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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 fruit[4], 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   diseases[5], 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 symptoms[11]. 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 fruits[12].

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 rots[13]. 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 susceptibility[14]. 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 colonization[15].

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 *Colletorichum gloesporoides* in avocado has been reported[16]. In addition,

52   a recent study[17] 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 10^5 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 LN_2. These time points were chosen

92   based on previous histological and microarray analysis of the *C. acutatum* infection on

93   strawberry fruits[12].

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 spectrometer[19]. 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 × 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 x 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-Lim[21], 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 isoforms[22]. The expression of target

133   genes were normalized with 1α (ef-1α) 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 µM of each primer, 3.25 µl of nuclease-free water,

136   and 2.5 µl of 1:12.5 dilution of cDNA, in a total volume of 12.5 µl. 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   method[23].   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 reports[14]. 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. acucatum* 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 biosynthesis[24] (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

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 species[11]. 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 fruits[12], whereas

310   for *B. cinerea* infection can occur at flowering stage but symptoms develop only at red fruit

311   stages[25]. 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 compounds[26].

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 fruits[11], 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 stages[28]. This hypothesis was later supported by several studies

328 addressing both the phenolic compounds antimicrobial properties[29] 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 ripening[32] and their role in plant defence[33], 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 ones[17]. 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 tissues[22]. 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 method[41],

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

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; H_2O, 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; H_2O, 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 1α* 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; 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.

*High molecular weight proanthocyanidins
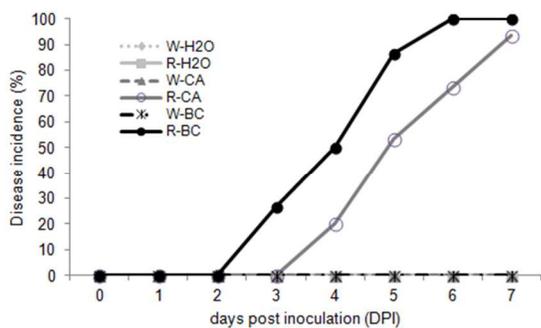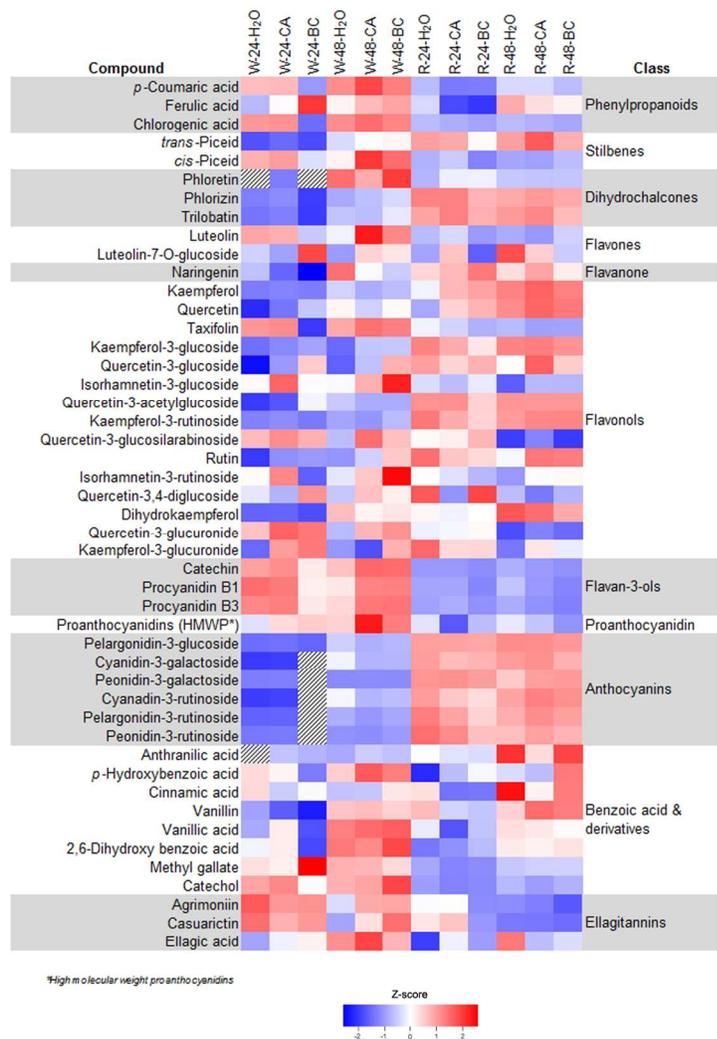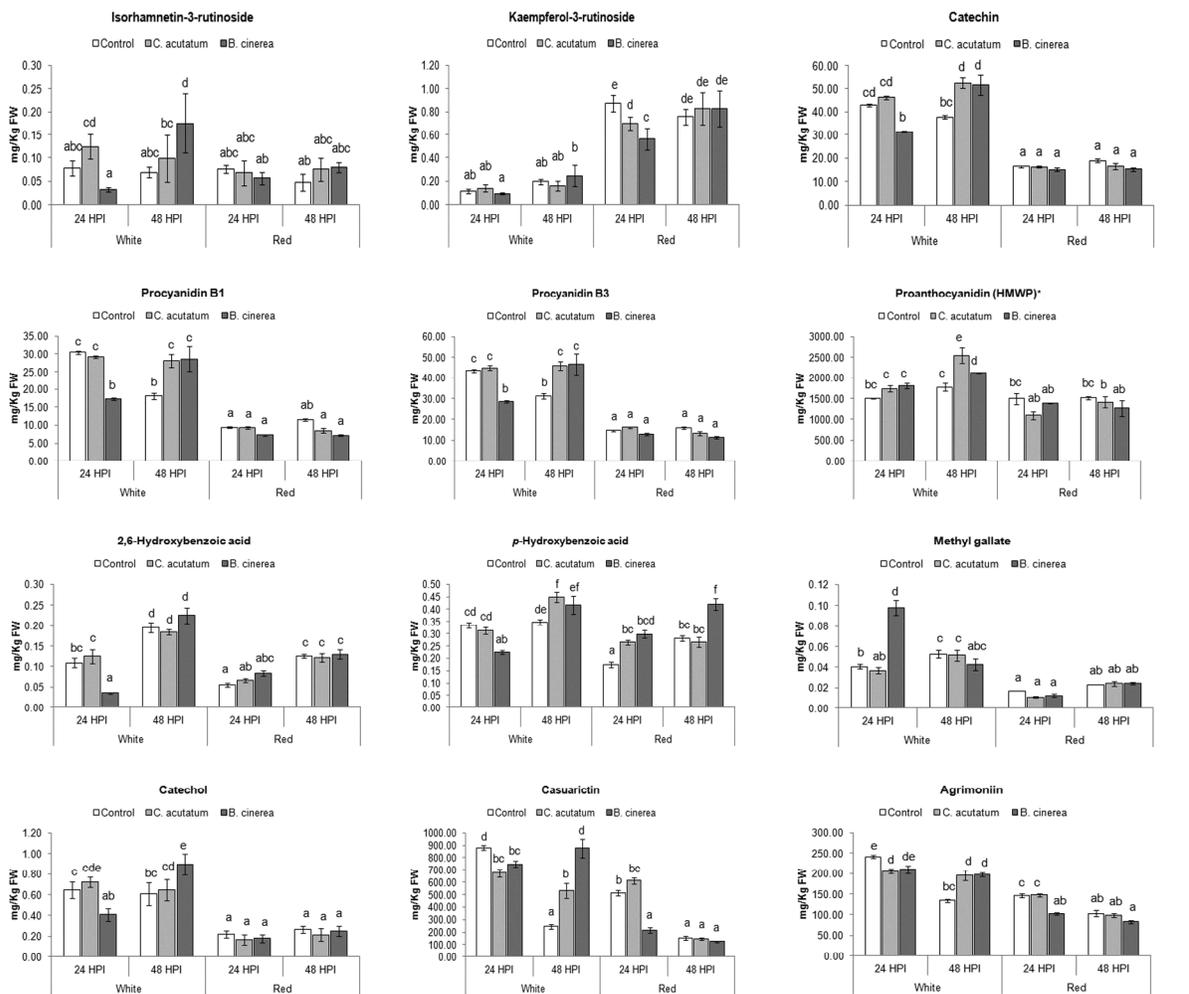
Figure 3.

*High molecular weight proanthocyanidins

Figure 4.
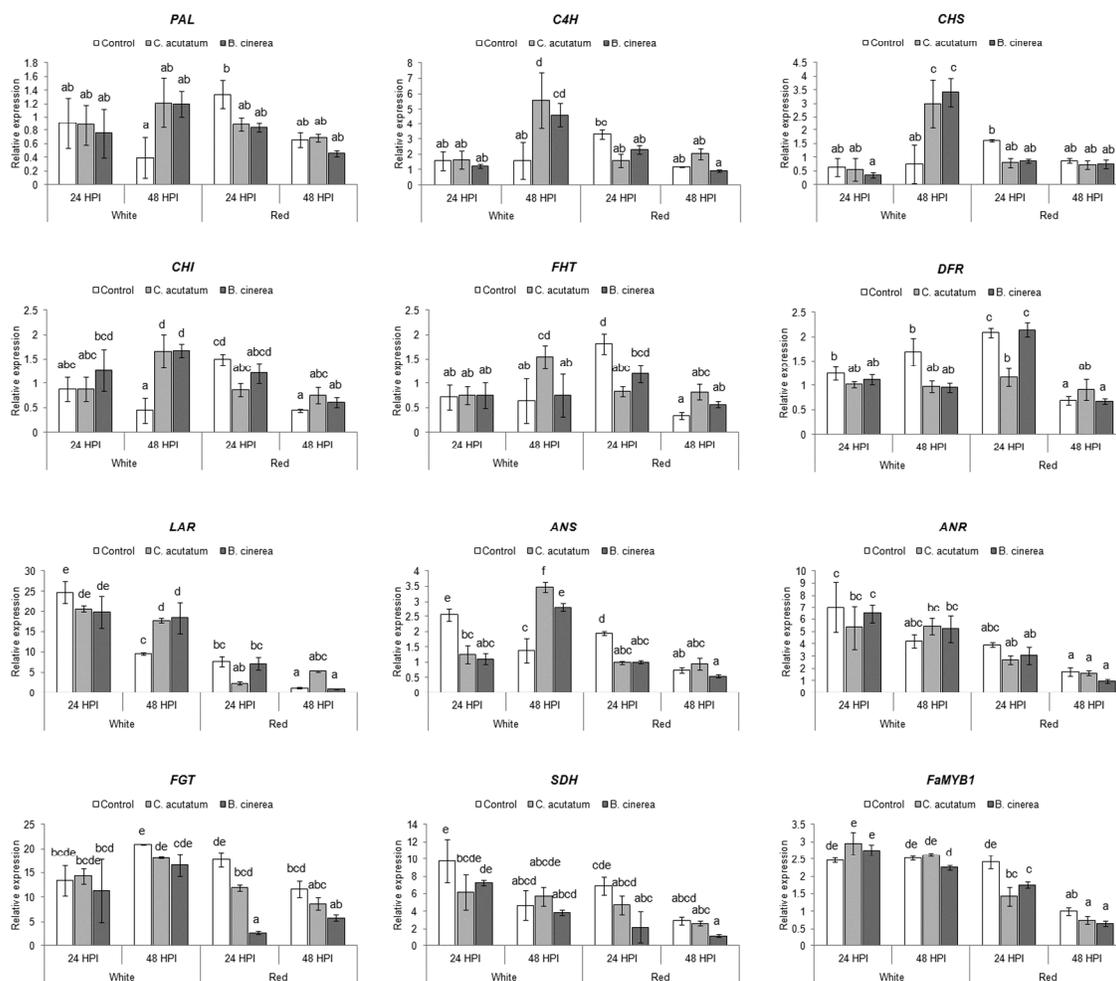
Figure 5.
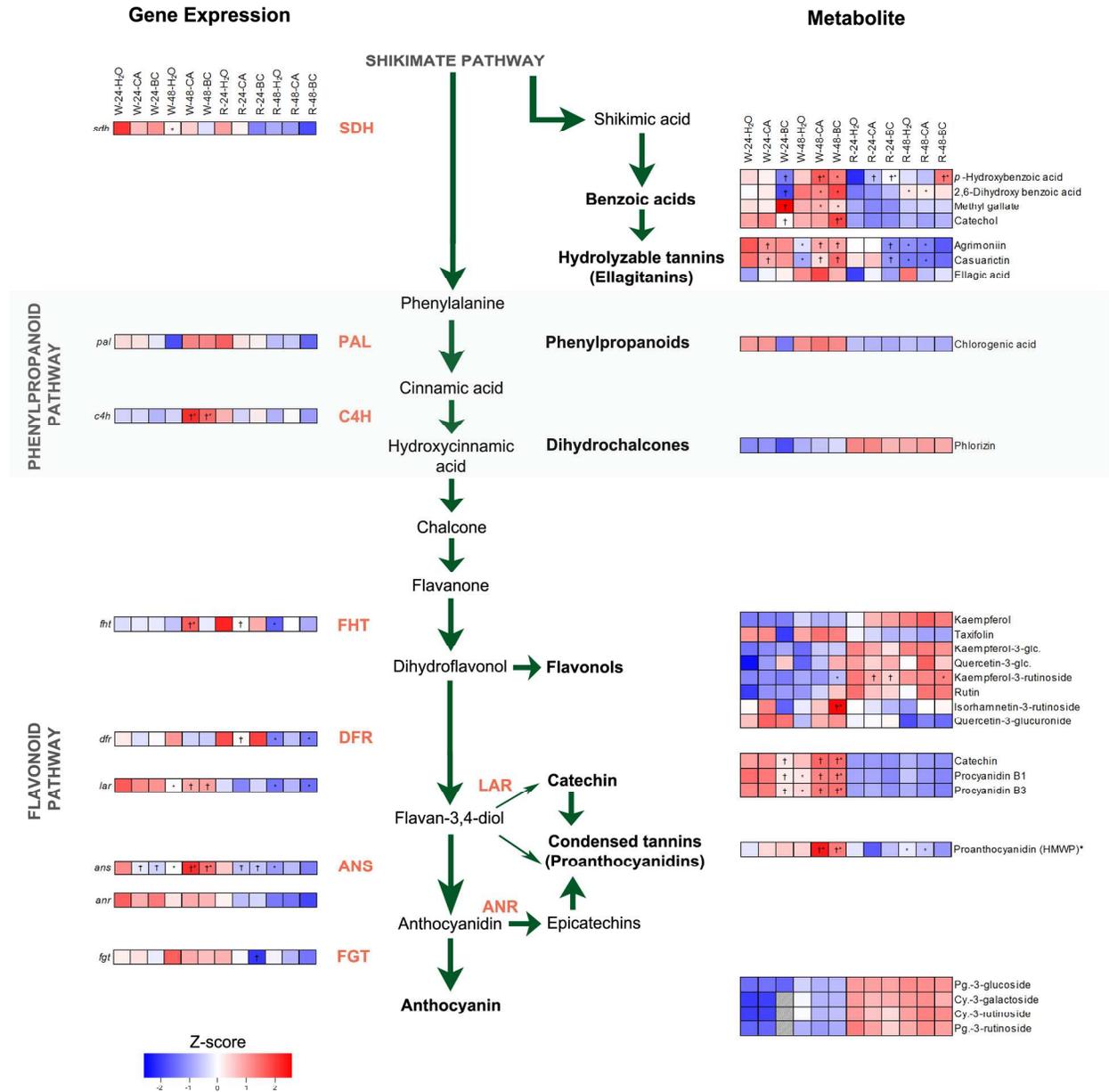
*High molecular weight proanthocyanidins

GRAPHIC FOR TABLE OF CONTENTS

White and red pathogen inoculated strawberry fruits were tested for their metabolimic response tp pathogen attack
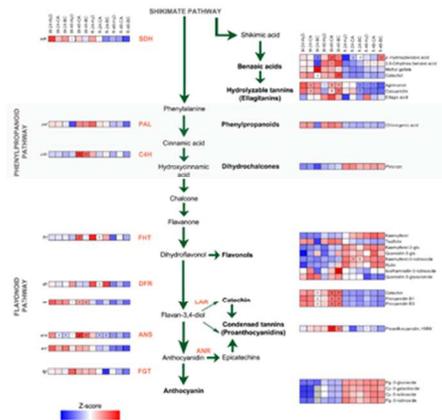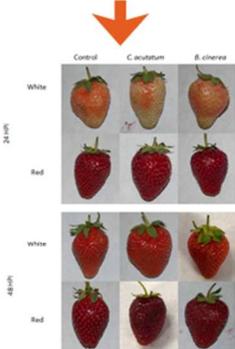
Metabolomic and gene expression profile of white and red strawberry fruits