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

Running head: FONTAINE et al.

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

Université de Lyon, Anses, INRAE, USC CASPER, Lyon, France

Correspondence

Séverine Fontaine; email: severine.fontaine@anses.fr

Keywords

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

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

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

1 Introduction

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

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.

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

2 Materials and methods

2.1 *Fusicoccum amygdali* material and sampling

In 2014, 2016 and 2018, a total of nine orchards were sampled, eight of which were almond orchards and one of which was a peach orchard (Figure 1). Sampling was performed in orchards where thiophanate-methyl treatments had been carried out. The samples consisted of 30 twigs with canker symptoms from 30 different trees spread throughout the orchard. Upon their arrival in the laboratory, twigs with canker symptoms were incubated in a humid chamber for 24 h, at 20°C (±1°C) in the dark, to stimulate the release of spore tendrils of 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 for the spore germination bioassays, and in addition, for three populations, monospore 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.

2.2 Fungicide sensitivity bioassays

2.2.1 Fungicides

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

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 10^4 mg/L.

2.2.2 Sensitivity of spore germination to carbendazim

For all but the 18-0296 orchards, a sufficient amount of spore solution was obtained after incubation in a chamber to perform the spore germination bioassays. For these eight orchards, 250 µl aliquots of the conidial suspension at 10^5 spores/ml were spread on the surface of Petri dishes filled with water agar (12.5 g/L) medium amended with 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 mg/L of carbendazim. Depending on the type of test, some of the concentrations in the full dose range were not included in the dose–response experiment. In these cases, bioassays were performed with the following active ingredient concentrations: 0, 0.01, 1, 10, 100 mg/L (File S1). After incubation for 48 h at 20°C (±1°C) in the dark, the mean germ tube length was estimated visually on approximately 100 conidia for each fungicide dose assayed. 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., fungicide-free medium).

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 geographic origin and tree species, as well as the resistance status of their respective *F. amygdali* population. The objective was to isolate resistant and sensitive strains, representative of the diversity of the spore populations resistance phenotypes and of the sampled host species. Aliquots of 250 µl of the conidial suspension at 10^5 spores/ml, obtained after incubation of the twigs in a humid chamber, were spread in Petri dishes. After 24 h at

20°C (±1°C) in the dark, 50 germinated spores per orchard were picked individually and placed on a potato dextrose agar (PDA) medium amended with chloramphenicol at 200 mg/L to limit bacterial contamination. These cultures from single spores of *F. amygdali* were 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, 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 spore cultures, with a diameter of 8 mm, were transferred to the PDA medium amended with fungicides. For each active ingredient and concentration tested, radial growth was measured after 7 days of incubation at 22°C (±1°C) with a photoperiod of 16 h of light and 8 h of dark. The growth rate was calculated as radial growth on the fungicide-spiked medium divided by radial growth on the control medium (fungicide-free).

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

To check whether there was cross-resistance to carbendazim and thiophanate-methyl, eight isolates sensitive to carbendazim (14-0020-084, 14-0020-085, 17-0193-001, 17-0193-002, 18-0063-011, 18-0075-001, 18-0286-003 and 18-0286-027) and two resistant isolates (16-0219-005 and 16-0219-025) were assayed in parallel for carbendazim and thiophanate-methyl 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 described above except that an additional concentration of 300 mg/L was added to the range of doses tested in an attempt to determine the EC_{50} values of the most resistant strains.

2.3 DNA extraction

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

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, 16-0219-25,16-0219-35 and 16-0219-37).

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

CATGAAGAAGTGGAGACGGGGGAA-3′) and Bt3a (5′-GCCAAGGGTCACTACACTGAGGGT-3′)/Bt1b (5′-GACGAGATCGTTCATGTTGAACTC-3′). Bt3a and Bt3b were the reverse complement of Bt2b and Bt1a primers from Glass and Donaldson (1995), respectively (File S2). Two separate PCR amplifications were carried out in a final volume of 50 µl containing: 5 µl of Fermentas Taq polymerase buffer, 0.5 µM each primer, 2 mM each dNTP, 25 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase, and 2 µl of template DNA solution at a concentration between 2 and 80 ng/µl. The PCR cycles consisted of an initial denaturation step at 95°C for 3 min, followed by 35 PCR cycles with a denaturation step at 94°C for 30 s, a primer annealing step at 58°C for 30 s and an elongation step at 72°C for 90 s. A final elongation step was performed at 72°C for 10 min. Sanger sequencing of the PCR products was performed by Genewiz (South Plainfield, NJ, USA). Sequencing analyses were undertaken using Bio-Edit software.

2.5 Data analysis

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

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

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 EC_{50}).

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):

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

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).

3 Results

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 EC_{50} values for carbendazim of all the populations were equal to or less than 0.1 mg/L. It was not possible to determine the EC_{50} of the 16-0219 population because at the highest carbendazim concentration (100 mg/L), the relative germination rate was around 70% (Figure 2).

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

All of these monospore cultures were also assayed for diethofencarb sensitivity. The EC_{50} values of isolates collected from orchards between 2016 and 2018 were between 17.9 and 79.7 mg/L. The sensitive reference isolate had a similar EC_{50} value of 48 mg/L (Figure 3b, File S3). To test for cross-resistance to carbendazim and thiophanate-methyl, eight isolates sensitive to carbendazim and two isolates resistant to carbendazim were selected for bioassays of sensitivity to thiophanate-methyl. The thiophanate-methyl EC_{50} values of carbendazim sensitive isolates (including the sensitive reference isolate) were between 0.200 mg/L and 0.627 mg/L. The thiophanate-methyl EC_{50} values of carbendazim resistant isolates were between 26.8 mg/L and 47.6 mg/L for 16-0219-25 and 16-0219-05, respectively (Table 1).

3.2 *β-tubulin* gene sequencing

Sequencing of the *β-tubulin* gene with two primer pairs produced a 1392 bp-long sequence for all the isolates analysed. A BlastX analysis of this fragment gave the highest score with the *Diaporthe helianthi* β-tubulin protein (identity 99.62%) with an overlap at amino acids 22 to 398 of the protein. The analyses of the DNA sequences revealed a mutation, resulting in 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 alanine in the nucleotide triplet (GAG→AAG) (File S4). The *β-tubulin* gene sequences of a wild and a mutant isolate were deposited in GenBank (accession numbers MG772818 and MG996732 for the sensitive and resistant isolates, respectively). Comparison of the β-tubulin sequences of *F. amygdali* with those of other species in family *Diaporthacea* (*Diaporthe ampelina, D. helianthi, Valsa mali*) revealed the presence of threonine instead of serine at position 32 and a valine instead of alanine at position 54 of β-tubulin.

4 Discussion

To our knowledge, this is the first report of resistance to thiophanate-methyl for *F. amygdali*. Previous studies on the sensitivity of *F. amygdali* to thiophanate-methyl using mycelial growth assays (Froelich & Schnabel, 2019) or to carbendazim using germination assays (Ji et al., 2013) have not found any evidence of resistance. The EC_{50} value of the reference isolate sampled before thiophanate-methyl was authorized is comparable to values reported for sensitive isolates (Ji et al., 2013), which confirms that these isolates can be used as a reference for sensitivity to thiophanate-methyl. The resistance levels detected in resistant isolates were high, but their EC_{50} values could not be estimated because 50% growth 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 sequenced carbendazim-resistant isolates including the two isolates assayed for thiophanate-methyl sensitivity. This mutation was not found in the investigated sensitive isolates (either the reference isolate or the isolates sampled in another location). This mutation leads to the substitution of glutamic acid with lysine at codon 198 (E198K). Identical substitutions have been linked to target site resistance to MBC in multiple other plant pathogen species: *B. cinerea* (Leroux et al., 2002; Yarden & Katan, 1993), *Venturia inaequalis*, *Monilinia fructicola*, *Penicillium* spp. and *Sclerotinia homoeocarpa* (Koenraadt et al., 1992). The presence of other mutations cannot be completely ruled out because, compared with other known *Diaporthacea* β-tubulin proteins (POS75197.1, KUI53087.1, AEG19576.1), we sequenced only 377 of the 447 amino acids in the protein.

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

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

Sensitivity to diethofencarb determined by dose–response mycelial growth bioassays for 63 isolates did not reveal cross-resistance to carbendazim as already reported in several species (*Pyrenipeziza brassicae*, Carter et al., 2013; *Colletotrichum gloeosporioides*, Lin et al., 2016*; B. cinerea*, Malandrakis et al., 2011; *M. laxa*, Malandrakis et al., 2012). Likewise, these assays revealed similar, high EC_{50} values for the sensitive reference (collected before diethofencarb authorization) and for all the other isolates screened, whether they were resistant or sensitive to carbendazim. Therefore, *F. amygdali* appears to be naturally resistant to diethofencarb. As already described for *B. cinerea*, the E198K substitution has no effect on natural insensitivity to diethofencarb in *F. amygdali*, unlike other substitutions such as E198A, which enhances sensitivity to diethofencarb in *M. laxa* (Malandrakis et al., 2012) and *B. cinerea* (Liu et al., 2016).

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

In addition to almond trees, *F. amygdali* can infect other tree species of economic interest, such as peach trees. Only one peach orchard was investigated in this study and the population tested was found to be sensitive to carbendazim. It is clearly important for the 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 metabolite in in vitro spore germination bioassays.

Acknowledgements

We would like to thank all the field workers who participated in sample collection. This project was partly funded and supported by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) via the tax on sales of plant protection products. Proceeds from this tax are allocated to ANSES to finance a "phytopharmacovigilance" (PPV) system for monitoring the adverse effects of plant protection products, pursuant to the French Act on the Future of Agriculture of 13 October 2014.

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.

References

Bai, Q., Zhai, L., Chen, X., Hong, N., Xu, W. & Wang, G. (2015) Biological and molecular characterization of five *Phomopsis* Species associated with pear shoot canker in China. *Plant Disease*, 99, 1704–1712.

Ballio, A., Chain, E.B., De Leo, P., Erlanger, B.F., Mauri, M. & Tonolo, A. (1964) Fusicoccin: a new wilting toxin produced by *Fusicoccum amygdali* Del. *Nature*, 203, 297.

Barrès B. (2020). Supporting data and code for: Report of a new 8 resistance to carbendazim in *Fusicoccum amygdali*, the causal agent of constriction canker of 9 peach and almond trees. *Zenodo*. http://doi.org/10.5281/zenodo.3473232.

Bienapfl, J. & Balci, Y. (2013) Phomopsis blight: a new disease of *Pieris japonica* caused by *Phomopsis amygdali* in the United States. *Plant Disease*, 97, 1403–1407.

Bruce, R.D. & Versteeg, D.J. (1992) A statistical procedure for modeling continuous toxicity data. *Environmental Toxicology and Chemistry*, 11, 1485–1494.

Carter, H.E., Cools, H.J., West, J.S., Shaw, M.W. & Fraaije, B.A. (2013) Detection and molecular characterisation of *Pyrenopeziza brassicae* isolates resistant to methyl benzimidazole carbamates. *Pest Management Science*, 69, 1040–1048.

Froelich, M.H. & Schnabel, G. (2019) Investigation of Fungi causing twig blight diseases on peach trees in South Carolina. *Plant Disease*, 103, 705–710.

Glass, N.L. & Donaldson, G.C. (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, 61, 1323–1330.

Jeschke, P. (2016) Propesticides and their use as agrochemicals. *Pest Management Science*, 72, 210–225.

Ji, Z., Zhang, H., Jin, J., Xiong, C. & Xu, J. (2013) Virulence and control efficacy in field of fungicides on *Phomopsis amygdali* of peach. *Journal of Fruit Science*, 30, 281–284.

377 Koenraadt, H., Somerville, S.C. & Jones, A. (1992) Characterization of mutations in the β-
378 tubulin gene of benomyl-resistant field strains of *Venturia inaequalis* and other plant
379 pathogenic fungi. *Phytopathology*, 82, 1348–1354.

380 Lalancette, N. & Polk, D.F. (2000) Estimating yield and economic loss from constriction
381 canker of peach. *Plant Disease*, 84, 941–946.

382 Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., et al. (2002) Mechanisms
383 of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Management*
384 *Science*, 58, 876–888.

385 Lin, T., Xu, X., Dai, D., Shi, H., Wang, H. & Zhang, C. (2016) Differentiation in
386 development of benzimidazole resistance in *Colletotrichum gloeosporioides* complex
387 populations from strawberry and grape hosts. *Australasian Plant Pathology*, 45, 241–
388 249.

389 Liu, S., Che, Z. & Chen, G. (2016) Multiple-fungicide resistance to carbendazim,
390 diethofencarb, procymidone, and pyrimethanil in field isolates of *Botrytis cinerea* from
391 tomato in Henan Province, China. *Crop Protection*, 84, 56–61.

392 Ma, Z. & Michailides, T.J. (2005) Advances in understanding molecular mechanisms of
393 fungicide resistance and molecular detection of resistant genotypes in phytopathogenic
394 fungi. *Crop Protection*, 24, 853–863.

395 Malandrakis, A., Markoglou, A. & Ziogas, B. (2011) Molecular characterization of
396 benzimidazole-resistant *Botrytis cinerea* field isolates with reduced or enhanced
397 sensitivity to zoxamide and diethofencarb. *Pesticide Biochemistry and Physiology*, 99,
398 118–124.

399 Malandrakis, A.A., Markoglou, A.N. & Ziogas, B.N. (2012) PCR-RFLP detection of the
400 E198A mutation conferring resistance to benzimidazoles in field isolates of *Monilinia*
401 *laxa* from Greece. *Crop Protection*, 39, 11–17.

402 R Core Team, (2020) R: A language and environment for statistical computing: R Foundation
403 for statistical computing (Version 4.0. 3) at http://www.r-project.org.

404 Ritz, C., Baty, F., Streibig, J.C. & Gerhard, D. (2015) Dose–response analysis using R. *PLoS*
405 *One*, 10, e0146021.

406 Ritz, C., Jensen, S.M., Gerhard, D. & Streibig, J.C. (2019). *Dose–response analysis using R.*
407 CRC Press.

408 Van Der Hoeven, N. (1997) How to measure no effect. Part III: statistical aspects of NOEC,
409 ECx and NEC estimates. *Environmetrics: The official journal of the International*
410 *Environmetrics Society*, 8(3), 255-261.

411 Varjas, V., Vajna, L., Izsépi, F., Nagy, G. & Pájtli, É. (2017) First report of *Phomopsis*
412 *amygdali* causing twig canker on almond in Hungary. *Plant Disease*, 101, 1674.

413 Yarden, O. & Katan, T. (1993) Mutations leading to substitutions at amino acids 198 and 200
414 of beta-tubulin that correlate with benomyl-resistance phenotypes of field strains of
415 *Botrytis cinerea*. *Phytopathology*, 83, 1478–1483.

416 Yin, L.F., Ma, Q.Y. & Chen, Y. (2011) Identification of peach constriction canker disease.
417 *Southwest China Journal of Agricultural Sciences*, 5, 026.

418 Young, D.H. (2015) Anti-tubulin agents. In: Ishii H and Hollomon DW (Eds.) *Fungicide*
419 *resistance in plant pathogens.* Tokyo: Springer, pp. 93–103.

Supporting Information

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

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

File S3 Estimated EC_{50} values and associated standard error based on dose–response bioassay for two active substances (carbendazim and diethofencarb) on the mycelial growth of isolates of *Fusicoccum amygdali*. Figure 3 is based on these results.

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

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.

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

Figure 3 Ranking of isolates of *Fusicoccum amygdali* according to their EC_{50} values in mycelial growth assays on media amended with (a) carbendazim and (b) diethofencarb. The isolates are arranged in order of increasing EC_{50}. The reference isolate, the isolates from the sensitive population and the isolates from the resistant population are depicted in blue, black and red, respectively. The different symbols stand for the different populations of origin of the isolates. The error bars indicate the associated approximate standard errors estimated 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, the error bars are not always visible.