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ABSTRACT

Strawberry (*Fragaria* x *ananassa*) fruit contain high concentration of health-promoting phenolic compounds, playing important role for the fruit ontogenic tolerance to fungi. In the highly susceptible cultivar Alba, the two major strawberry fungal pathogens, *Colletotrichum acutatum* and *Botrytis cinerea*, developed disease symptoms only at red ripe stages since immature fruits are tolerant to diseases. We analysed and compared the variation of 47 polyphenols in the surface of unripe and ripe Alba fruits upon 24 and 48 h of *C. acutatum* and *B. cinerea* infection, or mock-inoculation. Significant alteration in phenolic content was detected only in white infected fruit, with differences specific for each pathogen. The expression analysis of phenylpropanoid, flavonoid and shikimate pathway genes showed only in few cases a correlation with the relative metabolite abundance. The alteration in phenolic content and the lack of consistency with gene expression data is discussed in light of previously reported metabolome data of different susceptible and resistant strawberry genotypes.

KEYWORDS: *Fragaria* x *ananassa*, polyphenols, *Botrytis*, *Colletotrichum*, fungal quiescence, latent infection, ripening

INTRODUCTION

Besides its economic importance worldwide, strawberry *(Fragaria x ananassa)* crop is attracting much attention because of its nutritional benefits for human health since the fruit contains high levels of polyphenols with beneficial antioxidant, antibiotic and anti-inflammatory properties[1, 2, 3]. These phenolic compounds are present in strawberry fruits with concentration up to 40 mg per 100 g of fresh fruit[4], and make strawberry one of the most consumed fruit in the world. However, the soft and fleshy nature of strawberry fruit makes the crop highly perishable and susceptible to diseases[5], including a number of emerging threats affecting crop production in field[6, 7, 8].

Two of the most important diseases affecting strawberry fruits are anthracnose caused by *Colletotrichum acutatum*[9] and grey mould caused by *Botrytis cinerea*[10]. These pathogens are particularly insidious since, while infection can occur in flowers or immature stages of fruits, the disease symptoms are manifested at mature red stages when the fruit has reached its highest value. This phenomenon is attributed to the physico-chemical composition of immature fruits, which is not suitable for fungal growth; here pathogens can germinate and eventually develop early stages of colonization, but then they soon arrest their growth and survive as quiescent until the fruit is fully ripe. Once the fruit ripens, pathogens resume from the infection process and quickly invade the whole fruit and develop rot symptoms[11]. The infection strategy that *C. acutatum* displays on strawberry fruit has been studied on the susceptible cultivar Alba: at 24 h post-inoculation, pathogen growth is arrested in white fruits, whereas the pathogen already penetrated through intramural colonization in red fruits[12].

The involvement of secondary plant metabolites such as phenylpropanoids, flavonoids, benzoic acids and hydrolyzable tannins in defense during fruit development is well established. These compounds, collectively known as polyphenols, are produced during plant growth and

development and are also induced when plants are under stress. For instance, fungal growth inhibition is linked to the accumulation of polyphenols during pathogen infection, and is cited as one of the possible determinants for the low susceptibility of unripe fruit to fungal rots[13]. In particular for fruits of *Fragaria* spp., flavonoids such as proanthocyanidins and flavan-3-ols are actively synthesized in unripe fruit stages and diminish as the fruit ripens causing an increased fruit susceptibility[14]. For instance, the concentration of proanthocyanidins is correlated with varying susceptibility of different strawberry cultivars to *B. cinerea*[14]. Furthermore, it was proven that catechin, a major flavan-3-ol in immature strawberry receptacles, plays a key role in determining the infection strategy of *B. cinerea* from flower infection to ripe fruit colonization[15]. While no direct evidence of the involvement of phenolic compounds in the low susceptibility of immature strawberries to *C. acutatum* has been reported so far, the role of epicatechin in inhibiting the growth of *Colletorichum gloesporoides* in avocado has been reported[16]. In addition, a recent study[17] demonstrated that both susceptible and resistant strawberry cultivars exhibited a significant increase of flavan-3-ols and ellagic acid conjugates upon infection with *Colletotrichum nymphaeae.* Accumulation of ellagitannins was reported also in strawberry leaves infected with *Colletotrichum fragariae*. The isolated compound, sprayed on plants induced resistance against *Colletotrichum acutatum* and *Xanthomonas citri*[18].

With this knowledge, the present study was performed in order to evaluate the involvement of phenolic compounds in the ontogenic resistance of immature strawberry fruits to *C. acutatum* and *B. cinerea* in a susceptible genetic background. For this, a UHPLC system coupled with triple quadruple mass spectrometer (UHPLC MS/MS) and a spectrophotometric assay were used to quantify polyphenols in white and red strawberry fruits at 24 and 48 hours post-infection, in order to highlight the early fruit response determinants. Expression of the genes encoding for

different enzymes involved in the synthesis of phenolic compounds was also monitored in order to study and correlate the transcriptional and metabolic responses. The results allowed us to identify the compounds mostly responsive to each pathogen.

MATERIALS AND METHODS

Pathogens and plant material

Isolate Maya-3 of *Colletotrichum acutatum*[12] was grown on potato dextrose agar (Sigma) at 20°C for ten days. Meanwhile, B05.10 strain of *Botrytis cinerea* was grown on same conditions and exposed under UV light to facilitate sporulation.

Strawberry plants of cv. Alba, highly susceptible to several pathogens such as *Colletotrichum spp.*[12] and *Botrytis cinerea*, were tunnel-grown under conventional management practices in a local orchard (Cesena, Italy) and were maintained pesticide-free. The fruits were harvested 20 and 30 days after anthesis for the white and red berries, respectively and immediately brought to the laboratory for experiments. Fruits at white and red stages of ripening were used in order to compare fully tolerant and susceptible conditions.

Experimental set-up

For phenotypic assessment of the susceptibility of the strawberry Alba, three replicates of 10 fruits for each ripening stage were used and pathogen inoculated by dipping the fruits at a conidial suspension of 10^5 per mL for one minute. Another batch with the same number of fruits was dipped in water, serving as the control. Fruits were arranged in a lined-container and wrapped with polyethylene bags to maintain the relative humidity at level of 70%. Incidence of *C. acutatum* and *B. cinerea* on the fruits were monitored daily for seven days (red fruits) and for 14 days (white fruits). Disease incidence was expressed as the percentage of infected fruits over

the total number of samples in every treatment. Fruits were considered infected upon manifestation of symptoms.

For biochemical and molecular analyses of the phenolic content and gene expression profile, three replicates of 15 fruits for each ripening stage and for each type of pathogen infection were used. Fruits were inoculated as described above. After 24 and 48 hours of inoculation, the fruit surface (3 mm thick) was excised and immediately frozen in LN_2. These time points were chosen based on previous histological and microarray analysis of the *C. acutatum* infection on strawberry fruits[12].

Metabolomic analysis

Extraction of polyphenols. Phenolic compounds were extracted by homogenizing 30 g of strawberry fruits with a 50 mL acetone/water mixture (70:30 v/v) for 90 seconds. This was done twice, after which the volume adjusted to 120 mL. The extracts were centrifuged and the supernatant were stored in -20°C for subsequent analysis.

Targeted analysis of polyphenols with UHPLC-MS/MS. Samples for fruit polyphenol analysis were prepared accordingly as described by Gasperotti et al.[4]. An aliquot of 1 mL of the extract was initially dried and re-suspended in a 1 mL methanol/water mixture (50:50 v/v) with 1 mg/L rosmarinic acid as the internal standard. The analysis were performed with an ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) (Waters, Miliford, MA, USA) system coupled with triple quadruple (TQ) mass spectrometer[19]. The acquisition method was slightly modified, as two additional classes of compound were added to the analytical run: anthocyanins (eight compounds) and ellagitannins (four compounds). Ultra-performance liquid chromatography was performed employing a Waters Acquity UPLC system (Milford, MA, USA) coupled to a Waters Xevo TQMS (Milford, MA, USA) working in ESI ionisation mode.

Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40°C, with two solvents: A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). Calibration curves were prepared ranging from 5 x 10^{-3} to 20 mg/L with a proper relative standard reference for each analyzed compound. Information on MRM parameters are reported in Gasperotti et al.[4] and Vrhovsek et al.[19].

Bate Smith spectroscopy of high molecular weight proanthocyanidins (HMWP). HMWP were analyzed separately through the Bate Smith assay following the method described by Rigo et al.[20].

RNA preparation and qRT-PCR Analysis

RNA was extracted from frozen fruits samples upon grinding with mortar and pestle as described by Lopez-Gomez and Gomez-Lim[21], with minor modifications. The extracted RNA was visualized in agarose gel to determine their integrity and quantified with an ND-1000 UV spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA in a reaction of 20 μL with oligo-d(T) 17 as a primer using ImProm-II Reverse TranscriptaseTM (Promega, USA), following the provided protocol. The expression of genes belonging of the phenylpropanoid pathway, such as the *phenylalanine ammonia lyase* (*PAL*) and *cinnamate 4-hydroxylase* (*C4H*), and of the flavonoid pathway, such as *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*FHT*), *dihydroflavanol 4-reductase* (*DFR*) *anthocyanidin synthase* (*ANS*), *flavonoid-3-O-glucosyltransferase* (*FGT*), *leucoanthocyanidine reductase* (*LAR*), *anthocyanidin reductase* (*ANR*), and the expression level of a shikimate pathway gene, the *shikimate dehydrogenase* (*SDH*) and *FaMYB1* transcription factor gene, a negative regulator of the flavonoid biosynthesis, were analysed. These genes were amplified

using strawberry primers specific to the most expressed gene isoforms[22]. The expression of target genes were normalized with 1α (ef-1α) housekeeping gene. Amplifications were run in MX3000 thermocycler (Stratagene, CA, USA). Each reaction mixture contained: 1X of Platinum Sybr-Green Master mix (Invitrogen, Milan, Italy), 5 µM of each primer, 3.25 µl of nuclease-free water, and 2.5 µl of 1:12.5 dilution of cDNA, in a total volume of 12.5 µl. The following cycling conditions were used: an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, annealing step at a specific temperature for 30 sec (Supplemental Table 1), and an extension step at 72°C for 30 sec. Melting curve analysis was performed by monitoring the fluorescence from 55°C to 95°C every. Data were analyzed using MXPro QPCR Software version 3.0 (Stratagene, USA). Quantification was carried out using the relative standard curve method[23]. For each sample, three independent biological replicates were made and each replicate was run three times.

Statistical Analysis

The data were processed using the statistical package STATISTICA (version 7, Statsoft Inc., Tulsa, OK, USA). All metabolomic data were initially analyzed with factorial ANOVA at $p<0.05$ to determine the significant variations between the ripening stage, pathogen and post-inoculation time. Subsequently, one-way ANOVA was performed on the resulting polyphenols from the earlier analysis. Expression levels of genes from qRT-PCR were also subjected to one-way ANOVA. Separation of means was performed with Duncan's Multiple Range Test (DMRT) at $p<0.05$. For the gradient correlation of transcript levels and metabolite concentration, Z-scores were computed against the average of each gene expression or compound in all the conditions studied. Meanwhile, the heatmap was drawn with R software (version 3.2.2), accompanied with G-plot library.

RESULTS

Fruit susceptibility

No visible symptoms of infection by *Colletotrichum acutatum* and *Botrytis cinerea* were observed at 24 and 48 hours post-inoculation (HPI) in both white and red strawberries (cv. Alba) compared to mock-inoculated (dipped in water) ones (Figure 1, Supplemental Figure 1). In red fruits, symptoms of dark-brown lesions appear at three days post-inoculation (DPI) of *B. cinerea* and at four DPI of *C. acutatum*, with recorded incidence of 27% and 20%, respectively (Figure 1). At seven DPI, the lesions spread on most of the fruit surface in both types of infected red samples and, in fruits infected with *B. cinerea,* fungal egression occurs (Supplemental Figure 1). On the contrary, no symptoms were observed in inoculated white fruits at the same time points (Figure 1, Supplemental Figure 1) or later up to 14 days (not shown) despite the manifested red pigmentation.

Polyphenol profile in strawberry fruits during ripening and upon pathogen infection

A total of 47 compounds were detected from the surface of white and red strawberry fruits in inoculated or control conditions (Supplemental Table 2). Forty-six compounds were analyzed via targeted analysis with UHPLC MS/MS, while HMWP were quantified through Bates Smith spectrophotometric assay. The analyzed phenolic compounds belong to the following classes: benzoic acids and their derivatives, phenylpropanoids, stilbenes, dihydrochalcones, flavones, flavonone, flavan-3-ols, flavonols, anthocyanins, ellagitannins, and proanthocyanidin (Supplemental Table 2).

All these classes were found to vary upon ripening and pathogen infection (Figure 2). Considering the metabolite classes, proanthocyanidins are the most concentrated group in both

white and red fruits of Alba. In unripe strawberries, ellagitannins are the second most concentrated class, which is typical of this stage. On the other hand, anthocyanins are more concentrated in red fruits than ellagitannins due to ripening. Upon pathogen infection, an increase in polyphenol content is specifically noted in white fruits (Supplemental Table 2).

A more specific evaluation of individual polyphenols shows that the concentration of the compounds is affected by the ripening stage and pathogen infection (Figure 2). Upon ranking the abundance of compounds in each condition, it is revealed that HMWP are the most abundant polyphenol in strawberry fruits regardless of the ripening stage, presence and type of pathogen or the time after inoculation. Meanwhile, pelargonidin-3-glucoside is the second most concentrated polyphenol in all conditions of red fruits and in mock-inoculated white fruits at 48 HPI. This compound is the main anthocyanin in strawberry. Moreover, the increase of the ellagitannin casuarictin over pelargonidin-3-glucoside in infected white fruits of Alba suggests that this compound is involved in infection related response (Table 1).

Based on the other ranked compounds, it is apparent that the majority of polyphenols in red fruits do not exhibit differences upon pathogen infection. On the contrary, the abundance of the compounds in unripe Alba is greatly influenced by the presence of *C. acutatum* and *B. cinerea*. For instance, the flavan-3-ol catechin shows higher abundance in pathogen-inoculated white fruits than in control.

Variation of different classes of polyphenols in unripe and ripe strawberries upon fungal infection

In order to analyze the influence of the fruit ripening stage (R), of the fungal pathogen species (P) and of the post-inoculation time (T) on the variation of each polyphenol, a factorial ANOVA was performed on all analysed compound (Supplemental Table 3). Ten out of forty-seven (21%)

phenolic compounds were found to significantly vary between the pathogens, while 37 (79%) and 22 (47%) are significantly different between the ripening stage and the infection time, respectively (Table 2). Upon consideration of all three variables, 12 (26%) of the phenolic compounds were found to be significantly influenced (Table 2, PxRxT). These belong to the groups of benzoic acids and derivatives, ellagitannins, flavonols, flavan-3-ols, and proanthocyanidin.

Flavonols. Considering the total concentration, no significant variation of flavonol compounds was detected in strawberry fruits among different ripening stages or pathogen inoculation (Figure 2 and Supplemental Table 2). On the other hand, significant interactions between the pathogen, ripening stage and time were highlighted upon analysis of individual flavonol compounds (Table 2). In particular, the concentration of kaempferol-3-rutinoside is 73% higher in red fruits than in white. However, both *C. acutatum* and *B. cinerea* caused a decrease of this compound at 24 HPI infected red fruits, which could possibly be related to the susceptibility of red berries. Meanwhile, a significant accumulation of isorhamnetin-3-rutinoside is measured in white fruits inoculated with *B. cinerea* at 48 HPI (Figure 3).

Flavan-3-ols. Consistent with previous reports, control strawberry fruits exhibited a decrease in flavan-3-ols during ripening (Figure 2 and Supplemental Table 2). A decrease in flavan-3-ols is also detected in control white fruits from 24 to 48 HPI, possibly as postharvest effect on phenolic metabolisms. The infection with *C. acutatum* and *B. cinerea* influences the concentration of these polyphenols only in white fruits: *C. acutatum* infection does not lead to the flavan-3-ols decrease from 24 to 48 HPI, whereas *B. cinerea* first exhibited a decrease (24 HPI) and then an accumulation at 48 HPI. On the contrary, no variation is detected in pathogen inoculated red fruits compared to control.

Taken individually, catechin, procyanidin B1 and procyanidin B3 vary similarly to the total flavan-3-ols (Figure 3). The accumulation of flavan-3-ols exclusively in white infected fruits suggests that these polyphenols could be determinant for the low susceptibility of white fruit to pathogens.

Proanthocyanidins. The levels of strawberry proanthocyanidin found in our study decrease with ripening, similar to previous reports[14]. Within white fruits, the concentration of HMWP does not significantly vary upon 24 HPI with both pathogens. A significant increase of HMWP is detectable only in white fruit as late response (48 HPI) with both pathogen species. No significant differences were found in red fruits (Figure 2 and 3).

Benzoic acids and derivatives. Though present in relatively smaller concentrations, benzoic acids and their derivatives were found to have significant differences between fruit ripening stage, type of pathogen and time (Table 2 and Supplemental Table 2). In general, the concentration of total benzoic acids is significantly higher in white fruits than the red ones. Interestingly, this variation is independent from the pathogen infection in all the condition tested, except for *B. cinerea* inoculated white fruits at 24 HPI, where a significant decrease from control is measured. In red fruits on the other hand, the concentration of total benzoic acids and derivatives does not show any significant variation among the condition tested except for 48 HPI *B. cinerea* where these compounds are found significantly increased (Supplemental Table 2).

Out of the eight identified compound from the class, *p* Hydroxybenzoic acid and 2,6-Dihydroxy benzoic, methyl gallate, and catechol acid were found to be the only ones significantly contributing to the benzoic acids variation described above (Figure 3). It is noteworthy, that methyl gallate concentration increases in *B. cinerea* infection in white fruits at 24 HPI, which is in contrast to the general trend (Figure 3).

Ellagitannins. Total ellagitannins significantly decreases from 24 to 48 HPI, in both white and red control fruits of about 61% and 54%, respectively (Figure 2 and Supplemental Table 2). Infection with both fungal pathogens differently influences fruit ellagitannin concentration, depending on the ripening stage. In white fruits, ellagitannins remain stable from 24 to 48 HPI and do not decrease as in control. In contrast, a significant decrease in these compounds are detected in 24 HPI *B. cinerea* infected red fruits, but not in *C. acutatum* ones.

In the present study, casuarictin appears as the major ellagitannin compound, representing more than half of the total ellagitannin concentration in all the treatment conditions (Figure 2). Both casuarictin and agrimoniin change their concentration mirroring the total ellagitannins variations described above (Figure 3 and Supplemental Table 2).

Expression of genes of the polyphenol pathway during ripening and upon pathogen infection

The expression of genes encoding for enzymes involved in polyphenols biosynthesis was analyzed by RT-qPCR. Genes regulating the synthesis of flavan-3-ols and proanthocyanidins, such *ANS* and *LAR* showed higher expression at unripe stages, both infected or not (Figure 4); whereas the *FHT* gene, serving in the synthesis of early flavonol precursors, increases its expression in red control fruits. Interestingly, the expression of *FaMYB1* does not differ from white to red control fruits. In red fruits, the presence of pathogen infection does not seem to significantly alter the abundance profile of the transcript level of most of the genes. Particular decrease in the expression of few genes is exhibited only at 24 HPI. Upon *C. acucatum* infection, the transcript levels of *DFR*, which is involved in flavan-3-ols synthesis, and *FHT,* decrease, while *FGT* genes regulating anthocyanin production, together with *ANS,* decrease upon infection of *B. cinerea*. Meanwhile, in white infected fruits, only *ANS* exhibited a decrease in expression at

24 HPI in response to both types of pathogens. Contrary to this, major differences in gene expression are detected at 48 HPI: phenylpropanoid gene *C4H,* and the flavonoid genes *CHI* and *CHS* (Figure 4) significantly increase their transcript level in infected fruits independent of the type of pathogen. Similarly, *ANS* and *LAR* show a clear activation in transcription upon infection with both pathogens (Figure 4).

No significant differences in the expression of *PAL,* the gene encoding for the first enzyme in the phenylpropanoid pathway, were detected during ripening or upon infection. Similarly, no major differences are detected in the expression of *SDH,* the gene that regulates benzoic acid and ellagitannin biosynthesis[24] (Figure 4).

Metabolite and transcript profiles correlation

With the purpose of highlighting the possible correlations, the Z-scores of the phenolic compound concentration and of the expression level of the genes involved in their synthesis were calculated. It should be noted that the Z-scores, represented as colour changes, are standardized on the average value of each condition and do not take into account statistical significance (Figure 5). The phenolic compounds showing the most important variation along with the treatment condition or those with high concentration were considered (Supplemental Table 2).

Considering that the differences detected in the expression of *PAL* gene is not significant in any of the condition tested, and *C4H* only varies significantly only in white 48 HPI inoculated fruits, weak correspondence between gene expression and metabolite concentration is apparent for the phenylpropanoid pathway (Figure 5). With respect to the flavonoid pathway, the expression of *FHT* gene does not seem to influence the concentration of these metabolites. The expression of *FGT* gene, regulating the synthesis of anthocyanins, is not correlated likewise with any of the examined pelargonidin and cyanidin compounds. On the other hand, the expression of *LAR, ANS,*

and *ANR*, but not *DFR*, reflects fairly close the different concentrations of catechin, procyanidins and HMWP (Figure 5).

Finally for to the shikimate pathway, the higher expression of *SDH* gene in white fruits correlates with the higher concentration of most of the benzoic acid metabolites and ellagitannins (Figure 5).

DISCUSSION

The evolutionary role of fruit during ripening is dual since initially, it protects the embryo until seed becomes lignified, and later promotes seed dispersal into the surrounding environment. For this, unripe fruits have very efficient physical and chemical defence mechanisms so that the majority of fungal pathogens attacking these stages stop their growth and become quiescent. On the opposite, attractive colours and aromas develop in ripe fruits and defence barriers diminish, allowing animal dispersion of seeds and resumption of fungal pathogen growth

The impact of fruit ontogeny on the infection strategy of fungal pathogens has been studied and widely documented for several fruit species[11]. In particular for strawberry, the tolerance of unripe fruits to the two major fruit pathogens, *Colletotrichum acutatum* and *Botrytis cinerea* is well known: *C. acutatum* is found quiescent as appressorium in white immature fruits[12], whereas for *B. cinerea* infection can occur at flowering stage but symptoms develop only at red fruit stages[25]. Furthermore, aroma volatile compounds typical of ripe strawberry, such as furaneol, ethyl butanoate and *cis*-3-hexenyl acetate, clearly stimulate the growth of both these fungi, suggesting that fungal quiescence is not only dependent on inhibitory factors in the immature fruits, but also on ripeness-specific stimulating compounds[26].

In our study we confirm that the unripe fruit stages of a susceptible strawberry cultivar is tolerant to anthracnose and grey mould disease (Figure 1, Supplemental Figure 1). Contrary to unripe strawberry fruits that mature on the plant, the fruits harvested at unripe stages and inoculated with the *C. acutatum* or *B. cinerea* never developed symptoms of any fungal disease even as the fruits turned red. This could be due either to fungal death occurring at late time points, or also to the non-climacteric nature of strawberry. Unripe strawberry fruits are not able to satisfy all the physiological requirements needed to accomplish maturation, and these are probably required to restore fruit susceptibility and fungal growth.

Both pre-formed and induced factors have been addressed as involved in the tolerance of immature fruits[11], and phenolic compounds fall in both these categories playing roles as pre-formed (phytoanticipin) or induced (phytoalexin) defences[13, 27]. It is from 1989 the hypothesis that *B. cinerea* quiescence in strawberry green fruits was due to proanthocyanidins, which are particularly abundant at these stages[28]. This hypothesis was later supported by several studies addressing both the phenolic compounds antimicrobial properties[29] and their accumulation in different immature fruit species[30, 31].

The results presented here support a major role of polyphenols as pre-formed contribution to the disease tolerance of immature fruits (Figure 2). Indeed, according to previous reports on polyphenol variation during ripening[32] and their role in plant defence[33], we find that compounds such as flavan-3-ols, proanthocyanidins, benzoic acids and ellagitannins strongly decrease with ripening in the absence of pathogens. Consistently, genes such as *ANS* and *LAR*, regulating the synthesis of catechins and proanthocyanidins are down-regulated in red fruits (Figure 4). Conversely, the expression of the *FaMYB1* that negatively regulate the production of anthocyanins in *F.* x *ananassa*[34] does not vary during ripening. However, other *MYB* genes have

been recognized in strawberry[35, 36] that could be involved in the regulation of flavonoid gene expression. Interestingly, a recent study addressing the profile of phenolic compounds in strawberry fruits of different cultivars indicated that the level of flavan-3-ols in healthy fruits of a tolerant cultivar is much higher than in a susceptible one and that the differences between tolerant and susceptible cultivars laid more in the pre-existing phenolic profiles of than into the pathogen induced ones[17]. While supporting a key role for these compounds in fruit resistance, our data suggest that the mechanisms involved fruit ontogenic resistance are similar to those conferring genotype resistance.

Considering the ripening stages, pathogen, and post-infection time, most significant differences in the profile of individual polyphenols were detected at 48 HPI in white fruits, suggesting that fruit response to pathogens intensifies at this time (Figure 2 and Table 1). In particular, HMWP, the most concentrated polyphenol in strawberry fruits[4, 37] are also the most responsive to pathogen infections, increasing their level up to 44% with respect to control. The fact that the level of flavan-3-ols, proanthocyanidins and ellagitannins is maintained in 48 HPI white fruits infected with both pathogens, suggests that the fruit response to pathogens inhibits the normal postharvest metabolisms to maintain high the concentration of antimicrobial compounds. These alterations are probably related to the temporary tolerance of white fruits, independent of the susceptible genetic background. Accumulation of ellagitannins and ellagic acid conjugates as defense response have been reported in strawberry leaves, where these compounds can elicit hypersensitive response and salicylic acid mediated gene expression[18, 38], and in ripe fruits inoculated with *Colletotrichum nymphaeae*[17] or with *Colletotrichum simmondsii*[39].

Differently from *Colletotrichum*, *Botrytis* induces a decrease in benzoic acids (except methyl gallate), phenylpropanoid and flavan-3-ols exclusively associated to the early stage of the

interaction (24 HPI white fruits) (Figure 2). Since these compounds have all been recognized as active in immature defence response, their decrease at early stage of infection could be associated to a different infection strategy of the two fungi: *C. acutatum* has a general hemibiotroph *habitus*, while *B. cinerea* is a typical necrotroph. Therefore, it is reasonable that at early interaction steps they communicate differently with the host.

It is notable in red fruits that none of the two pathogens provoke important variation in metabolite profiles compared to control, except for few individual compounds that undergo a significant decrease (Supplemental Table 2). These ripe fruits contain lower levels of polyphenols, are fully susceptible to these pathogens and develop disease symptoms in few days (Figure 1). Thus, the decrease or lack of response in the concentration of phenolic compounds in this type of fruit is consistent with the establishment of a fully compatible interaction between the pathogen and its host. Furthermore, contrary to our findings, a significant increase in ellagic acid derivatives, flavan-3-ols and flavonols was recently reported not only in unripe but also in ripe strawberry fruits of the cultivar 'Asia' after infection with *C. nymphaeae* [40]. Provided that these results were obtained from a different strawberry cultivar, which already accounts for strong metabolic differences, it must be taken into consideration that only the external layers of the fruit were used to extract the phenolic compounds in our study and not the whole fruit; this was carried out with the purpose to examine only the tissues directly interacting with the pathogen during 24 and 48 HPI. Indeed, it is known that the spatial distribution of phenolic compounds inside the strawberry fruit tissue is discontinuous through the fruit section, with flavan-3-ols being much more abundant in the fruit core than into the external receptacle tissues[22]. This could explain the strong differences in the ellagitannins concentration found in our analysis with the data previously reported for the same cultivar utilizing the same method[41],

where casuarictin, agrimoniin and ellagic acid were found five to ten-fold less concentrated. A different pre-determined spatial organization of the phenolic compounds could meet a different functional requirement of these metabolites in the various parts of the fruit during ripening. However, defence-related compounds, such as benzoic acids, ellagitannins, flavan-3-ols and HMWPs, could also be induced to mobilize across the fruit layers at the site of infection upon pathogen perception. This hypothesis could also explain the disassociation between transcriptional activation of some of the regulatory genes and corresponding polyphenol accumulation that we have found in this study.

In conclusion, our results support a key role for phenolic compounds in the ontogenic fruit disease tolerance to two major postharvest strawberry diseases. However, further studies using cultivars with different level of disease susceptibility are needed to fully uncover the molecular mechanisms involved in unripe fruit tolerance. These can provide new important elements for the development of new cultivars less susceptible to *Colletotrichum* and *Botrytis*.

AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

SUPPORTING INFORMATION

Supplemental tables and figures as follows:

Supplemental Table 1: Primers used in qRT-PCR reaction. **Supplemental Table 2**: Mean concentration of polyphenols in white and red strawberry fruits inoculated with *C. acutatum* and *B. cinerea*. **Supplemental Table 3**: Factorial ANOVA summary of phenolic compounds. **Supplemental Figure 1**: White and red fruits of Alba strawberry inoculated with *C. acutatum,* or *B. cinerea* at 24 and 48 HPI and 7 DPI. **Supplemental Figure 2:** The shikimate, phenylpropanoid and flavonoid pathways.

REFERENCES

1. Giampieri, F.; Alvarez-Suarez, J. M.; Battino, M., Strawberry and Human Health: Effects beyond Antioxidant Activity. *Journal of Agricultural and Food Chemistry* **2014,** *62* (18), 3867-3876.

2. Forbes-Hernandez, T. Y.; Gasparrini, M.; Afrin, S.; Bompadre, S.; Mezzetti, B.; Quiles, J. L.; Giampieri, F.; Battino, M., The healthy effects of strawberry polyphenols: which strategy behind antioxidant capacity? *Critical Reviews in Food Science and Nutrition* **2015,** (just-accepted), 00-00.

3. Giampieri, F.; Forbes-Hernandez, T. Y.; Gasparrini, M.; Alvarez-Suarez, J. M.; Afrin, S.; Bompadre, S.; Quiles, J. L.; Mezzetti, B.; Battino, M., Strawberry as a health promoter: an evidence based review. *Food & Function* **2015,** *6* (5), 1386-1398.

4. Gasperotti, M.; Masuero, D.; Mattivi, F.; Vrhovsek, U., Overall dietary polyphenol intake in a bowl of strawberries: The influence of Fragaria spp. in nutritional studies. *Journal of Functional Foods* **2015,** *18*, 1057-1069.

5. Vincente, A.R.; Martinez, G. A.; Civello, P. M.; Chaves, A. R., Quality of heat-treated strawberry fruit during refrigerated storage. *Postharvest Biology and Technology* **2002,** *25* (1), 59-71.

6. Pope, T.; Gundalai, E.; Elliott, L.; Blackshaw, R.; Hough, G.; Wood, A.; Bennison, J.; Prince, G.; Chandler, D., Recording the movement of adult vine weevil within strawberry crops using radio frequency identification tags. *Journal of Berry Research* (Preprint), 1-10.

7. De Ros, G.; Conci, S.; Pantezzi, T.; Savini, G., The economic impact of invasive pest Drosophila suzukii on berry production in the Province of Trento, Italy. *Journal of Berry Research* **2015,** (Preprint), 1-8.

8. Wenneker, M.; Bergsma-Vlami, M., Erwinia pyrifoliae, a new pathogen on strawberry in the Netherlands. *Journal of Berry Research* **2015,** *5* (1), 17-22.

9. Buddie, A. G.; Martínez-Culebras, P.; Bridge, P. D.; García, M. D.; Querol, A.; Cannon, P. F.; Monte, E., Molecular characterization of Colletotrichum strains derived from strawberry. *Mycological Research* **1999,** *103* (4), 385-394.

10. Maas, J. L., *Compendium of strawberry diseases*. American Phytopathological Society (APS Press): 1998.

11. Prusky, D., Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology* **1996,** *34*, 413-434.

12. Guidarelli, M.; Carbone, F.; Mourgues, F.; Perrotta, G.; Rosati, C.; Bertolini, P.; Baraldi, E., Colletotrichum acutatum interactions with unripe and ripe strawberry fruits and differential responses at histological and transcriptional levels. *Plant Pathology* **2011,** *60* (4), 685-697.

13. Amil-Ruiz, F.; Blanco-Portales, R.; Muñoz-Blanco, J.; Caballero, J. L., The Strawberry Plant Defense Mechanism: A Molecular Review. *Plant and Cell Physiology* **2011,** *52* (11), 1873-1903.

14. Di Venere, D.; Linsalata, V.; Ippolito, A.; Nigro, F.; Arcuti, P.; Lattanzio, V., Endogenous phenolics, ripening and susceptibility of strawberry fruits (Fragaria ananassa Duch.) to post-harvest diseases. *Polyphenols Communication* **1998,** *98*, 459-460.

15. Puhl, I.; Treutter, D., Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of Botrytis cinerea in developing strawberry fruits. *J. Plant Dis. Prot* **2008,** *115*, 247-251.

16. Guetsky, R.; Kobiler, I.; Wang, X.; Perlman, N.; Gollop, N.; Avila-Quezada, G.; Hadar, I.; Prusky, D., Metabolism of the Flavonoid Epicatechin by Laccase of Colletotrichum gloeosporioides and Its Effect on Pathogenicity on Avocado Fruits. *Phytopathology* **2005,** *95* (11), 1341-1348.

17. Mikulic-Petkovsek, M.; Schmitzer, V.; Slatnar, A.; Weber, N.; Veberic, R.; Stampar, F.; Munda, A.; Koron, D., Alteration of the content of primary and secondary metabolites in strawberry fruit by Colletotrichum nymphaeae infection. *Journal of Agricultural and Food Chemistry* **2013,** *61* (25), 5987-5995.

18. Mamani, A.; Filippone, M.; Grellet, C.; Welin, B.; Castagnaro, A.; Ricci, J., Pathogen-Induced Accumulation of an Ellagitannin Elicits Plant Defense Response. *Molecular Plant Microbe Interactions* **2012,** *25* (11), 1430-1439.

19. Vrhovsek, U.; Masuero, D.; Gasperotti, M.; Franceschi, P.; Caputi, L.; Viola, R.; Mattivi, F., A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages. *Journal of Agricultural and Food Chemistry* **2012,** *60* (36), 8831-8840.

20. Rigo, A.; Vianello, F.; Clementi, G.; Rossetto, M.; Scarpa, M.; Vrhovšek, U.; Mattivi, F., Contribution of proanthocyanidins to the peroxy radical scavenging capacity of some Italian red wines. *Journal of agricultural and food chemistry* **2000,** *48* (6), 1996-2002.

21. López-Gómez, R.; Gómez-Lim, M. A., A Method for Extracting Intact RNA from Fruits Rich in Polysaccharides using Ripe Mango Mesocarp. *HortScience* **1992,** *27* (5), 440-442.

22. Almeida, J. R. M.; D'Amico, E.; Preuss, A.; Carbone, F.; de Vos, C. H. R.; Deiml, B.; Mourgues, F.; Perrotta, G.; Fischer, T. C.; Bovy, A. G.; Martens, S.; Rosati, C., Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (Fragaria × ananassa). *Archives of Biochemistry and Biophysics* **2007,** *465* (1), 61-71.

23. Livak, K. J., ABI Prism 7700 sequence detection system. *User bulletin* **1997,** *2*, 929-34.

24. Widhalm, Joshua R.; Dudareva, N., A Familiar Ring to It: Biosynthesis of Plant Benzoic Acids. *Molecular Plant* **2015,** *8* (1), 83-97.

25. Williamson, B.; Tudzynski, B.; Tudzynski, P.; van Kan, J. A. L., Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology* **2007,** *8* (5), 561-580.

26. Neri, F.; Cappellin, L.; Spadoni, A.; Cameldi, I.; Algarra Alarcon, A.; Aprea, E.; Romano, A.; Gasperi, F.; Biasioli, F., Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. *Plant Pathology* **2015,** *64* (3), 709-717.

27. VanEtten, H. D.; Mansfield, J. W.; Bailey, J. A.; Farmer, E. E., Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins". *The Plant Cell* **1994,** *6* (9), 1191-1192.

28. Jersch, S.; Scherer, C.; Huth, G.; Schlösser, E., Proanthocyanidins as basis for quiescence of Botrytis cinerea in immature strawberry fruits. *Zeitschrift fuer Pflanzenkrankheiten und Pflanzenschutz (Germany, FR)* **1989**.

29. Feucht, W.; Treutter, D., The role of flavan-3-ols and proanthocyanidins in plant defence. CRC Press: Boca Raton, FL, USA: 1999; pp 307-338.

30. Keller, M.; Viret, O.; Cole, F. M., Botrytis cinerea Infection in Grape Flowers: Defense Reaction, Latency, and Disease Expression. *Phytopathology* **2003,** *93* (3), 316-322.

31. Pezet, R.; Viret, O.; Perret, C.; Tabacchi, R., Latency of Botrytis cinerea Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *Journal of Phytopathology* **2003,** *151* (4), 208-214.

32. Aaby, K.; Mazur, S.; Nes, A.; Skrede, G., Phenolic compounds in strawberry (Fragaria x ananassa Duch.) fruits: Composition in 27 cultivars and changes during ripening. *Food Chemistry* **2012,** *132* (1), 86-97.

33. Halbwirth, H.; Puhl, I.; Haas, U.; Jezik, K.; Treutter, D.; Stich, K., Two-Phase Flavonoid Formation in Developing Strawberry (Fragaria × ananassa) Fruit. *Journal of Agricultural and Food Chemistry* **2006,** *54* (4), 1479-1485.

34. Aharoni, A.; De Vos, C. H.; Wein, M.; Sun, Z.; Greco, R.; Kroon, A.; Mol, J. N. M.; O'Connell, A. P., The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *The Plant Journal* **2001,** *28* (3), 319-332.

35. Medina-Puche, L.; Cumplido-Laso, G.; Amil-Ruiz, F.; Hoffmann, T.; Ring, L.; Rodríguez-Franco, A.; Caballero, J. L.; Schwab, W.; Muñoz-Blanco, J.; Blanco-Portales, R.,

MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of Fragaria× ananassa fruits. *Journal of experimental botany* **2014,** *65* (2), 401-417.

36. Medina-Puche, L.; Molina-Hidalgo, F. J.; Boersma, M. R.; Schuurink, R. C.; López-Vidriero, I.; Solano, R.; Franco-Zorrilla, J.-M.; Caballero, J. L.; Blanco-Portales, R.; Muñoz-Blanco, J., A R2R3-MYB transcription factor (FaEOBII) regulates eugenol production in ripe strawberry (Fragaria× ananassa) fruit receptacles. *Plant physiology* **2015**, pp-114.

37. Buendía, B.; Gil, M. I.; Tudela, J. A.; Gady, A. L.; Medina, J. J.; Soria, C.; López, J. M.; Tomás-Barberán, F. A., HPLC-MS Analysis of Proanthocyanidin Oligomers and Other Phenolics in 15 Strawberry Cultivars. *Journal of Agricultural and Food Chemistry* **2010,** *58* (7), 3916-3926.

38. Hukkanen, A. T.; Kokko, H. I.; Buchala, A. J.; McDougall, G. J.; Stewart, D.; Kärenlampi, S. O.; Karjalainen, R. O., Benzothiadiazole Induces the Accumulation of Phenolics and Improves Resistance to Powdery Mildew in Strawberries. *Journal of Agricultural and Food Chemistry* **2007,** *55* (5), 1862-1870.

39. Weber, N.; Schmitzer, V.; Jakopic, J.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Veberic, R., Influence of Colletotrichum simmondsii R. G. Shives & Y. P. Tan infection on selected primary and secondary metabolites in strawberry (Fragaria x ananassa Duch.) fruit and runners. *European Journal of Plant Pathology* **2013,** *136* (2), 281-290.

40. Weber, N.; Veberic, R.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Munda, A.; Jakopic, J., Metabolite accumulation in strawberry (Fragaria × ananassa Duch.) fruits and runners in response to Colletotrichum nymphaeae infection. *Physiological and Molecular Plant Pathology* **2015,** *92*, 119-129.

41. Gasperotti, M.; Masuero, D.; Guella, G.; Palmieri, L.; Martinatti, P.; Pojer, E.; Mattivi, F.; Vrhovsek, U., Evolution of Ellagitannin Content and Profile during Fruit Ripening in Fragaria spp. *Journal of Agricultural and Food Chemistry* **2013,** *61* (36), 8597-8607.

FIGURE CAPTIONS

Figure 1. Incidence of *C. acutatum* and *B. cinerea* in white and red fruits of Alba up to 7 DPI. For consistency, results are all presented in DPI, including disease incidence recorded at 24 and 48 HPI. W and R: white and red fruits; H_2O, CA and BC: mock-, *C. acutatum*-, and *B. cinerea*-inoculated fruits.

Figure 2. Concentrations of polyphenols expressed as Z-scores (bottom colored bar) in white and red strawberry fruits as affected by *C. acutatum* and *B. cinerea* infection visualized as a heat map. W and R: white and red fruits; 24 and 48: post-inoculation hours; H_2O, CA and BC: mock-, *C. acutatum*- and *B. cinerea*- inoculated fruits.

Figure 3. Variation of fruit polyphenols in white and red Alba strawberry upon infection of *C. acutatum* and *B. cinerea*. The 12 compounds found to be significantly influenced by fungal pathogens species (P), ripening stage (R) and post-inoculation time (T) are reported. Quantities are expressed as mg/Kg fresh weight (FW). Each data is an average of three biological replicates with its standard error. Means with the same letter are not significantly different at $p<0.05$ (DMRT).

Figure 4. Relative expression levels of genes in white and red strawberry fruits as affected by *C. acutatum* and *B. cinerea* inoculation. All values were normalized to the expression level of the *elongation factor 1α* housekeeping gene. Each data is an average of three biological replicates with its standard error. Means with the same letter are not significantly different at $p<0.05$ (DMRT).

Figure 5. Correlation of gene expression and metabolite concentration expressed as Z-scores in coloured boxes. Genes or compound that significantly vary based on the one-way ANOVA are designated in the boxes with an * (significance between 24 and 48 HPI), and/or a † (significance

567 with respect to control). W and R: white and red fruits; 24 and 48: post-inoculation hours; H_2O,

568 CA and BC: mock-, *C. acutatum*- and *B. cinerea*- inoculated fruits.

TABLES

Table 1. Top ten most abundant polyphenols in the different conditions tested (W and R: white and red fruits; 24 and 48: post-inoculation hours; H_2O, CA and BC: mock-, *C. acutatum*- and *B. cinerea*- inoculated fruits). Number within column represents the ranking position of each compound within the conditions, with 1 being the most abundant.

Compounds	Conditions											
	W-24-H_2O	W-24-CA	W-24-BC	W-48-H_2O	W-48-CA	W-48-BC	R-24-H_2O	R-24-CA	R-24-BC	R-48-H_2O	R-48-CA	R-48-BC
Proanthocyanidins (HMWP)	1	1	1	1	1	1	1	1	1	1	1	1
Casuarictin	2	2	2	3	2	2	3	3	3	3	3	3
Agrimoniin	3	3	3	4	4	4	4	4	4	4	4	4
Ellagic acid	4	4	4	5	5	5	5	5	5	5	5	5
Procyanidin B3	5	8	7	6	7	8	10	10	10	9	10	10
Quercetin-3-glucuronide	6	5	5	7	8	7	8	7	7	10	7	8
Catechin	7	6	6	8	6	6	9	9	9	8	9	9
Pelargonidin-3-glucoside	8	7	10	2	3	3	2	2	2	2	2	2
Procyanidin B1	9	9	8	9	9	9	—	—	—	—	—	—
Kaempferol-3-glucuronide	10	10	9	—	—	—	—	—	—	—	—	—
Cyanidin-3-galactoside	—	—	—	10	10	10	6	6	6	6	6	6
Pelargonidin-3-rutinoside	—	—	—	—	—	—	7	8	8	7	8	7

Table 2. Number of statistically significant compounds in each condition as influenced by of pathogen (P), ripening stage of strawberry fruit (R), and post-inoculation time (T), as tested with factorial ANOVA at $p<0.05$ and $p<0.01$.

Class of polyphenol	P	R	T	PxR	PxT	RxT	PxRxT
Benzoic acid and derivatives	3	6	4	3	4	5	4
Phenylpropanoids	1	3	2	2	0	2	0
Stilbenes	0	2	1	0	0	0	0
Dihydrochalcones	2	2	2	0	1	2	0
Flavones	0	1	0	1	0	0	0
Flavonone	0	1	1	1	0	1	0
Flavan-3-ols	0	3	1	0	3	0	3
Flavonols	3	13	5	2	4	2	2
Anthocyanins	0	2	2	0	0	2	0
Ellagitannins	0	3	3	2	3	0	2
Proanthocyanidins	1	1	1	1	1	0	1
TOTAL	10	37	22	12	16	14	12

FIGURES

Figure 1.

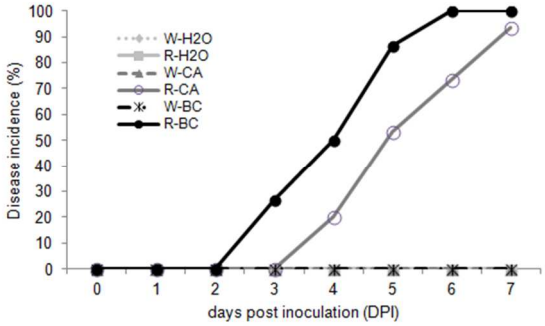


Figure 2.

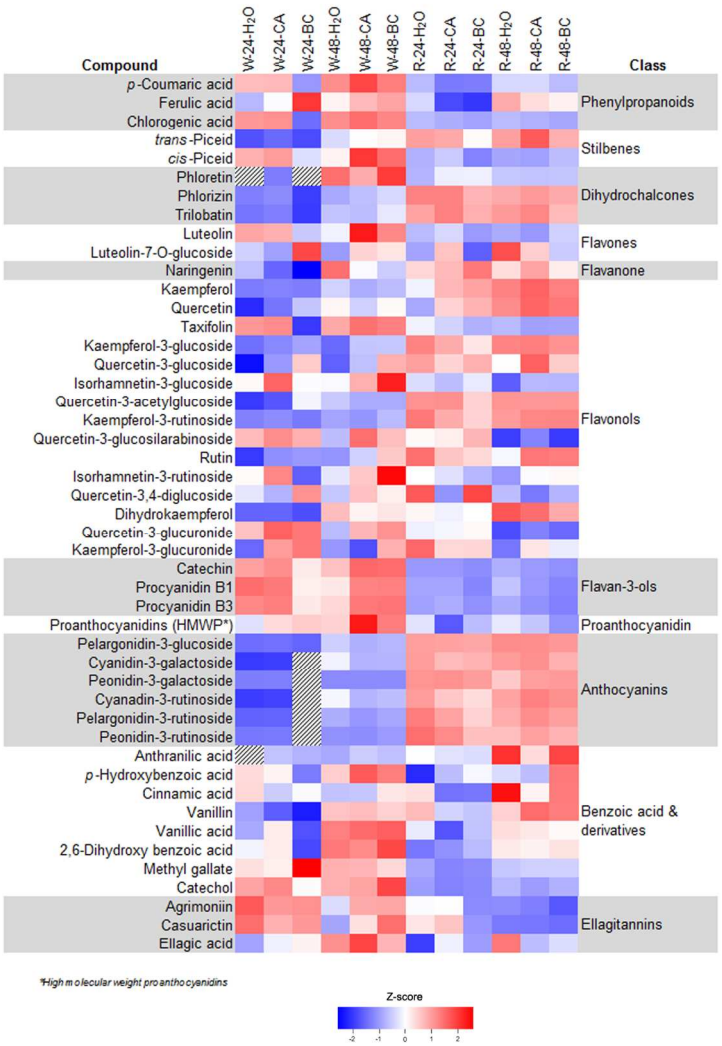
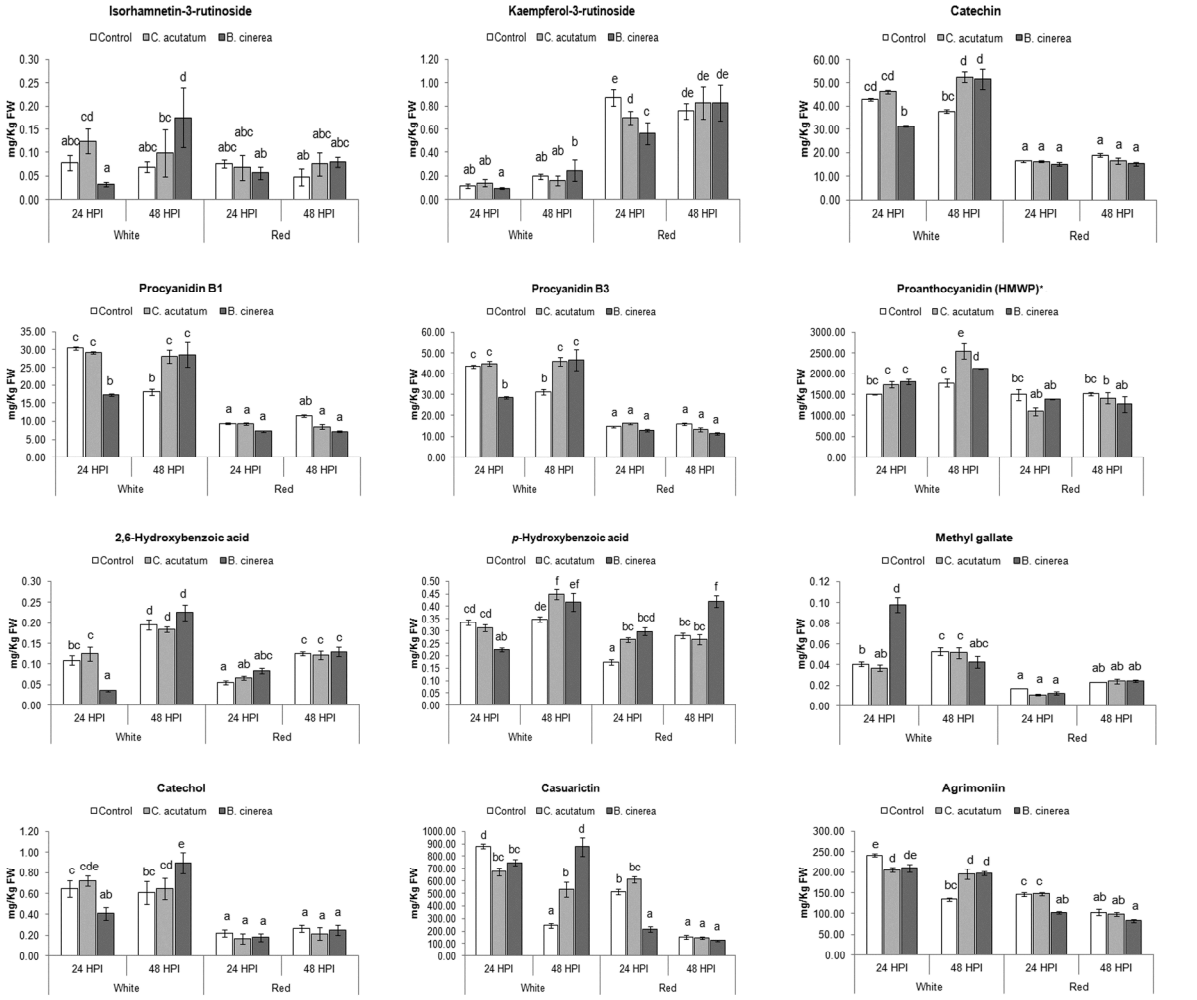


Figure 3.



*High molecular weight proanthocyanidins

Figure 4.

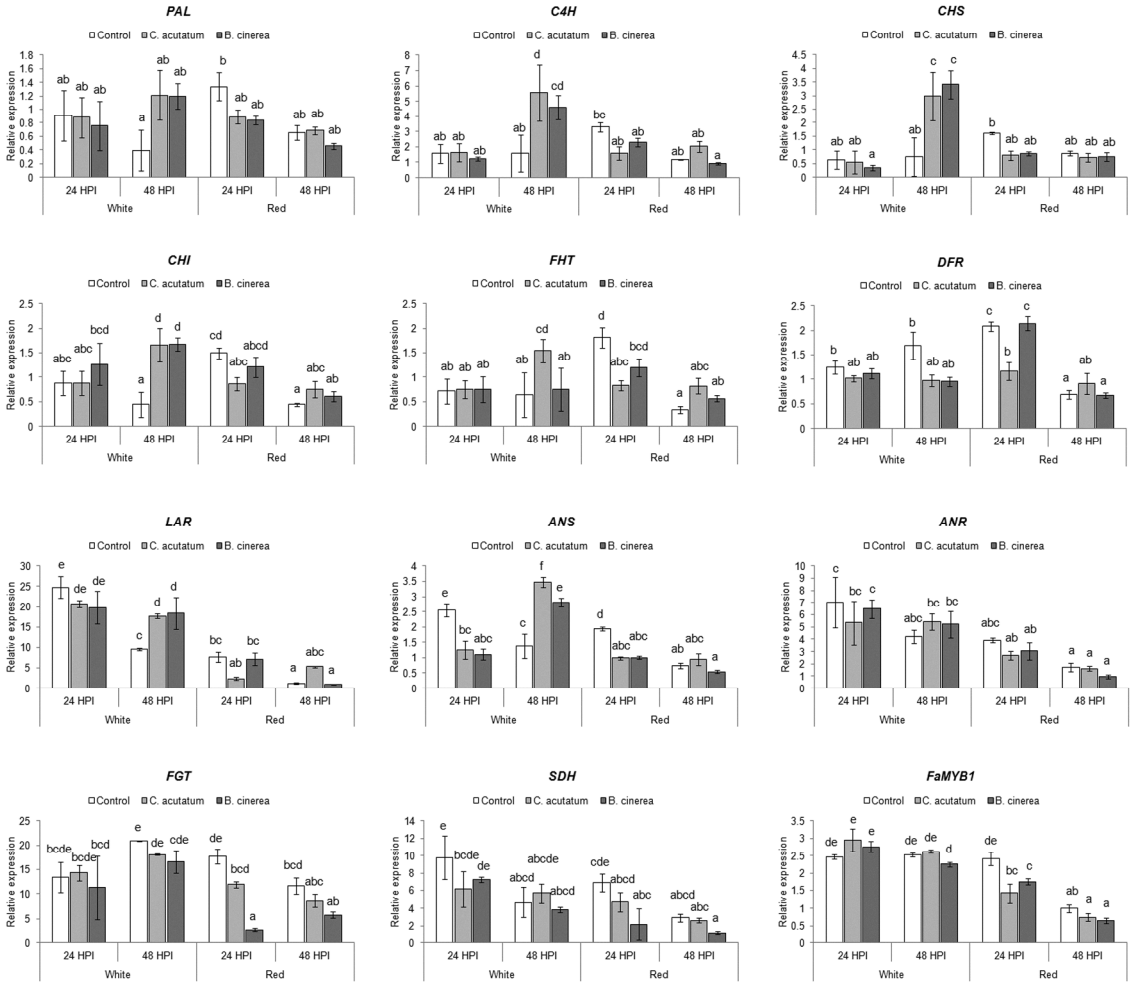
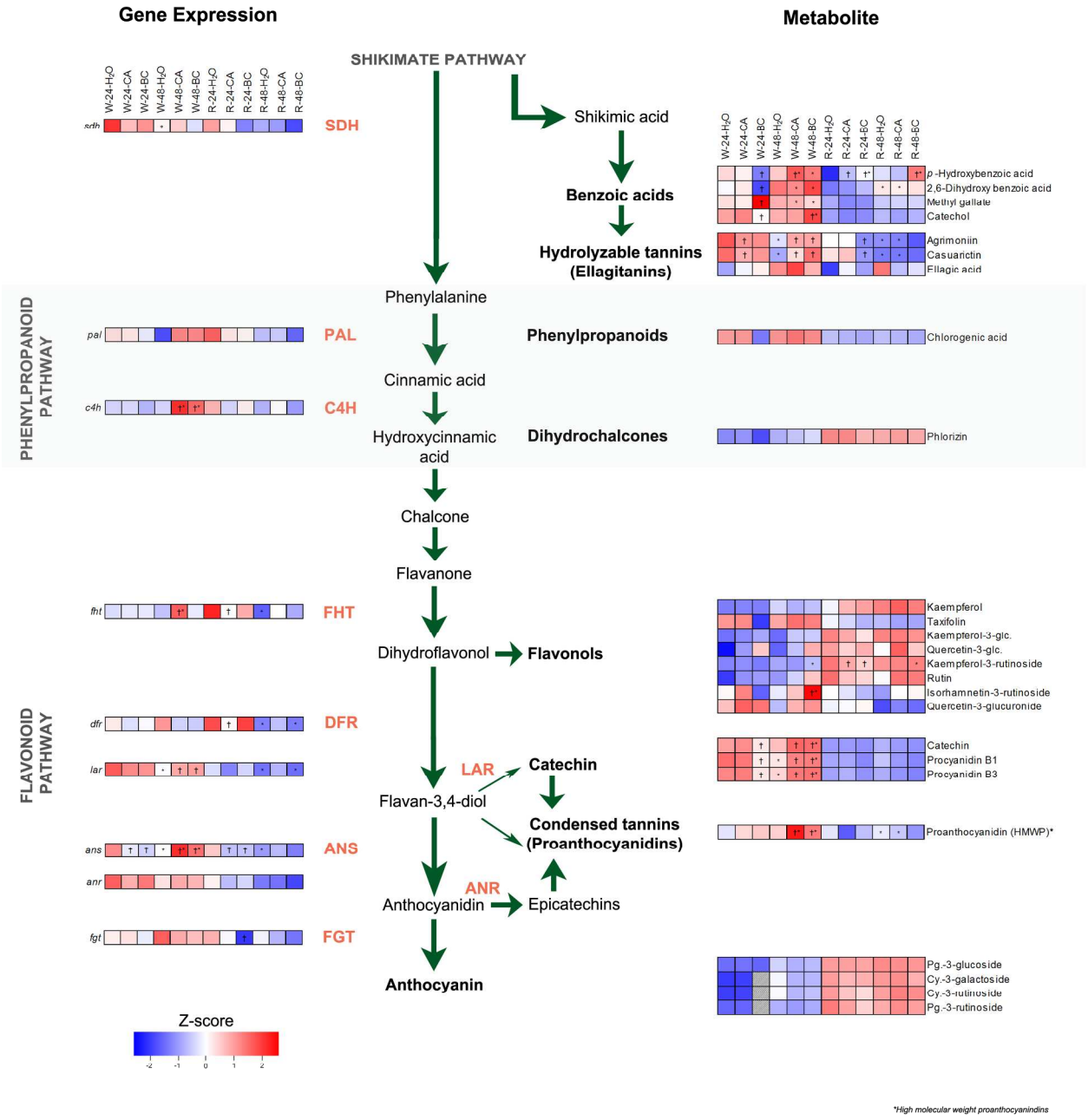

PAL
C4H
CHS
CHI
FHT
DFR
LAR
ANS
ANR
FGT
SDH
FaMYB1
Control
C. acutatum
B. cinerea
Relative expression
24 HPI
48 HPI
White
Red

Figure 5.


Gene Expression
Metabolite
SHIKIMATE PATHWAY
Shikimic acid
Benzoic acids
Hydrolyzable tannins (Ellagitanins)
Phenylalanine
Cinnamic acid
Hydroxycinnamic acid
Chalcone
Flavanone
Dihydroflavonol
Flavonols
Flavan-3,4-diol
Catechin
Condensed tannins (Proanthocyanidins)
Anthocyanidin
Epicatechins
Anthocyanin
PHENYLPROPANOID PATHWAY
FLAVONOID PATHWAY
SDH
PAL
C4H
FHT
DFR
LAR
ANS
ANR
FGT
Phenylpropanoids
Dihydrochalcones
p-Hydroxybenzoic acid
2,6-Dihydroxy benzoic acid
Methyl gallate
Catechol
Agrimoniin
Casuarictin
Ellagic acid
Chlorogenic acid
Phlorizin
Kaempferol
Taxifolin
Kaempferol-3-glc.
Quercetin-3-glc.
Kaempferol-3-rutinoside
Rutin
Isorhamnetin-3-rutinoside
Quercetin-3-glucuronide
Catechin
Procyanidin B1
Procyanidin B3
Proanthocyanidin (HMWP)*
Pg.-3-glucoside
Cy.-3-galactoside
Cy.-3-rutinoside
Pg.-3-rutinoside
Z-score
*High molecular weight proanthocyanindins

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