subsequently, the Petri dishes were returned to darkness for another 4 to 5 days. The temperature of the whole growth period was maintained at 20 °C. Conidia were harvested using sterile distilled water. The suspension was filtered through glass wool and conidia were pelleted by

centrifugation at 15,000 × *g* for 10 min and resuspended in sterile water. Conidia titers were determined microscopically using a Thoma counting chamber and the concentration was adjusted to 1×10^7 conidia mL^{-1} and diluted with sterile distilled water to a final concentration of 500 conidia μl^{-1}. Individual fruit were punctured with a sterile pipette tip to a depth of 2 mm on two opposite sides on the fruit equator. One side was inoculated with 2 µL of the conidia suspension as the infection; the other side was inoculated with 2 µL sterile distilled water as control (mock). The strawberries were placed in an incubation tray with a lid and stored at 12 °C and about 100% RH in darkness. The length and width of the fungal lesion was measured on day 3 to 5 post-inoculation with the lesion area (A) calculated as $A = \pi ab/4$ (a and b are the length and width, respectively).

3.2.5 Measurement of quality characteristics

Fresh weight, firmness and brix

Harvested fruit were weighed individually using a balance. Then the fruit was cut into two halves for firmness measurement. Fruit firmness was measured using a universal testing machine (Zwick Z2.5/TS1S, Ulm, Germany). Fruit were cut in half lengthwise and both halves were compressed at the equator with a probe (ø 2.5 mm) at a constant plunger speed (2.5 mm s^{-1}) to a fixed penetration depth (5 mm). The maximum force (N) was recorded during penetration of the fruit piece. Brix was measured by a refractometer (PAL-1, Atago, Japan) with a drop of juice squeezed out of the fruit from both two halves right after the firmness measurement.

Sugars

Fifteen mg of freeze-dried powder from the pooled samples was mixed with 5 mL 75% ethanol followed by shaking and incubation in a water bath at 80 °C for 20 min, then centrifuged at 8500 × *g* for 5 min at 4 °C. One milliliter supernatant was dried using a vacuum centrifuge (Savant SpeedVac SPD2010, Thermo Fisher Scientific) at 55 °C for 2.5 h. The pellet was resuspended with 1 mL distilled water and sonicated for 10 min, followed by centrifugation for 10 min at 4 °C at 14,800 × *g*. The supernatant was diluted 50 times with distilled water before analysis. The samples were loaded onto a High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Scientific), equipped with a CarboPac1 column (250 × 2 mm) eluted with 100 mM NaOH at a flow rate of 0.25 mL min^{-1} at 25 °C. Calibration of the HPLC was performed with glucose, fructose and sucrose standards (Sigma-Aldrich).

Ascorbic acid

Ascorbic acid content was analyzed by adding 0.2 g of the frozen powder from the pooled samples to 1 mL of 3.3% meta-phosphoric acid. The mixture was sonicated in darkness with ice water for 10 min and centrifuged at 10,000 × *g* and 4 °C for 10 min. The supernatant was filtered through a 0.45 µm cellulose filter prior to HPLC analysis according to Van Treuren et al. (2018). Calibration of the HPLC was performed with ascorbic acid standards (Sigma-Aldrich).

Anthocyanins

Frozen powder (0.5 g) from the pooled samples was dissolved in 1.5 mL 50% methanol containing 1% formic acid. The mixture was then centrifuged for 15 min at 10,000 × *g* and 4 °C. The supernatant was filtered through a 0.45 µm cellulose filter with the absorbance measured at 510 nm using a UV-Vis spectrophotometer (Varian Cary 4000, Canada). Pelargonidin-3-glucoside (Extrasynthase) was used as standard.

Proanthocyanidins

Proanthocyanidin content was quantified according to Prior et al. (2010). Twenty mg freeze-dried powder from the pooled samples was mixed with 10 mL extraction solution (75% acetone, 24.5% distilled water and 0.5% acetic acid). The mixture was vortexed for 30 s and placed into an ultrasonic bath for 30 min at room temperature. The mixture was then shaken for 1 h at room temperature, followed by centrifugation for 20 min at 8,500 × *g* and 12 °C. An aliquot of 70 µL of the supernatant was mixed with 210 µL acidified ethanol (12.5% HCl, 12.5% distilled water and 75% ethanol) containing 0.1% 4-dimethylaminocinnamaldehyde. The analysis was conducted using a plate reader (CLARIOstar Plus, BMG Labtech, Germany) and the absorbance of each well in the plate was read at 640 nm every minute, in total 30 min. Different concentrations (20, 50 and 100 µg mL^{-1}) of catechin (Extrasynthese) was used to quantify the content of proanthocyanidins.

Volatile compound detection by SPME/GC-MS and data processing

Extraction and detection of volatile metabolites were performed according to Tikunov et al. (2005) with slight modifications. Frozen powder of 0.5 g from the pooled samples was weighed into a 5 mL vial and 0.5 mL distilled water was added. The vials were closed and incubated at 30 °C water bath for 10 min. After adding 1 mL 100 mM, pH 7.5 EDTA/NaOH, 2.2 g solid $CaCl_2$ powder was mixed thoroughly to inhibit enzyme activities. One mL of the extract was transferred into a 10 mL crimp cap vial for headspace

SPME/GC–MS detection. Individual vials were randomized to avoid systematic memory effects and placed into a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). A 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre (Supelco, Bellefonte, USA) was exposed for 20 min to the vial headspace under continuous agitation and heating at 50 °C. The trapped compounds by SPME were desorbed into a Trace GC Ultra gas chromatograph (Thermofisher, Waltham, USA) injector for 1 min at 250 °C. Chromatographic separation was achieved on an Zebron ZB-5 (50 m × 0.32 mm × 1.05 µm) column (Phenomenex) with helium as the carrier gas (at constant flow 2 mL min^{-1}). The GC interface and MS source temperatures were 260 and 250 °C, respectively. The GC temperature program started at 45 °C for 2 min, was then increased to 250 °C at a rate of 5 °C min^{-1} and finally held at 250 °C for 5 min. Including oven cooling, the total run time was 60 min. Mass spectra in the 35 to 400 m/z range were recorded by an DSQII electron impact MS (Thermofisher) at a scanning speed of 2.8 scans s^{-1} and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using Xcalibur software (http://www.thermo.com). The raw data generated by SPME/GC-MS were processed by MetAlign™ software package (http://www.metalign.nl) for baseline correction, noise estimation and ion-wise mass spectral alignment. The VOCs mass spectra and quantitative ion fragments were extracted using MSClust software (http://www.metalign.nl) (Tikunov et al., 2012). VOCs were identified by matching mass spectra and the retention indices of the compounds extracted to the NIST mass spectral library using NIST MS Search software (http://www.nist.gov).

3.2.6 Statistical set-up and analysis

The whole experiment was performed twice, each time with one batch of plants distributed over four light treatments; each light treatment executed in two separate compartments. This results in 4 blocks (n = 4; 16 replicate plants per block). Data on plant height, flower number, days from anthesis to certain fruit stages and lesion area (approximately 30 fruit per block) that was analyzed using one-way analysis of variance (ANOVA). When fruit at different ripening stages were used for fruit firmness, brix and all phytochemical measurements, the experiment was analyzed as a split-plot design with light spectrum as main factor and fruit maturity as sub-factor. These measurements were performed on approximately 30 fruit per block from the turning or the red ripe fruit at harvest and the red ripe fruit after 3 days of storage respectively. The average value of each block was used for statistical analysis.

Statistical treatment effects were calculated at α=0.05 and assumptions for normality and homogeneity of residuals were evaluated with Bartlett's test and Shapiro-Wilk test, respectively at $P > 0.05$. Fisher's protected least significant difference (LSD) test was used as post-hoc test for the parameters with significant treatment effects. The analyses were performed in Genstat (19th Edition, VSN International Ltd., Hemel Hempstead, UK). Partial least squares discriminant analysis (PLS-DA) and heatmap were performed in R 3.5.1 (R Core Team, 2021) using the packages of mixOmics and heatmap.2 respectively.

3.3. Results

3.3.1 Plant morphology and fruit coloration

Adding FR to red and blue background light increased plant height, whereas changing the red to blue light ratio did not affect elongation (Figure 2A, B). Plants grown with additional FR developed more flowers than plants grown without FR in weeks 4 and 5, but by week 6 there was no difference anymore (Figure 2C). No difference in flower number was observed in plants cultivated under different red to blue light ratios.

The fruit skin showed about 2 to 3 d earlier coloration with FR during cultivation than in fruit cultivated without FR (Figure 2D). Red to blue light ratios did not affect the rate of skin coloration.

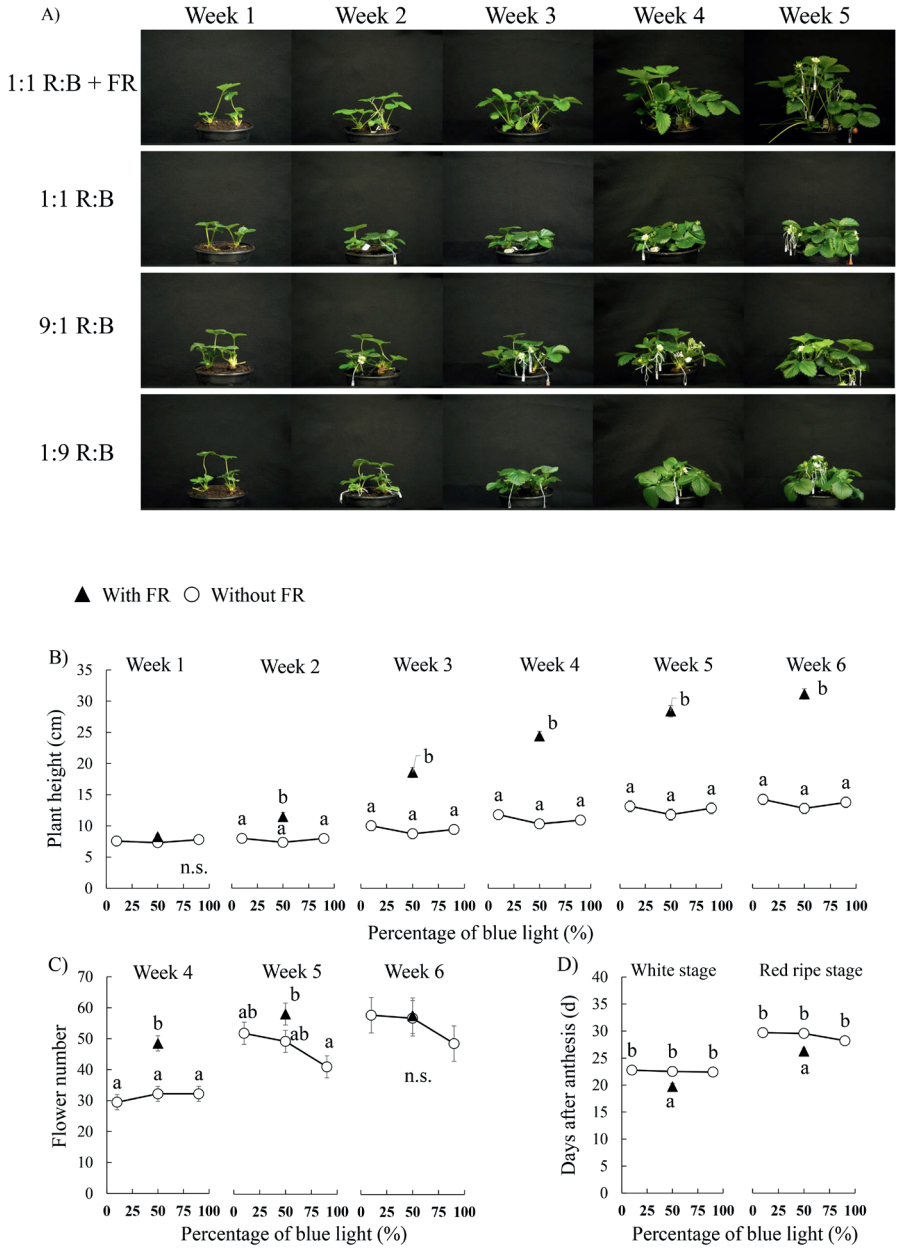

Figure 2 Plant morphology and fruit coloration of 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. A) Plant development from week 1 until week 5 under different light treatments, B) plant height recorded from week 1 until week 6, C) newly open flowers recorded in week 4, 5 and 6, D) days from anthesis until reaching the white and the red ripe stage. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Data represent means (n = 4 with 16 replicate plants per block) and error

bars represent standard error of means when larger than symbol size. Different letters denote significant differences for light spectra; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$).

3.3.2 Fruit fresh weight, dry weight percentage, firmness

There was no significant interaction between effects of light spectrum and fruit maturity on fresh weight, dry weight percentage and firmness (Figure 3). Fruit fresh weight and dry weight percentage increased whereas the firmness decreased from the turning to the red ripe stages. The fruit fresh weight generally decreased with decreasing red to blue light ratios; adding FR did not affect fruit fresh weight (Figure 3A). The dry weight percentage was not affected by different red to blue light ratios but was higher in fruit cultivated under additional FR (Figure 3B). Fruit firmness was reduced by the lowest red to blue light ratio and by additional FR light (Figure 3C).

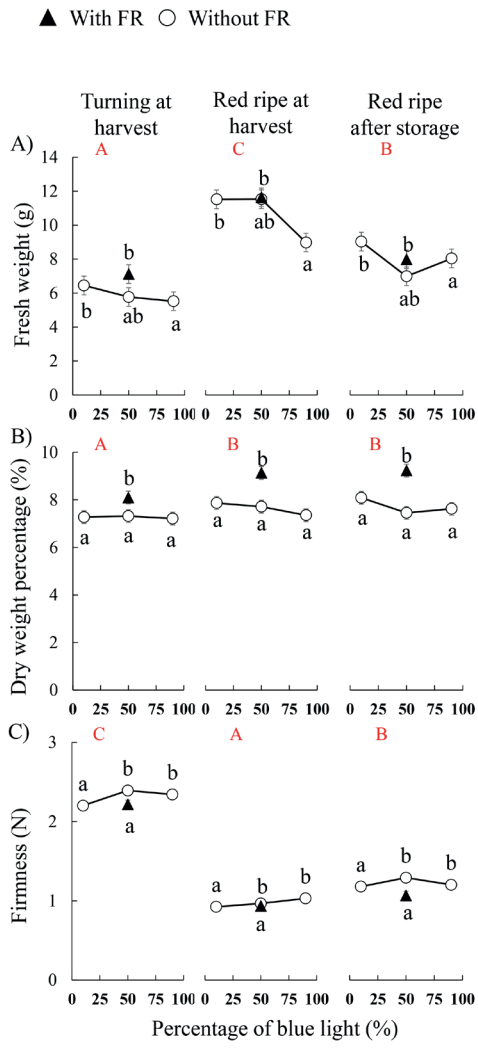

Figure 3 Fruit fresh weight, dry weight percentage and firmness of 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. A) Fresh weight, B) dry weight, C) firmness of fruit at the turning or the red ripe stage at harvest, or the red ripe after 3-d storage at 12 °C in darkness. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Data represent means (n = 4 with 16 replicate plants per block; each block contained approximately 30 replicate fruit). Error bars represent the standard error of means when larger than symbol size. The interaction between light spectrum and fruit maturity was not significant on fresh weight, dry weight percentage and firmness. Uppercase letters in red denote significant differences for fruit maturity and lowercase letters denote significant differences for light spectrum; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$).

3.3.3 Fruit soluble sugars and brix

There was no significant interaction between effects of light spectrum and fruit maturity on individual soluble sugars (glucose, fructose and sucrose) and brix of strawberries (Figure 4). In general, strawberry soluble sugar levels and brix values increased from the turning to the red ripe stage. After 3-d storage, glucose and fructose contents increased whereas the sucrose content decreased, which were associated with decreased brix values in red ripe strawberries. The contents of glucose and fructose levels as well as brix values were not affected by different red to blue light ratios but the sucrose content decreased with decreasing red to blue light ratios. Adding FR light generally elevated contents of glucose, fructose and sucrose as well as brix values of strawberry fruit.

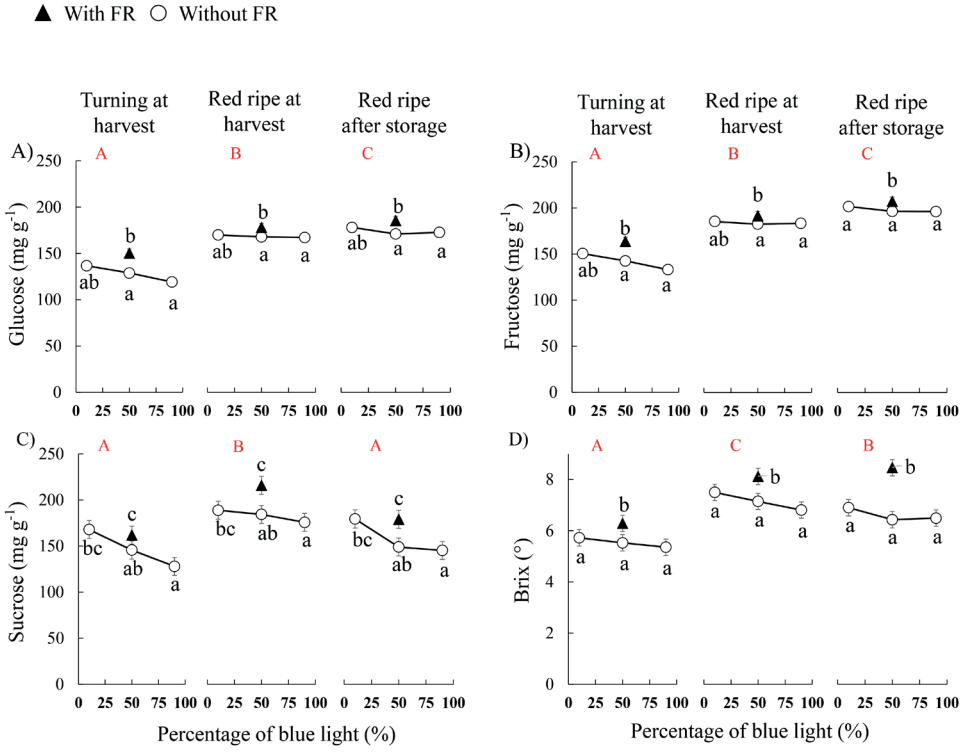

Figure 4 Soluble sugars (sucrose, glucose and fructose) and brix in 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. (A) Glucose, (B) fructose, (C) sucrose and (D) brix of fruit at the turning or the red ripe stage at harvest, or the red ripe after 3-d storage at 12 °C in darkness. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Data represent means (n = 4 with 16 replicate plants per block; each block contained approximately 30 replicate fruit). Error bars represent the standard error of means when larger than symbol size. The interaction between light spectrum and fruit

maturity were not significant on individual soluble sugars and brix. Uppercase letters in red denote significant differences for fruit maturity and lowercase letters denote significant differences for light spectrum; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$). Data are expressed on the base of per g dry weight.

3.3.4 Fruit proanthocyanidins, anthocyanins and ascorbic acid

There was no significant interaction between effects of light spectrum and fruit maturity on proanthocyanidin, anthocyanin and ascorbic acid contents of strawberry fruit (Figure 5). When strawberries ripened from the turning to the red ripe stage, the proanthocyanidin content decreased whereas the anthocyanin and ascorbic acid contents increased; no significant changes of all compounds were observed during 3-d storage compared to the levels at harvest. Low red to blue light ratios resulted in the elevated proanthocyanidin content whereas adding FR did not affect the proanthocyanidin levels (Figure 5A). In contrast, different red to blue light ratios had no effect on anthocyanin accumulation but additional FR light decreased the anthocyanin level (Figure 5B). The content of ascorbic acid increased with decreasing red to blue light ratios whereas decreased by adding FR (Figure 5C).

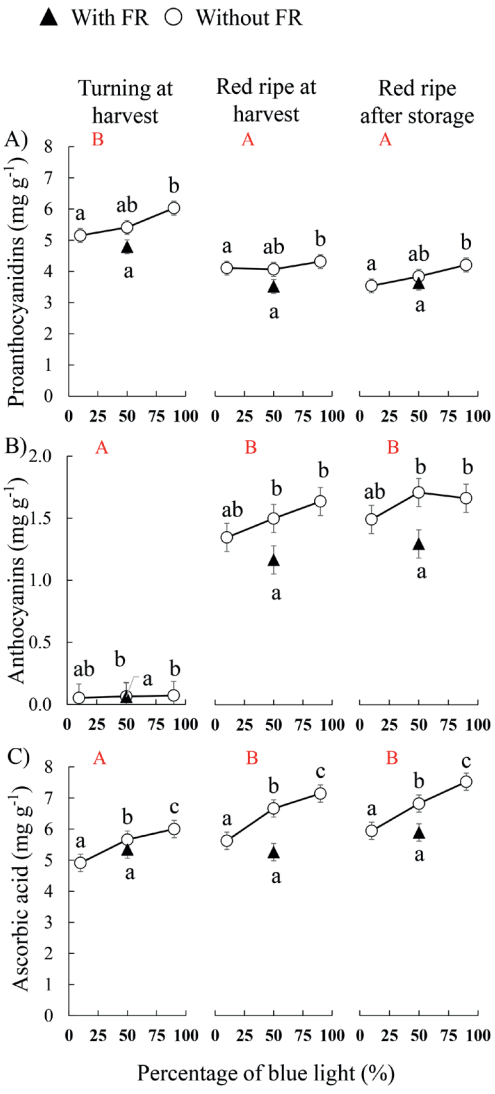

Figure 5 Proanthocyanidins, anthocyanins and ascorbic acid in 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. (A) Proanthocyanidins, (B) anthocyanins and (C) ascorbic acid of fruit at the turning or the red ripe stage at harvest, or the red ripe after 3-d storage at 12 °C in darkness. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Data represent means (n = 4 with 16 replicate plants per block; each block contained approximately 30 replicate fruit). Error bars represent the standard error of means when larger than symbol size. The interaction between light spectrum and fruit maturity was not significant on proanthocyanidin, anthocyanin and ascorbic acid contents. Uppercase letters in red denote significant differences for fruit maturity and lowercase letters denote significant differences for light spectrum; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$). Data are expressed on the base of per g dry weight.

3.3.5 Fruit volatile composition

Thirty-three volatile compounds, showing overlap between the two batches of strawberries harvested from plants with different planting dates, were tentatively identified; among others these included nine esters, nine volatile acids, seven alcohols and six aldehydes. An initial PLS-DA analysis on the log 10 transformed peak heights showed that volatile profiles from the red ripe fruit at harvest and red ripe fruit after 3-d storage greatly overlapped (data not shown). Only data of fruit sampled at the turning and the red ripe stages at harvest were therefore displayed (Figure 6). Four clusters were presented in the PLS-DA plot, in which the two ripening stages were separated along the 1st component (48% explained variation) and the two batches were separated along the 2nd component (28% explained variation) based on the variation in volatile production (Figure 6A). Within each cluster, data from different red to blue light ratios showed considerable overlap; the data from additional FR treatment grouped in the left bottom of the main clusters. Five volatile compounds along the 1st component with the highest loadings were (E)-2-hexen-1-ol acetate, methyl butanoate, methyl hexanoate, (Z)-3-hexen-1-ol, butyl butanoate (Figure 6B). Five volatile compounds along the 2nd component with the highest loadings were linalool, 2-ethylhexanoic acid, heptanoic acid, benzyl acetate, α-terpineol (Figure 6C). The differences in the volatile production from the two batches of plants were much greater than the differences caused by maturities and light treatments (Figure S1).

Volatile compounds that were significantly affected by light spectra are presented in Figure 7. Additional FR light generally elevated the production of several esters (Figure 7A-C, E), benzaldehyde (Figure 7D), 2-methylpropanoic acid (Figure 7G), which showed increasing trends with ripening. In contrast, the level of (Z)-3-hexen-1-ol in fruit at the turning stage was reduced by additional FR light (Figure 7F). Low red to blue light ratios resulted in higher abundance of (Z)-3-hexen-1-ol and (E)-2-hexenal (Figure 7H), but lower abundance of 2-methylpropanoic acid and benzyl acetate (Figure 7I).

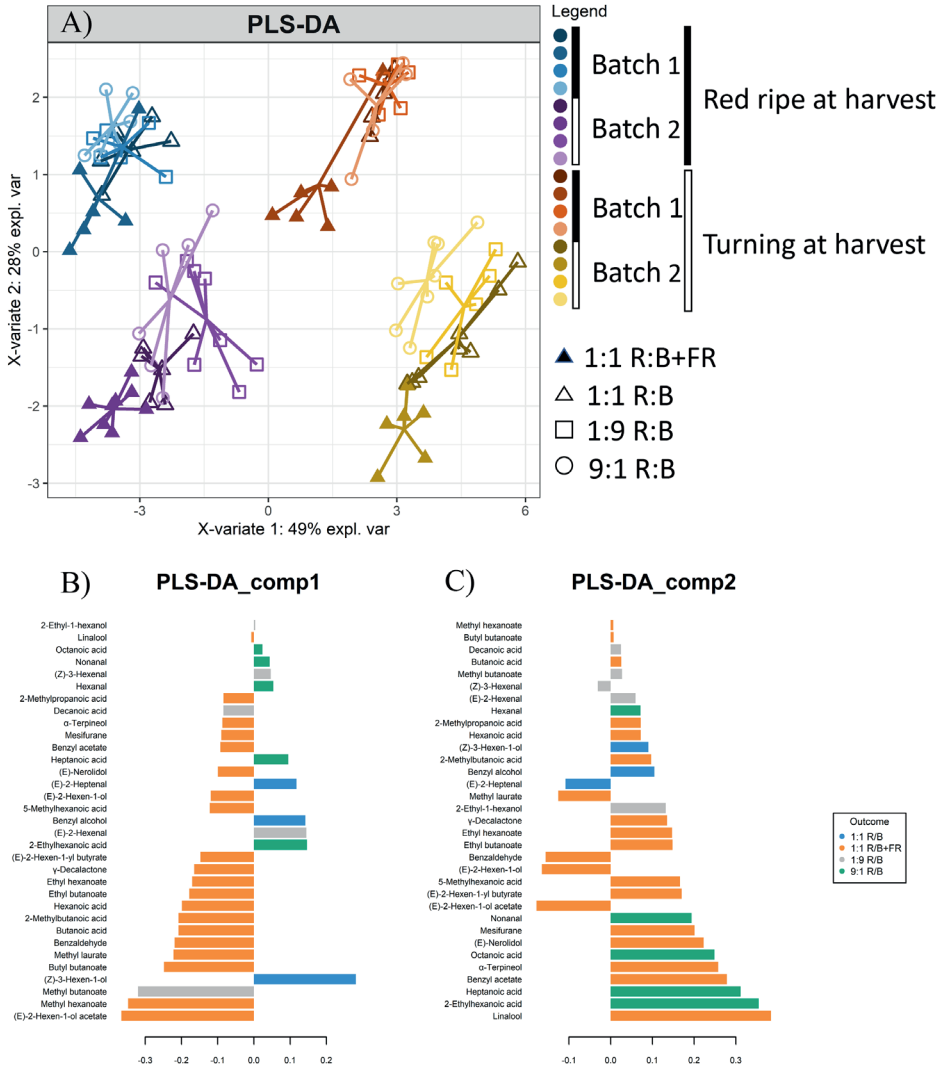

Figure 6 Volatile profiling of 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. (A) Partial least squares discriminant analysis (PLS-DA) of volatile profiling at the turning (orange- and yellow-colored points) and the red ripe stages at harvest (blue- and purple-colored points) from two plant batches. Individual data points represent individual harvests (5 to 10 fruit per harvest). Loading plots of first component (B) and second component (C) showing contribution of volatile compounds to clustering of data points. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} (700-800 nm). Light spectra are 10% red : 90% blue (1:9 R:B), 90% red : 10% blue (9:1 R:B), 50% red : 50% blue (1:1 R:B), 50% red : 50% blue : far-red (1:1 R:B+FR). Data used for PLS-DA are log10 transformed of peak height originated from SPME/GC-MS.

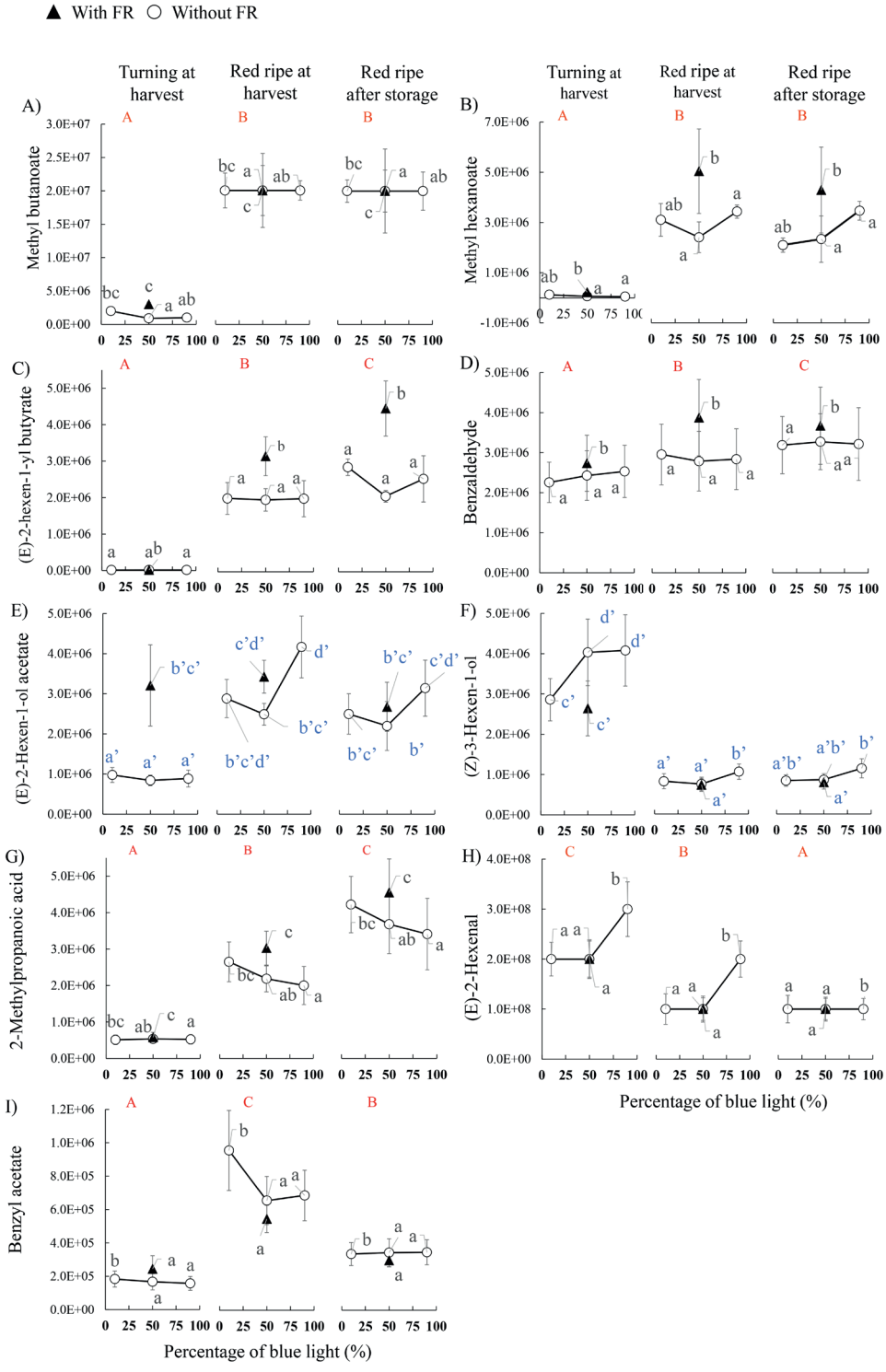
▲ With FR ○ Without FR
Turning at harvest
Red ripe at harvest
Red ripe after storage
A)
Methyl butanoate
B)
Methyl hexanoate
C)
(E)-2-hexen-1-yl butyrate
D)
Benzaldehyde
E)
(E)-2-Hexen-1-ol acetate
F)
(Z)-3-Hexen-1-ol
G)
2-Methylpropanoic acid
H)
(E)-2-Hexenal
I)
Benzyl acetate
Percentage of blue light (%)

Figure 7 Individual volatile compounds in 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. (A) Methyl butanoate, (B) methyl hexanoate, (C) (E)-2-hexen-1-yl butyrate, (D) benzaldehyde, (E) (E)-2-hexen-1-ol acetate, (F) (Z)-3-hexen-1-ol, (G) 2-methylpropanoic acid, (H) (E)-2-hexenal, (I) benzyl acetate of fruit at the turning or the red ripe stage at harvest, or the red ripe after 3-d storage at 12 °C in darkness. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Data represent means (n = 4 with 16 replicate plants per block; each block contained approximately 30 replicate fruit) of the peak height from SPME/GC-MS for individual volatiles and error bars represent the standard error of means. Data used for statistical treatment effects were log10 transformed). If the interactions between light spectrum and fruit maturity were not significant, uppercase letters in red denote significant differences for fruit maturity and lowercase letters denote significant differences for light spectrum; if the interactions between light spectrum and fruit maturity were significant, lowercase letters with apostrophes are used to denote the interaction effects; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$).

3.3.6 Fruit lesion development

Red ripe fruit were inoculated with *B. cinerea* spores immediately after harvest. Lesion started to expand three days post inoculation (Figure 8). Light treatments had no significant effects on the lesion area.

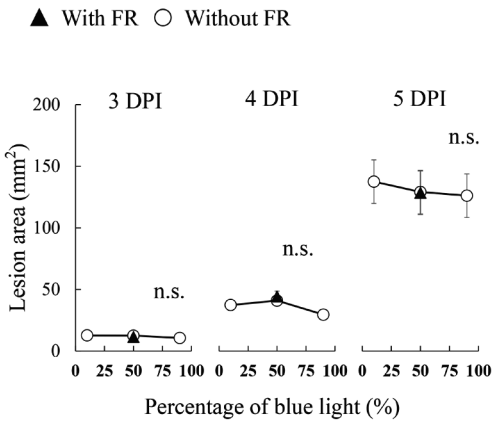

Figure 8 Lesion on 3, 4 and 5 d post *Botrytis cinerea* infection (DPI) of red ripe fruit at harvest in 'Furore' strawberry fruit in response to different red to blue light ratios and additional far-red light during cultivation. Light intensity was ~180 µmol m^{-2} s^{-1} (400-700 nm) with and without ~50 µmol m^{-2} s^{-1} far-red (700-800 nm). Inoculated fruit were stored at 12 °C in darkness. Data represent means (n = 4 with 16 replicate plants per block; each block contained approximately 30 replicate fruit). Error bars represent the standard error of means when larger than symbol size; n.s. denotes no significant differences. Significance is based on Fisher's protected least significant difference (LSD) ($P < 0.05$).

3.4 Discussion

3.4.1 Additional FR light leads to faster ripening, enhanced flavor and lower antioxidant levels in fruit

Strawberry plants cultivated under additional FR light showed elongated stems and a more open plant architecture (Figure 2A), similar to the shade avoidance syndrome (Franklin, 2008; Kalaitzoglou et al., 2019). The FR stimulation of leaf area and open plant architecture increases light interception (Kalaitzoglou et al., 2019). Adding FR light during cultivation results in higher accumulation of sugars in tomato leaves and fruit (Fanwoua et al., 2019; Courbier et al., 2020; Ji et al., 2020). Likewise, in our research, higher contents of glucose, fructose and sucrose were observed in red ripe strawberries cultivated under additional FR light (Figure 4). This was also reflected in a higher brix value of FR treated fruit (Figure 4D), indicating FR enhances fruit sweetness as brix is an indicator of sweetness (Jouquand et al., 2008).

In strawberry fruit, sucrose and abscisic acid (ABA) co-promote ripening by inducing the JA signaling pathway, thereby triggering cell wall disassembly (softening) and coloration (Jia et al., 2013, 2016; Luo et al., 2019, 2020). Earlier initiation of coloration (about one week) and more rapid coloration in fruit from additional FR light were observed (Figure 2D), and this was also reported in tomato fruit (Ji et al., 2020). However, we found a lower level of total anthocyanins in fruit cultivated under FR light (Figure 5B). This suggests that a rapid coloration of the fruit skin does not necessarily coincide with a high anthocyanin level of fruit at the red ripe stage. The phenomenon of FR elevating sugar levels but decreasing the anthocyanin content might be caused by upregulating the expression level of tonoplast monosaccharide transporter genes (*TMT*). In apple calli, overexpression of *MdTMT1* increased the contents of glucose and fructose in vacuoles, but reduced the availability of UDP-glucose and UDP-galactose that are required for the conversion of anthocyanidins to anthocyanins (Xu et al., 2020).

Changes in the volatile composition of fruit under additional FR light were also notable (Figure 6, 7), especially esters. Esters are mostly derived from the esterification of alcohols and acids by alcohol acyl-transferases (AATs) and the levels of most esters increase during strawberry ripening (Van de Poel et al., 2014). Production of different esters is restricted by different substrates that can be catalyzed by different AATs (Aharoni et al., 2000; Beekwilder et al., 2004; Cumplido-Laso et al., 2012). We observed that methyl butanoate, methyl hexanoate, (E)-2-hexen-1-ol acetate and (E)-2-Hexen-1-yl butyrate levels increased in fruit treated with additional FR (Figure7). Despite the fact

that fruit ripening on the plant took less time under additional FR light (Figure 2D), higher sugar and ester contents, as well as lower firmness (Figure 4C) indicating that additional FR light led to faster ripening and enhanced flavor in strawberry fruit compared fruit cultivated under 50% : 50% red : blue light.

In addition to the effects on fruit ripening, additional FR light decreased anthocyanin and ascorbic acid contents (Figure 5). These two compounds accumulate during strawberry ripening and are primary antioxidants in red ripe strawberries. These two compounds may be associated with JA-mediated responses as discussed above, which might explain that their contents are reduced by additional FR treatment.

3.4.2 A low red to blue light ratio leads to enhanced antioxidant but reduced sugar levels in fruit

Blue light can cause ROS production in plants (Lazzarin et al., 2021), which in turn may result in higher accumulation of antioxidants. Blue light may trigger JA-mediated defenses by decreasing GA levels, leading to stabilization of DELLA proteins and destabilization of JAZ (Zhao et al., 2007). The activated JA signaling pathway could also enhance the antioxidant capacity of fruit. In the current study, a low red to blue light ratio increased proanthocyanidin and ascorbic acid contents (Figure 5). The increase in the anthocyanin content, however, was not significant. Many studies have shown that blue light increases anthocyanin content in fruit tissues. However these studies did not distinguish effects of light spectra on anthocyanin accumulation by comparing different colors of light. In addition, the different harvest criteria also yielded different outcomes regarding antioxidant levels. Zhang et al. (2018b) observed that strawberries under blue light treatment showed the highest anthocyanin content 25 d after flowering compared to white or red light. Overall, low red to blue light ratios seem slightly enhance antioxidant levels of fruit.

Fruit cultivated under a low red to blue light ratio displayed a lower level of sucrose, higher levels of two primary GLVs (i.e., (E)-2-hexenal and (Z)-3-hexenol) (Figure 7F, H) and higher firmness (Figure 3C). These findings may indicate that a low red to blue light ratio resulted in less tasty fruit compared to a high red to blue light ratio.

3.4.3 Fruit susceptibility to *B. cinerea* is not affected by various light spectra despite changes in metabolites

It has been demonstrated that FR light counteracts JA-mediated defense responses whereas blue light enhances JA-mediated defense responses. According to our results,

fruit treated with additional FR ripened faster and had enhanced flavor but lower antioxidant contents. On the other hand, fruit treated with a low red to blue light ratio had higher antioxidant contents but lower sugar as well as more aldehyde contents. However, following inoculation of the harvested red ripe fruit, differences in *B. cinerea* lesion development were not observed either between with and without FR light treated fruit or between different red to blue light ratios (Figure 8). These indicate no or only a minor effect of light spectra during cultivation on red ripe fruit susceptibility to *B. cinerea* during postharvest storage.

The role of sugar levels in affecting the susceptibility to *B. cinerea* has been discussed in vegetative tissues. Higher fructose content may either decrease (Engelsdorf et al., 2013; Lecompte et al., 2017) or increase plant susceptibility to *B. cinerea* (Courbier et al., 2020). Increase in sucrose levels was proposed by Hu et al. (2019) to act as an early indicator for *B. cinerea* infection in strawberry leaves before the occurrence of visible symptoms. Differences in sugar content may not play an important role in inducing plant defenses or susceptibility in fruit as the contents of glucose, fructose and sucrose in e.g., strawberry fruit are ~12, ~22, and ~66 folds higher than the levels in leaves (Akšić et al., 2019). The relation between sugar levels and susceptibility to *B. cinerea* in red ripe strawberry genotypes was not significant (**Chapter 2**). We hypothesize that the subtle change of sugar contents in strawberries by light spectra does not alter the susceptibility to *B. cinerea*.

3.5 Conclusions

A low red to blue light ratio lowered the levels of soluble sugars and maintained higher (E)-2-hexenal, and (Z)-3-hexenol but increased antioxidant levels of fruit. Adding FR light on top of a 50% red : 50% blue light background during cultivation accelerated fruit ripening, reduced antioxidant levels and enhanced fruit sugar and ester levels, which improved fruit flavor quality. However, different light spectra during cultivation did not affect strawberry fruit susceptibility to *B. cinerea* in spite of altering sugar, antioxidant and volatile levels in fruit.

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Author contribution

HL, DHL, RES, LFM and EJW defined the research questions and designed the experiment. HL, DdJ, XH, SA and MW performed the chamber and lab work. HL performed data analyses. RdV provided the methodology and resource for certain measurements. HL wrote the manuscript with the help of EJW and LFM. DHL, RS and JvK critically revised the manuscript. All authors reviewed and approved the final manuscript.

Supplementary data

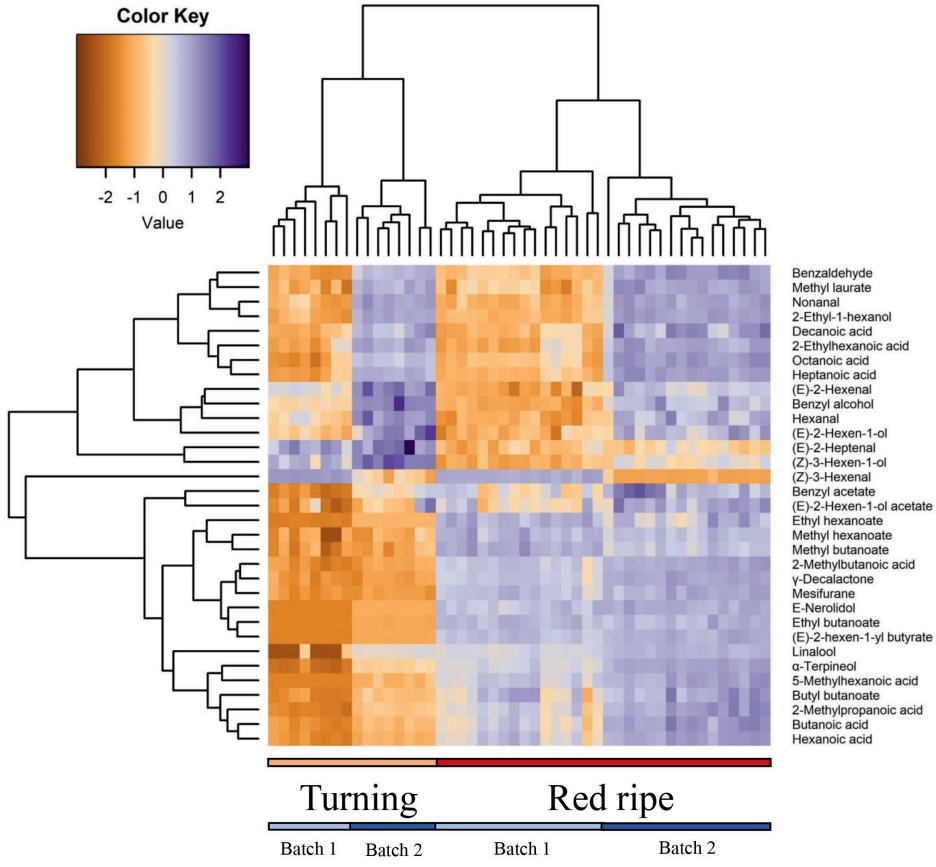

Figure S1 Heatmap of volatile profile in 'Furore' strawberry fruit in response to red, blue and additional far-red light during cultivation. The peak height data originated from SPME/GC-MS was log10 transformed prior to analysis. The hierarchical clustering analysis was computed by using the Ward's minimum variance method (ward. D2).

Chapter 4

High CO_2 reduces spoilage caused by *Botrytis cinerea* in strawberry fruit without impairing nutritional quality

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(submitted)

Abstract

High CO_2 (i.e., > 20 kPa) conditions are beneficial to suppress spoilage caused by *B. cinerea* in strawberry fruit, however, such conditions are often accompanied by discoloration, off-flavor production and faster softening. A stepwise increment of CO_2 levels has been proposed to alleviate injuries caused by high CO_2 in fruits. Here, we investigated whether the stepwise increment of CO_2, up to 30 kPa, under a reduced O_2 concentration, is beneficial to reduce spoilage caused by *B. cinerea* without inducing CO_2 injury symptoms in strawberries. We first selected optimal O_2 and CO_2 concentrations within the recommended settings (5 to 10 kPa O_2 with 15 to 20 kPa CO_2) that perform best in restricting spoilage caused by *Botrytis cinerea* using red ripe 'Sonsation' strawberries. We found that the higher O_2 (10 kPa) and CO_2 (20 kPa) conditions were most beneficial to prolong strawberry fruit shelf life. Subsequently we studied the performance of red ripe 'Arabella' strawberries stored at 5 °C under different CA conditions (10 kPa O_2 with either 0, 20 or 30 kPa CO_2). The CO_2 levels were achieved either within eight hours, or in a stepwise manner within the first four days of storage. 21 kPa O_2 and 0 kPa CO_2 served as control. Following storage for up to eleven days, spoilage incidence was assessed at 12 °C for five days. The application of high CO_2 (20 and 30 kPa) combined with 10 kPa O_2 greatly suppressed fruit spoilage during storage and subsequent shelf life. High CO_2 suppressed respiration, maintained a higher pH and led to higher firmness in treated fruit. The level of total sugars did not change, but during storage a substantial part of the sucrose was converted into glucose and fructose. High CO_2 stimulated the conversion of sucrose into reducing sugars, in particular fructose, which may result in sweeter fruit. High CO_2 did not affect the levels of ascorbic acid and anthocyanins. The stepwise increments of CO_2 did not result in beneficial effects compared to the static application of high CO_2. Our results show that 'Arabella' strawberries are highly tolerant to elevated CO_2 and can be stored under 30 kPa CO_2 to prolong the shelf life.

Keywords: *Fragaria × ananassa*, CA storage, shelf life, stepwise atmosphere, grey mold disease, pH, sugar and acid metabolism

4.1 Introduction

Strawberry fruit are highly appreciated due to their desirable quality characteristics, such as 'heart shape', shiny skin, full red color, sweetness, distinct aroma and abundant nutritional compounds (Madrid, 2020). However, the high perishability limits strawberry shelf life. Amongst pathogens, the ubiquitous fungi *Botrytis cinerea* caused the most losses in strawberry production (Petrasch et al., 2019a) and particularly in ripe fruit during postharvest storage. The strategy of combining low temperature and controlled atmosphere (CA, i.e., high CO_2 and low O_2 concentrations) has been widely used in the supply chain to retain product quality for market demand. For strawberry fruit, the postharvest condition of 5 to 10 kPa O_2 and 15 to 20 kPa CO_2 at 0 to 5 °C is recommended to extend its storage period (Kader, 2003). The suggested concentrations of O_2 (5 to 10 kPa) for strawberries decreased respiration without inducing fermentation thereby extending the storage life (Kanellis et al., 2009). Similarly, high CO_2 concentrations between 15 to 20 kPa further suppressed respiration, delayed ripening and senescence (Li et al., 2019), and may affect the acidity of strawberries (Rama and Narasimham, 2003). In addition, high CO_2 concentrations are fungistatic to *B. cinerea* since the fungus did not grow above 30 kPa CO_2 (García-Gimeno et al., 2002). However, high CO_2 levels, above 20 kPa, may also accelerate strawberry softening and impair coloration, taste and antioxidant levels (Wszelaki and Mitcham, 2000; Nakata and Izumi, 2020), thereby reducing commercial value.

Antioxidants such as ascorbic acid and anthocyanins may play a role in protecting fruit from fungal infections. Ascorbic acid showed inhibitory effects on *B. cinerea* growth in apple fruit (Davey et al., 2007; Bui et al., 2019) and tomato fruit (Bassolino et al., 2013; Zhang et al., 2013). High CO_2 levels may cause oxidation of ascorbic acid or inhibit the reduction of dehydroascorbic acid. For instance, 10 to 30 kPa CO_2 decreased the ascorbic acid content in berry species, especially strawberries (Agar et al., 1997; Shin et al., 2008). Similarly, the application of 20 to 40 kPa CO_2 to 'Selva' strawberries reduced fungal spoilage but induced discoloration and anthocyanin reduction, presumably caused by the increase in pH or decrease in co-pigmentation with flavonols and other phenolics (Gil et al., 1997a; Nakata and Izumi, 2020).

Soluble sugars and non-volatile organic acids contribute to fruit sweetness and acidity (Schwieterman et al., 2014) and these compounds are also substrates for respiratory metabolism (Saltveit, 2019). Soluble sugar contents and acidity of 'Camarosa' strawberries declined after a 3-d 20 kPa CO_2 treatment compared to fruit in ambient atmosphere (Bodelón et al., 2010). High CO_2 levels may lead to the production of

fermentative volatile compounds. The application of ambient oxygen combined with 20 kPa CO_2 to 'Camarosa' strawberries resulted in a shift in the synthesis of methyl- to ethyl esters, contributing to off-flavors (Pelayo-Zaldívar et al., 2007).

A novel CA strategy has been proposed recently to extend blueberry shelf life: the stepwise increment of the CO_2 level at the start of CA storage (Falagán et al., 2020). The stepwise increment of CO_2, reaching 10 kPa CO_2 in 3 or 7 d (under 5 kPa O_2) was applied. This resulted in a lower CO_2 production peak, a reduced spoilage incidence, higher firmness and higher ascorbic acid levels compared to static CA (5 kPa O_2 and 10 kPa CO_2) or ambient atmosphere conditions.

In the current research, we aimed to investigate if the stepwise increment of high CO_2 extends strawberry shelf life without damaging important quality characteristics such as firmness, color and nutrient contents. We first determined the spoilage incidence by *B. cinerea* in strawberry fruit as a function of O_2 and CO_2 levels, based on recommended settings (Kader, 2003). High levels of both O_2 (10 kPa) and CO_2 (20 kPa) resulted in lower spoilage incidences and similar levels of firmness and sweetness compared to the other tested O_2 and CO_2 levels. In a second experiment, we investigated the effects of both static and stepwise increments of CO_2 levels, up to 20 and 30 kPa, on the spoilage incidence, nutritional compounds and antioxidants during shelf life. The highest CO_2 level, 30 kPa, considerably lowered the spoilage incidence and respiration rate, without causing any nutrient or firmness loss in strawberries. However, stepwise increments of CO_2 levels did not provide benefits compared to the static CA storage at 20 or 30 kPa CO_2.

4.2 Materials and methods

4.2.1 Strawberry fruit and experimental setup

Exp I: Spoilage evaluation test using different CA settings

Strawberries (*Fragaria × ananassa* cv. Sonsation) were harvested in November, 2019 from a greenhouse located in Dongen, the Netherlands. Fruit were stored at 4 °C overnight and the next day (0 d) transported to the lab at Wageningen University and Research. Red ripe fruit (with calyxes attached) without mechanical damage or visible spoilage were selected and collected into round plastic cups (ø 10 × H 5 cm) covered with a transparent lid. The lid had nine small holes to allow sufficient gas diffusion. A total of 150 cups were packed. The initial weight of all cups with fruit was individually recorded on day zero. Six cups were used for chemical analyses on day zero. The

remaining 144 cups were distributed over six CA treatments with three blocks each. Each block contained six cups. In total, six 70 L stainless steel CA containers were used containing each 24 cups. CA containers were connected to a flow-through system flushing humidified gas mixtures at a flowrate of 250 mL min^{-1} for the duration of the experiment. Strawberries were subjected to the following CA treatments (balanced with N_2 in all treatments):

(i) Static 10 O_2 kPa + 10 CO_2 kPa;

(ii) Static 5 O_2 kPa + 15 CO_2 kPa;

(iii) Static 7.5 O_2 kPa + 15 CO_2 kPa;

(iv) Static 10 O_2 kPa + 15 CO_2 kPa;

(v) Static 10 O_2 kPa + 20 CO_2 kPa;

(vi) Control: Static 20 O_2 kPa + 0 CO_2 kPa.

The setpoints for CA treatments were reached within 5 to 8 h. Fruit were treated for 0, 3, 6, 9 and 12 d under the different CA conditions at 5 °C and ~100% relative humidity. Following CA storage, fruit were held for 3 d under ambient atmosphere at 12 °C and ~95% relative humidity. The CA containers were opened and closed within 2 min when strawberries had to be taken out for measurements with minimal disturbance of the atmosphere. For each CA container at each sampling time, six cups were used for measurements. Spoilage incidence, weight, firmness, and soluble solids content (SSC) were measured immediately after the CA treatments (3 cups per treatment) and subsequently after 3 d of shelf life at ambient temperature (3 cups per treatment).

Exp II: Physiological changes during static and stepwise increments of CO_2 levels

Strawberries (*Fragaria × ananassa* cv. Arabella) were grown in an open field tabletop system in Genderen, the Netherlands and harvested in August 2020. Fruit were cooled down for several hours and transported to the lab at Wageningen University and Research (day zero). Red ripe fruit (with calyx still attached) without mechanical damage and visible spoilage were selected and collected into paper punnets with approximately 25 fruit per punnet. A total of 98 punnets were packed. The initial weight of all punnets with fruit was individually recorded on day zero. Two punnets were used for chemical analyses on day zero. The remaining 96 punnets were distributed over six CA treatments with two blocks each. Each block contained 8 punnets. In total, twelve 70 L stainless steel CA containers were used with 8 punnets each. CA containers were connected to a flow-through system flushing humidified gas mixtures at a flowrate of

250 mL min^{-1} for the duration of the experiment. Strawberries were subjected to the following CA treatments (balanced with N_2 in all treatments):

(i) GCA30: 10 kPa O_2 with a stepwise increment to 30 kPa CO_2 (7.5 kPa per day in 4 d);

(ii) GCA20: 10 kPa O_2 with a stepwise increment to 20 kPa CO_2 (5 kPa per day in 4 d);

(iii) CA30: Static10 kPa O_2 + 30 kPa CO_2;

(iv) CA20: Static10 kPa O_2 + 20 kPa CO_2;

(v) CA0: Static 10 kPa O_2 + 0 kPa CO_2;

(vi) Control: Static 20 O_2 kPa + 0 CO_2 kPa.

The setpoints for CA treatments were reached within 5 to 8 h. Fruit were stored for 11 d under different CA conditions at 5 °C and ~100% relative humidity, thereafter they were held for 5 d under ambient atmosphere at 12 °C and ~95% relative humidity (shelf life). The CA containers were opened and closed within 2 min when strawberries were taken out for measurements. One individual CA unit (regarded as one replication) contained 8 punnets as described above. During the CA treatment fruit were sampled on d 4 and d 11; during the subsequent shelf life, fruit were sampled on d 3 (total period 14 d) and d 5 (total period 16 d). At each sampling point, one punnet per treatment was randomly taken for quality analysis, including fresh weight, firmness and biochemical compounds; another 4 punnets were used for non-destructive spoilage incidence evaluation on d 11 of CA storage and d 3 and d 5 of shelf life.

4.2.2 Respiration rate

In Exp II, the respiration rate of the fruit under the different CA conditions was monitored. For this, the outlet of each CA container was connected to a 2 L glass cuvette filled with approximately 600 to 750 g of fruit. In this way the cuvettes received the same gaseous conditions as the other stored fruit. At the start of the respiration measurement, the glass cuvettes were disconnected from the flow-through system and all valves were closed. Levels of O_2 and CO_2 were measured immediately after closure and again after about 6 h of incubation; from this the respiration rate was calculated. The calculation took into account the O_2 consumption rate, cuvette volume, fruit mass; the results were expressed in nmol O_2 kg^{-1} s^{-1}. CO_2 and O_2 were measured using a headspace gas analyzer (Checkmate 3, PBI Dansensor, Ringstead, Denmark).

4.2.3 Spoilage incidence and weight loss

Fruit showed typical *B. cinerea* infection symptoms (e.g., brown spots or visible grey mycelia). The spoilage incidence was expressed as the percentage fruit affected by

fungal infection. Weight of individual cup or punnet with fruit was recorded using a MS6002TS balance (Mettler-Toledo GmbH, Giessen, Germany) at harvest. Weight loss was expressed as the percentage of weight loss compared to the initial fruit weight, corrected for the weight of cups or punnets.

4.2.4 Firmness and brix

In Exp I firmness (limited compression) was measured using a FirmTech FT7 (UP GmbH, Ibbenbüren, Germany). Firmness was expressed as the force displacement (in g mm^{-1}) after about 1 mm compression of the fruit shoulder without calyx. Five fruit per replicate (cup) were used. The same fruit were used for brix measurement using a refractometer (PAL-1, Atago, Japan).

In Exp II fruit firmness (penetration) was measured using a universal testing machine (Zwick Z2.5/TS1S materials testing machine; Ulm, Germany). Fruit were cut in half lengthwise and both halves were compressed at the equator with a probe (ø 2.5 mm) at a constant plunger speed (2.5 mm s^{-1}) to a fixed penetration depth (5 mm). The maximum force (N) was recorded during penetration of the fruit piece. Fifteen strawberries per replicate (punnet) were used.

4.2.4 Dry weight percentage and pH

Fruit from one punnet (approximately 25 fruit) were cut into small pieces and immediately frozen in liquid N_2 and grounded into powder. Part of the frozen powder was freeze-dried for sugar and organic acid extraction and also for expressing all nutrients as per g dry weight. Frozen powder (0.3 g) was dissolved in 1.5 mL water, thoroughly shaken, and centrifuged at 21,000 × *g* at 20 °C for 15 min. The supernatant was used for pH measurement.

4.2.5 Ascorbic acid

Frozen powder (0.2 g) was thawed on ice in darkness with 1 mL ice cold 3.3% meta-phosphoric acid. Samples were sonicated for 10 min then centrifuged at 21,000 × *g* at 4 °C for 10 min. The supernatant was filtered through a 0.45 µm cellulose filter and injected into a HPLC system consisting of a GS50 pump (Dionex), a 340S UV-VIS detector (Dionex) and a MIDAS autosampler (Spark Holland) equipped with a ProntoSIL 120-3 C18 AQ, 250 × 3mm column (Knauer). The column was eluted with 400 µL L^{-1} H_3PO_4 + 2.5 mL L^{-1} MeOH + 0.1 mM EDTA in distilled water followed by a wash step with 30% acetonitrile in distilled water at a flow rate of 0.35 mL min^{-1} at 35 °C. Ascorbic acid was

detected at 243 nm. The system was calibrated using an authentic ascorbic acid standard (Acros Organics) prepared in 3.3% MPA.

4.2.6 Anthocyanins

Frozen sample (0.3 g) was mixed with 1.5 mL 50% methanol containing 1% formic acid extraction solvent. The supernatant was filtered through a 0.45 µm cellulose filter prior to injection into an HPLC system (Ultimate 3000, Dionex, Sunnyvale, CA, USA). Solvents used were: (A) 0.1% trifluoroacetic acid in distilled water, and (B) 0.1% trifluoroacetic acid in HPLC-grade acetonitrile, establishing the following gradient: 5-28% of B for 0-35 min, 28-75% of B for 35-37 min, isocratic 75% of B for 37-40 min, 75-5% of B for 40-42 min, and isocratic 5% of B for 42-50 min, using a flow rate of 0.8 mL min^{-1}. Separation was achieved using a C18 column (150 × 3 mm, 3 µm; HyPURITY™ C18, Thermo Scientific, New York, USA) and peaks were identified with a UV/Vis detector at 520 nm using pelargonidin 3-glucoside as an authentic standard (Extrasynthese).

4.2.7 Sugars, citric and malic acid extraction and measurement

Fifteen mg of freeze-dried tissue was mixed with 5 mL 75% ethanol followed by shaking and incubating at 80 °C in water bath for 20 min, then centrifuged at 8500 *× g* for 5 min at 4 °C. One mL supernatant was dried using a vacuum centrifuge (Savant SpeedVac SPD2010, Thermo Fisher Scientific) at 55 °C for 2.5 h. The pellet was resuspended in 1 mL distilled water and sonicated for 10 min, followed by centrifugation for 10 min at 4 °C at 14,800 *× g*. Before analyses, the supernatant was diluted with distilled water 50 times for sugars and 5 times for organic acids.

Soluble sugar measurements were carried out using a High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD; Dionex ICS5000, Thermo Fisher Scientific), equipped with a CarboPac1 column (250 × 2 mm) eluted with 100 mM NaOH at a flow rate of 0.25 mL min^{-1} at 25 °C. Quantification was performed using glucose, fructose and sucrose standards from Sigma-Aldrich.

Citric and malic acids in the extracts were analyzed using an IC system equipped with a Triathlon autosampler (Spark Holland), GS50 pump (Dionex), ED50A detector (Dionex) operating in the conductivity mode and an ASRS ultra II 2mm suppressor (Dionex). Anions were separated at 30 °C on an IonPac AS11HC (250 × 2 mm) column (Dionex), using the following multi-step gradients: 1 mM NaOH, 0 min; 1 mM NaOH, 1 min; 14 mM NaOH, 23 min; 30 mM NaOH, 31 min; 60 mM NaOH, 41 min at a flow rate of 0.38

mL min^{-1}. Quantification was performed using authentic DL-malic acid and citric acid standards from Sigma-Aldrich.

4.2.8 Statistical analysis

Treatment effects on all measured variables were tested using one-way analysis of variance (ANOVA) at each time point during the experimental period. Exp I was carried out with 3 blocks and Exp II was carried out with 2 blocks. In each block, replicate fruit were measured as an individual or a pooled sample, depending on variables. An average value of each block was used for statistical analysis. Homogeneity and normality of residuals in the ANOVA were tested using Bartlett's test and the Shapiro-Wilktest, respectively. Fisher's protected least significant difference (LSD) test was used as post-hoc test. All statistical analyses were performed in Genstat (19th Edition, VSN International Ltd., Hemel Hempstead, UK). All tests were conducted at α=0.05.

4.3 Results

4.3.1 Spoilage incidence of 'Sonsation' strawberry fruit

During CA storage, the spoilage incidence of CA-treated fruit was lower compared to control-treated fruit (Figure 1A). During shelf life, the spoilage incidence increased dramatically in fruit from ambient atmosphere and 10 kPa CO_2 + 10 kPa O_2 (Figure 1B). Fruit stored at 15 kPa CO_2 showed reduced spoilage incidence with increasing O_2 levels from 5 to 10 kPa. Under conditions of 10 kPa O_2, fruit treated with 15 and 20 kPa CO_2 showed the lowest spoilage incidence among all treatments. Overall brix values of fruit showed a decreasing trend during CA storage but did not show a further change during the 3-d subsequent shelf life; fruit firmness did not change during CA storage but showed a slight decrease during the 3-d subsequent shelf life period. The different treatments had no significant effects on the changes of brix values and the firmness of fruit (Figure S1).

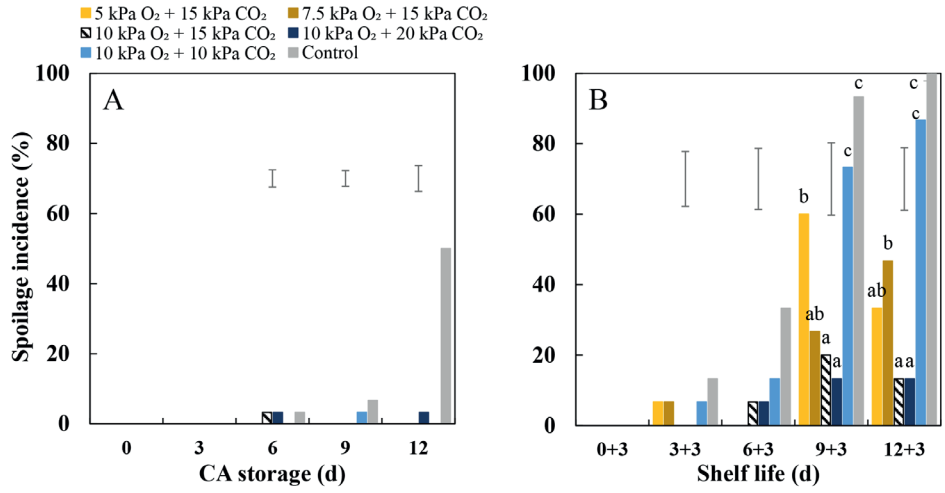

Figure 1 Spoilage incidence of 'Sonsation' strawberry fruit stored under static controlled atmosphere (CA) conditions and shelf life. (A) Spoilage incidence at different time points during CA storage at 5 °C and ~100% relative humidity. (B) Spoilage incidence at different time points of CA storage followed by 3-d shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. The first number is the days in CA storage and the second number the number of days in shelf life. 21 kPa O_2 and 0 kPa CO_2 served as control. Data represent means of 3 blocks (n=3) with five replicate fruit per block. The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05).

4.3.2 Respiration rate and spoilage incidence of 'Arabella' strawberry fruit

The fruit respiration rate was higher in CA0 and in control than in elevated CO_2 treatments (20 and 30 kPa), indicating that increased CO_2 levels suppressed respiration (Figure 2A). The respiration rate fluctuated during the first 5 d of the storage, regardless of treatments. After d 9, the respiration rate started to increase. This increase was smaller and not significant for fruit stored under 30 kPa CO_2 conditions. There were no apparent differences in respiration related to the immediate or stepwise application of high CO_2 (20 and 30 kPa).

Fruit stored under CA0 and control showed high spoilage incidence at the start of shelf life (Figure 2B). The spoilage incidence of CA0 overlapped with that of the control treatment, reaching about 58% spoilage at the end of the CA storage. In contrast, fruit stored under elevated CO_2 with 10 kPa O_2 treatments did not show any spoilage during the CA storage; during the subsequent shelf life at 12 °C and ambient atmosphere, fruit spoilage did occur in these treatments. After the 3-d shelf life, fruit stored under CA30

had the lowest spoilage incidence (32%), followed by GCA30 (53%), CA20 and GCA20 (over 60%). After 5 d of shelf life, spoilage incidence was above 80% in all treatments.

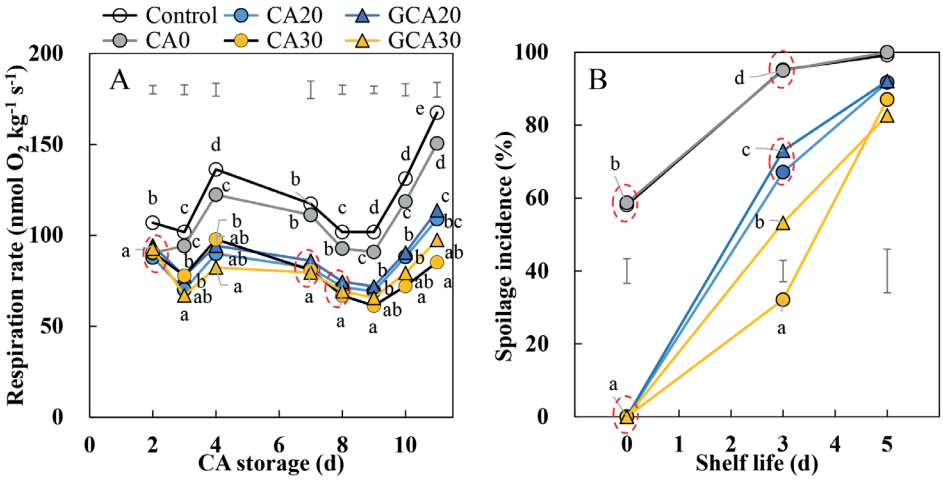

Figure 2 Respiration rate and spoilage incidence of 'Arabella' strawberry fruit during CA storage and shelf life. (A) Respiration rate during CA storage at 5 °C and ~100% relative humidity. (B) Spoilage incidence during shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. 21 kPa O_2 and 0 kPa CO_2 served as control. Data represent means of 2 blocks (n=2) with approximately 35 replicate fruit for respiration and approximately 25 fruit replicate fruit for spoilage measurements per block. The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05).

4.3.3 Sugar levels of 'Arabella' strawberry fruit

During the CA storage and shelf life, the glucose content did not show a clear trend (Figure 3A) whereas the fructose content increased (Figure 3B) and sucrose content decreased (Figure 3C). The total sugar content (glucose + fructose + sucrose) showed a downwards trend for all treatments (Figure 3D). No apparent differences in the changes in individual sugars among treatments were observed. However, the sucrose content in fruit from both the static and stepwise 30 kPa CO_2 treatments showed a more rapid decrease in the CA storage than the sucrose level in fruit from other treatments (Figure 3E). This indicates that under high CO_2 a more pronounced conversion of sucrose into glucose and, in particular, fructose occurred.

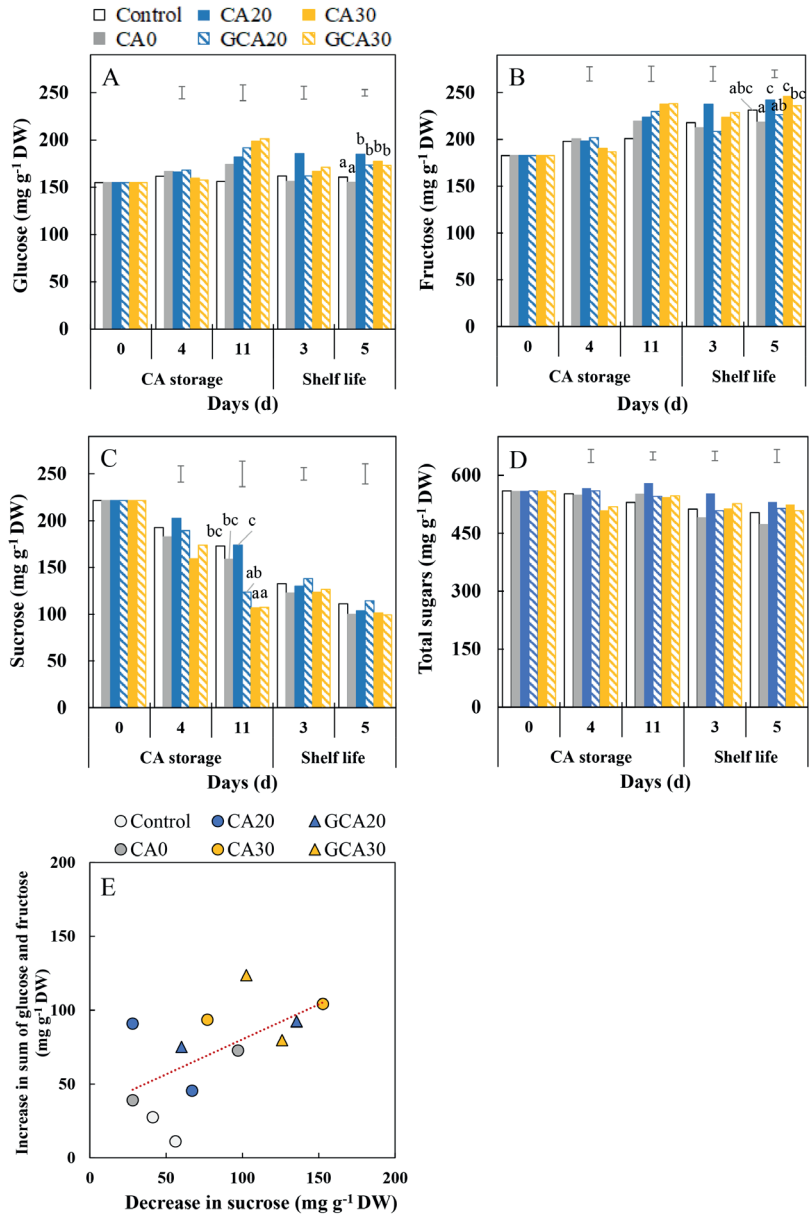

Figure 3 Soluble sugars of 'Arabella' strawberry fruit during CA storage and shelf life. (A) Glucose, (B) fructose, (C) sucrose and (D) total sugar (sum of glucose, fructose and sucrose) during CA storage at 5 °C and ~100% relative humidity and subsequent shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. Data represent means of 2 blocks (n=2; each block being a pooled sample of 20 replicate fruit). Different letters denote significant differences according to Fisher's protected LSD test (α=0.05). The error bars represent standard error of means. (E) Correlation between the increased content of glucose and fructose and the decreased content of sucrose between d 0 and d 11 during CA storage. Data represent individual blocks. 21 kPa O_2 and 0 kPa CO_2 served as control.

4.3.4 pH and organic acids of 'Arabella' strawberry fruit

Fruit pH increased from about 3.2 up to 3.7 with minimal differences between treatments (Figure 4A). Fruit from CA30 condition generally showed a slightly higher pH than the control and CA0 treatments. Citric acid did not show changes throughout the CA storage and shelf life, and no treatment effects were observed (Figure 4B). Malic acid content in fruit was 2.2-fold lower than citric acid at harvest and showed a slight decrease after fruit were transferred to the shelf life condition in all treatments. No differences with respect to the CA treatments were observed (Figure 4C). The increase in pH during the experimental period can be ascribed to the decrease in the two major acids; fruit pH during the CA storage was lower than during the subsequent shelf life, regardless of atmosphere composition. In addition, high CO_2 treatments led to a higher pH compared to control and CA0 treatments after fruit were transferred to ambient atmosphere (Figure 4D).

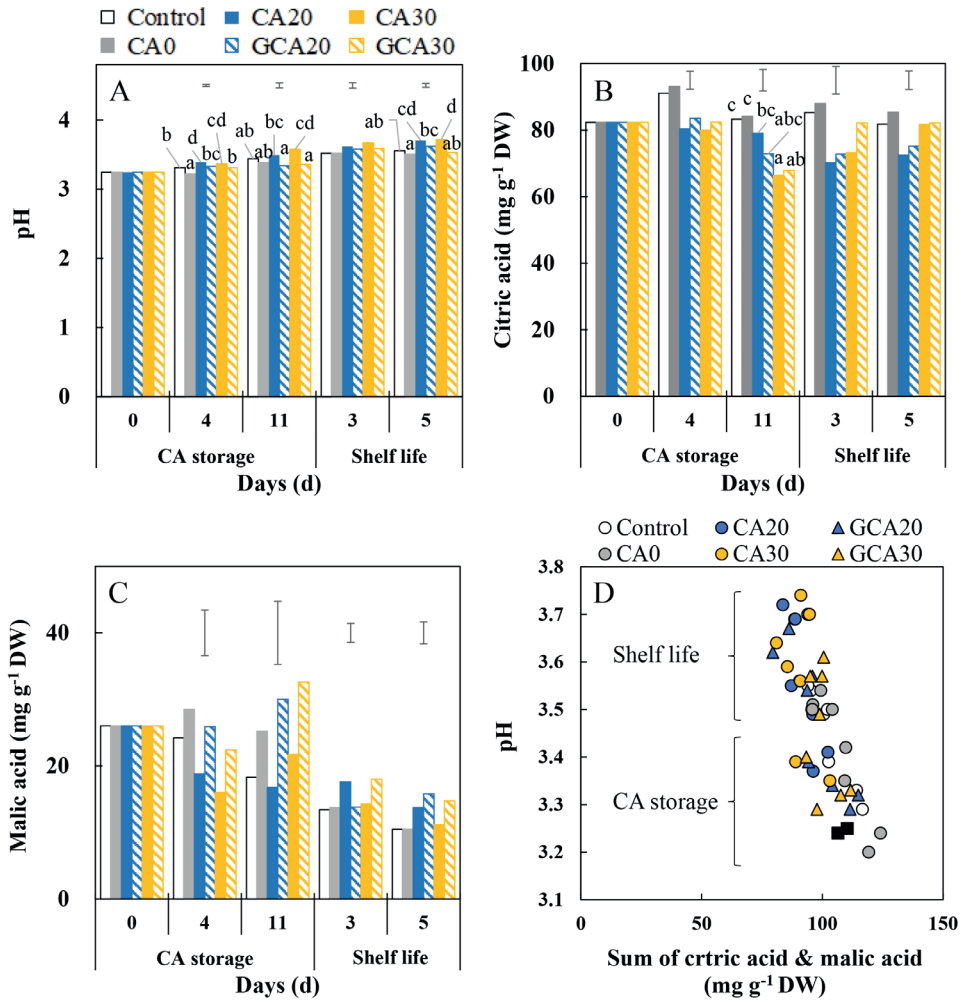

Figure 4 pH and main organic acids of 'Arabella' strawberry fruit during CA storage and shelf life. (A) pH, (B) citric acid and (C) malic acid during CA storage at 5 °C and ~100% relative humidity and subsequent shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. Data represent means of 2 blocks (n=2; each block being a pooled sample of 20 replicate fruit). The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05). (D) Correlation between pH and sum of citric- and malic acids of CA treatments during CA storage and subsequent shelf life. The closed black squares represent data of d 0. Data represent individual blocks. 21 kPa O_2 and 0 kPa CO_2 served as control.

4.3.5 Firmness and weight loss of 'Arabella' strawberry fruit

During CA storage, softening was observed only in fruit from the control atmosphere (Figure 5A). During the subsequent shelf life, softening was observed in fruit from control and CA0 treatments. Fruit from high CO_2 treatments (except for CA30) retained their firmness during

the shelf life and no superior effect of the stepwise elevation of CO_2 was observed. Fruit weight loss during the CA storage was minimal; in the subsequent shelf life, fruit weight loss increased up to a maximum of 3% and no differences were apparent between treatments (Figure 5B).

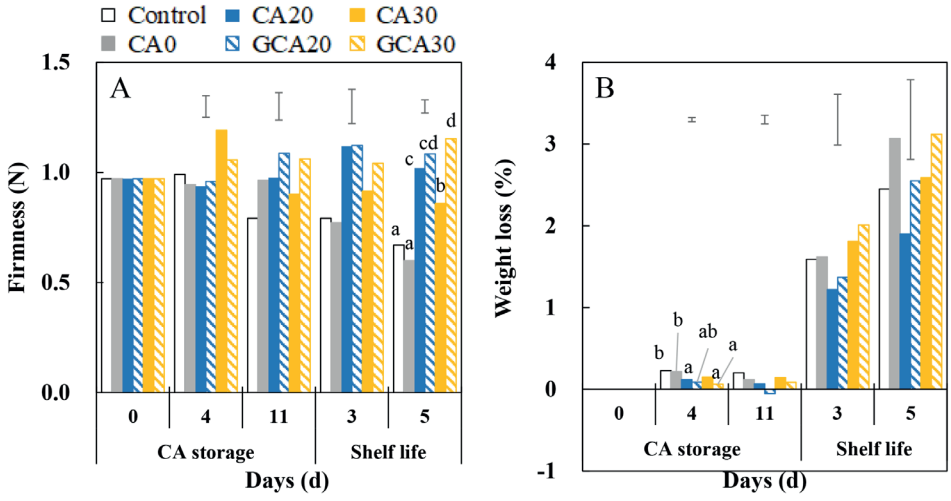

Figure 5 Firmness and weight loss of 'Arabella' strawberry fruit during CA storage and shelf life. (A) Firmness and (B) weight loss during CA storage at 5 °C and ~100% relative humidity and subsequent shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. Data represent means of 2 blocks (n=2; each block being averaged of 20 replicate fruit). The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05). 21 kPa O_2 and 0 kPa CO_2 served as control.

4.3.6 Ascorbic acid and anthocyanin levels of 'Arabella' strawberry fruit

Pelargonidin-3-glucoside and pelargonidin-3-malonyl glucoside were the major anthocyanins in 'Arabella' strawberries and the content of the former was lower (~75%) than the content of the latter at harvest (Figure 6A, B). Overall, pelargonidin-3-glucoside contents in fruit increased during the CA storage with decreasing trends during shelf life. There were no consistent differences between treatments. Similar trends were observed in pelargonidin-3-malonyl glucoside contents of fruit. Ascorbic acid levels of 'Arabella' fruit showed slightly increasing trends in fruit from control and CA0 treatments throughout CA storage and shelf life, whereas it did not change in fruit treated with elevated CO_2 levels where it maintained around 10 mg g^{-1} DW (Figure 6C). Collectively, until the end of shelf life, the antioxidants of fruit showed similar levels compared to fruit at harvest.

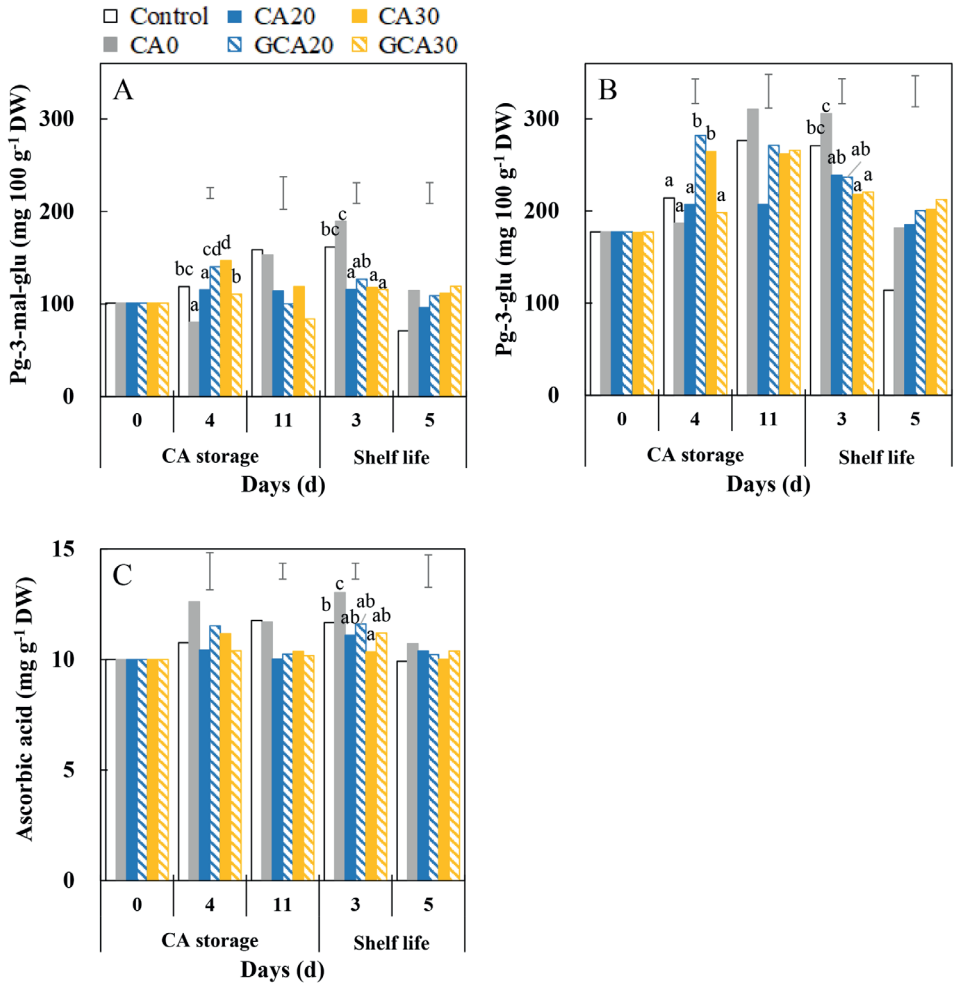

Figure 6 Anthocyanin and ascorbic acid contents of 'Arabella' strawberry fruit during CA storage and shelf life. (A) Pelargonidin-3-malonyl glucoside, (B) pelargonidin-3-glucoside and (C) ascorbic acid during CA storage at 5 °C and ~100% relative humidity and subsequent shelf life at 12 °C and ~95% relative humidity in ambient atmosphere. 21 kPa O_2 and 0 kPa CO_2 served as control. Data represent means of 2 blocks (n=2; each block being a pooled sample of 20 replicate fruit). The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05).

4.4 Discussion

4.4.1 Reduced O_2 and high CO_2 are simultaneously required for retarding fruit spoilage by *B. cinerea*

CA storage retains product quality via reducing O_2 and increasing CO_2 concentrations compared to ambient atmosphere. Generally, the lower the O_2 and the higher the CO_2 levels, the more the storage life can be extended by delaying senescence and ripening of commodities (Rama and Narasimham, 2003). However, this concept may not fully apply to strawberry fruit based on our observations. In Exp I, moderately decreased O_2 levels (5 kPa to 10 kPa) combined with 15 kPa CO_2 greatly decreased the spoilage incidence of 'Sonsation' strawberries, which was observed only after fruit were transferred to post-storage shelf life (Figure 1B). 10 kPa O_2 clearly performed better than 5 or 7.5 kPa O_2. When high CO_2 is applied to strawberry fruit, the risk of initiating fermentation increases, which leads to accumulation of acetaldehyde and ethanol, resulting in off-flavor (Rama and Narasimham, 2003; Kanellis et al., 2009). This could be prevented and/or attenuated by introducing higher O_2 into the environment. By contrast, Ke et al. (1991) found that decreasing concentrations of O_2 (down to 0.25% O_2 balanced with N_2 only) led to a lower spoilage incidence in 'Selva' strawberries at both 0 and 5 °C and did not affect the soluble solids content (brix), pH, or titratable acidity after 10-d CA treatments compared to air-treated fruit. However, this study also reported on higher ethanol content of fruit for this treatment compared to fruit from other treatments. It is not clear of these beneficial effects of ultralow O_2 persisted during the shelf life shelf life as authors only did quality measurements at the end of storage period.

We observed that increased CO_2 levels (10 to 20 kPa) combined with 10 kPa O_2 drastically lowered the spoilage incidence of fruit. Fruit firmness and brix did not exhibit differences during the CA storage and shelf life among treatments (Figure S1). Application of 20 kPa CO_2 in air did not induce fermentation of 'Selva' strawberries and resulted in a lower spoilage incidence (Ke et al., 1991). This shows that in order to benefit from CA storage O_2 levels should not be lower than 10 kPa and CO_2 should not be lower than 15 kPa.

Therefore, in Exp II we referred to the optimal CA condition of 'Sonsation' strawberries that showed a lower spoilage incidence without affecting fruit firmness and brix and applied 10 kPa O_2 in combination with different levels of CO_2 to 'Arabella' strawberries. Both conditions with 10 kPa O_2 and high (20 and 30 kPa) CO_2 greatly suppressed

spoilage confirming the results with 'Sonsation' fruit, indicating the two cultivars may respond to high CO_2 conditions similarly.

Note that in Exp II the condition of 10 kPa O_2 alone (Figure 2B, CA0) did not suppress the spoilage development compared to the control at the end of the CA storage. It seems high CO_2 combined with reduced O_2 is effective for reducing the spoilage via direct (i.e., inhibition of fungal growth) and indirect manners (i.e., suppression of fruit respiration). When high CO_2 is applied to strawberry fruit, the concentration of O_2 should not be too low, otherwise the additive effects of high CO_2 and low O_2 may induce fermentation.

4.4.2 High CO_2 affected sugar and acid metabolism

A CO_2 concentration higher than 30 kPa can lead to quality loss such as faster softening, berry discoloration and production of off-flavors. Elevating CO_2 in a stepwise manner may improve the adaption of products to high CO_2 compared to static CO_2 treatments such that the storage life and fruit firmness, color, nutritional compounds and antioxidants are retained (Falagán et al., 2020). Applying 10 kPa CO_2 and 5 kPa O_2 using either 3 or 7 d reaching final concentrations to blueberries resulted in a lower CO_2 production peak and a reduced spoilage incidence compared to static CA or ambient atmosphere conditions (Falagán et al., 2020).

In the current study, the respiration rate (Exp II) of fruit from high CO_2 treatments was reduced throughout the storage period compared to fruit from low CO_2 treatments (Figure 2A). However, the pattern was not affected by applying high CO_2 in a stepwise manner. Similarly, the spoilage incidence was greatly suppressed by high CO_2, but was not affected by the stepwise application. The suppressed respiration by high CO_2 is due to the inhibition of succinate dehydrogenase that catalyzes the conversion of succinate to fumarate at the tricarboxylic acid (Kanellis et al., 2009). Malic acid as the upstream metabolite of succinate and fumarate, was reduced by high CO_2 (20 kPa CO_2 in air) (Fernández-Trujillo et al., 1999; Ponce-Valadez et al., 2005). The observed decreasing trend of malic acid, especially in fruit treated with elevated CO_2 (Figure 4C), is in line with previous findings.

In strawberry fruit, sucrose is degraded via the invertase pathway to form fructose and glucose (Kanellis et al., 2009; Durán-Soria et al., 2020). We found that fructose and glucose in strawberries slightly increased during the experimental period in all CA treatments whereas sucrose decreased. High CO_2 treatments seemed to induce a larger conversion from sucrose to glucose and, in particular, fructose (Figure 3E). Bang et al.

(2019) also observed increases in glucose and fructose but a decrease in sucrose contents in strawberry fruit after 1 d treatment of fruit with 30 kPa CO_2 at 25 °C compared to the ambient atmosphere condition. The transcriptome analysis indicated that the invertase inhibitor was downregulated under 30 kPa CO_2 condition, triggering the activity of invertases; therefore more glucose and fructose are synthesized in fruit. In addition, as the total sugar content did, irrespective of CA treatments, not show a significant change during the whole experimental period, the increase in fructose content particularly (1.17 to 1.75 times sweeter as compared to sucrose) by high CO_2 may indicate that fruit sweetness was improved.

4.4.3 High CO_2 did not impair strawberry firmness and antioxidants or induce fermentation

A decreasing trend in firmness in control and CA0 treated fruit was observed, especially after fruit were returned to ambient atmosphere (Figure 5A). Firmness of fruit from high CO_2 treatments was maintained at approximately the same value at the end of shelf life compared to the firmness at harvest. The different firmness behaviors between 0 kPa CO_2 and high CO_2 corroborate that CO_2 treatments inhibit firmness loss of strawberries in a non-reversible manner (Harker et al., 2000). Fruit treated with static 30 kPa CO_2 tended to be softer compared to fruit from other high CO_2 treatments. This was associated with a relatively low level of malic acid in fruit from this treatment. This could be due to the adverse effect of long-term storage under static 30 kPa CO_2 as it was not observed under stepwise high CO_2 treatments.

Strawberry being firmer under elevated CO_2 is likely due to reinforcement of cell-to-cell bonding, which is associated with the increase in pH of the apoplast (Harker et al., 2000). According to our finding, the pH of 'Arabella' strawberries increased during the whole experimental period due to reduced content of organic acids, especially malic acid (Figure S2), which is in line with previous findings (Ke et al., 1991; Holcroft and Kader, 1999; Blanch et al., 2015). Both H^+ and HCO_3^- produced by solubilization of CO_2 could affect pH (Lucas, 1979; Bown, 1985). The presence of H^+ decreases pH whereas the uptake of HCO_3^- into cells increased apoplastic pH due to the presence of an OH^- efflux instead of an H^+ influx transport system (Laing and Browse, 1985). The increase in the pH of the apoplast probably enables Ca^{2+} rather than H^+ as the ion species that binds to negatively charged carboxyl groups of the cell wall (Harker et al., 2000) to promote linking of neighboring pectin polymers through the egg-box model (Demarty et al., 1984). The idea is in line with our observation. We observed that during high CO_2 treatment, fruit firmness went up whereas after fruit were transferred to ambient

atmosphere firmness slightly decreased (Figure 5C). By contrast, firmness continuously declined in fruit from control and CA0 treatments.

The differences in anthocyanin contents of fruit from different treatments during CA storage and shelf life were not consistent, presumably due to the biological variation in samples. Ascorbic acid contents in fruit did not change over the experimental period and no differences between control and different CA treatments were apparent. From these aspects, our findings in 'Arabella' strawberries are different from most of the studies where they found firmness loss, discoloration and antioxidant losses in strawberries treated with elevated CO_2 (Gil et al., 1997; Shin et al., 2008; S. K. Lee & Kader, 2000; Li et al., 2019).

Application of high CO_2 might induce fermentation. The extent of fermentation with increased CO_2 concentrations is cultivar-dependent in strawberries and some cultivars even did not produce fermentation metabolites (Fernández-Trujillo et al., 1999; Watkins et al., 1999; Pelayo-Zaldıvar et al., 2007). As the ratio of CO_2 production and O_2 consumption in fruit was near 1, both under 20 and 30 kPa CO_2 (data not shown), we assume that fermentative metabolism was not activated during CA storage. 'Arabella' strawberries therefore seem relatively tolerant to elevated CO_2. This could explain that although high CO_2 lowered the spoilage incidence, other quality properties, such as firmness, anthocyanins, ascorbic acid were hardly affected. The lower spoilage incidence not only resulted from the physiological effects of high CO_2 as we discussed above but may also be due to the direct suppression of high CO_2 on *B. cinerea* growth. *In vitro* inhibition tests showed that the *B. cinerea* colony diameter decreased with elevated CO_2 from 0 to 20 kPa and it could not grow when the CO_2 concentration was higher than 30 kPa (García-Gimeno et al., 2002). After fruit were transferred to the ambient atmosphere condition, the inhibitory effect was relieved, but the residual effect of elevated CO_2 still existed, which was observed in both experiments and in the two cultivars studied in our research.

4.5 Conclusion

The application of high CO_2 (20 and 30 kPa) combined with 10 kPa O_2 greatly suppressed fruit respiration, spoilage rates and softening during storage and/or subsequent shelf life. A more pronounced conversion of sucrose to glucose and, in particular, fructose was observed under high CO_2 conditions which might improve fruit sweetness. The stepwise increments of CO_2 did not show beneficial effects on overall fruit quality

compared to the static application of high CO_2. 'Arabella' strawberries can be stored under 30 kPa CO_2 for prolonged periods of time without loss of nutritional quality.

Author Contribution

HL, CZ and EW conceived and designed the experiments. HL and CZ conducted Exp I; HL and YY conducted Exp II. HL and YY analyzed the data. HL wrote the manuscript with the help of EW. RS and FYA provided critical comments to the overall structure of the manuscript. All authors reviewed and approved the final manuscript.

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Supplementary materials

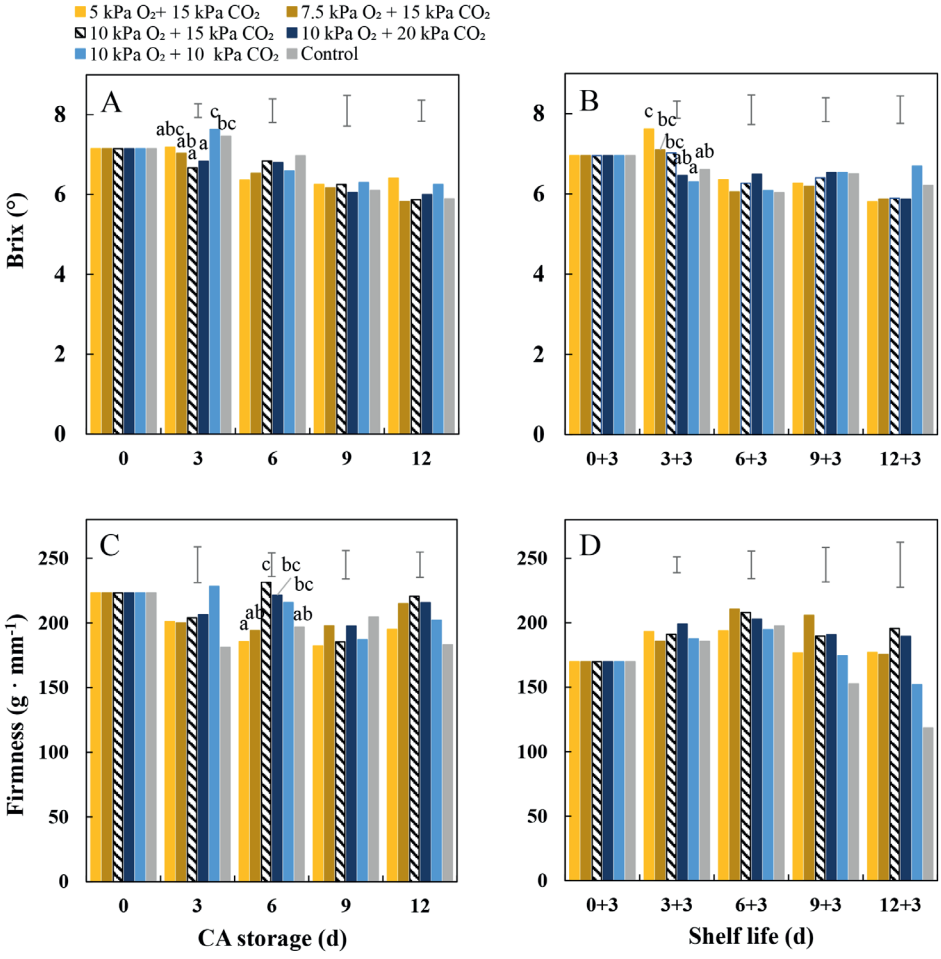

Figure S1 Brix and firmness of 'Sonsation' strawberry fruit stored under controlled atmosphere (CA) conditions and shelf life. (A) Brix and (C) firmness at different time points during CA storage at 5 °C and 100% relative humidity. (B) Brix and (D) firmness at different time points of CA storage followed by 3-d shelf life at 12 °C and 100% relative humidity in ambient atmosphere. 21 kPa O_2 and 0 kPa CO_2 served as control. Data represent means of 3 blocks (n=3) with five replicate fruit per block. The error bars represent standard error of means. Different letters denote significant differences according to Fisher's protected LSD test (α=0.05).

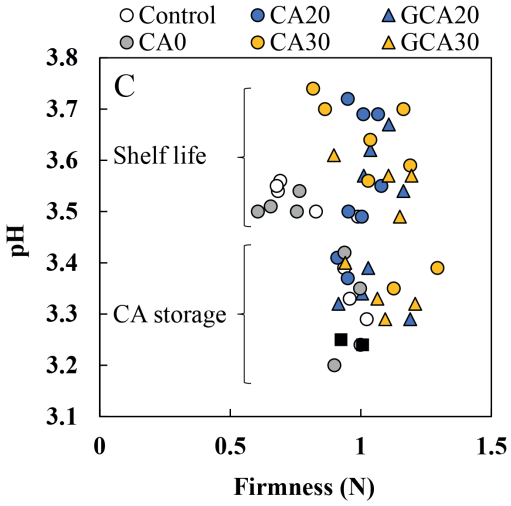

Figure S2 Correlation between pH and firmness of fruit from different CA treatments during storage and subsequent shelf life. The closed black squares represent data of d 0. Data represent individual blocks.

Chapter 5

Sensory, GC-MS and PTR-ToF-MS profiling of strawberries varying in maturity at harvest with subsequent cold storage

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Abstract

Harvesting strawberry fruit before they are fully ripe and allowing them to further ripen during postharvest cold storage is a common practice. The effect of these storage conditions on consumer liking is not well understood. The first aim of this study is to investigate the effects of maturity at harvest and subsequent cold storage on consumer liking, expressed as sweetness and aroma attributes, and volatile composition. The second aim of this study is to investigate whether volatile organic compounds (VOCs) can be used to predict consumer liking. Strawberries (*Fragaria × ananassa* cv. Lusa) were harvested either at the ¾ red stage or full red stage and stored at 4 °C for one, five or nine days. Strawberries were subjected to sensory profiling, color-, firmness-, GC-MS- and PTR-ToF-MS- measurements. The sensory profile of strawberries harvested at ¾ red stage showed lower sweetness and aroma than full red harvested strawberries. VOC analysis of these strawberries showed lower presence of volatile fatty acids, furanones and most esters even after nine days of cold storage, compared to full red strawberries. Strawberries harvested at full red stage showed the highest value for aroma attributes after one day of cold storage. Surprisingly, peak intensities of most esters (except for methyl butanoate and methyl hexanoate) and furanones were low on the first day, compared to ripe harvested fruit after longer storage. Ripe harvested fruit stored for nine days showed the highest peak intensities for most VOCs, but this did not correspond to the highest sensory aroma attributes. These fruits were judged with the lowest values for aroma attributes, perhaps related to the production of volatiles with off-flavors (acetaldehyde, ethyl acetate). PLS modelling showed that VOCs exist that are characteristic for both sweet and aromatic sensory attributes of 'Lusa' strawberries, based either on GC-MS (mainly volatile fatty acids) or PTR-ToF-MS analysis (mainly alcohol/ester fragments). This could lead to fast, non-destructive, selection of strawberries with high consumer liking using PTR-ToF-MS.

Keywords: consumer liking; expert panel; multivariate analysis; PLS modelling; volatile organic compounds

5.1 Introduction

Strawberry is the most commonly consumed berry fruit crop worldwide and is valued for its unique flavor and nutritional quality. Its overall liking is most affected by the sensory attributes sweetness and flavor intensity (Schwieterman et al., 2014). Volatile organic compounds (VOCs) are essential components of strawberry flavor, even though they only account for less than 0.01% of the fruit's weight (Yan et al., 2018). The volatilome of strawberry is one of the best studied of all fruit. Nevertheless, none of the identified VOCs were consistently present in all studies (Ulrich et al., 2018). The variation in volatile profiles is both complex and distinct due to genotype, growth conditions, ripeness at harvest, postharvest storage conditions and extraction techniques (Schwieterman et al., 2014; Li et al., 2015; Ulrich et al., 2018; Yan et al., 2018). In total, 979 volatile compounds have been identified in strawberry fruit. However, within strawberries of a single cultivar far fewer compounds are detectable (Ulrich et al., 2018). Less than twenty volatiles have a significant contribution to strawberry flavor based on concentration to sensory threshold ratio (Jetti et al., 2007). The furanones furaneol and mesifuran, due to their low sensory threshold values, provide the typical caramel-like, sweet, floral and fruity strawberry aroma (Zabetakis et al., 1999; El Hadi et al., 2013). Esters account for 25-90% of all strawberry volatiles and provide fruity and floral flavor (Yan et al., 2018). The most frequently identified esters in strawberry fruit are methyl hexanoate, ethyl hexanoate, ethyl butyrate and methyl butanoate (Ulrich et al., 2018), derived from esterification by alcohol acyl-transferases (AATs) (Duan et al., 2018). Fatty acid derived alcohols and aldehydes such as hexanal, E-2-hexenal, and Z-3-hexenol, are responsible for green, fresh notes in strawberry (Jetti et al., 2007; Du et al., 2011). Fatty acid-derived volatile aldehydes are generally produced from linoleic- and linolenic acid through the action of lipoxygenase (LOX) or hydroperoxide lyase (HPL). These aldehydes may then be converted to alcohols by alcohol dehydrogenases (ADHs) (Yan et al., 2018). Volatile fatty acids generally only affect the perceived aroma slightly, except for butanoic, 2-methylbutanoic and hexanoic acid, which are important contributors to strawberry aroma (Ménager et al., 2004; Du et al., 2011). Volatiles from other volatile pathways, such as terpenoids, benzenoids and sulphur compounds are also frequently reported (Yan et al., 2018).

Harvesting fruit before they are fully ripe, and allowing them to further color during postharvest storage, is a common practice for many fruits in the supply chain (Kader, 2008). This practice extends shelf-life, facilitates storage and transport and decreases the impact of harvesting and handling. Strawberry fruit of four cultivars reached the

longest shelf-life when harvested at the white to pink color stage (Rahman et al., 2016). Nunes et al. (2006) found that strawberries of three cultivars, harvested at the ¾ red stage developed the same level of soluble solids, ascorbic acid and total phenolics content during storage as strawberries harvested at the full red stage. However, Van de Poel et al. (2014) found lower sugar levels and lower overall volatile abundance for 'Portola' strawberries harvested at ¾ red stage compared to those harvested at the full red stage. Maturity at harvest is therefore likely an important factor affecting consumer liking of strawberries. How the liking of strawberries is affected by postharvest storage, during retail transport and home refrigeration, is currently mostly unknown. Total soluble sugars and total acidity, important factors determining sweetness (Magwaza and Opara, 2015) slowly decreased during storage (Li et al., 2015). It is clear though that the volatile composition changes during postharvest storage. During refrigerated storage aldehydes (E-2-hexenal, Z-3-hexenal, hexanal), hexanoic acid and the esters methyl butanoate and ethyl butyrate increased in red 'Sweet Charlie' strawberries (Ozcan and Barringer, 2011). Lower levels of total esters and total furanones, comparable levels of total acids, but higher levels of total terpenes were observed in cold stored red 'Akihime' strawberries compared to room temperature stored fruit (Li et al., 2015). Storage temperature and light conditions also interacted. Ester and furanone levels were comparable when white-pinkish 'Sweet Charlie' strawberries were stored for seven days either at 15 °C /dark, 25 °C/dark and 25 °C/light but lowest when stored at 15 °C in the light (Fu et al., 2017a). This indicates complex and distinct behaviour of the volatile composition during postharvest storage.

The first aim is to investigate the changes in consumer liking and the volatile composition during postharvest storage of 'Lusa' strawberries, harvested at either the ¾ red stage or the full red stage. Liking was assessed by a strawberry expert panel and the volatile composition by gas chromatography–mass spectrometry (GC-MS), the most often used technique to detect strawberry volatile compounds (Ulrich et al., 2018). The second aim is to investigate whether the changes in the volatile composition as affected by initial maturity and postharvest storage can also be assessed by proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS). PTR-ToF-MS enables fast and real-time monitoring of volatiles with high sensitivity without labor-intensive sample preparation (Majchrzak et al., 2018) but with the drawback that compound identification is more challenging and often not possible (Cappellin et al., 2012). We discuss the impact of harvesting strawberries either at the ¾ red stage or full red stage during cold storage on consumer liking and discuss the origin of the changes that

appear in the volatile pathways as assessed by GC-MS. Finally, we discuss the possibility to use VOCs as markers to predict consumer liking.

5.2 Material and methods

5.2.1 Plant material and storage conditions

Strawberries (*Fragaria × ananassa* cv. Lusa) were harvested either at the ¾ red stage or full red stage (indicated as 'unripe' and 'ripe', respectively) from a grower in Prinsenbeek, the Netherlands on May 10th, 2017. In total 75 punnets per maturity stage were harvested with each punnet consisting of about 500 g of undamaged and uniformly sized strawberries. Punnets were transported to the lab in Wageningen within approximately one hour after harvest. At the start of the next day the punnets were labelled and randomly assigned to six treatments; two initial maturity stages (unripe and ripe) and three storage times (day 1, day 5 and day 9) with 25 punnets per treatment. These were randomly assigned to five crates, each containing five punnets, with punnets in one crate considered a replicate. Eighty percent of the punnets were used for PTR-ToF-MS measurements and sensory analysis, the other twenty percent for color, firmness and GC-MS measurements. All punnets were stored in darkness at 4 °C and a relative humidity of 80%. Prior to measurements, fruits were placed at room temperature (20 °C) for six hours.

5.2.2 Trained expert panel

Following PTR-ToF-MS measurements, fruits were evaluated by a trained expert panel (Greenhouse Horticulture, Bleiswijk, the Netherlands) after transport from Wageningen to Bleiswijk in approximately one hour. Strawberries were evaluated at room temperature by nineteen panellists in individual booths illuminated with red light to minimise the effect of strawberry color differences. Evaluations took place during late afternoon and early evening sessions. Each panellist evaluated 21 attributes on a 1-to-100-point scale for six fruits per replicate for the unripe and ripe harvested fruits on day 1, day 5 and day 9 with data represented as average per replicate.

5.2.3 Color and firmness measurements

Strawberry skin color was measured using a LED color matching cabinet (IPSS Engineering, Wageningen, the Netherlands) containing a RGB camera (MAKO G-192C POE, Allied Vision, Stadtroda, Germany). Calyxes were removed prior to acquiring the images. A set of images was recorded for 15 individual fruits per punnet, from five

punnets per maturity stage and storage time. Two images were acquired, one from each side of 15 individual strawberries, randomly selected per punnet. Strawberries were placed on a blue holding tray and between images turned 180° over their proximal-distal axis. The acquired RGB images were calibrated using a 24-patch color card (ColorChecker Classic, XRite Europe GmbH, Regensdorf, Switzerland). Image analysis was carried out by using multi-threshold color image segmentation to remove the blue background and separate the individual strawberries in each image. Color data were transformed from the RGB to the HSV color model and expressed as the average Hue value of both sides of each strawberry. Due to the circular nature of the hue scale, hue values over 360° were expressed as negative values.

Firmness of the same strawberries that were selected for color measurements were measured using a FirmTech FT7 (UP GmbH, Ibbenbüren, Germany). Firmness was expressed as the average force displacement (in g mm^{-1}) between 70 and 250 g of force applied on the strawberry shoulder.

5.2.4 VOC analysis by SPME/GC-MS and data processing

Extraction and detection of volatile metabolites were performed according to Tikunov et al. (2005) with slight modifications as follows. Strawberries were cut in slices and immediately frozen in liquid nitrogen. The frozen slices were ground into powder with an analytical grinder (IKA A11, IKA, Staufen, Germany). Frozen strawberry powder (0.5 g) was added to 0.5 mL deionized water and incubated at 30 °C for 10 min. After adding 1 mL 100 mM, pH 7.5 EDTA/NaOH, 2.2 g solid $CaCl_2$ powder was mixed thoroughly to inhibit enzyme activity. One mL of the extract was transferred into a 10 mL crimp cap vial for headspace SPME/GC-MS detection. Individual vials were randomised to avoid systematic memory effects and placed into a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). A 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibre (Supelco, Bellefonte, PA, USA) was exposed for 20 min to the vial headspace under continuous agitation and heating at 50 °C. The trapped compounds by SPME were desorbed into a Trace GC Ultra gas chromatograph (Thermofisher, Waltham, USA) injector for 1 min at 250 °C. Chromatographic separation was achieved on an Zebron ZB-5 (50 m ×0.32 mm ×1.05 µm) column (Phenomenex) with helium as the carrier gas (at a constant flow of 2 mL min^{-1}). The GC interface and MS source temperatures were 260 and 250 °C, respectively. The GC temperature program started at 45 °C for 2 min, was then increased to 250 °C at a rate of 5 °C min^{-1} and finally held at 250 °C for 5 min. Including oven cooling, the total run time was 60 min. Mass spectra in the 35-400 m/z range were recorded by an DSQII electron impact MS (Thermofisher) at a scanning

speed of 2.8 scans s^{-1} and an ionization energy of 70 eV. The chromatography and spectral data were evaluated using Xcalibur software (Thermofisher). The raw data generated by SPME/GC-MS were processed by the MetAlign™ software package (http://www.metalign.nl) (Tikunov et al., 2012). VOCs were identified by matching mass spectra and the retention indices of the compounds extracted to the NIST mass spectral library using NIST MS Search software (http://www.nist.gov).

Per maturity stage and storage time five replicates were measured with each replicate consisting of pooled strawberry powder from one punnet per maturity stage and storage time. GC-MS analysis was carried out twice, three days apart from each other, on the same samples. Linear regression (using the lm package in R) was carried out to test whether a linear relationship existed between the intensities of all identified compounds from the first and second GC-MS analysis for all samples (five replicates per combination of maturity and storage time). Only the peak intensities of compounds showing a linear relation with a R^2adj > 0.85 were selected for further analysis. This means that only compounds that were identified twice and at comparable peak intensities were included. Peak intensities of these compounds were averaged and normalized.

5.2.5 VOC analysis by PTR-ToF-MS and data processing

PTR-ToF-MS analysis was carried out by placing two punnets in an airtight high-density airtight polyethylene (HDPE) drum (Engels Logistiek B.V., Eindhoven, the Netherlands) with septa (Suba-Seal, Sigma-Aldrich) mounted on the lids. After thoroughly flushing the drums with clean air for 2 min, the punnets were placed in the drums, the drums were closed and incubated for 2 h at 20 °C for accumulation of volatiles. Thereafter headspace volatiles were measured using a PTR Qi-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) set with the following conditions: 110 °C drift tube temperature, 3.8 mbar drift pressure, 900 V drift voltage, leading to an E/N ratio of about 120 Townsend (Td; 1 Td = 10^{-17} V cm^{-2}), where E corresponds to the electric field strength and N to the gas number density. Mass spectrometric data were collected over a mass range of 20-512 m/z using a flow rate of 60 mL min^{-1}. PTR-ToF-MS data was extracted using PTRwid (Holzinger, 2015). Noise reduction was done by averaging 20 consecutive and stable ToF spectra with subsequent baseline correction. Per maturity stage and storage time five replicates were measured. Each replicate consisted of the averaged data from two identical samples (two drums, each containing two punnets). Peak identification was carried out by combining data from Aprea et al. (2009) for raspberry and Farneti et al. (2015) for apple. Peak intensities were corrected for fruit

weight and normalized. No internal standard was added for both GC-MS and PTR-MS data processing purposes as many chemically diverse compounds were identified; an internal standard is only valid for quantification of one single class of compounds and using one would introduce errors for compounds of other chemicals classes.

5.2.6 Statistics

Statistical analyses were performed in R.4.0.2 (http://www.R-project.org/) with all tests conducted at a P value of 0.05. Color and firmness data were compared applying two-way analysis of variance (ANOVA), sensory attributes with one-way ANOVA. Homogeneity and normality were tested using Bartlett's test and the Shapiro-Wilk test, respectively. Tukey HSD test was used as post-hoc test for the variables with a significant treatment effect. Heatmaps were created with the help of the packages heatmap.2, gplots and RColorBrewer. The Pairwise Wilcox test (pairwise.wilcox.test) was used to identify differences in intensities due to maturity or storage time applying the DH correction (Benjamini and Yekutieli, 2001) for multiple testing. PCA plots and hierarchical clustering were conducted with the help of packages prcomp, survival, nnet, MASS, splines, ellipse and car. PLS modelling was carried out using the pls and mdatools packages applying leave-one-out cross validation. Permutation testing, to avoid overparameterization, was carried out using the function randtest, applying 5000 permutations. The PLS models were calibrated applying a VIP (Variables Important for Projection) score lower than 1 (Galindo-Prieto et al., 2014).

5.3 Results

5.3.1 Color and firmness development during storage

In unripe harvested fruit red coloration increased over storage time, as indicated by decreasing Hue values. Ripe harvested fruit did not change color over time (Figure 1A). Firmness decreased over time for unripe fruit whereas the firmness of ripe strawberries remained at the same level (Figure 1B). Both color and firmness values for the unripe fruits at day 9 did not reach those of ripe fruit at day 1.

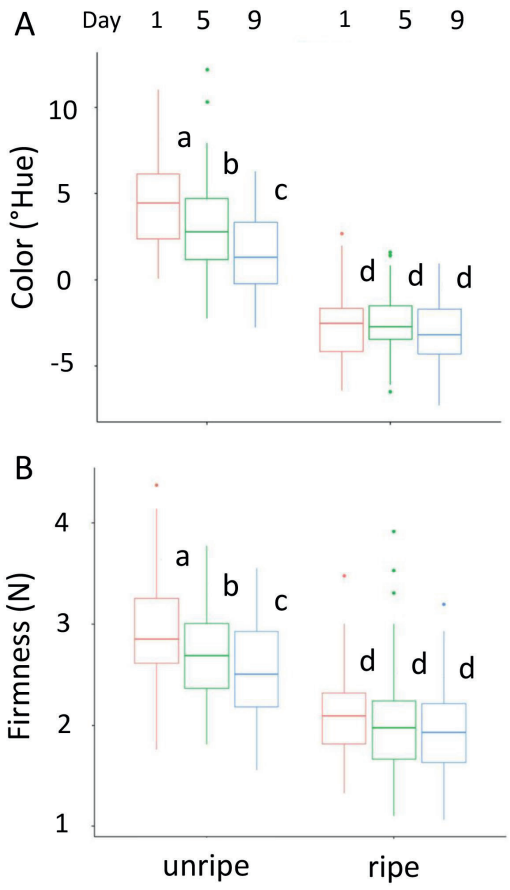

Figure 1 Color (A) and firmness (B) measurements for unripe and ripe harvested 'Lusa' strawberries after one, five and nine days of storage (4 °C) expressed as boxplots and analyzed by 2-way ANOVA with $P_{(ripeness)} < 0.001$ and $P_{(storage\ days)} < 0.001$ for both color and firmness measurements. Different letters indicate significant differences ($P < 0.05$). °Hue values over 360° were expressed as negative values. Data are means of five replicates (n=5) from one punnet, consisting of 75 measurements per combination of ripeness stage and storage time.

5.3.2 Sensory profiling

Mean scores of individual sensory attributes of unripe and ripe harvested fruits are shown per storage time, expressed as spider web charts (Figure 2). On day 1, unripe fruit showed significantly higher firmness, lower juiciness, lower sweetness, and higher sourness than ripe fruit (Figure 2A). In addition, sensory attributes related to aroma showed significantly lower values for unripe fruit on day 1, for example aroma presence, aroma liking, fruity aroma and strawberry aroma. These differences between unripe and ripe fruit increased on day 5 compared to day 1 (Figure 2B). In addition, significantly lower mealiness, higher firmness of the seeds, lower fruity (other) aroma and lower

flower aroma were observed in unripe compared to ripe fruit at day 5. On day 9, unripe fruit showed significantly higher firmness, lower juiciness, lower sweetness, and higher sourness than ripe fruits, similar to day 1 and day 5 (Figure 2C). Unripe compared to ripe fruit stored for nine days showed significant differences in flavor related attributes, such as lower aroma presence, higher green aroma, lower strawberry aroma and lower flower aroma. On day 9, differences between unripe and ripe fruit diminished compared to day 5 but were still larger than observed at day 1. For example, aroma liking, fruity aroma and fruity (other) aroma were similar in unripe and ripe fruit on day 9, but showed differences between unripe and ripe fruit on day 5. On day 9, significantly higher yeast and lower astringent attributes in ripe harvested fruit were observed.

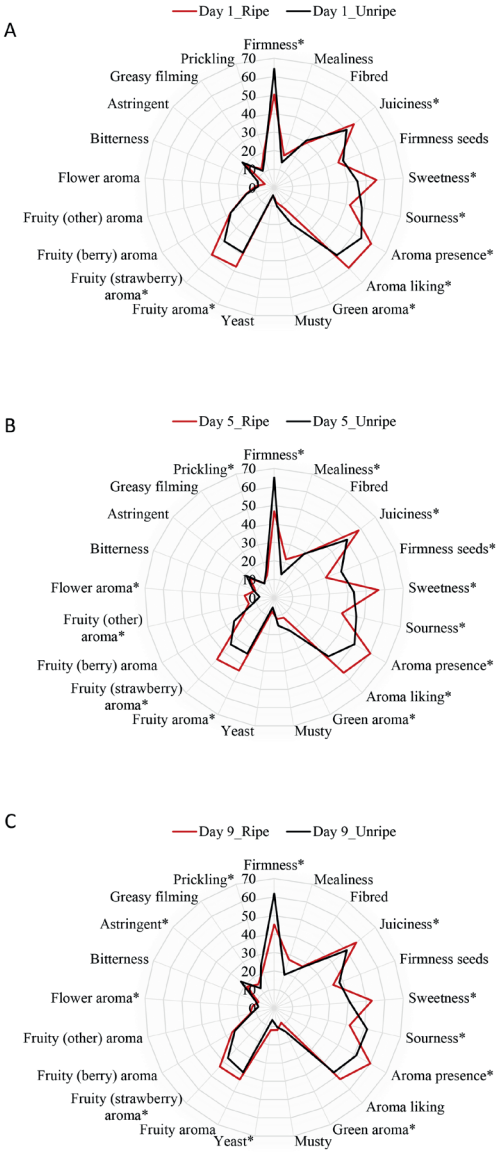

Figure 2 Sensory evaluation for unripe and ripe harvested 'Lusa' strawberries after one (A), five (B) and nine days (C) of storage (4 °C) expressed as spider plots. Asterisks indicate significant differences as indicated by 1-way ANOVA ($P < 0.05$).

5.3.3 Volatile profiling by GC-MS

Although 85 peaks were observed, only twenty-five passed the test of being present during two consecutive GC-MS runs with a similar peak intensity as detailed in the M&M section. Changes in relative peak intensities of these twenty-five compounds

during storage in unripe and ripe harvested fruit are represented as a heatmap. Hierarchical clustering, indicating similar VOC behavior, resulted in four groups of compounds (Figure 3). The first group consisted mainly of volatile aldehydes which showed relatively small changes regardless of initial maturity and storage time. The second group consisted of volatiles fatty acids and alcohols. The volatile fatty acids showed lower peak intensities for the unripe compared to ripe fruits. The alcohols, similar to the volatile fatty acids, showed lower peak intensities for the unripe compared to ripe fruits. The VOCs in the third group, mainly volatile fatty acids, and esters had low peak intensities in unripe compared to ripe fruits, regardless of storage time. In the last group, group 4, the main constituents were esters, with low peak intensities in unripe fruits. Ripe harvested fruits on day 1 had comparable peak intensities to those of unripe fruits. During storage of ripe fruit, all peak intensities increased.

Typical strawberry volatiles, furaneol (group 4) and mesifuran (group 3), had low peak intensities in unripe fruits. At the first day of storage of ripe fruit, furaneol peak intensities were low, but increased during storage. The peak intensities of mesifuran, at the start of the storage of ripe harvested fruit, were higher than in unripe fruit and continued to increase during storage. The PCA plot of the GC-MS data indicated that the total variability explained by the first and second principal components is almost 70% (Figure 4A). The 95% confidence ellipses indicate that there is a good separation between ripe and unripe fruit, and within the ripe harvested fruit, between storage days. Most volatiles present in the PC1 loading plot have positive loadings indicative of ripe fruit. The PC2 loading plot showed both positive and negative loadings, mostly indicative of the variation in storage days (Figure 4B). In other words, a range of volatiles characteristic for variation in storage days was observed in ripe fruit, whereas only E-2-hexenal was characteristic for unripe fruit at day 1.

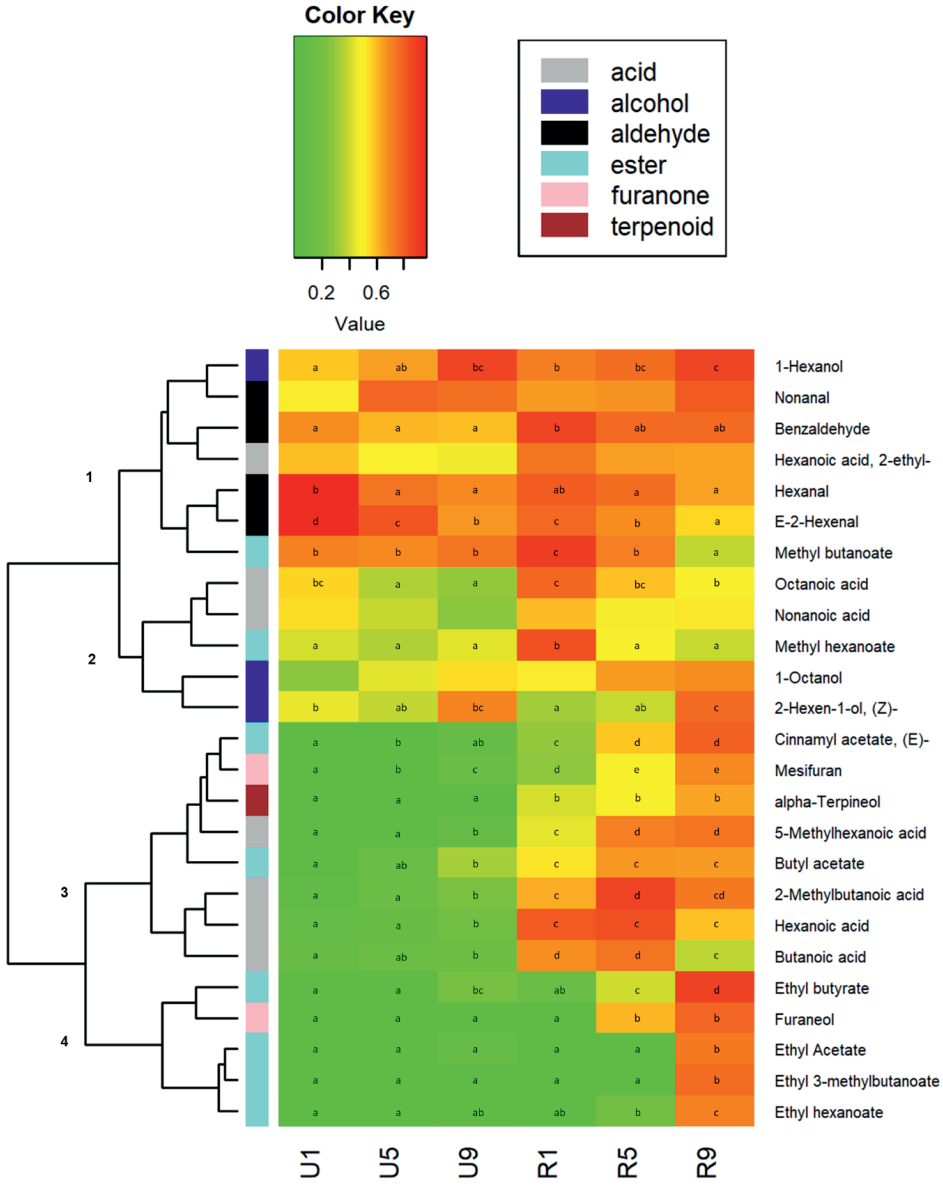

Figure 3 Heatmap, including hierarchical clustering, of the untransformed and normalized GC-MS data for unripe (U) and ripe (R) harvested 'Lusa' strawberries after one (1), five (5) and nine (9) days of storage (4 °C). Distinct letters indicate statistical differences depicted by the pairwise Wilcox test ($P < 0.05$). Data are means of five replicates (n=5) from one punnet.

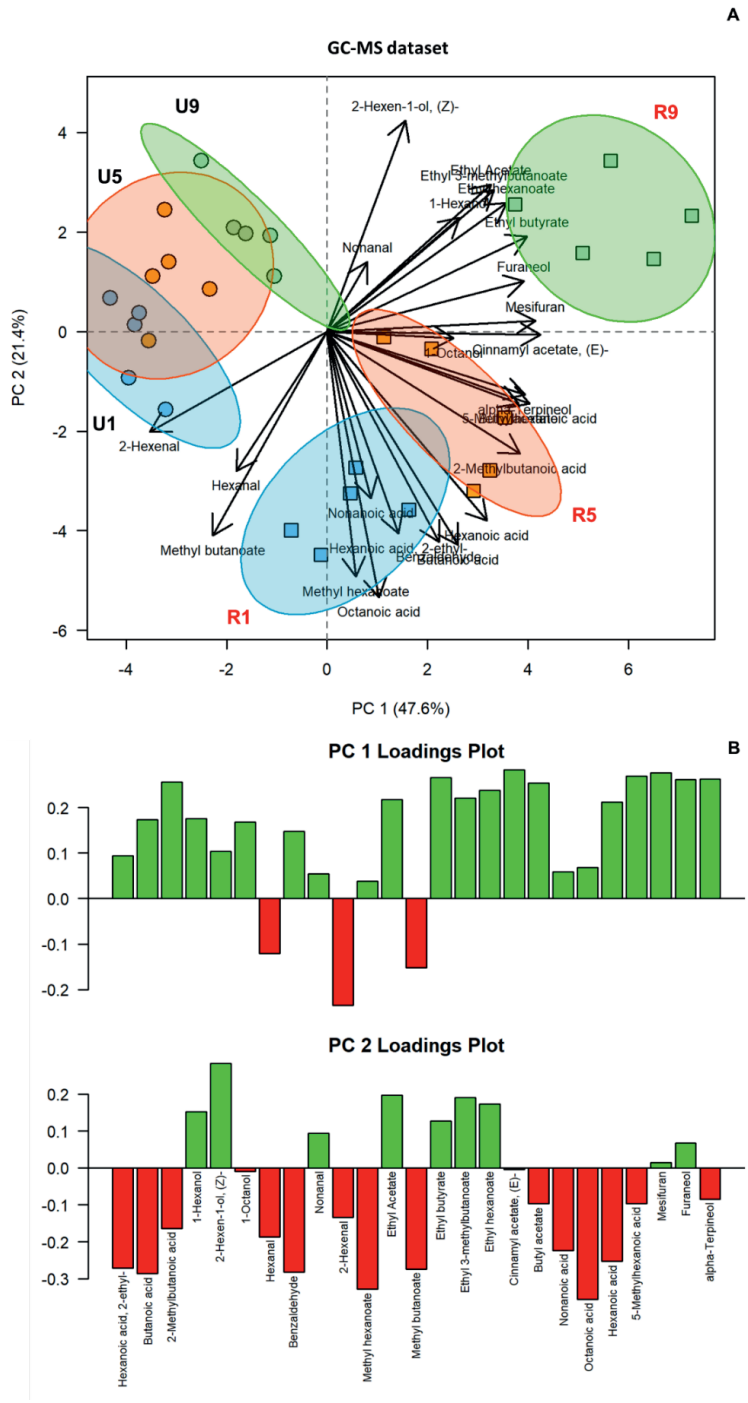

Figure 4 PCA plot (A) and loading plot (B) for unripe (U) and ripe (R) harvested 'Lusa' strawberries after one (1), five (5) and nine (9) days of storage (4 °C) based on GC-MS data.

5.3.4 Volatile profiling by PTR-ToF-MS

Changes in peak intensities of twenty-six fragments and compounds as measured by PTR-ToF-MS in unripe and ripe harvested fruit over time are represented as a heatmap (Figure 5). Hierarchical clustering indicates four main groups of compounds. Most groups consisted of a variety of fragment types. Fragments from alcohols, aldehydes (propanal and hexanal), and also esters are dominating in the first group. Most of the constituents of group 1 have lower peak intensities in unripe compared to ripe fruit, and hardly change over time. The second group consisted of fragments with peak intensities increasing over time. The third group consisted of fragments that, except for the C7H9+ fragment, did not show many changes as function of harvest maturity and storage time. The fourth group, consisting mostly of esters, and also acetaldehyde and ethanol, showed increasing peak intensities for unripe and ripe fruit over time.

The PCA plot of the PTR-ToF-MS data (Figure 6A) had similarities with the PCA plot for the GC-MS data (Figure 4A) with respect to the distribution of the 95% confidence ellipses. The separation between ripe and unripe fruit was well established, and within the ripe fruit there is a clear separation between storage days. The total variability explained by the first and second principal components is high at 84.3%. The PTR-ToF-MS PC1 loading plots showed mostly positive loadings, indicative of compounds and fragments characteristic for ripe fruits. The PC2 loading plot showed both positive and negative loadings, mostly indicative of the variation in storage days (Figure 6B). Similar as for the GC-MS PCA plot (Figure 4A), many compounds and fragments characteristic for variation in storage days in ripe fruit were observed, but not in unripe fruit. Only methanol was characteristic for unripe fruit.

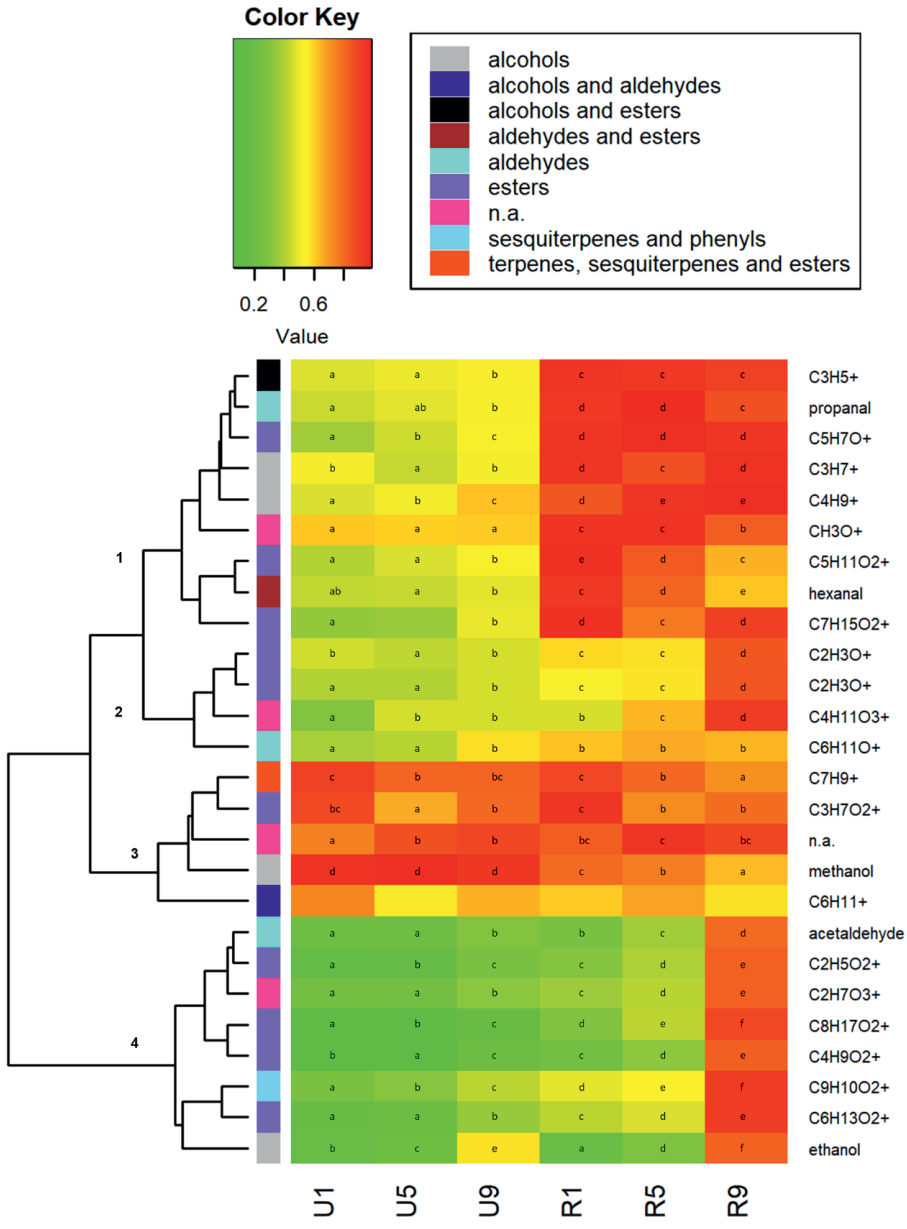

Figure 5 Heatmap, including hierarchical clustering, of untransformed and normalized PTR-ToF-MS data for unripe (U) and ripe (R) harvested 'Lusa' strawberries after one (1), five (5) and nine (9) days of storage (4 °C). Distinct letters indicate statistical differences depicted by the pairwise Wilcox test ($P < 0.05$). Data are means of five replicates (n=5) from four punnets.

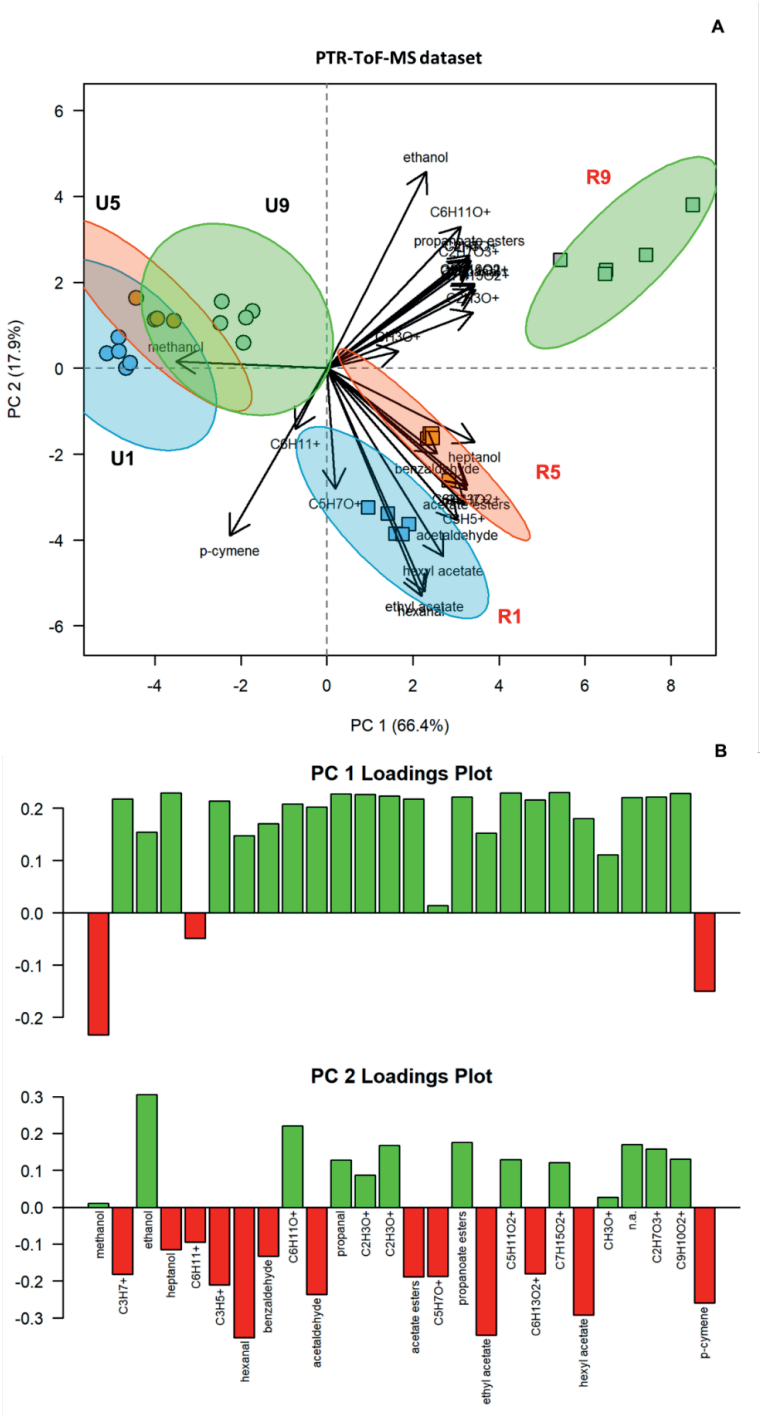

Figure 6 PCA plot (A) and loading plot (B) for unripe (U) and ripe (R) harvested 'Lusa' strawberries after one (1), five (5) and nine (9) days of storage (4 °C) based on PTR-ToF-MS data.

5.3.5 Prediction of consumer liking by PLS modelling of VOCs

Partial Least Square (PLS) regression modelling was carried out to predict the sweetness and aroma attributes based on the volatile composition. Consumer liking is determined by sweetness and flavor intensity. Flavor intensity was described as the retronasal olfaction complimenting sourness and sweetness intensities' contribution to taste (Schwieterman et al., 2014). Here, sensory attributes related to flavor intensity were recorded such as aroma presence, aroma liking, fruity aroma, fruity (strawberry) aroma (Figure 2). The average value of these attributes was regarded as aroma, describing flavor intensity. PLS models were built to predict sweetness and aroma based on peak intensities of volatiles gathered by either GC-MS or PTR-ToF-MS. The PTR-ToF-MS based PLS models showed higher explained variation of validation (R^2p) and lower values for the root mean square error of prediction (RMSEP) than GC-MS based PLS models (Table 1). Variables important for the prediction (VIP) of sweetness and aroma were often found to be in common (Table 2). Regression coefficients for sweetness and aroma, based on GC-MS data, showed a high correlation (R = 0.92), indicating high similarity between those PLS models.

Table 1 Performance of partial least square models using GC-MS and PTR-ToF-MS strawberry data of unripe and ripe harvested 'Lusa' strawberries after storage (4 °C). nLV, number of latent variables, root mean square error of calibration ($RMSE_C$) and validation ($RMSE_V$), root mean square error of prediction ($RMSE_P$), explained variation of calibration (R^2_c) and validation (R^2_p).

Sensory attribute	Analysis	Calibration		Prediction		nLV
		RMSEc	R^2_c	RMSEp	R^2_p	
Sweetness	GC-MS	2.6	0.84	3.1	0.78	2
Aroma	GC-MS	2.6	0.76	3.0	0.68	2
Sweetness	PTR-ToF-MS	2.4	0.87	2.8	0.81	3
Aroma	PTR-ToF-MS	2.0	0.86	2.6	0.75	4

Table 2 PLS analysis for the prediction of sweetness and aroma sensory attributes based on GC-MS and PTR-ToF-MS data for unripe and ripe harvested 'Lusa' strawberries during storage at 4 °C. Only variables there were significant ($P < 0.05$) are listed. VIP, variables important for projection.

Analysis	Attribute	Predictor	VIP	Regression coefficient		Attribute	Predictor	VIP	Regression coefficient	
				Value	Std.err.				Value	Std.err.
GC-MS	Sweetness	Hexanoic acid	1.60	0.15	0.03	Aroma	Hexanoic acid	1.62	0.14	0.03
		Butanoic acid	1.57	0.16	0.04		Butanoic acid	1.59	0.15	0.04
		2-Methylbutanoic acid	1.47	0.10	0.02		2-Methylbutanoic acid	1.38	0.08	0.02
		5-Methylhexanoic acid	1.38	0.09	0.02					
		α-Terpineol	1.29	0.07	0.03		α-Terpineol	1.19	0.04	0.04
		Butyl acetate	1.26	0.07	0.03					
		Benzaldehyde	1.21	0.11	0.04		Benzaldehyde	1.27	0.10	0.04
		Cinnamyl acetate, E-	1.18	0.04	0.02					
		Octanoic acid	1.09	0.14	0.03		Octanoic acid	1.30	0.15	0.03
		Methyl hexanoate	1.07	0.14	0.04		Methyl hexanoate	1.37	0.18	0.05
PTR-ToF-MS	Sweetness	CH3O+	1.42	0.22	0.07	Aroma	C5H11O2+	1.48	1.78	0.34
		propanal	1.33	0.16	0.05		C3H7+	1.36	2.99	0.56
		C3H5+	1.29	0.15	0.04		C7H15O2+	1.26	-2.02	0.62
		C3H7+	1.28	0.30	0.09		C5H7O+	1.25	-1.51	0.68
		ethanol	1.07	-0.38	0.08		C4H9+	1.15	1.04	0.47
		methanol	1.06	-0.32	0.15					

5.4 Discussion

5.4.1 Volatile fatty acids

Volatile profiles between unripe and ripe harvested fruits as measured by GC-MS analysis were quite different, especially with regard to the volatile fatty acids. Many volatile fatty acids showed higher (group 2 and group 3, Figure 3) peak intensities in ripe compared to unripe fruits, irrespective of storage duration. In a list of 54 strawberry volatiles, sorted on decreasing odor activity value (OAV), 2-methylbutanoic ('sour', 'cheesy', 'sweaty'), butanoic ('sour', 'cheesy') and hexanoic acid ('sweaty', 'cheesy') were placed on position 8, 15 and 18, respectively (Du et al., 2011). These volatile fatty acids are therefore not only important for perceived aroma, but also characteristic for differentiating unripe and ripe fruit. It is remarkable that these VOCs, with a generally negative perception, have higher peak intensities in ripe fruit, perhaps suggesting that they have a function in enhancing the characteristics of other fruity VOCs.

5.4.2 Esters

Esters observed in group 4 (Figure 3) had low peak intensities in unripe fruits, whereas intensities increased during storage in ripe fruit. Methyl butanoate, ethyl butyrate and ethyl hexanoate are part of this group and have high OAV values (3, 7 and 30 respectively (Du et al., 2011)). The peak intensities of these esters, present in ripe fruit at the start of the storage (day 1), were comparable with those in unripe fruit, and increased up to day 5, except for ethyl butyrate. The relatively low peak intensities for most esters at the first day of storage in ripe fruit might be due to low substrate availability that governs ester biosynthesis, next to AAT specificity (Yan et al., 2018). Not all ester peak intensities were low at the first day of ripe fruit; exceptions are methyl butanoate and methyl hexanoate. Methyl butanoate (group 1) and methyl hexanoate (group 2) showed decreasing peak intensities during storage in ripe fruits. These esters might therefore be important for the fruity and floral aroma of freshly ripe strawberry fruit, as most other esters are not yet synthesized.

5.4.3 Aldehydes and alcohols

All identified aldehydes are part of group 1 (Figure 3). Hexanal ('fresh', 'green') and E-2-hexenal ('grassy', 'pungent') are the aldehydes with the highest OAV (Du et al., 2011) and showed lower peak intensities over time, irrespective of harvest maturity (Figure 3). Propanal, another aldehyde, was only registered during by PTR-ToF-MS analysis, and

showed higher peak intensities in ripe compared to unripe fruit, irrespective of storage time (group 1, Figure 5). This might indicate that propanal is not further metabolized. Benzaldehyde was the only compound identified that originates from the shikimate pathway (Fu et al., 2017a). GC-MS analysis showed higher peak intensities of benzaldehyde in ripe compared to unripe fruits (group 1, Figure 3). Currently, not much is known about the volatile benzenoid biosynthetic pathway in strawberries (Yan et al., 2018). 1-Hexanol (group 1, Figure 3), 2-hexen-ol, and 1-octanol (group 2, Figure 3) all increased for both unripe and ripe fruit during storage. These alcohols have high odor thresholds compared to their aldehyde homologues (Schwab et al., 2008). Therefore they probably do not contribute much to the strawberry aroma (Larsen and Watkins, 1995). Methanol peak intensities decreased for long-stored ripe fruit, as measured by PTR-ToF-MS analysis (group 3, Figure 5). This might indicate that methanol is used for ester synthesis. In contrast, ethanol, and acetaldehyde ('green', 'apple' (Du et al., 2011)) levels increased during storage for both unripe and ripe fruit (group 4, Figure 5). Ethanol and acetaldehyde are fermentation products that accumulate over time in strawberry (Ponce-Valadez and Watkins, 2008).

5.4.4 α-Terpineol and furanones

α-terpineol was the only identified terpenoid during GC-MS analysis (group 3, Figure 3) with lower peak intensities observed in unripe strawberries, irrespective of storage time. Other terpenoids, like linalool and geraniol, often present in strawberry providing a fruity and floral aroma (Du et al., 2011) were not observed here.

Quinone oxidoreductase (FaQR) is the enzyme present in the last step of the furaneol biosynthesis (Yan et al., 2018). FaQR is strongly induced by ripening (Li et al., 2015). In the dark, increasing temperatures were accompanied by upregulated expression of *FaQR* (Fu et al., 2017a). The initial lower furaneol peak intensity for freshly harvested ripe fruit (group 4, Figure 3) might be due to the conversion of furaneol to mesifuran (group 3, Figure 3) by an O-methyltransferase (Yan et al., 2018).

5.4.5 The link between volatile profiles and consumer liking

Soluble sugar accumulation is prevalent during the last phase of the maturation of strawberry fruit, roughly doubling the fructose, glucose, and sucrose levels from turning to full red (Tian et al., 2012). One of the main enzymes responsible for sucrose accumulation is FaSS1, a sucrose synthase. FaSS1 is proposed to have an important role in strawberry ripening (Zhao et al., 2017). It can be hypothesized that ripe harvested fruit, unlike unripe harvested fruit, has sufficient sucrose levels to jumpstart the

production of a number of volatile fatty acids and esters during storage (Figure 3). Many esters, especially those present in group 4 (Figure 3), showed increasing peak intensities over time in ripe fruit, including ethyl butyrate, ethyl hexanoate and ethyl 3-methylbutanoate ('fruity, 'apple', 'pineapple'). These compounds have high OAV values. This could indicate that ripe fruit stored for 9 days would have the best volatile related sensory scores. However, this does not seem to be the case: flavor attributes aroma liking, fruity aroma, and fruity (strawberry) aroma were highest on day 1, and lowest on day 9 for ripe fruit (Figure 2A,C). This might be due to methyl butanoate and methyl hexanoate. These compounds had high peak intensities for ripe fruit on day 1, that subsequently decreased during storage (Figure 3). Aroma liking might be less dependent on the furanones, as especially furaneol showed comparable levels for unripe and ripe fruit on day 1 (Figure 3). Lower flavor attributes for ripe fruit might also be related to increasing peak intensities for ethyl acetate over time for ripe fruit. Ethyl acetate has a pineapple-like aroma at lower, but a non-pleasant chemical odor at higher concentrations (Larsen and Watkins, 1995). The fruity ester perception of long stored ripe harvested fruit might be masked by acetaldehyde (group 4, Figure 5) or due to the higher yeast score for long stored ripe fruit (Figure 2C), although no signs of *Botrytis cinerea* infection were observed.

5.4.6 Is it possible to predict consumer liking?

Consumers are willing to pay a premium for strawberries with both great appearance and flavor (CBI, 2019). The PCA plots indicated that a range of volatiles (Figure 4A) and fragments (Figure 6A) are present that differentiate between ripe and unripe harvested fruits and between storage times of ripe fruits. The position and shape of the confidence ellipses in the PCA plots derived from both GC-MS and PTR-ToF-MS data are similar, despite the obvious differences in measurement protocols and principles. The high correlation (R = 0.92) between regression coefficients of the GC-MS based sweetness, and aroma PLS models indicated that consumer liking can be predicted (Table 2) with both PLS models. The three compounds with the highest VIP scores for both sweetness and aroma were all volatile fatty acids: butanoic, 2-methylbutanoic, and hexanoic acid. These volatile fatty acids have been mentioned as important contributors to aroma (Ménager et al., 2004; Du et al., 2011). Fan et al. (2021a) investigated whether volatile and non-volatile markers exist for sweetness and consumer liking for fully red harvested strawberries within set of diverse cultivar and breeding selections. Amongst a list of GC-MS measured volatile compounds important for both sweetness and liking were multiple medium-chain fatty acid esters, such as

butanoic and hexanoic acid derived esters. In a sensory evolution study from the same authors sweetness and strawberry flavor were also linked to the presence of butanoic and hexanoic acid derived esters (2021b). Butanoic and hexanoic acid, compounds with high VIP scores in our study, were not identified, but likely serve as precursor for these acid derived esters. The PLS models for sweetness and aroma based on PTR-ToF-MS had only one VIP in common, the alcohol fragment C3H7+ (Table 2). Volatile profiling during ripening by PTR-ToF-MS was also carried out for avocado, banana, mango, and mangosteen (Taiti et al., 2015). It was indicated that PTR-ToF-MS might become a commercial standard tool to link volatile fingerprinting with consumer liking. Although PTR-ToF-MS is a non-destructive technique that is capable of fast, real-time monitoring, there are also drawbacks to incorporate PTR-ToF-MS devices in e.g., sorting and grading lines. One drawback is the current high equipment cost. Another, perhaps more troubling drawback is that the composition and abundance of strawberry volatiles is strongly cultivar dependent (Schwieterman et al., 2014). Volatile composition and abundance also depend on postharvest factors such as storage temperature and the use of coatings (Yan et al., 2018). Nevertheless, Ulrich et al. (2018) listed thirty strawberry volatiles that are mentioned at least twelve times in twenty-five studies. It might be of interest to scale up the approach proposed here for more cultivars and growing conditions to investigate whether there are common patterns in volatile production as measured by PTR-ToF-MS that can be linked universally to consumer liking.

5.5 Conclusions

Harvesting strawberries at the ¾ red stage lowered sweetness and aroma presence compared to harvesting full red ‘Lusa’ strawberries, irrespective of the storage time. Strawberries harvested at the ¾ red stage had lower presence of volatile fatty acids, furanones, and most esters, even after nine days of storage at 4 °C. Full red harvested strawberries received the highest consumer liking at day one of cold storage. The levels of many important esters and furaneol of ripe fruits harvested at day one were comparable with those of unripe harvested fruits, with the exception of methyl butanoate and methyl hexanoate. The lowest values for aroma attributes for full red strawberries were found at day nine, likely because volatiles with off flavors (acetaldehyde, ethyl acetate) were quickly increasing after day 5. PCA analysis of GC-MS and PTR-ToF-MS data indicated many compounds and fragments characteristic for variation in storage days in ripe fruit, but not in unripe fruit. PLS modelling identified VOCs exist that are characteristic for both sweet and highly aromatic ‘Lusa’ strawberries.

Based on GC-MS data these VOCs are mainly volatile fatty acids, whereas based on PTR-ToF-MS data these are mainly alcohol/ester fragments. This opens up the possibility for fast, non-destructive, selection of strawberries with high consumer liking using PTR-ToF-MS.

Author contribution

HL, BB, NO, FPdS and YT contributed to this work in data acquisition. FPdS and BB performed the experiment design. RS and HL performed the data analysis. RS and HL drafted the manuscript. EW and JV contributed to data interpretation and discussion. FPdS, EW, BB and JV critically revised and approved the manuscript.

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Chapter 6

General Discussion

Strawberry is a popular soft fruit worldwide due to its attractive appearance, aroma and nutritional value. However, strawberry fruit are highly susceptible to postharvest fungal infection, primarily caused by *Botrytis cinerea*. Breeding resistant strawberry genotypes is extremely challenging due to 1) the polygenic nature of resistance; 2) the octoploid genome structure of cultivated strawberry; 3) the functional redundancy of pathogenicity factors released by *B. cinerea*. Nevertheless, susceptibility is a quantitative trait which allows breeding for less susceptible genotypes resulting from the combination of certain physical and chemical characteristics of strawberry.

In this thesis, I aimed to explore the roles of physical and chemical characteristics of strawberry fruit (e.g., color, firmness, volatile compounds and soluble sugars) in susceptibility to *B. cinerea* and identify characteristics that could potentially serve as markers to assist in breeding for strawberry genotypes with reduced susceptibility.

To achieve these goals, I studied the subject from two angles. The first angle was at the genetic level, where I identified the association between the natural spoilage of strawberry genotypes and their metabolite profiles (**Chapter 2**). The second angle was to evaluate how fruit susceptibility was affected by pre- (**Chapter 3**) or postharvest (**Chapters 4&5**) practices and then to investigate the underlying physiological and metabolic characteristics that are associated with the trait.

In this general discussion, I firstly focus on the currently available approaches of screening for strawberry susceptibility to *B. cinerea* and bring forward a rapid screening method for future research, which can be exploited in breeding programs. Secondly, I focus on discussing the relevance of fruit ripening to susceptibility and defense associated with *B. cinerea* infection. To boost our knowledge on the less systematically studied strawberry-*B. cinerea* pathosystem, I refer to the more extensively studied tomato-*B. cinerea* pathosystem. The similarities and distinctions between these two fruit-*B. cinerea* interactions are of interest for both scientific and practical reasons. Thirdly, based on my experimental observations and information from other researchers, I discuss the possible roles of some strawberry metabolites in fruit-*B. cinerea* interactions. Finally, I provide suggestions for utilizing a systematic approach to unraveling the underlying mechanisms of the interaction between strawberry and *B. cinerea*.

6.1 Genotype screening for susceptibility to *B. cinerea* in strawberry

Breeding less susceptible genotypes is a sustainable cultural practice (requiring ~6 to 8 years of evaluation before a new cultivar is released) to reduce economic loss in strawberry production and to fulfill consumers' demands. To expedite releasing genotypes with low susceptibility, utilizing a streamlined approach to eliminating poorly performing genotypes and selecting superior genotypes is indispensable.

6.1.1 Available approaches

Most studies use ripe fruit as the target for screening, presumably because they have higher susceptibility than flowers and unripe fruit. Some studies focused on evaluating natural spoilage (i.e., without artificial inoculation under controlled laboratory conditions) of red ripe fruit from diverse genotypes during cultivation (e.g., greenhouse, open field) and/or postharvest storage within either one year or multiple years (Rhainds et al., 2002; Chandler et al., 2006; Seijo et al., 2008; Lewers et al., 2012, 2013). All these studies suggest that characterizing the intrinsic susceptibility of genotypes is largely hampered by preharvest conditions in different seasons and years.

In this thesis, two screening experiments for susceptibility among genotypes over two consecutive years (2018 and 2019) were conducted, with an overlap of five genotypes between the years (**Chapter 2**). Based on the data of the five overlapping genotypes, a parameter *k*, the spoilage rate, was found to be genotype-dependent. In 2020 and 2021, we again evaluated three genotypes used in the research described in **Chapter 2** exhibiting distinct susceptibility to *B. cinerea.* Natural spoilage of three of these genotypes performed consistently over three consecutive years (2019, 2020, 2021) (Unpublished data). The high consistency of the natural spoilage process in these three cultivars over multiple years suggests that their variation in the intrinsic susceptibility is indeed genetically determined.

Based on above studies, I summarize the pros and cons of natural spoilage assessment. The major pros include 1) a simple determination method (i.e., spoiled or not yet); 2) possibility to characterize primary and secondary infections. The major cons include 1) requiring a large number of fruit (minimum 15 to 20 fruit) per replication; 2) a long experimental period; 3) highly confounded by environmental conditions; 4) unpredictable natural infection occurrence; 5) few fruit may deteriorate due to other fungi other than *B. cinerea;* 6) difficulty in sampling for destructive measurements.

In recent years, other studies used artificial inoculation either with wounding (Petrasch et al., 2021) or without wounding (Bestfleisch et al., 2015) the exocarp to characterize the genotypic variation in strawberry fruit susceptibility to *B. cinerea*. I also tried to inoculate fruit without wounding, however, two reasons forced me to terminate this approach. First, I could not distinguish the infection of tissues resulting from natural spoilage or artificial inoculation, unless marking the inoculation sites, which was not favored as marking may result in mechanical damage of fruit. Second, more frequently, symptoms appeared from non-inoculated sites. Using the method, inoculation by wounding is more efficient and more synchronized for screening.

Based on my own experience and research by other authors, I summarize the pros and cons of artificial inoculation. The major pros include 1) requiring only a small number of fruit (minimum 5 to 10 fruit); 2) a short experimental period; 3) predictable infection development; 4) guarantee that individual fruit deteriorate due to *B. cinerea*; 5) possibility to sample the infection tissues at defined timepoints and stages of infection. The major cons include 1) complex quantification (i.e., lesion expansion, infection incidence); 2) wounding may mask potentially present fruit resistance traits that are mediated by reduced surface penetrability; 3) requiring significant working space to monitor the disease development for individuals.

6.1.2 Reflection on rapid screening for future use

The critical step to establish and optimize a rapid screening for susceptibility to *B. cinerea* in strawberry is to investigate which tissue at which developmental stage displays the largest variation among genotypes. This has not previously been investigated in strawberry. Spoilage of ripe strawberry fruit caused by *B. cinerea* results from latent infection of floral organs (e.g., stamens, petals) and/or manifest infection of receptacle tissues. Here I propose to assess the susceptibility of not only fruit tissues but also flower tissues.

For screening of fruit tissues, firstly, when evaluating the natural spoilage of red ripe fruit, primary and secondary infection should be clarified. The primary infection is characterized by fungal invasion of open flowers and invasion of the ovary, followed by a symptomless phase (i.e., quiescence) in which fungal growth is arrested by the presence of pre-formed chemical compounds in the floral tissue. The quiescent phase can last for a number of weeks and only switch to fungal outgrowth from internal fruit tissues during ripening, resulting in disease. The secondary infection is characterized by fungal invasion of the receptacle surface around the time of ripening. The occurrence of secondary infection can be hampered either by the penetrability of fruit skin, by

chemical defense responses in the invaded tissue, or by a combination of these factors, which jointly determine the susceptibility to *B. cinerea* of a given genotype. To detect the symptomless primary infection, plating out tissue sampled near the calyx on *Botrytis* selective media (BSM) is recommended for symptomless infection identification. BSM contain fungicides that are less toxic for *Botrytis* species and tannic acid that can be oxidized to show a brownish color (Ribereau-Gayon et al., 1980). The medium developed by Edwards and Seddon (2001) is specifically for characterizing *B. cinerea*. BSM is still being considered as an effective traditional method nowadays (Malarczyk et al., 2019) and used to identify *B. cinerea* infection in fruits such as grape (Haile et al., 2016) and raspberry (Kozhar and Peever, 2018, 2021). Notably, plating tissues on BSM gives a qualitative result (i.e., infected or not) under certain environmental conditions. Hence, the infection incidence should be considered as a parameter to compare the susceptibility among genotypes. Secondly, fruit can be used in an earlier stage to test the genotypic variation of susceptibility. Although it has been demonstrated that strawberry fruit susceptibility increases with ripening (Jersch et al., 1989; Haile et al., 2019), it is worthwhile to test whether unripe fruit show a larger variation of susceptibility among genotypes than ripe fruit. For instance, fruit in the turning stage (< 50% coloration of the fruit skin) are easier to recognize and could be better biologically synchronized in ripeness among genotypes.

For screening of flower tissues, assuming that flower susceptibility is positively correlated to fruit susceptibility, I propose to assess the natural infection (frequency of primary infection) of flowers by using BSM as reported in raspberry (Kozhar and Peever, 2018). Flowers at the post-pollination and the petal-fall stages can be used for screening by plating the flower parts on the BSM. If there is a good correlation between the susceptibility of flower and fruit, it would make screening of large populations feasible and would not need to wait until fruit development. Meanwhile, flower screening eliminates the effect of unsynchronized ripeness on susceptibility. Inoculation could also be applied to flowers. However, it is difficult to apply a consistent and controlled quantity of inoculum and to decide which part of flowers should be inoculated as most flower organs are potential sources of latent infection on fruit (Bristow et al., 1986). Haile et al. (2019) inoculated open flowers (7 days after anthesis) near the base of the receptacle, which gives rise to the edible part of strawberry fruit.

To quantify the severity of infection, molecular methods are recommended. For instance, a qPCR assay using β-*tubulin* and *actin* gene-specific markers to quantify *B. cinerea* biomass was reported by Gachon and Saindrenan (2004). A different protein-coding gene marker, *cutinase A* gene was effective for quantification of *B. cinerea* in

infected plants (Reich et al., 2016). More available molecular methods have been reviewed by Malarczyk et al. (2019).

6.2 The relevance of ripening to susceptibility and defense in fruit-*B. cinerea* interactions: from tomato to strawberry

Fruit exhibiting resistance at the unripe stage but susceptibility at the ripe stage to *B. cinerea* has been demonstrated in strawberries, tomatoes, grapes, raspberries (Haile et al., 2019, 2020; Kozhar and Peever, 2021; Silva et al., 2021). In recent years, a lot of knowledge has been gained about host-pathogen interactions on fruit through the study of the tomato-*B. cinerea* pathosystem (Cantu et al., 2008, 2009; Shah et al., 2012; Blanco-Ulate et al., 2013, 2014, 2016b; Petrasch et al., 2019b; Silva et al., 2021). Tomato and strawberry have been exploited as two fruit models to unveil the climacteric and non-climacteric ripening mechanisms, respectively. Hence, I will discuss currently available information derived from the tomato- and strawberry-*B. cinerea* pathosystems to elucidate the complex relations between ripening, susceptibility and defense.

6.2.1 Fruit susceptibility to *B. cinerea* increases with ripening

The transition from unripe to ripe fruit results in a markedly different physicochemical environment for colonization of fungi. In tomato, inoculation of mature green fruit with *B. cinerea* led to non-expanding necrotic rings around the inoculation sites whereas inoculation of red ripe fruit led to profuse mycelia growth and disease (Silva et al., 2021). In the same study, among three distinct non-ripening tomato mutants (*Cnr*, *rin* and *nor*), only *Cnr* fruit showed high susceptibility to *B. cinerea* in both mature green-like and red ripe-like stages. Different from *rin* and *nor* fruit, *Cnr* fruit have an altered cell wall structure (Ordaz-Ortiz et al., 2009), they displayed lack of defense responses against *B. cinerea* and produced strongly elevated levels of ethylene upon infection (Silva et al., 2021). These features might be the key factors leading to the high susceptibility of *Cnr* fruit regardless of their (lack of) ripeness. Tomato fruit susceptibility usually increases with ripening though not all ripening events are associated with increased susceptibility to *B. cinerea*.

Jersch et al. (1989) stated that infection of unripe strawberry fruit was limited to non-sporulating lesions. Terry et al. (2004) did not observe *B. cinerea* conidia production in artificially inoculated green strawberry fruit, but rather exclusively observed the development of white mycelia. However, the authors observed the production of

conidia on inoculated white fruit. In my own trial, both green and turning fruit could develop brown necrotic spots or white mycelia at three days post-inoculation at room temperature and ~100% relative humidity. However, I did not examine sporulation of *B. cinerea* on those green fruit during the trial. Unripe strawberry fruit that are contaminated with B. cinerea are common in the field (Mertely et al., 2018). Therefore, although strawberry fruit susceptibility evidently increases with ripening, unripe fruit may not be as resistant to *B. cinerea* as generally assumed.

In commercial practice of strawberry cultivation, the principal idea to prolong the shelf life of strawberry fruit is to retard ripening or respiration. Fruit are either harvested in an early stage or stored under a certain dedicated postharvest environment. Fruit that were harvested at the ¾ red stage showed lower natural spoilage during postharvest storage (Unpublished data of **Chapter 5**). We culivated strawberry under different light spectra and harvested fruit when they reached full red coloration (**Chapter 3**). Our chemical analyses indicated that, although the external color was uniform in fruit illuminated with or without far-red light, the levels of metabolites were different. Adding far-red resulted in higher levels of soluble sugars, volatile esters and acids, but lower levels of antioxidants (anthocyanins, proanthocyanidins and ascorbic acid). Despite changes in contents of the above metabolites which, in my opinion, caused variation in ripeness, fruit illuminated with far-red light did not differ from fruit illuminated without far-red light in their level of susceptibility to *B. cinerea*. It is likely that the subtle shifts in ripeness hardly affect susceptibility. I postulate that the interference of ripeness on determining genotype-dependent spoilage rates as described in **Chapter 2** is negligible. We succeeded in extending the shelf life of red ripe strawberry fruit by applying high CO_2 during storage (**Chapter 4**). In this experiment, fruit respiration was suppressed by high CO_2, which resulted in moderate changes in sugar and acid metabolism. High CO_2 may directly inhibit *B. cinerea* growth (Yahia and Sholberg, 2009). The dual effects on fruit and *B. cinerea* of high CO_2 together led to significantly extended shelf life of strawberries. The results shed light on the importance of respiratory metabolism, in delaying fruit spoilage to *B. cinerea* from ripe to senescence. Bangerth et al. (2012) showed differences in fruit respiration rates among strawberry cultivars throughout ripening. I recommend that future studies focus on differences in respiration between genotypes as a factor that affects fruit susceptibility to *B. cinerea,* which might eventually be developed into a screening tool for susceptibility.

Similar to tomato fruit, strawberry fruit susceptibility increases with ripening. However, the differences in susceptibility between unripe and ripe strawberry fruit seem less

substantial than that between mature green and red ripe tomato fruit. This indicates that infection of strawberry by *B. cinerea* is less affected by ripening compared to the clear dependence on ripening in tomato fruit. This may be related to the climacteric and non-climacteric ripening features of tomato and strawberry, respectively.

6.2.2 Promotion of fruit ripening by *B. cinerea*

Petrasch et al. (2019a) has pinpointed that *B. cinerea* may promote fruit ripening upon infection. In tomato fruit, unripe tomato fruit that was inoculated with *B. cinerea* showed premature expression of ethylene synthesis related genes (Cantu et al., 2009; Blanco-Ulate et al., 2013). Petrasch et al. (2019a) proposed that *B. cinerea* could promote early ripening by inducing ethylene production in tomato fruit. They further suggested that as strawberry is a non-climacteric fruit, the promotion of ripening by the fungus could be more related to abscisic acid as it has been reported that *B. cinerea* may release abscisic acid or compounds with a similar function as pathogenicity factors. In addition to hormones, ROS production by the fungus and the fruit may stimulate fruit ripening (Wang et al., 2017).

We showed that fruit inoculated with *B. cinerea* conidia (indicated as 'spore') might ripen faster than water-injected fruit (indicated as 'mock') during the sampling period (between 0 and 4 days post-inoculation) with two distinct strawberry genotypes (Figure 1, unpublished data). The results can generate ideas to target certain compounds and/or pathways. Moreover, the conclusion in **section 6.2.1** that only certain ripening events (e.g., cell wall degradation) promote fruit susceptibility to *B. cinerea*, lead me to propose that the ripening processes triggered by *B. cinerea* may be essential for fruit-*B. cinerea* interactions.

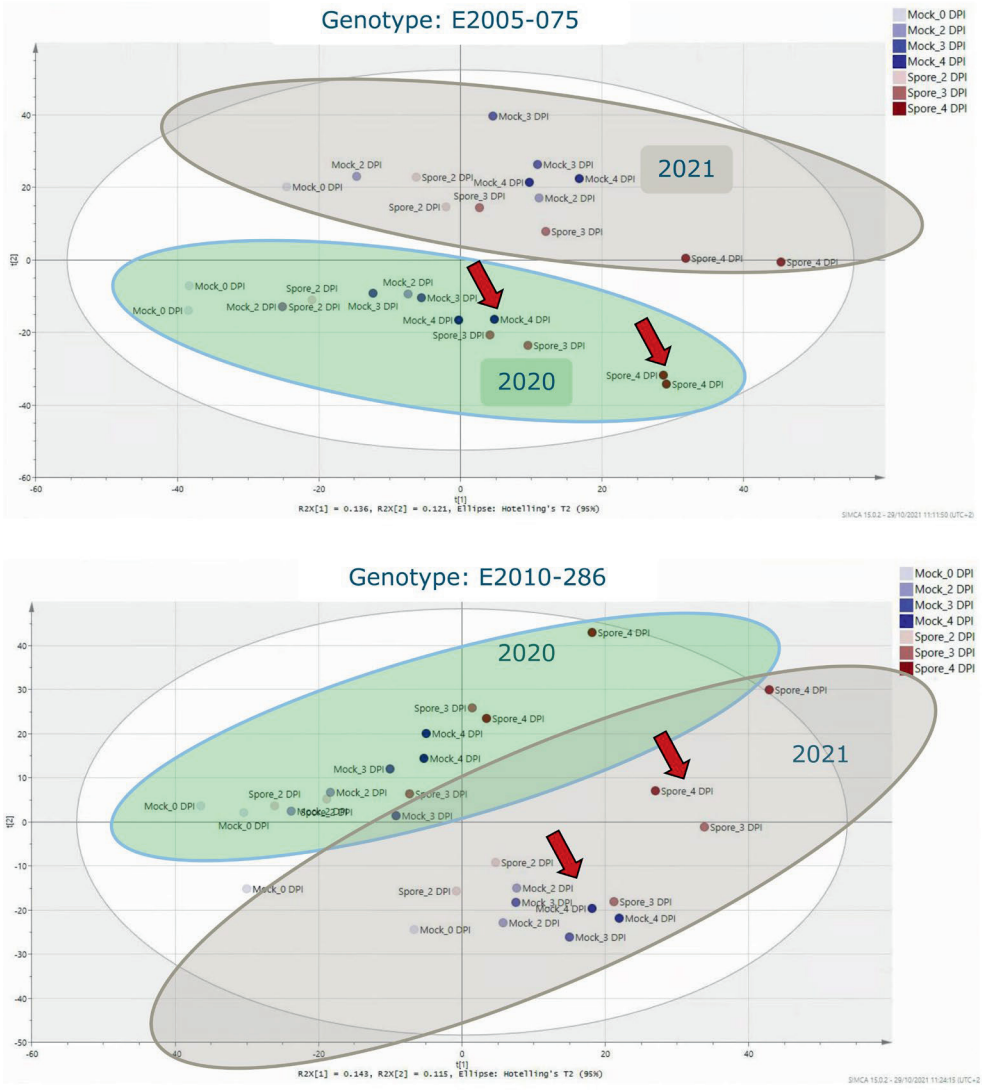

Figure 1 Principal component analysis of sample distribution from two strawberry genotypes ('E2010-286' and 'E2005-075') over two years based on LC-MS data. Each year had two data points as two replicates. Each data point was pooled from 10 fruit. Spore, samples inoculated with *B. cinerea* conidia; Mock, samples injected with sterile water. DPI, days post inoculation.

6.2.3 Susceptible ripe fruit respond to pathogens with a more diverse set of defense responses than resistant unripe fruit

In response to *B. cinerea* infection, Silva et al. (2021) proposed that red ripe tomato fruit appear to mount a more diverse defense response than mature green fruit early during infection. Firstly, more categories of defense genes were induced in inoculated

red ripe fruit than in inoculated mature green fruit. Secondly, genes that were uniquely induced in inoculated mature green fruit may be functionally redundant as they belong to a large gene family and their expression levels were rather low. The authors postulated that these genes do not significantly contribute to reducing the susceptibility in red ripe fruit. Thirdly, expression levels of genes that were induced in both inoculated mature green and red ripe fruit were similar and some genes were even more pronouncedly expressed in red ripe fruit.

In strawberry, a recent comparative transcriptome analysis using two genotypes with low and high susceptibility to *B. cinerea* showed that in both genotypes, the number of differentially expressed genes in green fruit was smaller than that in red fruit post-inoculation and the defense responses were genotype-dependent (Lee et al., 2021). In contrast, two other studies reported that defense responses of white fruit were faster and more pronounced than that of red ripe fruit. The first study conducted a targeted metabolic analysis on white and red fruit upon *B. cinerea* infection (Nagpala et al., 2016). Phenolic compounds can act as preformed (phytoanticipin) or induced (phytoalexin) defense compounds. Indeed, phenolic compounds were significantly induced (e.g., increases in catechin, proanthocyanidins and decreases in benzoic acids) in infected white fruit as compared to red fruit. This is in line with our observation that the levels of phenolic compounds of red ripe fruit remained unchanged after inoculation with *B. cinerea* conidia in three strawberry genotypes (Unpublished data). The second study performed a transcriptome analysis on *B. cinerea* inoculated white and red fruit (Haile et al., 2019). In this study, a larger number of differentially expressed genes was detected in white fruit than in red fruit, including genes with roles in secondary metabolism (e.g., flavonoid and terpenoid biosynthesis) and cell wall modification. In addition, genes encoding pathogenesis-related proteins were induced mainly in white fruit post-inoculation. It is very likely that strawberry fruit in the white stage may have more active defense responses than fruit in green or red stages.

6.2.4 Fruit rot may be promoted by decreases in preformed defenses and increases in susceptibility factors with ripening

Silva et al. (2021) assumed that preformed defenses decrease with ripening whereas susceptibility factors increase with ripening. They characterized defense genes that were highly expressed in non-inoculated mature green fruit compared to non-inoculated red ripe fruit. These genes may potentially play a role in the preformed defense and were mainly assigned to three categories: reactive oxygen species (ROS) response and detoxification, proteolysis, and the biosynthesis of secondary metabolites

(e.g., flavonoids, lignins, and terpenoids). They also suggested that genes that were highly expressed in non-inoculated red ripe fruit compared to non-inoculated mature green fruit may serve as susceptibility factors. These genes have functions in aldehyde biosynthesis, sugar metabolism, pectin degradation, and carotenoid biosynthesis.

In strawberry, similar and distinct defense responses or susceptibility factors are discussed in the next **section 6.3**.

6.3 Strawberry preformed defenses and susceptibility factors associated with *B. cinerea* infection

6.3.1 Does higher firmness indicate less susceptibility in fruit?

Fruit firmness reflects the skin penetrability upon fungal attack. We did not observe a correlation between strawberry fruit firmness and spoilage caused by *B. cinerea* (**Chapter 2**). It could be attributed to the methodology we used, i.e., acoustic response tests. Fruit firmness determined by this technique depends on fruit elasticity, shape as well as mass. Fruit penetration test showed little differences among fruit cultivated under different light spectra (**Chapter 3**) or stored under different controlled atmospheres (**Chapter 4**). Taken into account the experiments I did in the past, firmness differences in ripe strawberry fruit from different genotypes or treatments are very minor. Fruit firmness between two genotypes with contrasting susceptibility to *B. cinerea* showed a consistent difference in firmness with ripening, but it was much better quantifiable in an earlier fruit stage (Figure 2, unpublished data). To find a relation between fruit initial firmness and the susceptibility to *B. cinerea*, it may be better to compare firmness values of less ripe fruit. Furthermore, two genes that are involved in lignin metabolism were differentially expressed in strawberry genotypes with different levels of firmness (Salentijn et al., 2003). Lignin biosynthesis in several fruits is induced upon infection of *B. cinerea* for cell wall fortification (Haile et al., 2017, 2019; Silva et al., 2021). The association between fruit firmness and lignin biosynthesis as well as the susceptibility to *B. cinerea* should be further investigated.

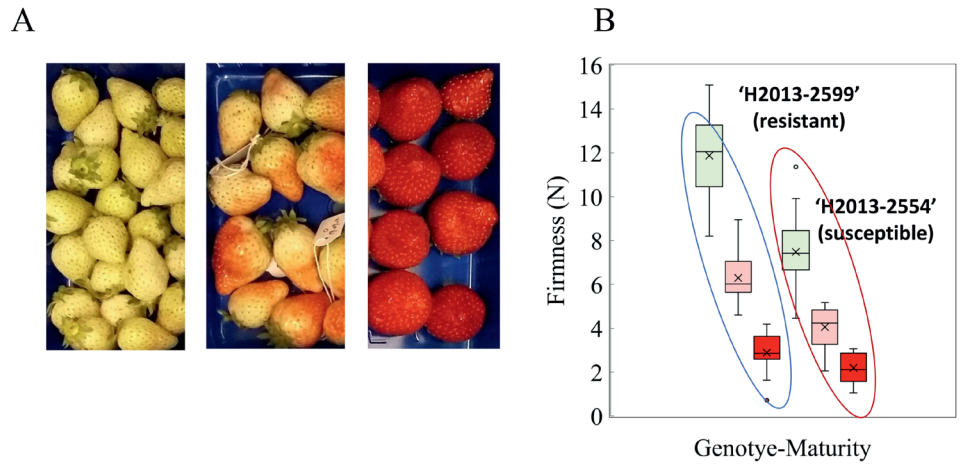

Figure 2 Firmness of two genotypes ('H2013-2599' and 'H2013-2554') in different ripening stages. green white, turning and red ripe stages. (A) Images of fruit at harvest; (B) penetration test in three stages of each genotype.

6.3.2 Do soluble sugars serve as susceptibility factors?

According to the model proposed by Silva et al. (2021), sugar accumulation is one of potential factors leading to high susceptibility of ripe fruit as a low sugar content was correlated to the low susceptibility of unripe fruit whereas a high sugar content was correlated to the high susceptibility of ripe fruit. The difference of sugar content between green and ripe fruit is about 2- to 4-fold in strawberry (Basson et al., 2010; Jia et al., 2011, 2013) and tomato (Gautier et al., 2008). Therefore, it would be necessary to experimentally estimate the threshold of the sugar content that permits *B. cinerea* to break the quiescent phase.

In red ripe strawberry fruit, we hypothesized that a higher sugar content is correlated to a more rapid spoilage process, which however was not confirmed in the 17 genotypes evaluated (**Chapter 2**). Additional far-red illumination during the cultivation elevated the total sugar content but did not affect the susceptibility of red ripe fruit (**Chapter 3**). In addition, the levels of the individual sugars did not differ between *B. cinerea* inoculated and water-injected fruit samples in strawberry genotypes with contrasting susceptibility (Unpublished data). The high sugar content in ripe fruit may already saturate *B. cinerea* demands. Meanwhile, a tendency was observed that a higher spoilage rate of a genotype was correlated to higher levels of fructose and glucose but not to the level of sucrose (**Chapter 2**). Effects of fructose and glucose stimulating *B. cinerea* growth were demonstrated in tomato leaves (Courbier et al., 2020). Not only could absolute contents of fructose and glucose increase the

susceptibility of fruit with ripening, the conversion of sucrose to fructose and glucose may also play a role. High CO_2 treatments potentially caused a more pronounced conversion of sucrose into glucose and fructose, corresponding to less spoilage during postharvest (**Chapter 4**).

Collectively, in unripe strawberry fruit, the soluble sugar content may contribute to facilitating infection by *B. cinerea*. In ripe fruit the absolute level of soluble sugars is very high such that any changes in total sugar or individual sugars do not affect infection progress anymore.

6.3.3 Is the role of non-volatile acids underestimated?

Acidity is an important fruit quality trait that is rarely studied in detail in strawberry fruit. As I describe in **Chapter 1**, changes in non-volatile organic acids varied among studies, which may blur our understanding of the roles of acids in interactions between strawberry fruit and *B. cinerea*. Centeno et al. (2011) investigated the effect of modifying malic acid metabolism on fruit ripening via targeted gene silencing approaches in tomato. In this study, transgenic tomato fruit containing low levels of malic acid in the green stage showed increased soluble sugar contents in the red ripe stage, which did not affect the susceptibility to *B. cinerea*. Transgenic tomato fruit with a higher level of malic acid in the green stage led to a decreased soluble sugar level in the red ripe stage, which increased susceptibility to *B. cinerea*. According to my hypothesis presented in **section 6.3.2** that the high sugar content is not required for interactions between ripe fruit and *B. cinerea*, it is likely that a higher content of acids results in higher susceptibility of ripe fruit. Malic acid contents of red ripe strawberries indeed showed a (weak) positive correlation to spoilage rates in 17 genotypes (**Chapter 2**).

Acidifying host tissues is one of the weapons that *B. cinerea* employs to conquer defenses of hosts (Müller et al., 2018). Two questions come to my mind, as most fruits are acidic (Manteau et al., 2003): why does *B. cinerea* still need to orchestrate its own acids to enhance acidification of host tissues? Does a small difference in fruit pH matter for infection? The first question was answered by two recent transcriptome analyses. Both studies reported that the oxalic acid metabolism of *B. cinerea* is activated during infection of grape and tomato fruits (Petrasch et al., 2019b; Haile et al., 2020). Müller et al (2018), however, pinpointed that the role of citric acid was more prominent than that of oxalic acid in acidification of apple fruit and for causing the lesion formation upon infection by *B. cinerea*. The second question was answered by one of my experiments. We confirmed the correlation between the acid content (citric acid plus

malic acid) and pH in fruit tissues (**Chapter 4**). High CO_2 conditions resulted in higher pH of fruit, which was associated with less spoilage. It seems that less acidic tissues are indeed less susceptible to *B. cinerea*.

Although *B. cinerea* releases its own acids as pathogenicity factors to infect hosts, low pH of strawberry fruit tissue may still play an important role in facilitating *B. cinerea* infection. I postulate that the low pH of unripe strawberry plays a role in our observation that they are more susceptible than generally assumed.

6.3.4 Complex roles of volatiles and antioxidants

The reasons for my focus on the roles of volatile organic compounds are because 1) they are highly related to consumer liking (Fan et al., 2021b, 2021a); 2) they might be better indicators for physiological ripeness than the external color; 3) some have been shown to inhibit (e.g., green leaf volatiles) or stimulate (e.g., esters) fungal growth (Hassan et al., 2015; Neri et al., 2015). When fruit were harvested in an earlier stage, the sweet and fruity flavor intensity indeed was reduced and could not reach that of ripe fruit even during postharvest (Van de Poel et al., 2014; **Chapter 5**). However, ripe fruit during the storage developed off-flavors related to senescence. Volatiles that indicated ripening correlated with the spoilage performance (**Chapter 5**). The high abundance of most esters, furanones and volatile fatty acids in ripe fruit therefore can be considered as potential susceptibility factors. However, additional far-red light illumination during cultivation induced faster accumulation of some esters and acids, but did not result in a faster spoilage as expected based on my hypothesis (**Chapter 3**). This could be attributed to the relatively minor differences in ripeness of fruit treated with and without far-red light. Among genotypes, two compounds, ethyl butanoate and 1-hexanol were associated with faster spoilage and may need more attention for future study (**Chapter 2**). Based on above findings, I propose that most volatile compounds in ripe strawberry fruit are associated with ripening but do not contribute to susceptibility to *B. cinerea*.

Jersch et al. (1989) showed that the total proanthocyanidin content did not change from the white-green to the red stage in 'Senga sengana' strawberries. However, they noticed that the inhibitory effect on *B. cinerea*-derived polygalacturonases by proanthocyanidins from fruit extracts was more pronounced in extracts from unripe strawberries than from ripe strawberries. They hypothesized that the decreased inhibitory effects resulted from the higher polymerization degree of proanthocyanidins in ripe strawberries instead of from their total contents. As the detection technique for proanthocyanidins has been improved nowadays, new evidence has rejected their

hypothesis. It was observed that in strawberry cultivars (including 'Senga sengana'), the content of total proanthocyanidins gradually decreased from the small green stage to the red stage while the mean degree of polymerization remained unchanged (Carbone et al., 2009; Schaart et al., 2013; Baldi et al., 2018). In addition, the increase of proanthocyanidins polymerization enhances their antioxidant activity (Heim et al., 2002), which might enhance strawberry defense against fungal infection. Therefore, the association between the mean degree of polymerization or the different composition levels of proanthocyanidins and the susceptibility to *B. cinerea* in strawberry genotypes should be further investigated.

Anthocyanins accumulated in transgenic tomato fruit limited *B. cinerea* growth on the fruit skin (Bassolino et al., 2013; Zhang et al., 2013). However, the extracts of transgenic tomato did not show inhibitory effects on *B. cinerea* growth *in vitro*. In apple fruit, the sun-exposed side that accumulated anthocyanin and other antioxidants exhibited smaller lesions compared to the shaded side (Bui et al., 2019). In strawberry, the correlation between anthocyanin contents of the whole fruit and spoilage rates was weakly correlated (**Chapter 2**). In the same study, the external red coloration was highly correlated with spoilage rates. All these together indicate that anthocyanins accumulated in the fruit skin may reduce its susceptibility to *B. cinerea*.

Although we did not observe a significant correlation between spoilage rates and levels of proanthocyanins, a positive correlation between proanthocyanidins and anthocyanins was observed (Figure 3, unpublished data of **Chapter 2**). This may suggest that enhancing expression levels of biosynthetic genes for the two compounds, such as *DFR* may reduce the susceptibility, however, this could result in stronger astringency.

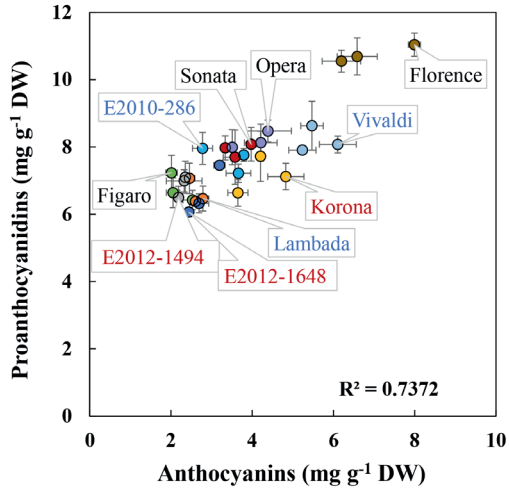

Figure 3 The correlation between levels of proanthocyanidins and anthocyanins among 10 genotypes of data in Chapter 2. Different colored-points represent different genotypes. The genotype names written in black showed medium spoilage rates; written in blue showed low spoilage rates; written in red showed high spoilage rates.

In apple fruit, the sun-exposed side that accumulated ascorbic acid, anthocyanin and other antioxidants showed smaller lesions compared to the shaded side (Bui et al., 2019). We did observe a weak positive correlation between ascorbic acid contents and fruit spoilage rates (**Chapter 2**). Ascorbic acid content of red ripe fruit inoculated with *B. cinerea* conidia did not show a clear increase or decrease during infection (Unpublished data). The role of ascorbic acid in strawberry susceptibility to *B. cinerea* still remains unclear.

It is very likely that antioxidants play important roles in interacting with *B. cinerea* during fungal penetration of the fruit surface. Therefore, I suggest future studies can investigate the distribution of targeted metabolites in fruit and also separately study the metabolite profiling of external (e.g., 2 to 5 mm of underlying tissues) and internal fruit tissues. The levels of measured variables could then be used to correlate with the susceptibility of whole fruit to *B. cinerea*.

6.3.5 Metabolite relationships

Sucrose promotes anthocyanin synthesis during strawberry fruit ripening (Jia et al., 2013; Luo et al., 2019, 2020). A model of sucrose promoting anthocyanin biosynthesis was proposed by Durán-Soria et al. (2020) overviewing currently available studies.

We found that glucose and fructose were negatively correlated with anthocyanin levels among 17 genotypes (**Chapter 2**). Increases in glucose and fructose contents and a decrease in anthocyanin content of fruit were observed under additional far-red light illumination during cultivation (**Chapter 3**). The association between these two sugars and anthocyanin has been investigated in apple calli by overexpression of a tonoplast monosaccharide transporter gene *MdTMT1*, which resulted in higher contents of glucose and fructose contents but a lower content of anthocyanins compared to wild type (Xu et al., 2020). MdTMT1 transports fructose and glucose into the vacuole (Wormit et al., 2006), meanwhile reducing the available amount of UDP-glucose and UDP-galactose, that are required for the conversion of anthocyanidins to anthocyanins (Xu et al., 2020).

Ascorbic acid may also affect anthocyanin biosynthesis. In strawberry, ascorbic acid and anthocyanins accumulate with ripening, which may assure to maintain the redox state of the cell (Cruz-Rus et al., 2011; Zhang et al., 2018a). A weak positive correlation between ascorbic acid content and anthocyanin content was observed in 17 genotypes (**Chapter 2**) and additional far-red light reduced both compounds in red ripe fruit (**Chapter 3**). Anthocyanins rarely accumulated in (vitamin C-deficient, *vtc*) *vtc1* and *vtc2* mutant plants when exposed to high light, suggesting that ascorbic acid-mediated redox reactions act upstream of anthocyanin synthesis (Page et al., 2012).

The metabolic competition between anthocyanin and lignin pathways was suggested to be regulated by the expression levels of peroxidase FaPRX27 (Ring et al., 2013). Strawberry genotypes with high levels of anthocyanins displayed low levels of lignin and low expression of *FaPRX27*. In addition, (E)-cinnamyl acetate levels were higher in *B. cinerea*-inoculated fruit tissues (Unpublished data) and its biosynthesis may interact with the lignin pathway. (E)-Cinnamyl acetate is an acetate ester resulting from the condensation of cinnamyl alcohol with acetic acid. Cinnamyl alcohol dehydrogenase is the final enzyme in the pathway to the monolignols, mainly coniferyl and sinapyl alcohols, from which polymeric lignin is derived (Kim et al., 2002).

The modification of a single trait can never lead to complete resistance. Understanding metabolite relationships and exploiting these in regulating fruit ripeness, susceptibility as well as defense may be more promising and effective in extending strawberry shelf life and improve other quality aspects such as taste and nutritional value.

6.4 Future perspectives

6.4.1 Methodology for systematic research on fruit-*B. cinerea* interactions

The significant economic loss in strawberry production by grey mold spoilage has been a problem for the industry for decades. Until now, it is partially addressed in commercial practice by cultivation of less susceptible cultivars, application of biological control agents and utilization of advanced postharvest technologies. Many researchers have studied possible infection and defense mechanisms in the interaction between fruit and the fungus or to test feasible practices that can alleviate spoilage in the supply chain. However, it is hard to compare the outcomes of available reports due to different experimental conditions, genotypes, protocols for the determination of susceptibility and the definition of 'shelf life'. Lack of systematic studies hampers the progress in understanding the interaction between strawberry and *B. cinerea*. I would like to bring forward some ideas to streamline future research.

Utilization of kinetic and regression modeling

Modeling the kinetics of biological processes enables a better understanding of the studied system. We applied kinetic modeling to the spoilage data of diverse genotypes originated from two years (**Chapter 2**). Notably, different evaluation systems were used to evaluate spoilage in these two years. The spoilage modeling allowed us to compare the spoilage progressions of genotypes and resulted in a genotype-dependent parameter, allowing screening for characteristics linked to the intrinsic, genetically determined susceptibility to *B. cinerea*. The biologically meaningful parameters acquired from the kinetic modeling enables comparison of studies on many aspects of fruit quality, such as susceptibility, firmness loss.

The scientific value of multivariate regression analyses is increasing in recent years. We applied partial least partial least-squares regression (PLSR) on sensory and instrumental data and we found that certain volatile organic compounds were important for sensory attributes (**Chapter 5**). Those volatile compounds could be potential biomarkers targeted for flavor-orientated breeding programs. Similarly, the regression model can also be taken into consideration for selection of biomarkers that predict low susceptibility.

Utilization of multi-omic technologies

Recent advances in multi-omic technologies increase the knowledge about the complexity of fruit metabolism and genetics, which is helpful for improving the storability and sensory quality of fruits, as demanded by consumers (Pott et al., 2021). Metabolomic and/or proteomic approaches enable to outline genotypes with quality-related characteristics. We can expect that deep sequencing combined with other omics platforms will increase our knowledge about the genetic architecture of quality traits, therefore improving fruit quality by breeding.

In this thesis, targeted and non-targeted metabolomics were used to explore the association between susceptibility to *B. cinerea* and metabolic status in strawberry fruit. We found compounds that were potentially linked to spoilage, such as anthocyanins, 1-hexanol, ethyl butanoate, (E)-cinnamyl acetate (**Chapter 2**; unpublished data). We plan to further study differentially expressed genes from the transcriptomic profiling of two genotypes with distinct susceptibility. For future research, a targeted metabolic study should focus on flavonoid, lignin, and terpenoid biosynthesis, of which the corresponding biosynthetic genes are all induced by *B. cinerea* (Haile et al., 2017, 2019; Lee et al., 2021; Silva et al., 2021).

Translating findings in tomato fruit to strawberry for quality improvement

In tomato, exploiting multiple omics and/or gene editing strategies, in conjunction with its genetic diversity has resulted in a considerable improvement in fruit yield and quality. Gaston et al. (2020) has brought forward that mining the data from various quality traits of tomato can be helpful to modify strawberry quality traits as cultivated strawberry is less studied and a genetically more complex species. The authors also highlighted the importance of utilizing woodland strawberry genotypes due to their wide genetic diversity. Once beneficial alleles have been identified and validated, the findings can be applied to cultivated strawberries via marker-assisted breeding or gene editing.

6.4.2 Reducing strawberry susceptibility to *B. cinerea* in practice

Here I list some recommendations to reduce strawberry fruit spoilage by *B. cinerea*.

Preharvest practices:

- Genotype selection.
  Breeding companies have been trying to develop less susceptible cultivars for commercial use. However, growers prefer to use old cultivars (e.g., 'Sonata', 'Elsanta' 'Sonsation' 'Malling Centenary' in Netherlands) for their predictable

cultivation and yield characteristics. It is always challenging to have a newly released cultivar widely accepted by growers. Therefore, the effective communication between growers and breeders needs to be improved.

- Indoor growing.
  Growing strawberry in the vertical farming system with additional far-red light may not only accelerate fruit ripening but also improve fruit sweetness. Far-red light induced open plant architecture may reduce Botrytis rot during cultivation.

Harvesting:

- Early harvest.
  Harvesting strawberry in an earlier stage is a common practice to extend fruit shelf life. This practice also allows consumers to extend fruit shelf life by storing in their own fridges but may be at the expense of taste.
- Robotic harvesting.
  Strawberry producers are concerned about the future availability of labor and the cost of labor for picking. Developing robotic harvesting may not only release the pressure from above two issues but also alleviate damage caused by manual harvesting. Xiong et al. (2020) has brought forward a novel obstacle-separation algorithm that allows the harvesting system to pick strawberries clustered together. The harvesting system has also been improved to enable the robot to pick directly into commercial punnets to avoid repacking.
- Detection of volatile profiling using PTR-ToF-MS.
  Volatile profiling can predict fruit flavor and shelf life. PTR-ToF-MS allows for acquiring the volatile profile of intact fruit, and can be achieved in many growth conditions (e.g., open field, greenhouse, climate chamber) or applied to fruit sorting lines.

Postharvest practices:

- Precooling.
  Rapid precooling is most effective practice in removing field heat of strawberry fruit and slowing down fruit metabolism right after harvest. Precooling includes forced-air cooling, room cooling, hydrocooling contact icing, and vacuum cooling, with different efficiencies of heat removal (Kuchi and Sharavani, 2019).
- UV light treatments.

UV light may act as fungicide killing *B. cinerea* and other fungal conidia on the fruit surface, which to some extent may reduce postharvest spoilage of strawberry fruit.

- Controlled atmosphere.
  High CO_2 combined with moderate levels of O_2 is effective in prolonging shelf life of strawberry fruit. However, each genotype may have its own threshold of high CO_2 tolerance, which should be tested before storage.
- Modified Atmosphere Packaging (MAP).
  MAP may retain a favorable environment within a sealed package until the product is sold, and it can also be used to delay ripening when combining with proper temperature ranges (Giuggioli et al., 2015). MAP is achieved by sealing fresh strawberries in polymeric film packages that alter O_2 and CO_2 levels within the package atmosphere. Combinations of polymeric and perforated films may be able to provide adequate O_2 and CO_2 fluxes for commodities with high respiration rates, such as strawberries. The optimal gas composition for strawberries of the MAP was found to be 2.5 kPa O_2 and 16 kPa CO_2 (Sandhya, 2010; Giuggioli et al., 2015).
- Edible Coatings.
  Edible coatings are known to improve fresh product quality as they act as selective barriers to produce modified atmospheres, reduce moisture transfer, delay spoilage and prevent the loss of volatile aroma compounds (Nayak et al., 2019). Natural polymers with film-forming ability, such as polysaccharides, proteins, and lipids, are used to create edible active coatings (Alvarez and Famá, 2017). The technique has been applied to extend strawberry shelf life and retain the overall fruit quality (Treviño-Garza et al., 2015; Riaz et al., 2021; Saleem et al., 2021). Given the remarkable effectiveness and security, edible coatings are a very promising tool for extending the shelf life of strawberries.

6.5 Concluding remarks

Fruit metabolic status determines their ripeness, defense and susceptibility. In this thesis, the association between fruit susceptibility to *B. cinerea* and the levels of key metabolites in strawberry was investigated. To achieve different metabolic statuses of fruit, different genotypes, ripening stages, and environmental factors were considered. Several fruit characteristics (e.g., intensity of red color of the fruit skin, the levels of some volatile compounds) in red ripe strawberry fruit were found to be (potentially) associated with either susceptibility or defense upon infection of *B. cinerea*.

To further validate the role of studied metabolites and unravel the role of other key metabolites (e.g., terpenoids) in fruit susceptibility and defense, future research may focus on metabolic fluxes combining transcriptome analyses in fruit-*B. cinerea* interactions. The fruit-*B. cinerea* infection model should not only consider fruit at different ripening stages but also diverse genotypes as well as fruit with climacteric and non-climacteric ripening features such as tomato and strawberry. Besides, not only the metabolites and/or genes of fruit should be monitored, but also those of *B. cinerea* should be considered to investigate the pathogenicity factors that the fungus releases. These comparative studies are helpful to boost our knowledge for understanding the relevance of ripening to susceptibility and defense in fruit-*B. cinerea* interactions and the infection strategies *B. cinerea* employs upon contacting fruit tissues.

Meanwhile, research on fruit-*B. cinerea* interactions and breeding programs take time (requiring ~6 to 8 years of evaluation before a new cultivar is released). Advancements in pre- and postharvest technologies should be continued to delay spoilage of strawberries and other fruit products by *B. cinerea* and other pathogens, with retaining or even improving fruit consumer liking. The effects of these technologies such as indoor UV lighting and high CO_2 concentrations on the fruit-*B. cinerea* pathosystems as well as the fungus' life cycle are worthwhile to be investigated in the future.

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Summary

Summary

The cultivated strawberry (*Fragaria × ananassa*) is a highly appreciated soft fruit owing to its organoleptic characteristics (e.g., shiny appearance, distinct aroma), nutritional quality (e.g., vitamins, minerals) and antioxidants (e.g., flavonoids). However, the high susceptibility of strawberry fruit to fungal diseases during cultivation and postharvest transport and storage results in huge economic loss for growers and retailers. Amongst pathogenic fungi, *Botrytis cinerea*, causing grey mold disease, is primarily responsible for strawberry fruit spoilage. Strawberry breeders have been trying to develop genotypes with reduced susceptibility. However, the polygenetic resistance and the octoploid genome structure of the cultivated strawberry hamper the breeding progress. Some cultivars such as 'Malling centenary' and 'Opera' are less susceptible to *B. cinerea* but are not highly appreciated by consumers' owing to the high firmness and/or acidity.

Targeting on fruit physical and chemical characteristics that are highly associated with the susceptibility to *B. cinerea* may be promising for breeding less susceptible genotypes. Notably, most fruit characteristics change with ripening, resulting in increased susceptibility of fruit from unripe to ripe stages. In this thesis, we aimed to acquire the knowledge on the association between strawberry fruit characteristics and their susceptibility to *B. cinerea*.

A general introduction of this thesis is presented in **Chapter 1**. Firstly, I introduced breeding priorities of strawberry from perspectives of both suppliers and consumers and I referred to the most serious issue that hampers strawberry production, i.e., Botrytis fruit rot. Secondly, I described how *B. cinerea* releases its pathogenicity factors to invade fruit tissues and further focused on how *B. cinerea* infects strawberry tissues specifically. Thirdly, I reviewed the roles of certain metabolites (e.g., sugars, acids, volatile compounds, antioxidants) by integrating currently available information on fruits (e.g., strawberry, tomato, grape, apple) and *B. cinerea* interaction studies. Fourthly, I briefly described environmental factors that may have an impact on fruit susceptibility. Finally, I described the main objectives of the thesis and presented a scheme that illustrates the complete research of this thesis.

Research on characterizing the association between shelf life and physical and chemical characteristics in strawberry fruit is presented in **Chapter 2**. Seventeen genotypes were

selected based on the contrasting susceptibility to *B. cinerea*. Physical characteristics such as color and firmness, targeted primary (e.g., soluble sugars, organic acids) and secondary metabolites (e.g., proanthocyanins, anthocyanins, volatiles) of red ripe strawberry fruit at harvest were determined and fruit spoilage during postharvest storage was assessed. The results showed that 1) the spoilage rate differed between genotypes, and this was consistent over two successive years; 2) the intensity of red coloration was negatively correlated to spoilage rates; 3) 1-hexanol and ethyl butanoate were positively correlated to spoilage rates; 4) antioxidants (e.g., anthocyanins and ascorbic acid) and soluble sugar levels were not correlated to spoilage rates.

Research on effects of light spectra on strawberry fruit susceptibility to *B. cinerea* is described in **Chapter 3**. Strawberry plants were grown in a climate chamber under different red to blue light ratios (1:9; 1:1; 9:1) and with (~50 µmol m^{-2} s^{-1}) or without far-red light. In all experiments a total photosynthetic photon flux density of ~180 µmol m^{-2} s^{-1} was applied. The results showed that 1) additional far-red light accelerated fruit ripening and improved fruit soluble sugar and esters levels, which may improve the flavor quality; 2) low red to blue light ratios decreased soluble sugar levels but increased the levels of two main green leaf volatiles in fruit; 3) the antioxidant levels were enhanced by low red to blue ratios whereas reduced by additional far-red light; 4) despite variations in ripeness, sugars, esters and antioxidant levels between fruit from different light spectra, fruit susceptibility to *B. cinerea* was not affected by either additional far-red or different red to blue light ratios.

Research on high CO_2 concentrations reducing strawberry fruit spoilage by *B. cinerea* without affecting nutritional quality is demonstrated in **Chapter 4**. Both static and stepwise increments of CO_2 concentrations, up to 30 kPa, were applied to red ripe strawberry fruit. The results showed that 1) high CO_2 concentrations did not induce fermentation or affected antioxidant levels of fruit; 2) high CO_2 concentrations effectively reduced spoilage by *B. cinerea*, probably resulting from lower fruit respiration rates and higher pH; 3) high CO_2 concentrations led to a higher conversion of sucrose into fructose and glucose, particularly fructose, which might enhance fruit sweetness. Overall, there was no beneficial effect of the stepwise increase in CO_2 compared to the static application.

Research on effects of maturity at harvest and subsequent cold storage on consumer liking is described in **Chapter 5**. The effects of harvest maturity (¾ red and red ripe) and subsequent cold storage (4 °C, up to nine days) on consumer liking were investigated.

Summary

The volatile detection was conducted on homogenized fruit juice extracts (using SPME/GC-MS) and on intact fruit (using PTR-ToF-MS). Following PTR-ToF-MS measurements, fruits were evaluated by a trained expert panel. The results showed that 1) strawberry fruit harvested at the ¾ red stage had lower sweetness and aroma presence compared to fruit harvested at the red ripe stage, irrespective of the storage time.; 2) red ripe fruit showed highest aroma at the beginning of cold storage; 3) fruit sweetness and aroma were well predicted by PTR-ToF-MS and SPME/GC-MS obtained volatile profiles.

A general discussion of this thesis is presented in **Chapter 6**. Firstly, I reviewed the available knowledge on screening for strawberry susceptibility to *B. cinerea*. Based on the literature and my own experiments and experience, I provided some suggestions for breeders on rapid screening of strawberry genotypes for susceptibility to *B. cinerea*. Secondly, I extended the discussion to the relevance of ripening to susceptibility and defense in interactions between fruit and *B. cinerea* by comparing tomato-*B. cinerea* and strawberry-*B. cinerea* pathosystems. Thirdly, I discussed the roles of main metabolites (as mentioned in above research chapters) in susceptibility and defense. Finally, I proposed some ideas on a systematic investigation of fruit-*B. cinerea* interactions and listed the effective practices that extend strawberry shelf life.

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About the author

Hua Li (李桦) was born on 5th Nov 1991 in Xi'an, Shaanxi. She never imagined herself as a plant scientist as she grew up in the city. In 2010, she started pursing her bachelor's degree at Horticulture department, Northwest Agriculture & Forestry University. Fortunately, she discovered her research interest, which is postharvest product physiology and technology. Due to her outstanding performance during this time, she was accepted into a five-year PhD thesis ('Reducing chilling injury in kiwifruit') at the same university without having to take any entrance exams. However, after the first year of the PhD project, she decided to pursue a doctoral degree abroad. In 2017, she obtained her master's degree and was successful in applying for a scholarship from her home country and traveled abroad to begin her PhD studies. She came to NL on 23rd Nov 2017 and officially started the project, which is presented as a thesis, on 1st Dec 2017. Her research output can be found through Google Scholar Hua Li. At this moment, she is looking for new challenges related to plant science and looking forward to a new chapter in her life. Feel free to reach out via huali0827@gmail.com.

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)

Writing of project proposal (4.5 ECTS)

- Environmental and genetic factors combining long shelf-life with superior quality attributes in strawberry (2018)

Post-graduate courses (9.8 ECTS)

- Basic statistics; PE&RC (2018)
- Design of experiment; PE&RC (2018)
- Multivariate analysis; PE&RC (2019)
- Mixed model; PE&RC (2019)
- Introduction to R and R studio; PE&RC (2019)
- 11th international workshop plant metabolomics; Leiden University (2019)
- Advances in plant and food metabolomics; WUR (2019)
- Tidy data transformation and visualization with R tidyverse and ggplot; PE&RC (2020)
- 16th Summer course glycosciences; online; VLAG (2021)

Deficiency, refresh, brush-up courses (6 ECTS)

- Genomics (2018)

Competence strengthening / skills courses (4.9 ECTS)

- Project and time management; WGS (2018)
- Scientific writing; WGS (2020)
- Career perspectives; WGS (2021)

Scientific integrity/ethics in science activities (0.8 ECTS)

- Ethics in plant and environmental sciences; WGS (2018)
- Scientific integrity; WGS (2019)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- PE&RC First year's weekend (2018)
- PE&RC Day (2018, 2019)

National scientific meetings, local seminars, and discussion groups (6 ECTS)

- FLOP: Frontier Literature in Plant Physiology (2018-2021)

International symposia, workshops and conferences (4.7 ECTS)

- Vertifarm workshop; Wageningen, the Netherlands (2019)
- IX International strawberry symposium; online; Rimini, Italy (2021)
- BotrySclero; online; Avignon, France (2021)

Lecturing / supervision of practicals / tutorials (0.6 ECTS)

- Postharvest physiology (2018)

BSc/MSc thesis supervision

- *Jing Yu*: unravelling the relationship between strawberry shelf life and firmness
- *Ruimin Cao*: role of Anthocyanins and Proanthocyanidins in varied shelf life behaviors of strawberry
- *Puspa Khanal Joshi*: effect of pre- and post- harvest LED lighting on shelf-life related quality attributes of strawberry in relation to *Botrytis cinerea* susceptibility
- *Yuwei Yin*: an elevated CO_2 level during CA storage extends strawberry shelf life but does not affect nutritional quality
- *Diede de Jager*: growing strawberries in vertical farms: the effect of red:blue ratio and additional far-red LED lights on plant morphology, fruit quality and fungal defences of strawberries
- *Mengxiao Wang*: effects of different LED light spectrum on the production of volatile organic compounds (VOCs) in strawberry (*Fragaria x ananassa*)
- *Xinhe Huang*: effect of red, blue and additional far-red light on volatile production and *Botrytis cinerea* infection of strawberry fruit
- *Salem Agboyinu*: the effect of blue, red, and far-red light on secondary metabolites and their related gene expression in strawberry fruit
- *Kangni Liu*: analysis of the strawberry cultivars showed different susceptibility to *Botrytis cinerea* regarding soluble sugars, ascorbic acid, and lignin

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