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2 **Thiophanate-methyl and carbendazim resistance in *Fusicoccum***
3 ***amygdali*, the causal agent of constriction canker of peach and**
4 **almond**

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10

11 **Keywords**

12 *β-tubulin* gene, fungicide resistance, *Fusicoccum amygdali*, in vitro bioassays, methyl-
13 benzimidazole-carbamates

14

15 In light of growing environmental concerns, surveys of fungicide resistance are needed to
16 ensure efficient control of fungi and avoid unnecessary treatments. Investigations of
17 fungicide resistance in *Fusicoccum amygdali* are scarce despite the economic impacts of this
18 pathogen in peach and almond orchards. Thiophanate-methyl has been registered for more
19 than 20 years to control *F. amygdali* but no resistance has been reported to date. This
20 propesticide is metabolized by fungi into carbendazim, a β -tubulin inhibitor. Sensitivity to
21 carbendazim of nine populations of *F. amygdali* from French orchards was assessed using

22 germination bioassays. Also, resistance levels of 63 strains isolated from four populations
23 were evaluated using mycelial growth assays. The underlying mechanism of resistance was
24 investigated by sequencing the *β-tubulin* gene, the molecular target of thiophanate-methyl, in
25 a set of isolates with different levels of sensitivity to carbendazim. Cross-resistance to
26 thiophanate-methyl and to another β -tubulin inhibitor, diethofencarb, was also assessed in
27 carbendazim-sensitive and -resistant strains. Isolates highly resistant to carbendazim were
28 found in one of the nine orchards studied. Sequencing showed that resistant phenotypes carry
29 a mutation leading to E198K substitution in the *β-tubulin* gene. Positive cross-resistance to
30 thiophanate-methyl was confirmed and no negative cross-resistance to diethofencarb was
31 identified in the phenotyped isolates, which were all resistant to this active substance. To our
32 knowledge, this is the first report of resistance to thiophanate-methyl in *F. amygdali*. The
33 high level of resistance of isolates sampled in one population is of concern, although the
34 limited geographical scope of resistance suggests its recent emergence.

35

36 1 Introduction

37 Constriction canker caused by *Fusicoccum amygdali* (syn. *Phomopsis amygdali*) is a disease
38 that affects peach (*Prunus persicae*) and almond (*Prunus dulcis*) trees in the Mediterranean
39 area, the United States, and more recently southern China (Bai et al., 2015; Yin et al., 2011)
40 and Hungary (Varjas et al., 2017). This fungus has also been reported to affect *Pieris*
41 *japonica* (Bienapfl & Balci, 2013) and *Pyrus pyrifolia* (Bai et al., 2015). The fungus causes
42 reddish-brown elongate lesions on twigs, the wilting of young twigs, and the desiccation of
43 young leaves, flowers, and fruits. Cankers appear in spring and grow until the end of summer.
44 Under favourable conditions, *F. amygdali* pycnidia can exudate tendrils of conidia (asexual
45 spores), which are released when it rains. The fungus penetrates through leaf abscission

46 wounds or bud scales and causes cankers. Furthermore, *F. amygdali* produces a toxin named
47 fusicoccin, which causes wilting of the affected organs (Ballio et al., 1964).

48 Major infections in peach orchards result in significant reductions in yield that, in
49 turn, lead to considerable economic losses (Lalancette & Polk, 2000). This disease can be
50 managed using prophylactic methods (choosing of less sensitive cultivars; pruning and
51 replacing trees in older orchards) and fungicide treatments. In France, control of constriction
52 canker in almond and peach trees is based on the use of three antifungal active substances:
53 dithianon, a multi-site fungicide, tebuconazole, a sterol demethylation inhibitor (DMI), and
54 thiophanate-methyl, an inhibitor of β -tubulin assembly. The latter fungicide belongs to the
55 MBC (methyl-benzimidazole-carbamates) class of fungicides. MBCs were the first anti-
56 tubulin fungicides marketed in the early 1970s. Resistance to MBCs quickly developed in
57 many fungal species: as of 2021, more than 100 fungal species have developed resistance to
58 these fungicides according to the Fungicide Resistance Action Committee FRAC
59 ([http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-](http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2)
60 [of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2](http://www.frac.info/docs/default-source/publications/list-of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2)). The main
61 resistance mechanism is target-site modifications (Ma & Michailides, 2005). Several
62 mutations affecting various codons of the *β -tubulin* gene have been identified. The most
63 frequent codon involved in target-site resistance to MBCs is codon 198 of the *β -tubulin* gene
64 (Ma & Michailides, 2005; Young, 2015).

65 In France, thiophanate-methyl has been used to control *F. amygdali* in almond and
66 peach trees for more than 20 years. Thiophanate-methyl is a biologically inactive compound
67 requiring structural transformation after its application to become an active pesticide. This
68 propesticide is metabolized in vivo to carbendazim by N-deacylation and cyclization
69 (Jeschke, 2016). Previously authorized prior to 2009, carbendazim is no longer approved for
70 field use in Europe due to its adverse effects on the environment and human health. It has

71 been replaced by MBC propesticides such as thiophanate-methyl and benzimidazoles. The
72 risk of *F. amygdali* developing resistance to thiophanate-methyl is regarded as high, and
73 fungicide treatments with this propesticide are limited to only one application per year. Over
74 the past few years, almond growers have reported difficulties in controlling *F. amygdali*,
75 mainly in Corsica, a French Mediterranean island.

76 To evaluate thiophanate-methyl sensitivity of *F. amygdali* populations from French
77 orchards, we performed spore germination and mycelial growth bioassays with the
78 thiophanate-methyl metabolite carbendazim. Carbendazim was used as a proxy for
79 thiophanate-methyl because the metabolization of this propesticide can take too long to
80 ensure a reliable assay. This is particularly true for spore germination bioassays that rarely
81 exceed two days. Therefore, to ensure a reliable assay, carbendazim must be used directly
82 instead of the registered propesticide. Mycelial growth assays were also carried out using
83 carbendazim to enable the comparison of results observed with both methods. A few mycelial
84 growth assays with carbendazim-sensitive and -resistant isolates were also performed with
85 thiophanate-methyl to confirm the cross-resistance between the propesticide and its
86 metabolite. Diethofencarb, another β -tubulin inhibitor, was included in our bioassays to
87 reveal potential cross-resistance with carbendazim, already outlined in other fungal species
88 (*Pyrenopeziza brassicae* - Carter et al., 2013; *Colletotrichum gloeosporioides* - Lin et al.,
89 2016; *Botrytis cinerea* - Liu et al., 2016, Malandrakis et al., 2011; *Monilinia laxa* -
90 Malandrakis et al., 2012). Cross-resistance to both active ingredients is caused by a
91 substitution affecting codon 198 or 200 of the β -tubulin gene. The changes can cause positive
92 or negative cross-resistance, depending on the substitution. Therefore, the β -tubulin gene of
93 strains was sequenced to determine the genetic markers of the different sensitivities observed.

94

95 2 Materials and methods

96 **2.1 *Fusicoccum amygdali* material and sampling**

97 In 2014, 2016 and 2018, a total of nine orchards were sampled, eight of which were almond
98 orchards and one of which was a peach orchard (Figure 1). Sampling was performed in
99 orchards where thiophanate-methyl treatments had been carried out. The samples consisted of
100 30 twigs with canker symptoms from 30 different trees spread throughout the orchard. Upon
101 their arrival in the laboratory, twigs with canker symptoms were incubated in a humid
102 chamber for 24 h, at 20°C ($\pm 1^\circ\text{C}$) in the dark, to stimulate the release of spore tendrils of
103 conidia extruded by the pycnidia. Next, the conidial tendrils were picked using a needle to
104 prepare a conidial suspension in sterile water with 10^5 spores/ml. This suspension was used
105 for the spore germination bioassays, and in addition, for three populations, monospore
106 cultures were also prepared from it (see section below).

107 For fungicide bioassays using the mycelial growth method, isolate CBS 428.64
108 ordered from the Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands) was used as
109 a sensitive reference strain. This strain was isolated from a peach canker lesion in France in
110 1964, six years before MBC fungicide use was first authorized in France. Thus, this strain
111 was considered as a reference for sensitivity to benzimidazole fungicides.

112 **2.2 Fungicide sensitivity bioassays**

113 **2.2.1 Fungicides**

114 Three active ingredients were tested: diethofencarb (Philagro), thiophanate-methyl (Sigma-
115 Aldrich) and carbendazim (BASF). The latter active substance was used for the spore
116 germination (with populations) and the mycelial growth (with individual isolates) bioassays.
117 Diethofencarb and thiophanate-methyl bioassays were performed only with individual

118 isolates using the mycelial growth method to investigate putative cross-resistance to
119 carbendazim. Stock solutions of both active ingredients were prepared in 96% ethanol at 10^4
120 mg/L.

121 **2.2.2 Sensitivity of spore germination to carbendazim**

122 For all but the 18-0296 orchards, a sufficient amount of spore solution was obtained after
123 incubation in a chamber to perform the spore germination bioassays. For these eight orchards,
124 250 μ l aliquots of the conidial suspension at 10^5 spores/ml were spread on the surface of Petri
125 dishes filled with water agar (12.5 g/L) medium amended with 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10,
126 30, 100 mg/L of carbendazim. Depending on the type of test, some of the concentrations in
127 the full dose range were not included in the dose–response experiment. In these cases,
128 bioassays were performed with the following active ingredient concentrations: 0, 0.01, 1, 10,
129 100 mg/L (File S1). After incubation for 48 h at 20°C ($\pm 1^\circ$ C) in the dark, the mean germ tube
130 length was estimated visually on approximately 100 conidia for each fungicide dose assayed.
131 The average elongation rate of the germ tube was calculated as the ratio of the average germ
132 tube length at the considered dose to the average germ tube length in the control (i.e.,
133 fungicide-free medium).

134 **2.2.3 Mycelial growth fungicide bioassays**

135 Mycelial growth assays were performed for three orchards (14-0020, 16-0219 and 18-0286)
136 and for the reference isolate (CBS 428.64). These orchards were selected based on their
137 geographic origin and tree species, as well as the resistance status of their respective *F.*
138 *amygdali* population. The objective was to isolate resistant and sensitive strains,
139 representative of the diversity of the spore populations resistance phenotypes and of the
140 sampled host species. Aliquots of 250 μ l of the conidial suspension at 10^5 spores/ml, obtained
141 after incubation of the twigs in a humid chamber, were spread in Petri dishes. After 24 h at

142 20°C ($\pm 1^\circ\text{C}$) in the dark, 50 germinated spores per orchard were picked individually and
143 placed on a potato dextrose agar (PDA) medium amended with chloramphenicol at 200 mg/L
144 to limit bacterial contamination. These cultures from single spores of *F. amygdali* were
145 grown at 22°C ($\pm 1^\circ\text{C}$) until the fungicide bioassay. Two active substances were assayed
146 based on mycelial growth: carbendazim and diethofencarb. For each active substance,
147 aliquots of stock solutions were incorporated into a PDA medium to provide final
148 concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 mg/L. Mycelial plugs from single
149 spore cultures, with a diameter of 8 mm, were transferred to the PDA medium amended with
150 fungicides. For each active ingredient and concentration tested, radial growth was measured
151 after 7 days of incubation at 22°C ($\pm 1^\circ\text{C}$) with a photoperiod of 16 h of light and 8 h of dark.
152 The growth rate was calculated as radial growth on the fungicide-spiked medium divided by
153 radial growth on the control medium (fungicide-free).

154 **2.2.4 Comparison of carbendazim and thiophanate-methyl sensitivity in mycelial** 155 **growth assays**

156 To check whether there was cross-resistance to carbendazim and thiophanate-methyl, eight
157 isolates sensitive to carbendazim (14-0020-084, 14-0020-085, 17-0193-001, 17-0193-002,
158 18-0063-011, 18-0075-001, 18-0286-003 and 18-0286-027) and two resistant isolates (16-
159 0219-005 and 16-0219-025) were assayed in parallel for carbendazim and thiophanate-methyl
160 sensitivity. Assays were performed using mycelia from isolated plugs stored at -80°C in 20%
161 of glycerol and subsequently grown for 6 days on PDA. Growth assays were performed as
162 described above except that an additional concentration of 300 mg/L was added to the range
163 of doses tested in an attempt to determine the EC_{50} values of the most resistant strains.

164 **2.3 DNA extraction**

165 Total DNA was isolated from frozen mycelia collected from cultures on PDA Petri dishes.
166 The mycelia (from 100 to 150 mg) were ground twice for 45 s at 30 Hz with one 4.76 mm
167 steel bead using a TissueLyser (Qiagen). Extraction was performed by incubation for 2 h at
168 60°C in 850 µl of preheated cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich)
169 buffer. The DNA purification step was performed by adding a chloroform-isoamyl alcohol
170 mixture (24:1; 570 µl), followed by centrifugation at 24,000 g for 10 min at 4°C to separate
171 contaminants in the organic phase from the nucleic acid in the aqueous phase. The organic
172 phase was removed and the DNA was precipitated by adding 2/3 (0.67) volume of
173 isopropanol to the aqueous phase with overnight incubation at -20°C. After centrifugation at
174 24,000 g for 10 min at 4°C, the DNA pellet was washed twice with 800 µl of 70% ethanol,
175 dried, and resuspended in 100 µl of ultrapure water. The DNA concentration and quality were
176 determined by measuring the absorbance spectrum from 200 to 300 nm using a NanoDrop
177 spectrophotometer (Thermo-Scientific).

178 **2.4 *β-tubulin* gene sequencing**

179 Previous studies have demonstrated target-site resistance to thiophanate-methyl (and its
180 metabolite, carbendazim) linked to mutations in the *β-tubulin* gene. Therefore, we sequenced
181 the *β-tubulin* gene in a set of isolates with different levels of sensitivity to carbendazim.
182 Sequencing was performed for eight different isolates: the reference isolate (CBS 428.64),
183 two isolates from the 14-0020 population (14-0020-10H and 14-0020-11D), and seven
184 isolates from the 16-0219 population (16-0219-5, 16-0219-14, 16-0219-18, 16-0219-25, 16-
185 0219-35 and 16-0219-37).

186 The *β-tubulin* gene was partially sequenced using two pairs of primers according to
187 Glass and Donaldson (1995). The primer pairs used were Bt2a (5'-
188 GGTAACCAAATCGGTGCTGCTTTC-3')/Bt3b (5'-

189 CATGAAGAAGTGGAGACGGGGGAA-3') and Bt3a (5'-
190 GCCAAGGGTCACTACACTGAGGGT-3')/Bt1b (5'-
191 GACGAGATCGTTCATGTTGAACTC-3'). Bt3a and Bt3b were the reverse complement of
192 Bt2b and Bt1a primers from Glass and Donaldson (1995), respectively (File S2). Two
193 separate PCR amplifications were carried out in a final volume of 50 µl containing: 5 µl of
194 Fermentas Taq polymerase buffer, 0.5 µM each primer, 2 mM each dNTP, 25 mM MgCl₂,
195 1.25 U of *Taq* DNA polymerase, and 2 µl of template DNA solution at a concentration
196 between 2 and 80 ng/µl. The PCR cycles consisted of an initial denaturation step at 95°C for
197 3 min, followed by 35 PCR cycles with a denaturation step at 94°C for 30 s, a primer
198 annealing step at 58°C for 30 s and an elongation step at 72°C for 90 s. A final elongation
199 step was performed at 72°C for 10 min. Sanger sequencing of the PCR products was
200 performed by Genewiz (South Plainfield, NJ, USA). Sequencing analyses were undertaken
201 using Bio-Edit software.

202 **2.5 Data analysis**

203 For the spore germination and mycelial growth bioassays, the analyses of dose–response
204 curves were carried out in a nonlinear regression framework using R statistical software (R
205 Core Team, 2020) and the drc package (Ritz et al., 2015). The response variable for the spore
206 germination bioassay in populations was the ratio of the average germ tube length at the
207 considered dose to the average germ tube length in the control. Because the ratios did not
208 always reach a lower plateau of 0%, the 50% absolute effective concentration (EC₅₀) was
209 estimated using a four-parameter log-normal dose–response model (Bruce & Versteeg, 1992).
210 The model was defined following equation B.16 in Ritz et al. (2019):

$$211 f(x, (b, c, d, e)) = c + (d - c)\Phi[b\{\log(x) - \log(e)\}] \quad (1)$$

212 where Φ is the cumulative distribution function of the standard normal distribution and the
213 parameters are b , the slope of the dose–response curve; c , the lower limit of the response; d ,
214 the upper limit of the response; and e , the dose producing a response half way between these
215 limits (or relative EC_{50}).

216 For the mycelial growth bioassays, the response variable was the growth rate at the
217 considered dose. The same framework employed for the spore germination bioassay was
218 used, except that the lower limit of the response was set to 0, resulting in a three-parameter
219 log-normal dose–response model (Ritz et al., 2019; Van Der Hoeven, 1997):

$$220 \quad f(x, (b, d, e)) = d * \Phi[b\{\log(x) - \log(e)\}] \quad (2)$$

221 where Φ is the cumulative distribution function of the standard normal distribution and the
222 parameters b , d and e are the same as in the previous equation above.

223 For both types of bioassay, the absolute EC_{50} and associated approximate standard
224 errors were estimated based on an after-fitting approach (Ritz et al., 2019) using the ED
225 function of the drc package (Ritz et al., 2015) with the type option set to ‘absolute’. For the
226 mycelial growth bioassays, the resistance factor (RF) was calculated by dividing the absolute
227 EC_{50} value of the studied isolate by the absolute EC_{50} value obtained from the reference
228 isolate (CBS 428.64). The data and script used to perform the analyses and produce the
229 figures are available in an online Zenodo repository (Barrès, 2020).

230

231 3 Results

232 3.1 Fungicide bioassays

233 The sensitivity of spore germination to carbendazim was determined for eight *F. amygdali*
234 populations. With the exception of one population (16-0219), the EC₅₀ values for
235 carbendazim of all the populations were equal to or less than 0.1 mg/L. It was not possible to
236 determine the EC₅₀ of the 16-0219 population because at the highest carbendazim
237 concentration (100 mg/L), the relative germination rate was around 70% (Figure 2).

238 The mycelial growth bioassays allowed us to determine the EC₅₀ values of the *F.*
239 *amygdali* isolates. The EC₅₀ value of the reference isolate (CBS 428.64) was 0.02 mg/L. Of
240 the 50 conidia isolated per population, 8, 24 and 30 pure mycelial strains were obtained for
241 orchards 14-0020, 16-0219 and 18-0286, respectively. All of the monospore cultures from
242 14-0020 (eight cultures) and 18-0286 (30 cultures) had EC₅₀ values below 0.09 mg/L,
243 corresponding to a maximum RF of 4.7 (Figure 3a, File S3). EC₅₀ values could not be
244 evaluated for any of the isolates from the 16-0219 orchard (24 cultures). These cultures had
245 high radial growth at the maximum concentration of carbendazim tested (100 mg/L) (Figure
246 3a, File S3). Indeed, relative to their growth without the addition of carbendazim, the
247 mycelial growth of these isolates was still between 55% and 90% at 100 mg/L carbendazim.

248 All of these monospore cultures were also assayed for diethofencarb sensitivity. The
249 EC₅₀ values of isolates collected from orchards between 2016 and 2018 were between 17.9
250 and 79.7 mg/L. The sensitive reference isolate had a similar EC₅₀ value of 48 mg/L (Figure
251 3b, File S3). To test for cross-resistance to carbendazim and thiophanate-methyl, eight
252 isolates sensitive to carbendazim and two isolates resistant to carbendazim were selected for
253 bioassays of sensitivity to thiophanate-methyl. The thiophanate-methyl EC₅₀ values of
254 carbendazim sensitive isolates (including the sensitive reference isolate) were between 0.200
255 mg/L and 0.627 mg/L. The thiophanate-methyl EC₅₀ values of carbendazim resistant isolates
256 were between 26.8 mg/L and 47.6 mg/L for 16-0219-25 and 16-0219-05, respectively (Table
257 1).

258 3.2 *β-tubulin* gene sequencing

259 Sequencing of the *β-tubulin* gene with two primer pairs produced a 1392 bp-long sequence
260 for all the isolates analysed. A BlastX analysis of this fragment gave the highest score with
261 the *Diaporthe helianthi* *β-tubulin* protein (identity 99.62%) with an overlap at amino acids 22
262 to 398 of the protein. The analyses of the DNA sequences revealed a mutation, resulting in
263 the substitution of glutamic acid with lysine at codon 198 (E198K) in all the isolates
264 sequenced from the 16-0219 orchard. The substitution was caused by a change of guanine to
265 alanine in the nucleotide triplet (GAG→AAG) (File S4). The *β-tubulin* gene sequences of a
266 wild and a mutant isolate were deposited in GenBank (accession numbers MG772818 and
267 MG996732 for the sensitive and resistant isolates, respectively). Comparison of the *β-tubulin*
268 sequences of *F. amygdali* with those of other species in family *Diaporthaceae* (*Diaporthe*
269 *ampelina*, *D. helianthi*, *Valsa mali*) revealed the presence of threonine instead of serine at
270 position 32 and a valine instead of alanine at position 54 of *β-tubulin*.

271

272 4 Discussion

273 To our knowledge, this is the first report of resistance to thiophanate-methyl for *F. amygdali*.
274 Previous studies on the sensitivity of *F. amygdali* to thiophanate-methyl using mycelial
275 growth assays (Froelich & Schnabel, 2019) or to carbendazim using germination assays (Ji et
276 al., 2013) have not found any evidence of resistance. The EC₅₀ value of the reference isolate
277 sampled before thiophanate-methyl was authorized is comparable to values reported for
278 sensitive isolates (Ji et al., 2013), which confirms that these isolates can be used as a
279 reference for sensitivity to thiophanate-methyl. The resistance levels detected in resistant
280 isolates were high, but their EC₅₀ values could not be estimated because 50% growth
281 inhibition was not reached for some of these strains at the highest concentration of

282 carbendazim tested. Consequently, it was not possible to calculate their RF value. However,
283 the ratio between the highest concentration tested and EC50 of the reference sensitive isolate
284 indicated a RF greater than 5000. This level of resistance suggests a target-site modification.
285 Comparison of growth assays performed with thiophanate-methyl and carbendazim confirms
286 a positive cross-resistance between the propesticide and its metabolite: carbendazim-resistant
287 isolates also showed high resistance to thiophanate-methyl (RF > 130) in in vitro bioassays.

288 The mutation affecting the codon 198 of the *β-tubulin* gene was found in all of the
289 sequenced carbendazim-resistant isolates including the two isolates assayed for thiophanate-
290 methyl sensitivity. This mutation was not found in the investigated sensitive isolates (either
291 the reference isolate or the isolates sampled in another location). This mutation leads to the
292 substitution of glutamic acid with lysine at codon 198 (E198K). Identical substitutions have
293 been linked to target site resistance to MBC in multiple other plant pathogen species: *B.*
294 *cinerea* (Leroux et al., 2002; Yarden & Katan, 1993), *Venturia inaequalis*, *Monilinia*
295 *fructicola*, *Penicillium* spp. and *Sclerotinia homoeocarpa* (Koenraad et al., 1992). The
296 presence of other mutations cannot be completely ruled out because, compared with other
297 known *Diaporthaceae* *β-tubulin* proteins (POS75197.1, KUI53087.1, AEG19576.1), we
298 sequenced only 377 of the 447 amino acids in the protein.

299 Of the six codons of the *β-tubulin* gene already identified as being involved in target
300 site resistance to MBC in other fungal species, four major codons were included in our partial
301 sequencing (codon 50 167, 198, 200). Mutations in the two missing codons (6 and 240) have
302 only been detected in a few species (substitutions H6Y in *M. laxa* and L240F in *Tapesia*
303 *yallundae* and *M. fructicola*; Ma & Michailides, 2005). Therefore, the sequencing presented
304 in this work covers the most important codons known to be associated with target-site
305 resistance to MBC. The analysis of the *F. amygdali* *β-tubulin* sequences revealed the
306 presence of threonine instead of serine at position 32 and a valine instead of alanine at

307 position 54 when compared with the β -tubulin gene of other *Diaporthaceae* species. Both
308 substitutions are caused by amino acids with similar physicochemical properties (threonine
309 and serine are hydrophilic and uncharged; valine and alanine are hydrophobic and uncharged)
310 and these residues are not known to have a functional role. Therefore, these substitutions
311 probably do not affect the benzimidazole sensitivity of *F. amygdali*.

312 Sensitivity to diethofencarb determined by dose–response mycelial growth bioassays
313 for 63 isolates did not reveal cross-resistance to carbendazim as already reported in several
314 species (*Pyrenopeziza brassicae*, Carter et al., 2013; *Colletotrichum gloeosporioides*, Lin et
315 al., 2016; *B. cinerea*, Malandrakis et al., 2011; *M. laxa*, Malandrakis et al., 2012). Likewise,
316 these assays revealed similar, high EC₅₀ values for the sensitive reference (collected before
317 diethofencarb authorization) and for all the other isolates screened, whether they were
318 resistant or sensitive to carbendazim. Therefore, *F. amygdali* appears to be naturally resistant
319 to diethofencarb. As already described for *B. cinerea*, the E198K substitution has no effect on
320 natural insensitivity to diethofencarb in *F. amygdali*, unlike other substitutions such as
321 E198A, which enhances sensitivity to diethofencarb in *M. laxa* (Malandrakis et al., 2012) and
322 *B. cinerea* (Liu et al., 2016).

323 The target-site resistance of *F. amygdali* to thiophanate-methyl that we reported in
324 this study was only found in one almond tree orchard in Corsica. Despite additional sampling
325 in the vicinity of the orchard in the following years, no other resistant isolates were identified.
326 The high resistance levels determined by in vitro bioassays may be problematic in the field.
327 Obviously, there is not always full agreement between in vitro and in vivo assays. However,
328 high RF values under laboratory conditions were observed with isolates from orchards in
329 which *F. amygdali* is difficult to manage, suggesting that resistance can lead to difficulties in
330 controlling the disease. More investigations, such as field trial assays, need to be performed
331 to evaluate the impact of the E198K mutation in orchards.

332 In addition to almond trees, *F. amygdali* can infect other tree species of economic
333 interest, such as peach trees. Only one peach orchard was investigated in this study and the
334 population tested was found to be sensitive to carbendazim. It is clearly important for the
335 production of this fruit to verify the sensitivity levels of *F. amygdali* populations in
336 Mediterranean orchards more thoroughly and monitor thiophanate-methyl resistance via its
337 metabolite in in vitro spore germination bioassays.

338

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346

347 Data availability statement

348 The data that support the findings of this study are openly available in repository zenodo at
349 <http://doi.org/10.5281/zenodo.3473232>.

350

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420

421 **Supporting Information**

422 **File S1** Range of carbendazim concentrations tested in spore germination bioassays for each
423 orchard population of *Fusarium amygdali*.

424 **File S2** Sequencing strategy for β -tubulin gene of *Fusicoccum amygdali* isolates. (a)
425 Genomic structure of the β -tubulin gene of *Neurospora crassa* and position and name of
426 primers pairs based on Glass and Donaldson (1995). Underlined primers are from Glass and
427 Donaldson (1995), black primers are primer sets used in this study. Bt3a and Bt3b are reverse
428 complement sequences of Bt2b and Bt1a, respectively. (b) partial sequence of the *F.*
429 *amygdali* β -tubulin gene. Grey boxes denote protein coding sequences (exons) and black
430 lines denote introns. The black numbers represent the nucleotide position of the obtained
431 sequence and the grey numbers correspond to the amino acid position according to the BlastX
432 alignment with *Diaporthe helianthi* β -tubulin protein.

433 **File S3** Estimated EC₅₀ values and associated standard error based on dose–response
434 bioassay for two active substances (carbendazim and diethofencarb) on the mycelial growth
435 of isolates of *Fusicoccum amygdali*. Figure 3 is based on these results.

436 **File S4** Multiple alignment of partial β -tubulin gene sequences of three isolates sensitive to
437 carbendazim and thiophanate-methyl and five resistant isolates. The location of the exons in
438 the sequence is indicated by the presence of grey boxes below the alignment. The nucleotides
439 of codon 198 are shown in bold and underlined. The single nucleotide polymorphism in the
440 resistant mutants is highlighted in red.

441

442 **Figure legends**

443 **Figure 1** Location within France of orchards sampled from 2014 to 2018. The different
444 symbols represent different sampling years. Orchards in which carbendazim-sensitive and -
445 resistant strains of *Fusicoccum amygdali* were detected are shown in grey and red,
446 respectively.

447 **Figure 2** Dose–response curves for the spore germination bioassay on populations of
448 *Fusicoccum amygdali* exposed to varying doses of carbendazim. The response variable is the
449 ratio of the average germ tube length to the average germ tube length in the control at the
450 considered dose. Sensitive and resistant populations are shown in black and red, respectively.

451 **Figure 3** Ranking of isolates of *Fusicoccum amygdali* according to their EC_{50} values in
452 mycelial growth assays on media amended with (a) carbendazim and (b) diethofencarb. The
453 isolates are arranged in order of increasing EC_{50} . The reference isolate, the isolates from the
454 sensitive population and the isolates from the resistant population are depicted in blue, black
455 and red, respectively. The different symbols stand for the different populations of origin of
456 the isolates. The error bars indicate the associated approximate standard errors estimated
457 using an after-fitting approach. The standard errors could not be estimated for the resistant
458 isolates. Due to the very small value of the standard errors of some of the sensitive isolates,
459 the error bars are not always visible.