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Sensitivity of *Podosphaera aphanis* isolates to DMI fungicides: distribution and reduced cross-sensitivity

Audrey Sombardier,[a,b] Marie-Cécile Dufour,[a] Dominique Blancard[a] and Marie-France Corio-Costet[a]*

Abstract

BACKGROUND: Management of strawberry powdery mildew, *Podopshaera aphanis* (Wallr.), requires numerous fungicide treatments. Limiting epidemics is heavily dependent on sterol demethylation inhibitors (DMIs) such as myclobutanil or penconazole. Recently, a noticeable reduction in the efficacy of these triazole fungicides was reported by strawberry growers in France. The goal of this study was to investigate the state of DMI sensitivity of French *P. aphanis* and provide tools for improved pest management.

RESULTS: Using leaf disc sporulation assays, sensitivity to myclobutanil and penconazole of 23 isolates of *P. aphanis* was monitored. Myclobutanil EC_{50} ranged from less than 0.1 to 14.67 mg L^{-1} and for penconazole from 0.04 to 4.2 mg L^{-1}. A cross-analysis and a Venn diagram showed that there was reduced sensitivity and a positive correlation between the less sensitive myclobutanil and penconazole isolates; 73.9% of isolates were less sensitive to a DMI and 47.8% exhibited less sensitivity to both fungicides.

CONCLUSION: The results show that sensitivity to myclobutanil and, to a lesser extent, penconazole has become less efficient in strawberry powdery mildew in France. Therefore, urgent action is required in order to document its appearance and optimise methods of control.

Keywords: cross-resistance; powdery mildew; DMI fungicide; sensitivity; myclobutanil; penconazole; strawberry

1 INTRODUCTION

Strawberry powdery mildew caused by the obligate parasite *Podosphaera aphanis* (Wallr.)[1] [formerly *Sphaerotheca macularis* f. *fragaria* (Harz)] occurs worldwide and affects all aerial plant tissues. Infections can cause severe losses in yield in temperate climates. In France, plastic tunnel and greenhouse soilless systems are common, as in the rest of Europe, and favour the growth of *P. aphanis*.[2] These production systems are associated with increased difficulties in controlling powdery mildew as compared with production in open fields.[3] Indeed, the development of powdery mildew is favoured by low light intensity, moderate-to-high relative humidity and temperatures ranging from 15 to 27 °C.[3] These production systems and the need to harvest throughout the year according to market conditions, with multiple harvests on double-cropping varieties, mean that many fungicide treatments (15–20) may be required to fight against pathogens such as *Botrytis cinerea* Pers., *Colletotrichum acutatum* Simmonds, *Alternaria alternata* (Fr.) Keissler and *Zythia fragariae* Laibach. In France, strawberry powdery mildew poses a real difficulty for both short cropping (one flowering cycle with one harvest period) and double cropping (two flowering cycles with two harvest periods). Currently, there is no curative method efficient enough to control soilless production in tunnels in France. As in Italy,[2] soilless production in tunnels generally requires at least six or eight and sometimes as many as 15 fungicide treatments per growing cycle.

Systemic single-site mode of action fungicides designed for strawberry powdery mildew in France include one QoI (quinone outside inhibitor) azoxystrobin, bupirimate, and two DMIs (14α-demethylase inhibitors), myclobutanil and penconazole, together with a contact fungicide, sulfur.[4] The DMI fungicides are a large class of sterol biosynthesis inhibitors that inhibit the sterol biosynthesis of most true fungi.[5] DMI fungicides inhibit the C_{14} demethylation of lanosterol or eburicol (24-methylene-24,25-dihydrolanosterol), an important step of sterol biosynthesis (ergosterol or methylene-24-cholesterol) in fungi.[6] The target enzyme is a cytochrome P450 14α-demethylase, leading to disruption of membrane function and dysregulation of the cell multiplication cycle.[7,8] Owing to their efficacy and specificity, these fungicides are particularly useful for controlling fungal

* *Correspondence to: Marie-France Corio-Costet, INRA, UMR Santé Végétale, 1065, ISVV, IFR 103, BP 81, 33883 Villenave d'Ornon, France. E-mail: coriocos@bordeaux.inra.fr*

a *INRA, UMR Santé Végétale, Villenave d'Ornon, France*

b *CTIFL, Centre Technique Inter-professionnel des Fruits et Légumes, Bergerac, France*

plant pathogens. In spite of a history of success in the past 20 years, resistance to some DMI fungicides has become common for plentiful fungal plant pathogens including *Blumeriella jaapii* Arx,[9] *Cercospora beticola* Sacc.,[10] *Colletotrichum cereale* Manns.,[11] *Monilinia fructicola* (Winter) Honey,[12] *Mycosphaerella fijiensis* Morelet,[13] *Mycosphaerella graminicola* (Fuckel) Schroter,[14] *Nectria haematococca* Berk. & Broome,[15] *Penicillium digitatum* (Pers.) Sacc.,[16] *Pyrenophora teres* Dreschler,[17] *Rhynchosporium secalis* (Oudem.) Davis,[18] *Sclerotinia homeocarpa* Bennett[19] and *Venturia inaequalis* (Cooke) Winter.[20,21] DMI fungicides are also among the most effective and widely used fungicides for powdery mildews, and loss of efficacy or resistance has been described for *Blumeria graminis* Speer,[22,23] *Erysiphe necator* Schw.[24–26] and *Podosphaera xanthii* Braun & Shishkoff.[27] Regarding sensitivity of strawberry powdery mildew to DMI fungicides, a reduction in nuarimol, triadimefon and fenarimol efficacy was noted in Belgium after 8 years of use, whereas penconazole still exhibited good efficacy.[28] In Japan, field trials exhibited a decreased sensitivity to bitertanol, fenarimol, triforine and triadimenol.[29] The intensive use of site-specific fungicides in agricultural production, which typically involves multiple applications over wide areas, represents a potent selective mechanism for increasing the frequency of fungicide-resistant isolates within populations. Depending on the stringency of selection pressure, sensitivity of *P. aphanis* populations may therefore shift gradually from baseline sensitivity to a distinct level of resistance.

After widespread application, the use of DMIs, of which myclobutanil was the first to be registered for strawberry in France in 1991, was limited to a maximum of three applications, and the alternation of myclobutanil and penconazole with fungicides having a different mode of action was strongly recommended. In spite of the recommendation not to use DMI fungicides more than 3 times per growing cycle, often at least three treatments are performed and sometimes as many as eight (range 2–8). Treatments still depend on whether short cropping (one growing cycle with one harvest) or double cropping (two growing cycles with two harvests) is being practised. Three years ago, a growers' survey carried out on 95 strawberry farms showed a reduced efficacy of DMI fungicides widely applied as preventive treatments to fight against *P. aphanis* (Pommier J-J, private communication, 2006). This resulted in a decrease in the average number of DMI treatments (−30%) on farms where the powdery mildew attacks were extensive in order to obtain better efficacy against *P. aphanis* (Bardet A, private communication, 2008). Moreover, preliminary results obtained after six DMI treatments in open-field cultivation showed a significant loss of efficacy, with attack intensity similar to those of trials without treatment. Furthermore, in treatment programmes including six DMI fungicides out of 11 treatments, a loss of efficacy was observed that was comparable with simple water microaspersion, which gave an increase in protection from 20 to 50% over that obtained with DMI fungicides. The authors suspected a loss of sensitivity of DMI fungicides to *P. aphanis*, which may be one of the factors explaining the difficulty in controlling this disease. However, cross-resistance between different DMI fungicides was not systematically noted, and as yet there have been no reports on myclobutanil and penconazole resistance in strawberry powdery mildew populations.

Resistance to DMI fungicides is under multigenic control, increasing gradually by the additive action of different resistant genes.[30–32] Various mechanisms are involved such as (i) mutations of the target-encoding gene (CYP51), which affect the binding affinity of CYP51 for DMIs,[23,31–36] (ii) overpression or increased copy number of *Cyp51* gene, leading to increased production of the target enzyme,[31,32,37–41] (iii) high expression of energy-dependent drug efflux proteins of the ATP-binding cassette (ABC) transporter family[32,42] and (iv) defection in Δ^{5-6} desaturase.[43,44]

To the best of the authors' knowledge, no reports have been published on sensitivity to penconazole and myclobutanil in *P. aphanis*. Investigating the potential presence of resistant isolates in strawberry crops in France was one of the major objectives of the present work, the overall objective being (i) to develop a reproducible method to assay individual *P. aphanis* isolates for sensitivity to DMI fungicides, (ii) to determine the distribution of sensitivities to DMI fungicides among isolates of *P. aphanis* and (iii) to investigate the degree of potential cross-resistance between two DMI fungicides commonly used to control strawberry powdery mildew in France.

2 MATERIALS AND METHODS

2.1 Isolate collection of *Podosphaera aphanis*

A total of 24 single-spore *P. aphanis* isolates collected in France between December 2006 and December 2007 at different seasons of the year in various production systems (open-field systems, soilless systems, soilless systems in heated tunnels) were used (Table 1). All samples were typically collected from crop systems that had been exposed to penconazole or myclobutanil several times (at least five DMI treatments) and where an efficacy problem had been observed (12 sampling sites) or had not been observed (12 sampling sites) 1 or more times in the 3 years. The powdery mildew from the infected leaves was transferred to the abaxial surface of cv. Darselect leaves by rubbing. The susceptible cv. Darselect was used for all experiments: maintenance, testing and single-spore isolates.

2.2 Plant material

Whole abaxial surfaces of detached leaves from two-month-old strawberry plants grown in the greenhouse at 25 °C with a 15 : 9 h light : dark photoperiod were disinfected by immersion (10 min) in calcium hypochlorite (32.5 g L^{-1}), rinsed and dried between two sheets of sterilised filter paper and placed in Petri dishes containing water agar (20 g L^{-1}) supplemented with benzimidazole (30 mg L^{-1}). Live plants at leaf stage 3–5 were produced at CTIFL Nîmes (France) in tubes in rooting medium (diameter 2 cm).

2.3 Single-spore and inoculum production and storage

The fungus was maintained on the lower surface of young leaves from Darselect cultivar as described in Section 2.4. From 24 collected samples from different localities, monocodial isolates were obtained by picking a single powdery mildew conidium from mildewed samples within a laminar flow hood using an eyelash fastened to a holder. Infections were established on 18 mm diameter leaf discs excised from surface-disinfected, powdery-mildew-free Darselect leaves. For each isolate, the procedure was conducted twice in succession over two generations of the fungus to ensure purity of the single-spore isolates.

After growth, *P. aphanis* isolates were inoculated under sterile conditions on the lower abaxial surface of strawberry leaves placed inside a Plexiglas spore-settling tower (25 × 25 × 60 cm high) by blowing conidia from sporulating leaves or discs using a pump connected to a flexible plastic tube terminating in a Pasteur pipette. The inoculated leaves in Petri dishes were removed from the tower and the lids were replaced. The inoculated leaves or discs

Table 1. Location, host, crop system and dates of collection of *Podosphaera aphanis* isolates

Isolate[a]	Agricultural region[b]	Cultivar	Crop system[c]	Month and year of isolation
ABA	Vaucluse (84)	Gariguette	Soilless	December 2006
AVI	Dordogne (24)	Mara des bois	Soilless	August 2006
ART*	Lot et Garonne (47)	Gariguette	Soilless	April 2007
BRA	Loir et Cher (41)	Cirafine	Soilless	July 2007
BRE*	Vaucluse (84)	Cléry	Soilless	October 2007
CAM	Lot et Garonne (47)	Gariguette	Soilless	May 2007
DAR1*	Lot et Garonne (47)	Darselect	Open field	September 2006
DAR18*	Lot et Garonne (47)	Darselect	Open field	September 2006
DOU*	Dordogne (24)	Gariguette	Soilless	April 2006
GUI	Vaucluse (84)	Cléry	Soilless	October 2007
HOR	Dordogne (24)	Mara des bois	Soilless	July 2007
LBP*	Landes (40)	Darselect	Open field	July 2007
LI1*	Lot et Garonne (47)	Gariguette	Soilless	April 2007
MAR*	Lot et Garonne (47)	Gariguette	Open field	August 2007
MEG	Vaucluse (84)	Gariguette	Field	November 2007
MEP	Vaucluse (84)	Pajaro	Field	November 2007
PAJ*	Lot et Garonne (47)	Darselect	Soilless	September 2006
PES	Vaucluse (84)	Pajaro	Field	April 2007
SAG	Lot et Garonne (47)	Gariguette	Soilless	April 2007
SIL	Lot et Garonne (47)	Gariguette	Soilless	April 2007
SOC*	Vaucluse (84)	Cléry	Soilless	October 2007
SOD*	Vaucluse (84)	Darselect	Soilless	October 2007
SOU*	Vaucluse (84)	Gariguette	Soilless	April 2007
VIR	Vaucluse (84)	Gariguette	Soilless	November 2007

[a] * Isolates collected in strawberry cultivations having one or two consecutive problems with their fungicide treatment programmes, including more than five DMI fungicide treatments.
[b] Production department with number of French departments.
[c] All crop systems were under shelter except for four open-field isolates (DAR, DAR18, LBP and MAR).

were placed in a growth chamber (mean temperature 22 °C, with 16 h day^{-1} light, 35 µm m^{-2} s^{-1}) for 15 days. The infected leaves were harvested, and *P. aphanis* conidia were used for inoculum production.

To store the different single-spore isolates, live plants were inoculated by rubbing their leaves with a piece of the mildew-infected leaf. After 8 days of incubation, live plants were placed under conservation conditions at a temperature of 9 ± 0.1 °C with a 16 : 8 h light : dark photoperiod. Successive transfers were performed every 8–12 weeks.

2.4 Determination of fungicide sensitivity

Assays were adapted from the technique described in detail by Délye *et al.*[24] and Debieu *et al.*[45] Briefly, cv. Darselect leaf discs (16 mm diameter) with their abaxial surface exposed were prepared as described in Section 2.3, taking care to excise them from areas of the leaves without major veins to minimise the aspect of raised butterfly wings. Excised discs were randomised and placed lower surface exposed on moist filter paper in Petri dishes, with eight repetitions for each concentration. Experiments were also independently duplicated. The formulated fungicides tested were two triazoles, myclobutanil [2-*p*-chlorophenyl-2-(1*H*-1,2,4-triazol-1-ylmethyl)hexanenitrile] 125 g L^{-1} EW (Systane 12E; Dow Agrosciences) and penconazole [1-(2,4-dichloro-*β*-propylphenethyl)-1*H*-1,2,4-triazole] 100 g L^{-1} EC (Topaze; Syngenta Agro). Fungicides were applied by spraying in water dispersions at required concentrations, using a hand-sprayer to spray 10 µL cm^{-2} leaf. Fungicide testing was performed at least twice with a range of nine concentrations adapted to the response of each isolate in order to obtain a dose–response curve (growth inhibition plotted versus fungicide concentration). Standard myclobutanil concentrations used were 0, 1, 2, 3, 4, 5, 8, 10 and 15 mg AI L^{-1}. According to the sensitivity of each isolate, a higher range of concentrations (0, 1, 2.5, 5, 10, 15, 20, 30 and 40 mg L^{-1}) and a lower range (0, 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5 and 3 mg L^{-1}) were also used. For very resistant or very sensitive isolates, a specific range was prepared. The standard penconazole range was 0, 0.5, 1, 2, 3, 4, 5, 8 and 10 mg AI L^{-1}. As previously, a higher range (0, 1, 3, 5, 6.5, 8, 10, 15 and 20 mg L^{-1}) and a lower range (0, 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5 and 3 mg L^{-1}) were also used. After 17 h incubation at 22 °C, the leaf discs were dried and inoculated in an inoculum tower as described in Section 2.3. The nine sets of eight discs were inoculated simultaneously in the same tower with *P. aphanis* conidia and incubated at 22 °C with alternating 16 h day^{-1} light. Inoculum density (400–600 conidia cm^{-2} leaf) was determined by placing a hematocytometer among the plates and counting the settled conidia. Petri dishes were sealed with plastic film, and inoculated discs were incubated for 8 days under the conditions described above. Eight days after inoculation, mycelial expansion on leaf discs was determined, using a stereomicroscope, by assessing the percentage of leaf disc surface covered with sporulating powdery mildew for each fungicide concentration. Measurements were made for each concentration, and the mean values for the eight replicates of leaf discs per treatment were used to calculate the relative growth (RG) [(values obtained for a concentration/value of control

Table 2. Sensitivity of *Podosphaera aphanis* to myclobutanil and penconazole

	Myclobutanil concentration (mg L^{-1}) (±SD)		Penconazole concentration (mg L^{-1}) (±SD)	
Isolate	EC_{50}[a]	MIC	EC_{50}[b]	MIC
ABA	3.75 (±0.35)	9.0 (±1.41)	1.25 (±0.07)	2.85 (±0.21)
AVI	0.5 (±0.14)	2.0 (±1.41)	0.58 (±0.11)	1.8 (±0.28)
ART	3.9 (±0.14)	10.7 (±0.35)	2.2 (±0.28)	6.6 (±0.14)
BRA	1.3 (±0.5)	3.0 (±0.7)	1.48 (±0.04)	2.6 (±0.14)
BRE	5.3 (±1.56)	13.7 (±53.18)	1.9 (±0.14)	6.0 (±0.71)
CAM	2.6 (±0.54)	5.5 (±0.25)	0.9 (±0.15)	2.5 (±0.21)
DAR1	4.75 (±0.71)	15 (±4.01)	2 (±0.3)	3.0 (±0.17)
DAR18	14.67 (±2.31)	31 (±1.73)	3.5 (±0.71)	9.0 (±0)
DOU	8.9 (±0.14)	15.5 (±0.71)	1.3 (±0.006)	2.0 (±0.16)
GUI	1.15 (±0.49)	2.35 (±0.49)	0.9 (±0.14)	2.1 (±0.14)
HOR	4.25 (±0.35)	10.75 (±3.89)	0.8 (±0.09)	2.0 (±0.14)
LBP	4.8 (±0.47)	12.5 (±1.98)	4.2 (±0.85)	9.5 (±0.21)
LI1	3.6 (±0.22)	8.5 (±1.25)	2.45 (±0.07)	5.15 (±0.21)
MAR	5.5 (±0.71)	16 (±1.41)	2.55 (±0.35)	10 (±0)
MEG	1.0 (±0.71)	2.35 (±0.64)	0.43 (±0.08)	1.0 (±0.22)
MEP	0.8 (±0.22)	3 (±0.7)	0.3 (±0.065)	0.8 (±0.19)
PAJ	3.0 (±2.26)	6.9 (±0.14)	2.9 (±0.14)	3.9 (±0.14)
PES	<0.1	<0.1	0.04 (±0.005)	0.15 (±0.018)
SAG	1.7 (±0.26)	5.33 (±1.53)	0.28 (±0.08)	0.5 (±0)
SIL	2.0 (±0.3)	6.5 (±0.35)	0.4 (±0.12)	1.0 (±0.25)
SOC	6.45 (±3.18)	26.0 (±1.41)	3.2 (±0.28)	7.9 (±0.14)
SOD	13.1 (±0.14)	21.5 (±2.12)	–	–
SOU	10 (±0)	23.0 (±8.49)	2.1 (±0.27)	9.8 (±0.18)
VIR	1.8 (±0.27)	5.0 (±0.5)	2.0 (±0.11)	5.0 (±0.21)

[a] Standard deviation of EC_{50} to myclobutanil ranged from 0 to 3.18 mg L^{-1}, with a mean SD of 20.44% of each value.
[b] The standard deviation of EC_{50} to penconazole ranged from 0.005 to 0.85 mg L^{-1}, with a mean SD of 13.51% of each value.

without treatment) × 100]. Dose–response curves for individual isolates were generated by plotting the RG values against $\log_{10}$ of the fungicide concentration used (Microsoft Excel 7.0, USA), and the $\log_{10}$ effective dose to reduce growth by 50% (EC_{50}) was calculated from the regression equation generated through the linear portion of the sigmoid curve. The minimal inhibitory concentration (MIC) was also calculated as described by Délye *et al.*[24]

Mean log EC_{50} values for each isolate for the two fungicides were analysed using ANOVA, whereas mean log EC_{50} values for the same fungicide between isolates or groups were compared using the Tukey *t*-test with $P < 0.05$ (Systat11 software, 2004, USA). Cross-sensitivity between myclobutanil and penconazole was assessed by regression analysis, comparing log EC_{50} values for individual isolates for myclobutanil and penconazole. The relative sensitivity factor (RSF) was calculated for different groups or isolates as RSF = EC_{50} of least sensitive isolate or group/mean EC_{50} of sensitive isolate or group.

3 RESULTS

3.1 Determination of myclobutanil sensitivity

Throughout the study, the isolates were tested independently at least twice to evaluate the EC_{50} and MIC (Table 2). EC_{50} values ranged from less than 0.1 mg L^{-1} to 14.67 mg L^{-1}. Three significant groups were identified (Table 3): a first group A of six isolates exhibiting an MIC ranging from less than 0.1 mg L^{-1} to 3 mg L^{-1}, with a hypersensitive isolate (PES) exhibiting an MIC more than 20 times lower than other isolates in this group (the mean EC_{50} was 0.81 ± 0.126 mg L^{-1} and the mean MIC was 2.13 ± 0.147 mg L^{-1}); a second group B of 14 isolates with EC_{50} ranging from 1.70 to 6.45 mg L^{-1} (the mean EC_{50} was 3.81 ± 0.39 mg L^{-1} and the mean MIC was 10.82 ± 1.52 mg L^{-1}); and a third group C of four isolates with EC_{50} ranging from 8.9 to 14.67 mg L^{-1} (the mean EC_{50} was 11.67 ± 1.34 mg L^{-1} and the mean MIC was 22.75 ± 3.19 mg L^{-1}) (Tables 2 and 3). The distribution of the 24 isolates showed a high frequency (0.74) of isolates with reduced sensitivity to myclobutanil (groups B and C) (Table 3). The RSF was 4 for group B versus group A, and 14.40 and 3.06 respectively for group C versus group A and for

Table 3. Sensitivity of *Podosphaerea aphanis* distribution to myclobutanil

Group[a]	*N*	Mean EC_{50} (mg L^{-1}) (±SEM)	Mean MIC (mg L^{-1}) (±SEM)	Frequency
A	6	0.81 (±0.126)[b]	2.13 (±0.147)[b]	0.25
B	14	3.81 (±0.39)	10.82 (±1.52)	0.58
C	4	11.67 (±1.34)	22.75 (±3.19)	0.17

[a] Isolates belonging to group A: PES, AVI, MEP, MEG, GUI, BRA; isolates belonging to group B: SAG, VIR, SIL, CAM, PAJ, ABA, LI1, ART, DAR, HOR, LBP, BRE, MAR, SOC; isolates belonging to group C: DOU, SOU, SOD, DAR18.
[b] Mean of EC_{50} and MIC calculated without PES isolate.

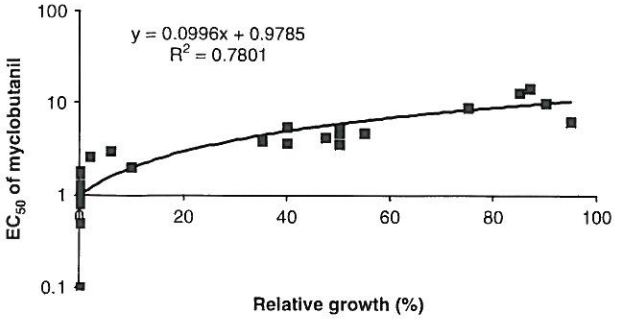

Figure 1. Relationship between relative growth (compared with control treatment) of individual isolates of *Podosphaera aphanis* on strawberry leaf discs treated with a single discriminatory dose of myclobutanil at 5 mg L^{-1} and EC_{50} values. The EC_{50} values are plotted on a logarithmic *Y* axis for better differentiation of the lower values.

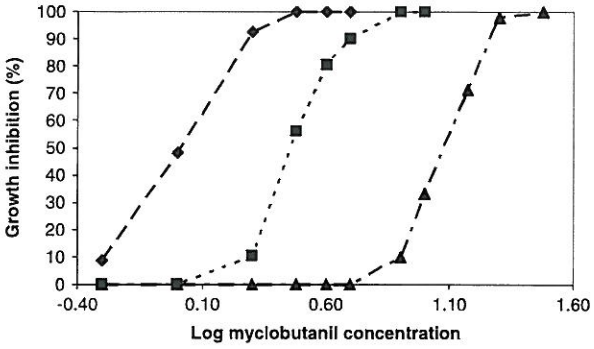

Figure 2. Dose responses to myclobutanil of three representative isolates of *Podosphaera aphanis*: –♦–, isolate BRA; --■--, isolate PAJ; --▲--, isolate SOU.

group C versus group B. The RSF between the most sensitive isolate (PES) and the most resistant one (DAR18) was higher than 146-fold.

To quantify isolate sensitivity to myclobutanil, a discriminating dose of 5 mg L^{-1} was used, reflecting a concentration higher than the mean EC_{50} and MIC of the sensitive group A (Fig. 1). Thus, the three groups were established after determining the best correlation between the relative growth values with myclobutanil EC_{50} values using all isolates. A significant relationship was described by the function (EC_{50} = 0.0996RG + 0.9785), with R^2 = 0.7801 ($P < 0.0001$). Finally, dose–response curves of representative isolates of each group unambiguously showed a large variation in dose responses to higher myclobutanil concentrations (Fig. 2).

3.2 Determination of penconazole sensitivity

Throughout this study, 23 isolates were tested independently twice to evaluate the EC_{50} and MIC (Table 2). EC_{50} values ranged from 0.04 to 4.2 mg L^{-1} and MIC values ranged from 0.15 to 9.8 mg L^{-1}. Six groups A′, AB, BC, CD, D and E were identified, with very sensitive group A′, groups of intermediary sensitivity AB, BC and CD and reduced-sensitivity groups D and E (Table 4). These groups showed a progressive distribution of the *P. aphanis* distribution. Group A′ contained six sensitive isolates, of which four were common with group A sensitive to myclobutanil. EC_{50} ranged from 0.04 to 0.58 mg L^{-1}, with a mean of 0.34 ± 0.074 mg L^{-1} and a mean MIC of 0.88 ± 0.229 mg L^{-1}. In this case, the

Table 4. Sensitivity of *Podosphaera aphanis* distribution to penconazole

Group[a]	*N*	Mean EC_{50} (mg L^{-1}) (±SEM)	Mean MIC (mg L^{-1}) (±SEM)	Frequency
A′	6	0.34 (±0.074)	0.88 (±0.229)	0.26
AB	3	0.87 (±0.035)	2.20 (±0.150)	0.13
BC	3	1.34 (±0.069)	2.48 (±0.250)	0.13
CD	3	2.3 (±0.300)	3.97 (±0.709)	0.13
D	6	2.40 (±0.188)	7.58 (±0.823)	0.26
E	2	3.85 (±0.347)	9.25 (±0.248)	0.09

[a] Isolates belonging to group A′: **PES, AVI, MEP, MEG, SIL, SAG**; isolates belonging to group AB: GUI, CAM, HOR; isolates belonging to group BC: BRA, ABA, DOU; isolates belonging to group CD: **PAJ, VIR, DAR**; isolates belonging to group D: **LI1, ART, BRE, MAR, SOC, SOU**; isolates belonging to group E: **DAR18, LBP**. In bold, isolates common with groups of myclobutanil sensitivity.

isolate PES also was at least tenfold more sensitive than the other isolates in this group. The three intermediary groups (AB, BC, CD) contained nine isolates which were spread among myclobutanil groups A, B or C. Group D exhibited EC_{50} ranging from 1.9 to 3.2 mg L^{-1}, with a mean of 2.40 ± 0.188 mg L^{-1} and a mean MIC of 7.58 ± 0.823 mg L^{-1}, and group E exhibited EC_{50} ranging from 3.5 to 4.2 mg L^{-1}, with a mean of 3.85 ± 0.347 mg L^{-1} and a mean MIC of 9.25 ± 0.248 mg L^{-1} (Tables 2 and 4). The frequency was 0.35 for the least sensitive isolates (groups D and E) and 0.26 for the most sensitive group. The frequency for the three intermediary groups AB, BC and CD was 0.39. Regarding the RSF between the most sensitive group A′ and the reduced-sensitivity groups D and E, it was 7.05 for group A′ versus group D and 11.32 for group A versus group E. It was 3.94 for group A′ versus group BC and 5.79 for group A′ versus group CD, representing a progressive variation in sensitivity in these groups. The RSF between the most sensitive isolate (PES) and the least sensitive (LBP) was 105-fold.

To quantify isolate sensitivity to penconazole, a discriminating dose of 2 mg L^{-1} was used, reflecting a concentration higher than the mean MIC of sensitive group A′ (Fig. 3). A significant relationship was described by the function EC_{50} = (0.0297RG + 0.6271), with R^2 = 0.8592 ($P < 0.0001$). Finally, dose–response curves of representative isolates of groups A′, BC and E showed

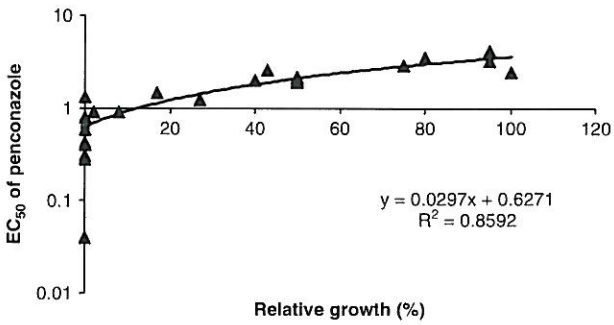

Figure 3. Relationship between relative growth (compared with the control treatment) of individual isolates of *Podosphaera aphanis* on strawberry leaf discs treated with a single discriminatory dose of penconazole at 2 mg L^{-1} and the $\log_{10}$ EC_{50} values. The EC_{50} values are plotted on a logarithmic *Y* axis for better differentiation of the lower values.

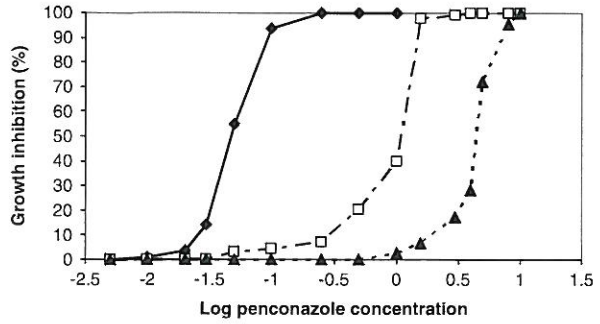

Figure 4. Dose responses of three representative isolates of *Podosphaera aphanis* to penconazole: –♦–, isolate PES; --□--, isolate ABA; --▲--, isolate LBP.

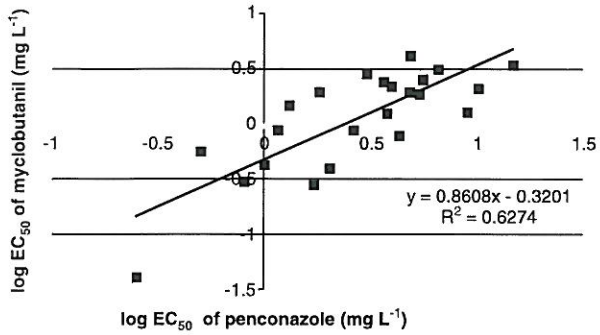

Figure 5. Relationship between sensitivity to myclobutanil and penconazole among 23 selected single-spore isolates of *Podosphaera aphanis*. The coefficient of determination and significance level are shown for the equation derived from regressing $\log_{10}$ EC_{50} values for myclobutanil on $\log_{10}$ EC_{50} values for penconazole.

a large variation in dose responses between the three isolates (Fig. 4).

3.3 Cross-sensitivity between myclobutanil and penconazole for isolates of *Podosphaeria aphanis*

The individual log EC_{50} values for each fungicide were regressed against the log EC_{50} values for the same isolates for the other fungicide and examined for correlation (Fig. 5). The linear regression analysis indicated that P values were low ($P < 0.0001$), with an R^2 value of 0.627, for these interactions, indicating a relationship between sensitivities to these two DMI fungicides. However, a Venn diagram of reduced sensitivity to myclobutanil and penconazole showed that eight isolates (DAR18, SOU, SOC, LBP, ART, LI1, PAJ, MAR) exhibited a high reduced sensitivity to the two fungicides, and that six isolates (DOU, HOR, ABA, CAM, SIL, SAG) were less sensitive to myclobutanil but sensitive to penconazole (Fig. 6). Three additional isolates belonging to group CD were significantly different from the sensitive group and presented an RSF_{EC50} of 5.75 for group A′ versus group CD. These isolates from group CD were less sensitive to both fungicides but had a twofold lower MIC than that of group D. Overall, 73.9% of the 23 isolates had high levels of reduced sensitivity to one DMI fungicide, and 47.8% had reduced sensitivity to both fungicides.

No significant difference was found between the isolates depending on south-west (departments 47, 24, 40) or east-west (84) geographic origin (Table 1). Similarly, there was no difference between crops, irrespective of their being heated under

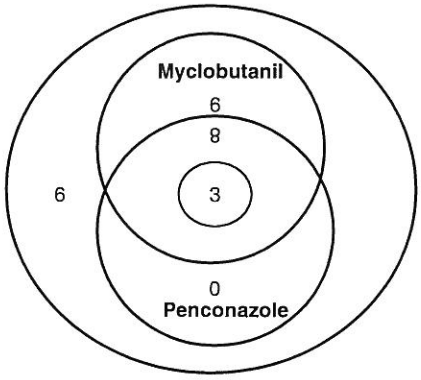

Figure 6. Venn diagram depicting the comparison of reduced sensitivity to myclobutanil and penconazole among 23 isolates of *Podosphaera aphanis*. Specific EC_{50} values classifying isolates as having reduced sensitivity to myclobutanil and penconazole were ≥ 1.7 and ≥ 2 mg L^{-1} respectively. The large circle represents the full set of 23 isolates tested for reduced sensitivity to both myclobutanil and penconazole. Each smaller circle represents the set of isolates with EC_{50} values classifying them as having reduced sensitivity to the fungicide indicated according to the above criteria. The small circle at the intersection between myclobutanil and penconazole circles represents the subgroup of three isolates that were more sensitive to both fungicides than the eight present in the major circle of the intersection. Numbers within the circles represent the number of individual isolates within each subset indicated. Six isolates were classified as being sensitive to both fungicides.

a shelter or not. Except for isolates obtained in an open field under shelter (DAR, DAR18, MAR and LBP), a significant difference was obtained ($P = 0.001$) with open-field isolates, which were much less sensitive to both fungicides than those from other crop systems.

4 DISCUSSION AND CONCLUSION

In this study, a simple bioassay on leaf discs was used to establish the state of fungicide sensitivity distribution of *P. aphanis* to two triazole DMI fungicides, myclobutanil and penconazole. The method has good reproducibility, basically depending on the age of the inoculum and on using homogeneous plant material (data not shown). A large variation in the population of *P. aphanis* in French strawberry crop systems towards reduced sensitivity to myclobutanil and, to a lesser extent, penconazole has been demonstrated. The distribution of the EC_{50} of 23 isolates shows a progressive variation depending on the different mechanisms involved in the resistance acquisition process. Isolates of *P. aphanis* showing reduced sensitivity to myclobutanil and penconazole were identified in all regions and in all crop systems, especially open-field systems. Discriminatory doses (5 or 2 mg L^{-1}) of myclobutanil or penconazole were selected that were greater than the mean MIC values and could be used to select highly resistant individuals from a large population or to detect significant shifts in the field population. The mean MIC values of the most sensitive groups to myclobutanil and penconazole were 2.13 and 0.88 mg L^{-1} respectively, indicating that penconazole was the most active azole tested. This is the first study to measure *P. aphanis* sensitivity to DMI fungicides in France, and the biological test developed is a useful tool to ascertain the level of sensitivity to such treatment in strawberry crops.

The present results complement previous studies on strawberry powdery mildew resistance to DMI fungicides. Bals and Gilles[28] observed a sharp decrease in the efficiency of some triazoles on

varieties very sensitive to powdery mildew, such as Porimella, Elvira, Bogota and Rapella, in greenhouse and plastic tunnels between monitoring in 1975 and 1985. Efficacy losses of fenarimol, triadimenol, nuarimol, imazalil and prochloraz ranged from 20 to 90%. It is presumed that intensive treatments (10–14 per season) have contributed to the development and the selection of less sensitive or resistant *P. aphanis* strains. The present study found a large variation in sensitivity to myclobutanil and penconazole in isolates collected from varieties like Gariguette, Mara des bois, Cirafine, Cléry and Darselect after more than 15 years of practice. Failure with DMI fungicides depended on the pathogen. For example, only 2 years after registration for cucurbit powdery mildew (*P. xanthii*), DMI fungicides exhibited loss of efficacy.[46] When resistant strains are present at an undetectable level, the pathogen population can quickly shift to predominantly resistant strains following a single fungicide application. In the case of strawberry powdery mildew in France, it is difficult to establish a sensitivity baseline from isolates clearly identified as representative of sensitivities found in unexposed wild-type populations. However, Okayama *et al.*[47] found that a myclobutanil concentration of 1.25 mg L^{-1}, i.e. a dose similar to 1.85 mg L^{-1}, which is the mean MIC dose of the most sensitive groups (A, AB and BC) tested here, fully controlled strawberry powdery mildew on leaflets, with 0% of leaflets diseased. Furthermore, by comparing the RSF found in *P. aphanis* between the most sensitive isolates and the least sensitive with those of other pathogens resistant to DMI fungicides, *P. aphanis* exhibited an RSF to myclobutanil of 4.70–14.40. The RSFs found for *P. aphanis* were similar to the relative resistance factors (RRFs) found with *Venturia inaequalis*,[48] *Colletotrichum cereale*[11] and *Erysiphe necator*[24,25,49–51] and ranged from 5.27 to 9.42. Regarding penconazole, RSFs varied from 5.75 to 9.17 and were close to RRFs reported for *V. inaequalis*[47] and *E. necator*.[24,48] Although the effect of shifts of *P. aphanis* to myclolobutanil and penconazole has not been precisely examined to date, the mean sensitivity variations observed, roughly 5–12-fold for myclobutanil and 6–9-fold for penconazole, were associated with the development of practical resistance to both fungicides. These data were similar to those for *C. cereale*[11] or *E. necator*.[24,25,49,50] Köller *et al.*[48] reported that control was compromised in orchards when the proportion of isolates resistant to fenarimol and myclobutanil exceeded ~40%. On the basis of Köller's criteria, the present authors classified isolates of *V. inaequalis*[48] or *E. necator*[51] as resistant to DMI fungicides and applied these classifications to *P. aphanis*. Thus, it is speculated that the 73.9% frequency of low sensitivity for myclobutanil and 47.8% for penconazole cause *P. aphanis* to be categorised as potentially resistant according to the terms of Köller *et al.*,[20] and this may represent a threshold with respect to the development of practical resistance to these fungicides. However, no monitoring was carried out to show that the general sensitivity profile of *P. aphanis* has been modified. Currently, potential strawberry powdery mildew DMI resistance is difficult to demonstrate without available baseline sensitivity measures. Only the known loss of efficacy of DMIs in strawberry cultivation might supply a probe for the presence of resistant isolates of *P. aphanis*. This is the case here only for the site providing isolates DAR1 and DAR18, where a real loss of efficacy of six straight treatments of DMI fungicides (four myclobutanil treatments and two penconazole treatments) was noted. On the other hand, with regard to the distribution of the isolates obtained from strawberry farms where the efficacy problem had been identified in the previous 3 years, none of the isolates was found in the sensitive groups (A for myclobutanil and A′ or AB for penconazole). However, the loss of efficacy in these cultivations could also have resulted from poor use of the fungicide. Nevertheless, the data obtained and comparisons with literature data on the presence or absence of resistant isolates in different pathogen populations suggest that resistant isolates are present in strawberry cultivations in France. This is of interest, because it seems that the resistance is not widespread, and it may be possible to obtain a better understanding of the resistance evolution in cultivations.

Indeed, as DMI resistance results from the modification of several interacting genes, pathogens exhibit a range of sensitivity to the two fungicides, depending on the number of gene changes. Different mechanisms, common or not, may be required to exhibit loss of sensitivity to myclobutanil and/or penconazole, with positive but not necessarily systematic cross-resistance. Thus, the least sensitive isolates to penconazole were also the least sensitive to myclobutanil, but the contrary was not true. The genetic correlation in resistance to both DMIs observed in this study agrees with results of previous studies also demonstrating cross-resistance among DMIs.[9–11,17,25,52] A consequence of cross-resistance is that using one fungicide in a chemical group (e.g myclobutanil) selects for strains less sensitive to other closely related fungicides (e.g. penconazole). This cross-resistance has important implications for DMI resistance in the field as well as for the practical management of resistance, and suggests that genetically correlated traits do not evolve independently. Currently, the presence of a single nucleotide polymorphism (SNP) in the $CYP51_{A1}$ gene is suspected, as demonstrated for other obligate pathogens, *B. graminis*[33] and *E. necator*,[34] and the present authors are going to sequence the CYP51 gene to determine the presence or absence of SNPs in the 14-demethylase gene, correlated or uncorrelated with loss of sensitivity to myclobutanil and penconazole. Nevertheless, other mechanisms are probably involved additively or synergistically with mutations in the CYP51 gene; this could explain the lack of perfect reduced cross-sensitivity between isolates to the two triazole fungicides. Thus, the practical significance of apparent differences in reduced cross-sensitivity among isolates can be determined conclusively only by monitoring shifts in sensitivity distributions and fungicide performances in field experiments.

Resistance of *P. aphanis* to DMI fungicides has not been previously demonstrated in France, in spite of reports by growers of poor powdery mildew control with myclobutanil and penconazole and the decrease in DMIs in the last 3 years (CASDAR survey 2005–2008) (Pommier J-J, private communication, 2006). The development of fungicide resistance in field populations may be influenced by the genetics of resistance, by any fitness costs associated with resistance and by the reproductive biology (sexual or asexual) of the pathogen. When resistance to a fungicide is detected, an alternative fungicide with a different mode of action should be used, and strict sanitation measures should be employed. For strawberry powdery mildew, only azoxystrobine, bupirimate and sulfur remain available in France if DMI fungicides are not used. Given the high risk of the development of resistance to QoIs, strawberry growers find themselves in a delicate situation, where the classic method of alternation and limitation of DMI use cannot be implemented. Indeed, fungicides that are systemic or have translaminar activity are needed to obtain adequate protection of abaxial leaf surfaces, where the conditions are more favourable for development of the pathogen than on adaxial surfaces. The development of resistance may not be detected when multisite-contact companion fungicides are used. Such

fungicides effectively control any resistant strains on adaxial leaf surfaces, but selection of resistant strains may still occur on abaxial surfaces where spray deposits may be inadequate. Given that numerous cleistothecia (sexual reproduction organs) were found in all regions, as with other pathogens, sexual reproduction probably plays a part in the spread of reduced sensitivity. One opportunity to limit the spread of reduced sensitivity may be to develop pest management methods based on the control of cleistothecia production or survival by carrying out sanitation measures or by cultivation rotation. However, the life cycle of the strawberry powdery mildew fungus needs to be better understood in order to manage resistance and assess resistance risk more effectively.[53–55]

Another possibility may be to register fungicides that are efficient against powdery mildew and have a different mode of action in order to provide flexibility, or to add elemental sulfur to DMI fungicides, as has been used to improve disease control in *Monilinia fructicola* populations that have started to show signs of reduced sensitivity to DMIs.[56] The efficacy of this strategy could be investigated in *P. aphanis* populations.

Current recommendations for managing fungicide resistance include using a range of fungicides within an integrated disease management programme that includes non-chemical practices, such as the use of resistant cultivars. In France, as in Italy, 6–15 treatments against strawberry powdery mildew are required for each growing cycle in soilless cultivation in tunnels. However, there are no cultivars with acceptable fruit quality, shelf life and resistance to other important diseases that are also resistant to powdery mildew.[2] The development of resistant varieties remains one of the main challenges in restricting the development of fungicide resistance and the intensive use of chemical compounds. However, local differences may exist, reflecting differences in usage patterns, potential genetic differences in pathogens and climatic conditions more or less favouring epidemics and selection pressure. Any IPM strategy should be thought through in terms of the population size at application and the potential impact on resistance management.

In conclusion, this study presents information on methods for monitoring *P. aphanis* sensitivity to penconazole and myclobutanil, evidence for reduced sensitivity to them and evidence for reduced cross-sensitivity between them. This is the first report of sensitivity distribution to DMI fungicides in *P. aphanis*. The information and methods presented here should allow for future monitoring of DMI resistance for this pathogen. The assay using a single discriminatory dose of fungicides was able to differentiate between isolates with EC_{50} values exhibiting a reduced sensitivity, and to detect significant shifts in field populations. The findings suggest that sensitivity to penconazole and myclobutanil is diminishing in strawberry crop systems in France, and that, while awaiting the advent of new molecules or other solutions, strawberry growers in France are faced with an uphill struggle against strawberry powdery mildew. The study lays the groundwork for monitoring shifts in *P. aphanis* to DMI fungicides and evaluating the effectiveness of resistance management programmes for this pathogen. Nevertheless, the impact of variable RSFs on the performance of particular DMI fungicides under field conditions is hard to predict, and in future the combination of sanitation measures, the choice of less sensitive cultivars and cautious treatments (alternation) and elicitation of plant defences may help to limit the appearance or spread of resistance. These results lead the authors to recommend the use of DMI fungicides with caution, depending on the sensitivity level of the crop, and the alternation of this treatment with sulfur after every second application, or the use of a single QoI fungicide treatment after combined DMI and sulfur treatment. DMI fungicides used to control *P. aphanis* should not be used at reduced rates in order to maximise efficiency. Furthermore, spray intervals should not be extended because the time period between two asexual cycles in the pathogen under optimal conditions can be as little as 5 days, and therefore this is an important factor for successful treatment.

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