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Efficacy of fungicides with various modes of action in controlling the early stages of an *Erysiphe necator*-induced epidemic

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Abstract

BACKGROUND: Limiting the use of fungicides is due to become an important issue in managing *Erysiphe necator* (Schwein) Burrill infections in vineyards. The authors determined how three fungicides currently used by vine growers could be managed to control the early stages of an *E. necator*-induced epidemic.

RESULTS: Leaf-disc bioassays and field experiments suggested that the protectant quinoxyfen induced minor disruption in *E. necator* development, but compounds with protectant and curative properties (tebuconazole and trifloxystrobin) caused significant, although different, disruption during *E. necator*-induced epidemics. Bioassays showed that each of the antifungals were most effective at different stages of fungal development, tebuconazole before sporulation and trifloxystrobin after sporulation of the colonies. Results from the bioassay also highlighted likely occurrences in the field, where several stages of fungal development are encountered simultaneously.

CONCLUSION: The present findings were complementary: leaf-disc tests showed when the fungicides were most effective at inhibiting *E. necator* infection cycles; the field trial provided results in terms of incidence and severity of disease on bunches without reference to the pathogenic cycle development. A protection strategy combining the different types of fungicide under study is suggested.

Supporting information may be found in the online version of this article.

Keywords: tebuconazole; trifloxystrobin; quinoxyfen; powdery mildew; leaf-disc bioassay

1 INTRODUCTION

Powdery mildew caused by the biotrophic ascomycete *Erysiphe necator* (Schwein) Burrill is the most widespread foliar disease affecting *Vitis vinifera* L. worldwide. Each year this pathogen is responsible for damage to grapevines, resulting in significant commercial losses. In addition to direct effects, such as *E. necator*-induced yield losses,[1] indirect effects, such as off-flavours, were also observed under some environmental or enological conditions that favour the development of microorganisms grown on berries previously infected with powdery mildew.[2] However, the mushroom-type odour characterising *E. necator* disappears after alcoholic fermentation.[3] *Erysiphe necator*-induced disease remains difficult to control for at least two reasons. First, there is currently no forecast system for epidemics initiated by ascospores that predicts the timing and the number of primary infections. Recent advances[4] suggest that a logistic model can predict disease changes and extension over time, but crop heterogeneity prevents the prediction of disease severity at the vine scale. Second, without expert sampling and careful monitoring of plants in the vineyard, early signs of the disease are difficult to detect during the first few days of the epidemic.

The *E. necator* infection cycle has been widely studied by various research groups. The grape–powdery mildew pathosystem is characterised by a polycyclic pathogen capable of explosive multiplication. Ascospores released from cleistothecia that overwinter in the bark of the vine[5] and new shoots colonised by resting mycelium that overwinter within dormant buds[6] are both potential sources of primary infection. Recent progress in the genetic characterisation of *E. necator* showed that two genetically distinct groups (coded A and B) of isolates overwinter in buds and are associated with various temporal niches.[7] Rapid and extensive colonisation of plants by group B isolates was associated with the most severe cases of disease. Sporulating lesions infecting new susceptible leaves resulted in secondary infections. Colonised

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leaves provide a source of infection for developing berries, but grape berries are only susceptible over a relatively short period.[8,9] Modelling has shown that high disease severity is associated with early infection[4] as a result of strong ontogenic resistance in both leaves[10] and bunches. Consequently, there is a direct relationship between the early development of disease in leaves and subsequent damage to grapes.[11]

Grower tolerance to *E. necator* infection varies, depending on where the vineyard is located, from zero tolerance to a situation in which up to 3% of berries can be infected with powdery mildew. Consequently, developing effective control methods against *E. necator* is crucial for viticulture. In recent years, biological controls using mycoparasite fungi, e.g. *Ampelomyces quisqualis* Ces.,[12] or elicitor treatments to induce mechanisms of disease resistance in plant tissues have constituted a new way of controlling powdery mildew. However, in spite of the evidence of the potential benefits of these alternative strategies,[13] inconsistencies in field performance have been frequently reported. An absence of registered compounds prevents these products from being commercialised for use in disease management. Thus, limiting powdery mildew growth typically relies on the use of chemical fungicides. At the present time, the overall strategy is to apply fungicides in a preventive way. Around seven fungicides are routinely used to treat plants in France; this costs the agricultural industry between 60 and 70 million euros per year. However, preliminary experiments suggested that fungicide treatment frequencies can be restricted and adapted to the type of epidemic, provided that fungicide treatments are made at key periods during an epidemic.[3,14,15] Because this implies that first treatments are applied to plots and/or plants previously infected with the pathogen, fungicides are therefore required to demonstrate curative properties to disrupt the epidemic. However, owing to changes in pesticide registration procedures, and societal and political pressures in the European Union (Directive 1107/2209/ EU), drastic reduction in fungicide applications has to be planned for. As a consequence, fungicide strategies are due to change in the future.

The objective of the present study was to test the efficacy of three fungicides frequently used by growers to control powdery mildew epidemics in vineyards. Here, they are specifically used to stop *E. necator* infection at an early phase before the onset of the epidemic. Quinoxyfen, tebuconazole and trifloxystrobin belong to three different chemical fungicide families, and they directly affect essential fungal functions, such as cell division (quinoxyfen),[16] sterol biosynthesis (tebuconazole) and respiration (trifloxystrobin).[17] The fungicides were tested (1) on leaves (leaf-disc tests), to assess their efficacy in reducing pathogen development at various stages in infection cycles, and (2) in the field on artificially inoculated plots, to assess their efficacy in controlling an initiated epidemic.

2 MATERIALS AND METHODS

2.1 Leaf-disc tests: plant materials

Cuttings were grown in a greenhouse (day/night natural photoperiod) and kept free from powdery mildew using an overnight sulfur lamp. Leaves (3–4 leaves/stage) were taken from six-week-old cuttings (cv. Cabernet Sauvignon). Leaves were sterilised by immersion in 5% aqueous sodium hypochlorite for 10 min and thoroughly rinsed 3 times in sterile distilled water. Leaf discs (26 mm diameter) were cut from the disinfected leaves and placed, upper side up, in petri dishes containing agar (20 g L^{-1}) and benzimidazole (30 mg L^{-1}).

2.2 Pathogenic fungal cultures

Monospore isolates of *E. necator* (biotype B – a different isolate each year) were sampled from vinestock leaves from a vineyard in the Bordeaux area before starting each experiment each year. The inoculum was bulked on Cabernet Sauvignon leaves, as described by Cartolaro and Steva.[18] Bioassay inoculations involved blowing spores from 12–14-day-old sporulating leaves onto the upper surface of discs by an air pump. The density of deposited conidia was 9 conidia mm^{-2}, and infected leaves were incubated at 22 °C in a growth chamber (12 : 12 h light : dark photoperiod).

2.3 Fungicide treatments: leaf-disc tests

Commercial formulations, tebuconazole 250 g L^{-1} EW (Corail, Bayer Crop Science), trifloxystrobin 500 g kg^{-1} WG (Flint, Bayer Crop Science) and quinoxyfen 250 g L^{-1} SC (Legend, Dow AgroSciences), were used on each of the leaf-disc tests. Fungicide concentrations were based on the manufacturer's recommendations: 1 g L^{-1} tebuconazole, 0.625 g L^{-1} trifloxystrobin and 0.5 g L^{-1} quinoxyfen. A quantity of 1 mL of fungicide per petri dish was sprayed through a Potter Burkard tower (8 bar pressure) onto the upper surface of leaf discs. Discs were air dried under sterile conditions and were incubated at 22 °C in the growth chamber. Controls in each test consisted of treatment with sterile water.

2.3.1 *Leaf-disc tests*

The effects of fungicide treatment were evaluated at various stages of pathogen development by applying the fungicide at the start of colony expansion (test 1: 48 h after inoculation), during colony growth (test 2: 4 days after inoculation, before sporulation) or on sporulating colonies (test 3: 13 days after inoculation). For each test, eight petri dishes (replicates) were sprayed with fungicide, with each petri dish containing 1 (test 1) or 2 (tests 2 and 3) leaf discs.

Test 1. Disease was assessed 5, 7, 9, 12 and 14 days after inoculation, and the number of spores was measured on day 14 for each of the eight replicates.

Test 2. Disease assessments were performed as in test 1 on one leaf disc of each replicate 4, 6, 8, 13 and 14 days after inoculation. The number of spores was measured on day 14. To measure the effects of presporulation fungicide treatment, during the secondary pathogen cycle, on day 14, the second disc was used as an inoculum source for a new set of eight petri dishes: each petri dish contained one leaf disc. The newly inoculated discs were similarly assessed for disease 4, 6, 8, 11 and 14 days after inoculation, and the number of spores was measured on day 14.

Test 3. The test was performed to measure the effects of a post-sporulating treatment on a secondary pathogen cycle: sporulating discs treated with fungicide were used as an inoculum source for a new set of eight petri dishes, each one with one leaf disc. Disease levels on each disc were assessed 6, 8, 10 and 14 days after inoculation, and the number of spores was measured on day 14.

2.3.2 *Leaf-disc tests: disease and spore assessments*

The disc area covered by the fungus mycelium was assessed visually for powdery mildew severity 4–5 times during the 2 weeks

after leaf inoculation. The area under the disease progression curve (AUDPC) was calculated as follows:

$$\text{AUDPC} = \sum_{i=1}^{n} \left(\frac{y_{i+1} + y_i}{2} \right) (t_{i+1} - t_i)$$

where y_i is the disease severity at the ith observation, t_i is the time (days after inoculation) at the ith observation and n is the total number of observations.

A particle counter (Coulter Counter® Multisizer™ 3; Beckman Coulter, Paris-Nord, France) was used to assess spore production. Sporulating discs were placed in vials filled with 20 mL isoton 2 (Beckman Coulter), and one drop of non-ionic dispersant (Nacconol 90F, Dispersant Type IIIA; Beckman Coulter), and shaken. Spore production was thus assessed by counting the number of particles with diameters between 17 and 37 µm in a sample of 500 µL.

2.4 Field experiments: experimental design and pathogenic inoculation

The 3 year experiment was conducted at the field experimental station in the vineyard area of the Institut National de la Recherche Agronomique (INRA) de Bordeaux, Gironde, France. Use was made of *Vitis vinifera* var. Cabernet Sauvignon 16-year-old vines that were grafted onto Fercal rootstock. The plant density was 5000 plant ha^{-1}, and the spacing was 1 m between vines and 2 m between rows. The vines were 'guyot' pruned, traditionally topped and trimmed, and were treated for downy mildew. A randomised complete block design with four replications per treatment was used. Field plots consisted of single 3 m long rows (three plants per plot). To minimise interplot interference, plots were separated from each other by a buffer zone consisting of plots with non-infected plants.

A shoot of the central vine was inoculated with a monospore strain in each block. Inoculations were performed once a year (year 1: 24 April; year 2: 11 May; year 3: 7 May) at BBCH stages 13/14. Inoculation was performed by dispersing spores from two sporulating leaf discs (18 mm diameter) placed at the top of a cellophane funnel attached around a shoot. Funnels were stapled, allowing air circulation, and were left in place for 24 h.

2.5 Field experiments: fungicide treatment and disease assessment

Fungicide spraying began 28 days after inoculation, and spraying was repeated 3 times at 14 day intervals. Fungicide solutions and concentrations were identical to those used for the bioassays. Fungicide solutions were applied onto each side of each row, using a pneumatic mistblower. Air/fungicide suspensions were discharged at a speed of 30 m s^{-1} (100 L ha^{-1}).

On the undersides of leaves, a visual assessment was made of the percentage of the leaf covered by hyphae in year 1 (18 leaves per plant) and year 2 (24 leaves per plant) before the first fungicide treatment, to ensure inoculation homogeneity. All bunches (except those belonging to the inoculated shoot) were then assessed for incidence and severity of disease *in situ*, by examining the bunch area infected. Vines were assessed at the bunch closure stage (77–79 BBCH) 14 days (year 1), 5 days (year 2) and 7 days (year 3) after the last spray.

2.6 Statistical analyses

For leaf-disc bioassays, analyses of variance were performed on AUDPC. If the assumptions necessary for the use of ANOVA were validated, comparison of means was performed using the Newmann–Keuls test (5% significance level). A repeated-measures ANOVA plus test was performed to compare the change in disease severity.[19] For field studies, analysis of variance was performed on the incidence and severity on bunches, with comparisons carried out using the Newmann–Keuls test (5% significance level). Data were analysed with GraphPad PRISM (San Diego, CA).

3 RESULTS

3.1 Leaf-disc test: disease assessment and spore production

3.1.1 Test 1 (Table 1, supporting information Fig. 1)

Vines were sprayed with fungicides 48 h after inoculation with *E. necator* (before fungal colony expansion). Inoculation with *E. necator* resulted in three stages of fungal development on leaves. No mycelial growth was reported, disease severity and AUDPC were equal to zero and no spores were detected after tebuconazole treatment. (Spores were not observed, but the Coulter Counter detected some particles with diameters between 17 and 37 µm). Disease severity (3%) was low 7 days after inoculation following trifloxystrobin treatment; however, disease severity then increased on a regular basis and reached 31.8% on day 14. This temporal change was different for tebuconazole, where there was a significant time × treatment interaction ($P < 0.0001$). The AUDPC value was low and was not statistically different from that associated with trifloxystrobin. Indeed, only a few spores were observed on leaves treated with trifloxystrobin. Quinoxyfen-treated leaves had significantly lower disease severity than control leaves. A regular increase in disease severity was observed in control and quinoxyfen-treated leaves over a 2 week period, but there was no time × treatment interaction ($P = 0.69$) (identical changes over time). In comparison with the leaf-disc controls, lower AUDPC values were obtained after treatment with quinoxyfen. However, these values were significantly higher than those obtained with the two other fungicides at the end of the 2 weeks. Quinoxyfen-treated leaves had several spores on day 14, but this value was significantly lower than that for control leaves.

3.1.2 Test 2 (Table 2, supporting information Figs 2a and b)

Leaves were treated with fungicides 4 days after inoculation with *E. necator*. The pathogen developed fungal colonies, but no sporulation was reported. Disease severity was between 22 and 28% at the start of test 2 whichever treatment was applied. Thereafter, changes in disease development were quite different, depending on which fungicide was being studied. There was a significant decrease in disease severity with tebuconazole treatment, from 25% (4 days post-inoculation) to 0% (day 13).

Table 1. Leaf-disc test. AUDPC and number of spores of powdery mildew formed on leaves (cycle 1) 14 days after *Erysiphe necator* inoculation. Fungicides were applied at the start of colony expansion (48 h after inoculation). According to the Newman–Keuls test, values followed by the same letter did not differ significantly ($P > 0.05$)

Treatment	Application dose (g AI L^{-1})	AUDPC	Number of spores ($\times 10^3$ cm^{-2})
Control		394 a	43.5 a
Tebuconazole	1	0 c	0.8 c
Trifloxystrobin	0.625	86 c	2.1 c
Quinoxyfen	0.5	354 b	27.3 b

Table 2. Leaf-disc test. AUDPC and number of spores of powdery mildew formed on leaves (cycles 1 and 2) 14 days after *Erysiphe necator* inoculation. Fungicides were applied during colony growth (4 days after inoculation, before sporulation). According to the Newman–Keuls test, values followed by the same letter did not differ significantly ($P > 0.05$)

Treatment	Application dose (g AI L^{-1})	Cycle 1 AUDPC	Cycle 1 Number of spores ($\times 10^3$ cm^{-2})	Cycle 2 AUDPC	Cycle 2 Number of spores ($\times 10^3$ cm^{-2})
Control		695.6 a	35.9 a	844 a	44.4 a
Tebuconazole	1	56.6 c	0.2 c	0 c	0.02 c
Trifloxystrobin	0.625	459 b	0.2 c	0 c	0.06 c
Quinoxyfen	0.5	442 b	10.7 b	637 b	36.9 b

Table 3. Leaf-disc test. AUDPC and number of spores of powdery mildew formed on leaves (cycle 2) 14 days after *Erysiphe necator* inoculation. Fungicides were applied to sporulating colonies (13 days after inoculation). According to the Newman–Keuls test, values followed by the same letter did not differ significantly ($P > 0.05$)

Treatment	Application dose (g AI L^{-1})	Cycle 2 AUDPC	Cycle 2 Number of spores ($\times 10^3$ cm^{-2})
Control		654 a	47.6 a
Tebuconazole	1	612 a	30.4 b
Trifloxystrobin	0.625	6 c	1.0 c
Quinoxyfen	0.5	361 b	23.2 b

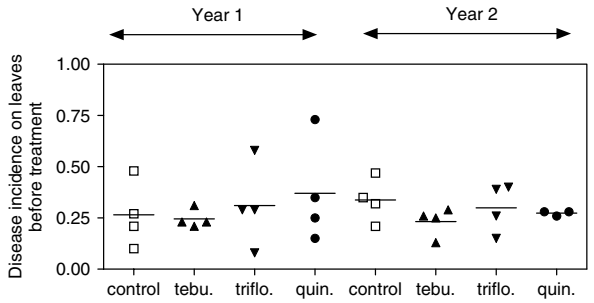

Figure 1. Field experiment. Disease incidence (proportion of diseased leaves) of powdery mildew on leaves before the fungicide treatments. Plant treatments: control = untreated; tebu. = with tebuconazole; triflo. = with trifloxystrobin; quin. = with quinoxyfen. For each treatment, the horizontal line refers to the average percentage. The four points are the values for four replicates.

No spores were observed and AUDPC values were lower. Consequently, there was no mycelium development (AUDPC equal to zero) or spore production during cycle 2 (Table 2, supporting information Fig. 2b). Trifloxystrobin and quinoxyfen have relatively similar effects on mycelium development, but very different effects on spore production. During test 2, disease severity in relation to both treatments increased from 24 to around 60% on day 14; it resulted in no significant difference between AUDPC values (Table 2). Both AUDPC values were significantly lower than that for the control. There were significant differences between the two fungicide treatments: several spores were produced on leaves treated with quinoxyfen, whereas none was produced on leaves treated with trifloxystrobin. Consequently, in cycle 2: (i) mycelia or spores were not formed on leaves treated with trifloxystrobin; (ii) on leaves treated with quinoxyfen 11 days after inoculation, the AUDPC value differed from the control but disease severity was equal. Sporulation and dense mycelial networks were observed on the leaf surface.

3.1.3 Test 3 (Table 3, supporting information Fig. 3)

Thirteen days after the first inoculation, sporulating discs were treated with fungicides and the spores were used to inoculate healthy leaves. Mycelial developments were similar, with no significant differences in AUDPCs, for control and tebuconazole replicates. Disease severity increased from 50% 6 days post-inoculation to between 95 and 100% on day 14. However, for the tebuconazole bioassay, the amounts of spores produced were lower than for control samples 14 days after inoculation.

In comparison with the control, the appearance of severe disease was significantly delayed in quinoxyfen-treated replicates, starting at levels of only 5% 6 days post-inoculation but reaching levels of 89% by the end of the experiment. Quinoxyfen AUDPC and trifloxystrobin AUDPC were both significantly lower than for the control and tebuconazole replicates. For all three fungicides, spore production was lower than in the control. In comparison with the two other fungicides, spores were rarely produced on trifloxystrobin replicates, and there was almost no disease development.

3.2 Field experiment: disease incidence and severity assessment

Disease incidence was assessed on leaves the day before they were treated with fungicides: there were no significant differences between blocks for years 1 and 2. The average incidence was 26.5% in year 1 and 33% in year 2 if compared with controls (Fig. 1) (not available on year 3). These findings indicated that the inoculation of leaves was successful and homogeneous, allowing a comparison of disease severity in bunches.

Throughout the 3 years of the study, more than 90% of bunches were diseased on controls during checks carried out at the bunch closure stage. However, disease severity was higher in year 1 (48%) and year 3 (64%) than in year 2 (26%). Two main results were obtained after the three fungicide trials: quinoxyfen induced a small decrease in disease incidence (28% in year 1, 36% in year 2 and 5% in year 3) and a moderate decrease in severity (86% in year 1, 72% in year 2 and 62% in year 3) in comparison with tebuconazole and trifloxystrobin. Disease severity in tebuconazole-treated leaves decreased by 98% in year 1, by 99% in year 2 and by 92% in year 3, and, in trifloxystrobin-treated leaves, disease severity decreased by 99% in year 1, by 99% in year 2 and by 91% in year 3 (Figs 2 and 3). Disease incidence was higher in years 1 and 3 after tebuconazole treatment than after trifloxystrobin treatment. The reduction in

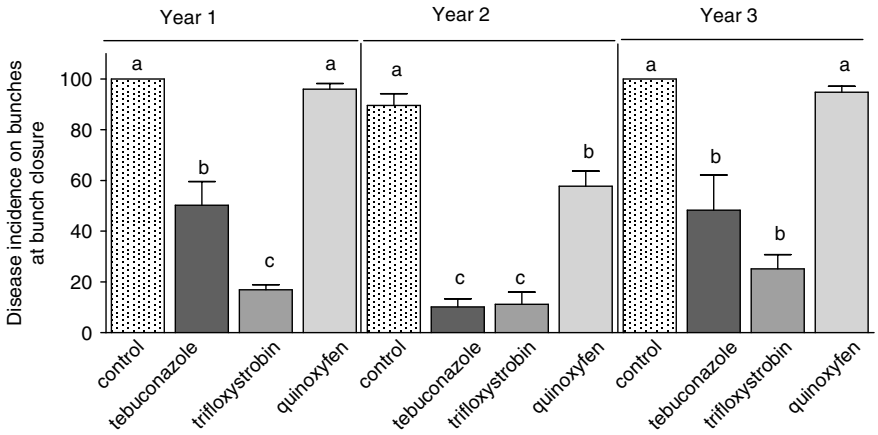

Figure 2. Field experiment. Disease incidence (percentage of diseased bunches) of powdery mildew on bunches at bunch closure (77–79 BBCH) 14 days (year 1), 5 days (year 2) and 7 days (year 3) after the last spray. For each year of experiment, according to the Newman–Keuls test, bars followed by the same letter did not differ significantly ($P > 0.05$).

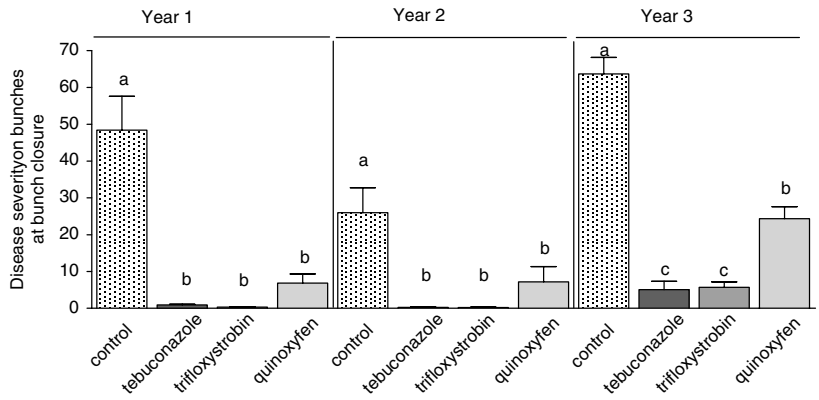

Figure 3. Field experiment. Disease severity (estimated diseased surface in percentages) of powdery mildew on bunches at bunch closure (77–79 BBCH) 14 days (year 1), 5 days (year 2) and 7 days (year 3) after the last spray. For each year of experiment, according to the Newman–Keuls test, bars followed by the same letter did not differ significantly ($P > 0.05$).

disease incidence was similar for the two fungicides in year 2 (Figs 2 and 3).

4 DISCUSSION

Significant changes in fungicide strategies are likely following the introduction of new pesticide registration procedures. These are expected to limit fungicide use and so become an important issue.[20] As the initial infection cannot be determined and most fungicides are likely to be used in vineyards already containing pathogenic spores, reducing fungicide use induces several limitations in the control of *E. necator* epidemics. Therefore, providing information on these key points is necessary in terms of vineyard protection. Two experiments were used, i.e. leaf-disc tests and field trials, to determine how three fungicides currently used by vine growers can be managed to control the invasion of *E. necator* during the early stages of an epidemic. The present experiments provided findings that were complementary: the leaf-disc tests highlighted the points at which the fungicides were most effective at breaking the *E. necator* cycles, and the field trial provided the final results in terms of disease incidence and severity on bunches, without reference to the pathogenic cycle development.

Scientists commonly use leaf-disc tests to monitor the sensitivity of fungal plant pathogens to various fungicides. They are useful for resistance monitoring programmes and for discriminatory dose screens. The present objectives for their use were different: the aim instead was to mimic the various initial stages of an *E. necator* epidemic: the start of colony expansion (test 1: 48 h after inoculation), colony growth before sporulation (test 2: 4 days after inoculation) and colony sporulation (test 3: 13 days after inoculation). All of these stages are usually observed in the field once an epidemic has been initiated. The present results were dependent on fungicidal properties: (i) the protectant fungicide (quinoxyfen) induced a slight decrease in *E. necator* development, but (ii) fungicides with protectant and curative properties (tebuconazole and trifloxystrobin) caused significant, although different, disruption of *E. necator* epidemics.

Quinoxyfen was less effective at controlling powdery mildew development than the other two fungicides. In the leaf-disc test, the AUDPC and spore production were reduced and a delay was always observed in the disease severity index if compared with the control. Quinoxyfen is a protectant that specifically controls powdery mildew pathogens in a number of hosts by interfering with germination or pre-infection development.[16] Quinoxyfen specifically targets the early stages of *E. necator* development, and there is much evidence for it affecting signal transduction

pathways.[21] Thus, the relatively poor performance of quinoxyfen in the leaf-disc sporulation assay was most likely related to the narrow timeframe during which it was required completely to inhibit the infection process. However, although spores that escape initial control with quinoxyfen proceed to form sporulating colonies, the number of newly formed spores was half that of the number of spores formed in control assays.

Tebuconazole and trifloxystrobin were the most effective at limiting *E. necator*-induced infection. These two fungicides with curative capabilities performed robustly in sporulation assays; this was probably due to the broad timeframe in which their activities were expressed. Nevertheless, the leaf-disc tests showed that each fungicide was most effective at different stages of fungal development.

Before sporulation of *E. necator* colonies, tebuconazole severely limited (48 h post-inoculation) or prevented mycelial growth (4 days post-inoculation). Under similar experimental conditions, trifloxystrobin had either a strong effect (48 h post-inoculation) or a slight effect (4 days post-inoculation) on mycelial growth. During development of fungal colonies; hardly any sporulation was observed after treatment with tebuconazole or trifloxystrobin, and consequently, when these two fungicides were applied to *E. necator* colonies before they had begun to sporulate, the epidemic could be stopped, as spores were not detected after fungicidal treatment.

After the sporulation of *E. necator* colonies: trifloxystrobin significantly limited the germination of treated spores. With tebuconazole-treated leaves, spores were able to germinate and form mycelia, which in turn produced several spores (results were similar to those obtained for control spores). Therefore, this study shows that trifloxystrobin treatment of *E. necator* sporulating colonies was effective at stopping the epidemic.

Based on the leaf-disc test, the present results suggest that, in the field, the protectant quinoxyfen will be unable to disrupt the *E. necator* epidemic, and, among the fungicides with protectant and curative properties, trifloxystrobin use should take precedence. Indeed, the fact that quinoxyfen, tebuconazole and trifloxystrobin respectively caused moderate, high and excellent control of *E. necator* in the field provided an indication that the results of the leaf-disc tests probably represent what really occurs in the field. With reference to the effectiveness of a leaf-disc test and how the results may be used, Green and Gustafson[22] report that the level of control provided by a protectant fungicide in a leaf-disc assay can be very sensitive to various factors such as spore density, compound application timing and compound distribution on the leaf surface, especially if the inoculum density is high. Therefore, in the case of a purely protectant fungicide, such as quinoxyfen, a leaf-disc sporulation assay may overestimate the sporulation level of the fungal pathogen isolate, as the spores that escape the initial effects of the fungicide proceed to form sporulating colonies. In the authors' opinion, the spore concentrations and the periods of intervention used here do not create this side effect; another specificity of the present leaf-disc test is that it is based on various stages of *E. necator* development.

Under field conditions, the percentages of diseased bunches (disease incidence) in quinoxyfen-treated leaves were relatively similar to those of the control; however, the fungicide did reduce disease severity (estimated diseased bunch surface in percentages) from 62 to 86% in bunches at the stage of bunch closure. This was probably due to the decreased number of spores formed after quinoxyfen treatment, as suggested by the test on leaves. The significant reduction in disease severity may also be explained by another factor: in the field, fungicides are applied to the organs infected by *E. necator*, but they are also applied to uninfected bunches and leaves, which further reduces the severity of the disease. Therefore, the combination of both effects is likely to decrease disease severity.

The reduction in disease incidence was higher with trifloxystrobin (from 75 to 88%) than with tebuconazole (from 50 to 89%) in years 2 and 3. However, for both fungicides, reduction in disease severity in bunches was similar (more than 90%) for all 3 years of the study. Tebuconazole may have been most effective at reducing disease severity in the field, as spores that escape the initial effects of the fungicide are then eliminated post-infection by the curative properties of the fungicide.

To conclude, if fungicides are applied to vineyards during an already initiated powdery mildew epidemic, their efficacy will depend on at least three factors: (i) the effects of the fungicide on the pathogenic inoculum colonising the leaves and the bunches; (ii) the effects on the various stages of *E. necator* development; (iii) the protection of further contamination of the healthy areas receiving various stages of treatment. The effectiveness of each of the fungicides for treating an already initiated powdery mildew epidemic was different, including their effects on the infection cycle. Interest in the leaf-disc test lay in its ability to help determine how a fungicide can be used to stop the growth of a pathogenic fungus. Another key factor was that these leaf-disc tests provided a picture of what really occurs in the field, where various stages of fungal development occur simultaneously.

Based on the present results, a protection strategy combining the different fungicide types under study can be proposed. Tebuconazole and trifloxystrobin, which have both protectant and curative properties, could be used in priority to control an *E. necator* epidemic at the early stages. The main value of the protectant quinoxyfen relies on its ability to be used after the two other fungicides to protect healthy leaves, and avoid the formation of an inoculum on the leaves. Finally, the present data will help in selecting which fungicide should be applied at the early stages of an *E. necator* epidemic.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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