

1 Title: **Metabolomics and biochemical approaches link salicylic acid biosynthesis to**
2 **cyanogenesis in peach plants**

3 Running title: **Salicylic acid biosynthesis link to cyanogenesis**

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20 Date of submission: June 8, 2017

21 Number of tables: 3

22 Number of figures: 8 (1 colour)

23 Word count: 4256

24 Supplementary data: 1 figure

30 **Highlight**

31 Mandelonitrile, and hence cyanogenic glycosides turnover, is involved in salicylic acid
32 (SA) biosynthesis in peach plants under control and stress conditions. A third pathway for
33 SA synthesis in peach is proposed.

34

35 **Abstract**

36 Despite the long-established importance of salicylic acid (SA) in plant stress responses
37 and other biological processes, its biosynthetic pathway has not been fully characterized.
38 The proposed SA synthesis originates from chorismate by two distinct pathways:
39 isochorismate and penhylalanine (Phe) ammonia-lyase (PAL) pathways. Cyanogenesis is
40 the process related to the release of hydrogen cyanide from endogenous cyanogenic
41 glycosides (CNglcs), and it has been linked to plant plasticity improvement. To date,
42 however, no relationship has been suggested between both pathways. In this work, by
43 metabolomics and biochemical approaches (including [¹³C]-labelled compounds), we
44 provide evidences showing that CNglcs turnover is involved, at least in part, in SA
45 biosynthesis in peach plants under control and stress conditions.

46 The main CNglcs in peach are prunasin and amygdalin, with mandelonitrile (MD),
47 synthesized from Phe, controlling their turnover. In peach plants MD is at the hub of the
48 suggested new SA biosynthetic pathway and CNglcs turnover, regulating both the
49 amygdalin and SA biosynthesis. MD-treated peach plants displayed increased SA levels via
50 benzoic acid (SA precursor). In addition, MD also provides partial protection against *Plum*
51 *pox virus* infection in peach seedlings. Thus, we proposed a third pathway, alternative to
52 the PAL pathway, for SA synthesis in peach plants.

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54 **Keywords:** cyanogenesis; mandelonitrile; metabolomics; peach; phenylalanine; *Plum pox*
55 *virus*; salicylic acid; salt stress

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58 **Introduction**

59 The plant hormone salicylic acid (SA) is the focus of intensive research due to its
60 function as an endogenous signal mediating plant defense responses against both biotic and
61 abiotic stimuli. In addition to its well-known role as a key signaling and regulatory
62 molecule in plant defense responses, SA also plays crucial roles in diverse biological
63 processes such as cell growth and development, seed germination, stomatal aperture, and
64 fruit yield, among others (Liu *et al.*, 2015; Rivas-San Vicente and Plasencia, 2011). Most of
65 the currently available information about the SA biosynthesis pathway comes from works
66 on *Arabidopsis* and other herbaceous plants (Chen *et al.*, 2009; Dempsey *et al.*, 2011). The
67 proposed SA synthesis originates from chorismate, the end product of the shikimate
68 pathway, by two distinct pathways: the isochorismate (IC) and the phenylalanine (Phe)
69 ammonia-lyase (PAL) pathways (Dempsey *et al.*, 2011). The PAL pathway uses Phe as
70 substrate, but its contribution to the total SA pool is minimal (ca. 5% of the total SA
71 synthesis). The IC pathway, on the other hand, accounts for the bulk of SA synthesis (ca.
72 95% of the total SA synthesis) (Chen *et al.*, 2009). Nevertheless, the biosynthetic pathway
73 of SA in plants has not been fully characterized yet (Dempsey *et al.*, 2011), and knowledge
74 regarding this topic is even scarcer in woody plants such as fruit trees.

75 Cyanogenic glycosides (CNgls) are specialized plant compounds (derived from
76 amino acids) that release toxic hydrogen cyanide (HCN) and ketones when hydrolyzed by
77 β -glycosidases and α -hydroxynitrilases in a process referred to as cyanogenesis (Gleadow
78 and Møller, 2014). The main cyanogenic glucosides in *Prunus* species are prunasin and
79 amygdalin, with mandelonitrile (MD) at the hub of its turnover (Sánchez-Pérez *et al.*,
80 2008). Whereas CNgls have traditionally been associated with protection against
81 herbivore and fungal attack, their role in other biological processes such as germination and
82 bud burst has been suggested (Gleadow and Møller, 2014). Nevertheless, endogenous
83 CNgls turnover may be highly species-dependent, and new functions for these molecules
84 remain to be elucidated. For example, secondary metabolites turnover could act dissipating
85 excess energy and providing reducing power in stress conditions (Neilson *et al.*, 2013;
86 Selmar and Kleinwachter, 2013). Moreover, CNgls may also be able to quench reactive
87 oxygen species such as H_2O_2 , suggesting a possible role for these glycosides during
88 unfavorable environmental conditions (Gleadow and Møller, 2014).

89 In peach plants, MD synthesized from Phe via cytochrome P450 enzymes (CYP79
90 and CYP71) is converted into benzaldehyde and HCN by mandelonitrile lyase (MDL)
91 activity, and benzaldehyde can be easily oxidized to produce benzoic acid (BA). In
92 addition, benzaldehyde and benzoic acid appear as intermediate precursors of SA
93 biosynthesis via the PAL pathway (Dempsey *et al.*, 2011; Ribnicky *et al.*, 1998). This fact
94 led us to consider a relationship between cyanogenesis and SA biosynthesis (Fig. 1).
95 Moreover, both SA and HCN are involved in thermogenesis events by the induction of the
96 alternative oxidase pathway, either directly (SA) or through the inhibition of cytochrome c
97 oxidase (HCN) (Taiz and Zeiger, 2010). In the present work, by feeding GF305 peach
98 (*Prunus persica* L.) micropropagated shoots and seedlings with MD and Phe, we have
99 accumulated strong evidence suggesting that MD could be metabolized into SA, linking the
100 SA biosynthetic and cyanogenic glucoside pathways in peach plants. Here we show that
101 MD could act as a hub controlling CNgles turnover and, at least in part, SA biosynthesis. It
102 therefore seems that a third pathway for SA synthesis is present in peach plants, being this
103 pathway functional under both control and stress conditions. This pathway is an alternative
104 to the PAL pathway for SA biosynthesis from Phe, and it is initiated by cytochrome P450
105 enzymes, similar to the indole-3-acetaldoxime pathway for auxin biosynthesis from
106 tryptophan (Mano and Nemoto, 2012).

107 Furthermore, MD levels could be involved in defense responses by increasing the
108 levels of SA and/or its interaction with oxidative signaling defense-induced pathways. To
109 assess this hypothesis we have also analyzed the levels of enzymatic and non-enzymatic
110 antioxidants in MD- and Phe-treated peach plants in addition to the expression of two genes
111 involved in redox signaling and the phenotypic scoring system used for evaluating
112 resistance and susceptibility to *Plum pox virus* (PPV) infection (Decroocq *et al.*, 2005).

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117 **Material and Methods**

118 ***Plant material***

119 The assays were performed on GF305 peach (*Prunus persica* L.) plants, both under
120 greenhouse and *in vitro* conditions. For *ex vitro* assays, after submitting the GF305 peach
121 seedlings to an artificial rest period in a cold chamber to ensure uniformity and fast growth,
122 seedlings were grown in 2 L pots in an insect-proof greenhouse and distributed to 3 batches
123 (control and MD- and Phe-treated) of 15 plants each. Two different experiments were
124 carried out in 2015 and 2016. Due to the fact that in the soil a small proportion of MD
125 could be dissociate non-enzymatically during the course of the experiment, plants were
126 irrigated twice per week with water (control) and 1 mM MD or 1 mM Phe for 7 weeks.

127 For *in vitro* assays [¹³C]-labelled compounds were used, and 200 µM MD- or Phe-
128 alpha[¹³C] (Campro Scientific GmbH, Germany) were added to the micropropagation
129 media during two sub-cultures. The micropropagated GF305 peach shoots were sub-
130 cultured at 4-week intervals for micropropagation (Clemente-Moreno *et al.*, 2011).

131 ***Metabolomics analysis***

132 The levels of Phe, MD, amygdalin, benzoic acid and SA were determined in *in vitro*
133 micropropagated shoots at the Metabolomics Platform at CEBAS-CSIC (Murcia, Spain).
134 Leaf samples from micropropagated shoots were extracted in 50% methanol, filtered in
135 PTFE 0.45 µm filters (Agilent Technologies) and analyzed using an Agilent 1290 Infinity
136 UPLC system coupled to a 6550 Accurate-Mass quadrupole TOF mass spectrometer
137 (Agilent Technologies). Standard curves for each compound were performed, and data
138 were processed using Mass Hunter Qualitative Analysis software (version B.06.00 Agilent
139 Technologies). Hormone levels in the leaves of MD-treated GF305 seedlings were
140 determined using a UHPLC-mass spectrometer (Q-Exactive, ThermoFisher Scientific) at
141 the Plant Hormone Quantification Platform at IBMCP (Valencia, Spain).

142 ***Extraction and enzymatic assays***

143 *In vitro* shoots and *ex vitro* leaf samples were homogenized with an extraction
144 medium (1:3, w/v) containing 50 mM Tris-acetate buffer (pH 6), 0.1 mM EDTA, 2 mM
145 cysteine, 0.2% (v/v) Triton X-100, 2% (w/v) polyvinylpyrrolidone (PVPP) and 1%

146 (w/v) polyvinyl-pyrrolidone (PVP). To determine APX, 20 mM ASC was added to the
147 extraction medium. The extracts were filtered through two layers of nylon cloth and
148 centrifuged at 13000 rpm for 10 min. The supernatant fraction was desalted on Sephadex
149 G-25 NAP columns equilibrated with the same buffer used for homogenization. For APX
150 activity, 2 mM sodium ascorbate was added to the equilibration buffer. The activities of the
151 ASC–GSH cycle enzymes, POX, CAT and SOD, were assayed as previously described
152 (Diaz-Vivancos *et al.*, 2008; Diaz-Vivancos *et al.*, 2013; Diaz-Vivancos *et al.*, 2006). MDL
153 activity was assayed by monitoring the increase of absorbance at 280 nm due to the
154 benzaldehyde released by the enzymatic hydrolysis of DL-mandelonitrile (Ueatrongchit *et*
155 *al.*, 2008; Willeman *et al.*, 2000). The enzyme solution was added to a 50 mM Tris-acetate
156 buffer pH 5 containing 0.1 mM mandelonitrile in a total volume of 1 ml. Protein
157 determination was performed according to the method of Bradford (Bradford, 1976).

158 ***Ascorbate and Glutathione analysis***

159 Leaf samples were snap-frozen in liquid nitrogen and stored at -80°C until use. The
160 frozen samples were homogenized in 1 ml 1 M HClO₄. Homogenates were centrifuged at
161 12 000 g for 10 min, and the supernatant was neutralized with 5 M K₂CO₃ to pH 5.5-6.
162 The homogenate was centrifuged at 12 000 g for 1 min to remove KClO₄. The supernatant
163 obtained was used for ascorbate and glutathione determination (Pellny *et al.*, 2009;
164 Vivancos *et al.*, 2010).

165 ***Gene expression***

166 RNA samples were extracted using a GF1-Total RNA Extraction Kit (Vivantis)
167 according to the manufacturer's instructions. The expression levels of *MDL*, *NPR1*, *TrxH*
168 and the reference gene *translation elongation factor II (TEF2)* (Tong *et al.*, 2009) were
169 determined by real-time RT-PCR using the GeneAmp 7500 sequence detection system
170 (Applied Biosystems, Foster City, CA, USA) (Faize *et al.*, 2013). The accessions and
171 primer sequences are as follows: *MDL1* (Y08211.1; forward 5'-gtttcgttgcaaagagggg-3';
172 reverse 5'-gctttagggagtcatttccttgc-3'); *NPR1* (DQ149935; forward 5'-tgcacgagctccttagtca-
173 3'; reverse 5'-cggttactgcgatcctaag-3'); *TrxH* (AF323593.1; forward 5'-
174 tggcggagttggctaagaag-3'; 5'-ttcttggcaccacaacctt-3'); and *TEF2* (TC3544; forward 5'-
175 ggtgtgacgatgaagatgatg-3'; reverse 5'-gaaggagaggggaaggtgaaag-3'). Relative quantification

176 of gene expression was calculated by the Delta-Delta Ct method, and the expressions of the
177 genes of interest were normalized with the endogenous control *TEF2*.

178 ***Statistical analysis***

179 The data were analyzed by one-way or two-way ANOVA using SPSS 22 software.
180 Means were separated with the Duncan's Multiple Range Test ($P < 0.05$). F-values and
181 probabilities associated with the main effects and possible interactions are indicated where
182 appropriate.

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185 **Results**

186 **SA biosynthesis from CNgles**

187 GF305 peach micropropagated shoots were fed with [^{13}C]Phe or with [^{13}C]MD. The
188 addition of these compounds to the culture media had no important effect in the growth of
189 the micropropagated peach shoots (Fig 2A). We determined the percentage of [^{13}C]-
190 labelled compounds from the total content of Phe, MD, BA and SA in the micropropagated
191 peach shoots treated with either [^{13}C]Phe or with [^{13}C]MD (Fig. 3). Due to the high
192 sensitivity of the UPLC-Quadrupole-TOF-MS system used for metabolomics analysis, we
193 detected basal levels (less than 10%) of [^{13}C]Phe, [^{13}C]MD and [^{13}C]SA in control
194 micropropagated shoots (Fig. 3). It is important to note that we only found [^{13}C]BA (one
195 SA precursor) in non-stressed [^{13}C]MD-fed micropropagated shoots. Of the total BA
196 detected in [^{13}C]MD-treated micropropagated shoots, nearly 40% was [^{13}C]-labelled. In the
197 presence of [^{13}C]MD, nearly 20% of the total SA quantified appeared as [^{13}C]SA.
198 Regarding the [^{13}C]Phe treatment, only a slight increase in the percentage of [^{13}C]MD was
199 observed in non-stressed *in vitro* peach shoots (Fig. 3).

200 After treatment, [^{13}C]Phe-fed micropropagated shoots showed a significant increase
201 in amygdalin (61%) and a non-significant increase in BA (Fig. 4). The [^{13}C]MD-fed
202 micropropagated shoots displayed a similar increase in amygdalin to that produced by the
203 [^{13}C]Phe treatment, indicating that the CNgles pathway is fully functional under our

204 experimental conditions. Interestingly, however, significant increases in BA and SA (of
205 about 80%) were only observed after the [^{13}C]MD treatment (Fig. 4).

206 Given the effect of MD treatments on SA levels in micropropagated peach shoots,
207 we also fed peach seedlings grown under normal physiological conditions in a greenhouse
208 with either MD or Phe. As observed in micropropagated peach shoots, irrigation with MD
209 or Phe had no significant effects on the growth of peach seedlings, which showed normal
210 growth in both the shoots and roots (Fig 2B). In this experiment, we analyzed the effect of
211 the 1 mM MD treatment on the hormone profile of leaf samples. Again, the MD treatment
212 produced an increase in SA levels (about 88%), similar to that noticed in *in vitro*
213 micropropagated peach shoots (Table 1). Furthermore, due to the well-known cross-talk
214 among plant hormones, MD affected also the levels of other hormones. The amount of
215 ABA, another stress-related plant hormone, increased up to 45%. In addition, the 1 mM
216 MD treatment also produced a significant increase in both the gibberellin GA1 and the
217 cytokinin dihydrozeatine (DHZ) in the same range (nearly 60%) (Table 1).

218 **SA biosynthesis and plant performance under stress conditions**

219 The level of SA was also determined in micropropagated peach shoots submitted to
220 both abiotic and biotic stresses. Abiotic stress was achieved adding 30 mM NaCl to the
221 culture media whereas *Plum pox virus* (PPV)-infected *in vitro* shoots (Clemente-Moreno *et*
222 *al.*, 2011) were used to assess the biotic stress condition. PPV is the causal agent of sharka
223 disease and the most destructive and detrimental disease affecting *Prunus* species
224 (Clemente-Moreno *et al.*, 2015). Regarding SA biosynthesis, under control conditions a
225 similar strong increase in the total content of SA was observed in NaCl- and PPV-stressed
226 micropropagated peach shoots (Fig. 5). Contrary to what observed in non-stressed peach
227 shoots, under both stress conditions [^{13}C]Phe increased SA, whereas [^{13}C]MD treatment did
228 not increase the total SA content (Fig. 5). Nevertheless, stressed [^{13}C]MD-fed
229 micropropagated shoots displayed increases in the percentages of [^{13}C]MD (45% and 148%
230 by NaCl and PPV infection respectively) and [^{13}C]SA (75% and 45% by NaCl and PPV
231 infection respectively) (Fig. 3).

232 In addition, according to salinity damage observed, MD-treatment had not a
233 significant effect on the *in vitro* shoots performance, whereas Phe seems to increase the salt

234 stress deleterious effect, as observed by the increase of leaves showing salinity injure per
235 micropropagated shoot (Fig. S1). On the other hand, it is well known that SA is a key
236 signaling molecule involved in systemic acquired resistance (SAR) and that it plays an
237 important role in plant defense against pathogens, including plant viruses (Vlot *et al.* 2009).
238 Micropropagated peach shoots, in spite of high virus content, did not show any PPV
239 infection symptoms (Clemente-Moreno *et al.*, 2011). Thus, we analyzed the effect of both
240 MD and Phe treatments (using non-labelled compounds) on PPV-infected peach seedlings
241 under greenhouse conditions. The presence of sharka symptoms in peach leaves was scored
242 for each plant according to a scale of 0 (no symptoms) to 5 (maximum symptom intensity),
243 a common test used in the evaluation of resistance to sharka (Decroocq *et al.* 2005; Rubio
244 *et al.* 2005). According to the mean intensity of symptoms in peach leaves, we observed
245 that MD-treated seedlings showed a significant decrease in PPV-induced symptoms (Fig.
246 6). This effect correlated with the increased SA levels found in MD-treated seedlings
247 (Table 1). In contrast, although the Phe treatments also reduced sharka symptoms, no
248 significant differences were observed compared with infected control seedlings (Fig. 6).

249 **Effects on antioxidative metabolism: the inhibition of H₂O₂-scavenging** 250 **enzymes in MD-treated seedlings**

251 The effects of both the MD and Phe treatments on the antioxidative metabolism of
252 *in vitro* peach GF305 micropropagated shoots were analyzed. ANOVA analysis showed
253 that the treatments had a significant effect on all the analyzed antioxidant enzymes except
254 for peroxidase (POX) and superoxide dismutase (SOD). Micropropagated peach shoots
255 treated with Phe displayed more significant increases in the enzymatic activities measured
256 than control plants. MD treatments, on the other hand, significantly increased
257 monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR),
258 catalase (CAT) and SOD activities (Table 2). It is interesting to remark that Phe-treated
259 plants seem to have more active ascorbate-glutathione (ASC-GSH) cycle enzymes than
260 MD-treated micropropagated shoots.

261 A different pattern was produced in GF305 seedlings. In this experiment, ANOVA
262 analysis indicated that the treatments had a significant effect on ascorbate peroxidase
263 (APX), glutathione reductase (GR), POX, CAT and SOD, but no significant effects were

264 observed in the ascorbic acid-recycling enzymes (MDHAR and DHAR) in any of the
265 treatments (Table 2). The MD treatment produced a significant decrease in APX, POX and
266 CAT activities (H₂O₂-detoxifying enzymes), which could be associated with the observed
267 increase in SA (Durner and Klessig, 1995; Rao *et al.*, 1997). The Phe treatment, on the
268 other hand, increased GR and SOD activity and decreased CAT activity (Table 2).

269 We also studied the effect of MD and Phe on the ascorbate and glutathione content
270 in leaf samples from peach seedlings. No oxidized ascorbate (DHA) was detected under our
271 experimental conditions, and only the reduced form (ASC) was measured (Table 3). We
272 observed that the treatments had a significant effect on ASC and oxidized glutathione
273 (GSSG) concentrations as well as on the redox state of glutathione. Both treatments,
274 particularly MD, decreased the ASC levels. In the case of MD, the decrease in ASC was
275 about 50%, whereas Phe decreased ASC levels by about 30%. The effect of Phe or MD on
276 reduced glutathione (GSH) was less pronounced: a 20% decrease was observed in both
277 treatments (Table 3). In parallel, a significant accumulation of the oxidized form of
278 glutathione (GSSG) was produced, mainly in Phe-treated plants, leading to a decrease in
279 the redox state of glutathione in both MD- and Phe-treated plants (Table 3).

280 **MDL1 activity and gene expression**

281 Once we determined that the MD treatment increased MD, BA and SA in
282 micropropagated peach shoots and SA levels in peach seedlings, we wanted to check if the
283 MD could stimulate and/or up-regulate the mandelonitrile lyase (MDL) activity and/or the
284 *MDLI* gene expression. MDL activity catalyzed the breakdown of MD into benzaldehyde
285 plus cyanide (Swain and Poulton, 1994b). Benzaldehyde could then be oxidized by
286 aldehyde oxidase into BA, an SA precursor. In micropropagated peach shoots, both the MD
287 and Phe treatments significantly increased MDL activity (Fig. 4). Regarding *MDLI* gene
288 expression, although a slight increase in gene expression was observed in MD- and Phe-
289 treated micropropagated shoots, differences were not statistically significant (data not
290 shown).

291 In peach seedlings, control and MD-treated plants had higher MDL activity levels
292 than Phe-treated plants. Moreover, MD-treated peach seedlings showed a slight increase in
293 MDL activity when compared with control plants, although differences were not

294 statistically significant (Fig. 7A). Nevertheless, MD-treated seedlings displayed a 3-fold
295 increase in *MDLI* gene expression (Fig. 7B).

296 **Gene expression of redox-related genes**

297 Due to the described role of SA in the induction of Non-Expressor of Pathogenesis-
298 Related Gene 1 (*NPR1*) expression and the role of thioredoxins (Trx) in the SA-induced
299 *NPR1* conformational changes (Dong, 2004; Tada *et al.*, 2008; Vieira Dos Santos and Rey,
300 2006), we also analyzed the effect of both treatments (MD and Phe) on the *NPR1* and *TrxH*
301 expression levels in peach seedlings. Whereas no significant changes in *NPR1* expression
302 were observed with either treatment (Fig 7C), *TrxH* expression was significantly induced
303 by Phe and MD treatments, with increases of about 42% and 23%, respectively (Fig 7C).

304

305

306 **Discussion**

307 In this work, we have accumulated strong evidence suggesting that mandelonitrile
308 could be metabolized into SA, linking the SA biosynthetic and CNgls pathways in peach.
309 Among this evidence, we observed increased levels of benzoic acid (BA) and SA as well as
310 enhanced MDL activity and *MDLI* gene expression in MD-treated peach plants. Our results
311 suggest that part of the total SA content in peach plants could be due to mandelonitrile, and
312 hence CNgls turnover. This possibility has not been described before in higher plants.
313 Mandelonitrile seems to act as a hub in this pathway controlling both the amygdalin and SA
314 biosynthesis. Similarly, in other plant species, the biosynthesis of the plant hormone auxin
315 from tryptophan via cytochrome P450 enzymes has been also described (Mano and
316 Nemoto, 2012).

317 The experiments with [¹³C]-labelled MD or Phe carried out using *in vitro*
318 micropropagated shoots revealed significant incorporation of ¹³C as [¹³C]SA and [¹³C]BA,
319 one of the SA precursors, in [¹³C]MD-treated micropropagated shoots. In fact, almost 40%
320 of total BA and 20% of total SA appeared as ¹³C-labelled compounds. In addition, and as
321 expected, an important increase in amygdalin content was observed in both treatments.
322 However, a possible route from Phe to BA cannot be ruled out because a slight increase in

323 BA was observed in [¹³C]Phe-treated micropropagated shoots, although this possibility
324 does not seem to be important for SA biosynthesis because no parallel increase in [¹³C]BA
325 or in SA was recorded. On the other hand, Phe is a ketogenic and glucogenic aminoacid,
326 and it is a precursor for a wide range of specialized natural compounds (Yoo *et al.*, 2013),
327 including CNgls, in peach plants. Moreover, treatment with [¹³C]Phe increased the
328 percentage of [¹³C]MD observed, highlighting the central role MD plays in controlling both
329 SA biosynthesis and CNgls synthesis and turnover.

330 It is known that during SA biosynthesis, benzaldehyde can be oxidized into BA in a
331 reaction catalyzed by aldehyde oxidase (Dempsey *et al.*, 2011). In addition, the results for
332 MDL activity and *MDLI* gene expression reinforce the possible role of MD in the increase
333 in SA via BA. Accordingly, we observed a high *MDLI* expression level to sustain
334 increased MDL activity when peach seedlings were treated with MD, but not in Phe-treated
335 plants. We do not know the exact cellular location in which these reactions take place. In
336 *Prunus serotina*, MDL was immunocytochemically localized in cell walls and vacuoles
337 (Swain and Poulton, 1994a). However, no studies of the sub-cellular localization of MDL
338 have been conducted in peach leaves. In previous works, due to the low contamination
339 levels obtained, we suggested an apoplastic localization of MDL (Diaz-Vivancos *et al.*,
340 2006), although a vacuolar localization should not be excluded.

341 Using a different analytical method than that used for micropropagated shoots, we
342 were also able to detect an increase in leaf SA content under MD treatment in peach
343 seedlings. Curiously, the SA increase observed was similar in both plant models (88% and
344 83% in peach seedlings and micropropagated shoots, respectively). The MD treatment,
345 besides increasing SA, also enhanced the concentration of ABA, GA1 and DHZ. It is
346 known that cross-talk among different hormonal signals is involved in different
347 physiological responses as well as in response to environmental challenges (Grant and
348 Jones, 2009; Micol-Ponce *et al.*, 2015; Yang *et al.*, 2013). Among plant hormones, ABA
349 and SA, along with JA and ethylene, play a major role in mediating plant defenses against
350 biotic and abiotic factors (Verma *et al.*, 2016; Yang *et al.*, 2013). Moreover, the interaction
351 of SA with other plant hormones regulating certain plant responses has been also reported
352 (Bari and Jones, 2009). For example, the role of cytokinins in modulating SA signaling in
353 biotic and abiotic stress responses is well documented (Choi *et al.*, 2010; Rivero *et al.*,

354 2009). GAs levels and signaling are also involved in plant defense (Zhu *et al.*, 2005), and
355 the interaction of auxins with SA can limit disease through the down-regulation of auxin
356 signaling (Wang *et al.*, 2007). ABA can also promote plant defense responses depending on
357 different factors, such as the pathogen, the development stage of the plant and the target
358 tissue (Yang *et al.*, 2013).

359 NPR1 is a key regulator in the signal transduction pathway that leads to SAR. The
360 induction or overexpression of the NPR1 protein leads to increased induction of *PR* genes
361 and enhanced disease resistance (Dong, 2004). Inactive NPR1 occurs in the cytosol as
362 oligomers, held together by disulfide bridges. SA-induced changes in the redox state can
363 lead to monomerization by the reduction of Cys residues via the action of TrxH (Tada *et*
364 *al.*, 2008) (Fig. 7). NPR1 monomers are translocated to the nucleus, activating defense
365 genes (Vlot *et al.*, 2009). Under our experimental conditions we were not able to detect any
366 *NPR1* induction, although the up-regulation of *TrxH* was noticed, suggesting its role in the
367 activation of NPR1 monomerization and thus in enabling the activation of defense genes.
368 Similarly, in a previous work, we were not able to detect any *NPR1* gene induction in
369 micropropagated peach shoots treated with benzothiadiazole, a SA analogous inducer of
370 SAR (Clemente-Moreno *et al.*, 2012). The interaction of SA with heme-containing
371 proteins, such as the H₂O₂-scavenger enzymes (CAT, APX or POX), can produce redox
372 stress, which can initiate the release of NPR1 monomers and their entry into the nuclei
373 (Durner and Klessig, 1995). Accordingly, we described a decrease in these enzymes in
374 MD-treated peach plants. Moreover, MD also decreased the levels of reduced ascorbic acid
375 and the glutathione redox state, leading to a more oxidized environment (Fig. 8). It has been
376 suggested that SA induces changes to the redox environment by modulating GSH levels
377 and reducing power, stimulating the plant defense responses (Herrera-Vasquez *et al.*, 2015;
378 Vlot *et al.*, 2009; Yang *et al.*, 2004). The known role of SA and ABA in the control of
379 stomatal closure (Khokon *et al.*, 2011) and the MD-induced SA and ABA levels (Table 1)
380 displayed in peach seedlings could led us to speculate that MD-treated peach plants could
381 tolerate situations of water and/or saline stress.

382 We have observed that the MD treatment induced partial protection against PPV
383 infection in peach seedlings. Based on our results, we can suggest that the partial protection

384 from PPV in MD-treated GF305 peach plants could be independent of *NPR1* induction.
385 Indeed, it is known that some SA-induced defense genes do not require *NPR1*, suggesting
386 that other proteins can be important in SA perception (Blanco *et al.*, 2005) (Fig. 8).
387 Moreover, the MD treatment increased DHZ levels, and this cytokinin has been found to
388 induce partial protection against *White clover mosaic virus* with no changes in the
389 expression of SA-responsive genes (Gális *et al.*, 2004). Moreover, in spite of its well-
390 known role on plant defense against pathogens (Alvarez *et al.*, 1998), SA has been
391 increasingly recognized as abiotic stress modulator via SA-mediated regulation of
392 important plant-metabolic processes (Khan *et al.*, 2015). Thus, MD levels and hence the
393 CNgls turnover could be involved in defense responses by increasing the levels of SA
394 and/or the interaction with oxidative signaling defense-induced pathways. On the other
395 hand, in micropropagated peach shoots, although MD treatment did not increase the SA
396 content under abiotic and biotic stress conditions (Fig. 5), data from Fig. 3 show that a
397 small amount of MD is metabolized to SA. Taking together, we suggest that under stress
398 conditions this new SA biosynthetic pathway contributes much less to the total amount of
399 SA than the PAL pathway.

400 We have therefore shown that the addition of a small molecule like MD can reveal
401 very useful information on the mechanisms of interaction of various hormones in
402 coordinating responses to environmental stress in plants. In conclusion, in this work we
403 provide strong evidence for a new SA biosynthetic pathway from MD in peach by using
404 two different plant models and different analytical approaches. Although we acknowledge
405 that additional genetic evidences would provide complementary data, the feasibility of
406 genetic approaches in peach plants is scarce nowadays. The MD molecule seems to act as a
407 hub in this novel pathway controlling amygdalin and SA biosynthesis (Fig. 8). The MD
408 treatment induced the gene expression of *MDL1* to maintain MDL activity. This result
409 reinforces the possible role of MD in the increase of SA via BA, which has been described
410 as an SA precursor (Dempsey *et al.*, 2011; Ribnicky *et al.*, 1998). In addition, there was
411 also a pleiotropic effect on other plant hormones (ABA, GA1, DHZ). MD, and therefore
412 SA, induced *TrxH*, but not *NPR1*. However, the effect of MD (or SA) in the antioxidative
413 machinery can induce redox stress, which can facilitate NPR1 monomerization and
414 therefore its effect on defense gene induction (Durner and Klessig, 1995). This argument is

415 supported by the partial protection against PPV induced by the MD treatment.
416 Alternatively, and based in our results, it is also possible that the partial protection against
417 PPV in MD-treated GF305 peach plants could be independent of *NPRI* induction, as
418 described by other authors (Blanco *et al.*, 2005). Nevertheless, because this new pathway
419 seems not to be relevant under stress conditions, at least under *in vitro* conditions, further
420 investigation will be required in order to elucidate how relevant is this new pathway for
421 plant performance.

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424 **Supplementary data**

425 **Fig. S1.-** Effect of [¹³C]MD or [¹³C]Phe on NaCl-stressed micropropagated peach shoots.

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428 **Acknowledgments**

429 This work was supported by the Spanish Ministry of Economy and Competitiveness
430 (Project AGL2014-52563-R). PDV and CP thank CSIC and UPCT, respectively, as well as
431 the Spanish Ministry of Economy and Competitiveness for their ‘Ramon & Cajal’ research
432 contract, co-financed by FEDER funds. We also acknowledge Prof. Manuel Acosta
433 Echeverría for his very useful commentaries and discussion.

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575 **Tables.**

576 **Table 1.** Effect of MD treatment on hormone levels in leaves of GF305 seedlings grown
577 under greenhouse conditions. Data are expressed as ng g⁻¹ FW except for SA levels (μg g⁻¹
578 FW). Data represent the mean ± SE of at least eight repetitions of each treatment. Different
579 letters indicate significant differences according to Duncan's test (P≤0.05).

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	Control	MD 1 mM
Stress-related hormones		
SA	3.73±0.24 b	7.01±1.16 a
ABA	996.88±14.95 b	1444.79±64.23 a
Gibberellins		
GA4	0.50±0.08	0.41±0.02
GA1	0.27±0.03 b	0.43±0.04 a
Citokinins		
DHZ	1.28±0.21 b	2.02±0.05 a
IP	0.69±0.08	0.64±0.04
Tz	1.31±0.10	1.33±0.08

Table 2. Effect of MD and Phe treatments on APX, MDHAR, DHAR, GR, POX, CAT, and SOD activities in *in vitro* GF305 shoots and in leaves of GF305 seedlings. APX, MDHAR, DHAR, and GR are expressed as nmol min⁻¹ mg⁻¹ protein. POX and CAT are expressed as μmol min⁻¹ mg⁻¹ protein, and SOD as U mg⁻¹ protein. Data represent the mean ± SE of at least five repetitions. Different letters in the same column indicate significant differences according to Duncan's test (P≤0.05). F-values from two-way ANOVA significant at the 99.9% (***), 99% (**), or 95% (*) level of probability (Treatment factor: Tto).

<i>In vitro</i> Treatment	APX	MDHAR	DHAR	GR	POX	CAT	SOD
Control	316.0 ± 48.0 b	866.2 ± 8.9 b	246.0 ± 18.5 c	342.8 ± 7.9 b	1453.8 ± 22.4 b	4.1 ± 0.1 b	36.2 ± 2.5 b
MD	257.0 ± 32.7 b	1539.6 ± 81 a	490.2 ± 29.6 b	387.0 ± 8.7 b	1600.4 ± 231.0 ab	7.2 ± 1.1 a	57.8 ± 8.1 a
Phe	490.9 ± 35.9 a	1538.6 ± 37.4 a	791.3 ± 1.2 a	489.6 ± 19.1 a	1935.9 ± 78.6 a	6.5 ± 0.4 a	56.0 ± 3.4 a
ANOVA				F-values			
Tto	8.8*	56.3***	190.7***	33.6**	3.8	5.7*	4.9

<i>Seedlings</i> Treatment	APX	MDHAR	DHAR	GR	POX	CAT	SOD
Control	344.9 ± 64.6 a	1262.9 ± 115.4 a	472.8 ± 51.6 a	180.4 ± 4.4 b	1291.8 ± 136.9 a	96.1 ± 6.8 a	119.1 ± 17.1 b
MD	198.2 ± 15.7b	1300.7 ± 13.6 a	405.7 ± 31.6 a	196.3 ± 16.0 b	969.1 ± 20.3 b	53.25 ± 2.2 c	107.9 ± 4.9 b
Phe	403.6 ± 22.1a	1106.6 ± 28.3 a	436.2 ± 18.2 a	283.8 ± 12.4 a	1323.7 ± 53.0 a	70.4 ± 1.9 b	198.3 ± 12.9 a
ANOVA				F-values			
Tto	9.2**	2.8	0.9	21.6**	5.8*	25.3**	17.9**

Table 3. Effect of MD and Phe treatments on reduced ascorbic acid (ASC) and glutathione content in the leaves of GF305 peach seedlings. Data represent the mean \pm SE of at least five repetitions. Different letters in the same column indicate significant differences according to Duncan's test ($P \leq 0.05$). F-values from two-way ANOVA significant at the 99.9% (***), 99% (**), or 95% (*) level of probability (Treatment factor: Tto).

Treatment	GLUTATHIONE (nmol/g FW)			
	ASC ($\mu\text{mol/g FW}$)	GSH	GSSG	Redox state
Control	15.2 \pm 1.2 a	223.6 \pm 17.6 a	13.0 \pm 1.2 c	0.94 a
MD	7.0 \pm 0.5 c	176.2 \pm 11.3 b	16.3 \pm 0.8 b	0.91 b
Phe	10.7 \pm 0.8 b	181.1 \pm 9.2 b	26.6 \pm 1.2 a	0.87 c
ANOVA		F-values		
Tto	18.3**	3.8	39.9***	20.3***

Figure legends.

Figure 1. Proposed SA biosynthetic pathway in peach plants. Blue arrows indicate the already described SA biosynthesis in plants (dot arrow, putative; CYP79 and CYP71, Cyt P450 monooxygenases; MDL1, mandelonitrile lyase), whereas red arrows show the new pathway suggested for peach plants.

Figure 2. Micropropagated GF305 peach shoots (A) and seedlings (B) grown under control conditions and in the presence of mandelonitrile (MD) and phenylalanine (Phe). *In vitro* shoots were micropropagated by incorporating 200 μM of either [^{13}C]MD or [^{13}C]Phe into the media, whereas peach seedlings were watered with 1 mM solution of MD or Phe. Neither of the treatments had effects on plant growth and development.

Figure 3. Percentage of [^{13}C]-phenylalanine, mandelonitrile, benzoic acid, and salicylic acid in non-stressed, NaCl-stressed and PPV-infected peach shoots micropropagated in the presence or absence of [^{13}C]MD or [^{13}C]Phe. Under control conditions, basal levels of [^{13}C]-mandelonitrile, phenylalanine and salicylic acid were observed, whereas [^{13}C]benzoic acid was only detected in [13C]MD-treated micropropagated shoots. Ions with an additional 1.0035 accurate mass and confirmation by isotopic distribution and spacing were defined as ions marked with ^{13}C . Data represent the mean of at least 20 repetitions of each treatment.

Figure 4. Total levels ($\mu\text{M g}^{-1}$ FW) of amygdalin, benzoic acid, mandelonitrile, phenylalanine and salicylic acid, and MDL enzymatic activity in micropropagated peach shoots in the presence or absence of [^{13}C]MD or [^{13}C]Phe. Data represent the mean \pm SE of at least 12 repetitions of each treatment. Different letters indicate significant differences in each graph according to Duncan's test ($P \leq 0.05$).

Figure 5. Total level ($\mu\text{M g}^{-1}$ FW) of salicylic acid in peach shoots micropropagated in the presence or absence of [13C]MD or [13C]Phe, submitted to salt stress (30 mM NaCl; A) or PPV infection (B).

Figure 6. Phenotypic scoring for evaluating the resistance/susceptibility to PPV infection (Decroocq *et al.*, 2005) and sharka symptoms in peach plants. Data represent the mean \pm SE of at least 18 repetitions (samples from two independent assays carried out in 2015 and

2016) of each treatment. Different letters indicate significant differences according to Duncan's test ($P \leq 0.05$).

Figure 7. Mandelonitrile lyase enzymatic activity (A), *MDLI* gene expression (B), and gene expression of *TrxH* and *NPR1* (C) in GF305 peach seedlings grown in the presence or absence of MD or Phe. Data represent the mean \pm SE of at least five repetitions of each treatment. Different letters indicate significant differences in each graph according to Duncan's test ($P \leq 0.05$).

Figure 8. Proposed roles of MD in peach plants. MD is involved in CNGlcs turnover and in SA biosynthesis. In addition, MD treatment leads to a more oxidized environment, which could modify the function of proteins such as those involved in the response to environmental stress conditions.

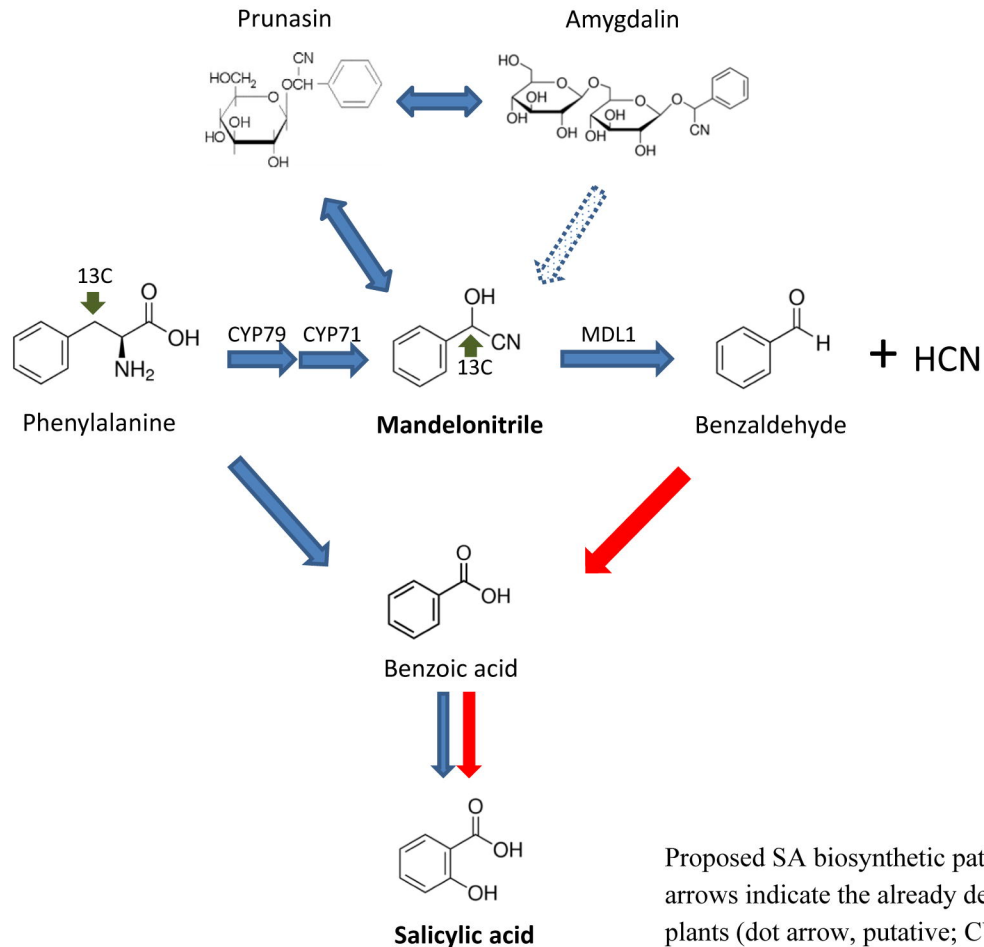


Fig. 1

Proposed SA biosynthetic pathway in peach plants. Blue arrows indicate the already described SA biosynthesis in plants (dot arrow, putative; CYP79 and CYP71, Cyt P450 monooxygenases; MDL1, mandelonitrile lyase), whereas red arrows show the new pathway suggested for peach plants.

A

Control



Phe



MD

B

Fig. 2. Micropropagated GF305 peach shoots (A) and seedlings (B) grown under control conditions and in the presence of mandelonitrile (MD) and phenylalanine (Phe). In vitro shoots were micropropagated by incorporating 200 μ M of either MD or Phe into the media, whereas peach seedlings were watered with 1 mM solution of MD or Phe. Neither of the treatments had effects on plant growth and development.

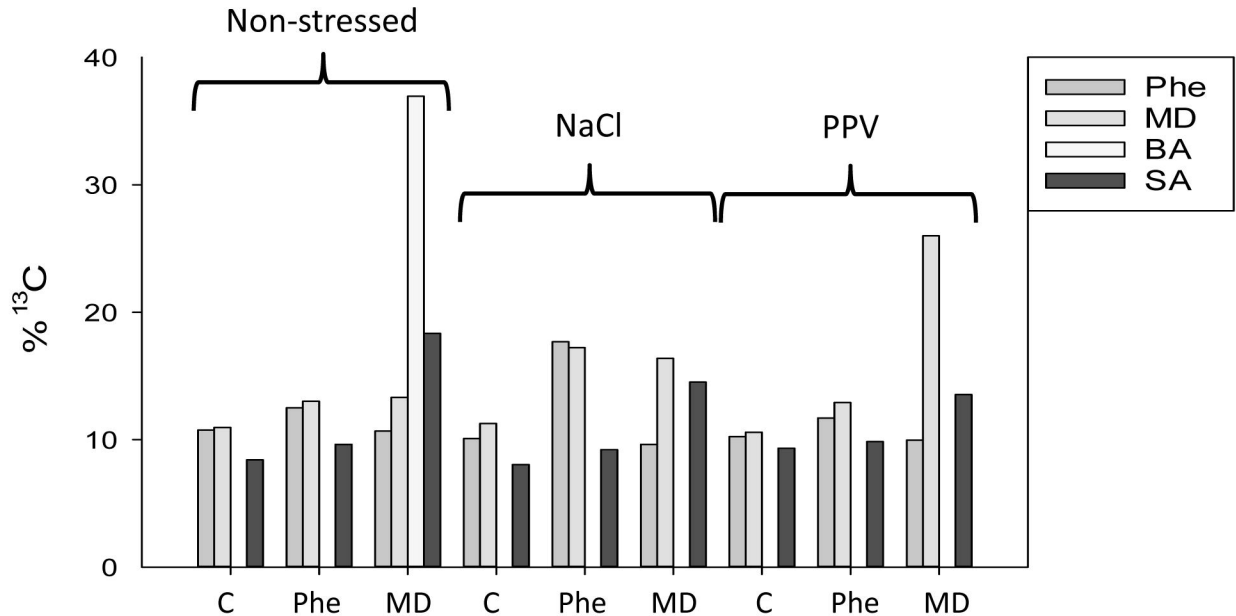


Fig. 3. Percentage of [^{13}C]-phenylalanine, mandelonitrile, benzoic acid, and salicylic acid in non-stressed, NaCl-stressed and PPV-infected peach shoots micropropagated in the presence or absence of [^{13}C]MD or [^{13}C]Phe. Under control conditions, basal levels of [^{13}C]-mandelonitrile, phenylalanine and salicylic acid were observed, whereas [^{13}C]benzoic acid was only detected in [^{13}C]MD-treated micropropagated shoots. Ions with an additional 1.0035 accurate mass and confirmation by isotopic distribution and spacing were defined as ions marked with ^{13}C . Data represent the mean \pm SE of at least 20 repetitions of each treatment

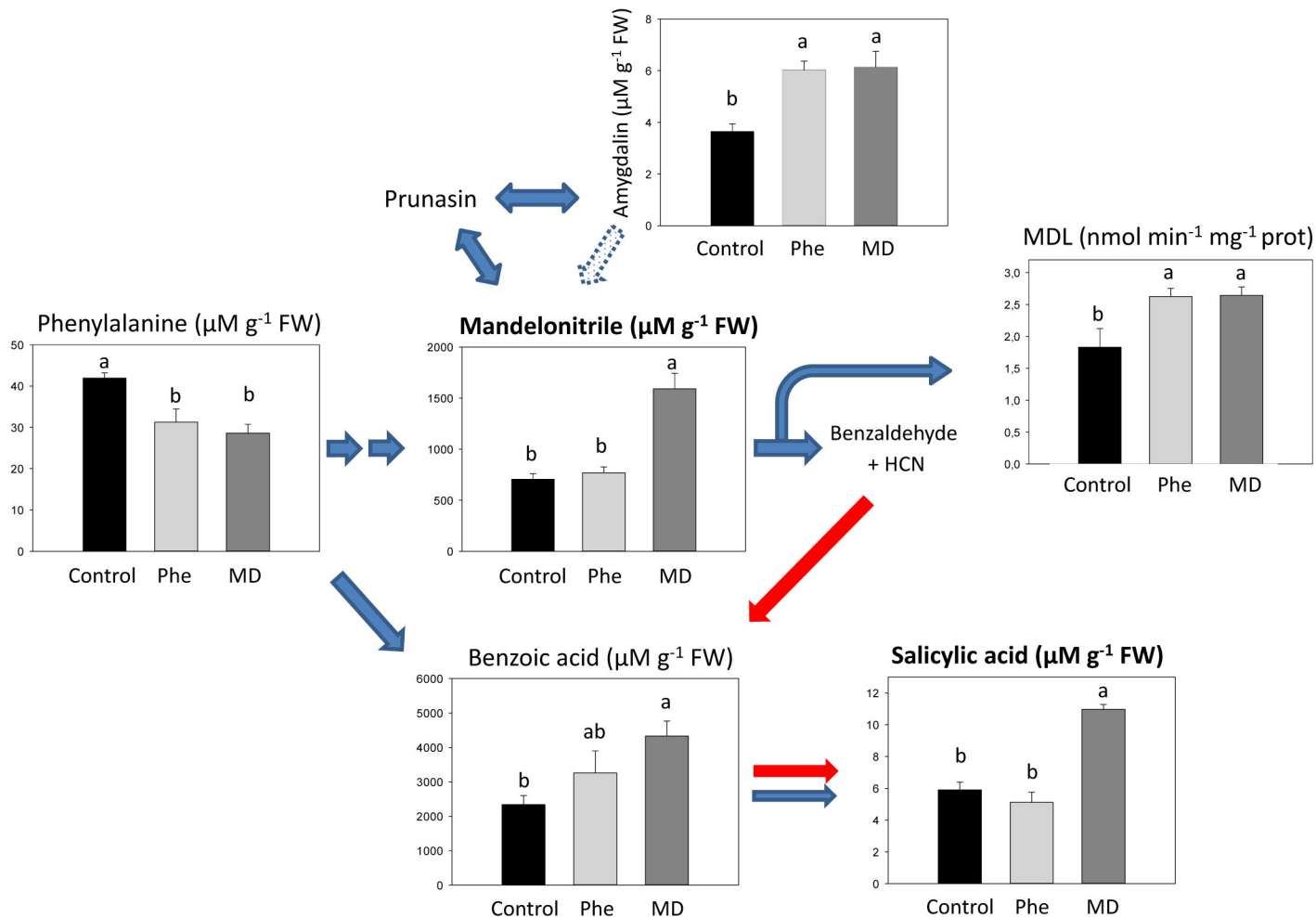


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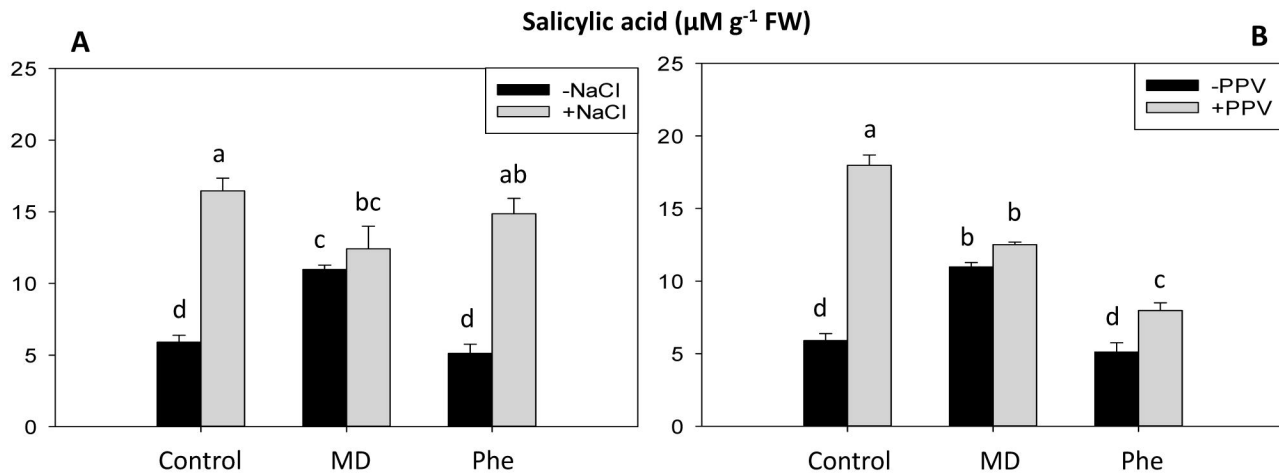


Fig. 5. Total level ($\mu\text{M g}^{-1}$ FW) of salicylic acid in peach shoots micropropagated in the presence or absence of $[^{13}\text{C}]\text{MD}$ or $[^{13}\text{C}]\text{Phe}$, submitted to salt stress (30 mM NaCl; A) or PPV infection (B).

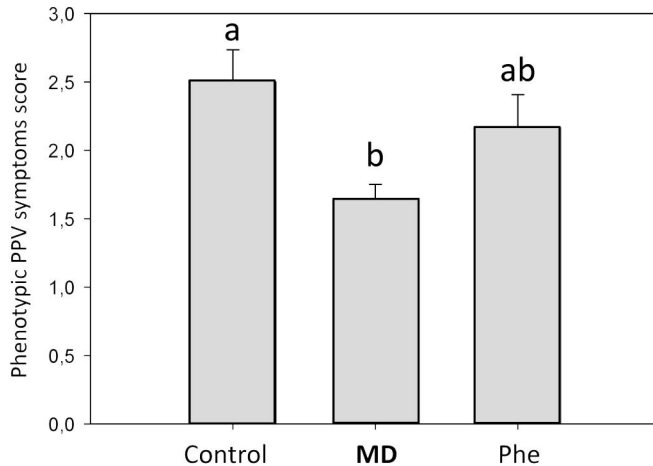


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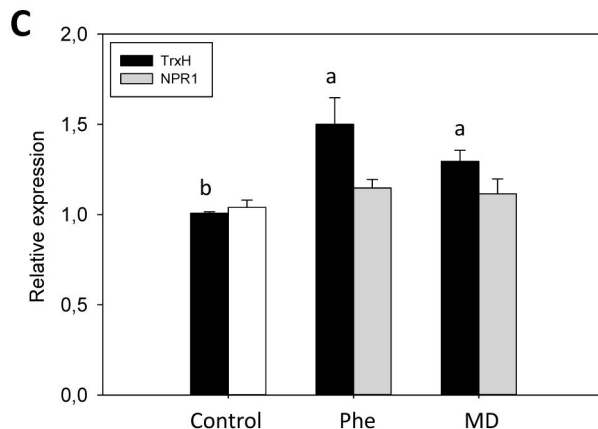
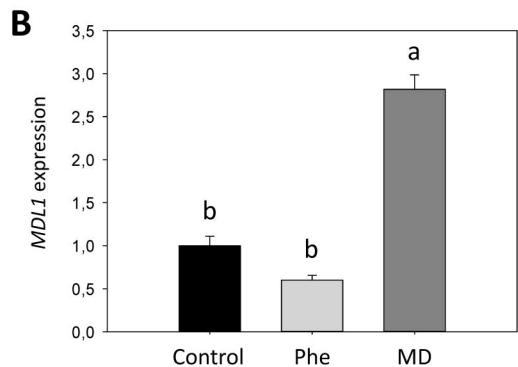
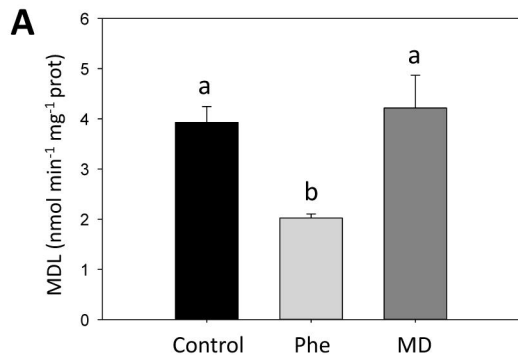


Fig. 7. Mandelonitrile lyase enzymatic activity (A), *MDL1* gene expression (B), and gene expression of *TrxH* and *NPR1* (C) in GF305 peach seedlings grown in the presence or absence of MD or Phe. Data represent the mean \pm SE of at least five repetitions of each treatment. Different letters indicate significant differences in each graph according to Duncan's test ($P \leq 0.05$).

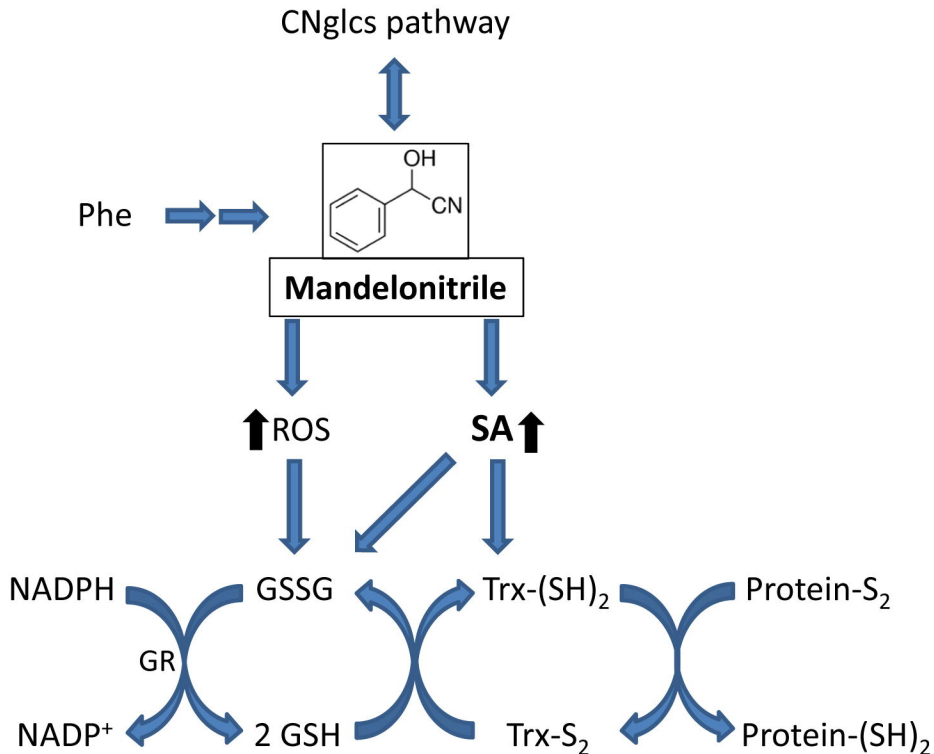


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