Monitoring of SDHI Resistance in California in the Grape Powdery Mildew Fungus *Erysiphe necator* Through Identification of Target-Site Mutations

By

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ABSTRACT OF THE THESIS

Monitoring of SDHI resistance in California in the grape powdery mildew fungus *Erysiphe necator* through identification of target-site mutations

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University of California Davis, 2021

Davis, California

Erysiphe necator is a primary pathogen on wine and table grapes that requires several fungicide applications for its effective control. However, there are indications that the pathogen is developing resistance to many of the fungicide classes used in its control in the US and other parts of the world. During the 2017-2020 growing seasons, 711 samples of *E. necator* were collected from wine and table grape vineyards of 18 counties of California. Collected samples were then analyzed for the presence of polymorphisms in the *sdhB, sdhC* and *sdhD* genes of *E. necator*, as mutations in these genes are known to mediate increased tolerance to the succinate dehydrogenase inhibitor (SDHI) class of fungicides. A large number of polymorphisms were discovered, six of which triggered the amino acid substitutions B-p.H242R/L, B-p.I244V, C-p.A83V, C-p.G169S and D-p.I71F that are known to confer SDHI resistance in other fungi. Among the 18 counties that were sampled, Napa and Sonoma were the two counties from which most samples with polymorphisms associated with SDHI resistance were obtained. During the 2017-2020 growing seasons, the B-p.H242R and B-p.I244V substitutions increased in frequency dramatically, whereas the other four polymorphisms were still present in low frequencies in the fungal population. Overall the data indicate that isolates of *E. necator* with SDHI-target-site mutations are already present in this state, which calls for a more cautious use of the SDHI fungicides.

Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work. The first part of result (i.e., in silico annotations of the SDH subunits) presented in this thesis is not an original writing product of myself but were based on a draft manuscript that is prepared by the PI Ioannis Stergiopoulos.

Acknowledgment

The accomplishment of this study has depended on countless relationships for which I feel deeply thankful. I want to give recognition to many whose contributions to the success of my research projects and to my intellectual growth were essential.

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Finally, I would like to give my deepest thoughts towards my first and dearest Buddhist teacher, who shows and lead me on the path of mindfulness, awakening, and enlightenment. May you rest in peace. Namo Shakyamuni Buddha.

"Enlightenment is when a wave realizes it is the ocean." – Thich Nhat Hanh.

Attribution

Part of sample handling from 2017 to 2019, prior to my fully take-over in the project, was performed by Guy Nicholas Mellan Robinson and my advisor Ioannis Stergiopoulos. Nathalie Aoun has contributed raw statistical analysis to this study.

Monitoring of SDHI resistance in California in the grape powdery mildew fungus *Erysiphe necator* through identification of target-site mutations

I. INTRODUCTION

Grapevine (*Vitis*) is grown widely in most California counties with a countless number of varieties for their deliciously succulent fruits, and for producing wine, raisins and juices. The European grape, *Vitis vinifera*, is highly susceptible to powdery mildew *Erysiphe necator* (formerly *Uncinula necator*), one of the most economically important fungal diseases in vineyards of the Pacific Northwest and worldwide (Gadoury et al., 2012). The pathogen is known to cause more severe symptoms in California's arid counties, especially in areas where over-the-canopy irrigation is used for protecting grapevines from frost damage.

1. A brief history of the grape powdery mildew

In general, "powdery mildew" is a common name for a complex worldwide collection of approximately 900 fungal species within 16 genera that infect thousands of plant species, including ornamental and horticultural plants. Grape powdery mildew was introduced to England from North America in 1845, before quickly spreading to France in 1847. Six years later, nearly six million grape growers from all French regions adopted additional routine sulfur applications on 2.5 million hectares of grape to tackle the powdery mildew infections. Since then, the pathogen remains one of the most damaging on grapes fungal pathogens with exceedingly negative impacts in all parts of the world where grapes are growing, including in California. Numerous efforts have been made by scholars to study the pathogen's biology and ecology, genetics, and molecular interaction with its host plant, in an effort to understand and thus manage the disease.

2. Fungal taxonomic classification

As mentioned earlier, "powdery mildew" is considered a complex species due to the wide host-range and the presence of asexual and sexual lifecycles of the fungus, rendering taxonomic classification very complicated. Grapevine powdery mildew is known as an obligate biotroph, proliferating on genera within the Vitaceae, including all the wild North American grapes in the genus *Vitis*, the grape-leaf ivy of the genus *Cissus*, the Virginia creeper, and Boston ivy from the genus *Parthenocissus*, the monk's hood vine of the genus *Ampelopsis,* and a lot more (Gadoury et al., 2012). Among the susceptible hosts, the European grape, V. *vinifera*, is the most economically important one as compared to the rest. Grape varieties that are highly susceptible to *E. necator* include Carignane, Thompson Seedless, Ruby Seedless,

Cardinal, Chardonnay, Cabernet Sauvignon, and Chenin blanc. Those that are more tolerant to the pathogen, include Petite Sirah, Zinfandel, Semillon, and White Riesling (Riaz et al., 2013; Fuller et al., 2014).

In the past, if the sexual stage of a fungus was known, then its classification would rely on its host range and the morphology of its sexual features, including the mature ascocarp, numbers of asci, and appendage tips' morphology. However, if only the asexual stage was known, identification was based solely on the host range (Smith et al., 2020). At a later stage, the identification was based on the comparison between the conserved regions of the internal transcribed spacer (ITS) sequences of ribosomal DNA, which correlates with conidial ontogeny and morphology (Gadoury et al., 2012). Based on its current taxonomic classification, the grape powdery mildew fungus is placed within the family Erysiphaceae, and is widely recognized as a species of *Erysiphe,* having the name *Erysiphe necator* (Schw.) (synonym *Uncinula necator* (Schw.) Burr.)

3. Disease symptoms of grape powdery mildew

The most recognized sign of the fungus is dusty white powder covering the upper side of the grape leaves, spreading to all other green succulent tissues, i.e., young stems, flowers, and fruits. All infected areas generally associate with the chlorotic tissue on the leaf. The initial stage of infections can be quickly spotted by the web-like growth of the fungus on the newly formed leaves. Younger colonies appear to be whiter than the senescent ones, which turn grey as they contain a higher amount of mature ascospores and many more dead mycelia than the younger counterparts. The infected colonies are roughly circular, ranging from a few millimeters to more than a centimeter in diameter. The fungal colonies grow by expanding the mycelia radically at the colony edges, appearing in large numbers in most of the cases, which subsequently merge with the adjacent ones to cover a large part of the leaf. Up until this point, infected leaves become rigid, chlorotic, thus developing multiple necrotic regions and falling off prematurely.

Green tissues are highly susceptible all year round, except during dormant seasons in some colder viticultural areas. Infections happen in leaves, shoots, and berries, especially when they are still young. By covering the leaves, the disease reduces the photosynthesis process, which in-turn reduces the sugar concentration in berries and results in crop loss. The pathogen also infects the berries, but this happens after their sugar content reaches 8% (Gadoury et al., 2003). The pathogen can also infect the stems, colonize dormant buds, and produce overwinter structures in the form of chasmothecia, which can start new disease epidemics in the new growing season. The timing of initial infection is very crucial, as it can determine the impact of powdery mildew on a particular crop, and it usually happens after the first spring rainfall. Thus, the infection rate of powdery mildew may vary greatly from one season to another.

5. Biology and morphology of *Erysiphe necator*

The epiphytic hyphae of grapevine powdery mildew are generally straight, flexuous but strong, hyaline to whitish in color. Measuring 4–5 μm in diameter, the average hypha is approximately 80 μm long. Their branching frequency, place, and angle occur randomly and are highly variable in pattern. Following germination, conidia produce germ tubes which eventually form an aspersorium, the main infection structure of the fungus. After entering the host, elongated hyphae, which extend from the lower side of the appressorium, penetrate the epidermal cell wall, eventually terminating in globose haustoria inside the host epidermal cell. The average haustoria are range from 10 to 25 μm and are the feeding structures of the fungus.

a. The fungal asexual structures and its cycle

In the ectoparasitic powdery mildew, the multiseptate conidiophores are commonly observed protruding perpendicularly to the host surface. Breaching does not occur in most cases. Producing from external mycelium, conidiophores are thin-walled as that of the primary mycelium but more expansive in diameter. They are 131 μm in length, but up to 350 μm, if found on the lower surface of a leaf and 154 μm on the upper surface. They are single hyaline, cylinder-ovoid in shape, and heavily packed together on the susceptible regions. Conidiophores are divided into a chain of conidia, with immature ones located closer to the base. Newly-made spores will be formed at the bottom of the previous spores, making them a single chain. Any conidia on chain produced during this process are clones of one another. In humid conditions, the membrane's turgor pressure between the two conidia is high. The connected membrane between the two conidia will remain rigid, hydrate, and hard to break, thus developing a longer chain than that in arid locations. Despite their relatively lower importance, inconspicuous fibrosin appears like tiny dusty things that can be clearly observed within an individual conidium with a magnified microscope, but not anymore after germination. During the growing season, the majority of grape-infected spores are conidia constantly produced by mycelia in uncountable events. Those conidia continue to germinate to mycelia which spread, infect, exploit and eventually produce even more asexual spores.

b. The fungal sexual structures and its cycle

Following the asexual cycle, the sexual one only occurs in early spring (i.e., before the asexual cycle) and late autumn (i.e., at the end of growing season). While the temperatures are unfavorable for the normal function of asexual cycle, the sexual one begins. Once hyphae of compatible mating types establish contact under favorable conditions for sexual processes to happen, chasmothecia formation is initiated within 24 hours. Attached to woody grape tissues or underground leaf litter, that hardy-overwinter-melanined structure provide protection and shelter for the asci hold within until the following spring. At the beginning of the next season, when the temperature gets warmer or whenever they get access to a

splash of water, chasmothecia crack their shell at a slit-like rupture of the ascus tip and release the asci forcefully as the asci absorb water, rapidly increasing their size. Like conidia, ascospores will germinate with a single germ tube after being released from the asci sac, as a result of which, another life asexual cycle begins.

6. Epidemiology of *Erysiphe necator*

a. Overwintering and dispersal conditions

As mentioned earlier, grape powdery mildew overwinters in the host as dormant haustoria, bud-colonized conidia, and sexual ascospores inside the protective chasmothecia. In general, conidia serve as the primary source of inoculum throughout the growing season, with ascospores only providing an additional secondary source to the overall infection. Dormant haustoria and conidia may initiate new infections at the start of the growing season but they tend to be wiped out by cold weather, which is why ascospores are the major source of inoculum in the following spring. Spring rains or high winds are two critical factors that help to disperse the conidial inoculum and new conidia can be produced from the initial colonies within 7-10 days.

b. Infectious conditions

Even though water is needed for ascospore release, excess humidity (over 85%) will not favor the growth of powdery mildew, since it is known to perform better in moderate conditions (Gadoury et al., 2010). Other harsh weather conditions, such as high heat, could actively reduce its viability. Recently, California has been experiencing more brutal and extensive heat-wave periods in mid-summer than in previous decades. Scorching heat relieves the severity of powdery mildew infections, as conidia and mycelia cannot survive periods of extended exposure to temperatures above 35°C. The optimum temperature for the release of *E. necator*'s ascospores from chasmothecia is 21 to 30°C (Gubler et al. 1999). Another factor that should be kept into consideration is leaf wetness. Generally, spores will be released in significant numbers after 24-hour exposure on wet leaves. The colder the temperature, the slower the spore release. In addition, moderate-to-high air moisture also positively influences the rate of ascospore release and germination process. More specifically, 2.5 mm of rain will wet the leaves and initiate the release of ascospores. The spores will get swollen immediately, and germination begins after 8-12 hours (Gadoury et al., 2010). However, the disease can spread uncontrollably under favorable conditions and without any management protocols.

7. Integrated pest management of grape powdery mildew

a. Sulfur and soft chemicals

Powdery mildew can be extremely difficult to manage and requires control programs to initiate early on in the season, ideally at the same time when the first leaves emerge. Even when symptoms are not present, spraying protectants regularly is shown to be more efficient than applying a few high-dose rounds of fungicides sparsely. The fungus is surprisingly persistent since most of its mycelia bury deep in the leaf's epidermal layer, thus making some commonly used fungicides ineffective. Until now, sulfur applications are still adopted by grapevine farmers as a regular protectant, especially in organic fields. In general, grapevine farmers apply sulfur dust every one or two weeks, depending on the weather conditions and the disease pressure. California growers too use sulfur regularly in order to control the pathogen. However, repetitive applications are required for reasonable control, and the application of sulfur at high temperatures causes phytotoxicity, which then burns plant soft tissues and impedes plant growth (Gubler et al., 1996). Many chemical companies have developed alternative "soft chemistry" products for use when the disease pressure is low to moderate. Potassium bicarbonate, narrow range oils, sulfur formulations, and biological controls are great alternatives that can be used effectively every year. These organic-rated protectants are also increasingly accepted by growers of table grapes. Additionally, one of the biggest advantages of using a mixture of these chemicals is that they slow down the development of resistance against synthetic fungicides. However, their effectiveness is dramatically reduced under high disease pressure (Stummer et al., 2003).

b. General synthetic fungicides

Synthetic fungicides are currently the main means to effectively control powdery mildew. However, their intensive use increases the risk for resistance development against them. Currently four fungicide groups are used against *E. necator*, i.e. the sterol C14α-demethylase inhibitors (DMIs) (FRAC group 3), the quinone-outside inhibitors (QoIs/Strobilurins) (FRAC group 11), the succinate dehydrogenase inhibitors (SDHIs) (FRAC group 7), and quinoxyfen (FRAC 13). Among these SDHIs and quinoxyfen are increasingly used in control of the pathogen, as resistance to both DMIs and QoIs has already developed. The California Department of Pesticide Regulation (CDPR) points out that an increase in synthetic fungicides application on Californian fields has been recorded since 2012. There are five fungicides, i.e., copper, quinoxyfen, tebuconazole, fluopyram, and pyraclostrobin which currently are the most commonly used products against *E. necator* in California. Over the past several years, the SDHI boscalid has also made it into the top five most commonly used fungicides; however, the increasing resistance in many fungal phytopathogens, such as *B. cinerea*, toward the chemical affects its predilection. Fortunately, several other synthetic chemicals, like fluopyram, cyflufenamid, flutriafol, tebuconazole

provide viable options for chemical rotations. Since 2018, grapevine farmers can use also pyriofenone, to control the pathogen (CDPR, 2020).

DMI fungicides, which include triazoles and imidazoles, are widely used in control a wide range of phytopathogenic fungi in agriculture. Their mode of action is based on disrupting fungal cell membranes by inhibiting the biosynthesis of ergosterol, thus hindering the growth of certain fungi. The DMI sensitivity of the fungus can range from extremely high (mostly like to be controlled by the fungicide) to extremely low (mostly resistant to the fungicide). This type of resistance is known as quantitative resistance and growers need several application trials in order to figure out the actual level of fungicide need to control the fungus on their fields and it will different between one to another. QoI fungicides are also effective against diverse fungi of vegetable crops. Their mode of action is very specific and based on inhibition of mitochondrial respiration, by interfering the function of Complex III in the electron transfer chain. However, because of its specific MOA, this fungicide group is highly vulnerable to fungicide resistance. Both DMIs and QoIs have been extensively used in crop protection, but this has led to resistance development against these compounds in many fungi, including in *E. necator* (Dufour et al., 2010; Rallos et al., 2016).

c. SDHIs fungicide and its mode of action

SDHIs (FRAC group 7) inhibit fungal mitochondrial respiration by binding to Complex II of the electron transfer chain, which is also known as the succinate dehydrogenase (SDH) or succinate:ubiquinone oxidoreductase (SQR) complex (Hägerhäll, 1997). Complex II is a heterotetrameric complex that is made up of the subunits SDHA, SDHB, SDHC, and SDHD (Gottlieb et al., 2005, Lemarie et al., 2011). Of these, SDHC and SDHD are membrane anchoring subunits, whereas SDHB and SDHA, are located within the mitochondrial cytoplasm (Gottlieb et al., 2005; Hägerhäll, 1997). Complex II plays an essential role in the Krebs cycle by coupling the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Specifically, two electrons are released during the oxidation of succinate to fumarate in SDHA and the conversion of FAD to $FADH_2$. Subsequently, three iron-sulfur clusters located in SDHB, transfer those free electrons through themselves and to the ubiquinone-binding sites formed in the middle of SDHC and SDHD complex (Gottlieb et al., 2005). Therefore, ubiquinones are reduced to ubiquinols, which are released and carry their electrons to Complex III in the Kreb cycle (Horsefield et al., 2005; Gottlieb et al., 2005).

Resistance to SDHIs is known to be mediated by point mutations in the SDHB, SDHC, and SDHD and at least 15 mutations have been described in various fungal pathogens. Notably, p.H242R/L/Y/V and it homologue substitutions was widely found in *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, *Corrynespora cassiicola, Zymoseptoria tritici* confering the resistance to boscalid. p.I244V and its homologue substitutions are possessed by phytopathogens, such as *Z. tritici, Didymella tenacity,* and

Stagonosporopsis citrulli, causing an increased tolerance to boscalid and fluxapyroxad. Carrying p.A83V or its homologue substitutions confer resistance to pydiflumetofen in *F. graminearum,* resistance to pydiflumetofen and several other SDHI fungicides, i.e., thifluzamide, fluxapyroxad, and sedaxane in *Didymella bryoniae*.

8. Rationale

From 2017 to 2020, samples of *E. necator* were collected from vineyards in 18 counties of California, and were screened for mutations in the *sdhB, sdhC,* and *sdhD* genes that could mediate resistance to SDHIs. Several mutations were identified of which a few have been preciously shown in other fungi to mediate increased tolerance against SDHIs. Notably, the frequency of some of these mutations increased over the years indicating a possible shift in the sensitivity of the *E. necator* towards SDHIs. The data present in this thesis will help devise more effective control management programs against *E. necator* and preserve the usability of SHDIs against the pathogen.

II. MATERIALS AND METHOD

1. *Erysiphe necator* sample collection

Samples (*N*=711) of *Erysiphe necator* were collected from different vineyard blocks, during the growing seasons from 2017 until 2020 across California (N_{2017}=*201*, N_{2018}=*170,* N_{2019}=*273,* N_{2020}=*103*). From all major grape growing regions, samples of 18 counties are included:

Northern California 11 counties:

- Mendocino (N_{total}=*66,* N_{2017}=*4,* N_{2018}=*16,* N_{2019}=*34,* N_{2020}=*12*)
- Trinity (N_{total}=N_{2017}=*2*)
- Lake (N_{total}=*7,* N_{2017}=*1,* N_{2018}=*2,* N_{2019}=*4*)
- Napa (N_{total}=*201,* N_{2017}=*116,* N_{2018}=*28,* N_{2019}=*47,* N_{2020}=*10*)
- Sacramento (N_{total}=*40,* N_{2018}=*7,* N_{2019}=*31,* N_{2020}=*2*)
- Sonoma (N_{total}=*87,* N_{2017}=*42,* N_{2018}=*32,* N_{2019}=*2,* N_{2020}=*9*)
- Yolo (N_{total}=N_{2019}=*5*)
- Marin (N_{total}=N_{2018}=*1*)
- Contra Costa (N_{total}=N_{2019}=*2*)
- San Joaquin (N_{total}=*18,* N_{2018}=*13,* N_{2019}=*5*)
- Solano (N_{total}=*15,* N_{2017}=*1,* N_{2018}=*2,* N_{2019}=*12*)

Southern California 7 counties:

- Fresno ($N_{total}=N_{2019}=16$)
- Kern ($N_{total}=42$, $N_{2018}=36$, $N_{2019}=6$)
- Kings ($N_{total}=N_{2019}=14$)
- Monterey ($N_{total}=26$, $N_{2017}=3$, $N_{2018}=18$, $N_{2019}=5$)
- San Luis Obispo ($N_{total}=58$, $N_{2017}=31$, $N_{2018}=14$, $N_{2019}=3$, $N_{2020}=10$)
- Santa Barbara ($N_{total}=71$, $N_{2019}=49$, $N_{2020}=22$)
- Tulare ($N_{total}=8$, $N_{2018}=1$, $N_{2019}=2$, $N_{2020}=5$)

2. DNA extraction method

Samples were either collected using Tough-Spots or Cotton Swab from infected leaves and grape clusters in commercial and research vineyards. Genomic DNA extraction of isolates was extracted using a modified Chelex extraction (Brewer et al., 2010). DNA quality and concentration were estimated using Qubit and checking absorbance at 230, 260, and 280 nm. DNA extracts were organized in 96-well plates and stored at -20°C until use.

3. Detection of mutations in *sdh* genes of *Erysiphe necator*

a. General PCR conditions for the majority of samples

The *Erysiphe necator* isolates were PCR amplified using REDTaq® (Sigma-Aldrich). The sequences of primers used for PCR amplification of the putative *SdhB, SDHC, SdhD* subunit genes are listed in Table 1. For one 20.0 µL PCR reaction, 1.0 µL of total genomic DNA was added to a master mix composed of 1.0 µL of each forward and reverse primer (10mM), 10.0 µL of REDTaq® (Sigma-Aldrich) premixed PCR Master Mix, and 7.0 µL of ddH_2O. The PCR cycling conditions were as follow: initial denaturation at 95°C for 3 min, followed by 39 cycles at 95°C for 30 sec, 61°C for 25 sec, 72°C for 1 min and a final extension at 72°C for 7 min. DNA amplicons were stored at -20°C until use. For separation, 10.0 µL of each PCR product was loaded on a 1.2% agarose gel (1.2g agarose in 100 ml 1x TAE buffer), stained with ethidium bromide, then run at 90V for one hour. The gel was then exposed under UV light and viewed by using ChemiDoc Imaging System (Bio Rad CAT#17001401). The gel pictures were used to determine the correct bands' intensity and size. PCR amplification product of SDHB gene using EnSDHB-F1 and EnSDHB-R1 is 1,003 bp. Thus, the PCR amplification product of the SDHC gene using EnSDHC-F1 and EnSDHC-R1 is 994 bp. Last, the PCR amplification product of the SDHD gene using EnSDHD-F1 and EnSDHD-R1 is 994 bp. PCR products were prepared in general skirted 96-well plates (Thermo Scientific) and sent out to

Eurofins Genomics LLC. in Louisville, Kentucky for Sanger sequencing. The sequencing reaction consists of 5.0 µL of ddH_2O, 5.0 µL of sequencing primer (10mM), and 1.0-2.0 µL of PCR products.

b. Improved PCR conditions for low-quality samples

For low-DNA samples, the primary PCR products were used as DNA templates for a secondary PCR (including the negative controls). The secondary PCR used REDTaq® (Sigma-Aldrich) to amplify primary PCR products. One PCR reaction was composed of 1.0 µl of the primary PCR product, 1.0 µL of each forward and reverse primer (10mM), 10.0 µL of REDTaq® (Sigma-Aldrich) MasterMix, and 7.0 µL of ddH_2O. The PCR cycling conditions were as follow 95°C for 3 min; 39 cycles at 95°C for 30 sec, 61°C for 25 sec, 72°C for 1 min; 72°C for 7 min. Successful high-quality amplification products were sent to Eurofins Genomics LLC. for Sanger sequencing with 5.0 µL of ddH2O, 5 µL of sequencing primer (10mM) (**Table 1**), and 2.0-4.0 µL of PCR products.

c. Analyze sequencing result

Using the MAX7 sequence alignment program, the generated sequences were compared against the template of SDHB, SDHC, SDHD subunits to investigate point mutations that might appear in some specific samples from the exact locations. The areas which have a high number of SDHIs mutations were summarized and reported to American Vineyard Foundation (AVF) yearly.

Table 1. List of primers used in the detection of allelic variations in *sdh* genes, related to the experimental procedures.

Primer name	Primer sequence	Melting temperature
EnSDHB-F1	CGC TCC TAT TCT CAA TTG AAT CC	Tm: 60.9°C
EnSDHB-R1	ATC CAG CTC GTC TTT TGT ACA G (original 5'-3' sequence: CTG TAC AAA AGA CGA GCT GGA T)	Tm: 60.1°C
EnSDHB-F2	CAT AGT ACT TGA ATC TCA AC	Tm: 52.3°C
EnSDHB-R2	AGA TTT GTT CTT TAC CCT AC (original 5'-3' sequence: GTA GGG TAA AGA ACA AAT CT)	Tm: 52.3°C
EnSDHC-F1	TTA CCA AAT ATC ACC CAC CCA C	Tm: 60.1°C
EnSDHC-R1	GAT TCT TAG AGA TGA AGT CTG CG (original 5'-3' sequence: CGC AGA CTT CAT CTC TAA GAA TC)	Tm: 60.9°C
EnSDHC-F2	TTT CCA ATT CCG TTG TTT C	Tm: 50.9°C
EnSDHC-R2	ACA GAA CAG CCT ATA CTG (original 5'-3' sequence: CA GTA TAG GCT GTT CTG T)	Tm: 51.4°C
EnSDHD-F1	GCT CCA CTC TAC AAT ACA ACT G	Tm: 60.1°C
EnSDHD-R1	TTC AAG CGT GAA TAT AGG TTG AAA G (original 5'-3' sequence: CTT TCA ACC TAT ATT CAC GCT TGA A)	Tm: 60.9°C
EnSDHD-F2	ATA GCA GAG AAC ATT TAC G	Tm: 50.9°C
EnSDHD-R2	GTT GAA AGC CTC AAC ATC (original 5'-3' sequence: GAT GTT GAG GCT TTC AAC)	Tm: 51.4°C

III. RESULTS

1. *In silico* annotations of the SDH subunits of *Erysiphe necator*

a. *E. necator* SDHB subunit

To screen for point mutations in the *E. necator sdhB, sdhC,* and *sdhD* genes, their sequences were obtained from NCBI and used as a reference for our studies. The 885 bp *E. necator sdhB* gene (EV44_g2973) is interrupted by a single 69 bp intron, which after splicing results in an 816 bp long coding sequence (cds) that encodes a 271 amino acid (aa) EnSDHB protein (KHJ35542.1). The protein contains a 26 amino acid long cleavable N-terminal mitochondrial targeting peptide (mTP), as is expected for a mitochondrial protein. EnSDHB contains three iron-sulfur clusters, i.e., the binuclear [2Fe-2S], tetranuclear [4Fe-4S], and trinuclear [3Fe-4S] cluster, which in turn facilitate electron transfer from succinate to ubiquinone. Several conserved among fungi residues are found inside the three iron-sulfur clusters, including 11 cysteine residues (C92, C97, C100, C112 C184, C187, C190, C194, C241, C247, C251) and residues involved in the formation of the Q_P site (P195, W198, W199, H242, I244) (**Figure 1**).

b. *E. necator* SDHC subunit

The 785 bp long *E. necator sdhC* gene (EV44_g2241) is interrupted by two introns of 99 bp and 119 bp in length, respectively which after splicing generate a 564 bp long cds that translates into a 187 aa long EnSDHC protein (KHJ35761). As for SDHB, the SDHC protein contains a 25 amino acid long cleavable N-terminal mTP peptide along with three transmembrane segments, in accordance with the localization of this subunit in the mitochondria's inner membrane. Several conserved among fungi residues are found within then SDHC subunit, including residues S82, R86 and H144 within the Q_P site and the heme *b* segments (**Figure 2**) and residues F96, F99 and F141, which create the Q_P site's hydrophobic environment (Sun et al., 2005).

c. *E. necator* SDHD subunit

The 706 bp *E. necator sdhD* gene (EV44_g2079) is interrupted by two introns of 55 bp and 50 bp, respectively, which after splicing generate a 600 bp cds. The translated 199 aa EnSDHD protein (KHJ35761) is predicted to contain a putative 28 aa long N-terminal mTP and three transmembrane helices. Residues D150 and Y151, which are essential for binding ubiquinone, and residues D119 and Y120 within the Qp pocket, are known to be conserved in the SDHD subunit of many organisms, including in the EnSDHD protein (**Figure 3**).

Figure 1. Multiple sequence alignment of SDHB subunits from various fungi. These fungi include *Erysiphe necator* (En, KHJ35542.1), *Saccharomyces cerevisiae* (Sc, PEJR01000114.1), *Alternaria alternata* (Aa, KJ426262.1), *Alternaria solani* (As, MN657255.1), *Botrytis cinerea* (Bc, KR866380.1), *Corrynespora cassiicola* (Cc, AB548739.1), *Fusarium graminearum* (Fg, JX869236.1), and *Zymoseptoria tritici* (Zt, JF916688.1). Polymorphic amino acids found in the SDHB subunit of *E. necator* and the rest of the plant pathogens in the alignment are highlighted in green. Highlighted and underlined in the alignment are the annotations of three iron-sulfur cluster domains found in the SDHB subunits. Due to the high-level of conservation, other species' iron-sulfur domains can be predicted. Conserved cysteine residues of the three clusters are bolded, and the residues engaged in the Q_P site formation are shown in red.

Figure 2. Multiple sequence alignment of SDHC subunits from various fungi. These fungi include *Erysiphe necator* (En, KHJ35761), *Saccharomyces cerevisiae* (Sc, LBMA01000011.1), *Alternaria alternata* (Aa, KJ637230.1), *Alternaria solani* (As, KC517314.1), *Botrytis cinerea* (Bc, KR705923.1), *Corrynespora cassiicola* (Cc, KZ678128.1), *Fusarium graminearum* (Fg, JX869217.1), and *Zymoseptoria tritici* (Zt, JX869229.1). Polymorphic amino acids found in the SDHC subunit of *E. necator* and the rest of the plant pathogens in the alignment are highlighted in green. Domain annotations of EnSDHC and ScSDH3p: three transmembrane (TM) helices are underlined in dark blue, residues forming Q_P and Q_D sites are in red and purple, respectively. Heme *b* binding residue is boxed.

Figure 3. Multiple sequence alignment of SDHD subunits from various fungi. These fungi include *Erysiphe necator* (En, KHJ30646), *Saccharomyces cerevisiae* (Sc, CP004698.2), *Alternaria alternata* (Aa, FJ437068.1), *Alternaria solani* (As, KC517315.1), *Botrytis cinerea* (Bc, KR705930.1), *Corrynespora cassiicola* (Cc, AB548745.1), *Fusarium graminearum* (Fg, JX869215.1), and *Zymoseptoria tritici* (Zt, JX869201.1). Polymorphic amino acids found in the SDHD subunit of *E. necator* and the rest of the plant pathogens in the alignment are highlighted in green. Domain annotations of EnSDHD and ScSDH4p: three transmembrane (TM) helices are underlined in dark blue, residues forming Q_P and Q_D sites are in red and purple, respectively. Heme *b* binding residue is boxed.

2. Type and frequency of polymorphisms identified in the *sdh* genes of *Erysiphe necator*

The *E. necator sdhB, sdhC,* and *sdhD* genes were PCR amplified and sequenced from 173, 183, and 163 samples, respectively in 2017, from 155, 143, and 164 samples, respectively in 2018, from 208, 193, and 206 samples, respectively, in 2019, and from 64, 58, and 53 samples in 2020, respectively in order to identify polymorphisms that could lead towards increased tolerance to SDHIs. The samples were collected from 8, 12, 16, and 9 California counties in 2017, 2018, 2019, and 2020 respectively, including all vital grape-producing ones.

A total of 32 DNA polymorphisms in the three *sdh* genes were identified in the samples, including 31 single nucleotide polymorphisms (SNPs) and a four bp deletion event (indel) in an intron sequence (**Table 2**). Out of the identified 31 SNPs, eight SNPs are located in introns, eight SNPs are silent substitutions that do not cause any amino acid changes, and fifteen SNPs induce non-synonymous amino-acid changes in one of the SDH subunits **(Table 2, Figure 4)**.

a. Type and frequency of polymorphisms identified in *EnsdhB*

Twelve DNA polymorphisms were detected in the *EnsdhB* gene, five of which were present within the gene's intron and seven within its cds. However, only five SNPs further induced an amino acid change in the translated protein. These were a change from serine to proline at codon 21 (p.S21P), a change from glutamic acid to aspartic acid at codon 94 (p.E94D), a change from histidine to arginine or leucine at codon 242 (p.H242R or p.H242L), and a change from isoleucine to valine at codon 224 (p.I244V).

In California (CA), the polymorphisms found in *EnsdhB* were present in 64% (n=455) of the 711 samples analyzed from 2017 to 2020. Excluding polymorphisms in introns and the ones triggering synonymous amino-acid changes, the non-synonymous amino-acid substitutions accounted for 17.9% (n = 127) of the 711 samples analyzed from 2017 to 2020, although individually, they were found in only a few samples. Among the polymorphisms that caused an amino acid substitution in the produced protein, the p.I244V substitution was the most abundant one as it was found in 9.8% (n = 70) of the samples analyzed, followed by the p.H242R substitution that was found in 7.0% (n = 50) of the samples, and the p.H242L substitution that was found in 0.7% (n = 5) of the samples. The p.S21P and p.E94D substitutions were each found in 0.1% (n=1) of the samples. When considering the distribution of these polymorphisms, the p.H242R substitution was detected in samples collected in 2017, 2018, 2019, and 2020 from ten counties in CA. The p.I244V substitution was detected in samples collected in 2017, 2019, and 2020 from seven CA counties, whereas the p.H242L substitution was detected only in five samples collected in 2018 from one county (Monterey), and with four out of the five samples coming from a single field (Lone Oak). The p.S21P substitution was only sampled once in 2018 from the Renteria field of Napa, CA. The p.E94D was only found once in 2019 from the UC HREC field in Mendocino, CA. Finally, polymorphisms that

caused silent substitutions or were present in introns were observed in very low frequencies of 0.11 to 4.99% (**Table 2, Figure 5**).

b. Type and frequency of polymorphisms identified in *EnsdhC*

A total of twelve polymorphisms were identified within the *EnsdhC* gene. These included a four-bps deletion event, two SNPs within the first intron of the gene, one SNP within the second intron, and eight SNPs within the *EnsdhC* cds. Of the later ones, six SNPs affected amino acid changes in this subunit, namely, a glycine to arginine substitution at codon 25 (p.G25R), an alanine to valine substitution at codon 83 (p.A83V), a threonine to isoleucine substitution at codon 98 (p.T98I), aspartic acid to asparagine substitution at codon 155 (p.D155N), a histidine substitution to aspartic acid at codon 112 (p.H112D), and glycine to serine substitutions at codon 169 (p.G169S) **(Table 2)**.

Polymorphisms found in *EnsdhC* were present in 35% (n=249) of the 711 samples analyzed from 2017 to 2020. Non-synonymous amino-acid substitutions were present in 33.9% (n = 241) of the 711 samples analyzed from 2017 to 2020. Of these, the p.G25R substitution was found in 25.9% (n = 184) of the samples and was sampled in all four years, thus making it the most abundant of all the polymorphisms identified in any of the three subunits. The p.A83V substitution was found in 2.7% (n = 19) of the samples and was sampled in all four years from a total of four counties (Napa, San Luiz Obispo, Sacramento, Solano). The p.D155N substitution was found in 4.4% (n=31) of the samples, and was sampled in 2019 and 2020 from two CA counties (Santa Barbara, Mendocino). However, all samples from Mendocino and Santa Barbara were originally collected from a single field. The rest of the polymorphisms identified in *EnsdhC* were individually present in less than 1% of the analyzed samples. Of the three remaining polymorphisms, the p.G169S was found in 0.7% of the samples and was sampled once in 2017 from one county (Napa), once in 2018 from one county (Sacramento), and twice in 2019 from two counties (San Joaquin, Napa). The p.H112D substitution was found in 0.1% of the samples and was sampled once in one county (Sonoma). Lastly, the p.T98I substitution was also found in 0.1% of the samples and was sampled only once in 2020 from one county (Sonoma) (**Table 2, Figure 6**).

c. Type and frequency of polymorphisms identified in *EnsdhD*

The survey of the allelic variation present in the *EnsdhD* revealed eight SNPs, all of which were present within its cds. However, only four of the SNPs caused amino acid substitutions, i.e., a change of lysine to glutamine at codon 37 (p.K37Q), a change of asparagine to aspartic acid at codon 69 (p.N69D), a change of isoleucine to phenylalanine at codon 71 (p.I71F), and a change of isoleucine to phenylalanine at codon 136 (p.I136F) **(Table 2)**.

Polymorphisms found in *EnsdhD* were present in 3.2% (n=23) of the 711 samples analyzed from 2017 to 2020. Non-synonymous amino-acid substitutions were present in 2.5% (n = 18) of the 711 samples analyzed from 2017 to 2020. Of these, the p.I71F substitution was found in 2.1% (n = 15) of the 711 samples analyzed, all of which were collected in 2018 and 2019 from three counties (San Joaquin, Fresno, Sonoma). The p.I136F, pN69D, and p.K37Q substitutions were all sampled only once (0.1% frequency) in 2018, 2019, and 2019, respectively and from one county (Sonoma, Sonoma, and Santa Barbara, respectively). Polymorphisms that caused synonymous changes in EnSDHD were even rarer, as they were detected in 0.7% (n = 5) of the 711 samples analyzed (**Table 2, Figure 7**).

3. Geographic distribution of non-synonymous polymorphisms in California

Collectively, the 771 samples were collected in 18 counties of California from 2017 to 2020. These counties are separated into two geographic regions: 11 counties from Northern California and seven counties from Southern California. The Northern counties consist of Mendocino, Trinity, Lake, Napa, Sacramento, Sonoma, Yolo, Solano, Contra Costa, Marin, and San Joaquin. Southern counties comprise Tulare, Kern, King, Monterey, San Luiz Obispo, Santa Barbara, and Fresno. Even though the Northern group comprises 11 counties, their collective land area is slightly more than half of the Southern group's area (15,695 square miles versus 29,598 square miles). However, the samples were not collected uniformly in every county across all four years. Some counties, for example, Napa, Sonoma and Mendocino, contributed the majority of the samples from 2017 to 2020, whereas the rest contributed only a few samples in specific years. For example, only two samples were collected from the Trinity county and only in 2017, whereas only five samples were collected from Yolo county and only in 2019. Thus, this study cannot accurately portray the distribution of polymorphisms in the EnSDH subunits across CA. Nonetheless, some preliminary conclusions can still be drawn regarding the emergence of SDHI target-site mutations in different counties.

Table 2. Polymorphisms detected in the *Ensdhb, Ensdhc,* and *Ensdhd* genes of *Erysiphe necator* from 2017 until 2020. Letter prefixes 'c' and 'p' refer to coding (cds) and protein sequences, respectively. Numbering is based on the reference sequences of the three genes deposited in NCBI GenBank. A forward slash is used to indicate a single nucleotide substitution (SNP) at the DNA level, whereas plus and minus symbols are used to number intronic nucleotides based on the closest flanking exon nucleotide. Specifically, a '+' indicates that the intronic nucleotide is closest to the 5' end of the closest exon and a '-' that is closest to the 3' end. An underscore shows the range of nucleotides or amino acids affected by insertions (ins) or deletions (del). Listed on the table is also the effect of non-synonymous substitutions on SDHI sensitivity in *E. necator* (if known) or other fungi. Noted that the table was adapted to cover the majority of samples, but not all, so the frequencies might change slightly. *En: Erysiphe necator*, *Aa: Alternaria alternata*, *As Alternaria solani*, *Bc: Botrytis cinerea*, *Cc: Corrynespora cassiicola, Zt: Zymoseptoria tritici, Fg: Fusarium graminearum.*

Single nucleotide polymorphism (SNP)	Induced amino acid substitution	Frequency (%)	Effect on SDHI resistance
sdhb			
c.61T/C	p.S21P	0.14	Unknown
c.183A/T	p.T61T	0.14	No effect (silent substitution)
c.282G/T	p.E94D	0.14	Unknown
c.396C/A	p.T132T	0.14	No effect (silent substitution)
c.725A/G	**p.H242R**	7.03	Induced resistance in *En, Aa, As, Bc, Cc and Zt*
c.725A/T	**p.H242L**	0.7	Induced resistance in *Aa, Bc and Zt*
c.730A/G	**p.I244V**	9.85	Induced resistance in *En and Zt*
c.342+4T/C	-	0.14	Possibly none (no change coding region)
c.342+13T/C	-	1.13	Possibly none (no change coding region)
c.342+16T/C	-	1.13	Possibly none (no change coding region)
c.342+20T/C	-	43.32	Possibly none (no change coding region)
c.412-20A/T	-	0.14	Possibly none (no change coding region)
sdhc			
c.73G/A	p.G25R	25.88	No effect on *En* resistance
c.207T/C	p.H69H	0.14	No effect (silent substitution)
c.248C/T	**p.A83V**	2.67	Induced resistance in *Zt* and *Fg*
c.288T/C	p.F96F	0.14	No effect (silent substitution)
c.293C/T	p.T98I	0.14	Unknown
c.334C/G	p.H112D	0.14	Unknown
c.463G/A	p.D155N	4.36	Unknown
c.505G/A	**p.G169S**	0.7	Induced resistance in *En*
c.40+20_23del TATC	-	0.42	Possibly none (no change coding region)
c.221+24G/A	-	0.14	Possibly none (no change coding region)
C.221+37T/C	-	0.14	Possibly none (no change coding region)
c.341-31T/A	-	0.14	Possibly none (no change coding region)
sdhd			
c.109A/C	p.K37Q	0.14	Unknown
c.205A/G	p.N69D	0.14	Unknown
c.211A/T	**p.I71F**	2.11	Induced resistance in *Zt*
c.406A/T	p.I136F	0.14	Unknown
c.54G/A	p.R18R	0.14	No effect (silent substitution)
c.147G/T	p.L49L	0.14	No effect (silent substitution)
c.267A/C	p.I89I	0.28	No effect (silent substitution)
c.438T/A	p.A146A	0.14	No effect (silent substitution)

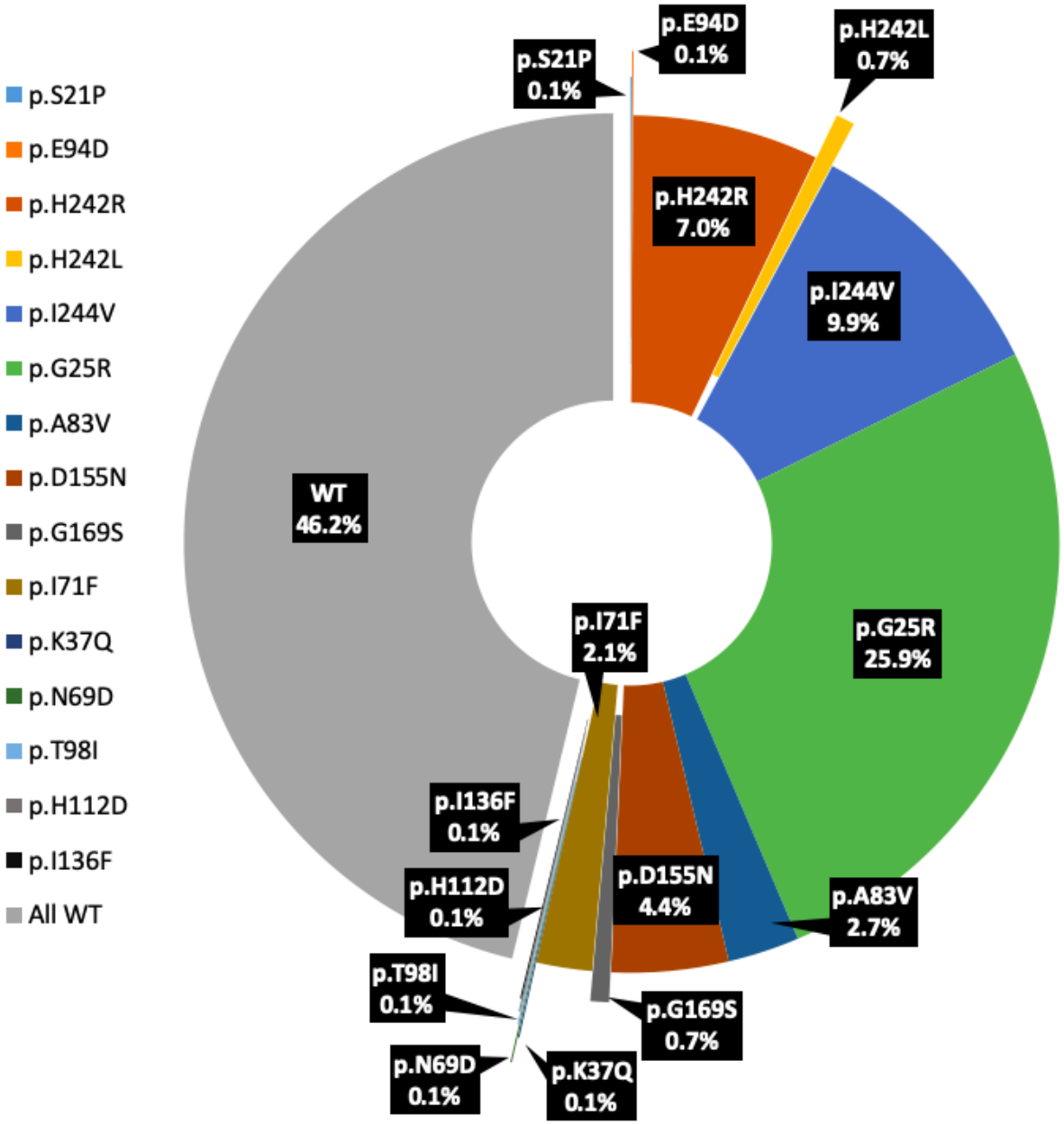


Figure 4. The frequencies of all amino acid substitutions identified in the SDHB, SDHC, and SDHD subunits of *Erysiphe necator* over the four years 2017-2020.

The pie chart shows the frequency percentages of polymorphisms identified from samples collected from 2017 until 2020 in 18 counties of California. The abbreviation 'WT' indicates the combined frequencies of all wild-type strains, intronic substitutions, and synonymous polymorphisms.

Each color code represents one different substitution found in SDH subunits. Their abbreviation names and corresponding frequencies shown inside each black box.

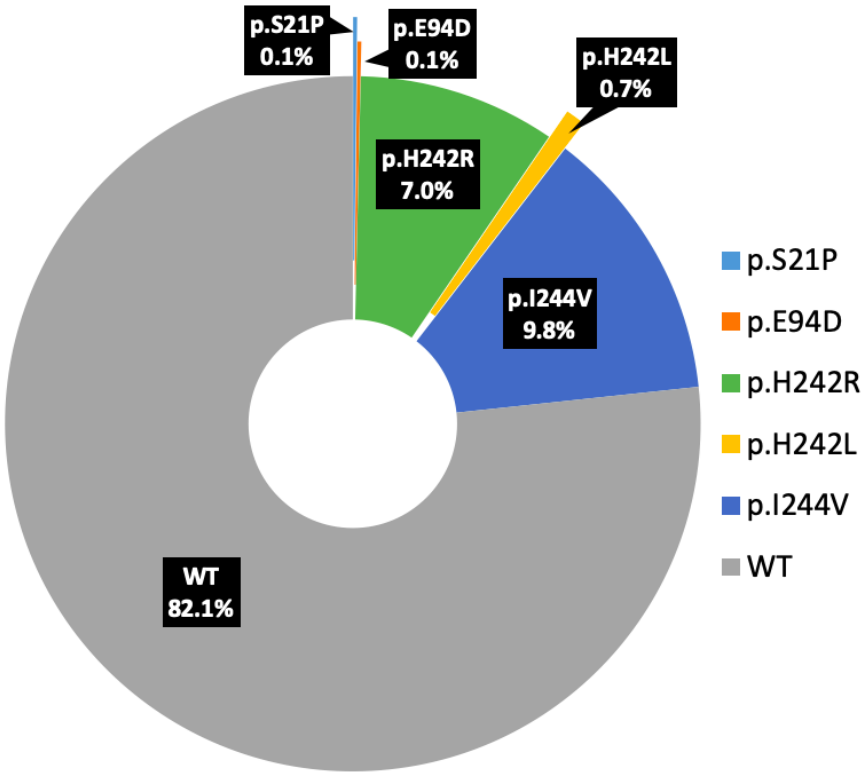


Figure 5. Frequencies of the major non-synonymous polymorphisms identified in the SDHB subunit of *Erysiphe necator* across the 18 Californian counties from 2017 until 2020. The abbreviation 'WT' indicates the combined frequencies of all wild-type strains, intronic substitutions, and synonymous polymorphisms. Each color code represents one different substitution found in the SDHB subunit. Their abbreviation names and corresponding frequencies shown inside each black box.

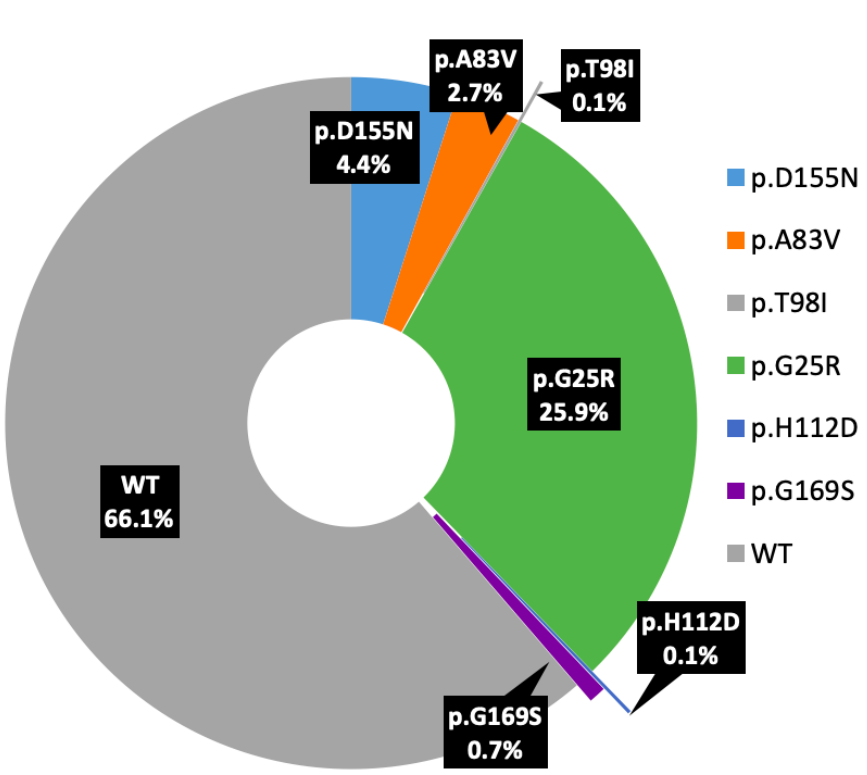


Figure 6. Frequencies of the major non-synonymous polymorphisms identified in the SDHC subunit of *Erysiphe necator* across the 18 Californian counties from 2017 until 2020. The abbreviation 'WT' indicates the combined frequencies of all wild-type strains, intronic substitutions, and synonymous polymorphisms. Each color code represents one different substitution found in the SDHC subunit. Their abbreviation names and corresponding frequencies shown inside each black box.

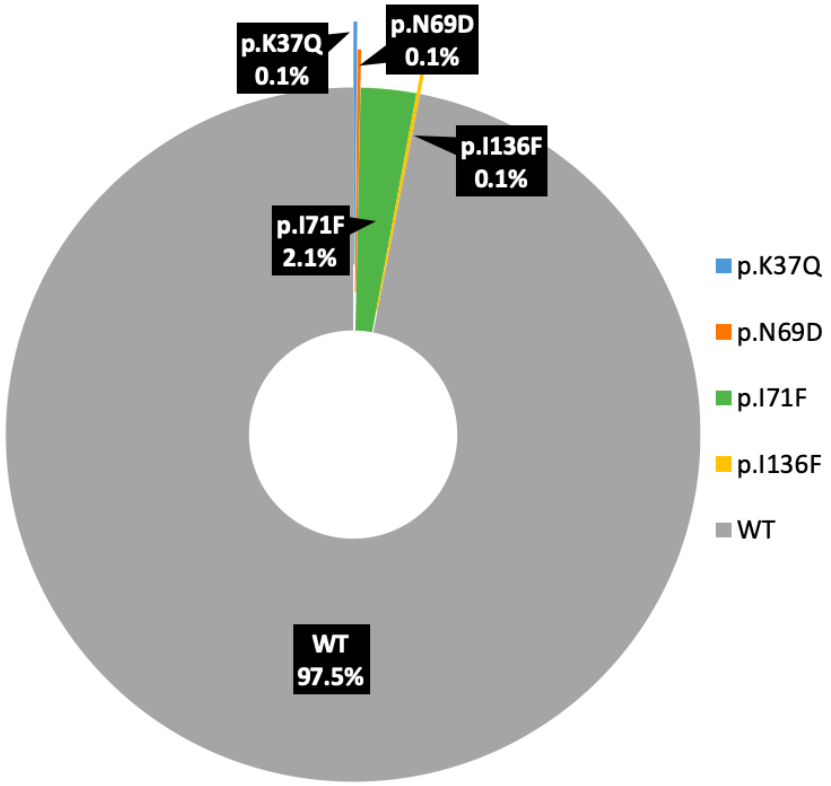


Figure 7. Frequencies of the major non-synonymous polymorphisms identified in the SDHD subunit of *Erysiphe necator* across the 18 Californian counties from 2017 until 2020. The abbreviation 'WT' indicates the combined frequencies of all wild-type strains, and synonymous polymorphisms. Each color code represents one different substitution found in the SDHD subunit. Their abbreviation names and corresponding frequencies shown inside each black box.

a. Polymorphisms in Northern California

A total of six non-synonymous amino acid substitutions conferring SDHIs resistance were identified in the *EnsdhB, EnsdhC* and *EnsdhD* genes in samples collected from Northern California. These six polymorphisms, i.e., B-p.H242R, B-p.I244V, C-p.A83V, C-p.D155N, C-p.G169S and D-p.I71F, accounted for 20.3% (n=91) of the 449 samples collected from this region from 2017 to 2020. The B-p.H242R substitution was found in 7.6% (n = 34) of the 449 samples analyzed for mutations in *EnsdhB* thus making it the most abundant of all the polymorphisms identified in Northern California. The polymorphism was found in six counties, i.e., Napa, Yolo, Sonoma, Solano, San Joaquin, and Contra Costa and was sampled in all four years (2017, 2018, 2019, and 2020). The B-p.I244V substitution was found in 6.0% (n = 27) of the population and was sampled in 2017, 2019 and 2020 from a total of five counties, i.e., Mendocino, Napa, Sacramento, Sonoma, and Lake. The C-p.A83V substitution was found in 3.8% (n=17) of the 449 samples analyzed for mutations in *EnsdhC* and was sampled in all four years from a total of three counties, i.e., Napa, Solano, and Sacramento. The C-p.D155N substitution was found in 0.7% (n=3) of the 449 samples analyzed for mutations in *EnsdhC* and was all sampled in 2019 from Roederer field of Mendocino county. The C-p.G169S substitution was found in 1.1% (n=5) of the 449 samples analyzed for mutations in *EnsdhC* and was sampled in three consecutive years (2017-2019) from a total of four counties, i.e., Napa, Sonoma, Sacramento, and San Joaquin. Lastly, the D-p.I71F substitution was found in 2.9% (n=13) of the 449 PCR samples analyzed for mutations in *EnsdhD* and was sampled in 2018 and 2019 from two counties, i.e., San Joaquin and Sonoma.

b. Polymorphisms in Southern California

Six non-synonymous amino acid substitutions conferring SDHIs resistance were identified in in the *EnsdhB, EnsdhC* and *EnsdhD* genes samples collected in Southern California. These six polymorphisms, i.e., B-p.H242R, B-p.H242L, B-p.I244V, C-p.A83V, C-p.D155N and D-p.I71F, account for 35.5% (n=93) of 262 analysed samples from 2017 to 2020. The B-p.I244V substitution was found in 15.6% (n = 41) of the 262 samples analyzed for mutations in in *EnsdhB*, thus making it the most abundant of all the polymorphisms identified in Southern California. The polymorphism was found in two counties, i.e., Santa Barbara and San Luis Obispo and in 2017, 2019, and 2020. However, all samples with the B-p.I244V substitution were collected from two farms in San Luis Obispo and one farm in Santa Barbara. The B-p. H242R substitution was observed in 6.1% (n = 16) of the samples and was sampled in all four years from a total of two counties., i.e., San Luis Obispo and Kern. The B-p.H242L substitution was found in 1.9% (n=5) of the samples and was sampled in 2018 from two farms in Monterey county. The C-p.D155N substitution was found in 10.7% (n=28) of the 262 samples analyzed for mutations in *EnsdhC* and was all sampled in two consecutive years (2019-2020) from Sierra Madre field in Santa Barbara county. The D-p.I71F substitution were identified in 0.8% (n=2) of the 262 samples analyzed mutations in in *EnsdhB* and

was only found in 2019 in one farm in Fresno county. Last, the C-p.A83V substitution was sampled only once (0.4%) in San Luis Obispo in 2017 (**Figure 8**).

c. Comparison between the two geographic regions

Collectively, despite having a land size that is twice as large as that of Northern California, Southern California accounted for roughly similar number of the polymorphisms. Over the years, one unique amino acid substitution (B-p.H242L) were identified in the Southern region and one novel substitution (C-p.G169S) were regconized in the Northern part. Interestingly, only four polymorphisms (B-p.I244V, B-p.H242R/L, and C-p.D155N) dominate the Southern fields and comprise 96.8% of all mutated samples (90 over 93 samples in total). Similarly, Northern California is overwhelmed by the four polymorphisms (B-p.H242R, B-p.I244V, C-p.A83V, C-p.D155N) and comprise 91.9% of all mutated samples (91 over 99 samples in total) (**Table 2, Figure 8**).

4. Temporal distribution of the mutations over four years

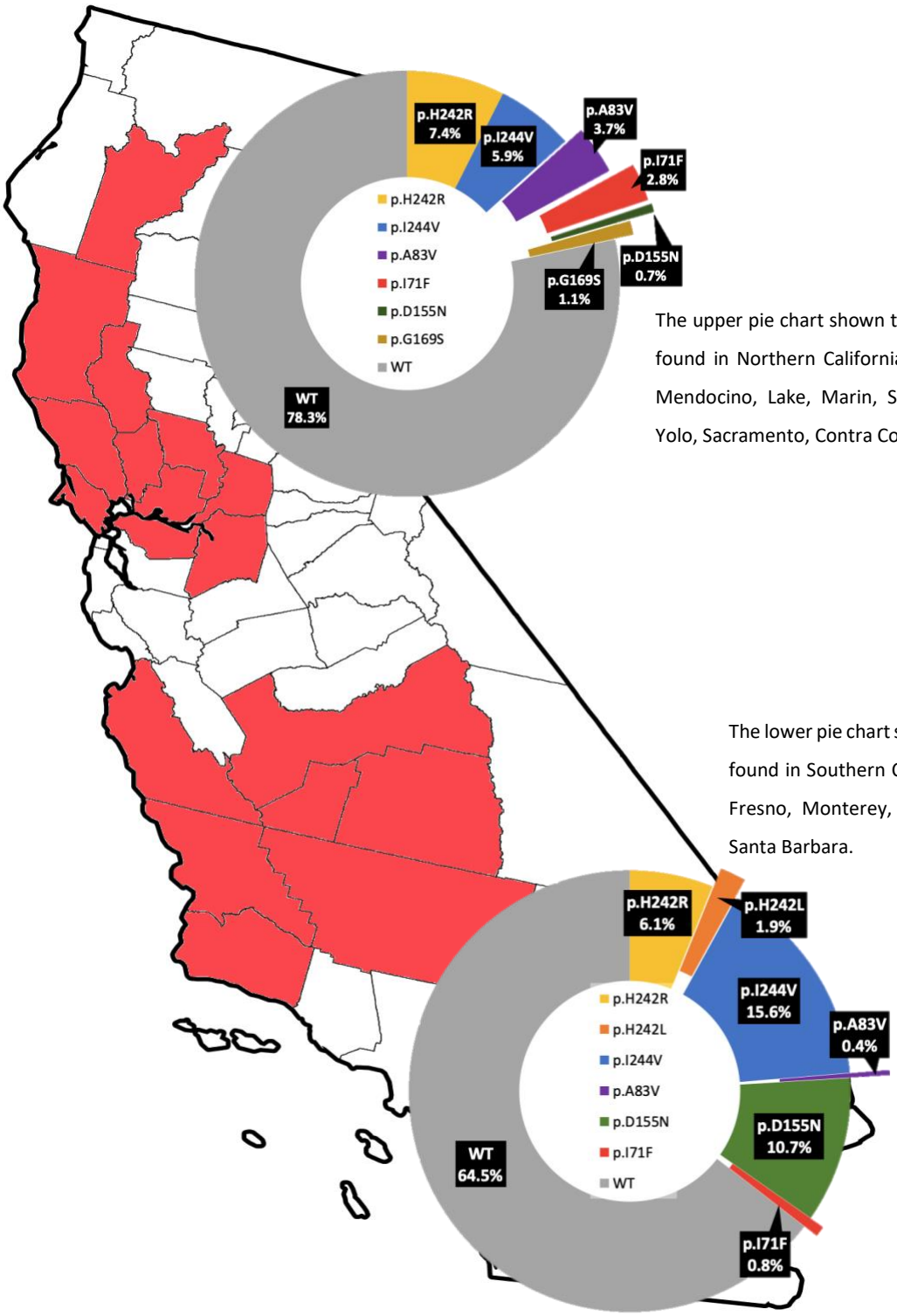
a. Overall analyze for all California counties

Many polymorphisms were identified only in a single year in the 2017-2020 period (e.g., B-p.S21P, B-p.H242L in 2018, C-p.H112D in 2017, D-p.I136F in 2018), suggesting that they are transient polymorphisms. However, other polymorphisms known to confer SDHIs resistance in fungi persist in field populations in low frequencies (<3.5%) and were found in several geographic locations. For instance, the C-p.A83V and C-p.G169S substitutions are in likely their emergence phase, whereas the B-P.I244V, B-p.H242R substitutions have probably moved to the selection phase, as indicated by their steady increase in frequency over the years. The C-p.D155N appeared in 2019, and its frequency has increased rapidly in 2020, thus becoming the second most abundant polymorphism (**Table 2, Figure 9**).

b. A close-up analysis of the Napa and Sonoma regions

Napa is the most famous US grape-growing region and thus most samples in our study were collected from vineyards in this county. Napa holds not only a wide variety of mutations but also hosts a plenty number of samples with mutations known to confer SDHI resistance in other fungi (i.e., B-p.H242R, B-p.I244V, C-p.A83V, and C-p.G169S). Specifically, the frequency of the p.H242R substitution stabilized to around 10% of the field population but the frequency of the B-p.I244V and C-p.A83V substitutions are on the rise.

Figure 8. Frequencies of the primary amino acid polymorphisms identified in the SDHB, SDHC, and SDHD subunits of *Erysiphe necator* in Northern and Southern California. The pie chart shows the frequency percentages of polymorphisms identified from samples collected in from 2017 to 2020 in 18 counties. The abbreviation 'WT' indicates the combined frequencies of all wild-type strains, intronic substitutions, and synonymous polymorphisms.



The upper pie chart shown the SDH polymorphisms found in Northern California counties, i.e., Trinity, Mendocino, Lake, Marin, Solano, Napa, Sonoma, Yolo, Sacramento, Contra Costa, San Joaquin.

The lower pie chart shown the SDH polymorphisms found in Southern California counties, i.e., Tulare, Fresno, Monterey, Kern, King, San Luis Obispo, Santa Barbara.

5. Temporal distribution of the mutations over four years

a. Overall analyze for all California counties

Many polymorphisms were identified only in a single year in the 2017-2020 period (e.g., B-p.S21P, B-p.H242L in 2018, C-p.H112D in 2017, D-p.I136F in 2018), suggesting that they are transient polymorphisms. However, other polymorphisms known to confer SDHIs resistance in fungi persist in field populations in low frequencies (<3.5%) and were found in several geographic locations. For instance, the C-p.A83V and C-p.G169S substitutions are in likely their emergence phase, whereas the B-P.I244V, B-p.H242R substitutions have probably moved to the selection phase, as indicated by their steady increase in frequency over the years. The C-p.D155N appeared in 2019, and its frequency has increased rapidly in 2020, thus becoming the second most abundant polymorphism (**Table 2, Figure 9**).

b. A close-up analysis of the Napa and Sonoma regions

Napa is the most famous US grape-growing region and thus most samples in our study were collected from vineyards in this county. Napa holds not only a wide variety of mutations but also hosts a plenty number of samples with mutations known to confer SDHI resistance in other fungi (i.e., B-p.H242R, B-p.I244V, C-p.A83V, and C-p.G169S). Specifically, the frequency of the p.H242R substitution stabilized to around 10% of the field population but the frequency of the B-p.I244V and C-p.A83V substitutions are on the rise.

6. Consideration of intronic and synonymous polymorphisms appearing in California

During the course of this research, intronic polymorphisms and silent substitutions have also been taken into consideration. Even though the effect of these mutations is not obvious, there could be a compelling reason for the abundance of the c.342+20T/C polymorphism in *sdhB*. Notably, intronic regions represent essential mutational targets. For example, very large introns contain multiple functional elements, including alternative splicing enhancers, silencers, and other regulatory elements. As a result, intronic mutations have often been underestimated, although mutations in these functional elements could alter gene expression and intron splicing. Most of the intronic variations found in the *sdh* genes of *E. necator* are SNPs, but there is also a deletion event (i.e., c.40+20_23del TATC).

Figure 11 shows the frequencies of four commonly found intronic polymorphisms in four years in *E. necator*. The polymorphism B-p.I244V's is also included as a reference. As seen in the figure polymorphisms c.342+20T/C accumulate in significant numbers, and are more abundant than the p.I244V one. Thus, these mutations may have a yet unknown effect on the fungus (**Table 3, Figure 11**).

7. One special case of non-synonymous polymorphism

Also shown in Figure 11 below, one special non-synonymous substitution (C-p.G25R), which is known to have no effect on SDHI resistance in *E. necator*. The polymorphism B-p.I244V's is also included as a reference. As seen in the figure the polymorphism C-p.G25R accumulates in a significant number, and is more abundant than the p.I244V one (**Table 3, Figure 11**). However, its frequencies exhibit a slowly decreasing trend over the four years.

sdhb				
Polymorphisms	**2017**	**2018**	**2019**	**2020**
p.S21P	-	0.59%	-	-
p.T61T	-	-	0.37%	-
p.E94D	-	-	0.37%	-
p.T132T	-	0.59%	-	-
p.H242R	**3.48%**	**6.47%**	**7.33%**	**11.65%**
p.H242L	**-**	**2.94%**	**-**	**-**
p.I244V	**6.47%**	**-**	**13.55%**	**19.42%**
c.342+4T/C	-	0.59%	-	-
c.342+13T/C	0.50%	2.94%	0.73%	-
c.342+16T/C	0.50%	2.94%	0.73%	-
c.342+20T/C	12.44%	71.76%	54.21%	12.62%
c.412-20A/T	-	-	0.37%	-

sdhc				
Polymorphisms	**2017**	**2018**	**2019**	**2020**
p.G25R	35.32%	24.71%	20.15%	15.53%
p.H69H	-	-	0.37%	-
p.A83V	**2.99%**	**1.18%**	**3.30%**	**1.94%**
p.F96F	0.50%	-	-	-
p.T98I	-	-	-	0.97%
p.H112D	0.50%	-	-	-
p.D155N	-	-	6.96%	11.65%
p.G169S	**1.00%**	**0.59%**	**0.73%**	**-**
c.40+20_23del TATC	-	1.18%	0.37%	-
c.221+24G/A	-	0.59%	-	-
C.221+37T/C	0.50%	-	-	-
c.341-31T/A	0.50%	-	-	-

sdhd				
Polymorphisms	**2017**	**2018**	**2019**	**2020**
p.K37Q	-	-	0.37%	-
p.N69D	-	-	0.37%	-
p.I71F	**-**	**7.06%**	**1.10%**	**-**
p.I136F	-	0.59%	-	-
p.R18R	0.50%	-	-	-
p.L49L	-	-	0.37%	-
p.I89I	-	-	0.73%	-
p.A146A	0.50%	-	-	-

Table 3. Frequency percentages of the all polymorphisms identified in the *sdhb, sdhc,* and *sdhd* genes of *Erysiphe necator* across the 18 counties of California from 2017 until 2020. The hyphen '-' indicates the absence of *E. necator* in sampling of the corresponding polymorphisms and that of the year, or the extinction of particular mutations in that particular year. Bolded polymorphisms indicate those conferring SDHIs resistance in grape powdery mildew and other fungal plant pathogens.

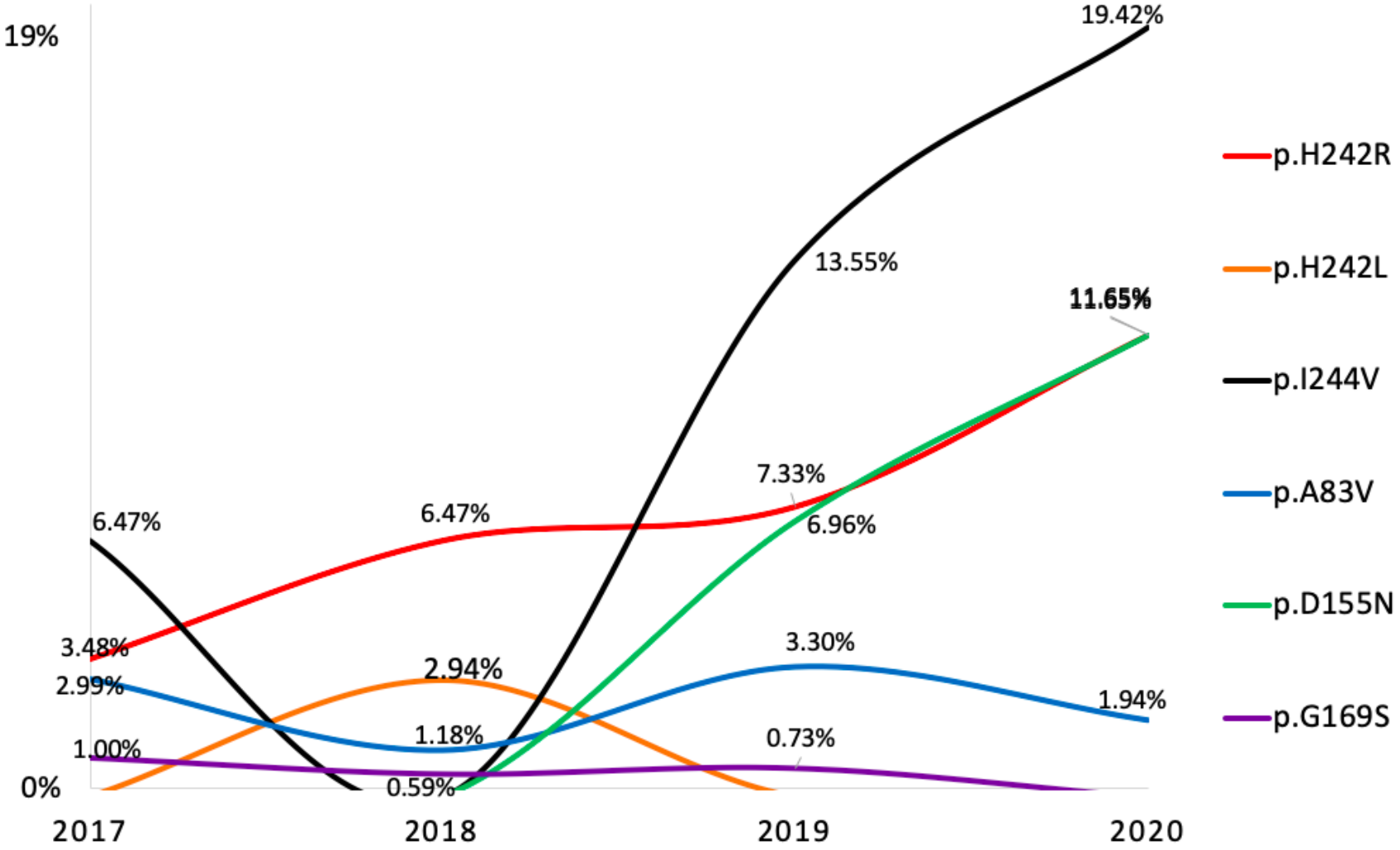


Figure 9. Frequency percentages of the polymorphisms identified in the *sdhb, sdhc,* and *sdhd* genes of *Erysiphe necator* across the 18 counties of California from 2017 until 2020.

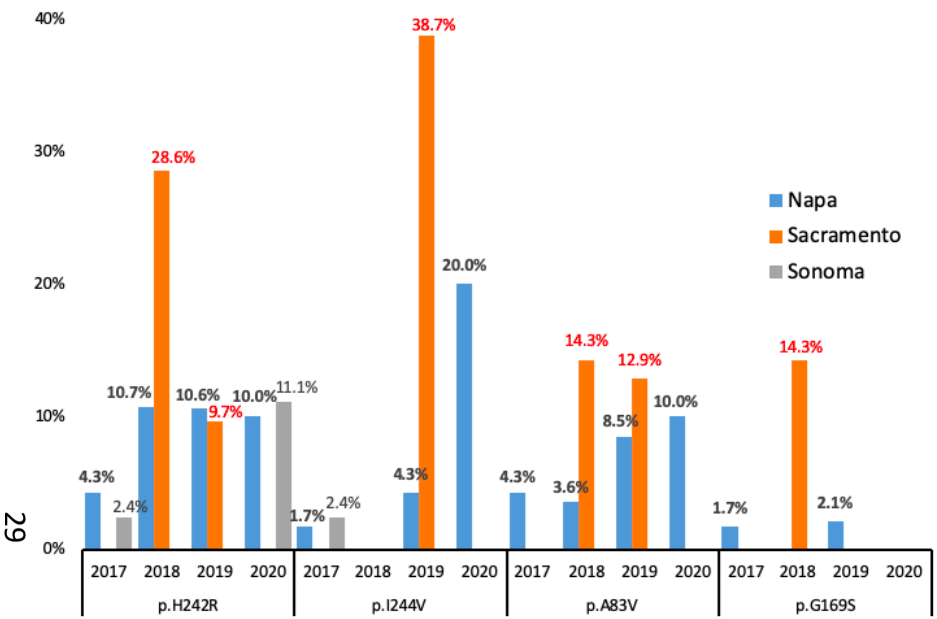


Figure 10. The percentages of the four major polymorphisms over four years in Napa, Sacramento and Sonoma. Four polymorphisms p.H242R, p.I244V, p. A83V, and p.G169S percentages are shown here. Napa county's one are shown in blue, Sacramento are in orange, and Sonoma are in gray.

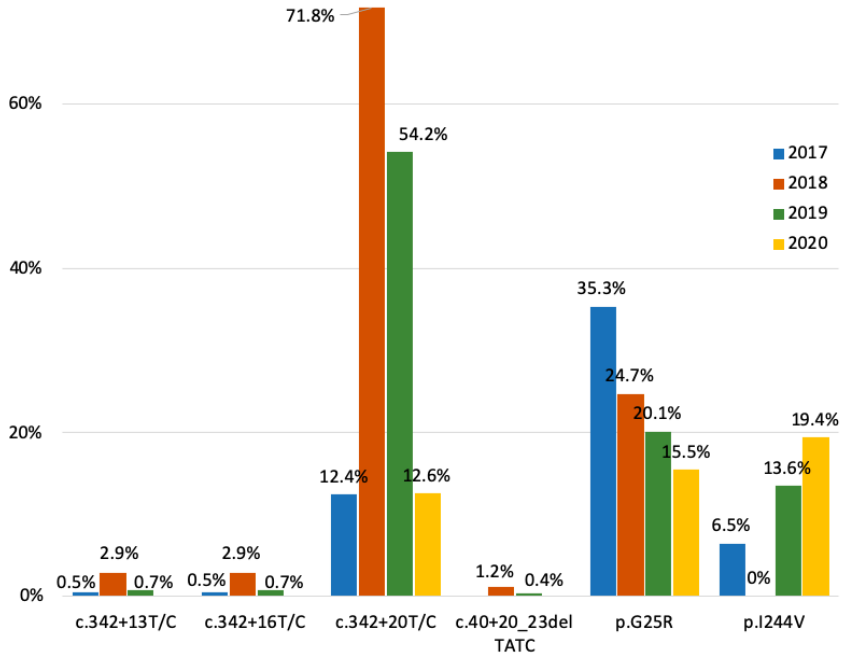


Figure 11. Frequency percentages of the intronic polymorphisms and some others identified in the *sdhb*, *sdhc*, and *sdhd* genes of *Erysiphe necator* across the 18 counties of California from 2017 until 2020. Noted that p.G25R have not had any known effect on SDHIs sensitivity, and the p.I244V is the most common strains which is known for its SDHIs resistance.

IV. DISCUSSION

Given the resistance is emerging against the major fungicide classes used in control of *E. necator*, grape growers should transition from using a single chemical class to applying mixtures of commercial fungicides in order to delay the development of resistance. California grape growers should avoid using more than one application of a fungicide with high risk for resistance development, and rotate to a fungicide with a different MOA. Additionally, it is advisable not to use fungicides of the same FRAC group for two consecutive applications in a single growing season. The practice of using the recommended by the manufacturer doses, will also help reduce the risk of resistance development. However, despite intensive efforts to prevent the development of fungicide resistance, DMI and QoI field resistance seems to have already developed in *E. necator*. Given this fact, the pressure for more frequent use of SDHIs will intensify, thus increasing the risk for resistance development against these fungicides as well.

1. The mechanism facts behind each mutants conferring resistance

a. How different polymorphisms found in EnSDHB wreck the MOA of SDHIs

Out of the three SDH subunits, the SDHB subunit is the most conserved one. SDHB, contains a ubiquinone binding pocket and a Q_P site, where SDHIs molecules actively bind and stop the electron transport chain from functioning properly (**Figure 1**). Amino acids residues residing within the binding site are highly conserved across many different prokaryotic and eukaryotic organisms (Hägerhäll 1997, Horsefield et al. 2006).

In EnSDHB numbering, tryptophan (W) at position 199 binds ubiquinone, and homologues to W224 of *Z. triciti*, W229 of *B. cinerea*, etc. and is further predicted to be binding directly to SDHIs. Compared to the ubiquinone molecules, SDHIs molecules bind deeper into the binding pocket and also make stronger interactions with several nearby amino acids (Sierotzki and Scalliet 2013; Stammler et al. 2015). However, there is none report of mutation at this direct binding site because the theoretical mutant would suffer a solid fitness penalty for carrying such mutation.

Nevertheless, proline at position 195, tryptophan at position 198, histidine at position 242 and isoleucine at position 244 (all in EnSDHB numbering) are more commonly mutated than W199 as a mean to overcome the effect of SDHIs. For example, Histidine 242, which is homologous to the H237 of *S. cerevisiae*, H272 of *B. cinerea*, and H267 of *Z. tritici*, is technically present in close proximity to the Q_P site's W199. The hydrogen bonding between the two is likely to be lost when Histidine 242 is substituted to Arginine, or Leucine (Sierotzki and Scalliet 2013; Stammler et al. 2015). Interestingly, Histidine 272 substitutions to different amino acids in *B. cinerea* (i.e, H272 to Y, R, L, or V) confer different levels of resistance to boscalid (Stammler et al. 2011; Stammler et al. 2015). Structurally, Histidine, plus Arginine are basic amino acids; and according to Britannica Encyclopedia, arginine has an overall charge of +1 at

physiological pH and its side chain is the most basic of all R groups (pKa value of 12.5). As the result, only the Arginine-substitution will partially leave the environment of the binding pocket site unchanged, thus making this type of mutation very common in the field population. Other than that, Leucine, and Valine are non-polar standard ones, and Tyrosine is an uncharged polar phenol derivative. Any substitution to Leucine, Valine or Tyrosine will more or less indirectly rearrange the Q_P site structure, and more important, alter the physiological pH values inside the pocket.

Thus, any differences in SDHI sensitivity level between the mutated strains are more dependent on the amino acid substitution and the host organism than the located distance of one amino acid to the binding site (Scalliet et al. 2012). Another example of a well-known mutation in SDHB happens at the Isoleucine at position 244 in EnSDHB numbering, which is homologous to the I239 of *S. cerevisiae,* I274 of *B. cinerea*, I269 in *Z. tritici*. That Isoleucine is also known to be present in close proximity to the Q_P site and exhibit an indirect interaction with SDHIs (Scalliet et al. 2012). Yamashita et al. 2018 also mentions the resistance toward fluopyram and fluxapyroxad, plus mild resistance to boscalid of the homologous *Z. tritici* I269V strain. Such resistance could be explained by considering Isoleucine and Valine structure, i.e., the similarity of those two non-polar amino acids' structure. Valine could partially play the role of Isoleucine in the interaction with SDHIs.

Put succinctly, p.S21P, p.E94D of EnSDHB either locate very close to the non-conservative amino-terminal end or reside in a very conservative iron-sulfur cluster domain. Such mutations are unlikely to cause any change in the SDHI resistance or suffer the fitness cost.

b. How different polymorphisms found in EnSDHC & EnSDHD wreck the MOA of SDHIs

SDHC and SDHD are less conserved than SDHB and each of these two subunits contains three hydrophobic transmembrane helices. Next to the Q_P site on the matrix side, biochemical and structural studies have pointed out the presence of a second distal binding site, Q_D site, located close to the intermembrane-space side. In addition to other iron-sulfur groups, heme *b*, which embeds into the transmembrane helices, is also crucially required for the electron transfer from succinate to ubiquinone. Heme *b* is also involved in many interactions with nearby residues (Sun et al, 2005). Even though substitutions happen frequently in the SDHC and SDHD subunits, some regions and specific amino acid residues remain highly conserved. Similarly to SDHB, residues in SDHC and SDHD residing within the two binding sites are also good examples.

In *E. necator*, serine at position 82 in SDHC, which is homologous to C-S83 in *Z. tritici* and C-S93 in *S. cerevisiae,* is located at the Q_P site and directly binds to ubiquinone. Likewise, Tyrosine at position 151 in EnSDHD, which is homologous to D-Y130 in *Z. tritici* and D-Y120 in *S. cerevisiae* also directly interacts with the SDHIs. One of the mutations in the EnSDHC subunit that potentially mediates resistance to SDHIs is the substitution C-p.A83V one, which is homologous to the C-A84V in *Z. tritici*. Alanine is the

second smallest nonpolar amino acid, whereas valine is also a small nonpolar one, which is why the substitution between these two amino acids might not cause significant changes inside the binding pocket, thus escaping the fitness penalty.

Interestingly, the Glycine 169 in EnSDHC is located in the third transmembrane helix which is not close to the binding pocket and not directly involved in the binding of SDHIs. In this study, only the C-p.S169G substitution was found in *E. necator*. However, the substitution of Glycine to Aspartic acid in position 169 was only reported in one unpublished European study of *Z. tritici*. Apparently, Aspartic acid is an acidic amino acid with an overall -1 charge, and Serine is a polar uncharged one; both amino acids are bigger in molar mass than Glycine and any substitution to each may lead to the structural rearrangements in the binding pocket. However, substitution to Aspartic acid causes lower physiological pH change to the environment of the Q_P site, probably leading to fitness penalty, which is why it is not frequently found in field population.

Surprisingly, the p.I71F substitution was found in *E. necator* and *Z. tritici*. The substitution it is present in an unknown domain inside SDHD. Even though there are no changes in physiological pH between the two amino acids, Phenylalanine and Isoleucine, the structure of the binding site might change slightly as a butyl group in the side chain of Isoleucine was replaced by a phenyl group in that of Phenylalanine. Additionally, due to the lack of toxicity assays, the function and relevance of the mutation still need to be determined.

Similarly, the p.D155N substitution has been found only in grape powdery mildew in California since 2019. No homologous mutation has been reported in any phytopathogens. The substitution happens in between the second and third transmembrane helices, several amino acids away from the heme *b*. Notably, the Aspartic acid at the position 155 is conserved among the eight organisms' SDHD, which indicates the substitution to Asparagine, a nonpolar amino acid, will cause an increase of overall physiological pH inside the binding pocket. There are not many differences between the side chains of the two, which might not cause any rearrangement in the binding site. Since being discovered the previous year, p.D155N carrying strain has not been tested with any toxicity assay; therefore, this mutation's function towards the overall SDHI resistance is still largely unknown.

Likewise, p.K37Q, p. N69D of EnSDHD, is also located in the non-conservative region close to the amino-terminal end and probably also does not confer any effects to the SDHI resistance. Briefly, p.T98I, p.H112D of EnSDHC, and p. I136F of EnSDHD happen at the highly conservative transmembrane helix II and the linkage between TM helix II and III, respectively. Any molecular changes occuring in those regions might suffer the fitness cost.

On the other hand, p.G25R is somewhat special among the non-synonymous mutations. It locates at the end of the 25-amino-acid long cleavable N-terminal mTP peptide; which will eventually be cleaved off. Thus, it is not likely to have any interaction with the transmembrane helices, binding sites, or heme *b*.

2. The current state of fungicide resistance in California

Although several non-synonymous mutations were identified in this study most were only found in low frequencies and in single years. Still, a few polymorphisms increased in frequency over the four years, including the B-p.H242R, B-p.I244V, C-p.A83V, and C-p.D155N ones.

a. p.A83V substitution

Sun et. al. (2020) found that the substitution of alanine-to-valine at the 83rd codon in the SDHC subunit mediates resistance to pydiflumetofen in *F. graminearum.* In Mao et. al. (2020), they suggested that pydiflumetofen resistance in *Didymella bryoniae* will confer cross-resistance between pydiflumetofen and several other SDHI fungicides, i.e., thifluzamide, fluxapyroxad, and sedaxane, but there was no cross-resistance between pydiflumetofen and other FRAC groups like tebuconazole and diethofencarb.

b. p.D155N substitution

The p.D155N substitution in the EnSDHC subunit was found in 6.96% of all samples collected in 2019 and its frequency quickly increased to 11.65% in 2020 (**Figure 6**). The steady increase suggests that the mutation has emerged and quickly become fixed in the population, suggesting that it might be affecting resistance to a particular SDHI.

c. p.I244V substitution

The p.I244V mutation in the EnSDHB subunit increased in frequency steadily and at higher rates as compared to the previous two. Indeed, this mutation was identified in many California counties and was found in 6.47% of all sample collected in 2017, 0% of the samples in 2018, 13.55% of the samples in 2019 and 19.42% of the samples in 2020 (**Figure 5**). According to some recent studies, phytopathogens, such as *Z. tritici, Didymella tenacity,* and *Stagonosporopsis citrulli,* possessing the p.I244V substitution have increased tolerance to boscalid and fluxapyroxad (Scalliet et al., 2012; Pearce et al., 2019).

d. p.H242R substitution

The p.H242R substitution in EnSDHB increased in frequency since 2017 and is now the second most abundant mutation in California (**Figure 6**). Among all the identified mutations in the SDHB subunit, the p.H242R substitution, is known to lower the sensitivity of several SDHIs in *E. necator* and other pathogens such as *B. cinerea, A. alternata, A. solani, Corrynespora cassiicola,* and *Z. tritici* (Avenot et al., 2008; Stammler et al., 2011; Veloukas et al., 2013; Gudmedstad et al., 2013; Mehl, 2017; Rehfus et al.,

2018, Cherrad et al., 2018). According to previous studies, the B-p.H242R substitution confers *E. necator* resistance to boscalid but no cross-resistance to other SDHIs (Cherrad et al., 2018).

e. p.G169S substitution

According to an unpublished study, the p.G169S substation confers mild resistance in *E. necator* towards boscalid and fluopyram, but not fluxapyroxad. G169 is also mutated to G169D, which leads to different resistance levels to SDHIs, namely benzovindiflupyr, boscalid, fluopyram, fluxapyroxad, isopyrazam, and penthiopyrad.

f. p.I71F substitution

The p.I71F in EnSDHD is a novel substitution as it has not been detected in other fungi so far. Thus, its effect on SDHI resistance is unknown.

3. The emergence of multiple mutations

Polymorphisms in the three SDH subunits could cumulate together under prolonged selection pressure. Indeed, more than one mutations in the three subunits were recorded. Double mutations were either present in two subunits or within the same subunit. It is a reasonable to assume that possessing multiple amino acid substitutions in one or more subunits could confer higher resistance to a wider range of SDHIs. Therefore, studying the effect of double mutations on *E. necator* sensitivity to SDHIs could bring more insights into this subject.

4. The good strategy for management powdery mildew

a. Trends in modeling and forecasting

Grape powdery mildew management is not trivial. Without any guidance, randomly spraying would be cost-intensive and time-consuming. In the attempt to keep powdery mildew infection in check, growers' only hope is to implement proper management practices at the right time. In this context, many attempts to model the growth and spreading mode of *E. necator* have been made, and the earliest one dates back to Delp's in 1954 at UC Davis (Delp, 1954). Later on, in 1980 at the same campus, Sall published her first-of-a-kind forecasting model to predict the efficacy of sulfur application on the fungus. Even though this model has come very handy to help time the sulfur applications, no integration is available for the assistance of fungicide applications. It was only in 1994 that a disease risk assessment was developed by Doug Gubler and his colleagues at UC Davis, before being validated in all California vineyards. The UC Davis powdery mildew risk assessment model consists of two stages: the ascospore stage and the conidiospore stage. Also referred to as the Gubler-Thomas model, its first ascospore stage can forecast

when the initial germination/infection begins, solely based on temperatures and leaf wetness periods. Once the infection begins, the model automatically switches to its second conidial stage and is based only on temperatures to forecast the reproductive rate of the pathogen. Armed with this helpful information, growers are better equipped in their fight against powdery mildew with the least amount of applications.

b. Grapevine powdery mildew management strategy

Table and wine grapes have a long history in California, starting in 1839 when the state's first vineyards were established in Northern California. Since then, grape cultivation expanded to most corners of California. As grapevine regions expand, the need for efficient pesticides to control plant pathogens becomes increasingly prominent. Traditionally, growers have used sulfur dust and soft chemicals as protectants, particularly in the beginning of the season when fungal spores' titration is still low, i.e., at the dormant stage, and during bud break. Then synthetic fungicides are applied several times (at least three times) throughout the rest of the growing season, i.e., full bloom, pre-close, and veraison.

Fungicides from common brands that are currently used in control of *E. necator* are listed in Table 4. Each chemical is recommended to be only applied once in the growing season and there is a need for at least five such applications. First, at least two applications of sulfur or mineral oils are useful at the beginning of the season when dormant grape buds are still intact until the first leaves emerge. Application timing should be based on the disease risk management model for optimal outcomes. Later on, when grapevines fully expand leaves and fungal spores spread throughout the field, at least three applications of different FRAC group fungicides should be aimed at the crucial timepoints: full bloom, pre-close, and veraison. A combination of two different MOA fungicides is preferred to prevent or slow down the *E. necator* resistance progress in all fungicides especially in SDHIs.

Table 4. Collection of highly effective fungicides in used to control *E. necator*. This table includes fungicides' trade names, MOA, and recommended spray timing.

Fungicide Trade name	Active ingredient	Class	FRAC group	MOA	Resistant Risk	Activity	Timing
Lime sulfur	-	Minerals	M2	multi-site	low	contact	Dormant/bud break
Sulfur	-	Minerals	M2	multi-site	low	contact	Dormant/bud break
Mineral oil	-	Oil	NC	multi-site	low	contact	Dormant/bud break
Luna Experience	fluopyram/ tebuconazole	DMI/SDHI	3/7	single-site	medium	systemic	Full bloom/Pre-close/Veraison
Aprovia Top	benzovindiflupyr/ difenoconazole	SDHI/DMI	7/3	single-site	high	systemic	Full bloom/Pre-close/Veraison
Inspire Super	difenoconazole/ cyprodinil	DMI/AP	3/9	single-site	medium	systemic	Full bloom/Pre-close/Veraison
Luna Sensation	fluopyram/ trifloxystrobin	SDHI/QoI	7/11	single-site	high	systemic	Full bloom/Pre-close/Veraison
Luna Tranquility	fluopyram/ pyrimethanil	SDHI/AP	7/9	single-site	medium-high	systemic	Full bloom/Pre-close/Veraison
Merivon	pyraclostrobin/ fluxapyroxad	SDHI/QoI	7/11	single-site	high	systemic	Full bloom/Pre-close/Veraison
Pristine	pyraclostrobin/ boscalid	SDHI/QoI	7/11	single-site	high	systemic	Full bloom/Pre-close/Veraison
Quintec	quinoxyfen	Quinolines	13	single-site	medium	contact	Full bloom/Pre-close/Veraison
Oso	polyoxin-D	Polyoxin	19	single-site	medium	contact	Full bloom/Veraison
Vivando	metrafenone	Benzophenone	U8	single-site	high	contact	Full bloom/Pre-close/Veraison

V. CONCLUSION

In summary, this work has provided a big picture of most, if not all, polymorphisms detected in grape powdery mildew's sdh genes in the majority of California vineyards. Single nucleotide polymorphisms mediate in the SDHB, SDHC, and SDHD subunits resistance to SDHIs, and at least fifteen non-synonymous substitutions have been reported from 18 CA counties. A few well-known polymorphisms conferring SDHI resistance increased in frequency over the four years, including the B-p.H242R, B-p.I244V, C-p.A83V, and C-p.D155N ones, which reflect the fact that SDHI resistant strains become fixed into the *E. necator* field population after four years. Few intronic mutations and an indel mutation were found in several consecutive years in significantly high numbers, even though they are not expected to cause any effect on SDHI resistance. Further experiments, i.e., toxicity assays, should be done to understand better the effect of every (ideally) detected polymorphisms found on the fields. With that in mind, fungicide spaying programs could be tailored for different regions to cope with emerging fungicide resistance in California vineyards.

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