Characterization of fungicide resistance in grape powdery and downy mildew using field trials, bioassays, genomic, and transcriptomic approaches: quinoxyfen, phosphite, and mandipropamid

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ABSTRACT

Development of fungicide resistance in fungal and oomycete pathogens is a serious problem in grape production. Quinoxyfen is a fungicide widely used against grape powdery mildew (*Erysiphe necator*). In 2013, *E. necator* isolates with reduced quinoxyfen sensitivity (designated as quinoxyfen lab resistance or QLR) were detected in Virginia. Field trials were conducted in 2014, 2015, and 2016 at the affected vineyard to determine to what extent quinoxyfen might still contribute to disease control. Powdery mildew control by quinoxyfen was good, similar to, or only slightly less, than that provided by myclobutanil and boscalid in all three years. The frequency of QLR in vines not treated with quinoxyfen declined only slowly over the three years, from 65% to 46%. Information about the mode of action of quinoxyfen is limited; previous research suggests that quinoxyfen interferes with the signal transduction process. We profiled the transcriptomes of QLR and sensitive isolates in response to quinoxyfen treatment, providing support for this hypothesis. Additional transcriptional targets of quinoxyfen were revealed to be involved in the positive regulation of the MAPK signaling cascade, pathogenesis, and sporulation activity. Grape downy mildew (*Plasmopara viticola*), another important grape pathogen, is commonly controlled by phosphite fungicides. A field trial and laboratory bioassays were conducted to determine whether *P. viticola* isolates from vineyards with suspected control failures showed reduced sensitivity against phosphite fungicides. Prophyt applied at 14-day intervals under high disease pressure provided poor downy mildew control in the field. Next-generation sequencing technologies were utilized to identify 391,930 single nucleotide polymorphisms (SNPs) and generated a draft *P. viticola* genome assembly at ~130 megabase (Mb). Finally, field isolates of *P. viticola* collected from a Virginia vineyard with suspected mandipropamid control failure were bioassayed. The EC_{50} values of the isolates were >240 $\mu g.ml^{-1}$ for mandipropamid, well above the field rate. The *PvCesA3* gene of two resistant isolates was sequenced revealing that these isolates had a GGC-to-AGC substitution at codon 1105, the same mutation that has been found associated with CAA resistance elsewhere.

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GENERAL AUDIENCE ABSTRACT

Powdery and downy mildew are two diseases of grapes that can cause large yield losses, and are usually controlled by regular fungicide applications. Development of fungicide resistance has been a growing challenge. Quinoxyfen is a protectant fungicide commonly used against powdery mildews. Unusual grape powdery mildew isolates that grew well on quinoxyfen-treated plants in the laboratory (designated as quinoxyfen lab resistance or QLR) were detected in a Virginia vineyard. In 2014, the first year of this study, 65% of powdery mildew isolates from parts of this vineyard that received no further quinoxyfen treatments had the QLR type of resistance, and this declined only slowly to 46% by the third year. Field trials were conducted in 2014, 2015, and 2016 to determine the efficacy of quinoxyfen in the presence of QLR. Powdery mildew control by quinoxyfen on both grape clusters and leaves was similar to, or only slightly less, than that provided by the standard anti-powdery mildew fungicides myclobutanil and boscalid in all three years. In order to gain a better understanding of the mode(s) of action and resistance mechanism(s) of quinoxyfen, gene expression of QLR and sensitive isolates, both in the presence and absence of quinoxyfen, was analyzed by nucleic acid sequencing. This study confirms previous research suggesting that quinoxyfen interferes with the important biological process signal transduction, and revealed additional gene targets of quinoxyfen.

The phosphites are a group of fungicides commonly used to control grape downy mildew. Control failures after phosphite application have occasionally been suspected, and downy mildew isolates from vineyards with and without suspected control failures were tested in laboratory bioassays to determine if any level of resistance could be demonstrated. There was a limited range of sensitivity, and none of the isolates showed a notable loss of sensitivity. A field trial was conducted to determine the efficacy of one phosphite fungicide, Prophyt, applied at 14-day intervals under conditions favorable for disease development. Prophyt provided poor downy mildew control, suggesting that it has to be applied more frequently. Next-generation sequencing technologies were utilized to identify genetic markers for clade identification and generated a draft genome assembly of grape downy mildew, which improves the understanding of grape downy mildew genome. Grape downy mildew isolates collected from a vineyard in Virginia where mandipropamid provided poor control of downy mildew were bioassayed. The isolates tolerated mandipropamid rates well above the field rate, showing that they were indeed resistant. The mutation that confers mandipropamid resistance on other continents was found in the *PvCesA3* gene of two resistant isolates.

DEDICATION

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Chapter 1. Background on grape pathogens *Erysiphe necator* and *Plasmopara viticola*

1.1. *Erysiphe necator*

Grape production is an economically important industry in Virginia. In 2014, Virginia ranked 7th in the nation, with a grape production estimated at 8,800 tons. This represented an increase of 18% from 2013. Based on utilized production, the average price received per ton was estimated at $1,850.00 in 2014 (Lidholm 2015).

Erysiphe necator, which causes grapevine powdery mildew, is one of the world's most widespread grapevine pathogens. The disease originated in eastern North America, and was transported with commercial grapevines/rootstocks to Europe in the 1840s, and soon became a focus of disease management in all grape-growing regions (Gadoury, 2012). The pathogen can infect grapevines at a wide range of growing stages with the stages from early bloom to berry touching being the most critical ones requiring disease management (Halleen and Holz 2001). One of the most common signs of this disease is the whitish colonies that can develop on both sides of the leaves. At early developmental stages of grape powdery mildew, mycelium starts to grow and adhere to leaf surfaces and produce chains of conidia. In the absence of chemical control, colonies will increase rapidly in size and number under favorable weather conditions. Severely infected leaves may become brittle resulting in premature defoliation. The pathogen can also infect dormant buds causing "flag shoots" in the next growing season, where new shoots heavily coated with fungal growth arise from the infected buds (Cortesi et al. 2004). If infected later in shoot development, the shoots will develop brown to black irregular blotches with indistinct margins. Young inflorescences and berries are the most susceptible. Berries can be infected from immediately after bloom through 4 weeks post-bloom and quickly become covered in conidia, giving a grayish appearance. The epidermal tissue will stop growing when severely infected, which may result in berry splitting as young fruit expand, giving rise to infection by secondary parasitic organisms. Severe infections on the rachis can also lead to premature cluster failure (Gadoury et al. 2015).

Powdery mildew reduces the winter hardiness and the survival rate of dormant buds (Pool et al. 1984). Photosynthesis will be impaired due to mildew coverage on the leaf and premature defoliation, resulting in low carbon dioxide fixation (Dhillon et al. 1992). Infection of

inflorescences lead to poor fruit set, fruit drop and darkening of the berries, and greatly reduced yield (Thind et al. 2004). Fruit infection reduces wine quality, causing increased acid concentration and off flavors. Ruptured berries often lead to secondary infection (Halleen and Holz 2001).

Disease progression is initiated at temperatures as low as 10°C, and is promoted by warm temperatures, with an optimum of 25°C (Wilcox et al. 2015). Spherical ascocarps (chasmothecia), start to develop on the leaf surface as early as late July and last throughout the season. Chasmothecia are at first light yellow and gradually turn black when mature, and then are washed off the leaves by rain and overwinter in the cracks of the bark. The ascospores are released as primary inoculum and first infect leaves growing nearby when free water is available. High relative humidity promotes germination, and the conidia do not need the presence of free water to infect the host. In areas with mild winters, mycelium and conidia may survive in dormant buds; the overwintered mycelium and conidia are activated at bud break and cause flag shoots. In areas with severe winters, the infected buds are less likely to survive than the healthy buds. Conidia serve as secondary inoculum and are transmitted mostly by the wind. Free water such as rainfall may reduce disease development by washing off the conidia and disrupting mycelial growth (Gadoury et al. 2015).

The classification of powdery mildew genera was largely based on the characteristics of the teleomorph stage as of the end of the 20th century (Glawe 2008). This system first emerged in 1851 and was commonly used by many later plant pathologists (Burrill and Earle 1887; Salmon 1900). However, teleomorphic structures are not always available to aid identification, and anamorph characteristics have increasingly been studied to aid in identification of powdery mildew fungi (Zaracovitis 1965, Glawe 2008). Braun (1987) re-categorized the powdery mildews in 1987 by introducing subfamilies, tribes, and genera. Aside from the morphological attributes of the anamorph and teleomorph, some other structural features were taken into consideration such as the location of the mycelia (endophytic or epiphytic), and the formation of the conidia (in chains or singly) (Braun 1987). Even so, some distinctions can be difficult to observe, for instance, whether conidia are produced in true chains, or singly on the tip of conidiogenous cells, forming pseudochains (Heffer et al. 2006).

The old taxonomic system of powdery mildews has also significantly changed based on gene sequencing and host specificity data. In the new taxonomy, powdery mildew genera have been grouped into five tribes. The tribe *Erysipheae* was further subdivided into subtribes based on ascocarp appendages (Saenz and Taylor 1999; Saenz et al. 1994; Wang et al. 2006). Today, the most widely accepted name of grape powdery mildew is *E. necator* Schw. based on the phylogenetic information inferred from the internal transcribed spacer (ITS) of ribosomal DNA sequences combined with conidium and conidiogenesis morphology (Braun et al. 2002; Saenz and Taylor 1999).

Various fungicides have been developed and put in use to control *E. necator* (Table 1.1). With the advent and intensive use of single-site fungicides, resistant *E. necator* isolates have emerged in grape growing regions worldwide, resulting in increased disease management cost and severe yield loss (Hollomon et al. 1997a). Resistance has been reported to most of the major mildewcide groups, such as the MBC fungicides (methyl benzimidazole carbamates) (Pearson and Taschenberg 1980), DMI fungicides (demethylation inhibitors) (Steva et al. 1990), QoI fungicides (quinone outside inhibitors) (Wilcox et al. 2003), and the aryl-phenyl-ketone fungicides (metrafenone) (Kunova et al. 2016). Additionally, resistant isolates have also been documented in cucurbit powdery mildew (*Podosphaera xanthii*) against SDHI fungicides (succinate dehydrogenase inhibitors) (Miyamoto et al. 2010), and in wheat powdery mildew (*Blumeria graminis*) against azanaphthalenes (quinoxyfen) (Hollomon et al. 1997b) implying a high potential of resistance development in grape powdery mildew to the latter group.

1.2. Quinoxyfen

Quinoxyfen is a surface-mobile protectant fungicide developed by Dow AgroSciences. It has specific efficacy against powdery mildews. Quinoxyfen belongs to the azanapthalene fungicide group, FRAC code 13 (FRAC 2017). The other fungicide in this fungicide group is proquinazid; cross resistance of this fungicide with quinoxyfen has been reported in *E. necator* (Genet and Jaworska 2009).

The resistance risk of quinoxyfen was evaluated. Three hundred and forty *Blumeria graminis* isolates, mainly from Scotland and England from 1991 to 1996, were tested to determine the baseline sensitivity (Hollomon et al. 1997b) by measuring the germ tube length and the

formation rate of appressoria in bioassays. The ED_{50} ranged from <0.001 mg/L to 0.016 mg/L and the median ED_{50} was between 0.001 and 0.005 mg/L. Mutants with ED_{50} values >0.016 mg/L were isolated at a very low frequency in the field. On the other hand, resistant isolates were generated in the lab. Interestingly, the resistant isolates were all defective to some extent and could only survive on quinoxyfen-treated leaves (Hollomon et al. 1997b). Bernhard *et al*. monitored the sensitivity of wheat powdery mildew (*Blumeria graminis*) to quinoxyfen in Western Europe for five years starting in 1995. The mean EC_{50} ranged from 0.039 mg/L to 0.071 mg/L (Bernhard et al. 2002).

Green et al. (2005) documented the baseline sensitivity of *E. necator* by testing 56 isolates from Europe and South Africa. The EC_{50} ranged from <0.03 mg/L to 2.6 mg/L. A discriminatory dose of 50 mg/L was used in this study. They also reported that a leaf disc sporulation assay may overestimate the frequency of isolates with significantly reduced sensitivity to compounds such as quinoxyfen, which act exclusively as protectant fungicides, especially when the inoculum density is high (Green and Gustafson 2006). Isolates with high quinoxyfen resistance (EC_{50}~100 mg/L) were documented in Europe (Genet and Jaworska 2009), but no published reports on their practical significance appear to be available beyond the annual meeting minutes of the FRAC AZN working group (Kemmitt et al. 2014). Field trials in western New York in 2010 and 2011 showed poor control of grape powdery mildew, which was attributed to "hypothesized but not confirmed resistance" (Wilcox 2012). In September 2013, a commercial vineyard in western Virginia reported difficulties with PM control during the season, and quinoxyfen-resistant powdery mildew isolates were collected at this site. These isolates grew normally on leaves and plants treated with full label rates (3 oz in 100 gallons, 50 mg/L) (Colcol and Baudoin 2016). Isolates of grape powdery mildew with this type of reduced quinoxyfen sensitivity will be designated as quinoxyfen lab resistance or QLR in this report.

Publications on quinoxyfen resistance in grape powdery mildew are rather limited. However, resistance has emerged in some other powdery mildew species. Davey and McGrath (2006) determined baseline sensitivity in cucurbit powdery mildew (*Podosphaera xanthii*), 15 isolates in 2004 and 21 isolates in 2005 were tested in the state of New York. Thirteen isolates were able to grow at 1 mg/L quinoxyfen and seven isolates were able to grow at 10 mg/L. In 2005, 13 out of the 20 isolates tested grew at 1 mg/L and seven isolates were able to grow at 5 mg/L quinoxyfen.

The observed variation in resistance levels suggested that there might be a risk of resistance development in the pathogen population to this fungicide over time (Davey and McGrath 2006). In 2010, 96 isolates were tested. The percentage of isolates tolerating 10 mg/L quinoxyfen increased to 47%, and within these isolates, 42% were tolerant to 40 mg/L quinoxyfen. The reduced sensitivity was discovered to increase quickly. In 2013, 100% of the tested isolates were found to tolerate 10 mg/L, 27% isolates tolerated 40 mg/L, and 13% isolates tolerated 80 mg/L (McGrath 2013).

Little is known about the specific mode of action of quinoxyfen. In the early studies (Wheeler et al. 2000; Wheeler et al. 2003), quinoxyfen was shown to interfere with germination and penetration of the pathogen into the host. Even though some conidia did germinate, their germ tubes became abnormal, grew longer and their tips failed to swell and remained hypha-like, while normal elongated germ tubes are usually 20 μm long and would form a swollen tip with a distinct hooked apical lobe. Quinoxyfen had no activity against *Blumeria graminis* (*Bgh*) once the primary appressorium had formed or the pathogen had penetrated into the host, indicating that quinoxyfen only inhibits the events of early germination and infection (Gustafson et al. 1999).

Wheeler et al. (2000) reported that quinoxyfen-resistant isolates have an increased ability to germinate on artificial surfaces compared to sensitive isolates, indicating that the mutant conidia may not receive the correct signals associated with host recognition, and suggesting that quinoxyfen may put a stop to the host recognition procedure of the pathogen. A later study (Wheeler et al. 2003) further substantiated this conclusion. Differential display-reverse transcription PCR (DDRT-PCR) identified a gene transcript in wild-type conidia, which was much less transcribed in conidia from quinoxyfen-resistant mutants. The identified mRNA encodes a Ras-like GTPase activating protein (GAP), which may interact with a small molecular weight Ras-type GTP binding protein. RT-PCR of mutant and wild-type isolate in different stages showed that even though *gap* gene expression was not detected in the mutant conidia, it was actually expressed in the later stages of the infection process (Wheeler et al. 2003).

Lee et al (2008) proposed that quinoxyfen plays a more important role in interfering with the germ tube differentiation rather than germination. Additional evidence showed that quinoxyfen might be involved in host recognition and penetration. Quinoxyfen significantly inhibited wild

type *Bgh* germination on plant-derived surfaces (barley epidermis), but had less effect on less plant-like surfaces (artificial surfaces). Meanwhile, quinoxyfen did not inhibit mutant germination on any surface, inferring that fungal enzymes present in the conidial extracellular matrix (ECM) may degrade components of the plant cuticle to release breakdown products, which can be perceived by the conidia as part of the signaling pathway related to germination. Quinoxyfen can also inhibit the activity of serine esterase, which is the key protein that hydrolyzes cutins and waxes (Feng et al. 2011), in the wild-type but not in mutant isolate. A gene encoding cutinase protein in mutant isolate was also found overexpressed in the presence of quinoxyfen, implying that resistance is mediated by overexpression of cutinase.

1.3. *Plasmopara viticola*

Plasmopara viticola, the causal agent of grapevine downy mildew, is an oomycete phytopathogen, which was introduced to Europe in the late 19th century, where it soon became a serious plant disease. Grape downy mildew is one of the diseases that made the culture of European wine grapes (*Vitis vinifera*) impossible in North America until the advent of modern fungicides (Gessler et al., 2011). The asexual fruiting structures of *P. viticola* are sporangia, which produce zoospores, which have two flagella and are very motile in water. Similar to other oomycete pathogens, *P. viticola* has diploid nuclei during the vegetative phase, with cell walls consisting of cellulose, which is quite distinct from the pathogens in the kingdom Fungi (Gadoury et al. 2015).

Plasmopara viticola is heterothallic, meaning that it needs the presence of two mating types to generate the sexual fruiting body. It overwinters in soil or leaf litter as oospores, which form after the fusion of the antheridium and the oogonium (Francesca et al. 2006). Oospores start to develop from late summer to autumn. The oospores require moist conditions to survive and can tolerate temperatures as low as -20 °C. The germination of oospores requires free water and generally takes place in moist soil. Oospores germinate in the spring to form sporangia that release zoospores. Zoospores are transmitted by rain-splashing. Once released, zoospores tend to swim in water and loose the flagella afterwards and sense the host and infect through stomata. Under moist conditions, zoospores encyst and start to develop germ tubes. The germ tubes infect the leaves by entering the stomata and develop intercellular hyphae and intracellular haustoria in the tissue. Sporangia produced on the resulting leaf lesions serve as the secondary infection

source and could last throughout the whole season (Ronzon and Clerjeau, 1988). Rainfall and temperatures are the two key factors that affect the disease initiation and development. The disease is also promoted by high moisture in the air and soil. When a mild and wet winter is followed by a wet spring and a warm and wet summer, the epidemic of downy mildew occurs rapidly. Sporulation requires at least 4-5 h of leaf wetness. However, excessive rainfall would act adversely by washing off the sporangia and prevent them from infecting the leaves (Gadoury et al. 2015).

Fungicides that work against *P. viticola* (oomycete) overlap to only a limited extent with fungicides that work against fungi (Table 1.2). However, resistance to many oomyceticide groups has also become a challenge to oomycete management. *Plasmopara viticola* isolates resistant to metalaxyl (phenylamides group) have been reported in Europe and since then widely discovered in grape growing region worldwide (Fourie 2017; Leroux and Clerjeau 1985; Wicks et al. 2005). Broad-spectrum QoI fungicides were widely used to provide control of both true fungi and oomycetes at the cost of a rapid resistance development (Gullino et al. 2004). Resistance to carboxylic acid amides (CAA), which is conferred by a recessive major nuclear gene, is also well documented (Gisi et al. 2007). Publications on resistance against phosphite fungicides are limited. The only report of phosphite resistance in grape downy mildew is from India, where the resistance was at a high level (Khilare 2003).

1.4. Phosphite fungicides

There are a number of terms that have been used to describe the compounds of this fungicide group. Phosphite refers to the ester, alkali salt or metal salt of phosphorous acid (Guest and Grant 1991). Phosphonate is the disassociated ion of phophonic acid which is also a fungicidal compound. In this dissertation, the terms phosphite and phosphonate will be used interchangeably. The active ingredients of the phosphite fungicide products are usually mono- and/or di-potassium salts of phosphorous acid. Once they have entered the host, these compounds will eventually be hydrolyzed into PO_3^{-3}, which serves as the actual fungitoxic ingredient. The salts of phosphorous acid are as effective as the acid itself (Fenn and Coffey 1984, 1985). Fosetyl-Al, which is essentially an ester of phosphorous acid, was registered as active ingredient of Aliette and became a widely used protectant (Cook et al. 2006; Landschoot and Cook 2005). If applied at high rates and without buffer, aluminum ions may be a second

active ingredient, since $A1^{3+}$ ions are also inhibitive on zoospores and mycelium growth at high concentrations (Bompeix and Saindrenan 1984; Muchovej et al. 1980). Table 1.3 lists the terms that have been used to describe phosphite fungicides and other phosphorus-related compounds (Table 1.3).

Aside from the only report of phosphite resistance in grape downy mildew, from India (Khilare 2003), a variety of publications have documented phosphite resistance in many other oomycete plant pathogens. Phosphonate-resistant mutants were acquired in *Phytophthora* spp. by treating the organism with mutagenic chemicals, for instance, *P. capsici* (Bower and Coffey 1985), *P. palmivora* (Dolan and Coffey 1988) and *P. parasitica* (Fenn and Coffey 1989). Bower and Coffey (1985) obtained two mutants tolerant to both phosphorous acid and fosetyl-Na, with tolerance evident both *in vitro* and *in vivo*. Sanders et al. (1990) found laboratory-induced isolates of *Pythium aphanidermatum* resistant to fosetyl-Al and metalaxyl. In California, field isolates of lettuce downy mildew (*Bremia lactucae*) were documented to be resistant to field rates of fosetyl-Al (Brown et al. 2004). Decreased phosphite sensitivity of *P. cinnamomi* on avocado was found in the field with increasing intensity of phosphite application (Dobrowolski et al. 2008).

Phosphite compounds were once being used as a substitute for phosphorus fertilizer for the sake of improving plant growth, because the salts of PO_3^{-3} were then considered more economic by having a slower release of phosphorus. However, a number of studies have shown that phosphite compounds cannot be taken up by the crops as nutritious elements (Thao and Yamakawa 2008; Thao et al. 2008a; Thao et al. 2008b). In an experiment where phosphite was mixed with phosphorus at different ratios and applied to the host roots at either high or low phosphorus level, phosphite compounds showed an inhibitory effect on plant growth if applied at a high level (Thao et al. 2008a).

In the 1960s to 1970s, a variety of phosphite compounds were introduced to the field due to their herbicidal or fungicidal activity. Some early investigators demonstrated that phosphorous acid has essentially no fungitoxicity (Vo-Thi-Hai and Ravise 1979). The data were later questioned because the researchers failed to consider that phosphonate and phosphate compete for transporter binding sites and share the same carrier system, which indicates that the presence of

phosphate may reduce the intake of phosphonate (Leconte et al. 1990). Although higher phosphonate concentrations are needed to inhibit phosphate uptake, only very low concentrations of phosphate are sufficient to inhibit phosphonate uptake (Griffith et al. 1989). Phosphonate compounds were thereafter found effective against oomycetes, especially *Phytophthora* spp. and *P. viticola* (Fenn and Coffey 1984; Guest and Grant 1991), and thus were brought back into plant disease management.

The spectrum of this group of fungicides extended to many fungal pathogens. Aliette not only acts against *Peronosporales* spp., including *P. viticola*, it was also reported to inhibit *Monilinia fructicola (Guest 1984)*, *Colletotrichum lindemuthianum* (Abu-Jawdah 1983), and Alfalfa Mosaic Virus in beans (Abu-Jawdah and Kummert 1983). Phosphonate is active against *Fusarium oxysporum in vitro* (Davis and Grant 1996) in a variety of host species such as cucumber and tomato (Yamada et al. 1986). *Rhizoctonia solani* on wheat was well controlled by phosphonate as a soil treatment (Wehner et al. 1987). Phosphite compounds inhibit the infection of bean by rust (*Uromyces phaseoli*) (Rusuku et al. 1984).

Coffey and Bower (1984) observed sensitivity variation not only between various species of *Phytophthora* but also between different isolates of the same species. Generally, phosphite fungicides are mostly effective against mycelium growth and have limited effect on sporulation. They are usually used as a component of the spray programs to limit infection. Phosphite compounds have high mobility in both xylem and phloem in the plants. However, they are not as effective as other systemic fungicides such as metalaxyl and mandipropamid and need higher application rates in the field (Guest and Grant 1991; Landschoot and Cook 2005; Thao and Yamakawa 2009).

The mode of action of the phosphites has been debated. The hypothesis of an "indirect" mechanism originated from the early studies that assumed phosphonates have no effect on pathogen growth *in vitro* but have efficacy in the field (Sanders et al. 1983; Vo-Thi-Hai and Ravise 1979, Nemestothy and Guest 1990). The indirect-effect hypothesis proposed that phosphite might interfere with the biosynthesis of some phytoalexins related to plant innate resistance (Saindrenan et al. 1988a; Saindrenan et al. 1988b). This conclusion was called into question by the discovery that the effect of phosphonate on pathogens depends on the

phosphorus status of the medium (Guest 1984; Guest and Grant 1991). Andreu et al. (2006) discovered that an increase in resistance to potato late blight was detected in tubers of phosphonate-treated potato plants after harvest. On the other hand, the "direct" effect hypothesis was supported by the appearance of the resistant mutants of *Phytophthora capsici* and *P. palmivora* both *in vivo* and *in vitro* (Bower and Coffey 1985; Sanders et al. 1990). Fenn and Coffey (1985) revealed that an inhibitor of phenylalanine ammonia-lyase and thus phenyl-propanoid biosynthesis also reduced the uptake of phosphonate by *P. capsici in vitro* (Fenn and Coffey 1985). In *Phytophthora* species, phosphonate fungicides caused the accumulation of polyphosphate and pyro-phosphate, both of which act in phosphorus metabolism. ATPs were thought to be transferred to other metabolic pathways in response to the accumulation of polyphosphate and pyro-phosphate and leading to a decrease in the growth of the pathogen (Niere et al. 1994). Similarly, in *P. palmivora* several enzymes in the glycolytic pathway and hexose monophosphate bypass are found to be inhibited by phosphonate (Stehmann and Grant 2000).

1.5. Next-generation sequencing technology

In the past decade, next-generation sequencing (NGS) technology has brought the realm of genomics and genetics to revolution. Next-generation sequencing platforms, with the capability of producing an enormous volume of data within a short period of time, have also driven a drastic reduction in sequencing cost. These systems are high-throughput and cost-efficient, providing an extensive range of applications, many of which were unachievable only a few years ago (Ekblom and Wolf 2014; Metzker 2010). The development of genetic marker-based molecular techniques has been greatly accelerated, allowing the identification of single nucleotide polymorphisms, copy number variation, structural rearrangements, selective sweeps, haplotype/genotype, and repetitive gene regions to trace signatures of selection, provide insights into population history, population structure and its association to evolutionary challenges (Davey et al. 2011). With the application of sequencing-based technologies, transcriptomic studies have come to a higher resolution, where low-level transcripts can be quantitatively detected with no prior knowledge of the particular gene/transcript, providing annotation information of alternative splicing and sequence variation within the transcript (Bolton et al. 2016; Mair et al. 2016; Zhao et al. 2013).

To date, whole genomes of over 1000 organisms have been sequenced (Berglund et al. 2011). High-quality and well-annotated genome sequences are important resources for host-pathogen systems, including grapevine and its associated pathogens (such as *E. necator*, and *P. viticola*). Previously, unknown plant resistance alleles have been identified based on genome data and a limited genetic diversity of R genes in grapevines was discovered (Borneman et al. 2013). Focused NGS work on the grape powdery mildew pathogen has provided better understanding of both pathogenicity and fungicide resistance (Jones et al. 2014). A genomics approach has led to the discovery of at least two origins of gene mutations conferring resistance to quinone outside inhibiting (QoI) fungicides (Chen et al. 2007) and the following discovery of the selective sweep signature of fungicide resistance evolution in grape downy mildew (Delmas et al. 2017). In addition, large-scale studies on comparative genomics have been performed to compare the effector complement from closely related oomycete species including grape downy mildew, providing a unique viewpoint and an improved understanding of oomycete pathogenicity, host specificity and evolution (Mestre et al. 2016).

As an important component in plant disease control, fungicide resistance management has made great contributions to reducing unnecessary crop losses by avoiding futile but costly applications. A better knowledge of fungicide resistance at the genomic level could benefit practical control and resistance management recommendations by taking the population divergence into account and by generating a more accurate estimation of resistance frequency for disease control. Additionally, pathogen genomes can assist to narrow down the range of the genomic targets of fungicides with unknown modes of action for future studies and thus contribute to the development of novel fungicides. The open database of the genomic data developed as part of the project will be a resource of genetic markers for targeting emergent resistance and tracking future resistance evolution.

Tables and figures

Table 1.1. Fungicides against grape powdery mildew

FRAC code (Year)	Group name	Target site	Compound name	Trade name
3 (1980)	DMI (Demethylation inhibitors)	C14- demethylase in sterol biosynthesis (*erg11/cyp51*)	difenoconazole	Revus Top (+mandipropamid)
			myclobutanil	Rally
			tebuconazole	Elite, Orius, Tebuzol
			tetraconazole	Mettle
			triflumizole	Procure, Viticure
			tebuconazole	Adament (+trifloxystrobin)
			fenarimol	Rubigan, Vintage
7 (2003)	SDHI (Succinate dehydrogenase inhibitors)	Complex II: succinate-dehydrogenase	boscalid	Endura
			fluopyram	Luna
			benzovindiflupyr	Aprovia
9 (1998)	Anilino-pyrimidines	Methionine biosynthesis, *cgs* gene (proposed)	cyprodinil	Inspire Super (+difenoconazole)
11 (1996)	QoI (Quinone outside inhibitors)	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (*cytb* gene)	azoxystrobin	Abound
			kresoxim methyl	Sovran
			trifloxystrobin	Flint
			pyraclostrobin	Pristine (+ boscalid)
13 (2003)	Azanaphthalenes	Signal transduction (mechanism unknown)	quinoxyfen	Quintec
U6 (2012)	Phenyl-acetamide	unknown	cyflufenamid	Torino
U8 (2011)	Aryl-phenyl-ketone	Actin disruption (proposed)	metrafenone	Vivando
M02 (~1850)	Inorganic	Multi-site	sulfur	Various

Table 1.2. Fungicides against *Plasmopara viticola*

FRAC code (Year)	Group name	Target site	Compound name	Trade name
4 (1990)	PA-fungicides (Phenylamides)	RNA polymerase I	metalaxyl/ mefenoxam	Ridomil
11 (1996)	QoI-fungicides (Quinone outside Inhibitors)	Complex III: cytochrome bc1 (ubiquinol oxidase) at Qo site (*cytb* gene)	azoxystrobin	Abound, Quadris Top (+difenoconazole)
			pyraclostrobin	Pristine (+boscalid)
			fenamidone	Reason
			famoxadone	Tanos (+cymoxanil)
			trifloxystrobin	Flint
21 (2010)	QiI-fungicides (Quinone inside Inhibitors)	Complex III: cytochrome bc1(ubiquinone reductase) at Qi site	cyazofamid	Ranman
22 (2001)	Benzamides	ß-tubulin assembly in mitosis	zoxamide	Gavel (+mancozeb)
27 (1998)	Cyanoacetamide oxime	Unknown	cymoxanil	Tanos (+famoxadone)
33	Phosphonates	Unknown	phosphite	Prophyt, Phostrol
40 (1990)	CAA-fungicides (Carboxylic acid amides)	Cellulose synthase	mandipropamid	Revus, Revus Top (+difenoconazole)
			dimethomorph	Forum
43 (2008)	Benzamides	Delocalisation of spectrin-like proteins	fluopicolide	Presidio
45 (2013)	QoSI fungicides (Quinone outside inhibitor, stigmatellin binding type)	Complex III: cytochrome bc1 (ubiquinone reductase) at Qo site, stigmatellin binding sub-site	ametoctradin	Zampro (+dimethomorph)
M03 (1987)	Dithiocarbamates	Multi-site	mancozeb	Manzate, Dithane
M04 (1992)	Phthalimides	Multi-site	captan	Captan
M01 (~1880)	Inorganic	Multi-site	copper	Various

Table 1.3. Phosphorous acid and related compounds

Name	Symbol	Remark
Phosphorus	P	The chemical element
Phosphoric acid	H_3PO_4 (Pi)	P-fertilizer
Phosphate	$PO4^{3-}$	The disassociated ion of phosphoric acid
Phosphorous acid	H_3PO_3 (Phi)	Fungicidal compound
Phosphite	PO_3^{3-}	The disassociated ion of phosphorous acid, fungicidal compound
Phophonic acid	$C\text{-}PO(OH)_2$ or $C\text{-}PO(OR)_2$	Organophosphorus acids containing both PO_3^{-3} and C-P bond, fungicidal compound
Phosphonate	$C\text{-}PO^{2+}$	The disassociated ion of phophonic acid, fungicidal compound

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Chapter 2. Evaluation of quinoxyfen resistance of *Erysiphe necator* (grape powdery mildew) in a single vineyard in Virginia, USA

Abstract

The protectant fungicide quinoxyfen has been used against grape powdery mildew (*Erysiphe necator*) in the U.S. since 2003. In 2013, isolates of grape powdery mildew with reduced quinoxyfen sensitivity (here designated as quinoxyfen lab resistance or QLR) were detected in a single vineyard in western Virginia. Field trials were conducted in 2014, 2015, and 2016 at the affected vineyard to determine to what extent quinoxyfen might still contribute to disease control. Powdery mildew control by quinoxyfen was similar to, or only slightly less, than that provided by myclobutanil and boscalid in all three years. In 2016, early- versus late-season applications of quinoxyfen were compared to test the hypothesis that early-season applications were more effective, but the result suggests that differences were small and not significant. Treatments with four quinoxyfen applications followed by one myclobutanil and one boscalid application provided only slightly less control than treatments containing two quinoxyfen applications followed by two myclobutanil and two boscalid applications. In 2016, two early-season applications of quinoxyfen provided slightly but not significantly better control than two mid-season or late-season quinoxyfen applications. Metrafenone and benzovindiflupyr generally provided excellent powdery mildew control. The frequency of QLR in vines not treated with quinoxyfen slowly declined from 65% in 2014 to 46% in 2016. Further research is needed to explain how, despite this QLR frequency, quinoxyfen applied to grapes in the field was still able to effectively control powdery mildew.

Introduction

Erysiphe necator Schwein., the causal agent of grapevine powdery mildew, is a damaging pathogen widespread in all grape growing areas (Dhillon et al. 1992; Gadoury et al. 2012; Gadoury et al. 2015; Halleen and Holz 2001). Powdery mildew infections can lead to poor fruit set, premature fruit drop, berry cracking, increased acid concentration, and off flavors of wines. When berries crack, the ruptured diseased berries also have increased susceptibility to secondary infection by other pathogenic organisms such as *Botrytis cinerea* (Halleen and Holz 2001; Thind et al. 2004). The biology, epidemiology and management of grapevine powdery mildew have been recently reviewed (Gadoury et al. 2015). In the western part of Virginia, USA,

overwintering of the pathogen in buds appears to be uncommon or absent due to low winter temperatures, but chasmothecia are commonly observed as the source of overwintering inoculum (Gadoury et al. 2015).

Quinoxyfen (trade name: Quintec) is a surface-mobile protectant fungicide developed by Dow AgroSciences. It has specific activity against powdery mildews of a variety of crops, and has been available for use on grapes in the United States since 2003. Details of its mode of action are still somewhat uncertain, but, as with other single-site powdery-mildew fungicides, the potential for the development of resistance has been a concern. Laboratory mutants and field isolates of *Blumeria graminis* (barley powdery mildew) with reduced sensitivity to quinoxyfen were described by Hollomon et al. (1997) and Wheeler et al. (2003), but had reduced sporulation. Green and Duriatti (2005) documented the baseline sensitivity of grape powdery mildew, and reported EC_{50} values ranging from 0.008 to 2.6 mg/liter. Green and Gustafson (2006) used a discriminatory dose of 50 mg/liter (within the field application rate of 25 to 120 mg/liter) to select less-sensitive isolates of the grape powdery mildew pathogen, and detected isolates with EC_{50} above 30 mg/liter. Six years after the commercialization of quinoxyfen, grape powdery mildew field isolates with high quinoxyfen resistance (EC_{50} up to nearly 100 mg/liter) were reported in Europe (Genet and Jaworska 2009). "Strongly adapted" isolates were discovered annually in Europe since then (Kemmitt et al. 2017). The 2016 annual meeting minutes of the azanaphthalene (AZN) working group of the Fungicide Resistance Action Committee (FRAC) indicated that "adapted isolates able to grow actively at a discriminatory dose of either proquinazid or quinoxyfen which controls baseline isolates have been detected across Europe" (Kemmitt et al. 2017). The frequency and the degree of adaptation of these isolates varied significantly between monitored regions within countries and also from year to year over the period of monitoring from 2003 to 2016 (Kemmitt et al. 2017). However, implications for disease control in the field appear to have not been reported beyond the annual meeting minutes of the FRAC AZN working group. The 2011 AZN working group minutes (no longer available online) included the statement "Field performance is not affected when the products have been used according to label recommendations. There were no field performance issues to proquinazid or quinoxyfen", but this statement was omitted in the minutes of subsequent years (Kemmitt et al. 2013, Kemmitt et al. 2014, Kemmitt et al. 2017). Field trials in western New York conducted during 2010 and 2011 revealed reduced control of grape powdery mildew by quinoxyfen, which

was attributed to "hypothesized, but not confirmed resistance" (Wilcox and Riegel 2012a, b, c).

In September 2013, difficulties with powdery mildew control were reported in a commercial vineyard in western Virginia by the grower. The grower had applied quinoxyfen in certain blocks for eight consecutive years, with four applications in each of the most recent three seasons. The powdery mildew isolates collected at this site grew well on both detached leaves and potted plants treated with full label rates (3 to 6.6 fluid oz./acre, equivalent to 60 to 130 mg a.i./liter if applied at 100 gal./acre or 935 liters/ha) (Colcol and Baudoin 2016). Such isolates appeared to be present at high frequency, and had not previously been observed in Virginia.

To test the hypothesis that efficacy of quinoxyfen would be reduced in the affected vineyard, we conducted studies from 2014 to 2016 to determine: (1) the frequency of quinoxyfen resistance in the of grape powdery mildew pathogen population in the affected area of Virginia; (2) the extent to which the field efficacy of quinoxyfen against grape powdery mildew was reduced; and (3) whether the number of applications and the application timing would affect the degree of control.

Materials and Methods

Field trials

The powdery mildew isolates studied had a high level of quinoxyfen resistance in laboratory and potted plant assays as described by Colcol and Baudoin (2016). Since the current study found only a slight loss of efficacy in the field, we will refer to these isolates as quinoxyfen lab resistant or QLR isolates.

Field trials were conducted in three separate seasons at the western Virginia vineyard (hereafter referred to as "QLR vineyard") where QLR grape powdery mildew isolates had been collected in the fall of 2013 (Colcol and Baudoin 2016). In 2014, a trial was set up in two rows of 'Chambourcin' grapes with plots consisting of four vines; the trial included five treatments (Table 2.1), each replicated four times, in a randomized complete block design. The early-season (pre-bloom) grower spray program consisted of mancozeb and sulfur, and trial applications were initiated at bloom. A five-spray quinoxyfen (Quintec, Dow AgroSciences, 73 g a.i./ha) program was compared with a boscalid (221 g a.i./ha, Endura, BASF Corporation, Research Triangle

Park, NC)-myclobutanil (84 g a.i./ha, Rally, Dow AgroSciences) rotation supplemented with a low dose of sulfur (897 g a.i./ha, Microthiol Disperss, UPI, King of Prussia, PA), and with metrafenone (224 g a.i./ha, Vivando, BASF Corporation, Research Triangle Park, NC). A treatment of quinoxyfen plus boscalid/myclobutanil+sulfur was included to determine whether quinoxyfen might still add efficacy to a partially effective treatment. Treatments of all plots (including the controls where no anti-powdery mildew fungicide was applied) were supplemented with 0.3% Prophyt (potassium phosphite, 54.5% active ingredient, Helena Chemical Company, Collierville, TN) and mancozeb (841 g a.i./ha, Dithane 75DF Rainshield, Dow AgroSciences, Indianapolis, IN) at early applications, or, at later applications, Prophyt plus azoxystrobin (179 g a.i./ha, Abound, Syngenta Crop Protection, Greensboro, NC) for control of downy mildew and/or black rot (powdery and downy mildew at this location were QoI resistant). Application volume of the first two sprays was 650 liters/ha, of the remainder 750 liters/ha. Nine days after the first trial spray, two of the blocks were mistakenly treated by the vineyard manager with boscalid plus sulfur. In order to compensate, the remaining blocks were sprayed 5 days later with the same mixture, and trial sprays were resumed 13 days later. Severity of powdery mildew on grape clusters was evaluated on 29 July, before berries started to change color, which makes it increasingly difficult to evaluate powdery mildew cluster infection; two evaluators each rated 15 clusters on each side of each plot (60 cluster ratings per plot) by estimating the percentage of the surface covered by visible powdery mildew. The last treatment application was made on 16 August, and foliar disease severity was rated on 14 September.

In 2015, two parallel field trials were conducted at the same vineyard. One trial was set up in two rows of Chambourcin with plots consisting of four vines; the other was set up in a row of Pinot noir approximately 200 m distant from the Chambourcin plots with four or five vines per plot (Table 2.2). Each trial consisted of the same five treatments, five applications, and four replicates, and the same application volumes as in 2014. The early-season grower spray program consisted of mancozeb and sulfur. Treatment applications were supplemented with Prophyt (0.4%) and mancozeb (841 g a.i./ha) at early applications or Prophyt plus azoxystrobin (179 g a.i./ha) at later applications for control of downy mildew and black rot. In 2014 and 2015, applications were made with a Solo model 410 backpack mistblower.

In 2016, the field trial was set up in two rows of Pinot noir with plots consisting of four or five

vines (Table 2.3), and treatments were applied with a Jacto XP416 backpack sprayer with a TeeJet 8003VS XR nozzle. Six fungicide treatments, plus an untreated control, were compared, each replicated four times and receiving six applications during the season, at the same application volumes as in previous years. One added treatment was benzovindiflupyr (Aprovia, Syngenta, 63 g a.i./ha), a new powdery mildew fungicide registered in the U.S. in 2015. Four treatments included quinoxyfen rotated with myclobutanil+sulfur and boscalid+sulfur; these treatments varied in number and timing of quinoxyfen applications. The pre-bloom grower spray program consisted of mancozeb and sulfur, as in 2014 and 2015. Trial applications included Prophyt (0.5%), mandipropamid (Revus, 146 g a.i./ha, Syngenta), mancozeb (841 g a.i./ha), or captan (1.12 kg a.i./ha, Captan 80WDG, Albaugh, Inc., Ankeny, IA) for control of downy mildew. Azoxystrobin (179 g a.i./ha) was included in the first three applications of all treatments for control of black rot.

Statistical analyses

Statistical analyses were performed using the Fit Y by X platform of JMP 10 (SAS Institute, Cary, NC). In many comparisons, control plots contributed a much greater level of variance than the other treatments, violating the assumptions of analysis of variance (ANOVA); in such situations, control plots were excluded from ANOVA and post-hoc Tukey's HSD (P =0.05) to determine whether the effects of the remaining treatments were significantly different from each other. Pairwise comparisons with the control were made by t-tests assuming unequal variance.

Isolate collection and maintenance

Isolates were collected from the QLR vineyard and several nearby locations during the 2014 to 2016 growing seasons. They were maintained and bioassayed on greenhouse-grown young leaves collected from positions 3 to 5 from the tip of the shoot of the susceptible cultivar 'Chardonnay' as described previously (Colcol and Baudoin 2016). Leaves were surface-disinfested by immersing in a 20% bleach solution with Tween 20 (1 or 2 drops per 100 ml bleach solution), then blotted with paper towel in a laminar flow hood, and placed on 1% water agar with sterile utensils. Powdery mildew-infected leaves from the field were brought to the lab in ziplock bags, and were gently rubbed on greenhouse-grown surface-disinfested young leaves in a laminar-flow hood. The inoculated leaves were incubated for 7 to 10 days at room temperature (22 to 26°C) to allow spores to develop. Single chains of conidia were transferred

individually from the developed colonies to surface-disinfested leaves using acupuncture needles and the plates were incubated for 7 to 10 days. Conidia from these subcultures were used in bioassay to test the sensitivity of *E. necator* isolates.

Bioassay

Since quinoxyfen dose has little effect on inhibition of QLR isolates (Colcol and Baudoin 2016; Lee et al. 2008; Wheeler et al. 2000; Wheeler et al. 2003), a discriminatory concentration of 30 mg/liter was used in this study based on previous results (Colcol and Baudoin 2016). Formulated quinoxyfen used in biological assay was provided by Dow AgroSciences LLC.

Surface-disinfested young grape leaves as described above were cut into strips of approximately 15 × 40 mm, the leaf pieces were randomized, and either immersed in sterile water (control) or quinoxyfen suspension of 30 mg/liter for 45 min. They were then gently blotted with a clean paper towel in a laminar-flow hood and plated onto 1% water agar using sterile utensils, three strips per plate. Non-treated and quinoxyfen-treated leaf pieces in separate plates were inoculated with conidia from one single-spored subculture, at four locations per leaf piece. The inoculated cultures were incubated for 7 to 10 days at room temperature (25°C) under fluorescent lights with a 14-h photoperiod. The number of colonies in each plate was recorded. Single-spored isolates producing more than three colonies from 12 inoculation points on quinoxyfen-treated leaf tissue while producing more than six colonies growing on non-treated leaf tissue were categorized as "QLR" isolates; isolates producing no colonies on quinoxyfen-treated plates while producing more than six colonies in non-treated plates were considered "sensitive"; usually, the number of colonies was either high or zero, and borderline responses were followed by a re-test.

Results

Powdery mildew severity was fairly low in the 2014 field trial. In contrast with laboratory results with leaf discs or potted plants (Colcol and Baudoin 2016), quinoxyfen provided very good powdery mildew control in the field, and was as effective or almost as effective (foliar evaluation, difference not significant) as the other treatments (Table 2.1). The treatment comprised of quinoxyfen alone provided 99% control of cluster disease severity and 92% control of foliar symptoms, whereas the other treatments provided 89-94% control of cluster disease and 96-100% control of foliar infection.

In 2015, little powdery mildew developed in the Chambourcin plots until late in the season. No powdery mildew was detected on 28 July, 15 days after the third application, and only one cluster with a very small amount of infection was seen on 12 August. Some foliar infection developed later, and the 5 September rating, 53 days after the last application, revealed low disease levels (all treatments means below 0.4% of the leaf surface) and no statistically significant treatment differences (data not shown).

Both cluster and foliar powdery mildew symptoms developed in the 2015 Pinot noir trial (Table 2.2). At the 14 July cluster rating, after three treatment applications, all treatments still provided statistically indistinguishable control, although quinoxyfen, with 84% control, was numerically the least effective of the fungicide treatments. A foliar rating on 12 August, 15 days after the fifth application, revealed distinctly more powdery mildew in the quinoxyfen plots (60% control of foliar colony numbers) than in the other treatments (97-98% control for treatments including boscalid), but all were different from the non-treated control ($P \leq 0.007$). Control of foliar severity by quinoxyfen was only 45% on 23 August, 25 days after the last application. Metrafenone and the boscalid/myclobutanil rotation plus sulfur treatments still had minimal disease levels at that time.

In 2016, at the cluster ratings on 14 and 20 July (Table 2.3), after three treatment applications, efficacy of treatments that included quinoxyfen was statistically the same and numerically only slightly less than efficacy of the best treatments, such as benzovindiflupyr, metrafenone, and myclobutanil/boscalid (T2 compared to T6 and T7 on 14 July; T2 and T5 compared to T6 on 20 July). At the foliar rating on 3 September (Table 2.3), 15 days after the sixth application, metrafenone and benzovindiflupyr both provided complete powdery mildew control. Treatments with four quinoxyfen applications (T2) provided slightly less control of powdery mildew (94% control) than treatments with two early-season (T3) or mid-season (T4) quinoxyfen applications where the other applications were replaced by a myclobutanil/boscalid alternation plus sulfur (96-99% control). Two late-season applications (T5) were statistically indistinguishable from the other quinoxyfen treatments.

To determine QLR frequency, powdery mildew isolates were collected in this vineyard in each of the three seasons. The number of single-spored isolates from quinoxyfen-treated plots in 2014 was too small to allow conclusions, but the data from 2015 and 2016 indicate that a regular quinoxyfen application increased the frequency of the quinoxyfen QLR, as would be expected (Table 2.4). The QLR frequency in untreated areas of the vineyard slowly declined from 65% in 2014 to 46% in 2016.

In 2014, powdery mildew isolates were collected at two additional vineyards, one about 8 km to the west, the other one about 16 km to the northeast of the QLR vineyard. Four potted "sentinel" Chardonnay vines were stationed at each of these locations; two of them were sprayed on a regular basis with 10 mg a.i./liter quinoxyfen and the remainder were non-treated controls. These plants were visited approximately every 14 days, and powdery mildew was collected as disease developed. A very limited number of powdery mildew colonies developed on the quinoxyfen-treated plants, whereas powdery mildew became moderately (northeast location) to severe (west location) on non-treated plants. At the northeast location 5 out of 31 isolates tested, or 16%, were able to grow on quinoxyfen-treated leaves, at the west location 2 of 42 isolates tested, or 5%, were QLR. Surprisingly, isolates with reduced sensitivity appeared to be just as likely or more likely to be collected from non-treated (6 QLR isolates out of 51 total) than from quinoxyfen-treated plants (1 QLR isolate out of 22).

Discussion

Fungicide resistance presents a major challenge to the management of grape powdery mildew in many grape production regions. *Erysiphe necator* has developed resistance to a series of major fungicide groups, which seriously limits growers' management options and puts increased selection pressure on modes of action not yet affected by resistance. Although quinoxyfen resistance ("strongly adapted isolates") has been known in several European countries for over a decade, we could find no published data documenting their impact on disease management.

Erysiphe necator isolates with QLR were previously found to keep their QLR when maintained in the laboratory on treated as well as untreated leaves (Colcol and Baudoin 2016), and this was confirmed by maintaining single-spored isolates for another 10-12 generations (14 days per

generation) and bioassaying them every two to three generations (data not shown). In the field, in competition with sensitive isolates, the QLR frequency in non-treated grapevines in the affected vineyard slowly declined from 65% in 2014 to 46% by 2016 (Table 2.4). Some of this decline may be due to immigration from non-treated vineyards or wild grapevines, and the slow decline suggests at most a slight fitness penalty in the absence of selection by fungicide.

Due to the presence of a 46-65% frequency of isolates that can grow and sporulate on leaves and plants treated with field rates of quinoxyfen (Colcol and Baudoin 2016), we expected a substantial impact on quinoxyfen's efficacy. However, our field-test results showed only a small reduction in the efficacy of quinoxyfen when compared to fungicides such as myclobutanil, boscalid, and metrafenone. In all three years, quinoxyfen provided cluster protection almost as effective as the other treatments, although there was some reduction in efficacy against foliar infection observed late in the season. In the 2016 field test, treatments that included four quinoxyfen applications provided only slightly reduced control compared to treatments with two quinoxyfen applications (Table 2.3, only the differences between T2 versus T3 and T4 were statistically significant).

The AZN Working Group of the Fungicide Resistance Action Committee (FRAC) has monitored for quinoxyfen resistance annually since 2003, and slightly to strongly adapted isolates have been widely found in Europe since 2006. The AZN Working Group has not reported any performance issues in the areas where the adapted isolates were discovered, but it is unclear to what extent this judgment is based on replicated field trials, and/or whether efficacy may have been due to mixing and rotating with other modes of action, as required or recommended by the label. On the other hand, reduced efficacy attributed to "hypothesized but not confirmed resistance" was observed in field trials conducted in New York state in two consecutive years (2010 and 2011). A quinoxyfen solo spray program provided good control of disease severity on clusters but only modest control of leaf disease severity and showed no control of disease incidence on either tissue (Wilcox and Riegel 2012a, b, c). This is somewhat similar to our field data, especially those from 2015.

Green and Duriatti (2005) and Green and Gustafson (2006) documented baseline sensitivity of 56 unexposed *E. necator* isolates shortly after quinoxyfen had been put into the market, and reported an EC_{50} range of 0.008 to 2.6 mg/liter. *Erysiphe necator* isolates with considerably reduced sensitivity (EC_{50}>2.6 mg/liter, approximately 10 to near 100 mg/liter) have been discovered across Europe (Genet and Jaworska 2009; Green and Gustafson 2006), with variable frequencies (Kemmitt et al. 2017), but no specific frequency data were provided. Colcol and Baudoin tested 160 *E. necator* isolates sampled from Virginia and nearby states against quinoxyfen and all the isolates were sensitive; the tested isolates all showed strongly reduced or no growth on leaf tissue treated with concentrations of 0.3 or 3 mg/liter (Colcol and Baudoin 2016), indicating that QLR isolates were not common, and that the population of 46% to 65% QLR *E. necator* isolates in the QLR vineyard is unusual in Virginia.

Our 2014 and 2015 field data, as well as the Wilcox and Riegel data cited above, suggested that quinoxyfen tends to perform better in controlling powdery mildew on grape clusters than on leaves. Given that cluster symptoms occur during early stages of berry development while foliar symptoms usually develop later in the season, we hypothesized that quinoxyfen was more effective on clusters because the efficacy of quinoxyfen declines late in the season. In 2016, we compared treatments with different quinoxyfen application timing, but differences based on the timing of two applications were small and not statistically significant (Table 2.3, T3 versus T4 and T5). Numerically, the late applications resulted in only slightly more foliar mildew in the late-season evaluation. Therefore, the hypothesis that quinoxyfen works better if applied early found only very weak support in the 2016 data.

Isolates tolerating high quinoxyfen levels have been documented for other powdery mildew species. The risk of developing resistance to quinoxyfen was first evaluated in *B. graminis* years before the product was commercialized (Hollomon et al. 1997). The baseline ED_{50} ranged from <0.001 mg/liter to 0.16 mg/liter, and median ED_{50} was between 0.001 and 0.005 mg/liter. Mutants with ED_{50} values >0.16 mg/liter (one reported at >5 mg/liter) were isolated at a very low frequency in the field. Mutants were also acquired by chemical treatment in the lab (Hollomon et al. 1997). In 2002, Bernhard et al. (2002) reported sensitivity of wheat powdery mildew against quinoxyfen as monitored from 1995 to 2000 in Western Europe. The mean EC_{50} ranged from

0.039 mg/liter to 0.071 mg/liter, showing no shift or adaptation in sensitivity of *B. graminis* at that point (Bernhard et al. 2002). However, isolates with reduced sensitivity were detected in Germany since 2001, 3 years after its commercial introduction in 1998, and in northern France starting in 2003 (Walker et al. 2006). In France, they made up 22-36% and up to 66% of the population, depending on the region. However, in field trials in most regions, quinoxyfen remained one of most effective fungicides when applied preventatively early in the season, but in the Champagne region efficacy reductions on *B. graminis* were observed, but were variable and not consistent (Walker et al. 2006).

In addition, cucurbit powdery mildew (*Podosphaera xanthii*) strains that tolerated quinoxyfen at 40 and 80 mg/liter have been reported from cucurbit fields in New York (McGrath 2013), and more recently, in 2015, isolates that tolerated 200 mg/liter, which is approximately the maximum field rate for the crop (McGrath 2017). However, quinoxyfen was still as effective as the other treatments in the trial plots where the isolates were collected, which was attributed to selection having occurred during that growing season, but not fast enough to affect efficacy. This is not a viable explanation in our grape experiments where the QLR isolates were present throughout three seasons.

It is unclear why quinoxyfen is still able to provide good control of powdery mildew in the field while over 50% of the isolates present cannot be controlled with field rates on lab-treated leaves or potted plants. Field conditions as such do not appear to harm the isolates as shown by their persistence in the field, but perhaps field conditions (temperature and humidity fluctuations and extremes, UV radiation, etc.) combined with the presence of quinoxyfen may have a harmful effect. Further research is needed to clarify this.

Despite the fact that the type of quinoxyfen resistance described in our report had only a modest effect on the fungicide's efficacy in the vineyard, prudence supports continuing to follow the recommendations to the growers made by FRAC (Kemmitt et al. 2017) and to use quinoxyfen with caution: to apply early in the season, preventatively, before significant disease development occurs; to apply no more than three times per season, with no more than two consecutive

applications; to mix quinoxyfen with fungicides of other modes of action; and to closely monitor the efficacy of quinoxyfen and switch to other fungicides when powdery mildew control deteriorates.

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Tables and figures

Table 2.1. Powdery mildew severity on grape clusters and leaves, Chambourcin, 2014.

	Severity, %			
Treatment [x]	Cluster [y] 29 July		Leaves [y] colonies/2 min 14 September	
Non-treated control	14.8		83.1	
Quinoxyfen	0.2	a [z]	6.6	a [z]
Boscalid or myclobutanil, + sulfur	1.7	a	3.3	a
Metrafenone	2.4	a	0.4	a
Quinoxyfen + (boscalid or myclobutanil) + sulfur	0.9	a	1.3	a

[x] Quinoxyfen (Quintec, Dow AgroSciences), 73 g a.i./ha; boscalid, 221 g a.i./ha; sulfur, 897 g a.i./ha; myclobutanil, 84 g a.i./ha; metrafenone, 224 g a.i./ha; Applications were carried out on 12 June (60% bloom), 26 June, 9 July, 23 July (approaching veraison), and 16 August. Anti-downy and -black rot materials were applied to all treatments, and sulfur, 897 g a.i./ha was applied to all plots on 7 August.

[y] Cluster severity: percent of surface area covered by powdery mildew, estimated for 30 clusters per plot evaluated separately by two evaluators. Leaf infection: number of powdery mildew colonies on leaves per 2-minute search for each side of each plot.

[z] Analysis of treatment differences with control excluded, to exclude the larger control variance from the within-treatment variance. Data in a column with the same letter are not significantly different (Tukey's HSD, $P = 0.05$). In addition, all treatments were significantly different from the control in pairwise t-tests with unequal variances ($P<0.05$).

Table 2.2. Powdery mildew severity on clusters and leaves, Pinot noir, 2015.

Treatment [v]	Cluster severity, % [w] 14 July	Foliar colonies/ 4 min [x] 12 August	Foliar severity, % [y] 23 August
Non-treated control	10.8	139.5	5.3
Quinoxyfen	1.7 a[z]	55.6 a[z]	2.9 a[z]
Boscalid or myclobutanil, + sulfur	1.1 a	3.1 b	0.1 b
Quinoxyfen + (boscalid or myclobutanil) + sulfur	0.5 a	4.0 b	0.1 b
Metrafenone	0.1 a	0.1 b	0.0 b

[v] Rates as in Table 2.1. Applications were carried out, on 2 June (10% bloom), 15 June, 29 June (late pea-size), 14 July (berry touch), and 28 July (veraison).

[w] Cluster disease severity: 30 clusters per plot evaluated separately by each of two evaluators. Data not connected by the same letter are significantly different (Tukey's HSD, $P = 0.05$).

[x] Foliar colonies were counted in a 2-minute search by two evaluators, one on each side of the row.

[y] Foliar disease severity as percent of leaf surface, 65 leaves were evaluated per plot.

[z] Analysis of treatment differences with control excluded, to exclude the larger control variance from the within-treatment variance. Data in a column with the same letter are not significantly different (Tukey's HSD, $P = 0.05$). In addition, all treatments were significantly different from the control in pairwise t-tests with unequal variances ($P<0.05$).

Table 2.3. Powdery mildew severity on clusters and leaves, Pinot noir, 2016.

			Disease severity, %		
	Before 20 July [x]	After 20 July [x]	14 July Clusters [y]	20 July Clusters [y]	3 September Leaves [z]
T1	Control	Control	1.4 a	9.6 a	37.0
T2	Q, Q, Q	Q, M+S, B+S	0.2 b	2.0 b	2.2 b
T3	Q, Q, M+S	B+S, M+S, B+S	--	--	0.1 d
T4	M+S, B+S, Q	Q, M+S, B+S	--	--	0.4 cd
T5	M+S, B+S, M+S	B+S, Q, Q	--	0.7 b	1.4 bcd
T6	Mf, Mf, Mf	Mf, Mf, Mf	0 b	0.1 b	0 d
T7	Bv, Bv, Bv	Bv, Bv, Bv	0.1 b	--	0 d

[x] Q = quinoxyfen; B = boscalid; S = sulfur; M = myclobutanil; Mf = metrafenone; Bv = benzovindiflupyr. Rates as in Table 1, benzovindiflupyr, 63 g a.i./ha. Applications were carried out on 9 June (45% bloom), 22 June (approaching BB-sized berries), 6 July (approaching cluster closing), and 20 July (early veraison), 5 August, and 19 August. The three applications before 20 July are listed separately from the three applications after 20 July, as the cluster ratings were performed after the first three applications, while the leaf rating was performed after all six applications.

[y] Cluster severity based on 50 clusters per plot (14 July, 8 days after the third anti-powdery mildew spray) or 40 clusters per plot (20 July 2016, 14 days after the third anti-powdery mildew spray); Within a column, data followed by the same letter are not significantly different (Tukey's HSD, $P = 0.05$).

[z] Foliar severity based 60 leaves per plot, rated 15 days after the fifth anti-powdery mildew spray. Analysis of treatment differences with control excluded, to exclude the larger control variance from the within-treatment variance. Within a column, data followed by the same letter are not significantly different (Tukey's HSD, $P = 0.05$). In addition, all treatments were significantly different from the control in pairwise t-tests with unequal variances ($P<0.05$).

Table 2.4. Average QRL frequencies in non-treated areas and quinoxyfen-treated plots of the quinoxyfen-resistance-affected vineyard, 2014-2016.

	QRL frequency		
	2014	2015	2016
Untreated vines	65% (n=124)	50% (n=224)	46% (n=100)
Quinoxyfen-treated plots	50% (n=18)	81% (n=170)	86% (n=99)

Chapter 3. RNA-seq profiles of quinoxyfen-sensitive and -resistant isolates of *Erysiphe necator* after treatment with quinoxyfen identify gene induction

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Abstract

Grape powdery mildew, caused by the obligate biotrophic fungus *Erysiphe necator*, is a major disease in grape production regions worldwide. Quinoxyfen is an efficacious protectant for controlling powdery mildews, although its mode of action remains uncertain. *Erysiphe necator* isolates with resistance to quinoxyfen were reported after quinoxyfen became widely used. To better understand the effect of quinoxyen at the transcriptome level, we profiled the transcriptomes of quinoxyfen-resistant and -sensitive isolates in response to quinoxyfen or distilled water (control) treatment. Sequencing data were analyzed following a standard computing pipeline (Trinity + DESeq2) to reveal information on the putative genetic target(s) of quinoxyfen and to identify transcripts involved in the resistance response. This study supports previous research suggesting that quinoxyfen interferes with the signal transduction process. Additionally, we identify previously undescribed transcriptional targets of quinoxyfen that involve the positive regulation of the MAPK signaling cascade, pathogenesis and sporulation activity. Future work will validate some of these candidates *in situ* and identify transcripts that are associated with resistance to quinoxyfen.

Introduction

Grape powdery mildew, caused by the obligate biotrophic fungus *Erysiphe necator*, is an economically important disease widespread across grape-growing regions worldwide (Gadoury et al. 2012; Glawe 2008; Pearson and Gadoury 1992). Chemical control remains a major component of integrated plant disease management, and in recent decades the control of powdery mildew has increasingly relied on the use of fungicides with single-site mode of action (Delye et al. 1997; McGrath 2001), resulting in increasing risks of fungicide resistance. Quinoxyfen (5,7-dichloro-4-(p-fluorophenoxy) quinoline), introduced by Dow AgroSciences, is an efficacious protectant which specifically controls plant pathogens of the powdery mildew group. To date, resistance to quinoxyfen has been documented for several powdery mildew pathogens, including barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) (Hollomon et al. 1997),

cucurbit powdery mildew (*Podosphaera xanthii*) (McGrath 2015), and grape powdery mildew (Green and Gustafson 2006). Grape powdery isolates with high level of resistance in laboratory tests were reported in Virginia (Colcol and Baudoin 2016).

The obligate nature of the powdery mildews makes it difficult to obtain sufficient biological material for detailed investigations, and knowledge on the mode(s) of action and resistance mechanism(s) of quinoxyfen remain limited. Quinoxyfen has been documented to interfere more with early-stage infection events such as germ tube differentiation and appressorium formation rather than the germination and germ tube elongation process (Gustafson et al. 1999; Longhurst et al. 1996). Wheeler et al. pointed out that quinoxyfen prevents the germinating conidia of barley powdery mildew from perceiving host recognition signals by preventing the depletion of GTPase-activating proteins (GAPs), and therefore inhibits the downstream signal transduction required for germination and appressorium formation (Wheeler et al. 2000; Wheeler et al. 2003). Additionally, Lee et al. (2008) showed that quinoxyfen may interfere with cutinase activity/host recognition signaling. A cutinase protein encoding gene (*CUT1*) was found overexpressed in a quinoxyfen-resistant isolate, implying that quinoxyfen resistance may be mediated by overexpression of cutinase. Quinoxyfen was also found to have inhibiting effect on serine esterase activity. Serine esterase is considered as the key enzyme that hydrolyzes cutins and waxes in order to assist the penetration of the host (Lee et al. 2008).

The high throughput, rapid and cost-effective RNA-Seq is one of the most widely used next generation sequencing (NGS) technologies (Ozsolak and Milos 2011; Wang et al. 2009). The RNA-Seq technology enables us to identify candidate genes responsive to certain biological phenomena by tracking the expression differences. To better understand the effect of quinoxyen at the transcriptome level, we profiled the transcriptomes of quinoxyfen-resistant and -sensitive isolates in response to quinoxyfen or distilled water (control) treatment. Sequence data were analyzed following a standard computing pipeline (Trinity + DESeq2) to reveal information on the putative genetic target(s) of quinoxyfen and to identify transcripts involved in the resistance response.

Material and methods

Isolates and culture maintenance

Resistant and sensitive *E. necator* isolates were collected from a resistance-affected area in Virginia in 2014-2016. All isolates were grown on detached leaves as described by Colcol and Baudoin (2016). Young leaves (3rd-5th from the tip of the shoot) of the susceptible cultivar Chardonnay were used for isolate maintenance. Leaves were surface-disinfested by immersing in a 20%-bleach solution containing Tween 20 (1-2 drops per 100 ml bleach solution), followed by blotting the leaves with clean paper towels in a laminar flow hood. Powdery mildew-infected leaves or berries were taken from the field, brought back to lab, and rubbed on surface-disinfested grape leaves prepared as described above. The inoculated leaves were kept at room temperature (22-26°C) for 7-10 days to allow colonies to develop, and single chains of conidia were then transferred individually using acupuncture needles to develop pure isolates. The sensitivities of the single-spored isolates were then characterized using a discriminatory dose screen of 30 mg/L. One single-spored resistant isolate (QR10) and one single-spored sensitive isolate (QS7) were selected for future use. QS7 is sensitive to quinoxyfen, showing no growth when inoculated on leaves treated with 30 mg/L quinoxyfen, while QR10 showed normal growth on such treated leaves.

RNA preparation and sequencing

A factorial experiment was devised wherein sensitive and resistant isolates were inoculated with sterile paint brushes onto quinoxyfen-treated (30 mg/L) and non-treated leaves, respectively, and were incubated for 24 hours. Samples from the four treatments were labeled as: SW (sensitive-water treated), SQ (sensitive-fungicide treated), RW (resistant-water treated), and RQ (resistant-fungicide treated). Water treatment was considered as the control. Incubated conidia were harvested following the method of Cadle-Davidson et al. (2010). RNA extraction was performed using RNeasy Mini Kits (Qiagen). Each treatment contained three biological replicates. Approximately 200 ng RNA was obtained per replicate. The extracted RNA samples were sequenced by the Illumina HiSeq 2000 platform with 50X coverage to detect important but rarely expressed variants.

Reads mapping to the generated reference genome

Sequenced raw reads were trimmed with Trimmomatic v0.35 (Bolger et al. 2014) to remove adaptor sequences and low-quality reads from the raw data. Reference transcriptome assembly

was conducted by Trinity v2.3.2 (Haas et al. 2013). Reads indexing and aligning were conducted with Bowtie v2.2.6 (Langmead and Salzberg 2012) and paired-end reads alignment to the *de novo* assembly reference transcriptome was done with Trinity.

Quantification of gene expression level and differential expression analysis

Before differential expression analysis, RSEM v1.2.25 (Li and Dewey 2011) was used to estimate the abundance of the mapped transcripts. DESeq2 v3.2.3 (Love et al. 2014) was used to conduct differential expression analysis cross treated/untreated conditions and resistant/sensitive isolates. A threshold of 0.1 was set for adjusted P-value and a threshold of 1 was set for the log2-fold change of the differential expression level change. The Trinotate pipeline, including BLAST (Love et al. 2014) and Gene Ontology (GO) databases (Love et al. 2014), was used to annotate the differentially expressed genes.

Results

Transcript level changes after quinoxyfen treatment

Across the four treatments, a total of 81,457 transcripts with nonzero read counts were obtained by DeSeq2, of which 2,064 genes were upregulated and 1,433 genes were down-regulated. The number of differentially expressed (DE) transcripts, after filtering at a threshold of log2fold changes ≥ 1 and $P \leq 0.1$, is summarized in Table 3.1. Hereafter, we refer to the DE genes that belong to the resistant isolates treated with quinoxyfen as the RQ group, DE genes that belong to the sensitive isolate with quinoxyfen treatment as the SQ group, and DE genes that belong to both the RQ and SQ groups as the SQ_RQ group. Thirty-six DE genes were found to solely belong to the RQ group, and are regarded as important genes related to quinoxyfen resistance (Figure 3.1). Thirteen genes out of this group were identified by the GO database, with 9 genes being upregulated and 4 genes downregulated. A hundred and nineteen genes were found responsive to quinoxyfen treatment in resistant or sensitive isolates (Figure 3.1). Of these 119 genes, 62 genes are differentially expressed only in the SQ group; 34 of these genes are downregulated and 28 are upregulated. Fifty-six genes are differentially expressed in both resistant and sensitive isolate under quinoxyfen treatment (SQ_RQ group), of which 54 genes were downregulated and two were upregulated. Genes from both groups were annotated by gene ontology (GO) database.

A hundred and thirty-two significant DE transcripts were found by contrasting RW and SW treatment in the absence of quinoxyfen treatment (RW_SW, Figure 3.2). In contrast, 435 DE transcripts in RQ were obtained in the presence of quinoxyfen treatment, including 399 downregulated and 36 upregulated DE transcripts (RQ_SQ), indicating that the gene expression pattern is greatly changed in the resistant isolate. Compared to RW and SW isolates, quinoxyfen treatment induced a large number of gene expression changes in both RQ and SQ isolates (Figure 3.3). In the sensitive isolate, 121 DE transcripts were obtained after quinoxyfen treatment, of which 89 were downregulated and 32 were upregulated (SQ_SW), whereas 33 transcripts were differentially expressed between RQ and RW (RQ_RW), indicating the quinoxyfen resistant isolate is more adapted to the fungicide treatment with fewer genes and biological processes affected by quinoxyfen treatment (Figure 3.3).

Classification of differentially-expressed genes in response to quinoxyfen

The GO annotations were categorized into three major functional groups (cellular component, molecular function, and biological process). The distributions of gene functions of the DE genes of predicted by GO database are shown in Figure 3.4, Figure 3.5, and Figure 3.6.

In group RQ (Figure 3.4), five transcripts (TRINITY_DN10567_c0_g1, TRINITY_DN20530_c0_g1, TRINITY_DN20771_c0_g1, TRINITY_DN22301_c0_g2, TRINITY_DN9443_c0_g1) out of 11 are located in the cellular component of "integral component of membrane" or "plasma membrane". The rest of the genes are mainly distributed in mitochondrial matrix, endoplasmic reticulum, and cytoplasm. Four transcripts (TRINITY_DN10567_c0_g1, TRINITY_DN12841_c0_g2, TRINITY_DN18858_c0_g1, TRINITY_DN3460_c0_g2), all upregulated, are involved in the molecular function of NAD(P)H dehydrogenase (quinone) activity. NAD(P)H dehydrogenase interferes with the electron transportation which leads to ATP synthesis (Melo et al. 2001). Six transcripts (TRINITY_DN10567_c0_g1, TRINITY_DN18289_c0_g2, TRINITY_DN20771_c0_g1, TRINITY_DN22301_c0_g2, TRINITY_DN61256_c0_g1, TRINITY_DN7151_c0_g1) are involved in amino acid, iron and oxygen transport biological processes.

There were 10 annotated DE genes in group SQ (Figure 3.5), the major cellular component categories including plasma membrane (TRINITY_DN18243_c0_g1), extracellular region (TRINITY_DN13247_c0_g1), and integral component of membrane (TRINITY_DN10215_c0_g1, TRINITY_DN12102_c0_g1). TRINITY_DN12102_c0_g1, located in integral component of membrane, is also involved in sporulation resulting in cellular spore/sorocarp development with a reduction of transcription level by 5.7 times. One transcript (TRINITY_DN10563_c0_g2, log2FoldChange = 5.53) was located in "structural constituent of cuticle" while TRINITY_DN18931_c0_g2 (log2FoldChange = -3.06) was involved in cell wall organization process.

Out of the 17 genes that were annotated with GO terms in group SQ_RQ (Figure 3.6), the top three cellular components were plasma membrane (TRINITY_DN12381_c0_g1, TRINITY_DN12381_c0_g1, TRINITY_DN21976_c0_g1), extracellular region (TRINITY_DN17149_c0_g1, TRINITY_DN20421_c0_g1, TRINITY_DN20756_c0_g1), and cytoplasm (TRINITY_DN14924_c0_g1, TRINITY_DN20421_c0_g1, TRINITY_DN21189_c0_g1). Two transcripts (TRINITY_DN10003_c0_g1, TRINITY_DN20421_c0_g1), which are involved in pathogenesis, were downregulated 10 and 16 times respectively. One of the transcripts located at the plasma membrane (TRINITY_DN21976_c0_g1) was involved in small GTPase mediated signal transduction/GTPase activity/GTP binding, with the expression level decreased by 9 times.

Discussion

This study confirms that quinoxyfen interferes with signal transduction not only in small GTPase mediated signal transduction but also in positive regulation of MAPK cascades. The MAPK ((mitogen-activated protein kinases) cascades are key signaling pathways that could stimulate an array of cellular processes, including proliferation, differentiation, apoptosis and stress response (Plotnikov et al. 2011; Xu 2000). Additionally, quinoxyfen also interferes with genes involved in pathogenesis, and sporulation activities.

Previous studies documented that the depletion of a gene encoding GTPase-activating protein (GAP) during early infection would trigger the interplaying Ras G-protein to transmit the signals needed for germination and appressorium formation in mildew infection (Hall and Gurr 2000;

Hall et al. 1999; Kinane et al. 2000; Zhang and Gurr 2001). GAPs are a family of regulatory proteins that can bind to activate guanine nucleotide-binding proteins (G proteins) and stimulate GTPase activity, leading to the termination of signal transmission. Ras proteins are a group of GTP-binding, monomeric G proteins with low molecular weight, which interplay with GAPs and function as molecular switches empowered by the interchange of a GDP-bound form or a GTP-bound form (Cox et al. 2014; Wittinghofer et al. 1997). In barley powdery mildew, Wheeler et al. (2003) first demonstrated the possible effect of quinoxyfen on signal transduction during early infection events of *Blumeria graminis* f. sp. *hordei* (*Bgh,* barley powdery mildew). In their study, a gene transcript (*gap,* encoding a GTPase activating protein) was identified to be highly expressed in wild type (WT) isolates treated with quinoxyfen while being absent in WT isolates without quinoxyfen treatment, implying that quinoxyfen can disrupt the degradation of the GAP signal in WT *Bgh* isolates. In addition, *gap* was not expressed in resistant isolates with or without quinoxyfen treatment, indicating the depletion of *gap* was maintained throughout the early infection stage in the resistant isolate, leaving infection by resistant isolates unaffected by quinoxyfen (Wheeler et al. 2000; Wheeler et al. 2003). Similarly, in the current study, a transcript (TRINITY_DN21976_c0_g1, GO:0005525; GO:0003924; GO:0007264, log2FoldChange = -3.18) was downregulated 9.1 times in both sensitive and resistant isolates treated with quinoxyfen. The GO annotation of this transcript indicates that it is involved in "GTP binding and GTPase activity" in the biological process of "small GTPase mediated signal transduction". We speculate that the identified transcript in the present study encodes one of the Ras proteins or similar proteins with GTP binding function. In contrast to *gap* being expressed at different levels in the resistant and sensitive isolate in Wheeler's study, the TRINITY_DN21976_c0_g1 is suppressed in both resistant and sensitive isolate. This could presumably be explained by the fact that, even though Ras proteins and GAPs interact to stimulate the GTP hydrolysis activity, genes encoding Ras proteins and GAPs are not necessarily induced or suppressed at the same level. It cannot be confirmed whether the *gap* gene reported in Wheeler's study is the same transcript we found in our data, since the sequence of the previously reported *gap* transcript was not published.

Lee et al. (2008) pointed out that a gene that encodes *Bgh* cutinases (*CUT1*) was expressed eight times higher in resistant *Bgh* conidia than in sensitive *Bgh* conidia, implying that quinoxyfen resistance may be mediated by overexpression of cutinase specifically when the conidia germinate. Cutinases present in the conidial extracellular matrix (ECM) have the ability to

degrade the host cuticle to release breakdown compounds that can then be perceived by the conidia as part of the host surface cues, leading to the initiation and differentiation of germ tubes (Carver and Gurr 2006; Zhang et al. 2005). Cutin monomers released by cuticle degradation have been shown to induce the differentiation of germlings to form hooked germ tubes on a non-host surface, which supports a link between host perception and signal transduction via cutinase in barley powdery mildew (Lee et al. 2008). The sequence of the *CUT1* transcript is not published. In an attempt to search for *CUT1* in the current data, we blasted the closest published sequence of a homologous *CUT1* gene from the rice pathogen *Magnaporthe grisea* (Sweigard et al. 1992) against the current RNA-seq data, but no significantly DE transcripts were found that resemble *Magnaporthe grisea CUT1*.

A number of DE transcripts were found to be located in the extracellular region. Three DE transcripts (TRINITY_DN17149_c0_g1, log2FoldChange = -2.78; TRINITY_DN20421_c0_g1, log2FoldChange = -4.00; TRINITY_DN20756_c0_g1, log2FoldChange= -3.28) in the extracellular region in both quinoxyfen-resistant and -sensitive isolates (SQ_RQ group) were found to be all downregulated. The aforementioned transcripts are involved in glutaminase activity, pathogenesis, and oxidation-reduction process respectively. A transcript (TRINITY_DN13247_c0_g1, GO:0008237, GO:0005576, log2FoldChange = 4.56), located in the extracellular region, was upregulated in the SQ group. A possible explanation is that quinoxyfen may target or at least interact with the extracellular region in both sensitive and resistant isolates. TRINITY_DN13247_c0_g1 is also involved in metallopeptidase activity, which may be related to the abnormality of the germ tube and the incapability of forming functioning appressoria caused by quinoxyfen treatment (Lee et al. 2008; Wheeler et al. 2003). Detailed functions of metallopeptidases in phytopathogenic organisms have not been well documented yet. In general, metallopeptidases are reported to play a key role in catalyzing hydrolysis of peptide bonds in extracellular protein catabolism (Turner 2003). In humans, matrix metallopeptidases (MMPs) are capable of degrading most of the structural components of the extracellular matrix, triggering the process of bone resorption (Bu et al. 2010). A transcript (TRINITY_DN12102_c0_g1, log2FoldChange = -2.50) related to “sporulation” was also found downregulated in the SQ group.

Quinoxyfen has been reported to reduce the expression level of Pkc1, Pkc-like and Cpka,

showing that it may influence the cellular signal transduction cascades (Lee et al. 2008; Wheeler et al. 2003). In the current study, a transcript in group RQ (TRINITY_DN10567_c0_g1, log2FoldChange = 3.47) was involved in the positive regulation of a stress-activated MAPK cascade. MAPKs are key components of eukaryotic signal transduction cascades present in phytopathogenic fungi. Two transcripts (TRINITY_DN10003_c0_g1, TRINITY_DN20421_c0_g1) related to pathogenesis were both downregulated in the SQ_RQ group, implying that quinoxyfen may interfere with the capability of host recognition and early infection. It is also worth noting that four transcripts (TRINITY_DN10567_c0_g1, log2FoldChange = 3.47, TRINITY_DN12841_c0_g2, log2FoldChange = 3.06, TRINITY_DN18858_c0_g1, log2FoldChange = 4.16; TRINITY_DN3460_c0_g2, log2FoldChange = 2.82) in the RQ group and one transcript (TRINITY_DN12102_c0_g1, log2FoldChange = -2.50) in the SQ group were annotated to be involved in NAD(H)/NADP(H)-related oxidation-reduction activity.

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Tables and figures

Table 3.1. Number of DE transcripts of RQ, SQ_RQ and SQ group

Group	Number of DE transcripts	Number of induced transcripts	Number of suppressed transcripts	Number of GO annotated transcripts
RQ	36	23	13	13
SQ_RQ	56	3	53	17
SQ	62	28	34	10

Figure 3.1. DE transcripts of RW, RQ, and SQ treatment contrasting with SW treatment

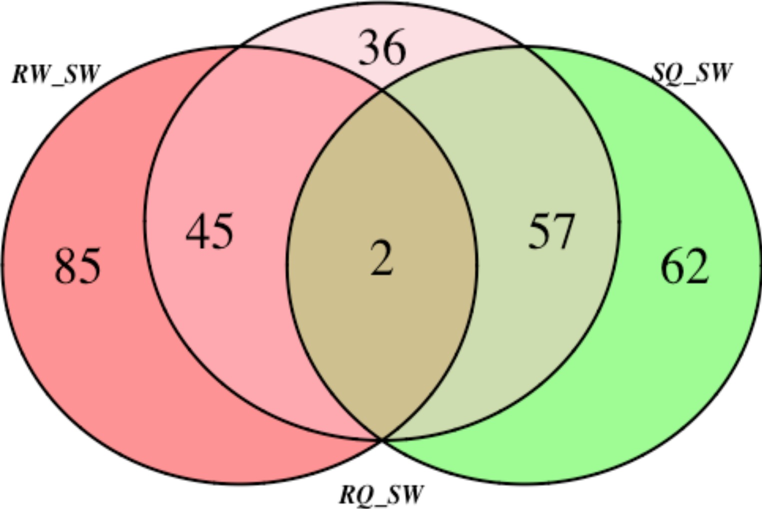


Figure 3.2. DE transcripts of RQ contrasting SQ and RW contrasting SW

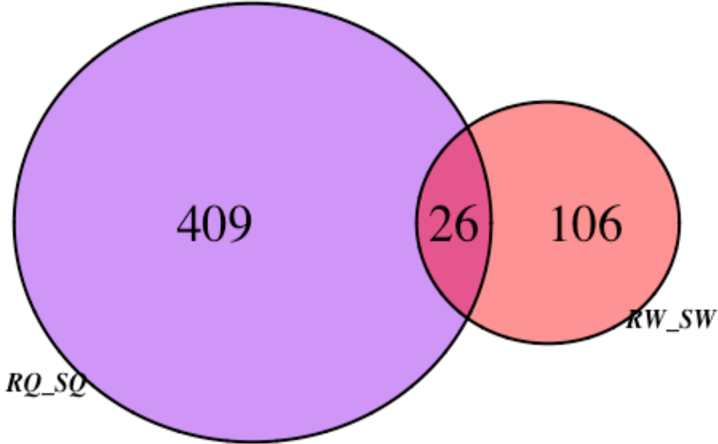


Figure 3.3. DE transcripts of SQ contrasting SW and RQ contrasting RW

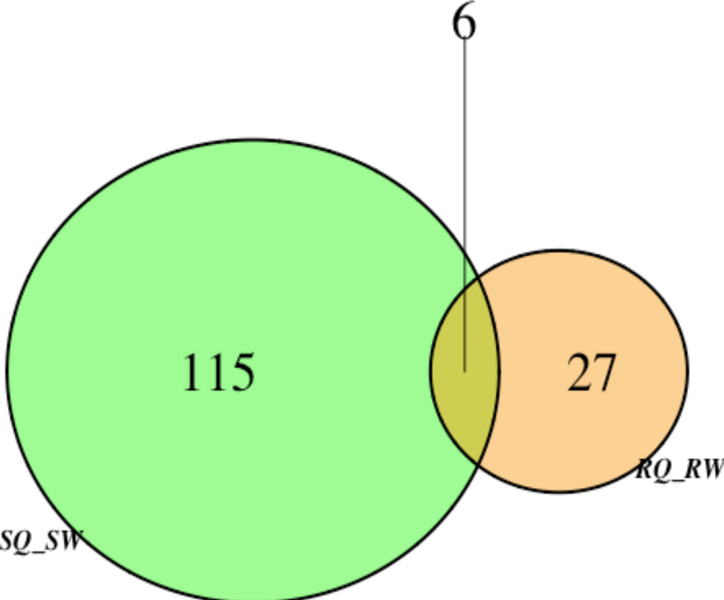


Figure 3.4. Functional annotations of transcripts in RQ treatment

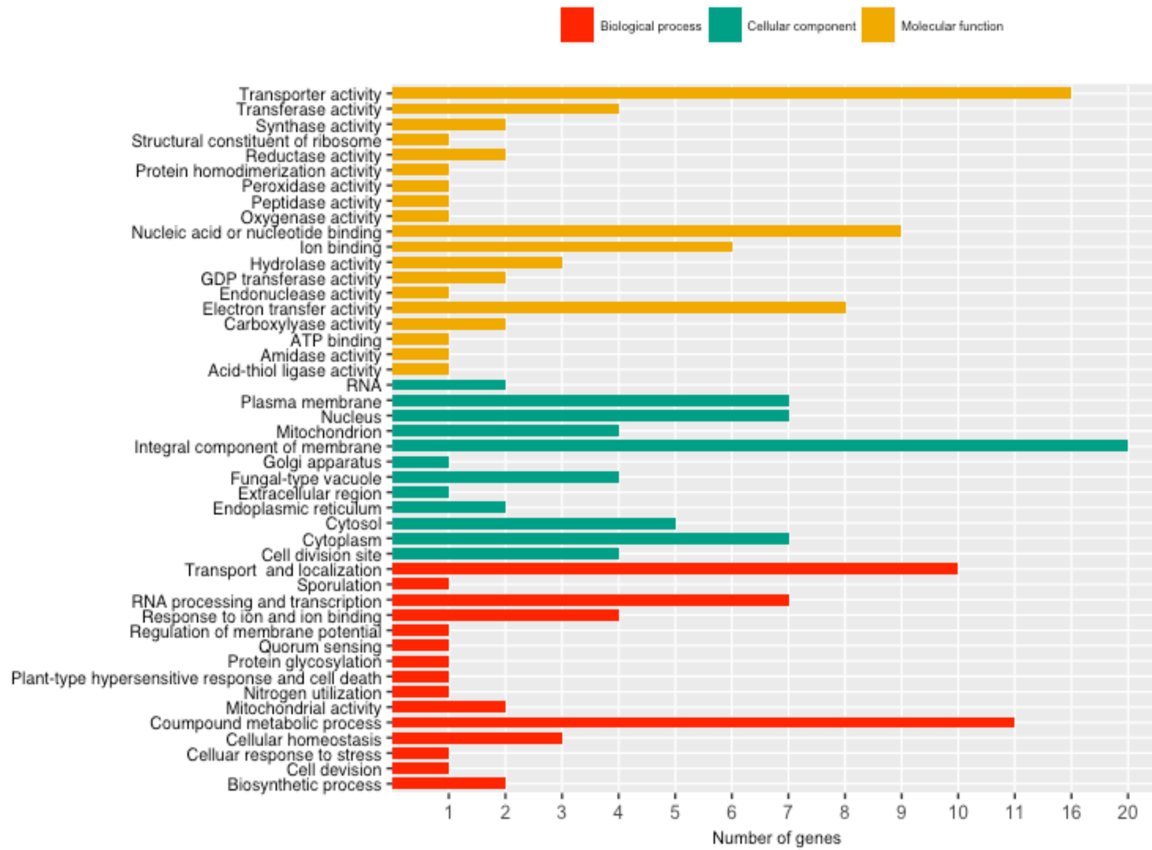


Figure 3.5. Functional annotations of transcripts in SQ treatment

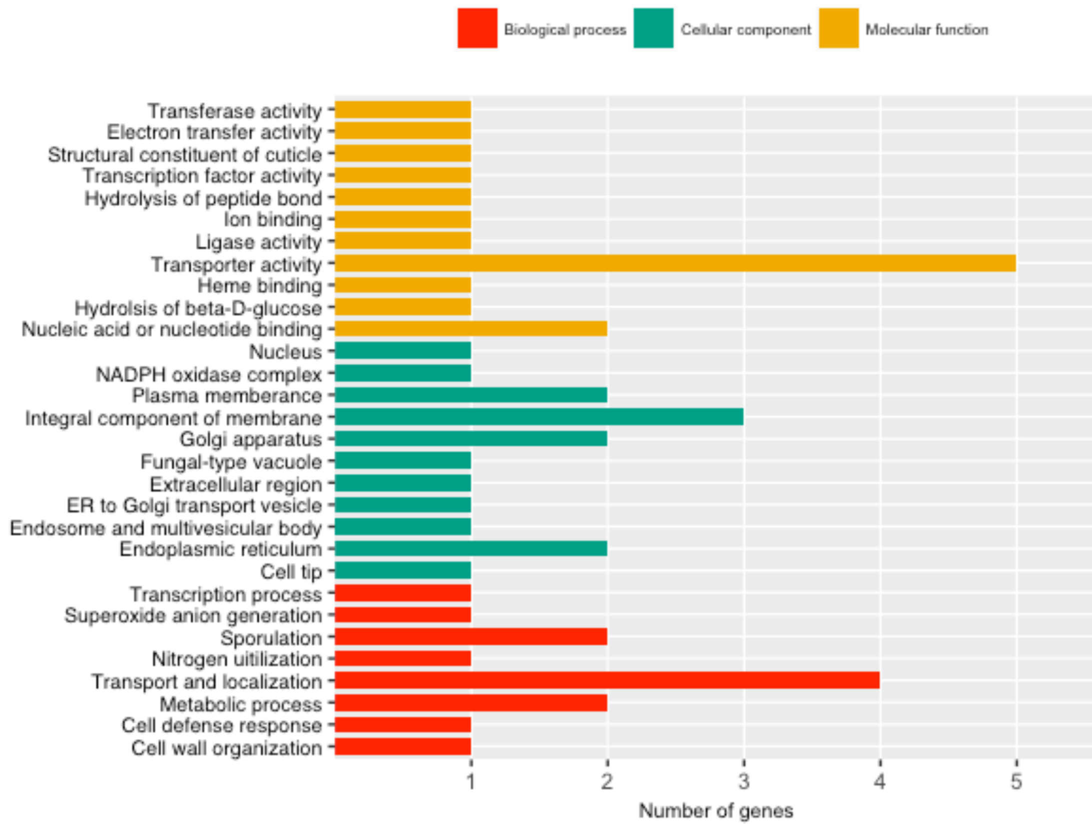


Figure 3.6. Functional annotations of transcripts in SQ and RQ treatment

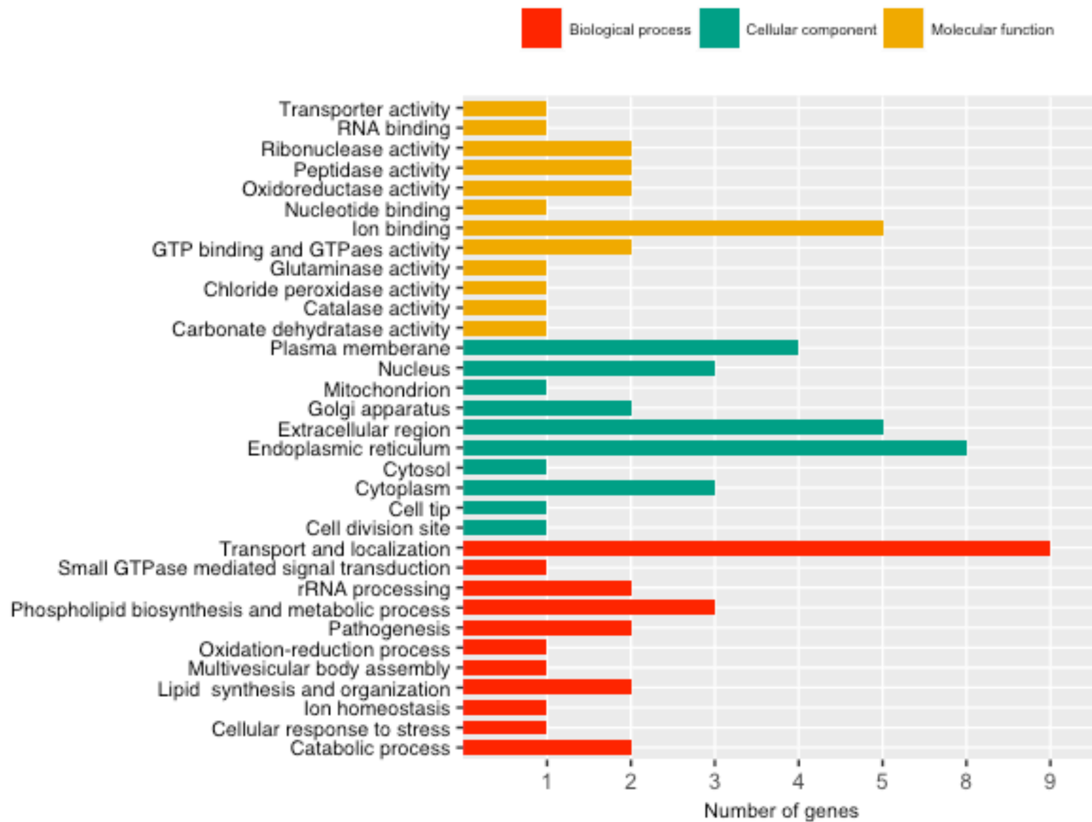


Figure 3.7. DE transcript counts from four conditions, indicate a strong condition effect and good reproducibility of the three biological replicates carried out in the RNA-Seq.

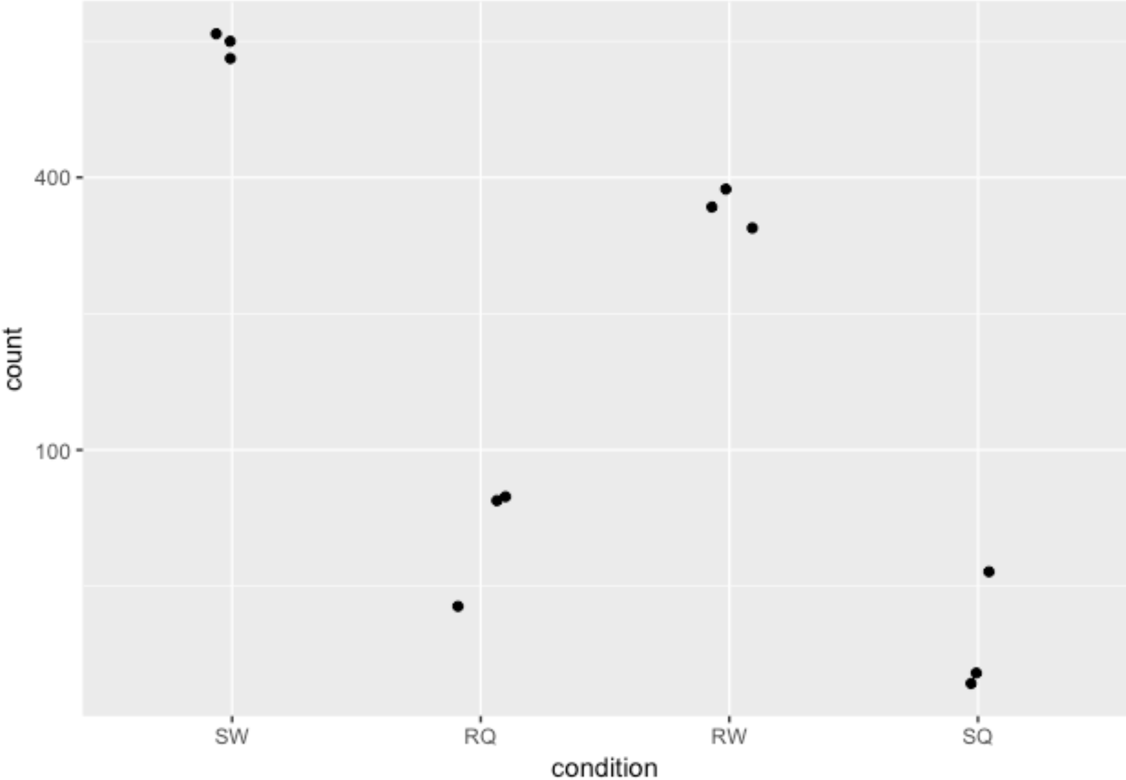


Figure 3.8. Expression level PCA shows a well-segregated pattern corresponding to four treatments. The most variation (sensitive/resistant effect) is indicated by x axis. The second most variation (quinoxyfen/water effect) is indicated by y axis.

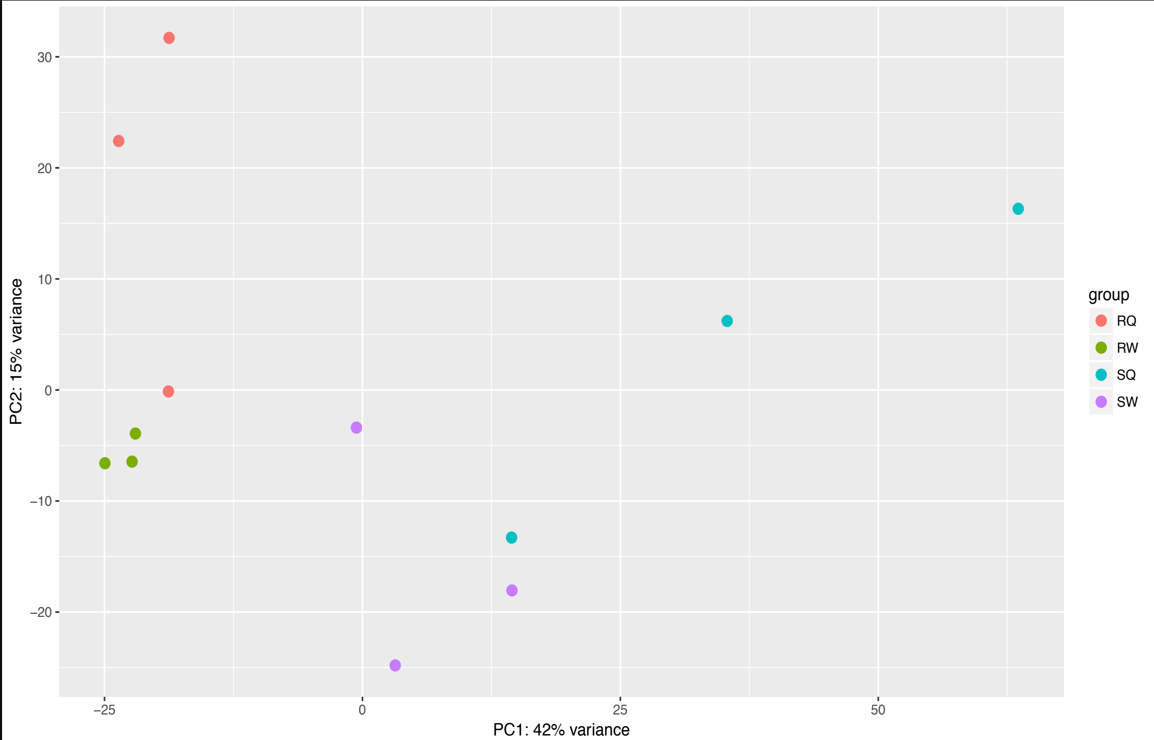
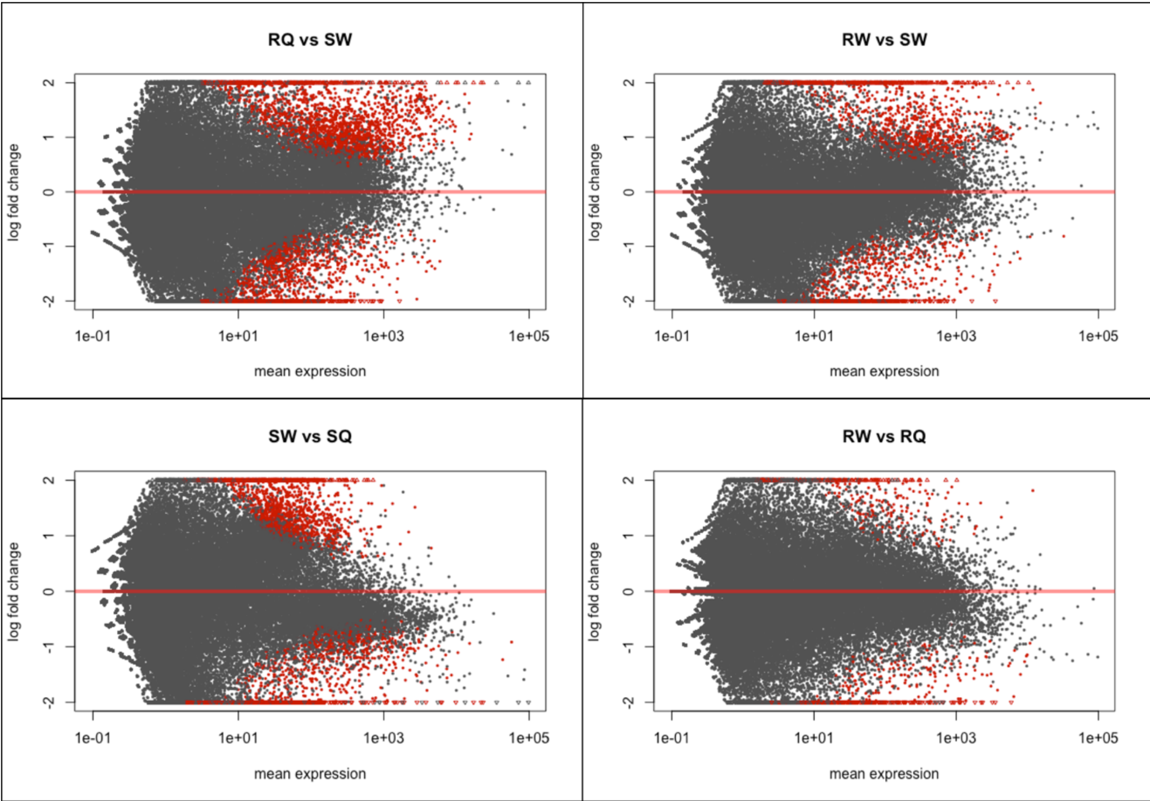


Figure 3.9. MA plots showing a large quantity of DE transcripts (indicated by red dots) were detected by contrasting RQ, RQ and SQ to SW (control). A smaller number of DE genes were detected by contrasting RW and RQ.



Chapter 4. Assessment of Variability of Grape Downy Mildew Sensitivity to Phosphonates

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Abstract

Grape downy mildew, caused by the obligate oomycete *Plasmopara viticola* is a widespread grape disease that can lead to severe yield loss in many years. Phosphite (e.g. Prophyt) fungicides are widely used against downy mildew diseases. Recently, several growers and consultants in Virginia and nearby states have raised the suspicions that phosphite products may not be as effective as they used to be. Grape downy mildew field samples were taken from vineyards across Virginia, and were bioassayed against Prophyt to monitor potential resistance development. A field trial was conducted in 2016 at a western Virginia commercial vineyard to determine whether phosphite fungicides had started to lose efficacy in the field. The bioassay results indicated that none of the isolates had a notable loss of sensitivity. Isolates from the 2016 field trial plots were bioassayed along with the reference isolate, some isolates were not as sensitive as the reference isolates, but the reduction in sensitivity was small. The field trial results indicated that Prophyt applied at 14-day intervals provided poor downy mildew control in the field.

Introduction

Grape downy mildew (*Plasmopara viticola*) is a major grape disease severely affecting the yield and quality of the grape production. Phosphonates or phosphites (salts of phosphorous acid), are widely used against downy mildew and are considered at low risk of fungicide resistance. However, a few cases of resistance of various pathogens to this fungicide group have been reported over the years. Phosphite-resistant mutants have been acquired by using a mutagenic agent to treat pathogens, such as *Phytophthora capsici* (Bower and Coffey 1985), *Phytophthora palmivora* (Dolan and Coffey 1988), *Phytophthora parasitica* (Fenn and Coffey 1989) and *Pythium aphanidermatum* (Sanders et al. 1990). As to field resistance to this group of fungicides, lettuce downy mildew field resistance was documented in California, where isolates were completely resistant to field rates of fosetyl Al (Brown et al. 2004). Reduced fungicide performance was also documented against Phytophthora root rot of avocado after increasing application intensity of this fungicide group (Dobrowolski et al. 2008). The only report of phosphonate resistance in grape downy mildew is from India, with resistance at a high level (Khilare 2003).

In recent years, several growers and consultants in Virginia and nearby states have raised the suspicion that phosphonate products (e.g. Prophyt) may not be as effective as they used to be. In 2012, a downy mildew sample was collected in Virginia that was able to develop a considerable amount of sporulation on plants that were treated with 0.2% Prophyt (0.5% being the maximum labeled concentration) while very little disease developed on plants treated with 0.4% Prophyt (unpublished results). This downy mildew isolate was maintained through several transfers on similarly treated plants, with the amount of downy mildew gradually decreasing. In 2015, Prophyt was used as the downy mildew control agent in our powdery mildew field trial at a western Virginia commercial vineyard. Despite Prophyt being applied every 14 days, the trial rows experienced a serious downy mildew outbreak after four applications, suggesting that loss of efficacy may have started to develop at this site. We hypothesized that fungicide resistance to phosphonates can develop, but that it may not be a stable type of resistance, and it may be maintained only under constant selection pressure. This hypothesis is not the only possible scenario; the lettuce downy mildew field resistance in California was apparently stable in individual isolates (Brown et al. 2004), although the problem in the field disappeared after use intensity of phosphonate fungicides declined (S. Koike, personal communication).

Materials and methods

Isolate collection and maintenance

Isolates were collected from vineyards throughout Virginia in 2014-2016. The isolates were maintained and bioassayed using young leaves from positions 3rd-5th from the tip of the shoot of the susceptible cultivar 'Chardonnay'. Leaves were surface-disinfested by immersing in water solution containing 20% bleach and Tween 20 (1 or 2 drops per 100 ml bleach solution), followed by blotting with paper towels in a laminar flow hood before plating on 1% water agar with sterile utensils. Downy mildew-infected leaves were taken from the field and brought back to the lab in ziplock bags. The sporangia were washed off and transferred onto surface-disinfested young leaves placed with the abaxial surface up in Petri dishes containing 1% water agar. The newly inoculated leaves were incubated overnight at room temperature (22-26°C) and were then allowed to dry off in a laminar flow hood. The dried-off leaves were incubated for another 5-6 days with alternating periods of 13 h light and 11 h dark to allow colonies to develop. Single sporangiophores were transferred individually from the developed

colonies to new surface-disinfested leaves using fine-tip tweezers and the plates were incubated as described above. Sporangia from these subcultures were used in bioassays to test the sensitivity of *P. viticola* isolates.

Field trial

In 2016, a field trial was set up to examine the efficacy of Prophyt in comparison with other downy mildew fungicides at the same location where we found the low efficacy of Prophyt in 2015. A randomized complete block design was used in this field trial. The field trial was set up in two rows of Pinot noir with plots consisting of 4 or 5 vines. Six fungicide applications were carried out on a 14-day schedule. Each treatment was replicated four times. Trial applications included Prophyt (0.5%, maximum label concentration, Helena Chemical Company), mandipropamid (Revus, 146 g a.i./ha, Syngenta), mancozeb (841 g a.i./ha), or captan (1.12 kg a.i./ha, Captan 80WDG, Albaugh, Inc., Ankeny, IA). Azoxystrobin (Abound, 179 g a.i./ha) was included in the first three applications of all treatments for control of black rot (downy mildew at this location had been found to be resistant to azoxystrobin in the past). Quinoxyfen was rotated with myclobutanil (84.0 g a.i./ha, Rally, Dow AgroSciences) + sulfur (841 g a.i./ha, Microthiol Disperss, UPI, King of Prussia, PA) and boscalid (221 g a.i./ha, Endura, BASF Corporation, Research Triangle Park, NC) + sulfur (841 g a.i./ha) for powdery mildew control. The pre-bloom grower spray program consisted of mancozeb and sulfur. Downy mildew-infected leaves were sampled from the trial plots for bioassays.

Bioassay

The assay was conducted as a completely randomized design with three replicates. Surface-disinfested leaves were cut into leaf pieces of 1.5 cm by 1.5 cm and five leaf pieces were placed in one Petri dish containing 1% water agar with the abaxial surface upward. The downy mildew isolates were tested against Prophyt at concentrations of 0%, 0.0125%, 0.025%, 0.05%, 0.1%, and 0.2 %. Fungicides were applied to the lower surface of the leaf pieces using a Preval sprayer (Precision Valve Corporation). Ten µl of sporangial suspension (10^4 sporangia ml^{-1}) was inoculated in the center of each leaf disc. The inoculated leaf discs were incubated as described in “Isolate Collection and Maintenance”. After 6 to 7 days, the numbers of growing sporangiophores were evaluated with a dissecting microscope. The numbers were expressed as a percentage of the sporangiophore number in the untreated control, and plotted against the log_{10}

fungicide concentration to calculate the EC_{50} value.

Potted-plant experiment

To test the hypothesis that resistance to phosphite fungicides can gradually develop if under fungicide selection pressure, a potted-plant experiment was conducted. Potted grape plants (susceptible cultivar 'Catawba') were sprayed with either 0.2% Prophyt or water, allowed to dry, and inoculated by spraying the leaf undersides with sporangial suspensions (10^4 sporangia ml^{-1}) of one of two bulk samples (infected leaves taken directly from the field without single-sporing). RS33 was obtained from Prophyt-treated vines, whereas Y4 was sampled from non-phosphite-treated plots. Both samples were taken 15 days after the sixth application of the season. The inoculated plants were incubated overnight in a mist chamber, and then incubated at room temperature to allow downy mildew to develop. After incubation of 10-15 days, plants were incubated overnight under humid conditions to induce sporulation, and sporangia were washed off to re-inoculate a new plant treated similarly as before. Every re-inoculation was considered as one "generation". Downy mildew sensitivity was bioassayed immediately after the two samples were obtained from the field (Generation 0), in the 5th generation, and in the 10th generation.

Statistical analyses

The range, mean, median, standard deviation of the disease severity of the field trial treatments were calculated and analysis of variance (ANOVA) conducted using the Fit Y by X platform of JMP 10 (SAS Institute, Cary, NC). In many comparisons, control plots contributed a much higher level of variance than the other treatments, violating the assumptions of ANOVA; therefore, control plots were excluded from ANOVA for some comparisons. Post-hoc Tukey's tests (P =0.05) were used to determine whether the effects of the remaining treatments were significantly different from each other.

Results and discussion

Field trial

At the first leaf rating on July 14, 14 days after the 3rd application, all treatments provided significant downy mildew control (Table 4.1). The Prophyt treatments (T2 and T4) had higher disease levels than the non-Prophyt treatments (T3 and T5), but the differences were not

significant. On August 13, 8 days after the 5th spray (Table 4.2), treatments consisting of Prophyt applications only (T2) were significantly less effective than the other treatments. Treatments where some of the Prophyt applications were replaced by mandipropamid (T4, T5, T6) performed better. The mancozeb/captan/mandipropamid rotation (T3) provided moderate disease control. On September 3, 15 days after the sixth spray, for mature leaves (Table 4.3), the Prophyt-only treatment (T2) performed almost as poorly as the non-treated control (T1) whereas the mancozeb/captan/mandipropamid rotation (T3) still provided moderate disease control, and treatments including additional mandipropamid applications were significantly more effective (T4 and T5). Disease on younger leaves (Table 4.3) was somewhat less severe than that of mature leaves, with the Prophyt-only treatment (T2) providing the least control, and the other treatments (T3, T4, T5, and T6) all having better efficacy with no significant differences from one another. The results indicate that Prophyt at 14-day intervals provided poor downy mildew control in the field. This result is in accordance with the field trial carried out in New York State (Wilcox and Riegel 2006), where the efficacy of Prophyt treatment (2-4 pints per 50-100 gallons, equivalent to approximately 0.5%) was compared to the efficacy of mancozeb treatment (Dithane Rainshield 75DF, 4.00 lb/acre) under high disease pressure. The Prophyt treatment provided 38-41% control of cluster infection, and 71-74% control on leaf disease severity, while mancozeb provided 66% control of cluster infection and 81% control on leaves, which was significantly higher than that of the Prophyt treatment (Wilcox and Riegel 2006). In our trial, Prophyt at the last two ratings provided 16-35% control, compared to 60-72% control for the mancozeb-captan-mandipropamid treatment.

Downy mildew sensitivity to Prophyt

Isolates collected from vineyards throughout Virginia were bioassayed to screen for possible differences in sensitivity to phosphite fungicides. Isolate SuD1, which was collected in 2006, was included for comparison since it was collected before use of phosphite or phosphonate fungicides became common. The reproducibility of the assay is illustrated in Table 4.4. The EC_{50} values are shown in Table 4.5 and Figure 4.1. None of the tested isolates showed a notable loss of sensitivity. Isolates from the 2016 field trial plots were bioassayed along with the reference isolate, some isolates were not as sensitive as the reference isolates, but the reduction in sensitivity was small.

Potted-plant experiment

One bulk sample taken from a non-phosphite-treated field trial plot (Y4) and one bulk sample from a Prophyt treated plot (RS33) were cultured on potted Catawba plants either treated with 0.2% Prophyt or distilled water. Isolates were bioassayed every five generations. The initial (Generation 0) EC_{50} of sample Y4 (0.033) was similar to that of sample RS33 from Prophyt treated plot (0.027) (Table 4.6). The EC_{50} value of RS33 slightly increased in the period of ten transfers (Table 4.6); however, EC_{50} values of Y4 fluctuated without showing an increase. There appeared to be no consistent loss of sensitivity of either Y4 and RS33 on either the Prophyt-treated or the nontreated plants (Table 4.6).

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Tables and figures

Table 4.1. Downy mildew leaf infection rated on July 14, 2016 (8 days after the third anti-downy mildew spray)

	Treatments [x]	Foliar severity, % [y]	
T1	Non-treated control	35.5	
T4	3 Prophyt	10.3	b[z]
T2	3 Prophyt	7.1	bc
T3	Mancozeb, captan, mandipropamid	3.1	bc
T5	3 mandipropamid	0.8	c

[x] Prophyt (0.5%), mandipropamid (Revus, 146 g a.i./ha), mancozeb (841 g a.i./ha), or captan (1.12 kg a.i./ha, Captan 80WDG). T2 and T4 contained the same Prophyt applications until the third spray, but differed after the third spray.

[y] 30 leaves per plot evaluated separately by two evaluators.

[z] Analysis of treatment differences with control excluded, to exclude the larger control variance from the within-treatment variance. All treatments significantly different from control by t-test with unequal variances. Data not connected by the same letter are significantly different (Tukey's HSD, $P = 0.05$).

Table 4.2. Downy mildew leaf infection rated on August 13, 2016 (8 days after the fifth anti-downy mildew spray)

	Treatments [x]	Leaf infection % [y]	
T1	Non-treated control	57.9	
T2	Prophyt	37.9	a [z]
T3	2 Mancozeb, 2 captan, 1 mandipropamid	19.7	b
T4	3 Prophyt, 2 mandipropamid	15.0	b
T6	Prophyt / mandipropamid rotation	7.1	bc
T5	3 mandipropamid, 2 Prophyt	7.0	c

[x] Prophyt (0.5%), mandipropamid (Revus, 146 g a.i./ha), mancozeb (841 g a.i./ha), or captan (1.12 kg a.i./ha, Captan 80WDG). Treatments were set up as follows: T1, non-treated control; T2, six Prophyt applications; T3, mancozeb, mancozeb, captan, captan, mandipropamid, mandipropamid; T4, three Prophyt applications followed by three mandipropamid applications; T5, three mandipropamid applications followed by three Prophyt applications; T6, Prophyt, mandipropamid, Prophyt, mandipropamid, Prophyt, mandipropamid.

[y] Leaf infection, 30 leaves per plot evaluated separately by two evaluators.

[z] Analysis of treatment differences with control excluded, to exclude the larger control variance from the within-treatment variance. Data not connected by the same letter are significantly different (Tukey's HSD, $P = 0.05$). T2 is significantly different from T1 by t-test with unequal variances ($P= 0.05$).

Table 4.3. Downy mildew infection of older, mature leaves and younger leaves rated on September 3, 2016 (15 days after the sixth anti-downy mildew spray) (see Table 1 for treatment codes).

	Treatments [x]	Leaf infection % [y] mature leaves		Leaf infection % [y] young leaves	
T1	Non-treated control	64.5	a [z]	39.0	a [z]
T2	Prophyt	54.2	a	26.3	b
T3	mancozeb or captan or mandipropamid	26.0	b	10.8	c
T6	Prophyt or mandipropamid	17.5	bc	12.6	c
T5	3 mandipropamid, 3 Prophyt	12.8	c	13.2	c
T4	3 Prophyt, 3 mandipropamid	10.4	c	8.5	c

[x] Prophyt (0.5%), mandipropamid (Revus, 146 g a.i./ha), mancozeb (841 g a.i./ha), or captan (1.12 kg a.i./ha, Captan 80WDG). Treatments are described in Table 4.2.

[y] Leaf infection, 30 leaves per plot evaluated separately by two evaluators.

[z] Data not connected by the same letter are significantly different (Tukey's HSD, $P = 0.05$).

Table 4.4. Reproducibility of the bioassays of *Plasmopara viticola* (n=3) against Prophyt.

Isolate	Mean EC_{50} (%)	CV(%)	95% CI lower level	95% CI upper level
C4Y	0.028	54.317	0.011	0.046
GRS28	0.022	53.188	0.009	0.035
SUD1	0.007	44.093	0.004	0.012

Table 4.5. EC_{50} value of downy mildew isolates against Prophyt

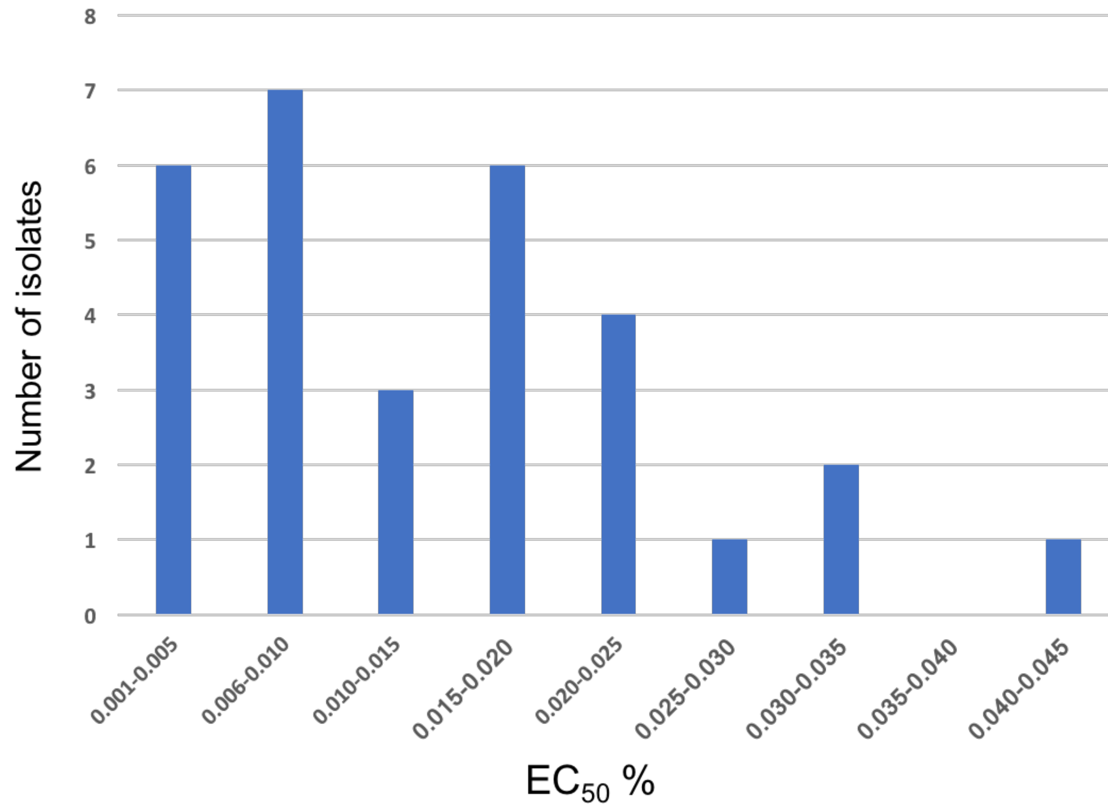
Isolate	Location	Sampling time	EC50 (%)	95% Confidence Interval
FLB	Florida	August, 2013	0.001	0-0.002
FLC	Florida	August, 2013	0.011	0.008-0.014
GHCh	Free Union, Virginia	October, 2013	0.002	0.002-0.003
GHV	Free Union, Virginia	October, 2013	0.006	0.005-0.008
Mar	Warrenton, Virginia	October, 2014	0.002	0.001-0.003
Medcel	Warrenton, Virginia	October, 2014	0.007	0.003-0.01
MJCab	Crozet, Virginia	October, 2013	0.025	0.023-0.027
MJCh	Crozet, Virginia	October, 2013	0.004	0.003-0.005
MJTA	Crozet, Virginia	October, 2013	0.006	0.002-0.009
Mu1	Brownsburg, Virginia	July, 2014	0.006	0.005-0.007
Mu2	Brownsburg, Virginia	July, 2014	0.003	0.001-0.004
RoCH	Raphine, Virginia	September, 2013	0.005	0-0.01
RoPin	Raphine, Virginia	September, 2013	0.009	0.007-0.011
SuD1	Greene Co, Virginia	2006	0.018	0-0.005
Wil1	Lynchburg, Virginia	July, 2015	0.020	0.01-0.029
Wil2	Lynchburg, Virginia	July, 2015	0.018	0-0.004
WinCh1	Winchester, Virginia	August, 2014	0.018	0.013-0.022
WinCh2	Winchester, Virginia	August, 2014	0.018	0.003-0.004
C6R	Raphine, Virginia	August, 2015	0.018	0.015-0.021
C4Y	Raphine, Virginia	August, 2015	0.022	0.016-0.029
E4Y	Raphine, Virginia	August, 2015	0.045	0.038-0.052
ERS28	Raphine, Virginia	August, 2015	0.013	0-0.037
F4Y	Raphine, Virginia	September, 2015	0.032	0.03-0.033
F13Y	Raphine, Virginia	September, 2015	0.023	0.017-0.029
F36Y	Raphine, Virginia	September, 2015	0.033	0.026-0.041
F8RS	Raphine, Virginia	September, 2015	0.006	0.001-0.01
F11RS	Raphine, Virginia	September, 2015	0.012	0.009-0.016
GRS8	Raphine, Virginia	September, 2015	0.027	0.011-0.043
GRS28	Raphine, Virginia	September, 2015	0.009	0.008-0.011
GRS33	Raphine, Virginia	September, 2015	0.018	0.009-0.027

Table 4.6. EC_{50} values of downy mildew isolates maintained on potted plants treated or not treated with 0.2% Prophyt

		Generation 0		Generation 5		Generation 10	
Isolate [z]	Prophyt treatment	EC_{50} (%)	95% confidence interval	EC_{50} (%)	95% confidence interval	EC_{50} (%)	95% confidence interval
Y4	0	0.033	0.026-0.041	0.041	0.023-0.059	0.020	0.013-0.027
Y4	0.2%			0.044	0.020-0.068	0.022	0.015-0.029
RS33	0	0.027	0.011-0.043	0.042	0.028-0.056	0.020	0.005-0.036
RS33	0.2%			0.024	0.011-0.038	0.036	0.014-0.059

[z] RS33 was obtained from Prophyt-treated vines, Y4 from non-phosphite-treated plots, both were taken 15 days after the sixth application of the season.

Figure 4.1. Frequency distribution of EC_{50} values of Prophyt for *P. viticola*.



Chapter 5. Whole genome sequence and single nucleotide polymorphisms identification of *Plasmopara viticola*

Dr. David Haak was one of the co-principal investigators for the grants that supported this research and contributed to the experimental design and data analysis for this research.

Abstract

Grape downy mildew, caused by the oomycete *Plasmopara viticola*, is a destructive plant pathogen native to North America. *Plasmopara viticola* is a prominent example of rapid adaptation, in which it has rapidly evolved in response to new evolutionary challenges. Previously, use of genetic markers has revealed that *P. viticola* is a complex of at least five cryptic species (hereafter designated as clades). Next-generation sequencing and bioinformatics technologies were used in this study to identify single nucleotide polymorphisms (SNPs) in the search of evolutionary important signatures, and to evaluate the adaptive potential of populations. A total of 391,930 SNPs was identified in this study. A draft *P. viticola* genome assembly of ~130 megabase (Mb) was generated and increases the understanding of the *P. viticola* genome.

Introduction

Grape downy mildew, caused by the oomycete *Plasmopara viticola*, is a destructive viticultural disease which causes large yield loss in grape production (Gessler et al. 2011). *P. viticola* is native to North America and was introduced to Europe in the late 19th century through infected grape seedlings, and thereafter rapidly spread to other grape-growing regions worldwide (Gobbin et al. 2006). *P. viticola* is a heterothallic oomycete (Wong et al. 2001), and its sexual reproduction takes place when the gametes of two mating types (A1 and A2) are present in the same leaf and fuse to form thick-walled oospores. The asexual cycle of *P. viticola* generates sporangia, which release zoospores to infect leaves through stomata.

Plasmopara viticola, favored by its large population size and short generation times, is a prominent example of rapid adaptation, in which it has rapidly evolved in response to new evolutionary challenges such as fungicide application and host shift or expansion (Blum et al. 2010; Corio-Costet et al. 2011; Leroux and Clerjeau 1985). *Plasmopara viticola* can deploy various strategies to facilitate the adaptation to the selective pressure imposed by fungicides. One

example is that the point mutation conferring resistance to quinone outside inhibitors (QoIs) was documented to have occurred twice independently based on phylogenetic data of isolates sampled across Europe (Chen et al. 2007). Recurrent mutations conferring resistance against QoI and carboxylic acid amide (CAA) fungicides without fitness costs were documented more recently (Delmas et al. 2017).

Genetic markers have been utilized in numerous studies revealing a generally medium to low genotypic diversity of most European *P. viticola* populations under investigation. Individuals within a population tend to mate randomly but mostly at the vineyard level, indicating a lack of gene flow between populations (Fontaine et al. 2013; Gobbin et al. 2003). In some grape-growing regions, the *P. viticola* populations showed a predominance of one genotype, with the remaining genotypes showing low clonal reproduction level and narrow dispersal range within one or several vines (Rumbou and Gessler 2004). Higher genetic diversity within populations and low gene flow between populations were also discovered in other regions (Gobbin et al. 2006; Gobbin et al. 2007; Gobbin et al. 2005). More recently, a genealogy study of four genes (ITS, 28S, TUB, ACT) demonstrated that *P. viticola* in North America is actually a complex of at least five cryptic species (clades A to E) with morphological and biological variation of sporangial size and host speciation (Rouxel et al. 2013; Rouxel et al. 2014). It is worth noting that only clade B appears to be present in Europe (Rouxel 2012).

Next-generation sequencing and bioinformatics technologies have greatly accelerated the progress of plant pathogen genomics. As the foundation of oomycete genomic studies, an increasing number of oomycete genomes have been published, including *Plasmopara halstedii* (Sharma et al. 2015), *Hyaloperonospora arabidopsidis* (Baxter et al. 2010), *Phytophthora sojae* (Tyler et al. 2006), *Phytophthora infestans* (Haas et al. 2009), and *Pythium ultimum* (Lévesque et al. 2010). As for the pathogen of interest in the current study, two draft genome sequences of *P. viticola* have been released recently, one for an isolate collected in France (INRA-PV221) (Dussert et al. 2016), the other collected in China (JL-7-2) (Yin et al. 2017). The increasing availability of genome-wide data greatly facilitates the search for evolutionary important variations and the evaluation of the adaptive potential of populations fulfilled by marker-based measures (Fontaine et al. 2013; Matasci et al. 2010), provides insights into evolutionary transitions by means of comparative genomic analysis (McCarthy and Fitzpatrick 2017; Zhang et

al. 2014), and deepens the understanding of host-pathogen interactions by exploring effector complement (Kämper et al. 2006).

Numerous genome-scale analyses have identified and predicted hundreds of genes that may encode proteins that could potentially function as effectors (Stam et al. 2013; Stassen et al. 2012; Wang et al. 2011). In oomycetes, two primary classes of cytoplasmic effectors: the RXLR effectors and crinkling and necrosis (CRN) effectors have been described. RXLR effectors seem to present only in the Peronosporales group, while CRN effectors have been described in all oomycetes under investigation (Kamoun 2006). RXLR effectors contain a characteristic "arginine-any amino acid-leucine-arginine" N-terminal motif, usually followed by a motif (D)EER motif., where the RXLR motifs may act as signal peptides that regulate the translocation of effectors. Similarly, CRN effectors feature a conserved LXLFLAK motif at the N-terminal, which have been described as essential to the trafficking of the effectors (Mestre et al. 2016). Both RXLR and CRN effectors have been demonstrated to have functions of suppressing plant immunity (Anderson et al. 2015). The prediction, identification and functional characterization of effectors can provide insightful perspectives of mechanisms of host resistance, pathogenesis of plant pathogens, and evolutionary transitions of host/pathogen populations.

Materials and methods

Isolate collection and DNA preparation

Two downy mildew samples, FLC (previously identified as clade C, or *vinifera*, Rouxel et al 2013; Rouxel et al 2014) and JS (previously identified as clade B or *aestivalis*) were collected from Florida and Maryland respectively in 2012-2013. Sporulating downy mildew leaves were taken from the field and mailed to the lab in ziplock bags. The sporangia were washed off and transferred onto surface-disinfested young 'Chardonnay' grape leaves (positioned 3rd-5th from the tip of the shoot) placed with the abaxial surface up in Petri dishes containing 1% water agar. The newly inoculated leaves were incubated overnight at room temperature (22-26°C) and were then allowed to dry off in the laminar flow hood. The dried-off leaves were incubated for another 5-6 days with alternating periods of 13 h light and 11 h dark to allow colonies to develop. Single sporangiophores were transferred individually from the developed colonies to new surface-disinfested young leaves placed in water agar dishes, and the plates were incubated as described above. The subculture derived from a single sporangiophore was considered as an

isolate. Subcultures were propagated to acquire sufficient material (sporangia and sporangiophores) for the following DNA extraction. Genomic DNA was extracted according to the protocol of the Qiagen DNeasy Plant Mini Kit, except that the 65 °C water bath incubation was increased from 5 min to 15 min.

Whole genome sequencing, genome assembly and quality assessment

The sequencing was carried out at the Duke Center for Genomic and Computational Biology using 8 μg of genomic DNA. A mate-pair library (insert size 2 kb) and a pair-end library (insert size 270 bp) was constructed and sequenced on an Illumina HiSeq 2000/2500 generating reads of 100 bp. Adapters and leading/trailing low-quality sequences were trimmed off using Trimmomatic v0.35 (Bolger et al. 2014). The quality of the trimmed reads was assessed by FastQC v0.11.4 (Andrews et al. 2010). *De novo* assembly of FLC and JS was conducted using BUSCO v2.0 (Simão et al, 2015).

Variant calling and phylogenetic analysis

Processed reads alignment was carried out using BWA–MEM mode from the Burrows Wheeler Alignment, which is programmed in Python. The generated bam files were preprocessed and indexed by Samtools v1.3 (Li et al. 2009), read group information was added to the bam files via the bamaddrg package of BamTools (Barnett et al. 2011). Metrics and statistics of the alignment were generated by Picard v 2.0.1supported by GATK forum (Van der Auwera et al. 2013). Single nucleotide polymorphisms (SNPs) were extracted using Freebayes v1.0 (Garrison and Marth 2012). Consensus sequences were called and converted to FASTA format from identified SNPs followed a GATK pipeline, and the phylogeny tree was generated by standard RAxML v8 (Stamatakis 2006) and visualized by Phylogenetic tree (newick) viewer of ETE Toolkit (http://etetoolkit.org/treeview/). Variant annotation and effect prediction were conducted using SnpEff v3.4 (Stamatakis 2006) based on the *Plasmopara halstedii* genomic gene models.

Results and discussion

A mate-pair library of 2 kb insert size, and a pair-end of library of 270 bp insert size were constructed and sequenced to 130x coverage. The genome assembly of FLC was covered by 17,551 contigs over 1000 bp, while 19,938 contigs were highly fragmented. The total length of the FLC genome was 81,286.9 kb with an N50 scaffold length of 5.7 kb. The genome assembly

of JS was covered by 10,132 contigs over 1000 bp, while 15,385 contigs were highly fragmented. The total length of the JS genome was 59,524.6 kb with an N50 scaffold length of 10.2 kb. The *de novo* assemblies of FLC resulted in 457,291 scaffolds spanning 138 Mb, while the assembly of JS resulted in 448,909 scaffolds, spanning 130 Mb.

To identify genome-wide polymorphisms in the nuclear genome, a European isolate (INRA) was included in variant calling to provide phylogenetic information on a larger geographical scale. The genomes of the FLC, JS, and INRA isolates were aligned to a closely related and well-annotated species *Plasmopara halstedii*, which is the causal agent of sunflower downy mildew. By calling the genomic variants from the alignment, 391,930 SNPs, 2,031 insertions, 1,727 deletions and 287,665 multiple nucleotide polymorphisms were identified across the above-mentioned isolates, with a rate of 0.106 variant per kb.

Polymorphisms were extracted and the gene function effect impact was annotated with SnpEff using the annotation of *P. halstedii* (http://dataportal-senckenberg.de/database/metacat/rsharma.26.4/bikf). Within the detected variants, 3,690 (0.15%) variants were predicted to be of high-impact effect by SnpEff, which indicates that the variants are assumed to have disruptive impact in the protein, probably causing protein truncation, loss of function or triggering nonsense mediated decay; 169,428 (7.02%) variants were of moderate-impact effect (non-disruptive variants that might change protein effectiveness, such as missense variants, and inframe deletion), 395,366 (16.39 %) variants were of low-impact effect (mostly harmless or unlikely to change protein behavior, such as synonymous variants), while the majority of variants (1,844,448, 76.44%) were predicted in modifier genomic regions (non-coding variants or variants affecting non-coding genes with no evidence of impact, such as downstream gene variants). Additionally, 56,163 variants (17.91%) were missense, 443 variants (0.14%) were nonsense variants, and 256,934 (81.93%) were silent variants. The missense/silent SNPs ratio was 0.23. Among the detected variants, 917,647 (38.03%) variants were located in downstream regions; 808,744 (33.52%) variants were located in upstream regions; 560,488 (23.23%) variants were located in exon regions; 10071 (0.42%) were located in intron regions, 107,986 (4.48%) variants were located in intergenic regions. A limited number of variants were located in splice site regions: 470 (0.019%), 565 (0.023%) and 6,960 (0.288%) variants were located in splice site acceptor region, splice site donor region, and

splice site region respectively (Figure 5.1).

The phylogenetic tree revealed that FLC (Florida isolate) and JS (Maryland isolate) displayed a sister clade relationship. In addition, INRA grouped with FLC and JS but is not as close to either FLC or JS as they are to each other, while *P. halstedii* placed outside of the *P. viticola* group (Figure 5.2). SNPs density indicates evenly distributed SNP counts across the various scaffolds of the genome (Figure 5.3).

In closing, the reference genome is an important tool in the molecular genetic studies on *Plasmopara viticola* for the scientific community and serves as a resource for more rapidly identifying genetic markers associated with various phenotypes including fungicide resistance. The current study utilized high-depth sequencing and multi-library data generating a draft *P. viticola* genome assembly, which is at ~130 megabase (Mb), guided by the available reference genome, and increases the information available about the *Plasmopara viticola* genome over what has been available in the previously published genomes (Dussert et al. 2016; Yin et al. 2017). Genome-wide single nucleotide polymorphism identification was carried out. A VCF file containing the detected SNPs that could be potentially used for clade identification in a future study was generated. The genomes, genomic markers, annotation data and raw data will be made publicly available.

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Tables and figures

Figure 5.1. Number of effects by region

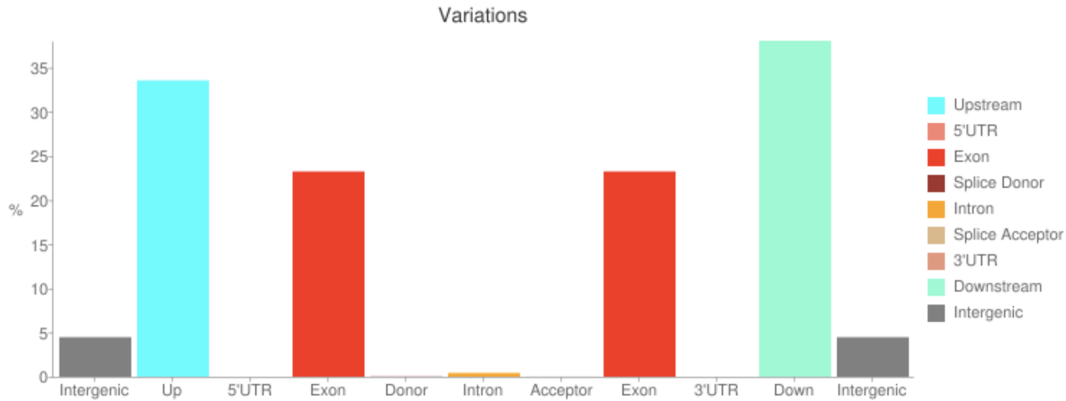


Figure 5.2. Genomic phylogeny determined based on pairwise SNP differences in the alignments of JS, FLC, INRA and *P. halstedii*. Branch lengths are proportional to the number of segregating sites that differentiate each pair of isolates.

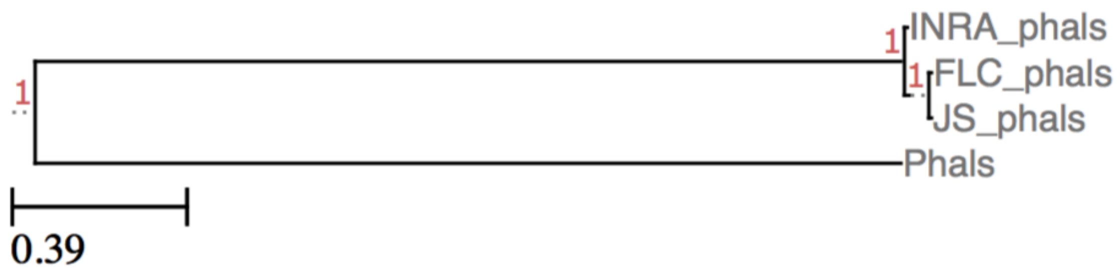
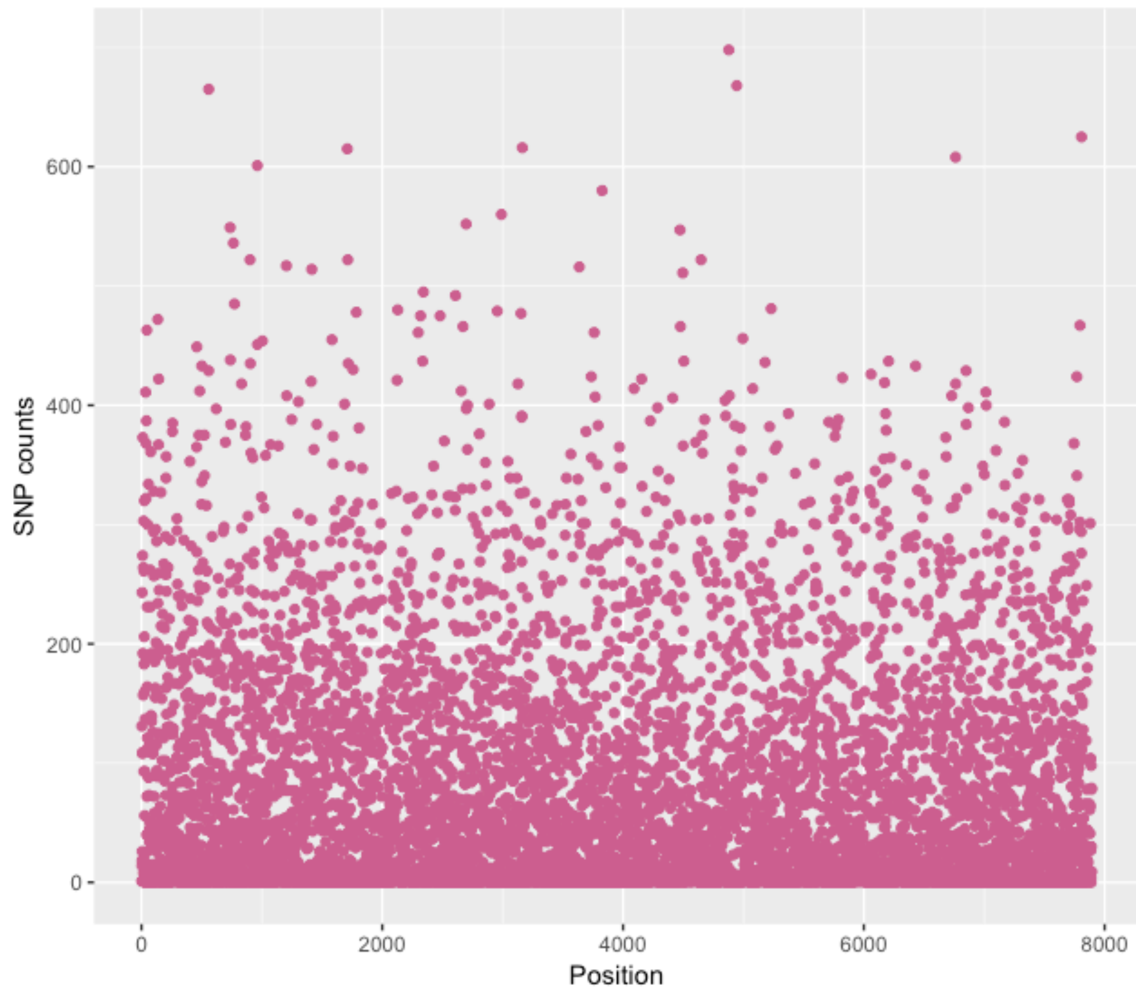


Figure 5.3. SNP density across the genome alignment of JS, FLC, and *P. halstedii*



Chapter 6. Occurrence of CAA Fungicide Resistance in *Plasmopara viticola* Isolates from a Vineyard in Virginia, United States

Plasmopara viticola, the causal agent of grapevine downy mildew, is an oomycete plant pathogen that has the potential to cause huge yield loss in humid grape production regions worldwide. Chemical fungicides play an important role in controlling grape downy mildew, but *P. viticola* has developed resistance to several groups of fungicides, including quinone outside inhibitors (QoI), phenylamides, and carboxylic acid amides (CAA). In Europe, resistance of grape downy mildew to CAA fungicides was reported almost 15 years ago (Blum et al. 2010). Control failure of CAA fungicides was also reported from vineyards in India recently (Sawant et al. 2016), and resistant isolates have been detected in Japan (Aoki et al. 2013) and China (Zhang et al. 2016). Eight field isolates of *P. viticola* were collected in October 2016 from a vineyard in Virginia, where mandipropamid (Revus) was reported to have provided poor control of downy mildew. The spray program in this vineyard included three Revus application in the 2016 growing season. Diseased leaf samples were brought to the lab in Ziploc bags and incubated overnight in a moist environment at room temperature (~23°C) to obtain sporulation. A sensitive isolate of *P. viticola*, collected before CAA fungicides were widely used in Virginia vineyards, was included as a reference isolate. Sensitivity to mandipropamid (Revus 23.4% SC, Syngenta Crop Protection, Greensboro, NC, United States) was determined by a leaf disc bio-assay method (Herzog and Schüepp 1985) with minor adjustments. Solutions of 200, 20, 2, 0.2, 0.02, and 0 $\mu g.ml^{-1}$ (active ingredient) concentrations were prepared by appropriate dilution of the 1,000 $\mu g.ml^{-1}$ stock suspension using sterile distilled water. The experiment was conducted as a completely randomized design with three replicates. Fungicides were applied to the lower surface of ~15-mm leaf discs using a Preval sprayer (Precision Valve Corporation). Six hours later, 10 µl of sporangial suspension (10^4 sporangia ml^{-1}) was inoculated on the center of each leaf disc. The inoculated leaf discs were incubated at 22°C with alternating periods of 13 h light and 11 h dark. After 6 to 7 days, the areas of the lesions were measured and the numbers of sporangiophores were estimated using a dissecting microscope. Readings were expressed as a percentage of the untreated response, and plotted against the log_{10} fungicide concentration to calculate the EC_{50} value. The EC_{50} value of the reference *P. viticola* isolate was <0.2 $\mu g.ml^{-1}$, showing sensitivity to mandipropamid. The EC_{50} values of the eight isolates from the commercial vineyard were >240 $\mu g.ml^{-1}$ for mandipropamid, which was well above the field rate

(146 $g.ha^{-1}$ or 156 µg $a.i.ml^{-1}$ if applied by dilute spraying at 1000 $liters.ha^{-1}$), illustrating their insensitivity. Additionally, the 144 bp *PvCesA3* gene of two resistant isolates was amplified (Taq 2X Master Mix, New England Biolabs) and sequenced (Eurofins Genomics). The sequencing data showed that the PCR products amplified from resistant *P. viticola* isolates had a GGC-to-AGC substitution at codon 1105 of the *PvCesA3* gene, the same mutation that has been found responsible for CAA resistance in other regions (Aoki et al. 2011; Blum et al. 2010). In the 2017 growing season, we identified two other locations where similar strains were collected, one 120 km to the north and one over 250 km to the southwest from the original site. Further mandipropamid resistance monitoring of downy mildew in commercial vineyards in Virginia is needed to define the geographic extent of this emerging resistance.

Literature cited

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