agrochemicals

MDPI

Article

First Identification of P230L and H134R Mutations Conferring SDHIs Resistance in *Stemphylium vesicarium* Isolated from an Italian Experimental Pear Orchard

Katia Gazzetti [1], Massimiliano Menghini [2], Irene Maja Nanni [2], Alessandro Ciriani [3], Mirco Fabbri [2], Pietro Venturi [2] and Marina Collina [2,*]

[1] Council for Agricultural Research and Economics (CREA), Research Centre for Genetics and Bioinformatics, Via Paullese 28, 26836 Montanaso Lombardo, Italy; katia.gazzetti@crea.gov.it
[2] Department of Agricultural and Food Sciences (DISTAL), University of Bologna, 40127 Bologna, Italy; massimilian.menghin2@unibo.it (M.M.); irenemaja.nanni2@unibo.it (I.M.N.); mirco.fabbri6@unibo.it (M.F.); pietro.venturi4@unibo.it (P.V.)
[3] Control Union Italia Srl, P.le F. Il Macedone 89, 00124 Rome, Italy; aciriani@controlunion.com
* Correspondence: marina.collina@unibo.it

Abstract

Since the late 1970s, brown spot of pear (BSP), a fungal disease caused by *Stemphylium vesicarium* (Wallr.) Simmons, has been one of the most important pear fungal diseases in Italy. To protect orchards from BSP, frequent fungicide application is essential throughout the period spanning petal fall to the onset of fruit maturation. In Italy, boscalid was the first succinate dehydrogenase inhibitor (SDHIs) fungicide authorised against BSP; subsequently, penthiopyrad and fluxapyroxad were authorised against the disease. In 2016 and 2017, SDHI compounds were applied against BSP as solo products at the University of Bologna's experimental farm, showing a reduction in efficacy. *Stemphylium vesicarium* strains were isolated from leaves and fruit, and sensitivity assays and molecular analyses were performed. In vitro tests confirmed resistance to SDHIs, and two specific single-nucleotide polymorphisms were discovered, SDHB P230L and SDHC H134R, both leading to amino acid substitutions in succinate dehydrogenase subunits and confirming the resistant phenotype.

Keywords: brown spot of pear (BSP); fungicides; SNPs; efficacy reduction; disease control; monitoring

check for updates

Academic Editor: Ioannis Vagelas

Received: 15 July 2025
Revised: 22 August 2025
Accepted: 26 August 2025
Published: 29 August 2025

Citation: Gazzetti, K.; Menghini, M.; Nanni, I.M.; Ciriani, A.; Fabbri, M.; Venturi, P.; Collina, M. First Identification of P230L and H134R Mutations Conferring SDHIs Resistance in *Stemphylium vesicarium* Isolated from an Italian Experimental Pear Orchard. *Agrochemicals* **2025**, *4*, 15. https://doi.org/10.3390/agrochemicals4030015

Copyright: © 2025 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Stemphylium forms a monophyletic genus in the Pleosporaceae family, Dothideomycetes class, and Pleosporales order. In this genus, a multi-gene phylogeny based on (parts of) the internal transcribed spacer (ITS), *gapdh* and *cmdA* gene regions distinguish 28 species [1]. *Stemphylium vesicarium* (Wallr.) E.G Simmons (teleomorph: *Pleorospora allii*) is a filamentous fungus that causes diseases in non-crop plants, herbaceous crops and fruit trees [2–7]. Brown spot of pear (BSP), caused by *S. vesicarium*, is an economically relevant disease of *Pyrus communis* L., with a similar or even higher incidence than apple scab in European pear-growing regions [8]. BSP was first discovered in 1975 in the Emilia-Romagna region of Italy on cv 'Abbé Fétel' [8], but since the 1980s, the disease has spread throughout the main pear production regions in Europe [5,9–12].

The disease cycle is characterised by two phases: saprophytic and pathogenic. During the saprophytic phase, both ascospores of *P. allii* and conidia of *S. vesicarium* permanently

colonise plant debris on the ground of the pear orchard or in neighbouring orchards [11]. Although the ascospores cause infection in pear fruit and leaves, their most important role is considered as overwintering on plant debris, resulting in mycelia that produce conidia, becoming the airborne inoculum that infects pear trees during the growing period [5,13–15]. Symptoms consist of necrotic lesions that generally first appear on leaves in April, and on other pear aerial organs, mainly fruit, from May to July; infections can occur throughout the whole season [13]. Infection development requires temperatures between 20 and 25 °C and a minimum of 6 h of wetness [16]. Under optimum conditions, half of the conidia germinate in 1 h [17,18], releasing SV-I and II, which are host-specific toxins (HSTs) [19]. SV-I and II HSTs induce ultrastructural changes in the plasmodesmata of the plasma membrane of susceptible European pear leaf cells [20], and their specificity determines differences in the susceptibility of pear cultivars to BSP. Several widely cultivated pear cultivars, including Abbé Fétel, 'Conference', 'Passe Crassane' and 'Alexandrine', are highly susceptible to BSP [9,13,21,22]; young leaves and immature fruit are particularly vulnerable compared to mature tissues [22].

Cappai et al. [23] identified a major quantitative trait locus (QTL) for susceptibility at the lower end of linkage group 15 in Abbé Fétel pear, with a putative susceptibility gene positioned 2 cM from the lower end of linkage group 15. In 2019, the first genome sequence resource for *S. vesicarium* was released, offering new opportunities to investigate the fungal lifestyle, epidemiology, molecular plant–pathogen interactions, and fungicide resistance [24]. BSP control strategies need to consider agronomical practices, such as avoiding water stagnation and overhead irrigation, proper tree fertilisation, orchard ground sanitisation, and plant debris removal [11,13]. Even treatment of the orchard litter has been proposed to reduce ascospores and conidia release, including the application of a product based on an antagonistic microorganism, such as *Trichoderma* [25], and mechanical treatment that involves tillage on the entire surface of the pear orchard [4]. Moreover, the BP15 synthetic antimicrobial peptide is a good candidate to be further developed as a fungicide for controlling BSP in the field [26], but currently, fungicide application remains the most efficient method of controlling BSP. Once conidia germinate, they release SV toxins [19], and then, necrosis appears on affected plant tissue. Disease control is based on the application of preventive fungicide spray every 7–14 days [27–30]. Forecast models, such as PAM cast [31] and BSP cast [22,32], have been developed to determine the effect of environmental parameters on the pathogen or the disease. Compared to fixed schedules at moderate to low disease levels, BSP cast reduces fungicide use by 30–40% in commercial orchards [32] and is currently used in Italy to help in scheduling fungicide sprays.

To protect orchards from BSP, many fungicide applications are required from petal fall to fruit ripening because of the infection strategy of *S. vesicarium* [8,30]. In addition to the high environmental and economic impact, intense fungicide use can lead to resistance phenomena. In the early 1990s, problems with BSP control using procymidone were reported in some areas of Northern Italy, and a monitoring study of *S. vesicarium* populations collected in Po Valley started in 1995, identifying field isolates' sensitivity to dicarboximides, phenylpyrroles, and strobilurins. *Stemphylium vesicarium* was shown to have developed field resistance to key products containing dicarboximides, and, in a few cases, fludioxonil [33,34] and strobilurins [35]. The deployment of novel compounds with diverse modes of action (MoAs) is therefore crucial to broaden the spectrum of effective fungicides against BSP and to mitigate the development of resistance. In chronological order, the last class of fungicides introduced was that of the succinate dehydrogenase inhibitors (SDHIs), which have been described to strongly bind to the ubiquinone-binding site formed by the SDHB, SDHC, and SDHD subunits [36], and thus, physically block access to ubiquinone, consequently preventing the catalysis of succinate [37]. At the end of 2006 season, boscalid

was the first SDHI fungicide authorised in Italy against BSP. To date, BSP control in the field also relies on broad-spectrum next-generation SDHIs, such as penthiopyrad, fluxapyroxad, and fluopyram, mixed with various partners (e.g., tebuconazole, difenoconazole, and fosetyl–aluminium) [38].

Because of their specific MoA, SDHIs are classified by the Fungicide Resistance Action Committee (FRAC) as fungicides at medium risk for developing resistance; thus, they require proactive resistance management strategies [39]. Since 2015, FRAC has reported the existence of substitutions conferring SDHI resistance in the B subunit (B-P225L, B-H272Y/R), as characterised in field samples of *S. vesicarium* collected from asparagus [40]. In 2009, in the Plant Pathology Laboratory at the University of Bologna, the in vitro baseline sensitivity of *S. vesicarium* to boscalid was determined in 57 isolates collected between 1995 and 2006 in commercial orchards located in the main pear-growing region of Northern Italy [41]. Moreover, 43 isolates collected in the same area before the market introduction of boscalid were tested for sensitivity to the new SDHI compounds, showing half-maximal effective concentration (EC50) values lower than 0.5 mg/L [42]. Monitoring studies and efficacy tests are still ongoing, now including the newest SDHI fungicides.

In the present study, the SDHI compounds currently authorised for BSP control were applied as solo products in a pear experimental orchard in 2016 and 2017 to evaluate the efficacy and disease incidence. In vitro sensitivity assays were also performed on fungal strains obtained from symptomatic fruit collected within the treated plots. Due to the poor performance of the fungicides, molecular investigations were carried out to identify mutations conferring SDHI resistance.

2. Materials and Methods

2.1. Field Experiments

The trials were conducted during the 2016 and 2017 seasons in a research pear orchard (cv. Abbè Fétel, BSP susceptible, 26-year-old plants) of the Department of Agricultural and Food Sciences (DISTAL) of the University of Bologna, located in Altedo in the Emilia-Romagna region. In the experimental orchard, the SDHI product application began in 2003 with boscalid mixed with pyraclostrobin and continued with the same mixture until 2012 when the fluopyram + tebuconazole mixture was used for the first time. After 2013, fluxapyroxad and penthiopyrad alone were tested. In all seasons, the products were applied at 10-day intervals from fruit setting to pre-harvest for a total of 10–15 applications. The air temperature, relative humidity, total precipitation and leaf wetness were recorded every 5 s and summarised every 15 min by a weather station (DigitEco s.r.l.) located 200 m from the orchard (latitude N 44°38′57″, longitude E 11°29′39″, altitude 13 m).

The commercial formulations of boscalid (Cantus WG, BASF), penthiopyrad (Fontelis SC, DuPont) and fluxapyroxad (Xemium, BASF) were tested in 2016 and 2017, while fluopyram (Luna privilege, Bayer) was only tested in 2017. In both years, compounds were tested as solo products, and cyprodinil + fludioxonil (Switch WG, Syngenta) was used as the reference treatment. All fungicides were applied at recommended doses for commercial usage (Tables 1 and 2) and 1000 L/ha using a hand-operated knapsack sprayer at 7–8-day intervals. Treatments were arranged in a randomised block design with plots of 6–7 plants, replicated 4 times. In 2016, plots received 16 applications, the first on 21 April and the last on 9 August. A total of 10 sprays were applied in 2017, the first on 9 May and the last on 7 August.

Table 1. List of commercial products, relative active ingredients, and application rates used in the blocks during the trial in 2016.

Block No.	Commercial Product	Active Ingredient (Concentration)	Rate (g or mL/100 L)
1	Cantus WG	boscalid (50%)	27 g
2	Fontelis SC	penthiopyrad (200 g/L)	75 mL
3	Xemium	fluxapyroxad (300 g/L)	20 mL
4	Switch WG	cyprodinil + fludioxonil (37.5 + 25%)	80 g

Table 2. List of commercial products, relative active ingredients, and application rates used in the blocks during the trial in 2017.

Block No.	Commercial Product	Active Ingredient (Concentration)	Rate (g or mL/100 L)
1	Cantus WG	boscalid (50%)	27 g
2	Fontelis SC	penthiopyrad (200 g/L)	75 mL
3	Xemium	fluxapyroxad (300 g/L)	20 mL
4	Luna Privilege	fluopyram (500 g/L)	28 mL
5	Switch WG	cyprodinil + fludioxonil (37.5 + 25%)	80 g

All fruit was collected from each plot, and disease assessments were performed on all fruit on 25 August in 2016 and 2017. The results were statistically analysed using analysis of variance (ANOVA), and averages were compared with Duncan's test ($p = 0.05$), utilising the SGWIN package. The fungicide efficacy was calculated using Abbott's formula, and the disease incidence of the pathogen was evaluated for both years.

2.2. Isolation of S. vesicarium

From each block of the experimental orchard, 13–15 symptomatic pear fruits were collected (Figure 1a). Colonies from a symptomatic area of each fruit were isolated as described in Alberoni et al. [34] and are reported as isolates (Figure 1c); thus, we collected 13–15 isolates per block to obtain a population (Figure 1b). In 2016, a total of 7 populations were obtained: 5 in July, 1 from each treatment and 1 from the untreated block; and 2 in August, 1 from the untreated block and 1 from the fluxapyroxad treatment block. In 2017, a total of 6 populations were collected in July, 1 from the untreated block, and 1 from each treatment block. We randomly obtained monoconidial isolates (MIs) (Figure 1d) from isolates 1, 4, and 14 collected in August 2016 from the block treated with fluxapyroxad. MIs were produced as described by Alberoni et al. [34].

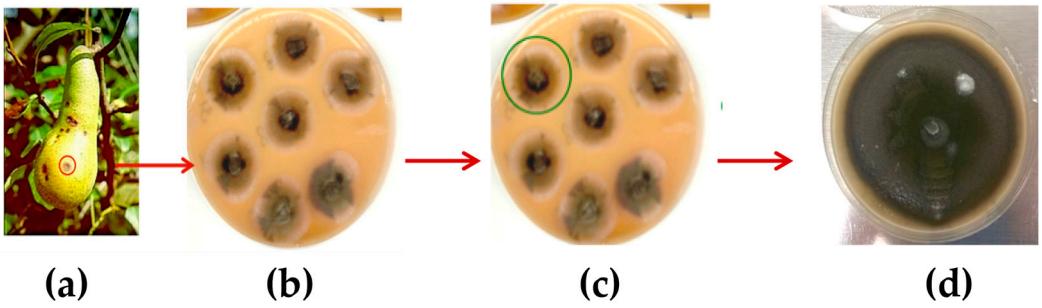


Figure 1. Symptomatic pear fruit (**a**); population (**b**); isolate is highlighted in green (**c**); monoconidial cultures (**d**).

Populations, isolates, and MIs were maintained on V8 juice agar [20% V8 (Campbell Soup TM, Continental foods, Rijkssweg, Belgium), 1.5% agar (BBL™ Agar Grade A, Becton, Dickinson & Company), 0.4% calcium carbonate (Sigma Aldrich, Germany) in dH_2O]. V8 agar was amended with 50 mg/L streptomycin sulphate after autoclaving. The cultures were incubated at 23 °C with a 12 h photoperiod for 4 days, followed by 23 °C with exposure to 12 h of NUV (BL-368 nm) for 3 days to facilitate conidia formation.

2.3. In Vitro Sensitivity Tests

Sensitivity assays to boscalid, fluxapyroxad, penthiopyrad, and fluopyram were performed on conidial suspensions using the spectrophotometric method. All SDHI compounds were tested with an analytical standard (PESTANAL, Merck KGaA, Darmstadt, Germany). For each compound, six concentrations (0, 0.02, 0.05, 0.5, 1, and 2.5 mg/L) of the active ingredient (a.i.) were prepared in YBA liquid medium (10 g yeast extract, 10 g bacto peptone, 20 g sodium acetate in 1 L dH_20). In the well of a 96-well microtiter plate, 50 µL of fungicide solution was added to 50 µL of conidial suspension (final conidia concentration of 2×10^4/mL). Conidia were obtained from 7-day-old colonies of *S. vesicarium* populations, isolates, or MIs grown on V8 juice agar plates as previously described. Briefly, several millilitres of sterile distilled water were added to the colony surface, which was then gently scraped using a sterile spatula to release conidia. The resulting suspension was filtered through a 100-µm mesh to eliminate mycelial fragments. For each tested culture (T0), four replicate wells were prepared for each concentration.

The plates were incubated at 23 °C, with a 12 h photoperiod and shaking at 450 rpm (T1) for 2 days. Growth was evaluated using a spectrophotometer (NanoQuant Infinite M200 Pro, TECAN Austria GmbH) by determining the difference in absorbance between T1 and T0 at 405 nm. EC50 values were calculated using probit analysis. The assays were repeated twice, and the mean was reported as the final value. The EC50 results are reported in this work as indicated in Table 3 in accordance with the sensitivity observed in baseline studies [43].

Table 3. Notation of EC50 values calculated by probit analysis and strain sensitivity definition. For EC50 results less than 5 mg/L, the EC50 value is always specified.

EC_{50} Value (mg/L)	Strain	Notation
<2.5	Sensitive	value (mg/L)
<5	Resistant	value (mg/L)
<20	Resistant	>2.5
>20	Resistant	>>2.5

2.4. DNA Extraction and Downstream Procedures for the Determination of Mutations

Stemphylium vesicarium populations, isolates and MIs were previously harvested by filtration from liquid culture obtained by growing in 250 mL flasks containing 50 mL of Czapek–Dox media inoculated with a conidial suspension (1×10^4/mL) and incubated at 23 °C in the dark in an orbital shaker (VDRL stirrer mod. 711/D+) at 100 rpm for 7 days. Genomic DNA was purified using a cetyltrimethylammonium bromide (CTAB)-based method with modifications [25].

The amplification of the putative genes encoding Sdh B, C, and D subunits was achieved using the oligonucleotides designed on the flanking region of each putative gene. These specific primers were designed by Perl Primer v1.1.17 and are listed in Table 4, together with those for the amplification of the fragment encoding the Fer4_17 domain belonging to the Sdh B subunit.

Table 4. Oligonucleotides used for amplification of *S. vesicarium* genes encoding SdhB, SdhC, SdhD and SdhB-Fer4_17 domains.

Name	Target	Sequence (5′-3′)	Amplicon Size (bp)
SdhB_for	*sdh B*	CACTCTCCACTCCGCACTAC	1192
SdhB_rev	*sdh B*	TGTCTGGTGACACTCGCTG	1192
SdhC_for	*sdh C*	AGCAGAGTAATGCGAAGGCA	863
SdhC_rev	*sdh C*	GGCTTGACACATGGAGGATC	863
SdhD_for	*sdh D*	CGAGGCATTGTCGTCAAC	788
SdhD_rev	*sdh D*	CCAAATTACACAGGCTGTATGCT	788
Fer4_17_for	*sdh B*	GTGGCTGAAGTACTAACGACCG	780
Fer4_17_rev	*sdh B*	ACCCTCGAGAGTCCACTCAC	780

All PCR assays were carried in a 50 µL volume containing 100 ng of genomic DNA, 5 µL 10X Ex Taq™ Buffer, 0.2 µM primers, 0.2 mM of each dNTP, and 1.25 U TaKaRa Ex Taq™ (Takara) with the following programme: 95 °C for 3 min; 34 cycles of 95 °C for 45 s, 61 °C for 45 s, 72 °C for 1 min; and 10 min at 72 °C. PCR products were purified using a GEL/PCR Extraction and Purification Kit (Fisher Molecular Biology, USA) and then sequenced using Sanger sequencing at Eurofins Genomics (Ebersberg, Germany) with the same oligonucleotides as for the corresponding PCR reaction.

Sequenced PCR products were assembled and aligned using Sequencher 5.6.4 (Gene Codes Corporation, USA) to the wild-type (WT) obtained from the MI 173-1a13FI1M3 (CBS-KNAW culture collection accession number: 145331) described by Gazzetti et al. [25].

Mutated *sdh* gene sequences were translated using DNAMAN version 4.15 to identify amino acid substitutions. Since there were no differences in the length of the fungicide target proteins, namely subunits b, c, and between *S. vesicarium sdh* genes and *P. teres* f. sp. *teres* orthologues, the amino acid numbering used in this study matches the mutation labels proposed by Mair et al. [43].

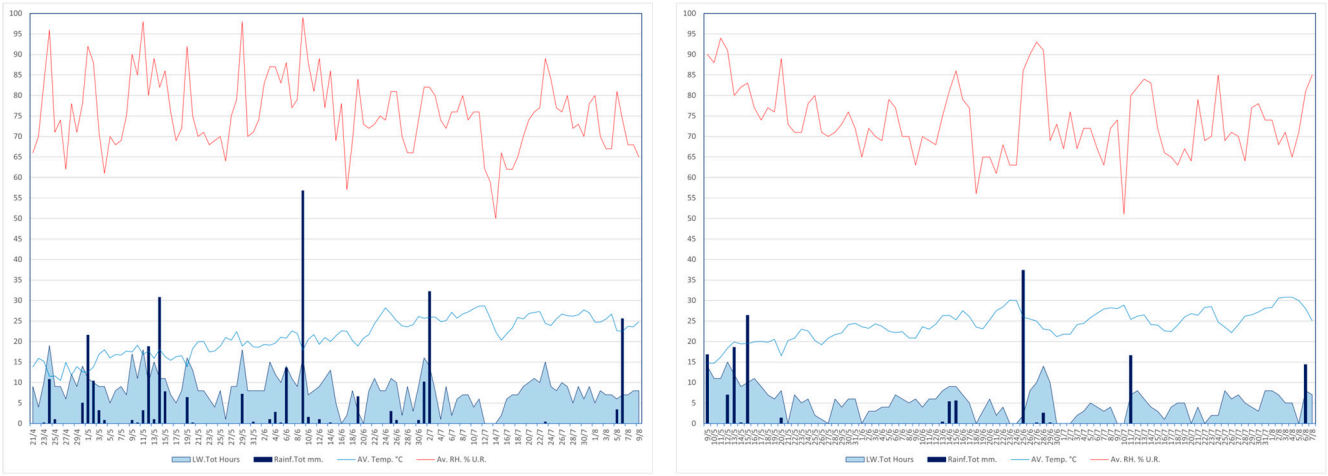
3. Results

3.1. Field Experiments

The weather in 2016 was characterised by high temperatures and low rainfall, especially in the summer. Rainfall was mainly concentrated in May and June, with three rainfall events exceeding 20 mm, which created high-humidity conditions favourable to the disease (Figure 2a).

In 2017, only two rainfall events occurred in April, both totalling less than 20 mm. Most of the rainfall was concentrated in May, resulting in the appearance of the first symptoms on vegetative organs. From the second half of May onwards, only three significant rainfall events were recorded: at the end of June, mid-July, and the beginning of August (Figure 2b).

In 2016, boscalid and penthiopyrad showed good efficacy, while the efficacy of fluxapyroxad was lower compared to the standard and the other SDHI treatments. The plot sprayed with fluxapyroxad exhibited a statistically significant difference in disease incidence percentage compared to other treatments, and no statistically significant difference compared to the untreated control (Table 5).



(a) **(b)**

Figure 2. The graphs represent the climate evolution through the detection of average temperatures (AV. Temp.) and relative humidity (Av. RH) of total hours of leaf wetness (LW) and rainfall (Rainf.), expressed in mm, during the 2016 (**a**) and 2017 (**b**) seasons.

Table 5. Percentages of disease incidence and treatment efficacy in 2016 and 2017.

Treatment	Disease Incidence (%) 2016	Efficacy (%) 2016	Disease Incidence (%) 2017	Efficacy (%) 2017
Untreated	25.8 a	-	59.3 a	-
Boscalid	6.5 b	74.8	68.4 a	0
Penthiopyrad	8.8 b	65.9	45.1 a	23.9
Fluxapyroxad	21.5 a	16.7	54.2 a	8.6
Fluopyram	-	-	66.6 a	0
Cyprodinil + fludioxonil	4.6 b	82.2	15.0 b	74.7

In each year, values of disease incidence followed by the same letter do not differ significantly (Duncan's test $p = 0.05$).

3.2. Sensitivity Assay

Sensitivity assays on populations isolated in 2016 (Table 6) showed EC50 values in the range of the baseline and similar to those of the untreated plot population, except the population sampled in the fluxapyroxad-treated plot. Regarding the EC50 values observed for the two populations collected in July and August from the plot treated with fluxapyroxad, they always overcame the 20 mg/L concentration of all compounds, detecting the emergence of in vitro resistance toward all tested SDHIs (Table 6).

The populations collected in 2017 from all treated plots showed EC50 values higher than 5 mg/L. The same sensitivity profile resulted from the biological assay of the population collected from the plot treated with fluopyram for the first time (Table 6).

To understand this resistance phenomenon, we tested the fluxapyroxad sensitivity of 15 isolates, comprising the population isolated in August 2016 from the plot treated with fluxapyroxad (Table 7). As expected, all isolates exhibited resistance to this fungicide. Furthermore, three isolates (no. 1, 4, and 14) representative of the population were tested against boscalid, penthiopyrad, and fluopyram. In particular, isolate 1 showed an EC50 of 4.21 mg/L for penthiopyrad but showed an EC50 of 5–20 mg/L for fluopyram. Regarding the sensitivity to boscalid, all three isolates showed high resistance, with EC50s > 20 mg/L. Similar results were obtained by isolate 4 for penthiopyrad, while the EC50 value for

fluopyram was between 5 and 20 mg/L. Isolate 14 showed a stronger resistance profile with an EC50 > 20 mg/L for all tested fungicides (Table 7).

Table 6. Mean EC50 (mg/L) values calculated for populations collected in 2016 and 2017.

	2016 (EC50 mg/L)				2017 (EC50 mL/L)			
	Boscalid	**Fluxapyroxad**	**Penthiopyrad**	**Fluopyram**	**Boscalid**	**Fluxapyroxad**	**Penthiopyrad**	**Fluopyram**
Untreated July	0.46	0.11	0.02	0.01	-	-	-	-
Untreated August	0.55	0.49	0.39	0.26	>2.5	>>2.5	>>2.5	>>2.5
Boscalid	0.43	0.43	0.3	-	>2.5	>>2.5	>2.5	>2.5
Penthiopyrad	0.77	0.5	0.49	-	>>2.5	>>2.5	>>2.5	>2.5
Fluxapyroxad July	>>2.5	>>2.5	>>2.5	>>2.5	-	-	-	-
Fluxapyroxad August	>>2.5	>>2.5	>>2.5	>>2.5	>>2.5	>>2.5	>>2.5	>>2.5
Cyprodinil + Fludioxonil	0.94	0.88	1.07	-	-	-	-	-
Fluopyram	-	-	-	-	>>2.5	>>2.5	>>2.5	>2.5

EC50 results notation: <5 mg/L = specified value; from 5 to 20 mg/L = >2.5; >20 mg/L = >>2.5.

Table 7. Mean EC50 (mg/L) values calculated for isolates belonging to the population collected in August 2016 from the plot treated with fluxapyroxad.

Isolates	**EC 50 (mg/L)**			
	Boscalid	**Fluxapyroxad**	**Penthiopyrad**	**Fluopyram**
1	>>2.5	>>2.5	4.21	>2.5
2		>>2.5		
3		>>2.5		
4	>>2.5	>2.5	>>2.5	>2.5
5		>>2.5		
6		>>2.5		
7		>>2.5		
8		>>2.5		
9		>>2.5		
10		>>2.5		
11		>>2.5		
12		>>2.5		
13		>>2.5		
14	>>2.5	>>2.5	>>2.5	>>2.5
15		>>2.5		

EC50 results notation: <5 mg/L = specified value; from 5 to 20 mg/L = >2.5; >20 mg/L = >>2.5.

To further investigate this resistance phenomenon, we tested the MIs against fluxapyroxad. For each isolate (no. 1, 4, and 14) we obtained 10 MIs, and we randomly tested three of them. All MIs showed EC50 values higher than the baseline range (Table 8).

Table 8. Mean EC50 (mg/L) values calculated for the three monoconidial isolates produced from isolates 1, 4, and 14 belonging to the population collected in August 2016 from the plot treated with fluxapyroxad.

Monoconidial Strains 2016	EC 50 (mg/L) Fluxapyroxad
1.1	>2.5
1.2	>>2.5
1.3	>>2.5
4.1	>>2.5
4.2	>>2.5
4.3	>2.5
14.1	3.47
14.2	2.36
14.3	1.34

EC50 results notation: <5 mg/L = specified value; from 5 to 20 mg/L = >2.5; >20 mg/L = >>2.5.

3.3. Identification of SDHB P230L and SDHC H134R Mutations

The Sdh B, C, and D subunits encoding genes were amplified from DNA belonging to the fluxapyroxad-treated plot collected in August 2016, from the 15 isolates that constitute it, and from three MIs obtained from isolates 1, 4, and 14. All amplified products were sequenced using the Sanger method, and their sequences were aligned with those of sensitive reference strain 173-1a13FI1M3.

Analysis of Sdh B, C, and D subunits encoding genes belonging to the fluxapyroxad-treated plot collected in August 2016 revealed two specific point mutations, leading to amino acid exchanges in B and C succinate dehydrogenase subunits. A target site mutation was detected in *sdh* b at nucleotide position 864, leading to a proline substitution to leucine at amino acid position 230 (Figure 3). In *sdh* c, we detected a single-nucleotide polymorphism (SNP) at nucleotide position 481, leading to a histidine substitution to arginine at amino acid position 134 (Figure 3). All isolates and MIs analysed carried only one of the two *sdh* mutations.

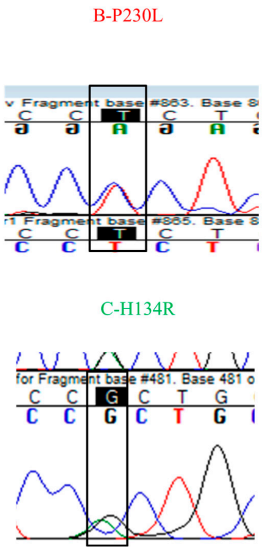


Figure 3. Output of the two electropherograms, highlighting the amino acid substitutions in sdhB (P230L), and sdhC (H134R).

In addition, the populations collected in 2017 from the plot treated with fluxapyroxad and fluopyram were analysed, and the results confirmed that they harboured the B-P230L and C-H134C R-alleles. No target-site mutations were observed in *sdh* d. The alleles detected in the strains studied are summarised in Table 9.

Table 9. *sdh* variants present in the population collected in August 2016 from the plot treated with fluxapyroxad and its derived isolates and monoconidial isolates and in the populations collected in 2017 from the plots treated with fluxapyroxad and fluopyram.

STRAIN	SNPs
Fluxapyroxad population August 2016	B-P230L, C-H134R
Fluxapyroxad isolates August 2016	
1	B-P230L
2	C-H134R
3	B-P230L
4	B-P230L
5	C-H134R
6	C-H134R
7	B-P230L
8	C-H134R
9	C-H134R
10	B-P230L
11	B-P230L
12	B-P230L
13	B-P230L
14	C-H134R
15	C-H134R
Fluxapyroxad monoconidial isolates 2016	
1.1	B-P230L
1.2	B-P230L
1.3	B-P230L
4.1	B-P230L
4.2	B-P230L
4.3	B-P230L
14.1	C-H134R
14.2	C-H134R
14.3	C-H134R
Fluxapyroxad population 2017	B-P230L, C-H134R
Fluopyram population 2017	B-P230L, C-H134R

4. Discussion

BSP, caused by *S. vesicarium*, is an economically significant disease of *Pyrus communis* L., and many fungicide applications are required from petal fall to fruit ripening to protect orchards from BSP [8,30]. In addition to the high environmental and economic impact, intense fungicide use can result in resistance phenomena. The introduction of

new chemicals with different MoAs is thus fundamental to increase the range of effective fungicides against BSP and to reduce the risk of resistance. In chronological order, the last class of fungicides introduced was that of SDHIs, classified by the FRAC as fungicides with medium risk for resistance development, thus requiring proactive resistance management strategies [39].

In 2016, in the area of the experimental orchard located in Altedo (IT), the emergence and persistence of the disease caused by *S. vesicarium* was strongly influenced by weather conditions. In the first 10 days of May, foliar symptoms, such as necrotic spots and vein necrosis, were evident. In early June, the disease also appeared on the fruit, following the rainfall and high temperatures recorded in late May and early June.

In 2017, disease progression was initially slow, but accelerated in July and August, partly because of overhead sprinkler irrigation. The efficacy of the fungicide fluxapyroxad showed a decline as early as 2016, which was confirmed in 2017. In addition, in the second year, a loss of efficacy was also observed for other fungicides belonging to the SDHI class, specifically boscalid, penthiopyrad, and fluopyram.

In all cases, no statistically significant differences in disease incidence were detected between SDHI-treated plots and untreated controls, but a significant difference was observed compared to the reference treatment (cyprodinil + fludioxonil). Notably, fluopyram had never been previously applied in the orchard, suggesting that the loss of efficacy may not be due to local selection pressure, but could involve other mechanisms, such as cross-resistance or an inherently reduced sensitivity of the pathogen [36]. This hypothesis was confirmed by in vitro analyses, which revealed resistance in *S. vesicarium* strains isolated in 2016, particularly in the strain obtained from isolated fruit in the plot treated with fluxapyroxad. These strains exhibited resistance to all tested fungicides, with EC50 values exceeding the resistance threshold. A similar result was observed in 2017, with resistant strains isolated from all plots.

Genes encoding the *sdhB*, *sdhC* and *sdhD* subunits were amplified from DNA extracted from the *S. vesicarium* population treated with fluxapyroxad and collected in August 2016. The analysis of the sequences obtained from the 15 isolates from this population and from three MIs derived from isolates 1, 4, and 14 and the subsequent comparison with the sensitive reference strain 173-1a13FI1M3 revealed two specific point mutations responsible for amino acid exchanges in the B and C subunits of succinate dehydrogenase (SDH) for the first time in *S. vesicarium* in pear; no mutations were observed in the *sdhD* gene. These mutations have been implicated in resistance development in other pathogens and in *S. vesicarium* isolates from alternative hosts, such as asparagus [40].

A mutation was observed in the sdhB gene at nucleotide position 864, resulting in the substitution of proline with leucine (P230L) in the B subunit of succinate dehydrogenase. Furthermore, a nucleotide variation was detected in the *sdhC* gene at position 481, leading to a histidine-to-arginine substitution (H134R) in the C subunit of succinate dehydrogenase. These mutations represent a novel finding in the context of SDHI resistance in *S. vesicarium*-infecting pear, as they had not been previously reported in this host–pathogen system.

All isolates and MIs analysed carried only one of the two mutations (P230L in sdhB or H134R in sdhC). Moreover, populations collected in 2017 from fluxapyroxad and fluopyram treatments confirmed the presence of these mutations, strengthening the hypothesis that they are directly related to SDHI fungicide resistance. In particular, the isolate with the P230L mutation exhibited an approximately 4-fold increase in EC50 compared to sensitive strains, while the H134R mutation conferred an approximately 2.5-fold increase in EC50 compared to the reference strain. These data suggest that the P230L mutation confers stronger resistance, significantly reducing the effectiveness of the fungicide, while the H134R mutation has a less pronounced but still relevant effect. Furthermore, in vitro

analyses revealed that fluopyram was compromised by the presence of both P230L and H134R mutations.

5. Conclusions

In conclusion, the results suggest that repeated SDHI fungicide application has led to the selection of resistant *S. vesicarium* strains, significantly impairing the effectiveness of these treatments. In vitro analyses confirm that the mutations detected in these strains play a key role in the onset and stabilisation of resistance to SDHIs. Sensitivity monitoring in commercial pear orchards has been conducted and remains ongoing. Despite the emergence of resistance, SDHI fungicides are still being used as part of integrated strategies, applied alternately and in mixtures with other products. This highlights the importance of continuous resistance monitoring, as carried out in this study, to track resistance development in plant pathogen populations.

To maintain effective disease control, it is crucial to optimise fungicide use by alternating SDHIs with products that have different modes of action (MoAs), and use them in mixtures. Additionally, exploring alternative control measures and integrating agroecological practices can help mitigate or delay resistance development, ensuring the sustainable management of fungal diseases in pear orchards.

Author Contributions: Conceptualisation, M.C. and K.G.; methodology, M.C., K.G., A.C., M.M., M.F. and P.V.; investigation, K.G., A.C. and M.M.; data curation, K.G., M.F., P.V. and M.M.; writing—original draft preparation, K.G., M.M. and I.M.N.; writing—review and editing, I.M.N., M.M., K.G. and M.C.; supervision, M.C. and I.M.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Conflicts of Interest: Author Alessandro Ciriani was employed by the company Control Union Italia Srl. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

a.i.	Active ingredient
BSP	Brown spot of pear
FRAC	Fungicide Resistance Action Committee
h	Hour
MoAs	Modes of actions
MIs	Monoconidial isolates
SDHIs	Succinate dehydrogenase inhibitors

References

1. Woudenberg, J.H.C.; Hanse, B.; van Leeuwen, G.C.M.; Groenewald, J.Z.; Crous, P.W. *Stemphylium* revisited. *Stud. Mycol.* **2017**, *87*, 77–103. [CrossRef]
2. Blancard, D.; Piquemal, J.P.; Gindrat, D. La stemphyliose de l'asperge. *Rev. Hortic.* **1984**, *248*, 27–30.
3. Basallote Ureba, M.J.; Prados-Ligero, A.M.; Melero-Vara, J.M. Aetiology of leaf spot of garlic and onion caused by *Stemphylium vesicarium* in Spain. *Plant Pathol.* **1999**, *48*, 139–145. [CrossRef]
4. Falloon, P.G.; Falloon, L.M.; Grogan, R.G. Etiology and epidemiology of *Stemphylium* leaf spot and purple spot of asparagus in California. *Phytopathology* **1987**, *77*, 407–413. [CrossRef]

5. Lamprecht, S.C.; Baxter, A.; Thompson, A.H. *Stemphylium vesicarium* on *Medicago* spp. in South Africa. *Phytophylactica* **1984**, *16*, 73–75.
6. Rossi, V.; Pattori, E.; Giosué, S.; Bugiani, R. Growth and sporulation of *Stemphylium vesicarium*, the causal agent of brown spot of pear, on herb plants of orchard lawns. *Eur. J. Plant Pathol.* **2005**, *111*, 361–370. [CrossRef]
7. Suheri, H.; Price, T.V. Infection of onion leaves by *Alternaria porri* and *Stemphylium vesicarium* and disease development in controlled environments. *Plant Pathol.* **2000**, *49*, 375–382. [CrossRef]
8. Ponti, I.; Cavanni, P.; Brunelli, A. Brown spot of pear: Aetiology and protection. *Inf. Fitopatol.* **1982**, *32*, 35–40.
9. Blancard, D.; Allard, E.; Brest, P. La stemphyliose du poirier ou "macules brunes". *Phytoma* **1989**, *406*, 35–37.
10. Heijne, B.; Van Mourik, J. Zwartvruchtrot op peer neemt toe. *Fruitt. Den Haag* **2001**, *91*, 18–19.
11. Llorente, I.; Moragrega, C.; Ruz, L.; Montesinos, E. An update on control of brown spot of pear. *Trees* **2012**, *26*, 239–245. [CrossRef] [PubMed]
12. Vilardell, P. *Stemphylium vesicarium* en plantaciones de peral. *Frutic. Prof.* **1988**, *18*, 51–55.
13. Llorente, I.; Montesinos, E. Brown spot of pear: An emerging disease of economic importance in Europe. *Plant Dis.* **2006**, *92*, 99–104. [CrossRef]
14. Llorente, I.; Moragrega, C.; Ruz, L.; Santamaría, G.; Vilardell, A.; Vilardell, P.; Montesinos, E. Basis for new strategies in integrated control of brown spot of pear (*Stemphylium vesicarium*, telemorpoh *Pleospora allii*). *IOBC/WPRS Bull.* **2010**, *54*, 35–39.
15. Rossi, V.; Pattori, E.; Bugiani, R. Sources and seasonal dynamics of inoculum for brown spot disease of pear. *Eur. J. Plant Pathol.* **2008**, *121*, 147–159. [CrossRef]
16. Montesinos, E.; Moragrega, C.; Llorente, I.; Vilardell, P.; Bonaterra, A.; Ponti, I.; Bugiani, R.; Cavanni, P.; Brunelli, A. Development and evaluation of an infection model for *Stemphylium vesicarium* on pear based on temperature and wetness duration. *Phytopathology* **1995**, *85*, 586–592. [CrossRef]
17. Cugier, J.P.; Humbert, W. Stemphyliose du poirier. Etude de la biologie du parasite et recherches des fongicides actifs. *Phytoma Déf. Vég.* **1991**, *431*, 47–50.
18. Montesinos, E.; Vilardell, P. Evaluation of FAST as a forecasting system for scheduling fungicide sprays for control of *Stemphylium vesicarium* on pear. *Plant Dis.* **1992**, *76*, 1221–1226. [CrossRef]
19. Singh, P.; Bugiani, R.; Cavanni, P.; Nakajima, H.; Kodama, M.; Otani, H.; Kohmoto, K. Purification and biological characterization of host-specific SV-toxins from *Stemphylium vesicarium* causing brown spot of European pear. *Phytopathology* **1999**, *89*, 947–953. [CrossRef]
20. Singh, P.; Park, P.; Bugiani, R.; Cavanni, P.; Nakajiama, H.; Kodama, M.; Otani, H.; Kohmoto, K. Effect of host- selective SV-toxin from *Stemphyilium vesicarium*, the cause of brown spot of European pear plants, on ultrasctucture of leaf cells. *J. Phytopathol.* **2000**, *148*, 87–93. [CrossRef]
21. Cavanni, P.; Ponti, I. Maculatura bruna del pero: Una micopatia sempre d'attualità. *Riv. Fruttic. Ortofloric.* **1994**, *12*, 37–42.
22. Montesinos, E.; Moragrega, C.; Llorente, I.; Vilardell, P. Susceptibility of selected European pear cultivars to infection by *Stemphylium vesicarium* and influence of leaf and fruit age. *Plant Dis.* **1995**, *79*, 471–473. [CrossRef]
23. Cappai, F.; De Franceschi, P.; Ciriani, A.; Collina, M.; Dondini, L. QTLs for susceptibility to *Stemphylium vesicarium* in pear. *Mol. Breed.* **2018**, *38*, 24. [CrossRef]
24. Gazzetti, K.; Diaconu, E.L.; Nanni, I.M.; Ciriani, A.; Collina, M. Genome sequence resource for *Stemphylium vesicarium*, causing brown spot disease of pear. *Mol. Plant-Microbe Interact.* **2019**, *32*, 935–938. [CrossRef]
25. Rossi, V.; Pattori, E. Inoculum reduction of *Stemphylium vesicarium*, the causal agent of brown spot of pear, through application of *Trichoderma*-based products. *Biol. Control* **2009**, *49*, 52–57. [CrossRef]
26. Puig, M.; Moragrega, C.; Ruz, L.; Montesinos, E.; Llorente, I. Controlling brown spot of pear by a synthetic antimicrobial peptide under field conditions. *Plant Dis.* **2015**, *99*, 1816–1822. [CrossRef]
27. Brunelli, A.; Di Marco, G.; Contarelli, G.; Ponti, I. Prove di lotta contro la maculatura bruna delle pere. *Atti Giornate Fitopatol.* **1984**, *2*, 203–212.
28. Brunelli, A.; Rovesti, R.; Di Marco, S.; Ponti, I. Attività di diversi fungicidi contro la maculatura bruna del pero. *Riv. Fruttic. Ortofloric.* **1986**, *1*, 51–54.
29. Ponti, I.; Brunelli, A.; Tosi, C.; Basaglia, M.; Bevilacqua, T.; Emiliani, G.; Cont, C.; Viccinelli, R. Verifica dell'attività di diversi preparati contro la maculatura bruna del pero. *Inf. Fitopatol.* **1993**, *43*, 45–52.
30. Ponti, I.; Brunelli, A.; Tosi, C.; Cavallini, G.; Mazzini, F. Chemical control trials on pear brown spot (*Stemphylium vesicarium*). *Atti Giornate Fitopatol.* **1996**, *2*, 165–172.
31. Llorente, I.; Montesinos, E. Development and field evaluation of a model to estimate the maturity of pseudothecia of *Pleospora allii* on pear. *Plant Dis.* **2004**, *88*, 215–219. [CrossRef]
32. Llorente, I.; Vilardell, P.; Bugiani, R.; Gherardi, I.; Montesinos, E. Evaluation of BSPcast disease warning system in reduced fungicide use programs for management of brown spot of pear. *Plant Dis.* **2000**, *84*, 631–637. [CrossRef]

33. Alberoni, G.; Collina, M.; Pancaldi, D.; Brunelli, A. Resistance to dicarboximide fungicides in *Stemphylium vesicarium* of Italian pear orchards. *Eur. J. Plant Pathol.* **2005**, *113*, 211–219. [CrossRef]
34. Alberoni, G.; Cavallini, D.; Collina, M.; Brunelli, A. Characterization of the first *Stemphylium vesicarium* isolates resistant to strobilurins in Italian pear orchards. *Eur. J. Plant Pathol.* **2010**, *126*, 453–457. [CrossRef]
35. Alberoni, G.; Collina, M.; Cavallini, D.; Brunelli, A. Field strains of *Stemphylium vesicarium* with a resistance to dicarboximide fungicides correlated with changes in a two-component histidine kinase. *Eur. J. Plant Pathol.* **2010**, *128*, 171–184. [CrossRef]
36. Sierotzki, H.; Scalliet, G. A review of current knowledge of resistance aspects for the next-generation succinate dehydrogenase inhibitor fungicides. *Phytopathology* **2013**, *103*, 880–887. [CrossRef]
37. Carolina Duarte Hospital; Tete, A.; Debizet, K.; Imler, J.; Tomkiewicz-Raulet, C.; Blanc, E.B.; Barouki, R.; Coumoul, X.; Bortoli, S. SDHI fungicides: An example of mitotoxic pesticides targeting the succinate dehydrogenase complex. *Environ. Int.* **2023**, *180*, 108219. [CrossRef] [PubMed]
38. Brunelli, A.; Fabbri, M.; Casagrandi, F.; Geminiani, E.; Troiano, P.P. Attività di recenti fungicidi inibitori della Succinato Deidrogenasi (SDHI) contro la maculatura bruna del pero. *Atti Giornate Fitopatol.* **2016**, *2*, 377–384.
39. Fungicide Resistance Action Committee SDHI Fungicides Page. Available online: https://www.frac.info/frac-teams/working-groups/sdhi-fungicides/#open-tour (accessed on 15 April 2025).
40. Fungicide Resistance Action Committee List of Species Resistant to SDHIs April 2015 Page. Available online: https://www.frac.info/media/k5yocwty/list-of-species-resistant-to-sdhis-april-2015.pdf (accessed on 15 April 2025).
41. Alberoni, G.; Cavallini, D.; Collina, M.; Brunelli, A. Baseline sensitivity of *Stemphylium vesicarium*, the causal agent of pear brown spot, to boscalid. *Commun. Agric. Appl. Biol. Sci.* **2009**, *74*, 797–800. [PubMed]
42. Ciriani, A.; Gazzetti, K.; Collina, M. Baseline sensitivity of *Stempylium vesicarium* of pear to SDHIs and fluazinam. In *Book of Abstracts: XXII National Congress of the Italian Society of Plant Pathology*; ETS: Pisa, ltaly, 2016; p. 85.
43. Mair, W.; Lopez-Ruiz, F.; Stammler, G.; Clark, W.; Burnett, F.; Hollomon, D. Proposal for a unified nomenclature for target site mutations associated with resistance to fungicides. *Pest Manag. Sci.* **2016**, *72*, 1449–1459. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.