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Polyphenols Variation in Fruits of the Susceptible Strawberry cv. Alba during Ripening and upon Fungal Pathogen Interaction and Possible Involvement in the Unripe Fruit Tolerance

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1 ABSTRACT

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

14

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

17 INTRODUCTION

18 Besides its economic importance worldwide, strawberry (*Fragaria x ananassa*) crop is attracting
19 much attention because of its nutritional benefits for human health since the fruit contains high
20 levels of polyphenols with beneficial antioxidant, antibiotic and anti-inflammatory properties^{1,2,3}.
21 These phenolic compounds are present in strawberry fruits with concentration up to 40 mg per
22 100 g of fresh fruit⁴, and make strawberry one of the most consumed fruit in the world. However,
23 the soft and fleshy nature of strawberry fruit makes the crop highly perishable and susceptible to
24 diseases⁵, including a number of emerging threats affecting crop production in field^{6,7,8}.
25 Two of the most important diseases affecting strawberry fruits are anthracnose caused by
26 *Colletotrichum acutatum*⁹ and grey mould caused by *Botrytis cinerea*¹⁰. These pathogens are
27 particularly insidious since, while infection can occur in flowers or immature stages of fruits, the
28 disease symptoms are manifested at mature red stages when the fruit has reached its highest
29 value. This phenomenon is attributed to the physico-chemical composition of immature fruits,
30 which is not suitable for fungal growth; here pathogens can germinate and eventually develop
31 early stages of colonization, but then they soon arrest their growth and survive as quiescent until
32 the fruit is fully ripe. Once the fruit ripens, pathogens resume from the infection process and
33 quickly invade the whole fruit and develop rot symptoms¹¹. The infection strategy that *C.*
34 *acutatum* displays on strawberry fruit has been studied on the susceptible cultivar Alba: at 24 h
35 post-inoculation, pathogen growth is arrested in white fruits, whereas the pathogen already
36 penetrated through intramural colonization in red fruits¹².
37 The involvement of secondary plant metabolites such as phenylpropanoids, flavonoids, benzoic
38 acids and hydrolyzable tannins in defense during fruit development is well established. These
39 compounds, collectively known as polyphenols, are produced during plant growth and

40 development and are also induced when plants are under stress. For instance, fungal growth
41 inhibition is linked to the accumulation of polyphenols during pathogen infection, and is cited as
42 one of the possible determinants for the low susceptibility of unripe fruit to fungal rots¹³. In
43 particular for fruits of *Fragaria* spp., flavonoids such as proanthocyanidins and flavan-3-ols are
44 actively synthesized in unripe fruit stages and diminish as the fruit ripens causing an increased
45 fruit susceptibility¹⁴. For instance, the concentration of proanthocyanidins is correlated with
46 varying susceptibility of different strawberry cultivars to *B. cinerea*¹⁴. Furthermore, it was
47 proven that catechin, a major flavan-3-ol in immature strawberry receptacles, plays a key role in
48 determining the infection strategy of *B. cinerea* from flower infection to ripe fruit colonization¹⁵.
49 While no direct evidence of the involvement of phenolic compounds in the low susceptibility of
50 immature strawberries to *C. acutatum* has been reported so far, the role of epicatechin in
51 inhibiting the growth of *Colletotrichum gloesporoides* in avocado has been reported¹⁶. In addition,
52 a recent study¹⁷ demonstrated that both susceptible and resistant strawberry cultivars exhibited a
53 significant increase of flavan-3-ols and ellagic acid conjugates upon infection with
54 *Colletotrichum nymphaeae*. Accumulation of ellagitannins was reported also in strawberry
55 leaves infected with *Colletotrichum fragariae*. The isolated compound, sprayed on plants
56 induced resistance against *Colletotrichum acutatum* and *Xanthomonas citri*¹⁸.
57 With this knowledge, the present study was performed in order to evaluate the involvement of
58 phenolic compounds in the ontogenic resistance of immature strawberry fruits to *C. acutatum*
59 and *B. cinerea* in a susceptible genetic background. For this, a UHPLC system coupled with
60 triple quadruple mass spectrometer (UHPLC MS/MS) and a spectrophotometric assay were used
61 to quantify polyphenols in white and red strawberry fruits at 24 and 48 hours post-infection, in
62 order to highlight the early fruit response determinants. Expression of the genes encoding for

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

66

67 MATERIALS AND METHODS

68 Pathogens and plant material

69 Isolate Maya-3 of *Colletotrichum acutatum*¹² was grown on potato dextrose agar (Sigma) at
70 20°C for ten days. Meanwhile, B05.10 strain of *Botrytis cinerea* was grown on same conditions
71 and exposed under UV light to facilitate sporulation.

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

78 Experimental set-up

79 For phenotypic assessment of the susceptibility of the strawberry Alba, three replicates of 10
80 fruits for each ripening stage were used and pathogen inoculated by dipping the fruits at a
81 conidial suspension of 10⁵ per mL for one minute. Another batch with the same number of fruits
82 was dipped in water, serving as the control. Fruits were arranged in a lined-container and
83 wrapped with polyethylene bags to maintain the relative humidity at level of 70%. Incidence of
84 *C. acutatum* and *B. cinerea* on the fruits were monitored daily for seven days (red fruits) and for
85 14 days (white fruits). Disease incidence was expressed as the percentage of infected fruits over

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

88 For biochemical and molecular analyses of the phenolic content and gene expression profile,
89 three replicates of 15 fruits for each ripening stage and for each type of pathogen infection were
90 used. Fruits were inoculated as described above. After 24 and 48 hours of inoculation, the fruit
91 surface (3 mm thick) was excised and immediately frozen in LN₂. These time points were chosen
92 based on previous histological and microarray analysis of the *C. acutatum* infection on
93 strawberry fruits¹².

94 **Metabolomic analysis**

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

99 ***Targeted analysis of polyphenols with UHPLC-MS/MS.*** Samples for fruit polyphenol analysis
100 were prepared accordingly as described by Gasperotti et al.⁴. An aliquot of 1 mL of the extract
101 was initially dried and re-suspended in a 1 mL methanol/water mixture (50:50 v/v) with 1 mg/L
102 rosmarinic acid as the internal standard. The analysis were performed with an ultra-performance
103 liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) (Waters, Miliford, MA,
104 USA) system coupled with triple quadruple (TQ) mass spectrometer¹⁹. The acquisition method
105 was slightly modified, as two additional classes of compound were added to the analytical run:
106 anthocyanins (eight compounds) and ellagitannins (four compounds). Ultra-performance liquid
107 chromatography was performed employing a Waters Acquity UPLC system (Milford, MA, USA)
108 coupled to a Waters Xevo TQMS (Milford, MA, USA) working in ESI ionisation mode.

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

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

118 **RNA preparation and qRT-PCR Analysis**

119 RNA was extracted from frozen fruits samples upon grinding with mortar and pestle as described
120 by Lopez-Gomez and Gomez-Lim²¹, with minor modifications. The extracted RNA was
121 visualized in agarose gel to determine their integrity and quantified with an ND-1000 UV
122 spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA in a reaction of
123 20 μL with oligo-d(T) 17 as a primer using ImProm-II Reverse TranscriptaseTM (Promega,
124 USA), following the provided protocol. The expression of genes belonging of the
125 phenylpropanoid pathway, such as the *phenylalanine ammonia lyase (PAL)* and *cinnamate 4-*
126 *hydroxylase (C4H)*, and of the flavonoid pathway, such as *chalcone synthase (CHS)*, *chalcone*
127 *isomerase (CHI)*, *flavanone 3-hydroxylase (FHT)*, *dihydroflavanol 4-reductase (DFR)*
128 *anthocyanidin synthase (ANS)*, *flavonoid-3-O-glucosyltransferase (FGT)*, *leucoanthocyanidine*
129 *reductase (LAR)*, *anthocyanidin reductase (ANR)*, and the expression level of a shikimate
130 pathway gene, the *shikimate dehydrogenase (SDH)* and *FaMYB1* transcription factor gene, a
131 negative regulator of the flavonoid biosynthesis, were analysed. These genes were amplified

132 using strawberry primers specific to the most expressed gene isoforms²². The expression of target
133 genes were normalized with 1 α (ef-1 α) housekeeping gene. Amplifications were run in MX3000
134 thermocycler (Stratagene, CA, USA). Each reaction mixture contained: 1X of Platinum Sybr-
135 Green Master mix (Invitrogen, Milan, Italy), 5 μ M of each primer, 3.25 μ l of nuclease-free water,
136 and 2.5 μ l of 1:12.5 dilution of cDNA, in a total volume of 12.5 μ l. The following cycling
137 conditions were used: an initial denaturation step at 95°C for 10 min, followed by 40 cycles at
138 95°C for 15 sec, annealing step at a specific temperature for 30 sec (Supplemental Table 1), and
139 an extension step at 72°C for 30 sec. Melting curve analysis was performed by monitoring the
140 fluorescence from 55°C to 95°C every. Data were analyzed using MXPro QPCR Software
141 version 3.0 (Stratagene, USA). Quantification was carried out using the relative standard curve
142 method²³. For each sample, three independent biological replicates were made and each
143 replicate was run three times.

144 **Statistical Analysis**

145 The data were processed using the statistical package STATISTICA (version 7, Statsoft Inc.,
146 Tulsa, OK, USA). All metabolomic data were initially analyzed with factorial ANOVA at
147 $p < 0.05$ to determine the significant variations between the ripening stage, pathogen and post-
148 inoculation time. Subsequently, one-way ANOVA was performed on the resulting polyphenols
149 from the earlier analysis. Expression levels of genes from qRT-PCR were also subjected to one-
150 way ANOVA. Separation of means was performed with Duncan's Multiple Range Test (DMRT)
151 at $p < 0.05$. For the gradient correlation of transcript levels and metabolite concentration, Z-scores
152 were computed against the average of each gene expression or compound in all the conditions
153 studied. Meanwhile, the heatmap was drawn with R software (version 3.2.2), accompanied with
154 G-plot library.

155

156 RESULTS**157 Fruit susceptibility**

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

168 Polyphenol profile in strawberry fruits during ripening and upon pathogen infection

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

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

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

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

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

196 **Variation of different classes of polyphenols in unripe and ripe strawberries upon fungal** 197 **infection**

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

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

207 **Flavonols.** Considering the total concentration, no significant variation of flavonol compounds
208 was detected in strawberry fruits among different ripening stages or pathogen inoculation (Figure
209 2 and Supplemental Table 2). On the other hand, significant interactions between the pathogen,
210 ripening stage and time were highlighted upon analysis of individual flavonol compounds (Table
211 2). In particular, the concentration of kaempferol-3-rutinoside is 73% higher in red fruits than in
212 white. However, both *C. acutatum* and *B. cinerea* caused a decrease of this compound at 24 HPI
213 infected red fruits, which could possibly be related to the susceptibility of red berries. Meanwhile,
214 a significant accumulation of isorhamnetin-3-rutinoside is measured in white fruits inoculated
215 with *B. cinerea* at 48 HPI (Figure 3).

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

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

228 **Proanthocyanidins.** The levels of strawberry proanthocyanidin found in our study decrease with
229 ripening, similar to previous reports¹⁴. Within white fruits, the concentration of HMWP does not
230 significantly vary upon 24 HPI with both pathogens. A significant increase of HMWP is
231 detectable only in white fruit as late response (48 HPI) with both pathogen species. No
232 significant differences were found in red fruits (Figure 2 and 3).

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

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

247 **Ellagitannins.** Total ellagitannins significantly decreases from 24 to 48 HPI, in both white and
248 red control fruits of about 61% and 54%, respectively (Figure 2 and Supplemental Table 2).
249 Infection with both fungal pathogens differently influences fruit ellagitannin concentration,
250 depending on the ripening stage. In white fruits, ellagitannins remain stable from 24 to 48 HPI
251 and do not decrease as in control. In contrast, a significant decrease in these compounds are
252 detected in 24 HPI *B. cinerea* infected red fruits, but not in *C. acutatum* ones.
253 In the present study, casuarictin appears as the major ellagitannin compound, representing more
254 than half of the total ellagitannin concentration in all the treatment conditions (Figure 2). Both
255 casuarictin and agrimoniin change their concentration mirroring the total ellagitannins variations
256 described above (Figure 3 and Supplemental Table 2).

257 **Expression of genes of the polyphenol pathway during ripening and upon pathogen** 258 **infection**

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

270 24 HPI in response to both types of pathogens. Contrary to this, major differences in gene
271 expression are detected at 48 HPI: phenylpropanoid gene *C4H*, and the flavonoid genes *CHI* and
272 *CHS* (Figure 4) significantly increase their transcript level in infected fruits independent of the
273 type of pathogen. Similarly, *ANS* and *LAR* show a clear activation in transcription upon infection
274 with both pathogens (Figure 4).

275 No significant differences in the expression of *PAL*, the gene encoding for the first enzyme in the
276 phenylpropanoid pathway, were detected during ripening or upon infection. Similarly, no major
277 differences are detected in the expression of *SDH*, the gene that regulates benzoic acid and
278 ellagitannin biosynthesis²⁴ (Figure 4).

279 **Metabolite and transcript profiles correlation**

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

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

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

295 Finally for to the shikimate pathway, the higher expression of *SDH* gene in white fruits correlates
296 with the higher concentration of most of the benzoic acid metabolites and ellagitannins (Figure
297 5).

298

299 **DISCUSSION**

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

306 The impact of fruit ontogeny on the infection strategy of fungal pathogens has been studied and
307 widely documented for several fruit species¹¹. In particular for strawberry, the tolerance of
308 unripe fruits to the two major fruit pathogens, *Colletotrichum acutatum* and *Botrytis cinerea* is
309 well known: *C. acutatum* is found quiescent as appressorium in white immature fruits¹², whereas
310 for *B. cinerea* infection can occur at flowering stage but symptoms develop only at red fruit
311 stages²⁵. Furthermore, aroma volatile compounds typical of ripe strawberry, such as furaneol,
312 ethyl butanoate and *cis*-3-hexenyl acetate, clearly stimulate the growth of both these fungi,
313 suggesting that fungal quiescence is not only dependent on inhibitory factors in the immature
314 fruits, but also on ripeness-specific stimulating compounds²⁶.

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

323 Both pre-formed and induced factors have been addressed as involved in the tolerance of
324 immature fruits¹¹, and phenolic compounds fall in both these categories playing roles as pre-
325 formed (phytoanticipin) or induced (phytoalexin) defences^{13, 27}. It is from 1989 the hypothesis
326 that *B. cinerea* quiescence in strawberry green fruits was due to proanthocyanidins, which are
327 particularly abundant at these stages²⁸. This hypothesis was later supported by several studies
328 addressing both the phenolic compounds antimicrobial properties²⁹ and their accumulation in
329 different immature fruit species^{30, 31}.

330 The results presented here support a major role of polyphenols as pre-formed contribution to the
331 disease tolerance of immature fruits (Figure 2). Indeed, according to previous reports on
332 polyphenol variation during ripening³² and their role in plant defence³³, we find that compounds
333 such as flavan-3-ols, proanthocyanidins, benzoic acids and ellagitannins strongly decrease with
334 ripening in the absence of pathogens. Consistently, genes such as *ANS* and *LAR*, regulating the
335 synthesis of catechins and proanthocyanidins are down-regulated in red fruits (Figure 4).
336 Conversely, the expression of the *FaMYB1* that negatively regulate the production of
337 anthocyanins in *F. x ananassa*³⁴ does not vary during ripening. However, other *MYB* genes have

338 been recognized in strawberry^{35, 36} that could be involved in the regulation of flavonoid gene
339 expression. Interestingly, a recent study addressing the profile of phenolic compounds in
340 strawberry fruits of different cultivars indicated that the level of flavan-3-ols in healthy fruits of a
341 tolerant cultivar is much higher than in a susceptible one and that the differences between
342 tolerant and susceptible cultivars laid more in the pre-existing phenolic profiles of than into the
343 pathogen induced ones¹⁷. While supporting a key role for these compounds in fruit resistance,
344 our data suggest that the mechanisms involved fruit ontogenic resistance are similar to those
345 conferring genotype resistance.

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

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

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

366 It is notable in red fruits that none of the two pathogens provoke important variation in
367 metabolite profiles compared to control, except for few individual compounds that undergo a
368 significant decrease (Supplemental Table 2). These ripe fruits contain lower levels of
369 polyphenols, are fully susceptible to these pathogens and develop disease symptoms in few days
370 (Figure 1). Thus, the decrease or lack of response in the concentration of phenolic compounds in
371 this type of fruit is consistent with the establishment of a fully compatible interaction between
372 the pathogen and its host. Furthermore, contrary to our findings, a significant increase in ellagic
373 acid derivatives, flavan-3-ols and flavonols was recently reported not only in unripe but also in
374 ripe strawberry fruits of the cultivar 'Asia' after infection with *C. nymphaeae*⁴⁰. Provided that
375 these results were obtained from a different strawberry cultivar, which already accounts for
376 strong metabolic differences, it must be taken into consideration that only the external layers of
377 the fruit were used to extract the phenolic compounds in our study and not the whole fruit; this
378 was carried out with the purpose to examine only the tissues directly interacting with the
379 pathogen during 24 and 48 HPI. Indeed, it is known that the spatial distribution of phenolic
380 compounds inside the strawberry fruit tissue is discontinuous through the fruit section, with
381 flavan-3-ols being much more abundant in the fruit core than into the external receptacle
382 tissues²². This could explain the strong differences in the ellagitannins concentration found in our
383 analysis with the data previously reported for the same cultivar utilizing the same method⁴¹,

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

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

397

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405 Notes

406 The authors declare no competing financial interest.

407

408 **SUPPORTING INFORMATION**

409 Supplemental tables and figures as follows:

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

416

417

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543

544 **FIGURE CAPTIONS**

545 **Figure 1.** Incidence of *C. acutatum* and *B. cinerea* in white and red fruits of Alba up to 7 DPI.
546 For consistency, results are all presented in DPI, including disease incidence recorded at 24 and
547 48 HPI. W and R: white and red fruits; H₂O, CA and BC: mock-, *C. acutatum*-, and *B. cinerea*-
548 inoculated fruits.

549 **Figure 2.** Concentrations of polyphenols expressed as Z-scores (bottom colored bar) in white
550 and red strawberry fruits as affected by *C. acutatum* and *B. cinerea* infection visualized as a heat
551 map. W and R: white and red fruits; 24 and 48: post-inoculation hours; H₂O, CA and BC: mock-,
552 *C. acutatum*- and *B. cinerea*- inoculated fruits.

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

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

564 **Figure 5.** Correlation of gene expression and metabolite concentration expressed as Z-scores in
565 coloured boxes. Genes or compound that significantly vary based on the one-way ANOVA are
566 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; H₂O,
- 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; H₂O, 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 ₂ O	W-24-CA	W-24-BC	W-48-H ₂ O	W-48-CA	W-48-BC	R-24-H ₂ O	R-24-CA	R-24-BC	R-48-H ₂ O	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.

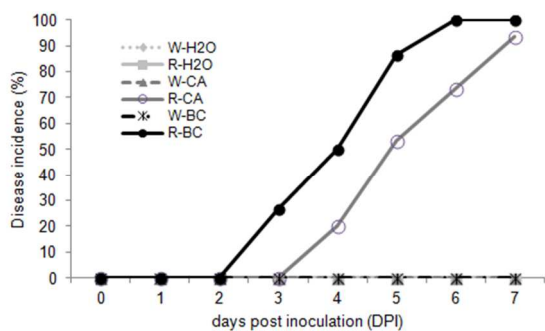


Figure 2.

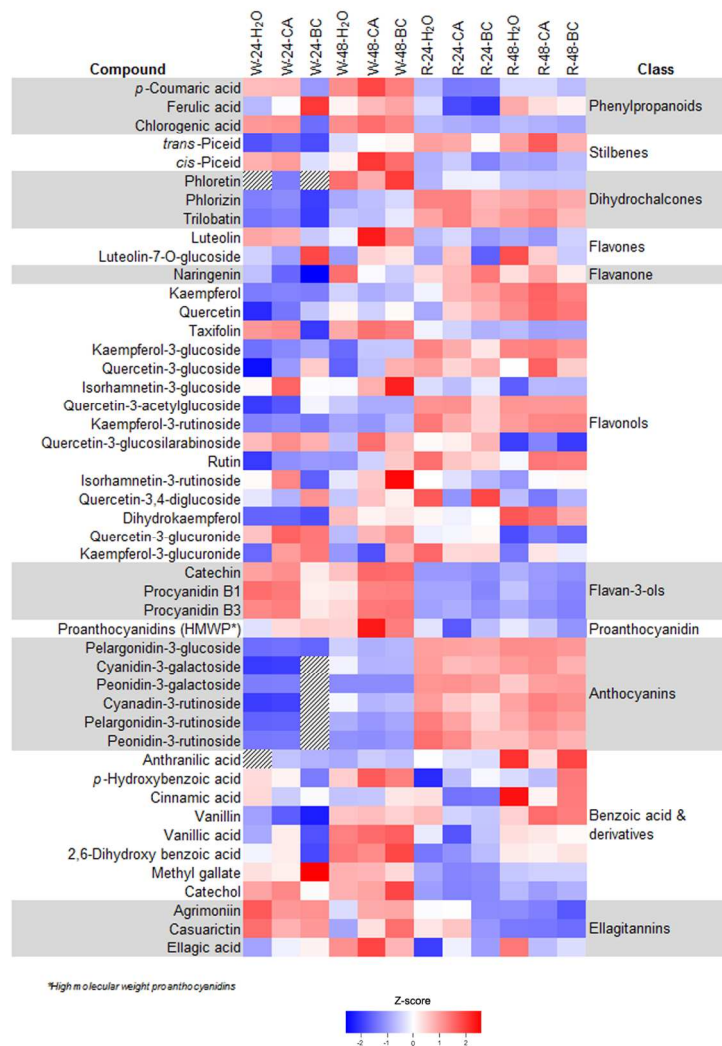
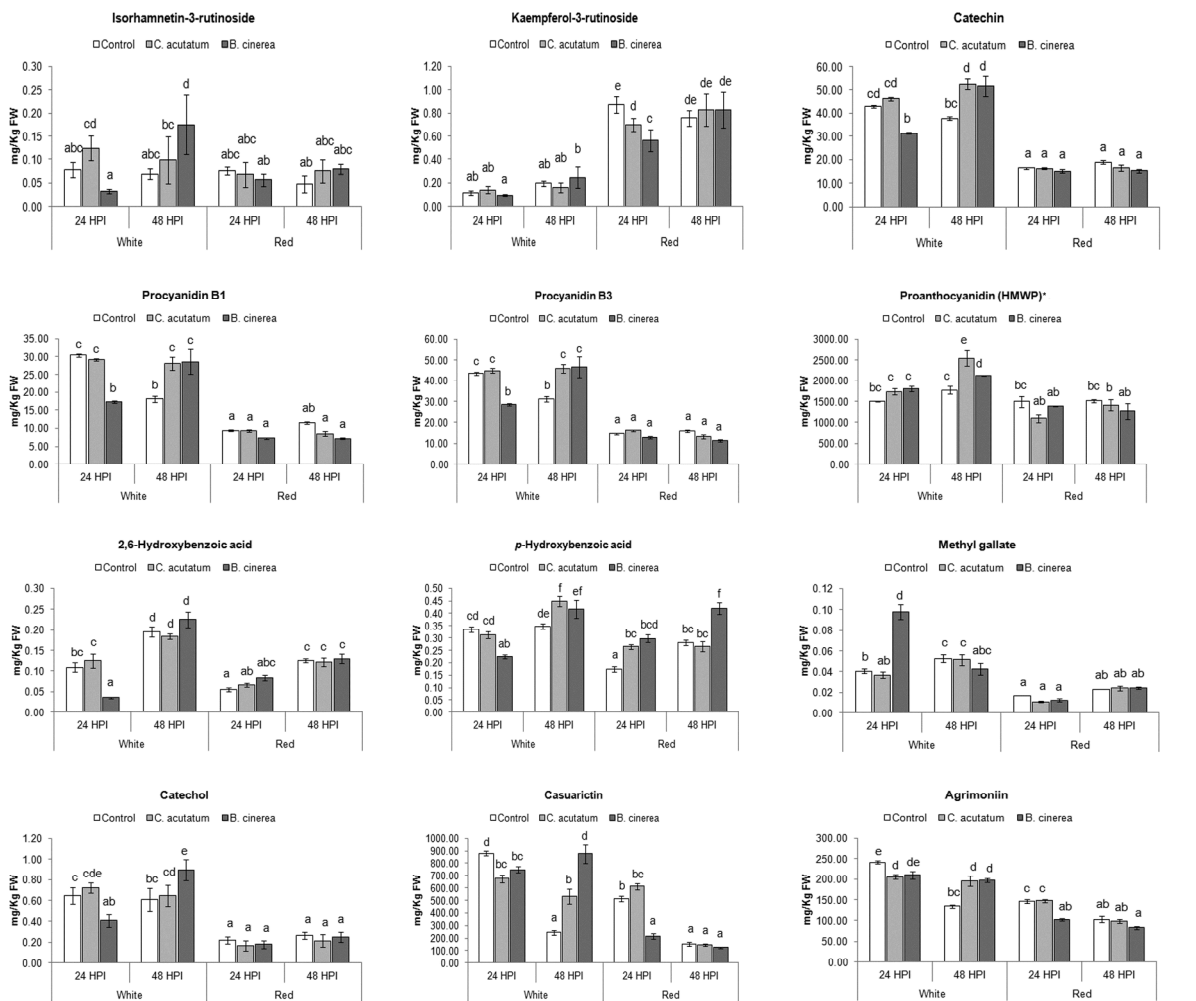


Figure 3.



*High molecular weight proanthocyanidins

Figure 4.

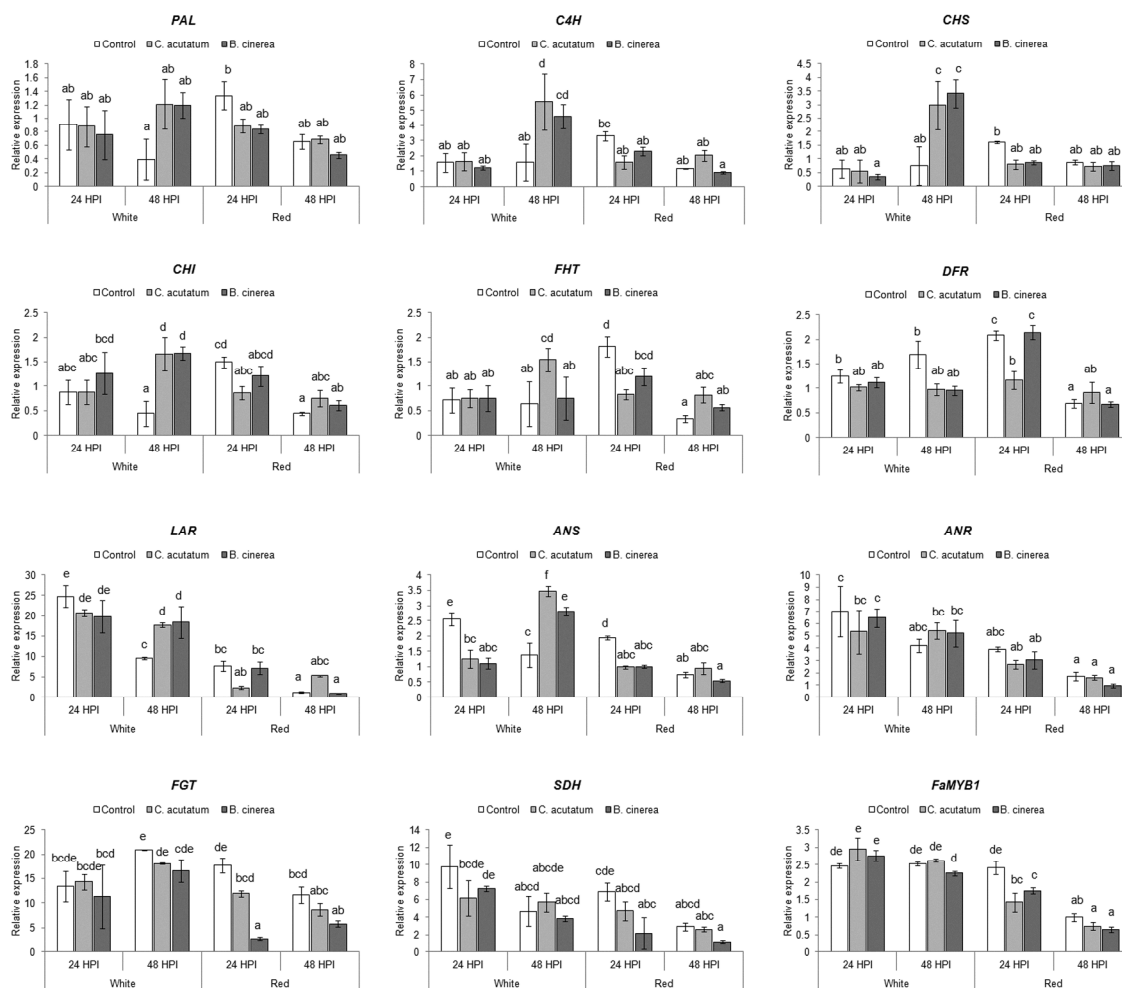
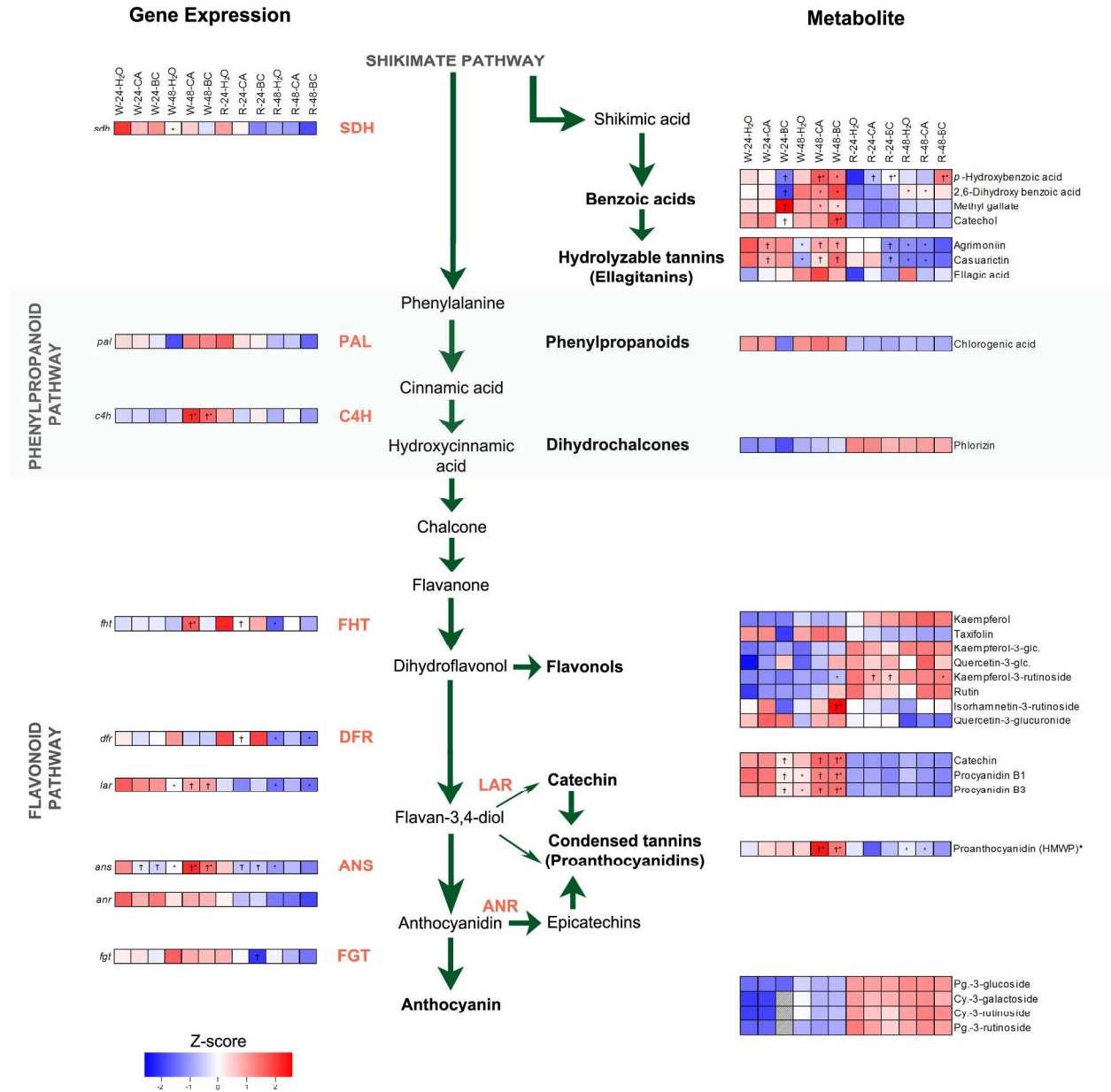


Figure 5.

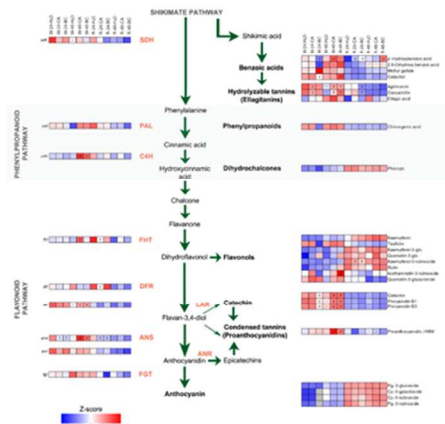
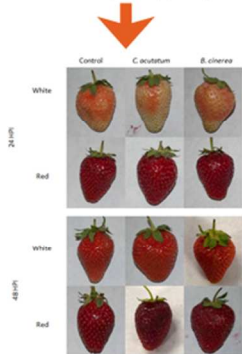


*High molecular weight proanthocyanidins

GRAPHIC FOR TABLE OF CONTENTS



White and red pathogen inoculated strawberry fruits were tested for their metabolomic response to pathogen attack



Metabolomic and gene expression profile of white and red strawberry fruits