

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

Séverine Fontaine, Laëtitia Caddoux, Florent Remuson, Benoit Barrès

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Séverine Fontaine, Laëtitia Caddoux, Florent Remuson, Benoit Barrès. Thiophanate-methyl and carbendazim resistance in Fusicoccum amygdali, the causal agent of constriction canker of peach and almond. Plant Pathology, 2022, 71 (4), pp.785-792. 10.1111/ppa.13525. hal-03551003

HAL Id: hal-03551003 https://hal.science/hal-03551003

Submitted on 1 Feb 2022

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- 1 Running head: FONTAINE et al.
- 2 Thiophanate-methyl and carbendazim resistance in *Fusicoccum*
- 3 *amygdali*, the causal agent of constriction canker of peach and
- 4 almond
- 5 Séverine Fontaine, Laëtitia Caddoux, Florent Remuson, Benoit Barrès
- 6 Université de Lyon, Anses, INRAE, USC CASPER, Lyon, France
- 7
- 8 Correspondence
- 9 Séverine Fontaine; email: severine.fontaine@anses.fr
- 10
- 11 Keywords
- 12 β-tubulin gene, fungicide resistance, Fusicoccum amygdali, in vitro bioassays, methyl-
- 13 benzimidazole-carbamates
- 14

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

wounds or bud scales and causes cankers. Furthermore, *F. amygdali* produces a toxin named
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-60 of-resistant-plant-pathogenic-organisms_may-2018.pdf?sfvrsn=a2454b9a_2). The main 61 resistance mechanism is target-site modifications (Ma & Michailides, 2005). Several mutations affecting various codons of the β -tubulin gene have been identified. The most 62 63 frequent codon involved in target-site resistance to MBCs is codon 198 of the β -tubulin gene 64 (Ma & Michailides, 2005; Young, 2015).

In France, thiophanate-methyl has been used to control *F. amygdali* in almond and peach trees for more than 20 years. Thiophanate-methyl is a biologically inactive compound requiring structural transformation after its application to become an active pesticide. This propesticide is metabolized in vivo to carbendazim by N-deacylation and cyclization (Jeschke, 2016). Previously authorized prior to 2009, carbendazim is no longer approved for field use in Europe due to its adverse effects on the environment and human health. It has been replaced by MBC propesticides such as thiophanate-methyl and benzimidazoles. The
risk of *F. amygdali* developing resistance to thiophanate-methyl is regarded as high, and
fungicide treatments with this propesticide are limited to only one application per year. Over
the past few years, almond growers have reported difficulties in controlling *F. amygdali*,
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 reveal potential cross-resistance with carbendazim, already outlined in other fungal species 87 88 (Pyrenipeziza 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 (±1°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 prepare a conidial suspension in sterile water with 10^5 spores/ml. This suspension was used 104 105 for the spore germination bioassays, and in addition, for three populations, monospore 106 cultures were also prepared from it (see section below).

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

carbendazim. Stock solutions of both active ingredients were prepared in 96% ethanol at 10⁴
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, 250 μ l aliquots of the conidial suspension at 10⁵ spores/ml were spread on the surface of Petri 124 125 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°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 tube length at the considered dose to the average germ tube length in the control (i.e., 132 133 fungicide-free medium).

134 **2.2.3 Mycelial growth fungicide bioassays**

Mycelial growth assays were performed for three orchards (14-0020, 16-0219 and 18-0286)
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 10⁵ 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 (±1°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 (±1°C) until the fungicide bioassay. Two active substances were assayed based on mycelial growth: carbendazim and diethofencarb. For each active substance, 146 147 aliquots of stock solutions were incorporated into a PDA medium to provide final concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 mg/L. Mycelial plugs from single 148 149 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 (±1°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).

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% of glycerol and subsequently grown for 6 days on PDA. Growth assays were performed as 161 162 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

Previous studies have demonstrated target-site resistance to thiophanate-methyl (and its metabolite, carbendazim) linked to mutations in the β -tubulin gene. Therefore, we sequenced the β -tubulin gene in a set of isolates with different levels of sensitivity to carbendazim. Sequencing was performed for eight different isolates: the reference isolate (CBS 428.64), two isolates from the 14-0020 population (14-0020-10H and 14-0020-11D), and seven 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, 1.25 U of *Taq* DNA polymerase, and 2 µl of template DNA solution at a concentration 195 196 between 2 and 80 ng/ μ l. 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
$$f(x, (b, c, d, e)) = c + (d - c)\Phi[b\{\log(x) - \log(e)\}]$$
 (1)

where Φ is the cumulative distribution function of the standard normal distribution and the parameters are b, the slope of the dose–response curve; c, the lower limit of the response; d, the upper limit of the response; and e, the dose producing a response half way between these limits (or relative EC₅₀).

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

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

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

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

230

231 3 Results

232 **3.1 Fungicide bioassays**

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

238 The mycelial growth bioassays allowed us to determine the EC_{50} 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 EC_{50} 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 EC_{50} 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 EC_{50} 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

Sequencing of the β -tubulin gene with two primer pairs produced a 1392 bp-long sequence 259 260 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 sequenced from the 16-0219 orchard. The substitution was caused by a change of guanine to 264 265 alanine in the nucleotide triplet (GAG \rightarrow 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. anygdali with those of other species in family Diaporthacea (Diaporthe 269 ampelina, D. helianthi, Valsa mali) revealed the presence of threonine instead of serine at 270 position 32 and a value instead of alanine at position 54 of β -tubulin.

271

4 Discussion

To our knowledge, this is the first report of resistance to thiophanate-methyl for F. amygdali. 273 274 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 EC_{50} 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 EC_{50} values could not be estimated because 50% growth 281 inhibition was not reached for some of these strains at the highest concentration of

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

The mutation affecting the codon 198 of the β -tubulin gene was found in all of the 288 289 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 (Koenraadt et al., 1992). The 296 presence of other mutations cannot be completely ruled out because, compared with other 297 known *Diaporthacea* 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 (Pyrenipeziza 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 EC_{50} 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 Mediterranean orchards more thoroughly and monitor thiophanate-methyl resistance via its 336 337 metabolite in in vitro spore germination bioassays. 338 Acknowledgements 339 We would like to thank all the field workers who participated in sample collection. This 340 341 project was partly funded and supported by the French Agency for Food, Environmental and 342 Occupational Health and Safety (ANSES) via the tax on sales of plant protection products. 343 Proceeds from this tax are allocated to ANSES to finance a "phytopharmacovigilance" (PPV)

344 system for monitoring the adverse effects of plant protection products, pursuant to the French

Act on the Future of Agriculture of 13 October 2014.

346

347 Data availability statement

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

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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 carbandazim 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**

Figure 1 Location within France of orchards sampled from 2014 to 2018. The different
symbols represent different sampling years. Orchards in which carbendazim-sensitive and resistant strains of *Fusicoccum amygdali* were detected are shown in grey and red,
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. Figure 3 Ranking of isolates of *Fusicoccum amygdali* according to their EC₅₀ values in 451 452 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 isolates. Due to the very small value of the standard errors of some of the sensitive isolates, 458 459 the error bars are not always visible.