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Tiago Camponogara Tomazetti

Piramidação de genes da Videira (*Vitis vinifera*) para resistência ao Mildio (*Plasmopara viticola*) e caracterização das interações genótipos e ambiente

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Piramidação de genes da Videira (*Vitis vinifera*) para resistência ao Mildio (*Plasmopara viticola*) e caracterização das interações genótipos e ambiente

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Coorientador: Prof. Leocir José Welter, Dr.

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Tiago Camponogara Tomazetti

Piramidação de genes da Videira (*Vitis vinifera*) para resistência ao Mildio (*Plasmopara viticola*) e caracterização das interações genótipos e ambiente

O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

Prof. Aparecido Lima da Silva, Dr.
Universidade Federal de Santa Catarina

Marco Stefanini, Dr.
Fondazione Edmund Mach – Trento – Itália

Marco Antonio Dalbó, Dr.
EPAGRI – Videira, SC

Prof. Miguel Pedro Guerra, Dr.
Universidade Federal de Santa Catarina

José Afonso Voltolini, Dr.
Universidade Federal de Santa Catarina

Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de doutor em Ciências.

____

Coordenação do Programa de Pós-Graduação

____

Prof. Aparecido Lima da Silva, Dr.
Orientador

Florianópolis, 2020

Este trabalho é dedicado aos meus pais, Luiz Elibio Tomazetti e Elza Maria Camponogara Tomazetti, minha namorada Márcia Denise Rossarolla e demais pessoas que participaram desta caminhada.

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"Verba volant, scripta manent"
Tacitus, Publius Cornelius (56 - 120)

RESUMO

O cultivo de uva possui marcante papel cultural em diversas regiões do mundo. Programas de melhoramento genético da videira tem baseado as seleções de genótipos superiores a partir da avaliação de caracteres de qualidade da uva e resistência a doenças, via seleção de locos gênicos associados à resistência (*R-locos*). Baseando-se na crescente demanda por cultivares viníferas com resistência a doenças, foi desenvolvida esta tese, com o objetivo de gerar e selecionar genótipos de videira PIWI com genes de resistência ao míldio em diferentes níveis de piramidação, conhecer a dinâmica genética populacional do míldio (*Plasmopara viticola*) nos vinhedos catarinense, bem como, descrever o desenvolvimento fenológico de genótipos PIWI nas condições climáticas de Santa Catarina. Para isto a tese foi dividida em cinco capítulos. O primeiro capítulo refere-se à seleção assistida por marcadores moleculares. Neste estudo foi verificado que existe efeito aditivo para a maior parte das interações genicas, o genótipo piramidado para os genes *Rpv1+Rpv3.1+Rpv10* apresentou o maior nível de resistência e o *Rpv10* foi o R-locos que proporcionou maior restrição ao crescimento do patógeno isoladamente. No segundo capítulo, foi avaliado a influência do ambiente e do hospedeiro, contendo diferentes *R-locos*, sobre a estrutura genética de populações de *P. viticola* no estado de Santa Catarina, Brasil. Foi demonstrado que a principal influencia sobre a estrutura genética em populações deste patógeno é do hospedeiro, mesmo havendo interações com o ambiente. Hospedeiros contendo genes piramidados apresentaram uma maior barreira para o estabelecimento das populações de míldio. No terceiro capítulo foi caracterizado a fenologia de cultivares PIWI, buscando-se conhecer demanda térmica de alguns cultivares que se destacam como potenciais para cultivo comercial. Foi observado que, em todos os genótipos avaliados a maior exegencia térmica está entre as fases fenológicas de florescimento e o *véraison*. A exigência térmica para completar o ciclo, na região dos vales da uva Goethe, onde foi acompanhado a soma térmica, variou entre 1260 °C dia (cv. Felícia) e 1454 °C dia (cv. Aromera). O quarto capítulo refere-se ao estudo realizado a partir da mensuração das áreas de folhas de 11 genótipos cultivadas em cinco ambientes distintos. Este estudo indicou a existência de interação entre genótipos e ambiente também para a área da folha. Além disto, foram gerados modelos lineares para predição da área foliar em 11 genótipos. No quinto capitulo esta relatado as atividades de pesquisa desenvolvindas junto à Unità di Genetica e Miglioramento Genetico della Vite (UGMGV), da Fondazione Edmund Mach (Trento, IT), no âmbito de cooperação em que foram desenvolvidas as atividades, foi gerado um estudo descrevendo um genótipo portador de resistência ao míldio, que foi

confirmado possuir o *Rpv27*, que herdou a partir da sua ancestralidade com *V. aestivalis*. A população segregante gerada a partir da autofecundação deste genótipo gerou indivíduos com segregação para a resistência ao míldio, oídio e black rot, indicando que seu uso é possível como doador de novas fontes de resistência genética a doenças em programas de melhoramento genético da videira.

Palavras-chave: *Vitis vinifera*; melhoramento genético; seleção assistida; resistência a doenças; ecofisiologia; PIWI

ABSTRACT

The cultivation of wine grapes has a marked cultural role in different regions of the world, being mainly linked to quality. Genetic improvement programs for the grapevine have based the selection of superior genotypes based on the evaluation of qualitative traits, such as absence of seeds, for table grapes, bunch architecture, as well as, resistance to diseases, via selection of R- loco. Based on this scenario and on the growing demand for disease-resistant wine cultivars, this thesis was developed, aiming to generate and select genotypes of PIWI grape with downy mildew resistance genes at different levels of pyramidation, to know the population genetic dynamics of the downy mildew (*Plasmopara viticola*) in the Santa Catarina vineyards, as well as describe the phenological development of PIWI genotypes in the Santa Catarina climatic conditions. For this, the thesis was divided into five chapters. The first chapter refers to the selection assisted by molecular markers, generating genotypes that carry pyramidized resistance genes. In this study it was verified that there is an additive effect for most of the genetic interactions, the pyramid genotype for the genes Rpv1 + Rpv3.1 + Rpv10 showed the highest level of resistance and Rpv10 was the R-locus that when only in the genome provided greater restriction to the growth of the pathogen. In the second chapter, the influence of the environment and the host, containing different R-loci, on the genetic structure of P. viticola populations in the state of Santa Catarina, Brazil, was evaluated. It has been shown that the main influence on the genetic structure in populations of this pathogen is that of the pathogen, even if there is interaction with the environment. It has also been demonstrated that hosts containing pyramid genes present a greater barrier to the establishment of downy mildew populations. In the third chapter, the phenology of PIWI cultivars was characterized. It was observed that, in all genotypes evaluated, the greatest thermal exegence is between the phenological phases of flowering and veraison. The thermal requirement to complete the cycle, in the region of the Goethe grape valleys, where the thermal sum was monitored, varied between 1260 ° C day (cv. Felícia) and 1454 ° C day (cv. Aromera). Leaf areas of 11 genotypes grown in five different environments were measured. This study also indicated the existence of interaction between genotypes and environment for the leaf area. In addition, linear models were generated to predict the leaf area for the 11 genotypes, based on the measurement of some dimension of the same. A study was also generated describing a genotype with resistance to mildew, which was confirmed to have Rpv27, which it inherited from its ancestry with *V. aestivalis*. The segregating population generated from the self-fertilization of this genotype generated individuals with segregation

for resistance to mildew, powdery mildew and black rot, indicating that its use is possible as a donor of new sources of genetic resistance to diseases in genetic improvement programs of the vine.

Keywords: *Vitis vinifera*; plant breeding; assisted selection; disease resistance; ecophysiology; PIWI

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Figure 1 Worldwide diffusion of powdery mildew (PM, panel A), black rot (BR, panel B), and anthracnose (AN, panel C). The relative bibliographic sources are reported in **(A)** PM was first reported in northeastern America in 1834 by Schweinitz. In 1845 it was introduced in Europe and less than 10 years later was affecting all the wine producing country of the Mediterranean region. In 1986, Amano published an outstanding review listing the countries all over the world where fungi causing PM were present at that time in relation with their specific plant hosts, including grapevine. Today PM can be considered a "worldwide grapevine disease", since it afflicts vineyards all over the world. **(B)** BR is native of northeastern America. In 1804 it was noticed in Dufour's vineyard (Kentucky) and it became epidemic in the second half of the 19th century in all the Great Lake Region, where the entire yield in many fields was lost. The first occurrence in Europe was recorded in 1885 by Viala and Ravaz in Southern France, then it spread all around the world, although without a huge economic impact. In 1989, BR showed an increasing presence in Switzerland, but it was in the 21th century that a second outbreak afflicted Europe, starting from Germany, where the economic losses were severe, to the Alps area (Ticino, Switzerland; Friuli and Veneto, Italy), Hungary and Romania. Regions with an unfavourable climate, as Scandinavia for cold and Mexico for dry weather, are considered BR "free" today. Interestingly, also in Australasia and Chile BR remains absent. **(C)** AN is considered one of the oldest known plant disease, since reference to it were reported in ancient Rome by Theophrastus (in *De causis plantarum*) and by Pliny the Elder (in *Naturalis historia*), dating back to the first century of the Christian era. Its European origin was also confirmed by the first report of the modern era in 1874 by De

Bary, in Germany. AN diffusion was not alarming until its arrival in Tropical areas, such as South America. Nowadays AN is again becoming a threat in Europe. 145

Figure 2 *Erysiphe necator* life cycle. Two overwintering strategies have been observed in *E. necator*. In areas with relatively mild winters, the fungus commonly overwinters as mycelium on leaf primordia within dormant buds. In the following spring mycelium activity resumes, resulting in the production of heavily infected and deformed shoots, called 'flag shoots'. The fungus sporulates on these shoots, producing a large number of conidia that are carried by the wind to healthy plant tissues. Alternatively, the fungus can overwinter as chasmothecium (syn. cleistothecium, a former term for this structure that is still widely used) in bark, on canes, leftover fruit, and on leaves on the ground. Chasmothecia form on the surface of heavily diseased tissues from mid-summer to autumn. During spring rainfall the chasmothecia open and release ascospores, which are spread by wind or raindrops to infect the lower leaves near where the chasmothecia have overwintered. Although free water is necessary to release ascospores, continued wetness is not required for subsequent spore germination and infection. At each new infection site, conidia and ascospores germinate and form an appressorium. From its lower surface a penetration peg develops, piercing the cuticle and entering through an epidermal cell where a haustorium is formed. Mycelium grows upon the surface of the plant epidermis and new conidia are produced within a few days, completing the cycle. Repetition of this cycle continues throughout the growing season resulting in a rapid increase in disease incidence (Wilcox et al., 2017).. 147

Figure 3 Fungal morphological characteristics and symptoms of powdery mildew (PM, panel **A**-**C**), black rot (BR, panel **D**-**G**), and anthracnose (AN, panel **H**-**K**). **(A)** PM on grapes (field). **(B)** PM on leaves (greenhouse). **(C)** PM leaf disc infection under different magnification (above); conidiophores and conidia on a leaf surface (below-left); mature (black) and immature (yellow) chasmothecia (below-right). **(D)** BR field symptoms on leaves and grape cluster. **(E)** *G. bidwellii* pycnidia on petiole and leaf (above), on berry with detail of cirri development under humid conditions (below). **(F)** Detail of *G. bidwellii* pycnidia and conidia under different magnification. **(G)** *G. bidwellii* isolate growing on culture media (above) and leaf symptoms after artificial infection (below). **(H)** AN field symptoms on a grape cluster, leaf and young shoot, along with detail about the typical "shot-hole" lesions on old infected leaf. **(I)** AN symptom details on berries. **(J)** *E. ampelina* colony on culture media. **(K)** *E. ampelina* acervulus releasing conidia and detail of conidia................. 149

Figure 4 *Guignardia bidwellii* life cycle. The fungus overwinters in mummified berries, retained on the vine or fallen to the ground, and on infected canes. Berry mummies host both pseudothecia, containing asci with ascospores, and pycnidia, with conidia, while canes and tendrils host pycnidia. Lesions capable of producing conidia can persist in the wood for at least two years. In spring, ascospores and conidia are released when pseudothecia and pycnidia become thoroughly wet; infection is therefore favored by frequent rainfall as the spores need water to be released and to germinate. Ascospores released from mummified berries are the most common form of primary inoculum. They are ejected actively from the asci during rainfall and are dispersed by wind currents (long distance dispersion). On the

contrary, conidia are exuded from the pycnidium in a white, mucilaginous cirrus from which they can be splashed away by rain (short distance dispersion). Primary infection from ascospores or conidia takes place on young, rapidly growing green tissues (growing leaves, inflorescences, fruit pedicels, berries). Adult leaves and ripe fruits, that have become fully expanded, are not susceptible to infection. Pycnidia are produced rapidly within the necrotic lesions found on leaves, shoots and berries, and, once mature and dampened by rain, they release the conidia which serve as secondary inoculum throughout the season. When the weather is moist, ascospores may be produced and released continually throughout spring and summer from mummies retained in the canopy, providing continuous primary infection, although most of them are discharged in the spring. In late summer, the sexual cycle initiates on infected berries and pseudothecia are formed (Wilcox et al., 2017)....................... 152

Figure 5 *Elsinoë ampelina* life cycle. The fungus overwinters mainly in infected canes as sclerotia, dense mycelial masses which are formed in autumn at the edge of lesions on shoots. In spring, sclerotia develop stromata on which, under humid conditions, produce abundant conidia. These conidia are then carried by rain or free water to young, rapidly growing green tissues (leaves, shoots, tendrils and young berries) where they germinate causing the primary infection. Conidia are by far the most important source of primary inoculum in spring. The fungus can also overwinter on infected berries, left on the vine or vineyard floor, as conidia or ascospores, which may also cause primary infections. Once the disease is established, the pathogen produces lesions upon which acervuli form and produce conidia which, dispersed by raindrops, serve as secondary source of inoculum for the rest of the growing season. During periods of humid weather, conidia can be released the entire spring and summer providing continuous infection. Heavy rainfall and warm temperatures are ideal for disease development and spread. At times pseudothecia, containing asci and ascospores, develop on the lesions (Wilcox et al., 2017) 155

LISTA DE ABREVIATURAS E SIGLAS

aLV: do inglês “average of lateral veins”

$\hat{f}$: índice de fixação

KDE: do inglês “Kernel density estimation”

L: do inglês “Length”

LA: do inglês “Leaf Area”

LDI: do inglês “Leaf Deformation Index”

LFDGV: Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal

PCA: do inglês “Principal Component Analysis”

PCR: do inglês “Polymerase Chain Reaction”

PIWI: do alemão “Pilzwiderstandsfähig”

R-loco: Loco gênico associado a regiões que conferem resistência a doenças

***Rpv1+Rpv3.1*:** Genótipo de videira contendo a piramidação de locos *Rpv1* e *Rpv3.1* associados com a resistência ao míldio.

***Rpv3.1*:** Genótipo de videira contendo o alelo 1 do loco *Rpv3* associado com a resistência ao míldio.

***Rpv3.1+Rpv3.2*:** Genótipo de videira contendo a piramidação dos alelos 1 e 2 do loco *Rpv3* associado com a resistência ao míldio.

Rpv3.3+Rpv10: Genótipo de videira contendo a piramidação de locos *Rpv3.1* e *Rpv10* associados com a resistência ao míldio.

srpv: genótipo de videira com ausência de locos associados a resistência genética ao míldio.

VIVC: do inglês “Vitis International Variety Catalogue”

W: do inglês “Width”

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1 ANTECEDENTES E JUSTIFICATIVA

1.1 CULTIVO DA VIDEIRA

A origem da videira é um tema ainda controverso, trata-se de uma das primeiras espécies a passar pelo processo de domesticação, sua história se confunde com a de diversas civilizações. Atualmente, são consideradas múltiplas origens e vias de domesticação. As modificações envolvidas na transformação do tipo silvestre (*Vitis vinifera* ssp. *sylvestris*) no tipo domesticado (*Vitis vinifera* ssp. *sativa*) ocorreram principalmente na Península Itálica e no Cáucaso (ARROYO-GARCÍA et al., 2006; GRASSI et al., 2006).

Estima-se que este processo começou entre 20 a 30 mil anos atrás e foi intensificado a aproximadamente 8.000 anos atrás. Neste período ocorreram em torno de 80 eventos de combinação alélica (reprodução sexuada). Assim, a principal diferença evolutiva entre as populações cultivadas ocorreu por meio do acumulo de mutações, gerando como consequência da síndrome da domesticação, acumulo de alelos deletérios no genoma (ARROYO-GARCÍA et al., 2006; GRASSI et al., 2006; ZHOU et al., 2017). As cultivares modernas de *Vitis vinifera* foram selecionadas ao longo da história, principalmente por influência do império romano e dos reinos da idade média, estabelecidos principalmente no continente europeu, buscando a produção de frutos com melhor qualidade assim como, melhores vinhos. Atualmente, muitas cultivares tem sua origem genética derivadas a partir das cultivares ‘Pinot’ e ‘Gouais blanc' (BOWERS et al., 1999).

A plasticidade fenológica das cultivares de videira é uma característica marcante da espécie, o que permite que a viticultura possa ser desenvolvida em diversas regiões, o que a torna a videira uma das espécies frutíferas mais cultivadas no mundo. Na safra de 2016 foram cultivados aproximadamente 7.096.741 ha com volume de produção alcançando 77.438.929 t da fruta (FAO, 2019). A cadeia produtiva da viticultura envolve comunidades em todos os continentes, o que torna o mercado viti-enológico um dos mais importantes setores comerciais em diversas regiões, com proporções globais (VIVIER; PRETORIUS, 2002). Atualmente, o cultivo comercial de videira está pautado em *Vitis vinifera* ssp. *sativa*, da qual existem castas para a produção de uva de mesa, sucos, uvas passa, entre outros. Entretanto, o principal uso comercial desta espécie é o mercado enológico, voltado para a produção de vinhos finos.

Mundialmente, a vitivinicultura tem sua raiz e principal mercado na Europa, principalmente Itália e França. Contudo, novas regiões têm apresentado expressivo crescimento da área cultivada e amadurecimento do mercado enológico, com destaque para os

Estados Unidos, Austrália, Chile e África do Sul (MORRISON; RABELLOTTI, 2017). No Brasil, a maior região produtora está situada no sul do país, e é caracterizada por verões quentes e úmidos, condições essas que favorecem o surgimento de doenças, em especial o míldio (*Plasmopara viticola*). Este aspecto dificulta o cultivo de variedades suscetíveis, devido à elevada quantidade de aplicações de agrotóxicos necessárias e as implicações causadas pelos impactos derivados do uso destes produtos (LAMICHHANE et al., 2018).

Modelos de simulações e observações em curso apontam para os impactos das mudanças climáticas, demonstrando que condições climáticas favoráveis para o desenvolvimento do patógeno serão ainda mais comum, em diversas regiões produtoras vitícolas do mundo (NAZIR et al., 2018; NEWBERY; QI; FITT, 2016; SALINARI et al., 2006). Assim, programas de melhoramento genético vem buscado mapear genes ligados ao comportamento fenológico da videira, visando desenvolver cultivares adaptadas as novas condições nos cenários apontados, buscando evitar a ocorrência de momentos mais sensíveis do ciclo fenológico, com os momentos mais suscetíveis a doença (ZYPRIAN et al., 2018). Para isto, faz-se necessário também, descrever a ocorrência dos ciclos fenológicos das cultivares, mesmo aquelas portadoras de locos associados com a resistência ao mildio (Rpv's) em diferentes condições climáticas de cultivo.

1.2 MÍLDIO (*Plasmopara viticola*)

O míldio é uma doença outrora presente somente no continente americano, mais precisamente no nordeste dos Estados Unidos das Américas e sudeste do Canada, região marcada pelo clima úmido e favorável ao desenvolvimento do patógeno. Foi introduzido no continente europeu através da entrada de mudas de videira, provenientes do centro de diversidade americano, sem passar por uma adequada barreira fitossanitária. A atualmente causa severas perdas na viticultura mundial (GOBBIN et al., 2006). É causado pelo oomiceto *Plasmopara viticola* (Berk. & Curt.), o qual é um parasita biotrófico obrigatório, que germina seus esporângios na face abaxial da folha e penetra no hospedeiro através dos estômatos. A interação molecular entre os organismos resulta na morte dos tecidos, causando perda de produtividade (AZIZ et al., 2006), além de alterar a expressão gênica induzindo a produção de substancias do metabolismo secundário em alguns genótipos (POLESANI et al., 2008).

Para ocorrer a germinação dos esporângios e penetração nos estômatos, é necessário haver condições climáticas favoráveis para o desenvolvimento do patógeno, como

temperatura entre 20 °C e 25 °C, elevada umidade relativa do ar e presença de filme de água livre na superfície dos tecidos.

O míldio reduz a produtividade do vinhedo, assim como deprecia a qualidade das bagas, pois, quando incide nas folhas, causa necrose foliar irreversível, seguida de desfolhamento, levando à queda na produtividade devido à redução da área fotossintetizante e produção de carboidratos. Quando incidente no cacho, compromete o uso das bagas para vinificação, prejudicando o vinhedo e em alguns casos inviabilizando o cultivo em determinadas regiões (OLIVEIRA et al., 2005).

Devido aos danos causados por esta doença, no último século, programas de melhoramento genético da videira, tem adotado estratégias de cruzamentos para desenvolver e selecionar genótipos com resistência a patógenos (BROWN et al., 1999).

1.3 MELHORAMENTO GENÉTICO DA VIDEIRA

O melhoramento genético vegetal baseia-se na seleção de plantas oriundas de cruzamentos realizados artificialmente entre indivíduos que possuem características de interesse distintas para formar novos indivíduos que compartilhem as características desejáveis de seus progenitores. As variedades oriundas do centro de diversidade europeu (*V. vinifera*) possuem melhor qualidade de uva para vinificação, contudo, apresentam elevada suscetibilidade a doenças e outros estresses bióticos ou abióticos. Por outro lado, espécies oriundas do centro de diversidade americano, ou asiático, que estão no *pool* gênico secundário, são fontes de resistência a doenças, como por exemplo o míldio. Contudo, é característica também destas espécies, o reduzido potencial para produção de vinhos finos com qualidade.

Para desenvolver novas variedades, que conciliem qualidade de vinho com resistência durável ao míldio, é necessário o cruzamento de acessos portadores de locos de resistência (R locus) com cultivares elite. Inicialmente, a partir da segunda metade do século XIX, cultivares hibridas foram desenvolvidas entre espécies americanas e a *V. vinifera*, estes foram conhecidos como híbridos americanos, desenvolvidos principalmente nos estados de Nova York e Vermont, devido a serem o centro de origem do patógeno e centro de diversidade americano da videira. Posteriormente, estes híbridos foram desenvolvidos por inúmeros melhoristas, principalmente franceses, dentre os quais destaca-se os híbridos desenvolvidos por Seibel, estes foram então denominados de híbridos franceses, ambos apresentam baixa qualidade enológica, devido à natureza selvagem das espécies americanas, doadoras dos genes

de resistência. A produção de híbridos foi abandonada como forma de combater as doenças a partir do da descoberta de moléculas químicas para este fim, permitindo o cultivo de cultivares *V. vinifera* sucetiveis mesmo com a presença dos patógenos. No entanto, no inicio da segunda metade do século XX, melhoristas alemães e húngaros se dedicaram a novas gerações de cruzamentos com estes materiais, gerando assim, cultivares que atualmente possuem elevada resistência a doenças, compartilhando elevado percentual do genoma de *V. vinifera*, devido ao elevado numero de retrocruzamentos realizados (TÖPFER et al., 2011).

Para inserir os R-locos em cultivares elite, são necessários diversos ciclos de retrocruzamentos modificados visando restaurar o genoma de *V. vinifera* na progênie. Por meio de sucessivos retrocruzamentos modificados efetuados entre os híbridos resistentes a doenças e a *V. vinifera* foram gerados cultivares modernos, combinando elevada qualidade enológica com resistência a doenças. Estes cultivares são conhecidos como PIWI (da palavra alemã *Pilzwiderstandsfähig*, que, em tradução livre, significa cultivares resistentes a doenças) (PEDNEAULT; PROVOST, 2016a; SIVČEV; SIVČEV; RANKOVIĆ VASIĆ, 2010).

Atualmente, os programas de melhoramento da videira estão focados na piramidação assistida por marcadores moleculares de R-locos (EIBACH et al., 2007a; MIGICOVSKY et al., 2016). A tendência da migração de cultivares tradicionais para PIWI também se deve a busca por produtos mais saudáveis, com menor impacto ambiental e econômico, bem como, redução de exposição do produtor e consumidor aos riscos de resíduos de agrotóxicos (GADOURY et al., 2012a; KAPUSTA; CEBULAK; OSZMIAŃSKI, 2017; MONTAIGNE; COELHO; KHEFIFI, 2016; NARDUZZI; STANSTRUP; MATTIVI, 2015a; PERTOT et al., 2017a).

1.4 SELEÇÃO ASSISTIDA POR MARCADORES MOLECULARES

A seleção de genótipos contendo genes piramidados somente é possível devido a estudos anteriores aplicados para o mapeamento de QTLs que permitem a localização genômica de distintas fontes de resistência. A localização de locos microssatélites (*Simple Sequence Repeat* – SSR) flanqueando QTLs de resistência, permite o uso destes na seleção assistida por marcadores moleculares. Para selecionar genótipos contendo genes piramidados, é necessário a genotipagem dos indivíduos com estes marcadores. Assim, a presença de alelos ligados e flaqueando locos de resistência torna possível a identificação de indivíduos com a presença de R-locos piramidados, evitando o efeito da interação com o ambiente para expressão dos caracteres.

Avanços na biologia molecular nas duas últimas décadas permitiram o mapeamento de R-locos, obtidos a partir do pool gênico secundário. Especialmente locos que conferem resistência ao míldio (*Plasmopara viticola*) e ao oídio (*Erysiphe necator*) foram descritos (AMRINE et al., 2015; DALBÓ et al., 2001; DIVILOV et al., 2018; FISCHER et al., 2004; OCHSSNER; HAUSMANN; TÖPFER, 2016; SCHWANDER et al., 2012; VENUTI et al., 2013; WELTER et al., 2007a). Estes estudos disponibilizaram marcadores moleculares ligados aos respectivos R-locos, oportunizando o desenvolvimento de estratégias de seleção assistida por marcadores moleculares (MAS – *Marker Assisted Selection*) para acelerar o processo de melhoramento genético da videira contra doenças (EIBACH et al., 2007a).

Dentre as fontes de resistência genética da videira para o míldio, destacam-se os acessos originários dos centros de diversidade americano e asiático (DI GASPERO et al., 2012; KOLEDA, 1975). Dentre os Rpv's oriundas do centro de diversidade americano, destacam-se o *Rpv1*, proveniente da *Muscadinia rotundifolia*, encontra-se localizado na região de 10,3 Mb do cromossomo 12 (MERDINOGLU et al., 2003) e o *Rpv3*, proveniente da *Vitis rupestris*, encontra-se localizado na região de 24 Mb no cromossomo 18 (BELLIN et al., 2009a; WELTER et al., 2007a). Este R-loco possui sete haplótipos que conferem resistência ao míldio (DI GASPERO et al., 2012), todavia, até o presente momento foram caracterizados o *Rpv3-1*, *Rpv3-2* e *Rpv3-3* (BELLIN et al., 2009a; VEZZULLI et al., 2019b; WELTER et al., 2007a).

No centro de diversidade asiático, mesmo com genótipos já documentados até mesmo anteriores às expedições de Vavilov, e incorporados pelos vários programas de melhoramento genético (soviético, austríaco, húngaro e alemão), somente nos últimos anos tem sido explorado em estudos que mapearam alguns R-locos a partir de *V. amurensis*, dentre estes destacam-se o *Rpv10* que está localizado na região de 3,7 Mb do cromossomo 09 (SCHWANDER et al., 2012) e o *Rpv12* que está localizado na região de 9,5 Mb do cromossomo 14 (VENUTI et al., 2013) conforme descrito na Tabela 1.

Em videira, algumas cepas desenvolveram mecanismos e reconhecimento e sinalização bioquímica capaz de identificar e promover a inibição da atividade dos patógenos, conferindo resistência a estes genótipos. Este fenômeno explica a existência de R-locos nas espécies do centro de diversidade americanao. Contudo, em espécies do centro de diversidade asiático também são encontrados R-locos a este patógeno (JÜRGES et al., 2009; SCHWANDER et al., 2012; STAUDT; KASSEMEYER, 1995; VENUTI et al., 2013).

Tabela 1. Locos *Rpv's* mapeados conhecidos até o momento, posição no cromossomo, marcadores associados e genitores da população utilizada para mapeamento, em sublinhado o genitor doador do *Rpv*. Dados adaptados de vivc.de (MAUL et al., 2020).

R-loco	Cromossomo	Posição (mb)	Marcador associado	Parentais	Espécie doadora	Referência
RPV1	12	10,3	VVIb32	Syrah x 28-8-78	*M. rotundifolia*	(MERDINOGLU et al., 2003)
RPV2	18			Cabernet Sauvignon x 8624	*M. rotundifolia*	(WIEDEMANN-MERDINOGLU et al., 2006)
RPV3	18		UDV112	Regent x Lemberger		(WELTER et al., 2007a)
		24,9 26,9	UDV305 VMC7f	Chardonnay x Bianca		(BELLIN et al., 2009a)
		26,9	VMC7f2	Regent x RedGlobe		(VAN HEERDEN et al., 2014)
RPV3.1		24,9 26,1	UDV305 UDV737	Seibel 4614	*V. rupestris*	(DI GASPERO et al., 2012)
		25,9 26,9	GF18-06 GF18-08	GF.GA-47-42 x Villard blanc	*V. rupestris*	(ZYPRIAN et al., 2016)
RPV3.2		24,9 26,1	UDV305 UDV737	Munson (Jaeger 70)	*V. rupestris* ou *V. lincecumii*	(DI GASPERO et al., 2012)
		25,9 26,9	GF18-06 GF18-08	GF.GA-47-42 x Villard blanc	*V. rupestris* ou *V. lincecumii*	(ZYPRIAN et al., 2016)
RPV3.3		24,9 26,1	UDV305 UDV737	Noah	*V. labrusca* ou *V. riparia*	(DI GASPERO et al., 2012)
		23,4 26,1	VVIN16 UDV737	Merzling x Teroldego	*V. labrusca* ou *V. riparia*	(VEZZULLI et al., 2019b)
RPV4	4	4,7 5,2	VMC7h3 VMCNg2e1	Regent x Lemberger		(WELTER et al., 2007a)
RPV5	9	4,0	VVIo52b	Cabernet Sauvignon x Gloire de Montpellier	*V. riparia*	(MARGUERIT et al., 2009)
RPV6	12	20,4	VMC8g9	Cabernet Sauvignon x Gloire de Montpellier	*V. riparia*	(MARGUERIT et al., 2009)
RPV7	7	11,4	UDV097	Chardonnay x Bianca		(BELLIN et al., 2009a)
RPV8	14	6,6	Chr14V015	*V. amurensis* Ruprecht x *V. amurensis* Ruprecht	*V. amurensis*	(BLASI et al., 2011)
RPV9	7	16,6	CCoAOMT	Moscato Bianco x *V. riparia* W63	*V. riparia*	(MOREIRA et al., 2011)
RPV10	9	3,7	GF09-46	GF.GA-52-42 x Solaris	*V. amurensis*	(SCHWANDER et al., 2012)

RPV11	5	4,5	VVMD27	Regent x Lemberger		(FISCHER et al., 2004)
			CS1E104J1 1F	Chardonnay x Bianca		(BELLIN et al., 2009a)
		4,1	VCHR05C	GF.GA-52-42 x Solaris		(SCHWANDER et al., 2012)
RPV12	14	8,0	UDV014	99-1-48 x Pinot noir	*V. amurensis*	(VENUTI et al., 2013)
		9,3	UDV304	Cabernet Sauvignan x 20/3	*V. amurensis*	
			rgvvin180			
		10,1	UDV370			
RPV13	12	10,0	VMC1g3.2	Moscato Bianco x *V. riparia* W63	*V. riparia*	(MOREIRA et al., 2011)
RPV14	5	20,2	GF05-13	GF.V3125 x Börner	*V. cinerea*	(OCHSSNER; HAUSMANN; TÖPFER, 2016)
RPV15	18			*V. piasezkii* (DVIT2027) x F2-35	*V. piasezkii*	(BOCIANOWSKI, 2013)
RPV16						Pap et al. *em preparo*
RPV17	8	11,7		*V. rupestris* B38 x Horizon		(DIVILOV et al., 2018)
RPV18	11	15,4		*V. rupestris* B38 x Horizon		(DIVILOV et al., 2018)
RPV19	14	29,5		*V. rupestris* B38 x Horizon	*V. rupestris*	(DIVILOV et al., 2018)
RPV20	6	0,9		Horizon x *V. cinerea* B9		(DIVILOV et al., 2018)
RPV21	7	2,1		Horizon x *V. cinerea* B9		(DIVILOV et al., 2018)
RPV22						Jiang et al. *em preparo*
RPV23						Jiang et al. *em preparo*
RPV24						Jiang et al. *em preparo*
RPV25	15	3,0	Marker561 375	Red Globe x Shuangyou	*V. amurensis*	(LIN et al., 2019)
		3,9	Marker549 779			
RPV26	15	14,7	Marker525 926	Red Globe x Shuangyou	*V. amurensis*	(LIN et al., 2019)
		15,0	Marker526 446			
RPV27	18	24,6	VVCS1H0 77H16R1	Norton x Cabernet Sauvignon	*V. aestivalis*	(SAPKOTA et al., 2019)
		26,0	UDV737			
RPV28						Bhattarai et al. *em preparo*

De acordo com a literatura, espécies do centro de diversidade asiático apresentam, nos R-locos, presença do domínio *coiled-coil* (CC) no N-terminal dos genes de reconhecimento patogênico NB-LRR, enquanto espécies americanas possuem o domínio *toll/interleukin-1 receptor-like* (TIR) no N-terminal destes genes. Assim, a resistência nas espécies asiáticas é mais conservada do ponto de vista evolutivo e adquirido provavelmente pela convivência com patógenos como *P. cissii* e *P. amurensis* (CANNON et al., 2002; DICK, 2002; VENUTI et al., 2013). Dos centros de diversidade da videira, somente o europeu não apresentou pressão de patógeno ao longo do ciclo de seleção de seu germoplasma, portando, as variedades européias (*V. vinifera*), produtoras de vinhos finos, necessitam de estratégias de cruzamentos para incorporar estes genes de resistência em seu pool gênico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Selecionar genótipos de videira PIWI com locos de resistência ao míldio em diferentes níveis de piramidação, conhecer a dinâmica genética populacional do míldio (*Plasmopara viticola*) nos vinhedos catarinense, bem como, descrever o desenvolvimento fenológico de genótipos PIWI nas condições climáticas de Santa Catarina.

2.2 OBJETIVOS ESPECÍFICOS

a) Avaliar populações segregantes para os R-locos *Rpv1*, *Rpv3* (alelos *3.1* e *3.3*) e *Rpv10*, que conferem resistência ao *P. viticola*, bem como, conhecer as interações genicas entre estes para o efeito de resistência a esta doença;

b) Caracterizar a diversidade genética e estrutura genética de populações de *Plasmopara viticola* no estado de Santa Catarina, Brasil e avaliar o efeito do ambiente e de diferentes locos de resistência sobre estes indicadores;

c) Descrever o comportamento fenológico e o plastocrono de genótipos contendo diferentes alelos de resistência ao míldio e oídio nas condições de cultivo na região do Vale da Uva Goethe e no planalto central de Santa Catarina;

d) Desenvolver, testar e validar modelos lineares para obtenção da área foliar em cultivares de videira PIWI implantadas em condições climáticas catarinenses.

e) Caracterizar novas fontes de resistência genética a doenças a partir de híbridos cultivados no Brasil.

3 CAPÍTULO I – PIRAMIDAÇÃO ASSISTIDA POR MARCADORES MOLECULARES DE R-LOCOS CONFERINDO RESISTÊNCIA AO MÍLDIO (*Plasmopara viticola*) EM VIDEIRA (*Vitis vinifera*)

Este capítulo encontra-se formatado para posterior submissão à publicação em forma de artigo científico em periódico especializado em genética vegetal

Piramidação assistida por marcadores moleculares de R-locos conferindo resistência ao míldio (*Plasmopara viticola*) em videira (*Vitis vinifera*)

TOMAZETTI, T.C. et al. Piramidação assistida por marcadores moleculares de R-locos conferindo resistência ao míldio (*Plasmopara viticola*) em videira (*Vitis vinifera*)

3.1 RESUMO

O objetivo com este estudo foi avaliar uma população segregantes para os R-locos *Rpv1*, *Rpv3* (haplótipos *3.1* e *3.3*) e *Rpv10*, que conferem resistência ao *P. viticola*, bem como, conhecer as interações gênicas entre estes para o efeito de resistência quando piramidados. A população foi gerada a partir do cruzamento entre o parental feminino Gf.2004.043.0015, possuindo os R-locos *Rpv1* e *Rpv3.1* e o parental masculino Bronner, possuindo os R-locos *Rpv3.3* e *Rpv10*, todos em segregação independente e em heterozigose. Os 105 indivíduos gerados foram genotipados para os R-locos presentes nos genitores, utilizando os marcadores SSR's *Sc34_8* e *Sc35_2, GF18-08* e *GF18-06,* e *GF-09-46* e *GF-09-48,* flanqueando os R-locos *Rpv1*, *Rpv3* e *Rpv10*, respectivamente. A avaliação da resistência foi realizada *in vitro* utilizando discos foliares coletados da terceira à quinta folha a partir do ápice, mantidos em placas de Petri com base de papel filtro umedecido. O isolado utilizado para a inoculação foi coletado a partir de cv. Pinot nero (*V. vinifera*) e a inoculação realizada via pulverização da solução de 10^5 esporângios mL^{-1} em cada placa de Petri contendo 4 discos foliares de 14 mm. Os discos foram avaliados de acordo com a escala OIV-452-1 aos sete dias após a infecção (DPI). Com base nos marcadores moleculares foram identificados indivíduos contendo as seguintes combinações dos genótipos-locos: Os genótipos mais frequentemente observados foram *srpv*, com 27 indivíduos, *Rvp3.1* com 20 indivíduos e *Rpv3.3* com 15 indivíduos, o genótipo piramidado com maior frequência foi *Rpv3.1+Rpv10*, observado em 14 indivíduos. Os níveis de resistência observados variaram de um a nove, sendo que os indivíduos sem R-locos (*srpv)* apresentaram maior suscetibilidade (1.3 na escala OIV-452-1), seguido pelo *Rpv3.3* e *Rpv1*. Em contraposição, os maiores níveis de resistência foram observados para o genótipo *Rpv1+Rpv3.1+Rpv10*. O genótipo *Rpv10* apresentou a maior resistência considerando somente os genótipos contendo R-locos não piramidados. As *Rpv3.1+Rpv3.3*, *Rpv3.3+Rpv10*, *Rpv1+Rpv3.3+Rpv10* e *Rpv1+Rpv3.1+Rpv3.3+Rpv10* não foram observadas.

Palavras-chave: *Vitis vinifera*; seleção assistida; melhoramento genético vegetal; Resistência a doenças.

3.2 INTRODUÇÃO

Programas de melhoramento genético vegetal têm por objetivo o desenvolvimento de cultivares superiores, combinando elevada produtividade, com qualidade e resistência a patógenos (NING; LIU; WANG, 2017). Contudo, estes objetivos são em muitas situações conflitantes entre si e a demanda por maior quantidade de alimentos faz com que programas

de melhoramento genético de diversas culturas tenham tradicionalmente priorizado a seleção de genótipos com maior produtividade, em detrimento das demais características (FOLEY et al., 2011; TESTER; LANGRIDGE, 2010).

Todavia, novos paradigmas têm levado os programas de melhoramento genético de diversas culturas a adotarem a seleção de materiais resistentes a pragas e a doenças, principalmente a partir da seleção assistida por marcadores moleculares (MAS) (TESTER; LANGRIDGE, 2010). Ainda neste contexto, estudos envolvendo a avaliação do papel de outros organismos têm demonstrado a importância de considerar a interação entre plantas e outros organismos durante o processo de melhoramento genético (JACOTT; MURRAY; RIDOUT, 2017; WEI; JOUSSET, 2017).

Quando se trata de espécies frutíferas, algumas peculiaridades devem ser destacadas. Principalmente a possibilidade de fixação de genótipos via propagação assexuada, para a maior parte das frutíferas, resultando em implicações práticas para os programas de melhoramento genético nestas plantas (WANG et al., 2017). Devido a esta característica, associada com o conhecimento atual do genoma de diversas espécies frutíferas, o uso de ferramentas moleculares para auxiliar no processo de seleção de genótipos tem se tornado cada vez mais frequente (IWATA et al., 2016). Especialmente a seleção de *quantitative trait loci* (QTL's) associados com a resistência a doenças, que tem possibilitado a seleção de indivíduos que contenham múltiplos QTL's piramidados (MIEDANER, 2016).

A videira é uma frutífera propagada comercialmente de modo assexuado, através de enxertia (GROHS et al., 2017), permitindo a fixação genotípica em uma única geração. O cultivo de uva para vinho possui marcante papel cultural em diversas regiões do mundo, estando ligado principalmente a qualidade, muitas vezes em detrimento do maior rendimento produtivo (FABRES et al., 2017; PONI et al., 2018; REYNOLDS et al., 2018).

Com o elevado nível de tecnologias empregadas para o melhoramento da videira (GRIMM et al., 2019) e com o advento do sequenciamento do genoma desta planta (JAILLON et al., 2007), aliado a diversos estudos de mapeamento de QTL's de interesse agronômico (DI GASPERO; FORIA, 2015; VEZZULLI; DOLIGEZ; BELLIN, 2019c). Programas de melhoramento genético da videira tem baseado as seleções de genótipos superiores a partir da avaliação de caracteres qualitativos, como ausência de sementes, para uvas de mesa (AKKURT; TAHMAZ; VEZIROĞLU, 2019), arquitetura do cacho (ZYPRIAN

et al., 2019), bem como, a resistência a doenças, via seleção de R-locos (SAIFERT et al., 2018; SCHNEIDER et al., 2019; VEZZULLI et al., 2019a).

A resistência a doenças tem recebido especial atenção de programas de melhoramento genético da videira devido a elevada suscetibilidade genética, a ataques patogênicos, presente na maior parte das cultivares de *V. vinifera* (ARMIJO et al., 2016). Dentre estas, especial atenção tem se dado a seleção de genótipos portando resistência ao míldio (BOVE; ROSSI, 2020), causado pelo oomiceto biotrófico obrigatório *Plasmopara viticola*, responsável por elevado impacto na viticultura (TOFFOLATTI et al., 2012).

O uso de marcadores moleculares, possibilitando a MAS é de grande importância para o melhoramento genético vegetal (DAS; PATRA; BAEK, 2017). Para a videira, o uso da MAS é efetiva para a piramidação de genes associados com a resistência a doenças, ou, até mesmo alelos do mesmo loco, como os haplótipos do *Rpv3* (EIBACH et al., 2007a). Apesar da MAS não ser uma ferramenta nova em programas de melhoramento genético da videira, o longo intervalo entre gerações para esta espécie, faz com que poucos genótipos sejam relatados piramidando R-locos provenientes de diferentes fontes. Atualmente, são conhecidos 27 QTL's no genoma da videira associados com a resistência a esta doença (www.vivc.de) (MAUL ET AL., 2020).

O *Rpv1* foi o primeiro loco de resistência mapeado para a videira, herdado a partir da espécie *Muscadinia rotundifolia* (2n = 40) oriunda do centro de diversidade americano, estando localizado no cromossomo 12 do genoma da videira (MERDINOGLU et al., 2003; WIEDEMANN-MERDINOGLU et al., 2006). Esta região genética é altamente repetitiva, com elementos transponíveis representando 49,8% da sequência entre os marcadores que flanqueiam este R-loco, destaca-se também a presença de uma família de proteínas putativas do tipo TIR-NB-LRR (toll interleukin 1 receptor nucleotide-binding leucine rich repeat), responsáveis pela resposta de hipersensibilidade, associada a indução da morte celular, como defesa contra patógenos biotróficos (SWIDERSKI; BIRKER; JONES, 2009; VAN ECK; BRADEEN, 2018), dentre outras 31 proteínas potencialmente funcionais (FEECHAN et al., 2013). Este loco está ligado também ao gene subtilisin-like protease SBT5.3 (XM_010659200.1), regulador da morte celular programada, reforçando a atuação deste mecanismo de defesa para este genótipo (FIGUEIREDO et al., 2016; FIGUEIREDO; SOUSA SILVA; FIGUEIREDO, 2018).

Mecanismo celular de defesa semelhante é relatado para o *Rpv3*, onde foram anotadas regiões genicas NB-LRR junto as LRR-kinase receptor-like como ativadoras da cascata de sinais que culmina na ativação dos genes PR (pathogenesis-related), principalmente o PR-1 e PR-2, resultando na reação de hipersensibilidade, via morte celular (CASAGRANDE et al., 2011). A rota de indução da morte celular é comum em diversos mecanismos de resistência ao míldio, todavia, efetores produzidos pelo patógeno, que atuam como supressores da morte celular programada são relatados na interação planta-patógeno (GÓMEZ-ZELEDÓN; SPRING, 2018; XIANG et al., 2016), podendo resultar na superação deste mecanismo de resistência para alguns isolados.

Assim como o *Rpv1*, o *Rpv3* é também do centro de diversidade americano. Tendo sido mapeado a parir de análise de QTL's, que relatou sua localização no cromossomo 18, proveniente da espécie *V. rupestris* (WELTER et al., 2007a). O *Rpv3* possui a particularidade de portar três haplótipos conhecidos conferindo respostas distintas de resistência, diferenciando-se dos demais R-locos descritos em videira para o míldio (DI GASPERO et al., 2012; FORIA et al., 2018). O *Rpv3* foi primeiramente descrito para as cvs. Regent (VIVC 4572) (VAN HEERDEN et al., 2014; WELTER et al., 2007a) e Bianca (VIVC 1321) (BELLIN et al., 2009b), contudo, a origem genética do haplótipo Rpv3.1, presente em ambas é herdado de um ancestral em comum, o híbrido interespecífico Seibel 4614 (MAUL et al. 2020). O haplótipo Rpv3.2 foi mapeado a partir da população segregante para o cruzamento GF.GA-47-42 x 'Villard Blanc' (VIVC 13081), sendo herdado a partir do híbrido interespecífico Seyval (VIVC 11558), na linhagem parental do genótipo GF.GA-47-42 (ZYPRIAN et al., 2016). O haplótipo Rpv3.3 foi finalmente descrito a partir de uma população segregante do cruzamento entre 'Merzling' (VIVC 4251) x 'Teroldego' (VIVC 12371), derivado do grupo fundador do híbrido interespecífico Noah (VIVC 8573) (VEZZULLI et al., 2019b).

Também via análise de QTL's foi localizado, na cv. Solaris (VIVC 20340), o R-loco *Rpv10*, localizado no cromossomo 9, herdado a partir do híbrido Severnyi (VIVC 11545), proveniente da espécie do centro de diversidade asiático *V. amurensis* (SCHWANDER et al., 2012). A presença de resistência ao *P. viticola* em espécies do centro de diversidade asiático, pode ser explicado principalmente pela presença de outros oomicetos com patossistema semelhante neste ambiente, como *P. cissii* e *P. amurensis* adaptados às respectivas espécies do gênero *Vitis* deste continente (DICK, 2002).

Isto sugere que os R-locos, que fornecem resistência contra o *P. viticola*, proveniente destas espécies apresentam resistência a diversas raças do patógeno, o que é verificado para o *Rpv12*, também mapeado a partir da *V. amurensis*, que apresenta maior eficiência da resistência em comparação ao *Rpv3* (VENUTI et al., 2013). A região gênica onde se encontra o *Rpv10* é também rica em genes NB-LRR, assim como os R-locos observados a partir de espécies americanas. Entretanto, a região é também apresenta um gene anotado como um fator de transcrição responsivo ao etileno *stress-activated* e uma proteína transmembranar ankyrin-like, genes ligados a SAR (*Systemic Acquired Resistance*) e reguladores da rota de sinalização dos genes PR's ligados à rota do ácido salicílico (CAO HUI et al., 1994; LE HENANFF et al., 2009; SCHWANDER et al., 2012; SHARMA; PANDEY, 2016)

Contudo, pouco se conhece a respeito da interação entre estes R-locos quando em combinações dentro do mesmo genoma. Portanto, o objetivo com este estudo foi avaliar populações segregantes para os R-locos *Rpv1*, *Rpv3* (alelos *3.1* e *3.3*) e *Rpv10*, que conferem resistência ao *P. viticola*, bem como, conhecer as interações genicas entre estes para o efeito de resistência a esta doença.

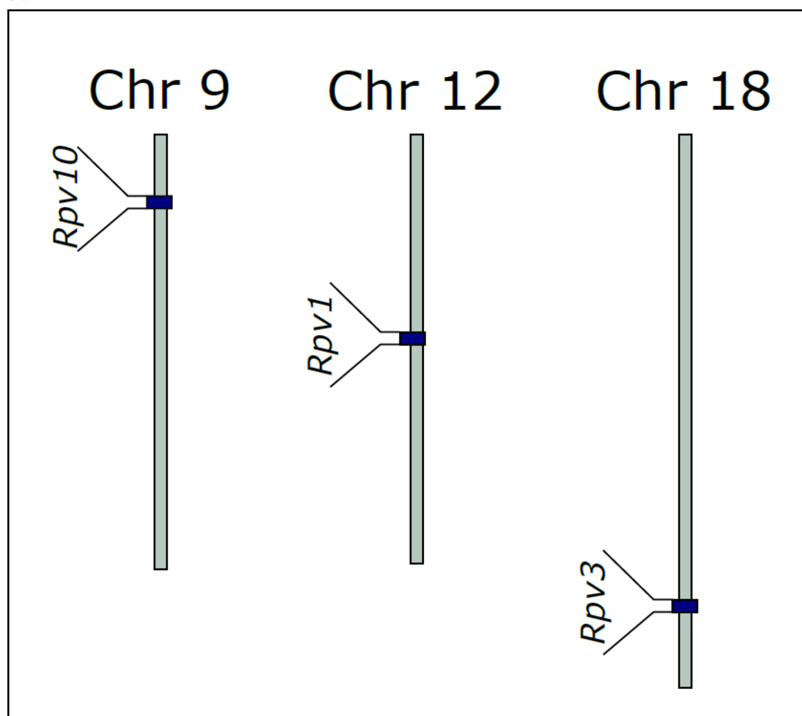
3.3 MATERIAL E MÉTODOS

3.3.1 Cruzamentos

A população segregante foi obtida a partir do cruzamento entre indivíduos contendo diferentes R-locos associados à resistência ao *P. viticola*. O parental feminino (Gf.2004.043.0015), receptor dos pólens possuí os R-locos *Rpv1* e *Rpv3.1*, enquanto o parental masculino, doador dos pólens, possui os R-locos *Rpv3.3* e *Rpv10*. Os R-locos *Rpv1*, *Rpv3* e *Rpv10* segregam independente entre si, conforme demonstrado na Figura 1, e estão em heterozigose nos parentais.

Os cruzamentos foram realizados no ciclo 2016/17, na área experimental da EPAGRI em Videira, bem como, na Área Experimental Agropecuária da UFSC, Campus de Curitibanos. A emasculação foi realizada poucos dias antes da abertura das flores, com o auxílio de uma pinça, e, posteriormente, as inflorescências foram ensacadas. Quando o estigma estava receptivo, o pólen foi aplicado aos estigmas com auxílio de um pincel e as inflorescências foram novamente ensacadas com envelope de papel manteiga, visando evitar a polinização cruzada.

Figura 1. Localização genômica dos R-loci presentes na população segregante utilizada neste estudo.



Fonte: Tomazetti, TC. (2020)

3.3.2 Preparo das sementes

A colheita foi realizada no ponto ideal de maturação das bagas, momento em que as sementes possuíam completo desenvolvimento fisiológico. Após a colheita, as bagas foram armazenadas em 4 °C até a extração das sementes que ocorreu em até 24 h. Para isto, as sementes foram retiradas e lavadas com água corrente, até a retirada completa da polpa que a envolvia. Em seguida, foram armazenadas em placas de petri com areia autoclavada, umedecida em água destilada esterilizada até o ponto de capacidade de campo (aprox. – 0.3 atm) em temperatura de 4 °C, por 2500 horas. Após este período as sementes foram lavadas em água corrente, tratadas com ácido nítrico [7%] por 20 min e postas a germinar em bandejas de 128 células, com substrato comercial em câmara úmida. O armazenamento das sementes, quebra de dormência, germinação e condução das plantas foi realizada na UFSC, Campus de Curitibanos.

3.3.3 Condução da população segregante

As plantas após formadas, apresentando em torno de cinco folhas verdadeiras, foram transplantadas para recipiente contendo 5 L, preenchido com substrato comercial e mantidas em casa de vegetação até as avaliações. Amostras foliares foram coletadas para isolamento do material genético e confirmação da herança dos alelos ligados aos locos associados com a

resistência ao *P. viticola*. Para a coleta, foi utilizado folhas jovens, dando prioridade para a primeira e segunda folha a partir do ápice de crescimento. Estas foram acondicionadas em embalagem plástica hermética, devidamente identificada, acondicionada em caixa de polipropileno expandido com blocos de gelo isolados por camadas de celulose, para manter a temperatura em aproximadamente 4 °C, até o seu transporte para o Laboratório de Fisiologia e Genética Vegetal (LFDGV), onde foi imediatamente armazenado em 20 °C até o momento do isolamento do DNA.

3.3.4 Extração de DNA

O protocolo CTAB (DOYLE; DOYLE, 1987) com algumas modificações foi empregado para o isolamento do DNA das amostras. Para isto, aproximadamente 500 mg do material vegetal fresco foi macerado com auxílio de um homogeneizador Precellys®, em 2 mL do tampão de extração CTAB (2% CTAB, 1,4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8,0, 2% PVP e 2% de β-Mercaptoetanol). O macerado foi incubado em banho maria a 60 °C por 30 minutos, com homogeneização aos 15 minutos, após esta etapa, a amostra foi centrifugada por 10 minutos 11.000 g, o sobrenadante foi aliquotado para novo microtubo [2 ml], tratado com 600 µl de CIA (Clorofórmio + álcool isoamílico [24:1]) e centrifugado por cinco minutos a 11.000 g. O sobrenadante foi transferido em novo microtubo [2 ml] e adicionado de 50 µl de CTAB [10%] e 600 µl de CIA, passando por nova centrifugação nas mesmas configurações da anterior. O sobrenadante foi isolado em novo microtubo [1,5 ml], sobre o qual foi adicionado em 2/3 do volume de álcool isopropílico gelado. A amostra foi mantida em -20 °C em *overnight* e então centrifugada a 11.000 g por 10 minutos. O precipitado foi lavado por duas vezes com 1000 µl de etanol 70% e uma vez com 1000 µl em etanol 100%. Entre as lavagens, a amostra foi centrifugada a 10.000 g por 10 minutos. Ao final da terceira lavagem, o precipitado foi seco e ressuspendido em 50 µl de tampão TE+RNAse A [10 µg ml^{-1}] (Invitrogen™), incubado por 60 minutos em 37 °C e acondicionado a 4 °C até sua quantificação via eletroforese e espectrometria.

3.3.5 Genotipagem

A eletroforese horizontal foi realizada em gel de agarose [0.8%], submerso em tampão TBE 1X e submetido a uma corrente de 90 V por 60 min. Antes do início da eletroforese, uma alíquota de 2 µL da amostra de DNA foi tratada com 3 µL de tampão de carregamento (10 mM Tris-HCl, pH 7.6; 0.03% azul de bromofenol, 0.03% xileno cianol, 60% glicerol e 60mM EDTA), contendo GelRed® [0.5%]. A revelação do gel foi realizada em transluminador (UVP© – MultiDoc-it Digital Imaging System), utilizando emissor de radiação

no comprimento ultravioleta. Adicionalmente, as amostras foram também quantificadas via espectrometria, utilizando o espectrofotômetro Nanodrop® 1000, nos comprimentos de onda de 230, 260 e 280 nm. As amostras de DNA degradadas ou com elevado grau de contaminação foram descartadas e novamente extraídas. As amostras extraídas e quantificadas foram diluídas em água destilada esterilizada para a concentração de 10 ng μL^{-1} e estas foram novamente diluídas para 1 ng μL^{-1}.

As reações de PCR foram realizadas em colaboração com a *Unità di Genetica e Miglioramento Genetico della Vite* (UGMGV) da Fondazione Edmund Mach (FEM – Trento, Itália). Foi utilizado o termociclador GeneAmp® PCR System 9700 (Applied Biosystem) e o mastermix comercial Kapa2G (Sigma-Aldrich), conforme recomendações do fabricante. O volume das reações foi de 10 µL, contendo 2 ng de DNA. Foram utilizados dois marcadores SSR's flanqueando cada um dos locos de resistência ao míldio empregados neste estudo (Bellin *et al.*, 2009; Merdinoglu *et al.*, 2003; Schwander *et al.*, 2012; Venuti *et al.*, 2013; Vezzulli *et al.*, 2019b; Welter *et al.*, 2007), conforme descrito na Tabela 1. As reações foram realizadas em multiplex, com marcadores para ambos os locos SSR's ligados ao mesmo R-loco. A programação térmica do multiplex foi desnaturação inicial de 3 minutos a 95 °C, seguido de 30 ciclos a 95 °C por 30 segundos, 60 °C por 30 segundos e 72 °C por 1 minuto, seguido de 15 minutos a 72 °C.

A amplificação foi primeiramente confirmada via eletroforese horizontal em gel de agarose [1,5%] e, posteriormente, diluídos em água destilada esterilizada na proporção de 1:1 ou 1:25, conforme o nível de concentração da fluorescência observado na eletroforese horizontal. Posteriormente, a genotipagem foi realizada via eletroforese capilar na plataforma ABI 3500 XL (Applied Biosystems), utilizando 10 µL de volume final, consistindo de 1 µL da reação da PCR diluída, 0,25 µL do marcador de tamanho molecular GeneScan® 600 LIZ (Applied Biosystems) e 8,75 µL de formamida (Applied Biosystems). O mix foi desnaturado por 5 minutos a 95 ºC e imediatamente mantido em gelo por ao menos três minutos, iniciando posteriormente o processo de eletroforese. A genotipagem foi realizada em eletroforese capilar, utilizando capilares de 50 cm, preenchidos com polímero POP7®, a detecção de fragmentos nos canais FAM, VIC e NED do analisador genético Applied Biosystems™ 3500xL. A leitura dos alelos para cada um dos locos foi realizada com o auxílio do Software GeneMapper™ v. 3.0.

Tabela 1. Marcadores microssatélites empregados na detecção dos alelos dos R-locos de resistência ao míldio (*Plasmopara viticola*) em videira.

Gene	Chr.	Loco	Sequência
Rpv1	12	*Sc34_8* F	TGGTTGCATCCCACTAAACA
		Sc34_8 R	ACTTTGGGTTCTTGGGCTTT
		Sc35_2 F	CCACACCAAAATTCCCAGAT
		Sc35_2 R	CAATCATCCATTGCCACCTT
Rpv3	18	*GF18-08* F	GACAATAGCGAGAGAGAATGGG
		GF18-08 R	AGTTGGCTAAAACCCTAGAGGC
		GF18-06 F	GGTCTCCTAGAAAGCCAAGCAA
		GF18-06 R	TCCCTTTTCCCCTTGTTCTCG
Rpv10	09	*GF-09-46* F	GAGAGATTTGAGGGATTGTTGG
		GF-09-46 R	ATCCACGTTTGTAGCCTTTTGT
		GF-09-48 F	TCTGGAAAGCACAGTAGAGAAGTG
		GF-09-48 R	ATGGAAGGAACCAATGCTAAGA

3.3.6 Fenotipagem para resistência ao míldio

No momento em que as plantas possuíam ao menos 10 folhas verdadeiras foram coletadas da terceira à quinta folha formada, a contar do ápice, para ser realizado o desafio do míldio *in vitro* empregando discos foliares. A coleta e condições de transporte das folhas foi realizado conforme descrito para a extração do material genético. As folhas foram transportadas até o LFDGV em caixa térmica, mantendo a temperatura de aproximadamente 5 °C, acomodadas em embalagens plásticas individuais com elevada umidade relativa, para assegurar a viabilidade das folhas.

Em até 24 horas após a retirada das folhas, foi realizado o desafio do patógeno nos discos folhares, conforme protocolo de Vezzulli *et al.* (2018), com algumas modificações. Primeiramente a superfície foliar foi desinfestada com hipoclorito de sódio [1%] por 2 minutos, seguido por tríplice lavagem em água destilada esterilizada, por 1 minuto em cada etapa da lavagem. Em seguida a superfície foliar foi seca em papel toalha e imediatamente os discos foliares foram excisados com auxílio de um furador de rolha de corte liso, com 14 mm de diâmetro interno. Em seguida os discos foram depositados com a face abaxial voltada para cima em placas de Petri previamente preparadas; foram acondicionados quatro discos em cada placa, sendo considerado cada placa como uma repetição e os discos como replicatas.

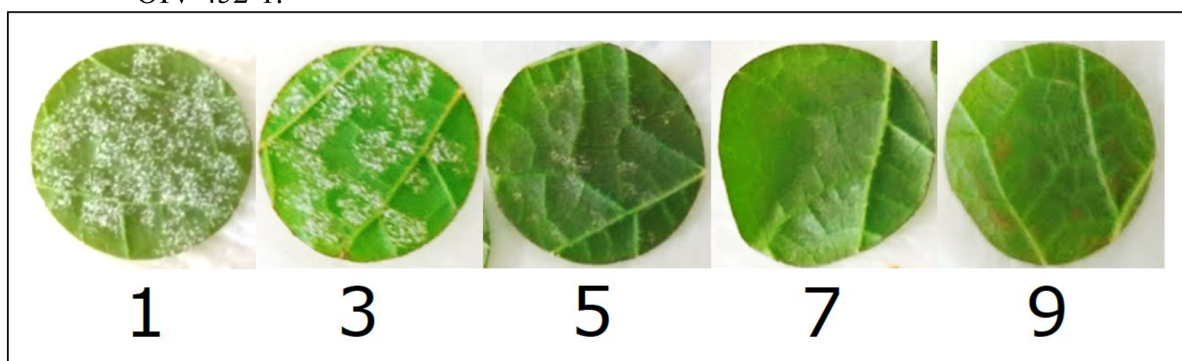
Foram utilizadas placas de Petri de vidro com 15 cm de diâmetro, que foram previamente esterilizadas em autoclave por 20 minutos a 121 °C, juntamente ao papel filtro e a água destilada. O papel filtro foi previamente cortado em discos de 14.5 cm de diâmetro. Após a autoclavagem, foram dispostos quatro discos de papel filtro em cada placa de Petri e

adicionado 6 ml de água destilada esterilizada. As placas foram então vedadas até o momento do uso como base para os discos foliares para o desafio com o *P. viticola*.

O inóculo do patógeno foi coletado a partir de esporulações frescas do patógeno em área de vinhedo com plantas da cv. Pinnot nero (*V. vinifera*), em Nova Trento-SC. Após a coleta os isolados foram transportados em temperatura de 4 °C para o LFDGV. A coleta dos esporângios foi realizada utilizando água destilada esterilizada (ADE) gelada (4 °C). A solução obtida foi quantificada utilizando câmara de contagem de células e ajustada para 10^5 esporângios mL^{-1} utilizando ADE para diluição. A inoculação do patógeno foi realizada a partir da pulverização de 100 µL desta solução em cada placa de Petri (25 µL $disco^{-1}$).

As placas foram então depositadas na ausência de luminosidade por 24 h a 22 °C. Em seguida as gotículas resultantes da inoculação foram removidas dos discos foliares com auxílio de papel filtro esterilizado. A partir deste momento as placas foram mantidas em B.O.D com temperatura de 22 °C em fotoperíodo de 16 h de luz. As avaliações foram realizadas em 7 dias após a inoculação (DPI). As avaliações realizadas foram visuais, utilizando a escala OIV 452-1, com valores atribuídos a resistência variando de 1 (resistência muito baixa ou inexistente) a 9 (resistência muito alta), com notas intermediárias de 3 (baixa resistência), 5 (resistência média) e 7 (elevada resistência), conforme ilustrado na Figura 2.

Figura 2. Escala de notas atribuídas a resistência da videira de acordo com a recomendação OIV 452-1.



Fonte: Tomazetti, TC. (2020)

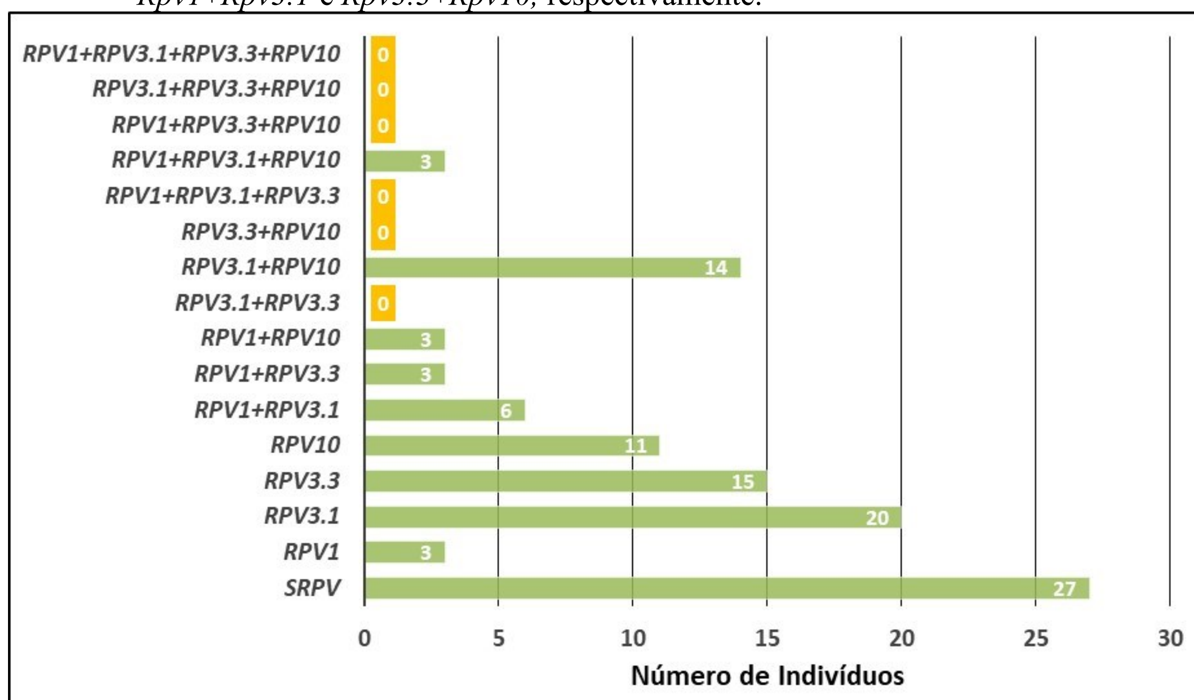
3.4 RESULTADOS

A partir do cruzamento, foram obtidas 184 sementes sadias, que após a quebra de dormência apresentaram 71,7% de germinação, gerando 132 plantulas, destas, 79,5% desenvolveram-se até a 10ª folha, permitindo o desafio com o patógeno e a genotipagem. Ao total, foram genotipados 105 indivíduos da população segregando para os R-locos *Rpv1, Rpv3* (haplótipos 1 e 3) e *Rpv10*. Destes, não foram observados indivíduos para os genótipos

Rpv3.1+Rpv3.3, *Rpv3.3+Rpv10*, *Rpv1+Rpv3.1+Rpv3.3*, *Rpv1+Rpv3.3+Rpv10, Rpv3.1+Rpv3.3+Rpv10* ou *Rpv1+ Rpv3.1+Rpv3.3+Rpv10*. Os genótipos mais frequentemente observados foram *srpv* (27 indivíduos), *Rvp3.1* (20 indivíduos) e *Rpv3.3* (15 indivíduos). Dentre os genótipos piramidados, o *Rpv3.1+Rpv10* (14 indivíduos) foi observado com maior frequência, conforme figura 3.

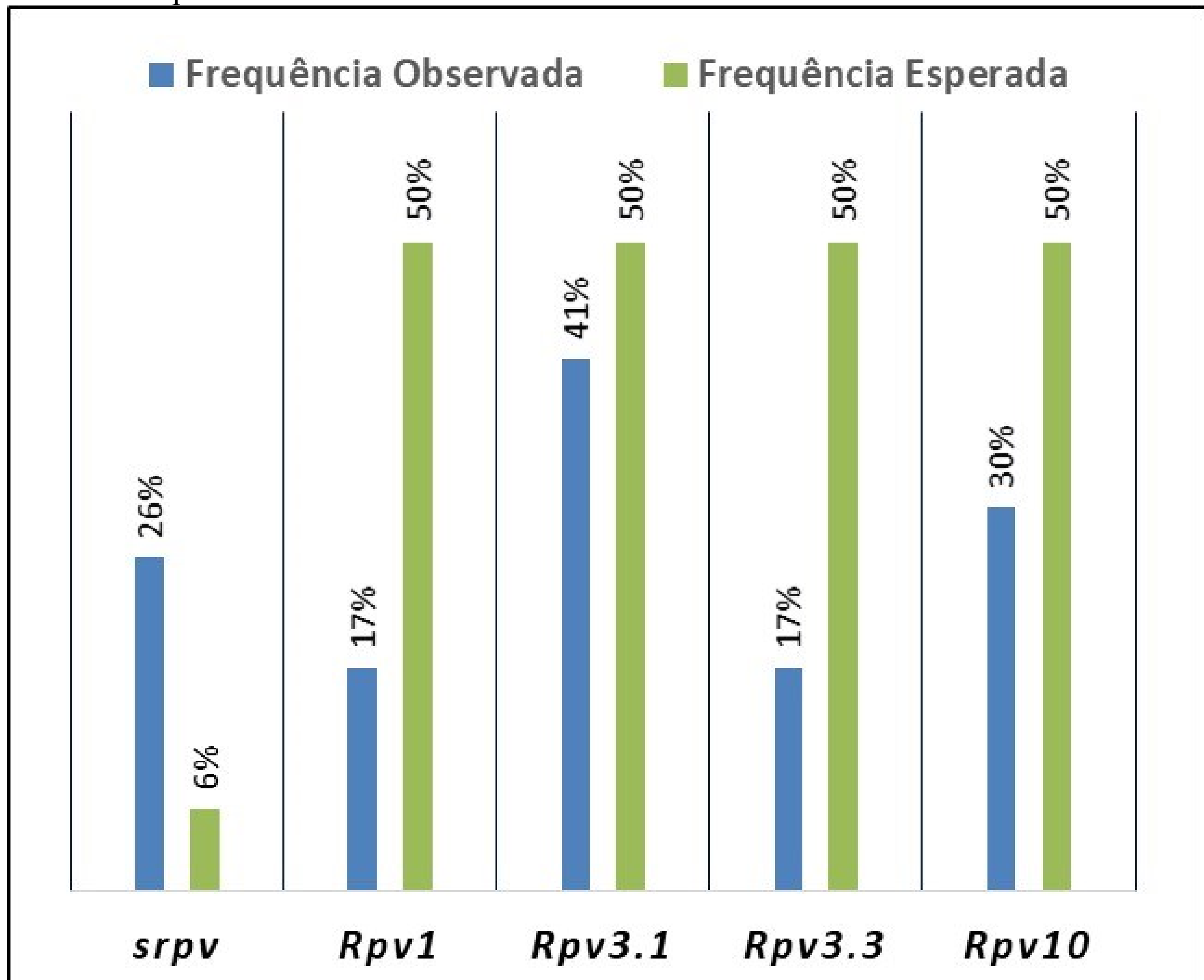
A diferença encontrada entre a distribuição genotípica esperada e a observada para os 16 genótipos possíveis de serem gerados neste estudo foram testadas utilizando o teste de aderência χ^2, demonstrando que há diferença significativa entre estas distribuições (Figura 5). A frequência de genótipos srpv observada foi superior a esperada para a segregação aleatória dos R-locos em 20 pontos percentuais (Figura 4). Todos os R-locos apresentaram frequência observada inferior a esperada. Destes, a maior frequência foi observada para o Rpv3.1, que esteve presente em 41% dos indivíduos, de forma isolada ou piramidada com outro R-loco.

Figura 3. Número de indivíduos obtidos para cada combinação de R-locos possível de populações segregantes obtidas através do cruzamento dois indivíduos contendo *Rpv1+Rpv3.1* e *Rpv3.3+Rpv10,* respectivamente.



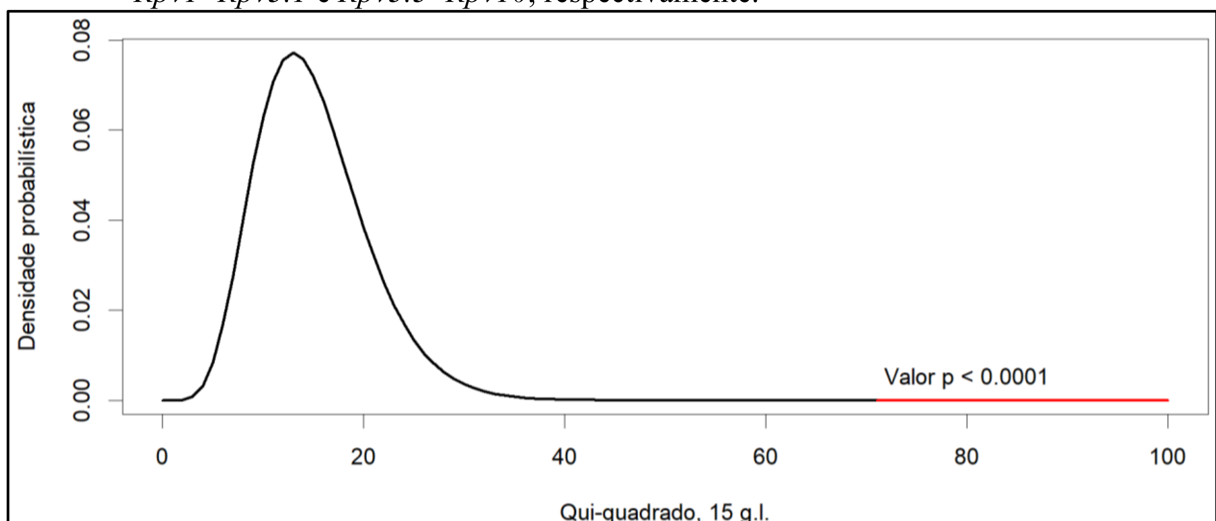
Fonte: Tomazetti, TC. (2020)

Figura 4. Frequências observada e esperada para os R-locos em população gerada a partir do cruzamento de dois indivíduos contendo *Rpv1+Rpv3.1* e *Rpv3.3+Rpv10*, respectivamente.



Fonte: Tomazetti, TC. (2020)

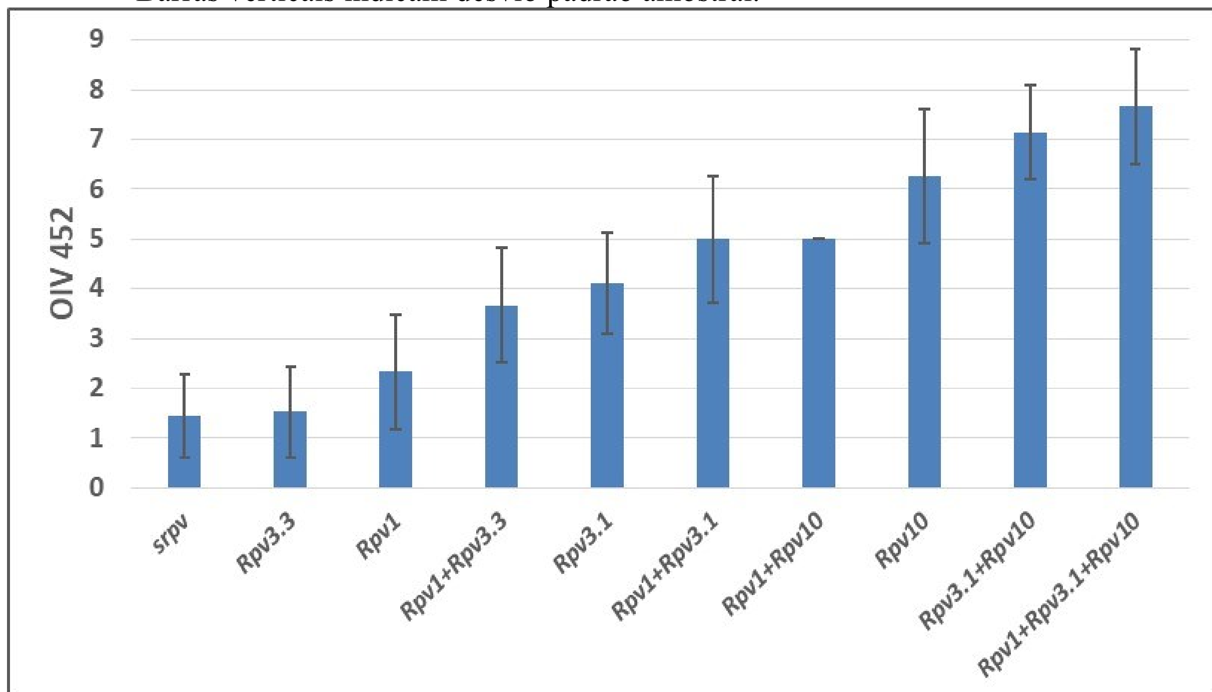
Figura 5. Distribuição χ^2 para testar a aderência das distribuições observada e esperada para os 16 genótipos esperados a partir do cruzamento de dois indivíduos de contendo *Rpv1+Rpv3.1* e *Rpv3.3+Rpv10*, respectivamente.



Fonte: Tomazetti, TC. (2020)

A resposta fenotípica ao *P. vitícola* demonstrou haver a interação gênica, com efeito aditivo, para todas as combinações de R-loci, exceto *Rpv1+Rpv10*. Quando analisado os indivíduos que contém somente os R-locos de modo isolado, menor resistência foi observada nos indivíduos contendo *Rpv3.3*, seguido pelo *Rpv1*. A piramidação entre estes dois R-locos apresentou efeito aditivo, no entanto, foi o genótipo piramidado que conferiu a menor resistência dentre os avaliados (Figura 6). Quando avaliado o efeito de resistência dos R-locos separadamente, o maior efeito foi observado em genótipos porntando o *Rpv10*, seguido do *Rpv3.1*, que conferiu resistência intermediária. As combinações de R-locos que retornaram os melhores resultados foram *Rpv3.1+Rpv10* e *Rpv1+Rpv3.1+Rpv10*, ambos com efeito aditivo. As combinações *Rpv1+Rpv3.1* e *Rpv1+Rpv10*, apresentaram resultados intermediários. De modo surpreendente, a resistência conferida pelo *Rpv10* foi reduzida quando combinada com *Rpv1*. De modo geral, *Rpv1* contribui pouco para o aumento da resistência quando piramidados.

Figura 6. Nível de resistência ao *Plasmopara viticola* obtido a partir da escala de um (ausência de resistência) a nove (resistência completa), conforme norma OIV 452. Barras verticais indicam desvio padrão amostral.



Fonte: Tomazetti, TC. (2020)

3.5 DISCUSSÃO

Dentre estes 3 R-locos utilizados neste trabalho, resultados contrastantes entre os observados e o relatado na literatura pode ser apontado para a atuação do *Rpv1*. Pois em estudo realizado com isolados de *P. viticola* europeus, genótipos portadores deste *R-loco* são

relatados como portadores de elevada resistência a este patógeno, sendo superior a *Rpv3*.1 (ZINI et al., 2019). Todavia, em trabalhos realizados a partir de isolados brasileiros, é relatado que o *Rpv1* porta baixa resistência contra o patógeno, sendo inferior a *Rpv3.1,* mesmo em genótipos homozigotos, portadores de duas cópias dente R-loco (SAIFERT et al., 2018; SÁNCHEZ-MORA et al., 2017).

A baixa frequência de indivíduos portando *Rpv1* também foi observada por (SÁNCHEZ-MORA et al., 2017). Este fato possivelmente está vinculado à origem deste R-loco, proveniente da *M. rotundifolia* (2n = 40) que possui maior distância filogenética da *V. vinifera* (2n=38) em comparação às demais espécies portadoras de R-locos, que possuem a cariótipo semelhante.

Efeitos aditivos da interação entre dois R-locos para a resistência ao *P. viticola* são relatados em alguns trabalhos (KOSEV et al., 2017; SAIFERT et al., 2018; ZINI et al., 2019). Todavia, a piramidação nem sempre resulta em ganhos genéticos, podendo em alguns casos, apresentar resposta contrária ao acréscimo da resistência genética (SAIFERT et al., 2018; ZINI et al., 2019). Este comportamento pode estar associado com o papel do *backgroud* genético, pois este possui elevada importância para a atuação dos R-locos, podendo resultar em alterações no perfil da resistência (FORIA et al., 2018). Estes resultados podem serem influenciados pela atuação de genes de suscetibilidade ao míldio, como Downy Mildew Resistant (DMR) (VAN DAMME et al., 2005) e DMR-like Oxygenases (DLO), cujo mapeamento e atuação não estão completamente elucidados, contudo podem estar envolvidos na variação da resposta de resistência dos genótipos (PIRRELLO et al., 2020).

Neste trabalho, foram avaliados os haplótipos o *Rpv3.1* e *Rpv3.3* e os resultados obtidos estão de acordo com o relatado na literatura para este gene. Neste estudo envolvendo a avaliação de 18 variedades búlgaras contendo diferentes combinações entre este e os R-locos *Rpv10* e *Rpv12*, provenientes de *V. amurensis*, foi relatado a baixa resistência genética conferida por *Rpv3*(KOSEV et al., 2017).

Para o loco *Rpv3*, o observado neste experimento, onde a resistência proveniente do haplótipo *Rpv3.1* foi superior àquela observada para o *Rpv3.3*, deve-se possivelmente ao primeiro haplótipo conferir resistência superior e melhor restrição do crescimento do patógeno, motivo também pelo qual ele tem maior presença em programas de melhoramento genético da videira (FORIA et al., 2018; ZINI et al., 2019).

As interações aditivas possuem papel importante na atuação de complexos gênicos, como os traços fenotípicos mapeados por QTL's (BOCIANOWSKI, 2013; HE et al., 2017), como exemplo dos locos de resistência a doenças em videira (Vezzulli *et al.*, 2019a), onde são relatados resultados do incremento da sinalização de proteínas associadas aos mecanismos de defesa da planta (NASCIMENTO-GAVIOLI et al., 2017). As observações deste experimento demonstram a atuação desta interação para os genótipos piramidando *Rpv1+Rpv3.3*, *Rpv1+Rpv3.1*, *Rpv3.1+Rpv10* e *Rpv1+Rpv3.1+Rpv10*, indicando que estas combinações são benéficas para o aumento da resistência genética ao míldio.

3.6 CONCLUSÕES

Efeito aditivo foi observado na maioria das interações genicas encontradas.

A piramidação de R-locos se demonstrou uma técnica efetiva para melhorar a resistência ao patógeno.

Baixo nível de resistência foi observado para os genótipos contendo srpv, *Rpv3.3* e *Rpv1*.

Os genótipos com maior nível de resistência foram os piramidados *Rpv1+Rpv3.1+Rpv10*, seguido de *Rpv3.1+Rpv10*.

Efeitos aditivos foram observados nos genótipos *Rpv1+Rpv3.3*, *Rpv1+Rpv3.1*, *Rpv3.1+Rpv10* e *Rpv1+Rpv3.1+Rpv10*.

O *Rpv10* foi o loco isolado que apresentou a maior resistência ao *P. vitocola*.

4 CAPÍTULO II - O EFEITO DO AMBIENTE E DE DIFERENTES *R-LOCOS* ASSOCIADOS À RESISTÊNCIA AO MÍLDIO SOBRE A ESTRUTURA GENÉTICA DE POPULAÇÕES DO *Plasmopara viticola*, AGENTE CAUSAL DO MÍLDIO DA VIDEIRA

Este capítulo encontra-se formatado para posterior submissão à publicação em forma de artigo científico em periódico especializado na área de genética

O efeito do ambiente e de diferentes R-locos associados à resistência ao míldio sobre a estrutura genética de populações do *Plasmopara viticola*, agente causal do míldio da videira

TOMAZETTI, T.C. et al. O efeito do ambiente e de diferentes R-locos associados à resistência ao míldio sobre a estrutura genética de populações do *Plasmopara viticola*, agente causal do míldio da videira

4.1 RESUMO

O objetivo com este trabalho foi avaliar o efeito do ambiente e de diferentes locos de resistência sobre a estrutura genética de populações de *Plasmopara viticola* no estado de Santa Catarina, Brasil. No total, foram estudadas 30 populações de *P. viticola* coletadas a partir de 11 cultivares de videira (*V. vinifera*) que foram agrupadas de acordo com os R-locos presentes (*srpv*, *Rpv3.1*, *Rpv3.1+Rpv3.2*, *Rpv3.1+Rpv3.3*, *Rpv1+Rpv3.1* e *Rpv3.3+Rpv10*), em 5 vinhedos distribuídos em diferentes regiões climáticas de Santa Catarina, Brasil (Urussanga, Videira, Curitibanos, São Joaquim e Água Doce). Em cada população foram amostrados 50 indivíduos, que foram genotipados para oito locos SSR's. A partir dos dados de genotípicos, foram estimados os índices de diversidade genética: 1) porcentagem de locos polimórficos ($\hat{P}_{99\%}$), 2) somatório do número de alelos, 3) número médio de alelos por loco ($\hat{A}$), 4) número médio de alelos por loco polimórfico ($\hat{A}_p$), 5) heterozigosidade esperada pelo equilíbrio de Hardy-Weinberg ($\hat{H}_E$) e 6) observada ($\hat{H}_O$), 7) índice de fixação ($\hat{f}$), 8) número de alelos exclusivos (*Ae*) e 9) número de alelos raros (*Ar*), além do *corrected Assignment Index* (AIc), os estimadores de fixação de Wright e o desvio de heterozigosidade. Com estes dados, foi realizada a análise multivariada, a partir da análise de componentes principais e da análise de densidade de Kernel. Os maiores índices de diversidade genética foram observados nas populações obtidas do hospedeiro suscetível (*srpv*). Dentre os genótipos contendo genes de resistência, maior diversidade genética foi encontrada para genótipos contendo *Rpv3*, principalmente para os haplótipos piramidados *Rpv3.1+Rpv3.3*. A presença de dois locos associados à resistência no mesmo genótipo reduz a diversidade genética do *P. viticola* neste hospedeiro, independentemente do vinhedo coletado. O índice F_{ST} demonstrou que a maior parte das populações dentro do mesmo vinhedo apresentaram diferenças significativas. Considerando o hospedeiro *srpv*, somente as populações *URU_Rpv3.1+Rpv3.2* e *URU_srpv* não se diferenciaram. As análises multivariadas demonstraram que as populações se agrupam em 3 *clusters* principais, com destaque para a população *URU_Rpv1+Rpv3.1* que apresentou um *cluster* isolado. Diferenças no grupamento também foram notadas para as populações a partir de hospedeiros com R-locos piramidados em comparação àquelas sem R-loco ou com somente os haplótipos do *Rpv3*. A diversidade genética em populações de *P. viticola* é influenciada pelo ambiente de cultivo e pela genética do hospedeiro.

Palavras-chave: *Vitis vinifera*; Pilzwiderstandsfähig; Diversidade genética; SSR’s; Doença da videira.

4.2 INTRODUÇÃO

O míldio da videira, doença causada pelo patógeno *Plasmopara viticola* (Berk & Curt) Berl, causa impactos econômicos expressivos e é considerado atualmente a principal causadora de danos em muitas das regiões vitivinícolas do mundo (GESSLER; PERTOT; PERAZZOLLI, 2011; TAYLOR; COOK, 2018). Sua origem está no norte do continente americano, todavia, foi disperso pelo mundo a partir do transporte de mudas entre os continentes, principalmente com a introdução de novos materiais na Europa na segunda metade do século XIX (GOBBIN et al., 2006). Atualmente, o patógeno está distribuído em todas as regiões produtoras mundiais, com elevada diversidade genética dentro das mais distintas regiões de cultivo (GOBBIN et al., 2006; PONS et al., 2018; TAYLOR et al., 2019; YIN et al., 2017; ZHANG et al., 2017).

O combate à doença nos vinhedos é realizado principalmente com a aplicação de fungicidas sintéticos (DERCKS; CREASY, 1989; GISI, 2005; GISI et al., 2007). Todavia a adoção desta prática por safras consecutivas a mais de meio século, com princípios ativos que pouco se diferenciam no mecanismo de ação, associado ao elevado polimorfismo genético do patógeno, proporciona o surgimento de raças resistentes ou tolerantes aos principais princípios ativos utilizados (FENG; BAUDOIN, 2018; FONTAINE et al., 2018; FURUYA et al., 2010).

Devido a este cenário, a melhor alternativa para conter o avanço do míldio é, associado ao uso de moléculas químicas, adotar o cultivo de variedades resistentes a esta doença (BISSON et al., 2002; ZANGHELINI et al., 2019b). Embora estudos genéticos populacionais tenham sido realizados extensivamente em ambientes de cultivo com hospedeiros suscetíveis e com a aplicação convencional de fungicidas, nenhum estudo foi realizado para conhecer o impacto da atuação de genes de resistência na estruturação genética de populações deste patógeno.

Este conhecimento é essencial para entender e desenvolver estratégias de combate da doença com maior eficácia e de efeito duradouro. Para isto, é importante a caracterização de populações do patógeno oriundas de climas favoráveis ao surgimento da doença. Estas condições climáticas são principalmente temperatura e umidade elevadas (ANGELOTTI et al., 2017). No Brasil, a principal região vitícola, situada ao sul do país, possui clima

caracterizado por verões quentes e úmidos, motivo pelo qual o míldio é a principal causa de danos na viticultura local, dificultando o cultivo de variedades suscetíveis, e necessitando do constante uso de moléculas químicas para o seu controle (LAMICHHANE et al., 2018).

Neste contexto, o objetivo com este trabalho foi avaliar o efeito do ambiente e de diferentes locos de resistência sobre a estrutura genética de populações de *Plasmopara viticola* no estado de Santa Catarina, Brasil.

4.3 MATERIAL E MÉTODOS

4.3.1 Amostragem dos isolados

Lesões de *P. viticola* foram coletadas na safra 2017/18 em vinhedos naturalmente infectados com o patógeno, em cinco regiões do estado de Santa Catarina. Cada lesão foi considerada como um único isolado, que foi amostrado a partir de uma esporulação característica da doença, de folhas com no máximo duas lesões, para cada um dos hospedeiros. Foi evitado a coleta de lesões da mesma folha, como forma de assegurar maior aleatoriedade na amostragem dos isolados. As lesões foram coletadas com auxílio de um furador de disco com 12 mm de diâmetro interno e mantidos individualmente em microtubos de 2 mL. Após a coleta, as amostras foram armazenadas em recipientes isolantes de polipropileno expandido, junto a blocos térmicos gelados, isolados por folhas de celulose, mantendo a temperatura próximo a 4 °C, para o transporte. Nestas condições, as amostras foram transportadas ao Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal (LFDGV), onde foram mantidos em – 20 °C até o momento da extração do material genético total.

4.3.2 Populações amostradas

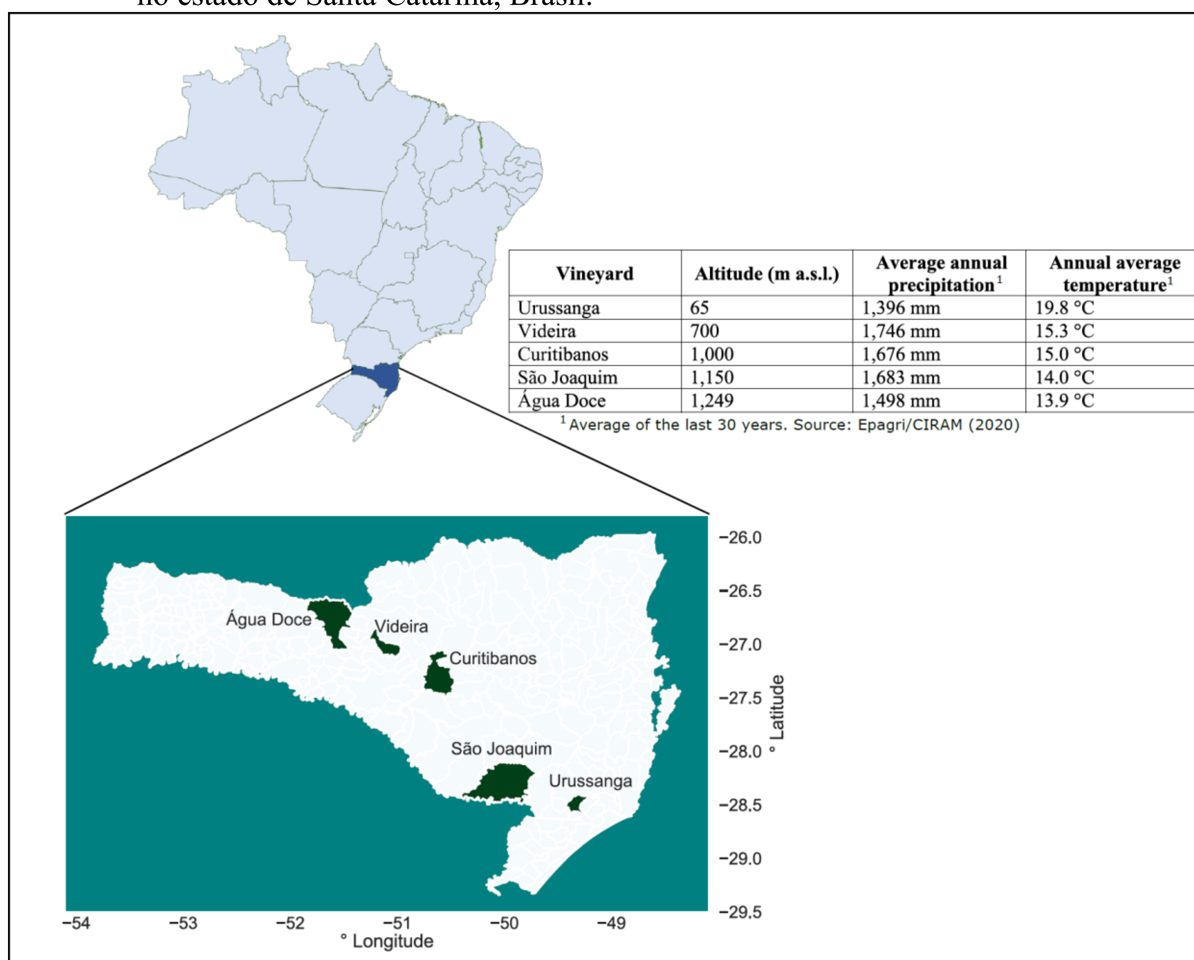
As coletas ocorreram em vinhedos experimentais localizados nos municípios de Urussanga (65 m a.s.l), Videira (700 m a.s.l), Curitibanos (1000 m a.s.l), São Joaquim (1150 m a.s.l) e Água Doce (1249 m a.s.l) (Figura 1). Os hospedeiros diferenciavam-se pelas combinações de locos de resistência ao míldio, conforme descrito na Tabela 1. Como controle suscetível foram coletadas lesões de um conjunto de cultivares *V. vinifera*, desprovidas de locos de resistência. Os hospedeiros foram agrupados pelo(s) R-loco(s) portados, como forma de diluir os efeitos do *background* genético, totalizando seis grupos de hospedeiros. Cada local x grupo de hospedeiro compôs uma população, totalizando 30 (sub)populações analisadas, nomeadas com o uma sigla contendo as 3 primeiras letras referente a localidade da coleta, seguida da informação do R-locos no qual se enquadra o respectivo hospedeiro.

Tabela 1. Cultivares hospedeiras que compõem as seis populações amostradas de *Plasmopara viticola* coletadas em cinco regiões do estado de Santa Catarina, Brasil.

R-locos	Cultivar
1. srpv	*Vitis vinifera* suscetíveis ao patógeno
2. Rpv3.1	Aromera, Calandro, Helios e Regent
3. Rpv3.1+Rpv3.2	Calardis Blanc
4. Rpv3.1+Rpv3.3	Felicia e Prior
5. Rpv1+Rpv3.1	Gf.2004.043.0015 e Gf.2004.043.0024
6. Rpv3.3+Rpv10	Baron e Bronner

Fonte: Maul et al. (2020).

Figura 1. Descrição dos cinco locais de coleta das populações de míldio da videira, realizadas no estado de Santa Catarina, Brasil.



Vineyard	Altitude (m a.s.l.)	Average annual precipitation[1]	Annual average temperature[1]
Urussanga	65	1,396 mm	19.8 °C
Videira	700	1,746 mm	15.3 °C
Curitibanos	1,000	1,676 mm	15.0 °C
São Joaquim	1,150	1,683 mm	14.0 °C
Água Doce	1,249	1,498 mm	13.9 °C

[1]Average of the last 30 years. Source: Epagri/CIRAM (2020)

Fonte: Tomazetti, TC (2020)

4.3.3 Extração de DNA

A extração do DNA foi realizada utilizando o protocolo CTAB (DOYLE; DOYLE, 1987) com algumas modificações (NANNI et al., 2016). A solução CTAB foi previamente aquecida a 65 °C por 60 min em banho-maria. O tecido congelado, contendo os isolados de míldio, foi previamente imerso em nitrogênio líquido e em seguida macerado com o auxílio de pistilo e almofariz. O tecido macerado foi transferido para um microtubo de 2 mL e em

cada amostra foram adicionados 500 μL da solução de CTAB aquecido, acrescentado de 2% de β–Mercaptoetanol imediatamente anterior ao uso. As amostras foram mantidas em banho-maria a 65 °C por 45 min passando por agitação a cada 15 min. Em seguida foram adicionados 500 μL de Clorofórmio álcool isoamilico (CIA) [24:1] e a amostra foi centrifugada por 5 min a 5.900 g. O sobrenadante foi separado em novo microtubo (2 mL) e novamente lavado com 500 μL de CIA (24:1). O sobrenadante foi então separado e adicionado de 500 μL de isopropanol para precipitação dos ácidos nucleicos, sendo mantido em *overnight* a -20 °C. A formação do *pellet* foi propiciada pela centrifugação das amostras, ainda em baixa temperatura, por 10 min a 15.000 g. A fase líquida foi descartada enquanto o *pellet* foi mantido e lavado uma vez com etanol 70% e, posteriormente, com etanol 100%, sendo as lavagens intercaladas por centrifugação por 5 min a 10.000 g. Finalmente, o *pellet* foi seco em banho seco a 37 °C até a completa evaporação do etanol e logo ressuspenso em 45 μL de tampão Triz-EDTA (TE, 10 mM Triz + 1 mM EDTA) + 5 μL de RNase A (10 mg ml^{-1}, Fermentas). A amostra foi então mantida a 37 °C por 45 minutos para a digestão do RNA presente. Para confirmação da qualidade do DNA extraído, cada amostra foi quantificada em eletroforese e espectrometria.

A eletroforese foi realizada em gel de agarose [0.8%], submerso em tampão TBE 1X e submetido a uma corrente de 90 V por 60 min. Antes do início da eletroforese, uma alíquota de 2 μL da amostra de DNA foi tratada com 3 μL de tampão de carregamento (10 mM Tris-HCl, pH 7.6; 0.03% azul de bromofenol, 0.03% xileno cianol, 60% glicerol e 60mM EDTA), contendo GelRed® [0.5%]. A revelação do gel foi realizada em transluminador (UVP© – MultiDoc-it Digital Imaging System), utilizando emissor de radiação no comprimento ultravioleta. Adicionalmente, as amostras foram também quantificadas via espectrometria, utilizando o espectrofotômetro Nanodrop® 1000, nos comprimentos de onda de 230, 260 e 280 nm. As amostras de DNA degradadas ou com elevado grau de contaminação foram descartadas e novamente extraídas a partir de um novo isolado da mesma população. As amostras extraídas e quantificadas foram diluídas em água destilada esterilizada para a concentração de 10 ng μL^{-1}.

4.3.4 Genotipagem

Ao final das extrações de DNA, foram obtidos materiais genéticos de 50 isolados para cada população (local x hospedeiro), totalizando 1500 indivíduos amostrados e genotipados. As reações em cadeia da polimerase (PCR) foram realizadas testando diversas combinações de marcadores em arranjos multiplexes, buscando a melhor combinação dos SSR's para o

genoma do *P. viticola* (ROUXEL et al., 2012). Os melhores resultados de amplificação foram obtidos com a combinação em dois multiplexes de quatro SSR's (M-I e M-II). Assim, este arranjo foi adotado para as genotipagens dos indivíduos. O M-I foi composto pelos marcadores para os locos *Pv83*, *Pv88*, *Pv103* e *Pv127*, todos em concentração de 0.08 µM (0.04 µM de cada oligonucleotideo, *forward* + *reverse*). O M-II foi composto pelos marcadores para os locos *Pv139*, *Pv142*, *Pv143* e *Pv147*, todos em mesma concentração que àqueles do M-I. O PCR MasterMix (QuatroG© Pesquisa & Desenvolvimento) foi utilizado para as reações de amplificação em ambos os multiplexes. A ciclagem térmica para o M-I foi configurada em 95 °C por 5 minutos, seguido de 35 ciclos de 95°C por 20 segundos, 54 °C por 20 segundos e 72 °C por 20 segundos, encerrando com 72 °C por 10 minutos. A ciclagem do M-II foi configurada em *touchdown*, iniciando a 95 °C por 5 min, seguido de 10 ciclos de 95 °C por 20 segundos, 55 °C por 20 segundos, com redução de 0.5 °C a cada ciclo e 72 °C, passando para 25 ciclos de 95 °C por 20 segundos, 50 °C por 20 segundos e 72 °C por 20 segundos, finalizando com 10 minutos a 72 °C (Tabela 2).

A confirmação da amplificação dos multiplexes foi realizada em eletroforese em gel de agarose [1.5%], conforme descrito para a quantificação, com a adição de um *ladder* com concentração de bandas conhecidas, possibilitando a estimativa da concentração dos produtos da PCR.

O produto das PCRs foi diluído em água destilada esterilizada na proporção de 1:1 ou 1:25, conforme o nível de concentração da fluorescência observada na eletroforese horizontal. Após diluído, foi utilizado 1 µL da amostra que foi adicionada em 8.75 µL de formamida e 0.25 µL de marcador de peso molecular para eletroforese capilar GeneScan® LIZ. As amostras foram então desnaturadas a 95 °C por 5 min e em seguida mantidas em gelo por ao menos 3 min. A genotipagem foi realizada em eletroforese capilar, utilizando capilares de 50 cm, preenchidos com polímero POP7® com detecção de fragmentos nos canais FAM, VIC e NED do analisador genético Applied Biosystems™ 3500xL. A leitura dos alelos para cada um dos locos SSR estudados foi realizada com o auxílio do Software GeneMapper™ v. 3.0.

4.3.5 Análise da diversidade e estrutura genética

Os dados genotípicos foram utilizados para inferir os índices populacionais. Foi testado o equilíbrio de Hardy-Weinberg (EHW) para cada um dos locos estudados, bem como os índices de diversidade alélica, como o número de locos polimórficos ($\hat{P}_{99\%}$), número total de alelos, número médio de alelos por loco ($\hat{A}$), número médio de alelos por loco polimórfico

($\hat{A}_p$), heterozigosidade esperada ($\hat{H}_E$) e observada ($\hat{H}_O$), número de alelos exclusivos e número de alelos raros. Adicionalmente foram também estimados o *corrected Assignment Index* (AIc) (GOUDET; PERRIN; WASER, 2002) e os índices de fixação F (WRIGHT, 1951) como o índice de fixação ($\hat{f}$) e a diferenciação entre subpopulações F_{ST} que foi calculado par a par para populações dentro do mesmo ambiente e diferenças estatísticas foram consideradas utilizando 10.000 reamostragens com reposição (*Bootstrap*). As estatísticas foram calculadas em linguagem R (R CORE TEAM, 2019), com o auxílio do pacote Hierfstat v.0.04-22 (GOUDET; JOMBART, 2015), utilizando as equações a seguir:

O AIc para o indivíduo *j* na amostra *k*, para *l* locos e *n* indivíduos é obtido pela seguinte equação:

$$AIc_{kj} = \log\left[\prod_{i=1}^{l} P_{kij}\right] - 1/n \sum_{j=1}^{n} \log\left[\prod_{i=1}^{l} P_{kij}\right]$$

A distribuição do AIc na população deve ser centrada em 0. Valores não estatisticamente diferentes de zero ou positivos indicam indivíduos residentes, ou seja, baixa probabilidade da presença de migrantes entrando na população. Valores negativos, significativamente diferentes de zero, indicam presença significativa de migrantes na população (GOUDET; PERRIN; WASER, 2002). O teste de significância utilizado nesta análise foi o teste t de Student, realizado em linguagem R (R CORE TEAM, 2019).

Os índices de fixação de Wright foram calculados conforme segue:

$$F_{IS} = \hat{f} = 1 - \frac{H_O}{H_S}$$

$$F_{ST} = 1 - \frac{H_S}{H_E}$$

Onde:

$$H_o = \sum_k \sum_i \frac{P_{kii}}{np}$$

$$H_S = \frac{\tilde{n}}{\tilde{n} - 1} \cdot \left[1 - \sum_i \overline{P_i^2} - \frac{Ho}{2 \cdot \tilde{n}}\right]$$

$$\tilde{n} = \frac{np}{\sum_k 1/n_k}$$

$$\overline{P_i^2} = \sum_k p_{ki}^2/np$$

Em que, H_O é a heterozigosidade observada na população, H_E é a heterozigosidade esperada pelo EHW com base na frequência alélica da população completa, H_S é a heterozigosidade esperada pelo EHW considerando o efeito de subpopulações.

A análise de componentes principais (PCA), associada a uma estimativa de densidade Kernel (KDE) com 10000 reamostragens, foram utilizadas para demonstrar a distribuição das populações baseadas em seus índices de diversidade populacionais. Para isto, foram considerados o número de locos polimórficos, número total de alelos, heterozigosidade esperada e observada, número de alelos exclusivos e número de alelos raros, AIc e $\hat{f}$. Estas variáveis foram primeiramente testadas para correlação par-a-par, visando evitar covariâncias enviesando a PCA. Assim, em casos de duas variáveis com correlação de Pearson superiores a 0.80, àquela com menor correlação com as demais variáveis foi mantida para a PCA, enquanto a outra foi descartada. Como forma de padronizar as unidades de medidas, todos os valores foram transformados por estandardização, utilizando para tanto a matriz de correlação (R). O tratamento dos dados e análises foram realizadas em linguagem R (R CORE TEAM, 2019), com a utilização dos pacotes "Vegan" (OKSANEN et al., 2019) e ggplot2 (WICKHAM, 2015). As reamostragens e a construção dos KDE's foi implementada em linguagem Python 3.7 utilizando as bibliotecas matplotlib.pyplot v. 3.1.1 e seaborn v.0.9.0 (WASKOM et al., 2018).

4.4 RESULTADOS

O intervalo de alelos observados foi, para a maioria dos locos SSR's, superior a àqueles observados nas populações utilizadas para descrever estes locos (ROUXEL et al., 2012), conforme pode ser observado na Tabela 2. A exceção foi o loco *Pv127*, onde não foram observados alelos polimórficos. Este foi o único dos oito locos SSR estudados que retornou alelo fixado nas 30 populações. Além deste, alelo fixado em todos os ambientes estudados foi observado apenas para o loco *Pv142* no hospedeiro *Rpv3.3+Rpv10* (Tabela 3). Para a população *URU_Rpv1+Rpv3.1*, alelo fixado foi observado para o loco *Pv103* e *Pv142*. Para este último loco, alelo fixado foi observado também nas populações *VID_Rpv3.1+Rpv3.2*, *CUR_Rpv3.1+Rpv3.2*, *SJO_Rpv3.1,* *SJO_Rpv3.1+Rpv3.2* e *ADC_ Rpv3.1+Rpv3.2* (Tabela 4).

Tabela 2. Marcadores SSRs empregados na genotipagem das 30 populações de míldio (*Plasmopara viticola*), coletadas em cinco localidades de Santa Catarina, Brasil.

	Loco	Sequência	TA[1] (°C)	Intervalo[2]		Fluorocromo[3]
Multiplex I	Pv83_F	TGCAGCATTGTTTCATCCAT	54	233	247	NED
	Pv83_R	ACACGGTACTTTGCGTTCCT				
	Pv88_F	AATACCAAAAATGGCCGTCA		196	222	FAM
	Pv88_R	ACTCTCTTGCCAGCACCATC				
	Pv103_F	TGACCTACCACCCATTTACCA		274	288	FAM
	Pv103_R	ACGGTCAGGTCAAAAGCAGT				
	Pv127_F	TTGAAAACGCGGATAGGAAC		233	233	VIC
	Pv127_R	GAACGTCCAGTTCGGATTGT				
Multiplex II	Pv139_F	GACCCGGACAATGGACTCTA	50 / 55	123	135	FAM
	Pv139_R	CCGCCATGTATTGAACAGTG				
	Pv142_F	TTATGCCACGCAAATCTCTG		198	214	FAM
	Pv142_R	AGGGCGAAATACGAGAGTGA				
	Pv143_F	CCTGAATAAAGCAACACGCA		104	138	NED
	Pv143_R	TTGGCAGCAAATTGTACGAC				
	Pv147_F	TCGACTACGAGTCCGAGAGG		196	214	VIC
	Pv147_R	TTCTAGCTCGACGAAGACCG				

[1]TA: temperatura de anelamento do multiplex; [2]Intervalo: Faixa de tamanho dos alelos observados neste estudo; [3]Fluorocromo: marcador utilizado no primer *forward* para identificação do amplicon em eletroforese capilar.

Tabela 3. Distribuição alélica em oito locos SSR's empregados na genotipagem de populações de *Plasmopara vitícola*, amostradas em hospedeiros contendo diferentes combinações de genes associados à resistência genética ao patógeno, implantados em cinco diferentes regiões de cultivo de videiras no estado de Santa Catarina, Brasil

Alelo*	Pv83	Pv88	Pv103	Pv127	Pv139	Pv142	Pv143	Pv147
				srpv				
A	0.008	0.448	0.050	1.00	–	–	–	0.198
B	0.112	0.470	0.174	–	–	–	0.100	0.002
C	–	–	0.024	–	0.046	–	–	0.000
D	0.106	0.016	0.004	–	0.386	0.020	–	0.000
E	0.646	–	0.046	–	0.026	0.748	0.002	0.000
F	0.126	0.004	0.702	–	0.074	0.232	0.338	0.800
G	0.002	0.062	–	–	0.468	–	0.052	0.000
H	–	–	–	–	–	–	0.088	–
I	–	–	–	–	–	–	0.420	–
				RPV3.1				
A	0.024	0.276	0.066	1.00	–	–	0.002	0.160
B	0.014	0.668	0.096	–	0.002	–	0.008	0.014
C	0.006	0.026	0.022	–	0.148	0.068	0.170	0.042
D	0.214	0.008	–	–	0.224	–	0.002	0.056
E	0.482	0.006	0.016	–	0.014	0.762	0.340	0.002
F	0.260	0.002	0.786	–	0.376	0.122	0.084	0.722
G	–	0.014	0.014	–	0.236	0.016	0.030	0.004
H	–	–	–	–	–	0.032	0.054	–
I	–	–	–	–	–	–	0.296	–
J	–	–	–	–	–	–	0.014	–
				Rpv3.1 + Rpv3.2				
A	0.052	0.430	0.150	1.00	0.122	–	–	0.078

B	–	0.488	0.068	–	0.002	–	–	–
C	0.004	0.012	–	–	0.208	–	0.164	0.044
D	0.314	0.014	–	–	0.134	–	0.016	0.104
E	0.534	–	0.008	–	–	0.988	0.056	0.004
F	0.096	0.004	0.774	–	0.322	0.012	0.328	0.748
G	–	0.052	–	–	0.212	–	0.152	0.022
I	–	–	–	–	–	–	0.284	–
------------------------------------ ***Rpv3.1 + Rpv3.3*** ------------------------------------								
A	0.060	0.388	0.062	1.00	0.022	–	0.002	0.188
B	0.020	0.556	0.132	–	0.002	–	0.034	0.002
C	0.006	0.008	0.022	–	0.120	0.038	0.108	0.030
D	0.254	0.004	0.006	–	0.256	–	0.004	0.038
E	0.580	0.006	0.024	–	0.026	0.852	0.194	–
F	0.080	–	0.748	–	0.298	0.098	0.180	0.740
G	–	0.038	0.006	–	0.276	0.006	0.086	0.002
H	–	–	–	–	–	0.006	0.070	–
I	–	–	–	–	–	–	0.312	–
J	–	–	–	–	–	–	0.010	–
------------------------------------ ***Rpv1 + Rpv3.1*** ------------------------------------								
A	0.014	0.320	0.054	1.00	–	0.034	–	0.132
B	0.012	0.648	0.066	–	–	0.012	–	–
C	–	–	–	–	–	–	0.074	–
D	0.170	0.024	–	–	0.106	–	0.086	–
E	0.696	–	–	–	–	0.704	0.030	–
F	0.108	–	0.880	–	0.394	0.250	0.412	0.868
G	–	0.008	–	–	0.500	–	0.012	–
I	–	–	–	–	–	–	0.386	–
------------------------------------ ***Rpv3.3 + Rpv10*** ------------------------------------								
A	0.062	0.356	0.142	1.00	0.130	–	–	0.096
B	0.054	0.512	0.130	–	–	–	–	–
C	–	0.030	0.006	–	0.214	–	0.120	0.060
D	0.238	–	–	–	0.092	–	0.046	0.144
E	0.532	0.016	–	–	–	1.00	0.052	0.018
F	0.114	–	0.722	–	0.394	–	0.336	0.664
G	–	0.086	–	–	0.170	–	0.138	0.018
I	–	–	–	–	–	–	0.308	–

*Dados consolidados das cinco unidades avaliadas, Urussanga, Videira, Curitibanos, São Joaquim e Água Doce.

Nos locos que apresentaram polimorfismo, a heterozigosidade observada variou entre 0.02 (na população *VID_Rpv1+Rpv3.1* no loco *Pv139*) a 1.00 (nas populações *URU_Rpv3.3+Rpv10*, *VID_Rpv3.1* e *ADC_Rpv3.3+Rpv10* no loco Pv139, na população *CUR_Rpv3.1+Rpv3.2* no loco *Pv88*, bem como, nas populações *SJO_Rpv1+Rpv3.1* e *ADC_Rpv1+Rpv3.1* no loco *Pv143*). A heterozigosidade esperada variou entre 0.04 (na população *URU_Rpv1+R3.1* no loco *Pv83*, *URU_Rpv3.3+Rpv10* no loco *Pv147* e na população *ADC_Rpv3.1+Rpv3.3* para o loco *Pv142*) a 0,82 (na população *VID_Rpv3.1+Rpv3.3* no loco *Pv143*), conforme pode ser observado na Tabela 4.

Não foi encontrado EHW para todos os locos em nenhuma das populações estudadas (Tabela 4). Considerando somente os locos polimórficos e isoladamente os locais, o equilíbrio foi observado em todos os hospedeiros nos locos *Pv103* e *Pv147* em URU, e no loco *Pv103* em CUR. Considerando isoladamente cada genótipo hospedeiro, o equilíbrio foi verificado na população *Rpv1+Rpv3.1* em todos os vinhedos para o loco *Pv147* e para o loco *Pv103* na população *Rpv3.1* (Tabela 4).

Tabela 4. Frequência de heterozigosidade observada e esperada para cada loco SSR nas 30 populações estudadas no estado de Santa Catarina, Brasil.

Loco	**srpv**	**RPV3.1**	**Rpv3.1+ Rpv3.2**	**Rpv3.1+ Rpv3.3**	**Rpv1+ Rpv3.1**	**Rpv3.3+ Rpv10**
			Urussanga			
Pv83	0.44/0.42**	0.42/0.33^{NS}	0.62/0.49^{NS}	0.54/0.44**	0.04/0.04^{NS}	0.74/0.59**
Pv88	0.90/0.54**	0.88/0.49**	0.96/0.57**	0.94/0.50**	0.24/0.21^{NS}	0.94/0.58**
Pv103	0.42/0.37^{NS}	0.40/0.39^{NS}	0.44/0.35^{NS}	0.38/0.39*	0.00/0.00§	0.38/0.32^{NS}
Pv127	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§
Pv139	0.92/0.62**	0.94/0.67**	0.90/0.68**	0.98/0.72**	0.94/0.52**	1.00/0.65**
Pv142	0.00/0.39**	0.00/0.47**	0.04/0.11**	0.00/0.37**	0.00/0.00§	0.00/0.00§
Pv143	0.74/0.72**	0.66/0.64**	0.66/0.68**	0.80/0.68**	0.48/0.67**	0.36/0.78**
Pv147	0.32/0.27^{NS}	0.36/0.30^{NS}	0.10/0.10^{NS}	0.40/0.32^{NS}	0.22/0.20^{NS}	0.04/0.04^{NS}
			Videira			
Pv83	0.50/0.47**	0.88/0.63**	0.66/0.55^{NS}	0.66/0.60^{NS}	0.28/0.24^{NS}	0.52/0.56^{NS}
Pv88	0.92/0.56**	0.34/0.28^{NS}	0.90/0.56**	0.64/0.44**	0.50/0.38*	0.98/0.57**
Pv103	0.38/0.36^{NS}	0.08/0.08^{NS}	0.32/0.31**	0.26/0.24**	0.08/0.08^{NS}	0.50/0.38*
Pv127	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§
Pv139	0.90/0.61**	1.00/0.71**	0.92/0.67**	0.94/0.75**	0.02/0.35**	0.92/0.64**
Pv142	0.00/0.35**	0.06/0.60**	0.00/0.00§	0.06/0.41**	0.14/0.53**	0.00/0.00§
Pv143	0.76/0.71**	0.66/0.69**	0.72/0.70**	0.68/0.82**	0.94/0.56**	0.70/0.77**
Pv147	0.46/0.36*	0.46/0.38^{NS}	0.28/0.24^{NS}	0.42/0.34^{NS}	0.30/0.26^{NS}	0.48/0.37*
			Curitibanos			
Pv83	0.42/0.42**	0.28/0.42**	0.54/0.42^{NS}	0.42/0.52**	0.48/0.37*	0.64/0.61**
Pv88	0.94/0.56**	0.22/0.20^{NS}	1.00/0.50**	0.88/0.53**	0.88/0.49**	0.60/0.53**
Pv103	0.44/0.41^{NS}	0.34/0.30^{NS}	0.36/0.31^{NS}	0.34/0.30^{NS}	0.10/0.10^{NS}	0.46/0.38^{NS}
Pv127	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§
Pv139	0.90/0.66**	0.44/0.64**	0.98/0.66**	0.90/0.76**	0.04/0.33**	0.90/0.68**
Pv142	0.00/0.35**	0.44/0.55**	0.00/0.00§	0.08/0.33**	0.12/0.53**	0.00/0.00§
Pv143	0.80/0.67**	0.56/0.43^{NS}	0.52/0.68**	0.72/0.75**	0.96/0.57**	0.74/0.69**
Pv147	0.50/0.38*	0.40/0.35^{NS}	0.42/0.40^{NS}	0.40/0.38^{NS}	0.24/0.21^{NS}	0.46/0.53**
			San Joaquim			
Pv83	0.50/0.48**	0.64/0.46*	0.62/0.49^{NS}	0.66/0.50^{NS}	0.66/0.44**	0.44/0.41^{NS}
Pv88	0.94/0.54**	0.64/0.59**	0.98/0.56**	0.74/0.53**	0.90/0.52**	0.88/0.66**
Pv103	0.46/0.40^{NS}	0.40/0.35^{NS}	0.26/0.27**	0.38/0.33^{NS}	0.24/0.22^{NS}	0.56/0.42^{NS}
Pv127	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§
Pv139	0.90/0.66**	0.88/0.72^{NS}	0.92/0.72**	0.82/0.71^{NS}	0.06/0.54**	0.80/0.63**
Pv142	0.00/0.35**	0.00/0.00§	0.00/0.00§	0.00/0.08**	0.20/0.51**	0.00/0.00§
Pv143	0.76/0.68**	0.30/0.33**	0.48/0.61**	0.32/0.56**	1.00/0.60**	0.76/0.63^{NS}
Pv147	0.38/0.31^{NS}	0.52/0.56**	0.64/0.58^{NS}	0.54/0.49^{NS}	0.26/0.23^{NS}	0.92/0.70**
			Água Doce			
Pv83	0.84/0.69^{NS}	0.80/0.73^{NS}	0.90/0.65**	0.82/0.74^{NS}	0.72/0.65^{NS}	0.82/0.66^{NS}
Pv88	0.86/0.67**	0.86/0.60**	0.82/0.66**	0.82/0.64**	0.80/0.62*	0.80/0.58*
Pv103	0.84/0.72**	0.72/0.61^{NS}	0.80/0.55**	0.92/0.66**	0.78/0.55**	0.88/0.52**

Pv127	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§	0.00/0.00§
Pv139	0.92/0.57**	0.92/0.71**	0.90/0.67**	0.84/0.68^{NS}	0.20/0.49**	1.00/0.65**
Pv142	0.00/0.51**	0.00/0.08**	0.00/0.00§	0.00/0.04**	0.22/0.45**	0.00/0.00§
Pv143	0.82/0.65**	0.72/0.72^{NS}	0.64/0.58^{NS}	0.68/0.77^{NS}	1.00/0.60**	0.78/0.58*
Pv147	0.34/0.29^{NS}	0.68/0.56^{NS}	0.76/0.63**	0.70/0.46**	0.30/0.26^{NS}	0.74/0.68*

^{NS} População em EHW para determinado loco, de acordo com a distribuição de probabilidade χ^2; * frequência genotípica fora do EHW (α=0.05); ** (α=0.01); §loco não polimórfico para a população.

A presença de locos polimórficos foi constantemente superior nas populações *srpv* e *Rpv3.1+Rpv3.3* em todos os vinhedos coletados, sendo que, 87.5% dos locos foram polimórficos em todos os locais de amostragem. Nas demais populações, em ao menos um dos vinhedos, foram encontradas proporções menores de locos polimórficos (Tabela 5). A população *ADC_Rpv3.1* apresentou maior número de alelos totais (37 alelos). No mesmo vinhedo a população *ADC_srpv* apresentou 34 alelos. Nos vinhedos de *URU*, *VID*, *CUR* e *SJO* a população *Rpv3.1+Rpv3.3* apresentou número de alelos superiores àqueles observados na população *srpv*. A população *Uru_Rpv1+Rpv3.1* apresentou o menor número de alelos totais (17). Além desta, as demais populações a partir deste hospedeiro, ou as populações *Rpv3.3+Rpv10* não apresentaram maior número de alelos em comparação as populações *srpv* em nenhum dos vinhedos amostrados (Tabela 5).

Apesar de não haver EHW na maior parte das populações, para a maioria dos locos, a heterozigosidade observada e esperada são semelhantes. Contudo, a taxa de heterozigotos observados é frequentemente superior àquela esperada pela aleatoriedade para a maior parte das populações, retornando índices de fixação negativos. Esta afirmação somente não é válida para as populações *VID_Rpv1+Rpv3.1* e *CUR_Rpv3.1* (Tabela 5).

A população *ADC_srpv* apresentou maior número de alelos exclusivos (4), seguido das populações *CUR_Rpv1+Rpv3.1* e *SJO_Rpv1+Rpv3.1* ambas com 3 alelos exclusivos. O hospedeiro *Rpv1+Rpv3.1* foi o único que apresentou alelos exclusivos em todos os vinhedos. Em contraponto, às populações *Rpv3.1* e *Rpv3.3+Rpv10* não apresentaram alelos exclusivos em todos os vinhedos (Tabela 5).

Maior número de alelos raros foi observado para a população *CUR_Rpv3.1+Rpv3.3*. O mesmo hospedeiro retornou o maior número de alelos raros também para os vinhedos *URU*, *VID* e *SJO*. No vinhedo de maior altitude, as populações com maior número de alelos raros foram *srpv* e *Rpv3.1*. Assim como para o número total de alelos, baixa frequência de alelos raros foram observados para os hospedeiros contendo R-locos piramidados e a população *VID_Rpv3.3+Rpv10* foi a única em que não foi encontrado alelos raros (Tabela 5).

A análise do AIc demonstrou que a entrada de migrantes foi significante em todas as populações localizadas no vinhedo *ADC*. Em contraste, nenhuma das populações de *VID* retornou valores significativamente negativos de AIc. Valores significativos para a entrada de migrantes são também observados nas populações *URU_Rpv3.1*, *URU_Rpv3.1+Rpv3.2*, *URU_Rpv3.1+Rpv3.3*, *URU_Rpv3.3+Rpv10*, *CUR_Rpv3.1*, *SJO_Rpv3.1+Rpv3.2* e *SJO_ Rpv3.1+Rpv3.3* (Tabela 5).

Tabela 5. Índices de diversidade genética, porcentagem de locos polimórficos ($\hat{P}_{99\%}$), somatório do número de alelos, número médio de alelos por loco ($\hat{A}$), número médio de alelos por loco polimórfico ($\hat{A}_p$), heterozigosidade esperada pelo equilíbrio de EHW ($\hat{H}_E$) e observada ($\hat{H}_O$), índice de fixação ($\hat{f}$), número de alelos exclusivos (Excl), número de alelos raros (Raros) e *corrected Assignment Index* (AIc)

Índice	srpv	Rpv3.1	Rpv3.1+ Rpv3.2	Rpv3.1+ Rpv3.3	Rpv1+ Rpv3.1	Rpv3.3+ Rpv10
Urussanga						
$\hat{P}_{99\%}$	87.5%	87.5%	87.5%	87.5%	62.5%	75.0%
nº alelos	27	22	24	28	17	22
$\hat{A}$	3.375	2.750	3.000	3.500	2.125	2.750
$\hat{A}_p$	3.857	3.143	3.429	4.000	3.400	3.666
$\hat{H}_E$	0.416	0.413	0.374	0.429	0.205	0.372
$\hat{H}_O$	0.468	0.458	0.465	0.505	0.240	0.432
$\hat{f}$	-0.123	-0.108	-0.243	-0.177	-0.171	-0.164
Excl.	–	–	–	1	1	–
Raros[1]	7	3	7	9	4	3
AIc	-0.510^{NS}	-2.707**	-1.544**	-2.013**	-0.400^{NS}	-1.987**
Videira						
$\hat{P}_{99\%}$	87.5%	87.5%	75.0%	87.5%	87.5%	75.0%
nº alelos	27	26	23	34	21	20
$\hat{A}$	3.375	3.250	2.875	4.250	2.625	2.500
$\hat{A}_p$	3.857	3.714	3.833	4.857	3.000	3.333
$\hat{H}_E$	0.426	0.421	0.380	0.451	0.300	0.410
$\hat{H}_O$	0.490	0.435	0.475	0.458	0.282	0.512
$\hat{f}$	-0.151	-0.032	-0.251	-0.014	0.058	-0.249
Excl.	2	–	–	–	2	–
Raros	6	6	3	11	6	–
AIc	3.186**	1.145**	0.295^{NS}	-0.027^{NS}	5.285**	-0.480^{NS}
Curitibanos						
$\hat{P}_{99\%}$	87.5%	87.5%	75.0%	87.5%	87.5%	75%
nº alelos	27	23	22	36	20	23
$\hat{A}$	3.375	2.875	2.750	4.500	2.500	2.875
$\hat{A}_p$	3.857	3.286	3.666	5.143	2.857	3.833
$\hat{H}_E$	0.431	0.356	0.373	0.445	0.326	0.428
$\hat{H}_O$	0.500	0.335	0.478	0.468	0.352	0.475
$\hat{f}$	-0.159	0.060	-0.282	-0.050	-0.080	-0.110
Excl.	2	–	–	1	3	–
Raros	6	4	3	14	5	1
AIc	1.195**	-1.311**	2.875**	-0.881^{NS}	4.865**	1.391**

			San Joaquim			
$\hat{P}_{99\%}$	87.5%	75.0%	75.0%	87.5%	87.5%	75.0%
nº alelos	27	28	28	33	20	23
$\hat{A}$	3.375	3.500	3.500	4.125	2.500	2.875
$\hat{A}_p$	3.857	4.667	4.666	4.714	2.857	3.833
$\hat{H}_E$	0.429	0.375	0.403	0.401	0.380	0.430
$\hat{H}_O$	0.492	0.422	0.488	0.432	0.415	0.545
$\hat{f}$	-0.149	-0.126	-0.209	-0.079	-0.091	-0.267
Excl.	1	–	1	–	3	–
Raros	6	9	8	13	2	1
AIc	2.773**	-0.351^{NS}	1.162*	-0.935*	3.424**	0.352^{NS}
			Água Doce			
$\hat{P}_{99\%}$	87.5%	87.5%	75.0%	87.5%	87.5%	75.0%
nº alelos	34	37	27	33	23	24
$\hat{A}$	4.250	4.625	3.375	4.125	2.875	3.000
$\hat{A}_p$	4.857	5.286	4.500	4.714	3.286	4.000
$\hat{H}_E$	0.511	0.502	0.467	0.499	0.502	0.628
$\hat{H}_O$	0.578	0.588	0.602	0.598	0.453	0.459
$\hat{f}$	-0.129	-0.171	-0.290	-0.197	-0.110	-0.368
Excl.	4	–	–	1	1	–
Raros	12	12	4	11	2	4
AIc	-2.110**	-4.946**	-2.769**	-3.726**	-1.963**	-3.012**

^{NS} indica médias não diferentes significativamente de zero; * indica diferença ao nível de 5% de probabilidade de erro tipo α; ** indica diferença ao nível de 1% de probabilidade de erro tipo α. [1] Alelos são considerados raros quando em frequência igual ou inferior a 5% do número de alelos da população.

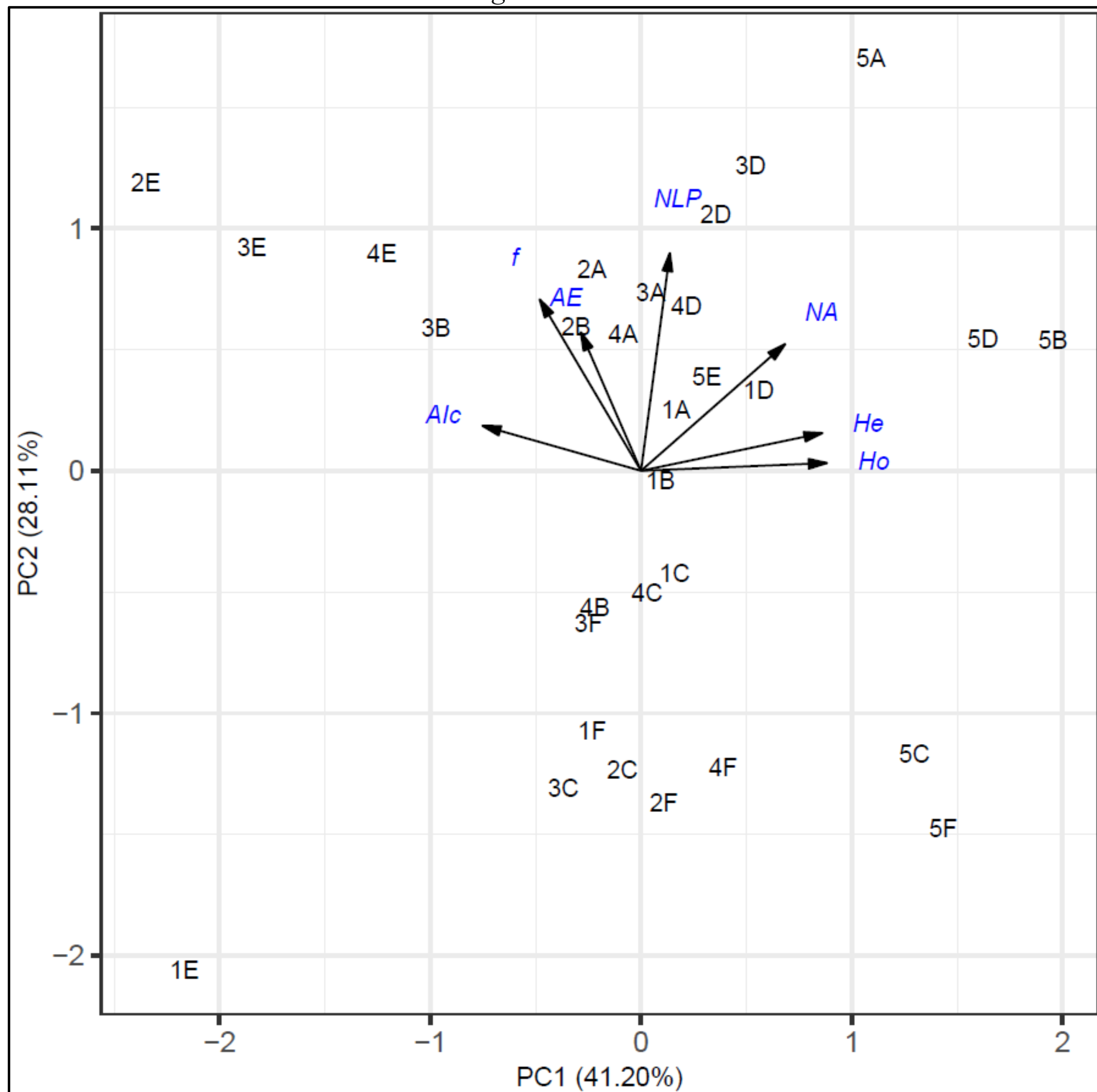
No vinhedo *CUR* todos os índices de F_{ST} foram significativos nas combinações par-a-par entre as populações, indicando que o efeito do hospedeiro atua significativamente na fragmentação destas. Para as demais unidades, as populações *URU_srpv* e *URU_Rpv3.1+Rpv3.2* não se diferenciaram significativamente, sendo este o único vinhedo em que alguma população não se diferenciou do hospedeiro *srpv*. Diferenças não significativas também foram observadas entre as populações *VID_Rpv1+Rpv3.1* e *VID_Rpv3.3+Rpv10*. Para estes mesmos hospedeiros diferenças também não foram observadas nos vinhedos *SJO* e *ADC*. Além destas, houve diferença significativa entre *SJO_Rpv3.1* e *SJO_Rpv3.1+ Rpv3.2*, bem como, entre *SJO_Rpv3.1* e *SJO_Rpv1+Rpv3.1* (Tabela 6).

Tabela 6. Divergência genética entre populações de *P. viticola* (F_{st}) cultivados em cinco ambientes distintos de Santa Catarina, Brasil e obtidos a partir de cinco hospedeiros contendo diferentes combinações de genes associados com a resistência genética ao patógeno, obtido através de 10.000 bootstraps

	RPV3.1	Rpv3.1+ Rpv3.2	Rpv3.1+ Rpv3.3	Rpv1+ Rpv3.1	Rpv3.3+ Rpv10
			Urussanga		
srpv	0.073**	0.021^{NS}	0.185**	0.128**	0.138**
RPV3.1		0.026**	0.140**	0.092**	0.151**
Rpv3.1+Rpv3.2			0.139**	0.093**	0.114**
Rpv3.1+Rpv3.3				0.142**	0.190**
Rpv1+Rpv3.1					0.034**
			Videira		
srpv	0.115**	0.074**	0.078**	0.138**	0.138**
RPV3.1		0.027**	0.164**	0.144**	0.139**
Rpv3.1+Rpv3.2			0.152**	0.052**	0.050**
Rpv3.1+Rpv3.3				0.246**	0.245**
Rpv1+Rpv3.1					0.004^{NS}
			Curitibanos		
srpv	0.247**	0.019**	0.074**	0.073**	0.082**
RPV3.1		0.167**	0.306**	0.268**	0.200**
Rpv3.1+Rpv3.2			0.091**	0.038**	0.034**
Rpv3.1+Rpv3.3				0.126**	0.158**
Rpv1+Rpv3.1					0.033**
			São Joaquim		
srpv	0.220**	0.148**	0.058**	0.083**	0.120**
RPV3.1		0.009^{NS}	0.245**	0.191^{NS}	0.192**
Rpv3.1+Rpv3.2			0.167**	0.123**	0.130**
Rpv3.1+Rpv3.3				0.090**	0.116**
Rpv1+Rpv3.1					0.020^{NS}
			Água Doce		
srpv	0.052**	0.068**	0.080**	0.114**	0.114**
RPV3.1		0.020**	0.069**	0.027**	0.046**
Rpv3.1+Rpv3.2			0.103**	0.085**	0.092**
Rpv3.1+Rpv3.3				0.088**	0.114**
Rpv1+Rpv3.1					0.046^{NS}

Na análise de correlação entre as variáveis para a PCA, foi observada covariância somente entre alelos raros e número de alelos (>0.80), assim, a variável alelos raros foi retirada da análise multivariada. A PCA representou 69.31% da variação e demonstrou que existe um agrupamento entre as populações coletadas entre diferentes vinhedos ou a partir de diferentes hospedeiros, contudo, existem sobreposições entre estas distribuições. A população *URU_Rpv1+Rpv3.1* apresentou maior distanciamento das demais, diferenciando-se das demais populações do mesmo hospedeiro principalmente pelo PC2. Este eixo representou 28.11% da variação e separou melhor as populações do mesmo hospedeiro, enquanto a separação de acordo com o vinhedo foi obtida pelo PC1, que representou 41.20% da variação (Figura 2).

Figura 2. Análise multivariada, realizada com populações de *Plasmopara viticola* coletados em vinhedos localizados em Santa Catarina, Brasil, nos municípios de Urussanga, Videira, Curitibanos, São Joaquim e Água Doce, enumeradas de 1 a5, respectivamente, de hospedeiros sem R-locos de resistência (*srpv*), *Rpv3.1*, *Rpv3.1+Rpv3.2*, *Rpv1+Rpv3.1 e Rpv3.3+Rpv10,* nomeados de “A” a “F”, respectivamente. *NLP*: Número de locos polimórficos; *NA*: número total de alelos; *He*: heterozigosidade esperada e *Ho*: observada; *f:* índice de fixação; *AE*: alelos exclusivos e *AIc*: *corrected Assignment Index.*



Fonte: Tomazetti, TC. (2020)

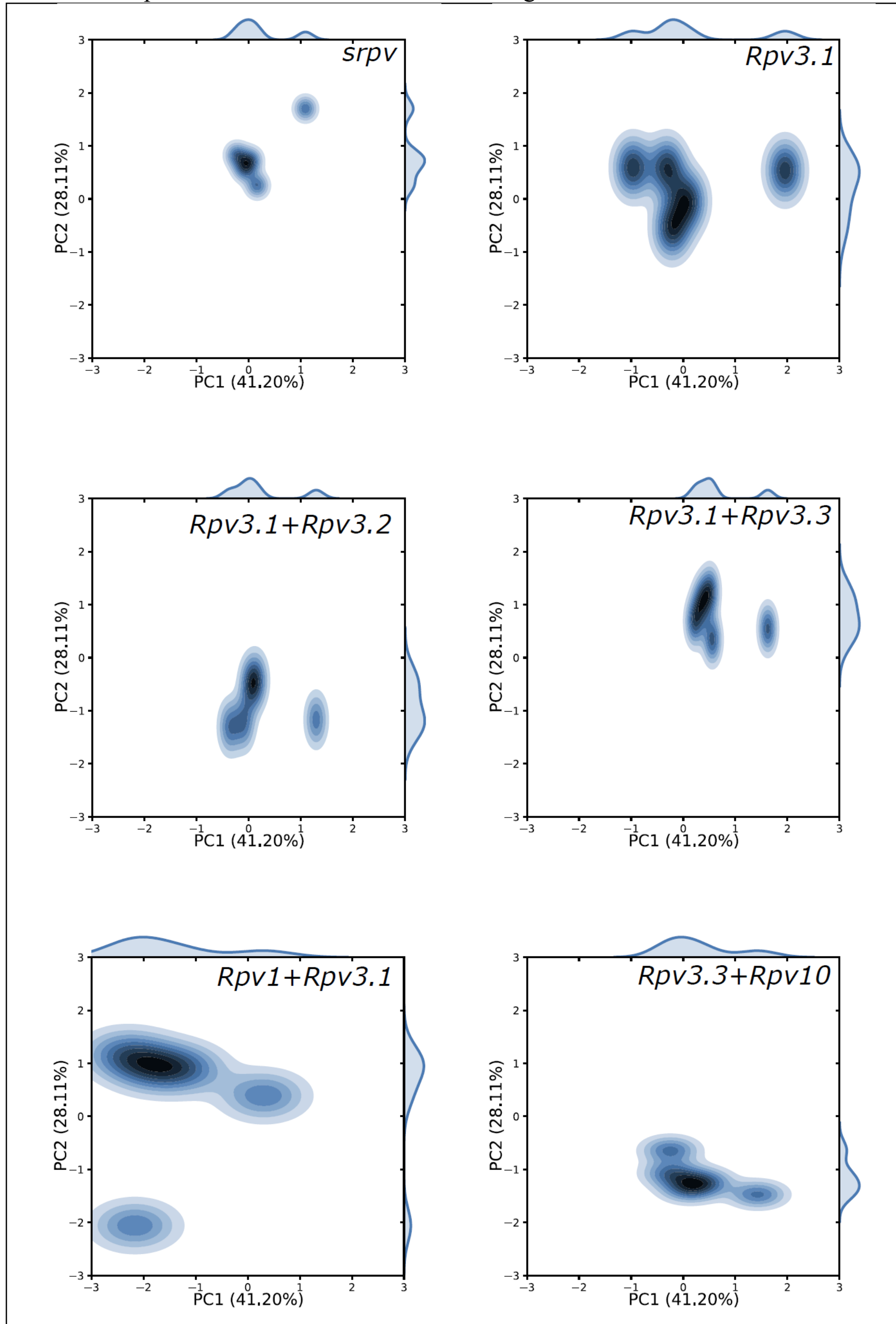
A análise de KDE, considerando o efeito hospedeiro, demonstrou que somente as populações a partir do *Rpv3.3+Rpv10* não apresentaram segregação devido ao vinhedo, pois, para estas populações todos os vinhedos foram agrupados no mesmo *cluster*. Para todos os demais hospedeiros, populações se dividiram em dois *clusters*, contudo, somente para o hospedeiro também piramidado *Rpv1+Rpv3.1* foi observado um *cluster* isolado para o

vinhedo de menor altitude (URU). Para os demais hospedeiros, as populações a partir dos vinhedos URU, VID, CUR e SJO formaram um único cluster, diferenciando-se somente das populações a partir do vinhedo ADC (Figura 3).

Considerando o agrupamento das populações pelo vinhedo, foi observado a formação de dois *clusters* para os vinhedos URU, VID e SJO. Para todos estes, um *cluster* foi composto pelas populações a partir dos hospedeiros *srpv*, *Rpv3.1*, *Rpv3.1+Rpv3.2*, *Rpv3.1+Rpv3.3* e *Rpv3.3+Rpv10*, enquanto *Rpv1+Rpv3.1* foi a única população a se diferenciar formando um segundo *cluster*. Mesmo para os vinhedos CUR e ADC, a população a partir do hospedeiro *Rpv1+Rpv3.1,* que não chegou a formar um segundo *cluster*, apresentou resultados distintos das demais, formando um sub-cluster visível para a população *ADC_ Rpv1+Rpv3.1* (Figura 4).

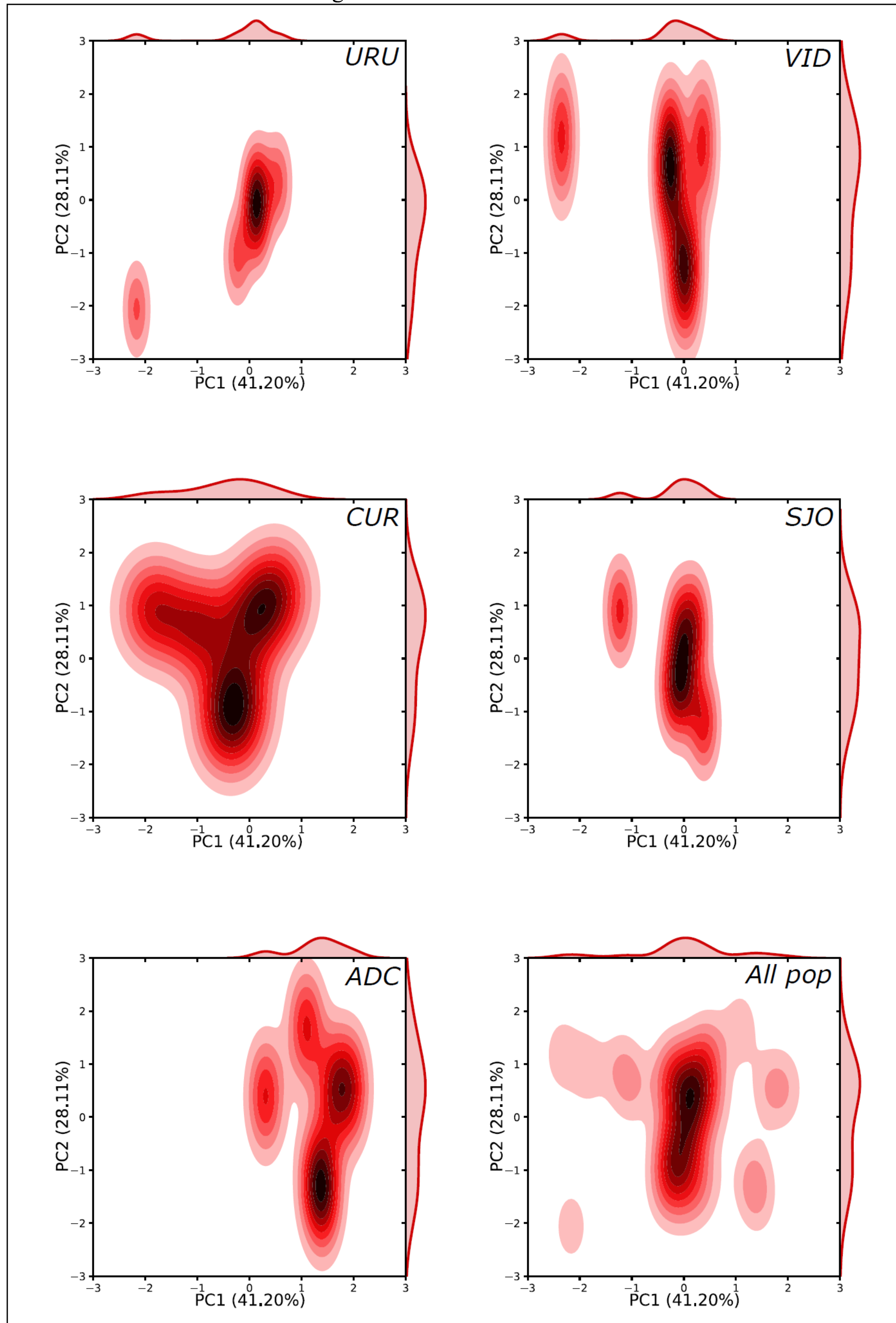
Observando todas as populações, três *clusters* são observados, sendo o maior destes, subdividido em três *sub-clusters*. O cluster com maior isolamento foi formado pela população *URU_Rpv1+Rpv3.1*, confirmando o observado para a PCA. Outro *cluster* foi formado pelas populações *ADC_ Rpv3.1+ Rpv3.2* e ADC_*Rpv3.3+ Rpv10*. O *cluster* principal é formado pelas demais populações, com a segregação dos *sub-clusters* supracitados, formados, um pelas populações *ADC_srpv*, *ADC_Rpv3.1* e ADC_*Rpv3.1+Rpv3.3*, outro pelas populações *VID_Rpv1+Rpv3.1*, *CUR_Rpv1+Rpv3.1*, *CUR_Rpv1+Rpv3.1* e *SJO_Rpv1+Rpv3.1*, o terceiro *sub-cluster* é composto por todas as demais populações (Figura 4).

Figura 3. Distribuição da densidade de populações de *Plasmopara viticola* coletadas em hospedeiros contendo diferentes R-locos associados à resistência a este patógeno a partir de vinhedos localizados em Santa Catarina, Brasil, nos municípios de Urussanga, Videira, Curitibanos, São Joaquim e Água Doce, utilizando valores da PCA a partir de estimadores da diversidade genética.



Fonte: Tomazetti, TC. (2020)

Figura 4. Distribuição da densidade de populações de *Plasmopara viticola* coletadas em vinhedos localizados em Santa Catarina, Brasil, nos municípios de Urussanga, Videira, Curitibanos, São Joaquim e Água Doce, a partir de hospedeiros *srpv*, *Rpv3.1*, *Rpv3.1+Rpv3.2*, *Rpv3.1+Rpv3.3*, *Rpv1+Rpv3.1* e *Rpv3.3+Rpv10* utilizando valores da PCA a partir de estimadores da diversidade genética.



Fonte: Tomazetti, TC. (2020)

4.5 DISCUSSÃO

A ausência de EHW em diversos locos microssatélites analisados no presente estudado também foram observados em populações de *P. viticola* coletadas de hospedeiros com diversos níveis de resistência ao patógeno, em 18 localidades distintas na China (LI et al., 2016). Os dados do presentes estudo contrastam, no entanto, com o observado em 34 populações do mesmo patógeno, hospedadas em videiras suscetíveis, cultivadas na Europa, onde é reportado predominantemente o EHW, não sendo relatado populações onde todos os locos estudados estavam em desequilíbrio (GOBBIN et al., 2006).

A afirmação da maior aderência ao EHW nas populações europeias é corroborada por um novo estudo realizado em populações de *P. viticola* distribuídas pelo mundo (TAYLOR et al., 2019). Neste trabalho, é relatada a avaliação da diversidade genética, mensurada via marcadores SSR's para cinco populações na América do Norte (EUA), duas na América do Sul (Brasil e Uruguai), uma na Europa (França) e seis na Austrália. Os resultados obtidos a partir destas populações apontam para a menor aderência ao EHW nas populações do oeste da Austrália, Queensland, América do Norte e América do Sul. Por outro lado, populações da França e sul da Austrália apresentaram aderência ao EHW na maior parte dos locos SSR's estudados. Os autores atribuem este resultado a possibilidade de acasalamento aleatório existente nas populações em equilíbrio. Todavia, o ponto fenológico do hospedeiro e a época de coleta não foram mencionados pelos autores, podendo haver distorções nestes resultados devido a fase sexuada do patógeno ocorrer predominantemente em temperaturas reduzidas na estação fria (GESSLER; PERTOT; PERAZZOLLI, 2011).

Os resultados relatados para a América do Sul estão de acordo com os verificados no presente estudo, bem como, os obtidos a partir de 26 vinhedos localizados na região sudeste do Brasil (CAMARGO et al., 2019). Utilizando como parâmetro a condição de EHW para a maior parte dos locos, conforme relatado pelos autores, somente as populações *URU_Rpv1+Rpv3.1*, *CUR_Rpv3.1* e *SJO_Rpv3.1* não sofrem forças evolutivas significativas que afetam o seu estado de equilíbrio. Para as demais populações neste estudo, eventos estão influenciando as suas frequências alélicas e, consequentemente, genotípicas.

Mesmo com desvios significativos do equilíbrio de EHW para as populações *srpv*, pode ser descartado a presença significativa de pressão de seleção nestas populações. Isto pois a diversidade genética, evidenciada pela proporção de locos polimórficos e quantidade superior de alelos, em praticamente todos os ambientes, apontam para ausência de barreiras

restringindo o crescimento do patógeno neste cluster. A presença de pressão de seleção positiva, implica na redução da diversidade genética nas populações em que esta ocorre (DERBYSHIRE et al., 2019; HAASL; PAYSEUR, 2015), indicando que esta força evolutiva é nula ou menor, nas populações *srpv* e *Rpv3.1*.

Esta informação é corroborada pelo número de alelos exclusivos, que, retornaram valores superiores para as populações *srpv* e *Rpv3.1+Rpv3.3*, com exceção da população *ADC_Rpv3.1*, contrastando com a baixa frequência em populações *Rpv1+Rpv3.1* ou mesmo a ausência de alelos exclusivos para as populações *Rpv3.3+Rpv10*, reforçando o possível efeito aditivo presente entre os respectivos R-locos piramidados nos hospedeiros. Mesmo padrão é observado para os alelos raros, contudo, para esta variável, somente a população *VID_Rpv3.3+Rpv10* não possuiu alelos raros, o que pode estar vinculado com o baixo número de alelos desta população (menor número de alelos dentre as populações para este genótipo hospedeiro).

Considerando somente os hospedeiros contendo R-locos piramidados, resultados conflitantes são apontados entre o número total de alelos, quantidade de alelos raros e alelos exclusivos. As populações *Rpv3.3+Rpv10* possuem constantemente maior número total de alelos em comparação à *Rpv1+Rpv3.1*, com exceção da *VID_Rpv3.3+Rpv10*. Soma-se a isto o maior número de alelos raros nas populações *Rpv1+Rpv3.1*, com exceção da população *ADC_Rpv1+Rpv3.1*, todos estes indicadores apontam para maior poder de restrição do desenvolvimento do patógeno pelo hospedeiro *Rpv1+Rpv3.1*. Todavia, as populações a partir deste hospedeiro possuem alelos exclusivos em todas as unidades avaliadas, que somente não são mais abundantes que as populações *srpv* em ADC, contrastando com a ausência de alelos exclusivos nas populações *Rpv3.3+Rpv10*.

Este comportamento pode indicar que um processo de seleção pode estar favorecendo o surgimento de populações especializadas que consigam superar os mecanismos de resistência do genótipo *Rpv1+Rpv3.1*. Portanto, o reduzido número de alelos é explicado devido ao processo de seleção genética, que conduz a presença massiva de alelos exclusivos, possivelmente favoráveis a superação da resistência deste genótipo. Para esta hipótese estar correta, estes alelos devem resultar em custo energético adicional para o patógeno, motivo pelo qual, não foi observado em populações *srpv,* conforme relatado no patossistema (BOLTON, 2009; WALTERS; HEIL, 2007).

E todas as populações estudadas observou-se a maior heterozigosidade observada em comparação à esperada. Este resultado demonstra que em todas as populações há uma tendência de seleção de indivíduos heterozigotos, possivelmente pelo efeito aditivo que favorece estes indivíduos em comparação àqueles que portam duas cópias do mesmo alelo. A seleção em favor dos heterozigotos pode ser atribuído também ao efeito do paradoxo de Parrondo, no qual, estratégias que resultam em menor fitness combinadas, podem produzir um fitness superior (CHEONG; KOH; JONES, 2019).

A maior heterozigosidade observada em relação a esperada reflete índice de fixação negativo, este valor por sua vez distorce o AIc. Assim, a utilização deste índice para formular hipóteses não é adequada neste cenário. Todavia, os valores observados para o AIc demonstram que o ambiente de cultivo apresenta influência sobre a entrada de migrantes nas populações. Os principais pontos a serem evidenciados é o reduzido número de populações com entrada de migrantes em CUR e SJO, onde somente foi observado este evento nas populações *CUR_Rpv3.1* e *SJO_Rpv3.1+Rpv3.3,* respectivamente, somado a ausência de imigrantes nas populações a partir de VID. Estes dados contrastam com o observado para URU e ADC, onde somente as populações *URU_srpv* e *URU_Rpv1+Rpv3.1* não possuíram presença significativa de migrantes.

As populações, mesmo convivendo em mesmo vinhedo apresentaram, em sua maioria, diferenças significativas entre si, demonstrando que o efeito do hospedeiro é significativo para causar barreira de fluxo de alelos entre os isolados. Resultados similares são relatados em estudo com diferentes populações coletadas em 11 locais, em clima subtropical no Brasil (CAMARGO et al., 2019). Contudo, neste estudo, mesmo comparando populações de diferentes ambientes, os autores relatam a presença de F_{ST} não significativo em algumas comparações par-a-par entre populações. Resultados similares de diferenciação entre populações de *P. viticola* são relatados para populações coletadas em diferentes vinhedos chineses (YIN et al., 2014; ZHANG et al., 2017). Contudo, os autores atribuem as diferenças entre as populações, às diferenças climáticas entre os pontos de ocorrência destas.

Não há relato na literatura de estudos baseados na comparação de populações de *P. viticola* coletado a partir de hospedeiros contendo diferentes combinações de R-locos, partilhando do mesmo ambiente de cultivo. Os dados obtidos neste trabalho, quando interpretados pela análise multivariada dos indicadores de diversidade populacional demonstram que as populações *Rpv3.3+Rpv10*, não apresentam diferenças significativas da diversidade entre si. Isto não foi observado para nenhum outro hospedeiro. Isto pode estar

associado com o fator de restrição deste genótipo, resultar em índices de diversidades que não variam de acordo com a unidade de coleta.

O efeito de ambiente de cultivo apresentou menor influência nos índices de diversidade genética das populações, evidenciando o efeito dos genótipos hospedeiros na formação dos *clusters* populacionais. Entretanto, não há relatos na literatura de estudos com a mesma abordagem para comparar os resultados obtidos neste estudo. Por fim, ressalta-se a importância de estudos genéticos similares acompanhando a evolução dos índices de diversidade genética nestas populações ao longo do ciclo da videira, buscando testar algumas das hipóteses levantadas com os dados obtidos neste trabalho, como a seleção para heterozigotos a partir de evento de segregação genética durante o inverno.

4.6 CONCLUSÃO

1. As populações obtidas a partir dos genótipos sem R-locos de resistência possuem maior diversidade genética;
2. Dentre os genótipos contendo R-locos de resistência, a maior diversidade genética foi encontrada para genótipos contendo *Rpv3*, principalmente para os haplótipos piramidados *Rpv3.1*+*Rpv3.3*;
3. O efeito de diferentes haplótipos para o *Rpv3* não apresenta efeito aditivo na redução da diversidade genética para as populações de *Plasmopara viticola* neste hospedeiro;
4. A presença de dois R-locos associados à resistência no mesmo genótipo reduz a diversidade genética do *P. viticola* nestes hospedeiros, independente do vinhedo coletado;
5. O efeito do ambiente de cultivo possui influência na diversidade das populações de *P. vitícola,* contudo, o principal efeito observado é devido a genética do hospedeiro.

5 CAPÍTULO III - PHENOLOGY AND THERMAL REQUIREMENT OF DISEASE RESISTANT GENOTYPES (PIWI) GROWTH IN GOETHE GRAPE VALLEY REGION BRAZIL

Este capítulo foi publicado em forma de artigo científico no periódico Acta Horticulturae

Phenology and thermal requirement of disease resistant genotypes (PIWI) growth in Goethe Grape Valley region Brazil

5.1 ABSTRACT

The Goethe Grape Valley (GGV) is a traditional South Brazilian region for grape and wine production mainly from hybrid vines. High temperatures and rainfall conditions hamper the cultivation of *Vitis vinifera* varieties susceptible to diseases, especially *Plasmopara viticola*. New PIWI (from the German word Pilzwiderstandsfähige) genotypes of *V. vinifera* with resistance to *P. viticola*, are under evaluation in this region. The aim of this work was to characterize the phenology and the thermal requirement of PIWI genotypes grown in GGV. The experiment was carried out at an experimental winery (28°32'S, 49°19'W, altitude 60 masl), established in 2016 and the evaluations were done in 2018 vintage. The evaluated PIWI varieties were Aromera, Baron, Calardis blanc and Felicia. Minimum and maximum air temperatures were recorded daily, using 10 °C as the down threshold temperature, 25 °C as the optimum temperature for development, and 35 °C as the upper threshold temperature of development. The evaluated phenological stages were bud break to flowering, flowering to *veraison* and *veraison* to maturity. The total thermal requirement mean was 1360 °C day; the variety Felicia showing the lower thermal requirement (1260 °C day), which contrasted with the Aromera that presented the higher thermal requirement (1454 °C day). Baron and Calardis blanc needed 1337 °C, and 1391 °C day, respectively to reach maturity. From bud break to flowering the average of the thermal requirement was 229 °C day, from flowering to *veraison* the mean was 679 °C day, and from *veraison* to maturity the mean was 452 °C day. The four evaluated varieties exhibited adaptive development in the field with thermal requirement compatible with the region of Goethe Grape Valley. The results also revealed the lower thermal requirement, and consequently shorter cycle to the Felicia variety, as well as the greater thermal requirement to the Aromera variety.

Keywords: *Vitis vinifera* L.; *Plasmopara viticola*; downy mildew; PIWI varieties; adaptation

5.2 INTRODUCTION

The Goethe Grape Valley (GGV) is a traditional South Brazilian region for grape and wine production mainly from hybrid vines. High temperatures and rainfall conditions hamper the cultivation of *Vitis vinifera* varieties susceptible to diseases, especially *Plasmopara viticola*. This is an obstacle to be overcoming aiming the development of the viticulture sector, especially for making fine wines, with lower environmental impact. Genotypes with high oenological quality and disease resistant are current targets of breeding programs. These genotypes are known by the German acronym PIWI (*Pilzwiderstandsfähige*), used to

designate varieties that combine high oenological quality with disease resistance (PEDNEAULT; PROVOST, 2016a; SIVČEV; SIVČEV; RANKOVIĆ VASIĆ, 2010).

The cultivation of PIWI varieties is essential not only in regions with climate favorable to pathogen development, as well as well as, in traditional regions of Europe, as a sustainable alternative for the oenological chain and the stakeholders (BUONASSISI et al., 2017). However, there are just a few reports in the specialized literature approaching the phenotypic adaptability of PIWI genotypes and none from Brazil.

Knowledge of phenotypic behavior of different varieties, as well as their adaptation in distinct environments plays a central role in order to define appropriate climatic regions, and to estimate the adaptability response in climate change scenarios (GARCÍA-MOZO; MESTRE; GALÁN, 2010; GORDO; SANZ, 2010; MARTÍNEZ-LÜSCHER et al., 2015). One of the most studied phenological responses is the thermal sum (°C day) required to overcome the phenological stages (WEBB; WHETTON; BARLOW, 2007). Knowing the thermal varietal requirement in each phenological phase, it is possible modeling the phenological behavior and the candidate regions for their growth (PARKER et al., 2011; WEBB; WHETTON; BARLOW, 2007).

The most used algorithm to calculate the thermal sum is only based in the lower threshold temperature (Lt) to predict the daily thermal sum, and accumulated thermal sum. However, to generate more robust models in a biological system, another thresholds need to be considered, namely the temperature of maximum development (optimum temperature, Opt), and the Upper threshold temperature (Ut) (ANDREUCCI et al., 2016; HATFIELD; PRUEGER, 2015).

In this context, the aim of the present work was use a robust model to evaluate the thermal sum required to overcome the phenological stages in PIWI varieties Aromera, Baron, Calardis blanc, and Felicia, cultivated in a traditional wine growing region (Goethe Grape Valley, Urussanga, Santa Catarina State, South Brazil), which present climatic conditions favorable to high natural pathogen pressure.

5.3 MATERIAL AND METHODS

Data were collected from the vintage 2017/18, using adult plants cultivated in an experimental vineyard established in 2015 (28°32’S, 49°19'W, and altitude 60 masl) in north-south rows (SMART, 1973), with row spacing of 2.5 m and 1.0 m in plant spacing. The plants

were conducted in espalier with spur pruning system using two to three nodes by spur. The varieties were grafted on rootstock Paulsen 1103 and the winter pruning being performed in the end of dormancy.

This region present, according to the classification proposed by Köppen, humid mesothermic climate characterized by hot summers and well distributed annual rainfall (VIEIRA; GARCIA; BRUCH, 2015). Climate monitoring consisted in data collected about daily maximum and minimum air temperature, using an automatic weather station placed in the experimental field.

The PIWI evaluated varieties were Aromera, Baron, Calardis blanc, and Felicia. Phenological monitoring was performed according to Baillod and Baggiolini scale (BAILLOD; BAGGIOLINI, 1993). The dates and duration of the phenological phases were marked observing the intervals between bud break to flowering, flowering to *veraison*, *veraison* to complete maturate and complete cycle, and from bud break to complete maturation.

The accumulated thermal sum was defined using an algorithm conditioned to vine cardinal temperatures and daily temperatures. Thus, the lower threshold temperature (Lt) was considered 10 °C, optimum development temperature (Opt) was considered 25 °C and upper threshold temperature (Ut) was considered 35 °C. The equation used in each thermal availability condition were(TOMAZETTI et al., 2015):

When TM < Lt

$$dTS = 0\ x\ 1\ day$$

When Tmean < Opt and Tm < Lt

$$dTS = [(T_M - L_t)\ x\ 0.5]\ x\ 1\ day$$

When Tmean < Opt and Tm > Lt

$$dTS = (T_{mean} - L_t)\ x\ 1\ day$$

When Tmean > Opt and TM < Ut

$$dTS = \{(Opt - L_t)\ x\ [(U_t - T_{mean})/(U_t - Opt)]\}\ x\ 1\ day$$

When Tmean > Opt and TM > Ut

$$dTS = [U_T - (U_T + T_m)\ x\ 0.5]\ x\ 1\ day$$

Where dTS is daily thermal sum (°C); Tm is the minimum air temperature; TM is the maximum air temperature; and Tmean is the mean air temperature. Lt, Opt and Ut is the vine

cardinal temperatures, respectively lower threshold temperature, optimum temperature and upper threshold temperature. The accumulated thermal sum (aTS, °C day) was obtained by summing the daily thermal sum over the period, Thus:

$$STa = \sum dTS$$

For proposes comparison with others works of the literature, the dTS also was calculated using the algorithm considering only the Lt, where:

When Tmean < Lt:

$$dTS = 0 \, x \, 1 \, day$$

When Tmean > Lt:

$$dTS = (T_{mean} - L_t) \, x \, 1 \, day$$

5.4 RESULTS AND DISCUSSION

The temperature in the Goethe Grape Valley during the evaluations, demonstrated that although the region with high temperature, most of time, the thermal condition are considered appropriate for the vine plant development. The daily mean temperature ranged from 15.6 to 27.9 °C; the absolute minimums ranged from 9.3 to 22.7 °C and the absolute maximums temperature ranged from 18.5 to 39.0 °C. Thus, the average observed temperature in the region, during the growing cycle was 21.2 °C, below the optimum temperature considered in this study. The daily thermal sum ranged from 4.1 to 15.0 °C day, and in the average, it was accumulated 10.9 °C day by day between bud break to complete maturation (Figure 1).

The date of the phenological phase's occurrence showed a homogenous bud break, with the exception of the 'Baron', which had the bud break late 14 days after the others. From the flowering, 'Felicia' proved to be the earliest; this behavior was maintained until the complete maturation, which occurred at the end of December, 18 days in advance of the 'Baron' that was the latest variety among the evaluated (Table 1).

When the cycle was monitored based on the energy accumulation needed to complete the stages, the lower thermal requirement was observed for 'Felicia' (1260 °C day) and the higher requirement was recorded for 'Aromera' (1454 °C day) (Figure 2). In all varieties, the range from flowering to *veraison* was the period which higher thermal accumulation requirement. In this phase, the lower thermal accumulation required was exhibited by

'Calardis banc' (569 °C day), contrasting with 'Baron' (747 °C day), the highest thermal accumulation required in this period.

The range from flowering to *veraison* and from *veraison* to complete maturation occurred on the hottest days in all varieties. The average of the maximum temperature in this period ranged from 28.4 °C for 'Felicia' to 29.3 °C for 'Baron' and the mean temperature around was 24 °C (Table 2). In the comparison between the algorithms to predict thermal sum, using all three cardinal temperature, or only the Lt (Table 3), it is evident the greater difference in the predict thermal sum only on warmer days, when the maximum temperature exceeded the Ut. According to previous information, these days are most frequent at the end of the cycle.

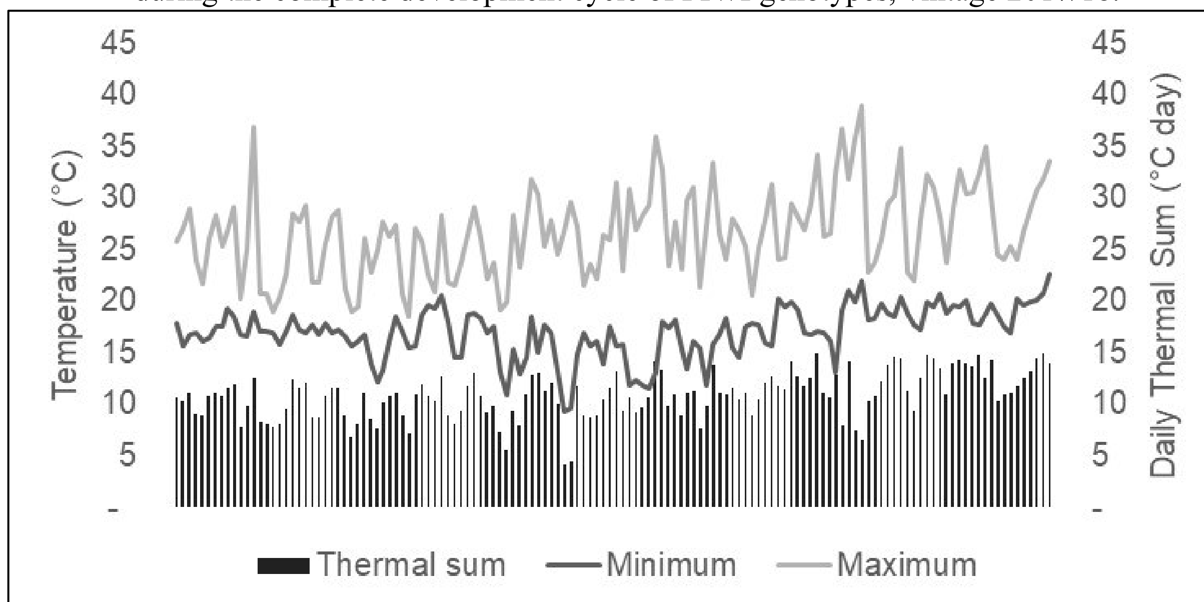
Table 1. Date of occurrence of the phenological stages on PIWI genotypes growing on Goethe Grape Valley region, vintage 2017/18

Events	Aromera	Baron	Calardis blanc	Felicia
Bud break	02/sep	16/sep	02/sep	02/sep
Flowering	30/sep	30/sep	30/sep	20/sep
Veraison	08/dec	10/dec	25/nov	25/nov
Maturity	13/jan	15/jan	08/jan	28/dec

Table 2. Mean temperature observed (°C) during the phenological stages on PIWI genotypes growth on Goethe Grape Valley region, vintage 2017/18

Temperature	Aromera	Baron	Calardis blanc	Felicia
		bud break to flowering		
Minimum	17.1	17.0	17.1	17.3
Mean	21.0	20.3	21.0	21.2
Maximum	24.8	23.6	24.8	25.1
		Flowering to *Veraison*		
Minimum	15.9	15.9	15.5	15.8
Mean	21.0	21.1	20.8	20.8
Maximum	26.1	26.3	26.1	25.8
		Veraison to Complete Maturation		
Minimum	18.4	19.0	18.4	18.1
Mean	24.0	24.2	23.5	23.3
Maximum	29.2	29.3	28.6	28.4
		Complete Cycle		
Minimum	16.8	17.0	16.8	16.6
Mean	21.8	21.9	21.8	21.5
Maximum	26.7	26.8	26.7	26.4

Figure 1. Minimum and maximum temperature (°C) reported on Goethe Grape Valley (lines), and the daily thermal sum (°C day) considering all cardinal temperature (columns) during the complete development cycle of PIWI genotypes, vintage 2017/18.



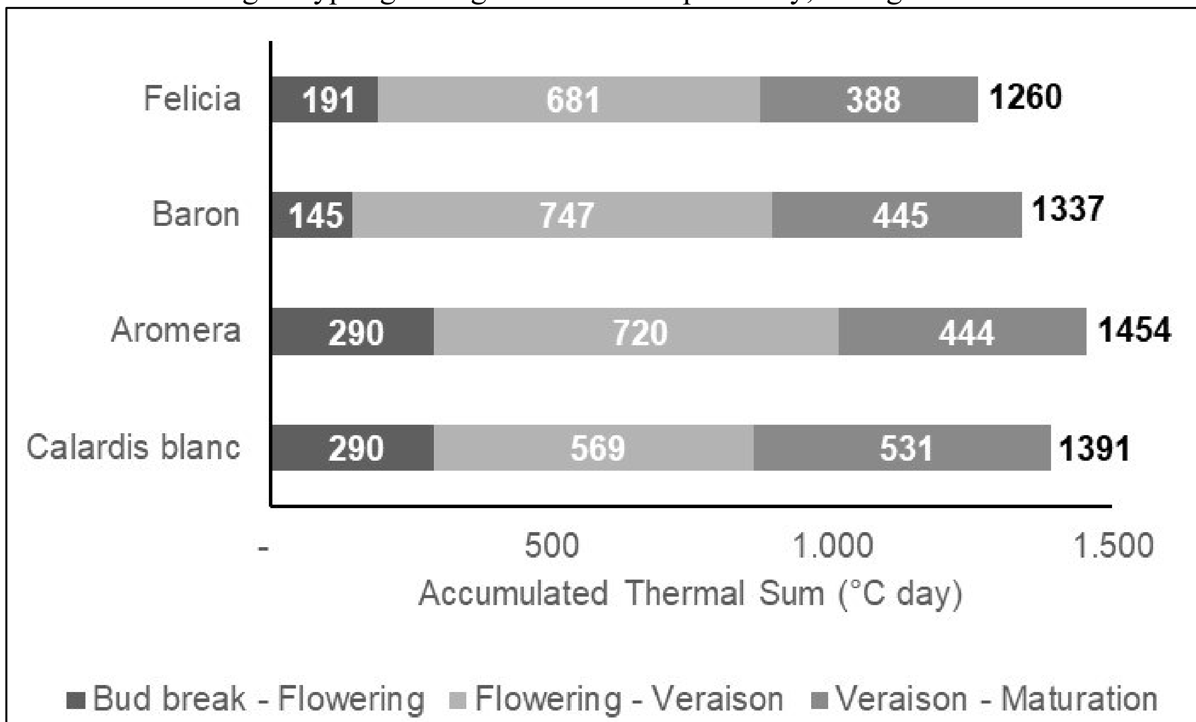
Fonte: Tomazetti, TC. (2020)

Table 3. Comparison between algorithms for estimated the daily thermal sum, using all cardinal temperature (Lt, Opt and Ut), and using only the Lt, for modeling the development in PIWI genotypes growth on Goethe Grape Valley, vintage 2017/18

Stage	Aromera	Baron	Calardis blanc	Felicia
		Algorithm using only Lt		
Bud break - Flowering	290	145	290	191
Flowering - *Veraison*	729	756	578	690
Veraison - Maturation	479	482	567	419
Total	1498	1384	1435	1300
		Algorithm using all cardinal temperature		
Bud break - Flowering	290	145	290	191
Flowering - *Veraison*	720	747	569	681
Veraison - Maturation	444	445	531	388
Total	1454	1337	1391	1260

[1]Lt: lower threshold temperature (10 °C), Opt: optimum temperature to plant development (25 °C) and Ut: upper threshold temperature (35 °C).

Figure 2. Accumulated thermal sum needed to overcome the phenological stage requirements in PIWI genotypes growing on Goethe Grape Valley, vintage 2017/18.



Fonte: Tomazetti, TC. (2020)

Many studies use the thermal sum to measure the development of the plant phenological stages (Barros et al. 2015 e Zeist et al. 2016). However, few describe the use of thermal sum methods considering the development cardinal temperatures (optimal and threshold temperature). These methods are more accurate and precise due the best correlation with the plant development (Porter; Gawith 1999; Rosa et al. 2009 e Tomazetti et al. 2015).

It should be stressed that there is little information about the phenology and thermal requirement in PIWI genotypes; thus, the results of the present study have limited basis for comparisons. Using the same algorithm to obtain the dTS in a warming region, and employing traditional *V. vinifera* varieties it was reported higher thermal accumulation in each phenological stage (Tomazetti et al. 2015). In that study the lower thermal accumulated dTS was recorded for 'Merlot' (1781 °C day), and the higher was observed for 'Cabernet Sauvignon' (1959 °C day). Similar results were reported to 'Syrah' growing in altitude regions, and with lower temperatures (SARTOR et al., 2017). However, these results reported by Tomazetti et al. (2015) and Sartor et al. (2017) contrast with those reported with traditional varieties growing in cooler region (BORGHEZAN et al., 2014), in which the thermal accumulation was similar to the reported in the present research.

Similar phenological cycle between PIWI variety Reberger and traditional variety Chardonnay was reported in an environment with lower temperature (KRAUS et al., 2018), as compared to the environment of the present research. However, the authors do not report the thermal sum needed in each phenological stage or in the complete cycle. Similar results of thermal sum requirement were reported on ‘Sangiovese’, in a region with lower thermal availability (GATTI et al., 2016).

The development of adjustment equations looking at to estimate the cardinal temperatures in vine, Zapata et al. (2017) using seventeen varieties of traditional grapevine, cultivated in Washington, USA, were the related temperature is lower than the reported in our work, found results of thermal accumulation similar to ours, until the *veraison*. These authors report that the Lt was theoretically below that commonly considered (10 °C), mainly in the initial phenological stages and increased on the final phenological stages independent of genotype. As a way to standardize the results, and in line with most of the work in this area we maintained Lt in 10 °C in the complete phenological cycle.

The data collected of complete phenological cycle on the evaluated PIWI genotypes area in agreement with those reported in the same region for the traditional Goethe variety. The short periods for vegetative development may contribute to a smaller leaf area increase, by the reduction of the number of leaves emitted (BORGHEZAN et al., 2017; ZEIST et al., 2017). Moreover, the reduced period between flowering and complete maturation shows a marked influence on the biochemical composition of berries (BORGHEZAN, 2017). Thus, the parameters of wine quality derived from PIWI varieties in this region need to be considered in future assessments of their *terroir* characteristics.

5.5 CONCLUSIONS

All four evaluated genotypes present appropriate development with thermal requirements compatible with the traditional grape varieties cultivated in the Goethe Grape Valley region.

Felicia variety with 1260 °C day has the lower thermal requirement for the complete development cycle. ‘Aromera’ shows the higher thermal requirement (1454 °C day).

The stage from flowering to *veraison* requires the higher thermal requirement in these genotypes.

6 CAPÍTULO IV – LINEAR MODELS FOR LEAF AREA ESTIMATION IN GRAPEVINE FUNGUS RESISTANT CULTIVARS AND THE ROLE OF GENOTYPE BY ENVIRONMENTAL INTERACTION

Este capítulo encontra-se formatado de acordo com a normativa do periódico “Sciencia Horticulturae”

Linear models for leaf area estimation in grapevine fungus resistant cultivars and the role of genotype by environmental interaction

TOMAZETTI, T.C.; et al. Linear models for leaf area estimation in grapevine fungus resistant cultivars and the role of genotype by environmental interaction

6.1 ABSTRACT

The main aims of this work was the development and validation of linear models for non-destructive estimation of leaf area (LA) in fungal resistant grapevine genotypes and the determination of phenotypic plasticity of the leaf traits in contrasting environments. The measurements were made in nine cultivars (Aromera, Baron, Bronner, Calandro, Calardis Blanc, Felicia, Helios, Prior, and Regent) and two advanced breeding selections, Gf.2004.043.0015 (ABS15) and Gf.2004.043.0024 (ABS24), cultivated in five contrasting edaphoclimatic conditions in Southern Brazil. One hundred leaves per genotype were randomly sampled in each location. Leaf length (L), width (W), average length of lateral veins (aLV), and LA were measured. The LA was obtained by the portable scanner of LA model ADC-AM 300. Eight mathematical models fitted by L, L^2, W, W^2, aLV, aLV^2, L W or L^2 W^2 aLV^2 were tested using simple and multiple linear regression to estimate the LA of each genotype. Additionally, we tested the generic model for LA estimation in *Vitis vinifera* and proposed the use of leaf deformation index (LDI) to measure the leaf asymmetry. The LA showed a significant genotype-environment interaction, however the genotypes played a major role to define the leaf shape. The betters linear models to estimate the LA for each genotype were: Aromera=0.99LW+2.76 (RMSE: 9.9 cm^2) , Baron=$0.24L^2+0.32W^2+0.47aLV^2+1.85$ (RMSE: 4.0 cm^2), Bronner=$0.44L^2+0.30W^2+0.46aLV^2+0.12$ (RMSE: 5.8 cm^2), Calandro=$0.82W^2-1.05$ (RMSE: 16.4 cm^2), Calardis Blanc=$0.75W^2-0.25$ (RMSE: 12 cm^2), Felicia=$0.78W^2-1.91$ (RMSE: 9.9 cm^2), ABS15=1.08LW-3.33 (RMSE: 23.6 cm^2), ABS24=0.85LW-0.42 (RMSE: 16.8 cm^2), Helios=1.06LW+3.07 (RMSE: 14.6 cm^2), Prior=1.05LW+3.60 (RMSE: 18.5 cm^2) and Regent=$0.11L^2+0.40W^2+0.77aLV^2-4.15$ (RMSE: 2.83 cm^2). The generic model was not adequate for eight genotypes evaluated. In this work, we provided precise, accurate and robust linear models to estimate the LA in nine fungal resistant cultivars and presented the relationship between these phenotypic traits with the environment.

Key words: *Vitis vinifera*; Pilzwiderstandsfähig; disease resistance; modeling; viticulture, leaf shape.

6.2 INTRODUCTION

Viticulture is one of the main agricultural activities around the world. In 2016, 7,096,741 ha were covered with vineyards and 77,438,929 t of grapes were produced worldwide (FAO, 2019). Currently, the vine is cultivated in various regions of the world, mainly based on *Vitis vinifera* cultivars. This species is consolidated in commercial cultivation in detriment of others *Vitis* spp., especially due to the fruit quality, appreciated in fresh consumption and winemaking (BURIN et al., 2014).

However, *V. vinifera* cultivars are highly susceptible to a series of fungal diseases. This is a major challenge for today's viticulture, because a huge amount of fungicides is required to produced high qualitative grapes (PIRRELLO et al., 2019). This fact was

evidenced by the unintentional introduction of North American endemic pathogens, in the second middle of the 19th century, causing severe losses in European viticulture (GOBBIN et al., 2006). Since then, breeding strategies are being applied aiming the development of fungal disease-resistant cultivars (BROWN et al., 1999; EIBACH et al., 2007b; VEZZULLI et al., 2018).

The first generation of cultivars developed by crossing American and/or Asian species with *V. vinifera* revealed low oenological quality, due to the wild nature of the resistance donors. Successive modified backcrossing between the selected disease-resistant hybrids with different *V. vinifera* cultivars, donor of quality attributes, increased the proportion of *V. vinifera* genome and led to modern cultivars which combine high oenological quality with disease resistance (TÖPFER et al., 2011). These are known as PIWI cultivars, an acronym of the German word *Pilzwiderstandsfähig*, that means fungus disease resistant (PEDNEAULT; PROVOST, 2016b; SIVČEV; SIVČEV; VASIĆ, 2010).

Currently, dozens of PIWI cultivars are available for cultivation especially in Europe (https://www.piwi-international.de/en/varieties.html). The European continent is the traditional and largest wine producer, and their Common Agriculture Policy (PAC) has clear environmental goals, aiming the promotion of organic farming and the responsible management of inputs, such as pesticides and fertilizers. The current regulations (2017/625 and 1107/2009) dealing with the topic promote the adoption of low environmental impact cultivation methods (EUR-LEX, 2009, 2017). Thus, the member countries are implementing policies to promote the PIWI cultivation and PIWI breeding programs (MONTAIGNE; COELHO; KHEFIFI, 2016).

This trend of migrating from traditional *V. vinifera* to PIWI cultivars is also driven by the increased demand on healthy products with lower environmental and economic impacts, as well as reduced exposure of producers and consumers to pesticide residues (GADOURY et

al., 2012b; KAPUSTA; CEBULAK; OSZMIAŃSKI, 2017; MONTAIGNE; COELHO; KHEFIFI, 2016; NARDUZZI; STANSTRUP; MATTIVI, 2015b; PERTOT et al., 2017b).

Despite of the economic, social and environmental positive impacts of PIWI cultivars, there are only few studies showing their adaptive potential in different growing regions worldwide. In Southern Brazil, PIWI cultivars are being tested in different edaphoclimatic conditions (BRIGHENTI et al., 2019; SOUZA et al., 2019; STEFANINI et al., 2019), where resistance scoring showed the potential of these cultivars to reduce the chemical input in the viticulture (ZANGHELINI et al., 2019a).

To advance with agronomical and ecophysiological researches the estimation of leaf area (LA) is critical. The estimation of LA is required, for example, to determinate light interception, leaf transpiration, photosynthesis, plant nutrition, ecological competition, plant-water relations, leaf cover, cultivation ecosystem, plant productivity (BESLIC; TODIC; TESIC, 2010; COSTANZA et al., 2004; ROUPHAEL et al., 2010; SILVESTRONI et al., 2018).

The LA may be estimated by destructive or non-destructive methods. Although destructive methods have high precision and accuracy, they are laborious and require leaf removal, not allowing to monitor the evolution of LA during the phenological cycle (BESLIC; TODIC; TESIC, 2010; JIANG et al., 2015). On the other hand, non-destructive methods have as great advantage the fact of not removing or damaging the leaves, allowing the monitoring of LA evolution along the phenological stages (BUTTARO et al., 2015; TONDJO et al., 2015). For non-destructive methods, various combinations of measurements and simple linear mathematical models have been utilized to estimate the LA in many species (CARVALHO; ALVES; BIANCO, 2017; CIRILLO et al., 2017; FASCELLA et al., 2018; KERAMATLOU et al., 2015; LIU et al., 2017).

In grapevine, the LA is commonly modeled by leaf length and width, using a simple linear regression to fit the observed LA with these traits (BUTTARO et al., 2015; WILLIAMS; MARTINSON, 2003a). However, another leaf traits can be considered to developed better models and test them by multiple linear regression, where the LA (dependent variable) is fitted with more traits (independents variables) in the same equation, such as $\widehat{LA} = b_0X_0 + b_1X_1 \ldots b_nX_n$ (CANKAYA et al., 2006). The employment of these complex models are currently enabled by automatized LA estimative by computational algorithms that run the data from several acquired traits and return the LA through these mathematical models (EASLON; BLOOM, 2014; HU et al., 2018; TECH et al., 2018).

Mathematical models can be evaluated in a virtually infinite number of parameters. Precision, accuracy and robustness are common terms to define the model quality. In this work, we considered precision as the measure of the model residues, commonly measured by correlation or root mean square error (RMSE); accuracy as the measure of the model bias (CONTURO et al., 1995; GRUBBS, 1973; KARNES; MARCH, 1993) and robustness as the characteristic of the model to represent the major variance of the data and consequently present the smallest biases, which means that a robust model should not have significant changes due another variables that are not in the model (BOX, 1979; CARROLL; PEDERSON, 1993; COPAS, 1988).

Models to estimate the LA can be developed with generalist or specific scope. Generalist models aim developing a general equation that can be applied to any cultivar (BUTTARO et al., 2015). In the other hand, specific models aim to obtain greater precision and accuracy in specific cultivars (TEOBALDELLI et al., 2019). Generalists models should consider larger data variability, once the LA vary according the genetic and environment effects (SCHEEPENS; FREI; STÖCKLIN, 2010). Specific models tend to be more precise

and accurate because they do not consider genetic variation, reducing the deviations contained in the models.

Specific models should be developed when the leaf traits present significant genotype x environment interaction (GxE). Under this scenario it is important that the data used to fit the models are collected in different environments, thus incorporating the morphologic variation induced by the environmental conditions. The leaf shape is a trait strongly influenced by GxE in several species, as *Capsicum annum* (CEMEK; UNLUKARA; KURUNC, 2011), *Ipomoea batatas* (GUPTA et al., 2020), *Campanula thyrsoides* (SCHEEPENS; FREI; STÖCKLIN, 2010), *Malus* spp. (MIGICOVSKY et al., 2018) and grapevine (BAUMGARTNER et al., 2020; DEMMINGS et al., 2019). Thus, the development of robust models to estimate the LA, should consider the GxE interaction, using data collected in different environment, thus enabling the application of the model for the specific genotype in several environments.

Cultivar-adjusted models are currently more used in *V. vinifera*, exploring the leaf shape differences between cultivars (BESLIC; TODIC; TESIC, 2010; BORGHEZAN et al., 2010; CIRILLO et al., 2017). Although there are LA estimation models available for traditional *V. vinifera* grapevine cultivars, none is yet available for PIWI cultivars. Besides that, the LA estimation models are frequently based on inappropriate statistics to select the better model (BUTTARO et al., 2015; MARTIN BLAND; ALTMAN, 1986). They are based mainly in the correlation between the estimated and observed LA. However, other statistic errors should be considered for model validation, such as root mean square error (RMSE), model bias, model efficiency, as well as the coefficients of significance (BUTTARO et al., 2015; SENTELHAS et al., 1997; VAN CALSTER et al., 2016). Additionally, it is fundamental to know how strongly the leaf traits are influenced by the genotype, environment

and GxE interaction, and consequently the model's applicability in different environment conditions.

Taking in consideration the increasing importance of PIWI cultivars worldwide and the absence of LA estimation models for these cultivars, the main objectives with this work were: 1) to develop and validate robust, precise and accurate linear cultivar-adjusted nondestructive models for LA estimation in PIWI cultivars; 2) to test the adjustment of a *V. vinifera* generic model for these cultivars; 3) to evaluate the GxE interaction of all leaf traits measured.

6.3 MATERIAL AND METHODS

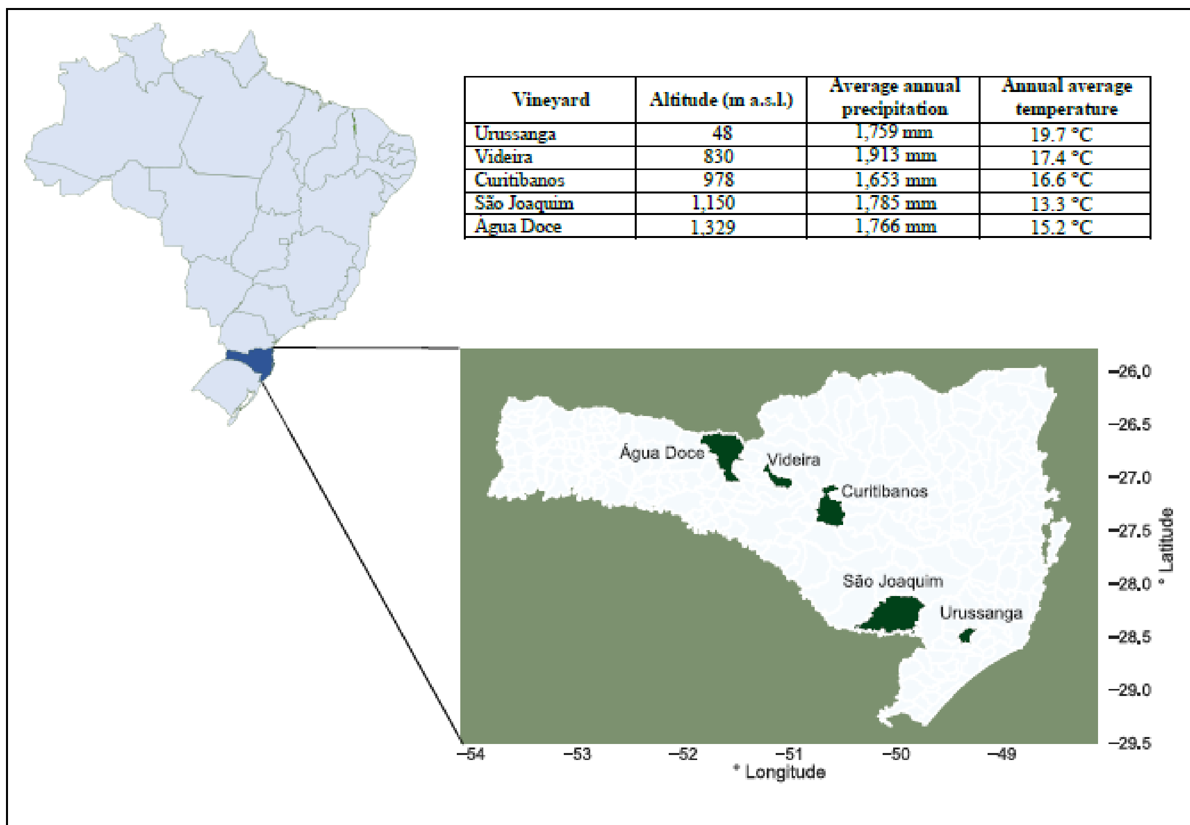
6.3.1 Plant material

LA estimation models were constructed for eleven PIWI genotypes as follow: 1) the white PIWI cultivars 'Aromera' (VIVC: 25400), developed by Innovitis (Bolzano, Italy); 'Bronner' (VIVC: 17129) and 'Helios' (VIVC: 17133), developed by Staatliches Weinbauinstitut Freiburg Abteilung Weinbau (WBI); 'Calardis Blanc' (VIVC: 22828) and 'Felicia' (VIVC: 20348) developed by Julius Kühn-Institut (JKI) – Institut für Rebenzüchtung Geilweilerhof, and 2) the red PIWI cultivars 'Baron' (VIVC: 20010) and 'Prior' (VIVC: 19993) developed by WBI, 'Calandro' (VIVC: 21797) and 'Regent' (VIVC: 4572), developed by JKI. In addition, the models were generated for two advanced breeding selections (ABS15 and ABS24), containing resistance genes pyramided, for breeding purposes.

6.3.2 Experimental vineyards

The PIWI genotypes were grown in five experimental vineyards located at different edafoclimatic regions of Santa Catarina State, Southern of Brazil. The vineyards were located at two Experimental Stations of Epagri, in the cities of Urussanga and Videira; at the Experimental Station of the Federal University of Santa Catarina (UFSC) in Curitbanos; and

two in wineries, Suzin Winery in São Joaquim and Villaggio Grando Winery in Água Doce. The vines were trained in vertical shoot positioning trellis (VSP) at spacing of 3.0 m x 1.2 m, grafted on 'Paulsen 1103' and pruned in double spur cordon. Climatic data were obtained from weather stations located near the vineyards and kindly provided by EPAGRI/CIRAM. The geographical location, altitude, annual average temperature and annual accumulated precipitation may be observed in Figure 1 and the climatic variables during the productive cycle are shown in the Figure 2.



Vineyard	Altitude (m a.s.l.)	Average annual precipitation	Annual average temperature
Urussanga	48	1,759 mm	19.7 °C
Videira	830	1,913 mm	17.4 °C
Curitibanos	978	1,653 mm	16.6 °C
São Joaquim	1,150	1,785 mm	13.3 °C
Água Doce	1,329	1,766 mm	15.2 °C

Figure 1. Geographic location and climatology conditions (mean of the last 30 years) of the five vineyards containing the eleven PIWI varieties evaluated in this work.

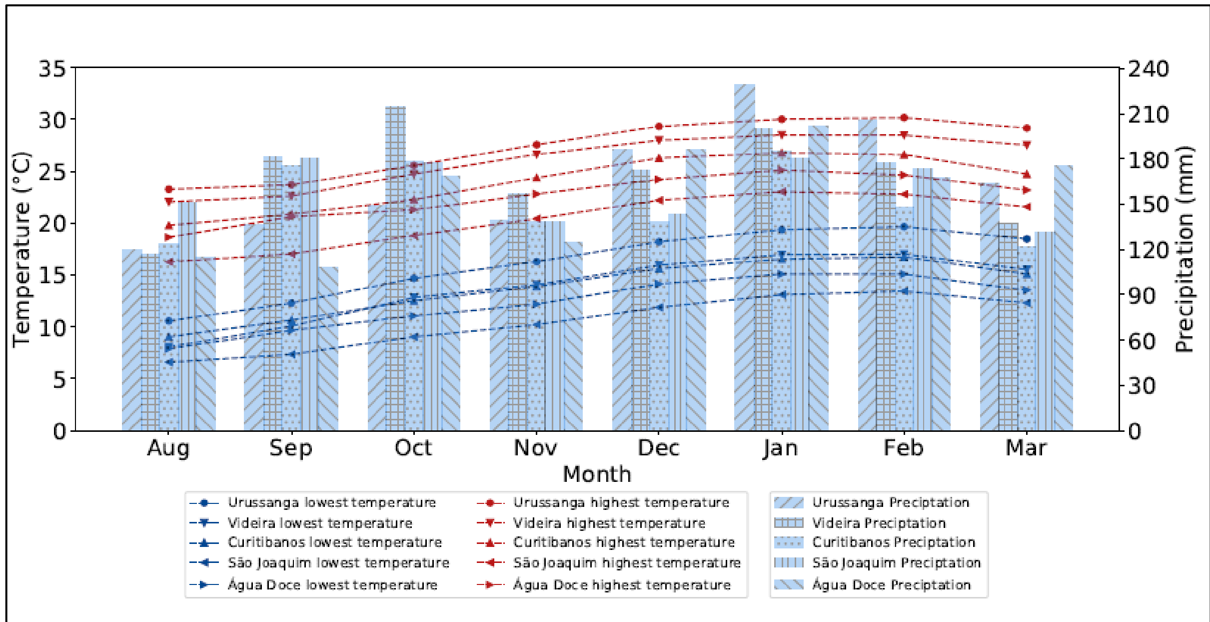


Figure 2. Climatologic conditions, that represents the mean of the last 30 years, in the five experimental vineyards during the period from august to march, covering the grapevine full productive cycle.

6.3.3 Data collection

The leaves were collected from adult plants in pre-veraison stage. One hundred leaves per cultivar from each vineyard were randomly collected in the productive cycle of 2016/17. All collected leaves were healthy and without anomalies and with at least 30 mm in length. The leaf traits measurements started immediately after the sampling.

For each leaf, the leaf length (L), the width (W) and the length of lateral veins (left and right) were measured using a millimeter ruler (Figure 3). The average length of lateral veins (aLV) was calculated by the average between left and right veins (LVL_r and LVL_l). The LA was determined by the portable leaf area meter ADC, model AM 30 (BioScientific, England).

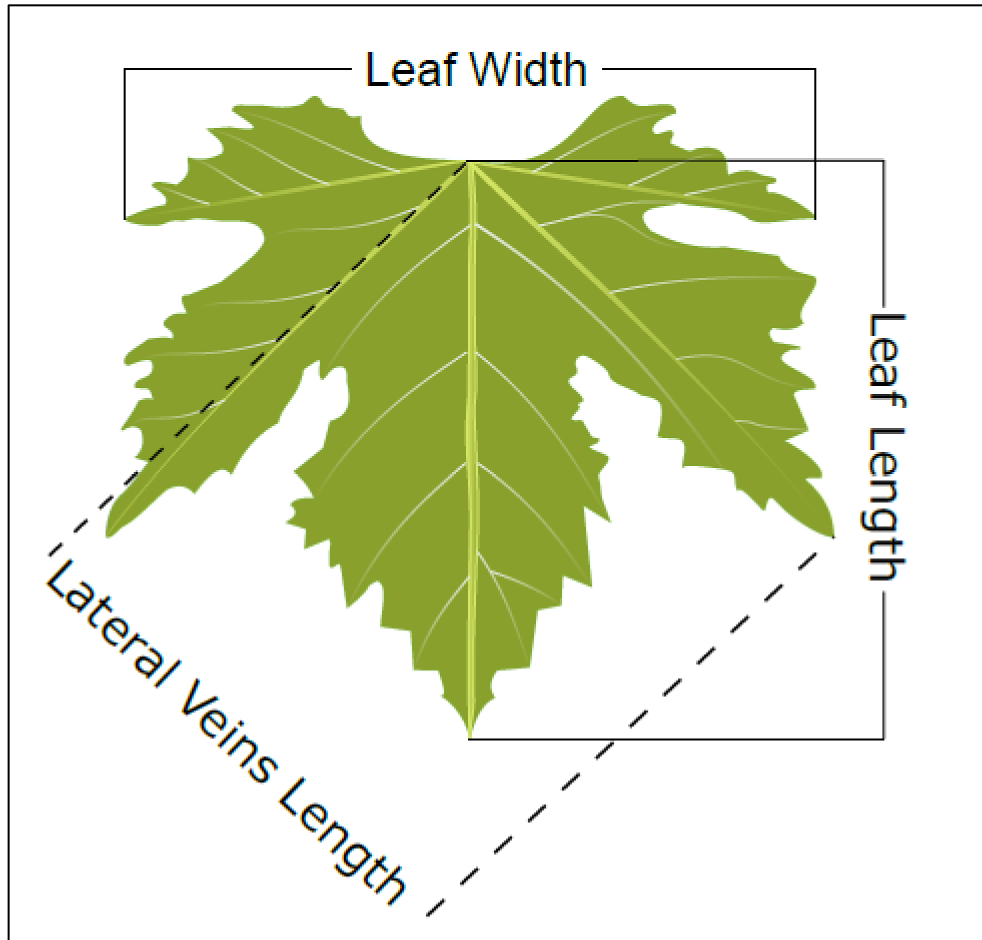


Figure 3. Graphical representation from the measured leaf traits used to develop mathematical linear models for the estimation of leaf area in eleven grape fungal resistant genotypes.

6.3.4 Models construction

The linear regressions used for the LA estimation was performed with 60% of the random data. Simple linear regression was fitted through the relationships between LA and L, L^2, W, W^2, aLV, aLV^2 or L·W. Moreover, a multiple linear regression (mLR) was fitted with L^2, W^2 and aLV^2. In total, seven simple linear regressions and one multiple linear regression models were tested. The significance of the angular and intercept coefficients, the coefficient of determination (R^2) and the square standard error (SSE) were obtained for each model. The generated models were considered for validation when their residuals presented normal distribution, tested with the Shapiro-Wilk test (α=0.05).

6.3.5 Models validation

The generated models were tested with the remaining 40% of the random data, ensuring the data independence. The estimated LA (eLA) obtained from each generated model, more the generalist model proposed by Buttaro et al. (2015), were compared with the observed LA (oLA), using the t test (α=0.05). The models were discarded when the eLA was significantly different from oLA. The remaining models were ranked by the error's statistics and user friendliness.

For ranking the validated models, it was considered the significance of the angular coefficient and intercept of the linear regression by eLA and oLA, as well as, the Pearson correlation coefficient (r) among eLA and oLA, Root Mean Square Error (RMSE), model bias (%) and efficiency. As user more friendly was considered the model that required a lower number of leaf traits that should be measured.

6.3.6 Leaf Deformation Index

To complement the allometric study, we proposed a methodology to calculate the leaf deformation index (LDI), which express the average difference of the lateral veins, right (LV_r) and left (LV_l) in mm cm^{-1}. Thus, the LDI was calculated by the equation $LDI = \frac{|LV_r - LV_l|}{aLV} x\ 10$.

6.3.7 Genotype x Environment Interaction

To avoid the collinearity we selected the variable LA, LDI and the ratio L W^{-1}, L aLV^{-1} and W aLV^{-1} for statistical analysis. These were contrasted using ANOVA (α=0.05) and when the variation was significant the Scott Knott (α=0.05) test was performed to understand genotype and environment effects. The same data were transformed to normalize the variation and employed to perform a Principal Component Analysis (PCA). The two principal components (PC1 and PC2) were used to plot a kernel density estimation (KDE). Thus, it was

possible to demonstrate the behavior and the phenotypic plasticity from all genotypes into each environment and for all environments into each genotype.

6.3.8 Statistical analysis

The statistical analysis was performed using the statistical program R v. 3.5.1 (R CORE TEAM, 2013), employing the packages SkottKnot (JELIHOVSCHI; FARIA; ALLAMAN, 2014), ggmap (KAHLE; WICKHAM, 2013), vegan (OKSANEN et al., 2019) and ggplot2 (WICKHAM, 2015). The kernel density estimation and graphics were constructed using the Python3.x language, using the libraries numpy, matplotlib v. 3.1.1 (HUNTER, 2007), pandas and seaborn v.0.9.0 (WASKOM et al., 2018).

6.4 RESULTS

6.4.1 Genotype and environment interaction (GxE)

The shortest L measured among all leaves was 3.0 cm from the cvs. 'Baron', 'Calardis Blanc' and ABS15, whereas the longest L was 18.2 cm from the genotype ABS24. The W ranged from 3.6 cm (ABS15) to 24.1 cm (ABS24), while the observed LA ranged from 9.0 cm^2 (cv. 'Baron') to 430.4 cm^2 (ABS15). The aLV ranged from 2.50 cm (cvs. 'Baron', 'Calardis Blanc', and 'Regent') to 17.90 cm (ABS24) and the ratio L W^{-1} ranged from 0.49 cm (cv. 'Prior') to 1.46 cm (cv. 'Felicia') (Figure 4). The LDI ranged from 0.00 mm cm^{-1} (obtained in few leaves of any cultivars) to 5.03 mm cm^{-1} reported from one leaf of cv. 'Aromera'.

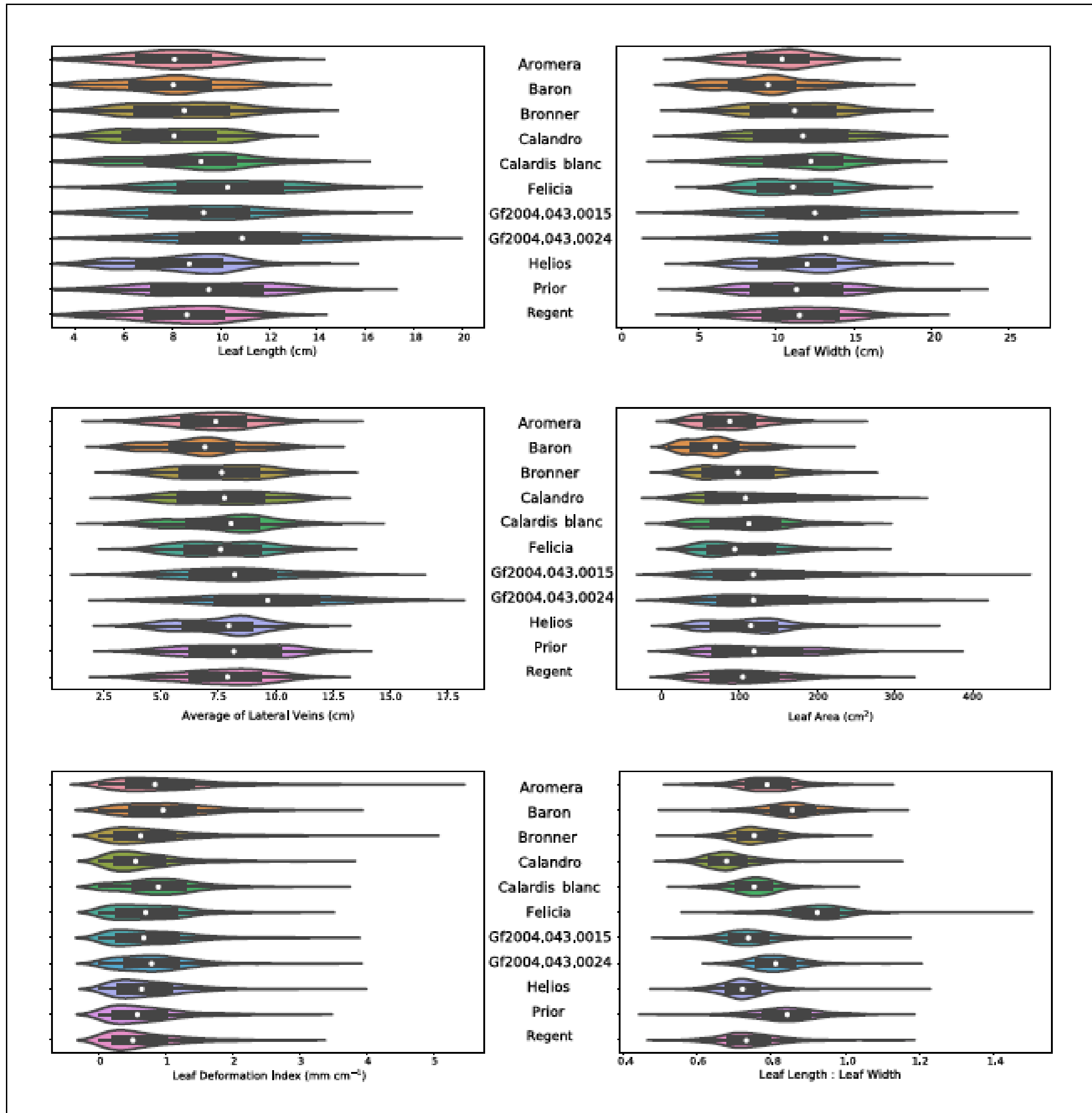


Figure 4. Values variation of the measured leaf traits from eleven fungal diseases resistant genotypes, cultivated in five locations of Santa Catarina State, Southern Brazil.

The relationship between the traits L W^{-1}, L aLV^{-1}, and W aLV^{-1}, as well as, the LDI and the LA are means to access the leaf shape. Quantifying the variation of these traits in divergent environmental conditions is important to better understand the magnitude of the genetic and environmental influences and their interaction on the leaf shape. Significant differences of all traits were observed between the genotypes and the environments, as well as, a significant GxE interaction occurred (Table 1).

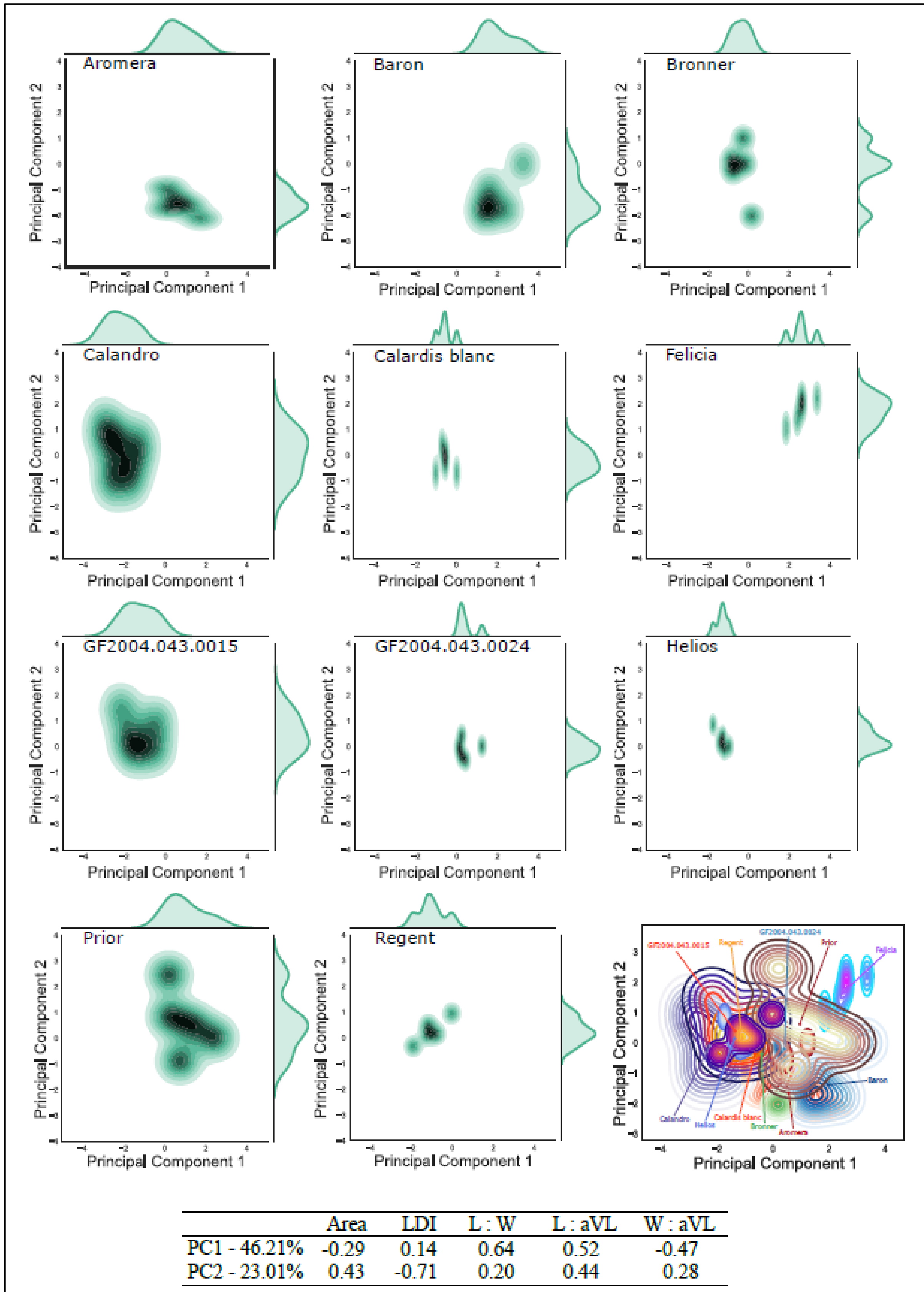
Table 1. ANOVA performed with allometric traits from leaves of fungal resistant genotypes (*V. vinifera*) grown in five locations of Santa Catarina State, Brazil. The measured traits used were leaf area, leaf deformation index (LDI), relationship between leaf length and width (L W^{-1}), length and average of lateral veins (L aLV^{-1}) and width and average of lateral veins (W aLV^{-1})

	Sum square	df	F	p value
		Leaf Area		
Genotype	1,635,937	10	58.15	< 0.01
Environment	1,667,849	4	148.21	< 0.01
Interaction GxE	1,201,854	40	10.68	< 0.01
Residual	15,318,250	5,445	–	–
		LDI		
Genotype	65.75	10	18.94	< 0.01
Environment	38.15	4	27.47	< 0.01
Interaction GxE	51.27	40	3.69	< 0.01
Residual	1,890.56	5,445	–	–
		L W^{-1}		
Genotype	22.65	10	456.25	< 0.01
Environment	2.26	4	113.60	< 0.01
Interaction GxE	1.15	40	5.80	< 0.01
Residual	27.03	5,445	–	–
		L aLV^{-1}		
Genotype	32.08	10	468.31	< 0.01
Environment	1.42	4	51.76	< 0.01
Interaction GxE	1.44	40	5.27	< 0.01
Residual	37.29	5,445	–	–
		W aLV^{-1}		
Genotype	19.91	10	194.77	< 0.01
Environment	1.83	4	44.66	< 0.01
Interaction GxE	2.95	40	7.21	< 0.01
Residual	55.65	5,445	–	–

The scott-knott test revealed that the smallest LA in Urussanga, Videira, Curitibanos, and Água Doce was reported from cv. ‘Baron’, but in Videira it didn’t differ from cvs ‘Aromera’ and ‘Regent’. In São Joaquim, the cvs. ‘Aromera’, ‘Bronner’ and ‘Calandro’ presented the smallest leaves (Table S1). The ABS15, ABS24, ‘Calandro’ and ‘Regent’ have shown the largest LA in the lowest (Urussanga) and also in the highest altitude (Água Doce). However, they didn’t differ from ‘Bronner’ in Urussanga and from ‘Calardis blanc’, ‘Felicia’ and ‘Prior’ in Água Doce. In videira, Curitibanos and São Joaquim, the largest LA was reported for ABS15, ‘Calandro’ and ‘Prior’, respectively.

The PCA expressed 69.2% of the variation in the two first dimensions. The cultivars were better separated by PC1, which expressed 46.21% of total variation, and was positively

loaded by $L\,W^{-1}$ and $L\,aLV^{-1}$ and negatively loaded by $W\,aLV^{-1}$ (Figure 5). The PC2 expressed 23.01% of total variation and segregated better the locations and was positively loaded by the ratio $L\,aLV^{-1}$ and leaf area, and negatively by LDI. The genotypes 'Calardis Blanc', 'Felicia', ABS24, 'Helios', and 'Regent' showed a lower allometric plasticity when compared to the genotypes 'Aromera', 'Baron', 'Bronner', 'Calandro', ABS15, and 'Prior' (Figure 6).



	Area	LDI	L : W	L : aVL	W : aVL
PC1 - 46.21%	-0.29	0.14	0.64	0.52	-0.47
PC2 - 23.01%	0.43	-0.71	0.20	0.44	0.28

Figure 5. Principal component analysis from eleven fungus resistant genotypes cultivated in five locations of Santa Catarina State, Brazil, using the leaf traits leaf area, leaf deformation index (LDI), ratio between leaf length and width ($L W^{-1}$), length and average of lateral veins ($L aLV^{-1}$), and width and average of lateral veins ($W aLV^{-1}$).

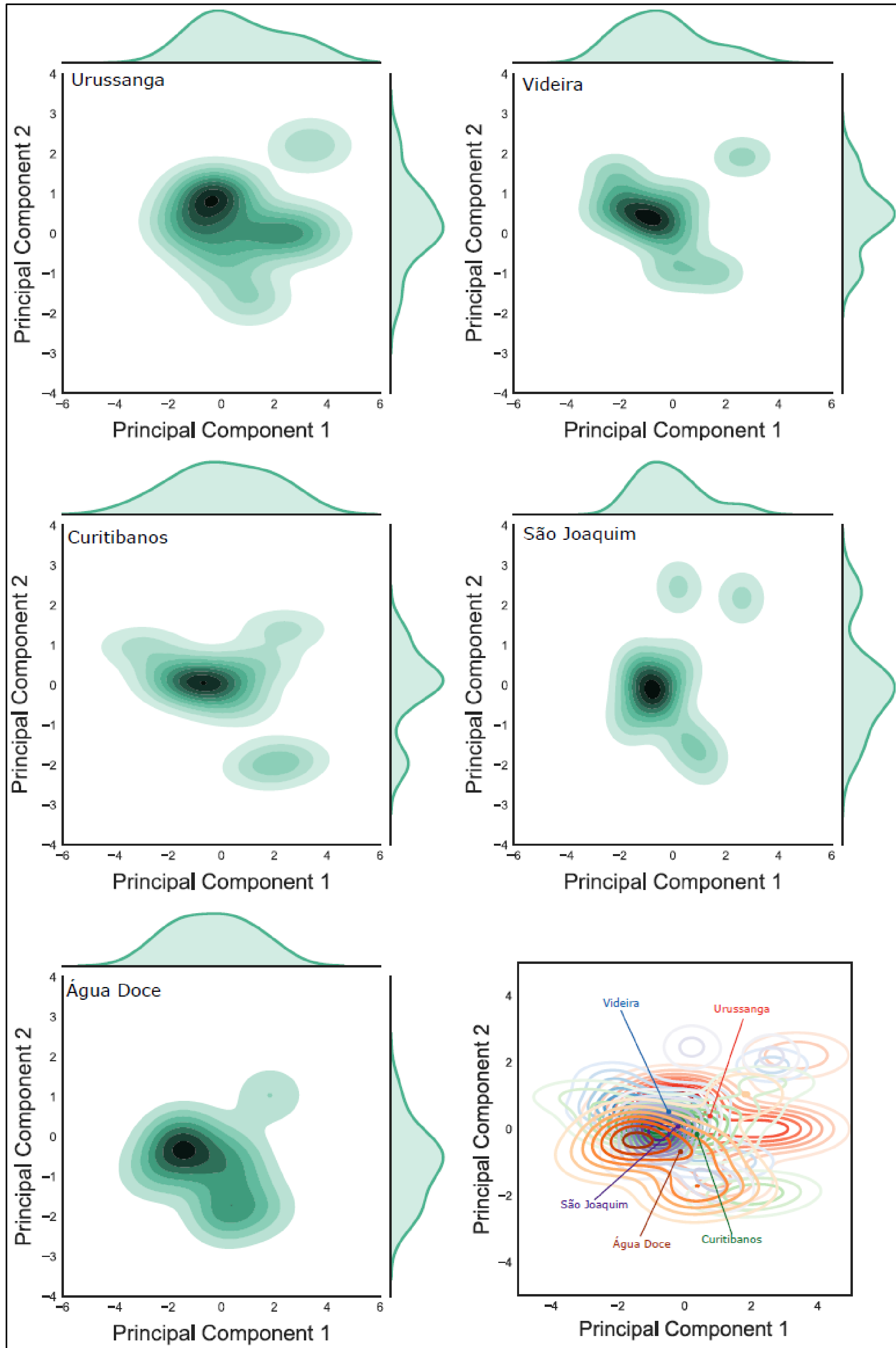


Figure 6. Principal component analysis from five locations of Santa Catarina State, Brazil cultivated with eleven fungus resistant genotypes using leaf traits, leaf area, leaf deformation index (LDI), ratio between leaf length and width (L W^{-1}), length and average of lateral veins (L aLV^{-1}), and width and average of lateral veins (W aLV^{-1}).

6.4.2 Fitted LA models

The generated models to estimate the LA in all evaluated genotypes presented angular coefficient significantly different from zero and few non-significant intercepts (Table S2_A). It is important to highlight the high coefficient of determination observed for all generated models, except those generated from the L for 'Calandro' and 'Regent'. The mLR models, fitted by L^2, W^2, and aLV^2, revealed that the effect of the L was only not significant at 1% in the cv. 'Regent' and had a negative significant coefficient in cv. 'Calandro', while W and aLV were significant at 1% for all genotypes evaluated (Table S2_B). These results corroborate with the lower coefficient observed for L in the simple linear regression models for the cvs. 'Regent' and 'Calandro', showing that it is not adequate to estimate the LA in these two cultivars.

6.4.3 LA models validation

From all models generated in this work, only the models obtained from L, L^2, W and aLV for cv. 'Baron' and from L and aLV for cv. 'Prior' were excluded from the validation because they returned eLA significantly different from the oLA (t test < 0,05; Table S3). The Buttaro general model returned non-significant differences between eLA and oLA only for 'Baron', 'Felicia' and ABS24 (Table S3) and were also validated.

The linear regression between oLA and eLA demonstrates the high correlation between these areas in models generated for any cultivar (Figure S1). The ranking of the validated models for each genotype is shown in Table S4. The model that best ranked to estimate the LA for each genotype is described in Table 2. Four models require one single measure. The W^2 was the best variable for the estimation of LA from de cvs. 'Calandro', 'Calardis Blanc', and 'Felicia', while L^2 don't returned the best model for any cultivars. Models fitted by L W returned the best LA estimation for the cvs. 'Aromera', 'Helios' and 'Prior' and the genotypes ABS15 and ABS24. Multiple linear regression was required for LA estimation from cvs. 'Baron', 'Bronner' and 'Regent'.

Considering the use of a single measure (L or W), in order to facilitate LA estimation, the use of W returned better models in all cultivars and genotypes, except from cv. 'Helios'. However, L cannot be used only in the cv. 'Baron'.

Table 2. Models to estimate the leaf area (LA) from eleven PIWI genotypes (*Vitis vinifera*) obtained from simple and multiple linear regression employing the measured leaf traits length (L), width (W) and average lateral veins (aLV). For each genotype two models are presented: 1) the best ranked model of all, and 2) the best ranked model considering the use of only one measure. The accuracy was determined by the bias (BIAS), the precision was determined by the correlation coefficient (r) and Root Mean Square Error (RMSE), while robustness was determined by angular coefficient and model efficiency (EF). The statistics were obtained in the models validation, comparing estimated and observed LA.

	Model	r	RMSE	BIAS	EF
	---------- Aromera ----------				
1	$LA = 0.99LW + 2.76$	0.98	9.88 cm^2	-3.68%	0.96
2	$LA = 0.77W^2 + 3.95$	0.96	13.05 cm^2	-1.77%	0.94
	---------- Baron ----------				
1	$LA = 0.24L^2+0.32W^2+0.47aLV^2+1.85$	0.98	3.98 cm^2	-0.34%	0.95
2	$LA = 0.73W^2+2.98$	0.97	11.34 cm^2	-0.21%	0.94
	---------- Bronner ----------				
1	$LA = 0.44L^2+0.30W^2+0.46aLV^2+0.12$	0.98	5.82 cm^2	0.20%	0.96
2	$LA = 0.75W^2+4.21$	0.98	14.15 cm^2	4.54%	0.95
	---------- Calandro ----------				
1	$LA = 0.82W^2 - 1.05$	0.99	16.35 cm^2	-1.32%	0.98
	---------- Calardis Blanc ----------				
1	$LA = 0.75W^2 - 0.25$	0.97	11.99 cm^2	-0.02%	0.93
	---------- Felicia ----------				
1	$LA = 0.78W^2 - 1.91$	0.98	9.90 cm^2	0.75%	0.97
	---------- ABS15 ----------				
1	$LA = 1.08LW - 3.33$	0.98	23.56 cm^2	-1.90%	0.95
2	$LA = 0.73W^2+3.88$	0.98	24.59 cm^2	-2.82%	0.96
	---------- ABS24 ----------				
1	$LA = 0.85LW - 0.42$	0.98	16.83 cm^2	0.09%	0.96
2	$LA = 0.66W^2+2.96$	0.98	18.66 cm^2	-0.68%	0.95
	---------- Helios ----------				
1	$LA = 1.06LW + 3.07$	0.97	14.57 cm^2	1.50%	0.95
2	$LA = 1.41L^2+5.68$	0.96	16.27 cm^2	0.05%	0.92
	---------- Prior ----------				
1	$LA = 1.05LW + 3.60$	0.97	18.51 cm^2	-2.21%	0.95
2	$LA = 0.84W^2+6.71$	0.96	22.27 cm^2	-2.19%	0.92
	---------- Regent ----------				
1	$LA = 0.11L^2+0.40W^2+0.77aLV^2-4.15$	0.98	2.83 cm^2	-1.70%	0.97
2	$LA = 0.73W^2+3.38$	0.98	13.07 cm^2	-2.49%	0.95

6.5 DISCUSSION

The allometric plasticity is the differential behavior presented by the same population or the same genotype in different environments and has an important role in genotypes adaptations (LANE et al., 2019; VITASSE et al., 2013; WEINER, 2004). Thus, to know the interaction between genotypes and the environment is important to predict the behavior of these genotypes in another climatic scenarios (LANDE; SHANNON, 1996).

In this investigation our results demonstrated a clear genotype-environment interaction of leaf morphology related traits and the LA, as described for *Eugenia calycina* (CARDOSO; LOMÔNACO, 2003) and *Berberis lycium* (RAHMAN et al., 2019), The PCA analysis revealed that the genotype has a larger effect than the environment on the leaf morphology.

Grapevine leaf traits are influenced by several genes (CHITWOOD et al., 2014; WELTER et al., 2007b). This pattern of inheritance is also described in other species, such as maize (CUI et al., 2017) and wheat (HUSSAIN et al., 2017). The quantitative nature of leaf traits explains the significant phenotypic variation promoted by the environment, as well, the genotype-environment interaction. But, it is also shown that for the other traits evaluated, such as $L\ W^{-1}$, $L\ aLV^{-1}$ and $W\ aLV^{-1}$, the genetic effect was superior to the effect of the environment, evidenced by the greater genetic influence on the variation, as well, as the separation of clusters from genotypes in multivariate analysis.

The Knowledge about the leaf morphological characteristics and their behavior in relation to the environment is of high importance for the development and adoption of new cultivation forms, especially for PIWI cultivar (KRAUS et al., 2018). Therefore, the availability of nondestructive methods of LA estimation is fundamental for studies related to crop grown, such as ecology, physiology, pathology and agronomic performance (COLAIZZI et al., 2017; FORRESTER et al., 2017; HANG et al., 2019; PUDUMALAR; MUTHURAMALINGAM; SHANMUGAPRIYAN, 2020; ZEIST et al., 2017). In this

investigation we developed simple, rapid, inexpensive, robust, precise, accurate and nondestructive LA models for eight PIWI cultivars. To our knowledge, these are the first models available to estimate the LA in PIWI cultivar.

Models for LA estimation with greater precision were generated from the measurement of the product between L and W from nine *V. vinifera* cultivars (BUTTARO et al., 2015). These results are also reported in this study for the cvs. ‘Aromera’, ‘Helios’ and ‘Prior’ and the genotypes ABS15, ABS24. Considering the addition of the aLV, and employing the mLR model, better results were obtained only in the cvs. ‘Baron’, ‘Bronner’ and ‘Regent’. Simple linear models, generated with only one leaf trait, returned better results for cvs. ‘Calandro’, ‘Calardis Blanc’, and ‘Felicia’, using W.

The use of the lateral veins and L was cited in the literature to estimated LA from cvs. ‘BRS-Violeta’ and ‘Cabernet Sauvignon’ (AMARANTE et al., 2009; MALAGI et al., 2010). However, these works used only the coefficient of determination (R^2) to choose the model, without an adequate validation. Even as L and W, for the present evaluated cultivars, also aLV generate models with reliability, precision and accuracy, with high efficiency and low bias (Table S3). High correlation between aLV dimensions and LA were also reported for ‘Sauvignon Blanc’ and ‘Cabernet Sauvignon’ (BORGHEZAN et al., 2010).

Taking in consideration the user-friendliness of the models for LA estimation, which is easier to measure only one dimension (L, W), the W return best ranked models for ten cultivars, while, only cv. Helios present the best ranked model using the L. These results contrast with those reported for *V. vinifera* hybrids with American species such as cvs. ‘De Chaunac’ and ‘Niagara’ where LA is best simulated by the L (WILLIAMS; MARTINSON, 2003b), probably due the differences on the leaf shape.

In addition to the fact that LVs measures can improve the robustness of LA estimation by the implementation of the mLR models, these measures can also be used to estimate the

LDI. The proposed methodology to describe the leaf asymmetry is new and can be helpful in research aiming to know the effect of some external factors, such as biotic or abiotic stresses, on the leaf shape. Although the environmental influence on the leaf shape is already known (CHITWOOD et al., 2014), this is the first research investigating the behavior of the leaf deformation.

Taking in consideration the practical aspects of the application of LA estimation models, using the measurement of only one dimension, it is recommended to use models from W^2 for all genotypes evaluated in this study. However, we also presented models with a greater robustness that can be employed in automated acquired measurement systems.

6.6 CONCLUSION

1. The trait(s) that returned the most adequate LA estimation model was different according to the genotypes evaluated.
2. For most of the genotypes, the best model for the LA estimation involved at least two measurements (traits), however for all genotypes the use of a single measurements maintained a high precision and accuracy.
3. In case of using a single measure for LA estimation, the leaf width is the most adequate for all genotypes evaluated, except for cv. ‘Helios’, for which leaf length was more adequate.
4. There was significant genotype-environment interaction for all leaf traits evaluated, reinforcing the importance of developing genotype-specific models.
5. The generic model could only be used to estimate the LA for cvs. Baron, Felicia and ABS24, however, even in these genotypes returned values with lower precision and accuracy.

6.7 ACKNOWLEDGEMENTS

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6.8 SUPPLEMENTARY MATERIAL

Table S1. Scott-Knott clustering test performed with allometric traits from leaves of fungal resistant genotypes (*V. vinifera*) grown in five locations of Santa Catarina State, Brazil. The measured used traits were leaf area, leaf deformation index (LDI), relationship between leaf length and width (L W^{-1}), length and average of lateral veins (L aLV^{-1}) width and average of lateral veins (W aLV^{-1})

Genotypes	Vineyard's locations				
	Urussanga	**Videira**	**Curitibanos**	**São Joaquim**	**Água Doce**
			----------------------- Leaf Area -----------------------		
Aromera	72.4 cB*	106.8 eA	102.5 dA	87.2 eB	78.4 bB
Baron	30.0 dC	99.6 eA	67.1 eB	107.4 dA	60.0 cB
Bronner	99.6 aB	120.3 dA	116.7 dA	95.7 eB	75.0 bC
Calandro	97.7 aC	122.2 dB	173.5 aA	93.2 eC	107.3 aC
Calardis Blanc	83.1 bC	138.5 cA	128.7 cA	109.7 dB	96.2 aC
Felicia	65.3 cC	112.3 dB	136.6 cA	106.1 dB	105.5 aB
Gf.2004.043.0015	106.6 aD	177.3 aA	146.0 bB	126.1 cC	107.3 aD
Gf.2004.043.0024	99.7 aC	156.8 bA	143.4 bA	148.5 bA	118.7 aB
Helios	86.0 bB	135.0 cA	120.6 dA	127.3 cA	85.1 bB
Prior	75.3 cD	139.1 cB	128.5 cB	172.7 aA	109.9 aC
Regent	104.5 aB	96.1 eB	113.9 dA	123.3 cA	105.2 aB
			----------------------- LDI -----------------------		
Aromera	0.93 aB	0.91 aB	1.17 aA	0.90 bB	0.98 aB
Baron	0.60 cB	1.02 aA	1.08 aA	1.17 aA	1.04 aA
Bronner	0.51 cC	0.74 bB	0.75 cB	0.90 bA	1.06 aA
Calandro	0.53 cB	0.56 bB	0.64 cB	0.80 cA	0.85 bA
Calardis Blanc	0.96 aA	0.90 aA	0.90 bA	0.91 bA	0.93 aA
Felicia	0.54 cC	0.74 bB	0.94 bA	0.73 cB	0.83 bA
Gf.2004.043.0015	0.65 bB	0.69 bB	0.81 cA	0.91 bA	0.83 bA
Gf.2004.043.0024	0.70 bB	0.88 aA	0.98 bA	0.75 cB	0.91 aA
Helios	0.68 bB	0.70 bB	0.89 bA	0.75 cB	0.65 bB
Prior	0.67 bB	0.67 bB	0.73 cB	0.47 dC	0.96 aA
Regent	0.51 cB	0.56 bB	0.73 cA	0.76 cA	0.75 bA
			----------------------- L W^{-1} -----------------------		
Aromera	0.81 eB	0.77 dC	0.84 bA	0.78 dC	0.75 dD
Baron	0.91 bA	0.85 bB	0.87 bB	0.83 bC	0.82 bC
Bronner	0.78 fA	0.75 dB	0.78 dA	0.74 eB	0.76 dB
Calandro	0.73 gA	0.68 fB	0.68 fB	0.70 gB	0.65 gC
Calardis Blanc	0.77 fA	0.76 dA	0.76 dA	0.75 eA	0.72 eB
Felicia	0.95 aA	0.93 aB	0.92 aB	0.92 aB	0.87 aC
Gf.2004.043.0015	0.77 fA	0.72 eB	0.73 eB	0.77 dA	0.72 eB
Gf.2004.043.0024	0.85 dA	0.80 cB	0.82 cB	0.81 cB	0.79 cB
Helios	0.73 gA	0.72 eA	0.73 eA	0.73 fA	0.71 eA
Prior	0.89 cA	0.81 cD	0.86 bB	0.84 bC	0.81 bD
Regent	0.79 eA	0.73 eC	0.76 dB	0.74 eB	0.69 fD
			----------------------- L aLV^{-1} -----------------------		

Aromera	1.10 eA	1.10 dA	1.12 cA	1.08 dB	1.08 dB
Baron	1.22 bA	1.18 bB	1.17 bB	1.14 cC	1.15 bC
Bronner	1.13 dB	1.10 dC	1.11 cC	1.17 bA	1.08 dC
Calandro	1.05 fA	1.03 fA	1.03 dA	1.04 eA	1.01 fB
Calardis Blanc	1.17 cA	1.16 bA	1.15 bA	1.14 cB	1.11 cC
Felicia	1.38 aA	1.36 aB	1.35 aB	1.38 aA	1.30 aC
Gf.2004.043.0015	1.17 cA	1.11 dC	1.10 cC	1.14 cB	1.10 dC
Gf.2004.043.0024	1.14 dA	1.11 dA	1.13 cA	1.12 cA	1.14 bA
Helios	1.10 eB	1.11 dA	1.12 cA	1.10 dA	1.08 dB
Prior	1.18 cA	1.13 cB	1.16 bA	1.17 bA	1.13 cB
Regent	1.12 dA	1.08 eB	1.11 cA	1.09 dB	1.04 eC
	------------------------ W aLV^{-1} ------------------------				
Aromera	1.37 dC	1.43 cA	1.33 eD	1.40 dB	1.44 cA
Baron	1.34 eB	1.39 dA	1.36 eB	1.38 dA	1.40 dA
Bronner	1.46 bB	1.47 bB	1.43 cC	1.57 aA	1.44 cC
Calandro	1.46 bC	1.52 aB	1.52 aB	1.50 cB	1.56 aA
Calardis Blanc	1.53 aA	1.53 aA	1.50 aA	1.52 bA	1.54 aA
Felicia	1.46 bB	1.47 bB	1.47 bB	1.51 bA	1.49 bA
Gf.2004.043.0015	1.52 aA	1.54 aA	1.52 aA	1.49 cB	1.54 aA
Gf.2004.043.0024	1.36 dC	1.39 dB	1.39 dB	1.38 dB	1.44 cA
Helios	1.51 aB	1.55 aA	1.54 aA	1.52 bB	1.54 aA
Prior	1.33 eB	1.40 dA	1.36 eB	1.40 dA	1.40 dA
Regent	1.43 cC	1.49 bB	1.47 bB	1.48 cB	1.53 aA

*Means follow by the same capital letter on the lines and lower-case letters in columns do not differ significantly by Scott-knott test (α=0.05).

Table S2_A. Parameter of the generated models based on linear regression between leaf area (LA) and leaf length (L), width (W), average of lateral veins length (aLV) and L W

Model	Coefficient Angular (b)	Coefficient Intercept (a)	R^2	SSE[1]	p value (SW)[2]
		Aromera			
LA = b L + a	18.02**	-58.17**	0.90	11.88	>0.05
LA = b L^2 + a	1.14**	7.84**	0.92	10.50	>0.05
LA = b W + a	14.79**	-62.36**	0.93	9.41	>0.05
LA = b W^2 + a	0.77**	3.95**	0.96	7.82	>0.05
LA = b aLV + a	20.49**	-63.49**	0.95	8.40	>0.05
LA = b aLV^2 + a	1.45**	4.43**	0.96	7.22	>0.05
LA = b L W + a	0.99**	2.76*	0.96	7.71	>0.05
		Baron			
LA = b L + a	13.34**	-39.98**	0.91	7.18	>0.05
LA = b L^2 + a	0.99**	1.59NS	0.93	6.21	>0.05
LA = b W + a	11.11**	-36.48**	0.95	5.03	>0.05
LA = b W^2 + a	0.73**	2.98**	0.96	4.60	>0.05
LA = b aLV + a	16.05**	-41.20**	0.94	5.81	>0.05
LA = b aLV^2 + a	1.43**	0.59NS	0.97	4.57	>0.05
LA = b L W + a	0.89**	0.41NS	0.98	3.86	>0.05
		Bronner			
LA = b L + a	20.58**	-73.29**	0.93	12.80	>0.05
LA = b L^2 + a	1.27**	3.57**	0.96	9.51	>0.05
LA = b W + a	15.84**	-73.78**	0.95	10.80	>0.05
LA = b W^2 + a	0.75**	4.21**	0.96	9.12	>0.05
LA = b aLV + a	24.19**	-83.39**	0.95	10.20	>0.05
LA = b aLV^2 + a	1.62**	0.57NS	0.97	8.44	>0.05
LA = b L W + a	0.99**	2.56**	0.98	7.55	>0.05
		Calandro			
LA = b L + a	30.09**	-116.53**	0.85	27.90	>0.05
LA = b L^2 + a	1.91**	-7.11*	0.89	23.90	>0.05
LA = b W + a	19.49**	-105.90**	0.94	18.20	>0.05
LA = b W^2 + a	0.82**	-1.05NS	0.98	10.60	>0.05
LA = b aLV + a	31.72**	-122.50**	0.93	19.50	>0.05
LA = b aLV^2 + a	2.05**	-9.49**	0.95	16.00	>0.05
LA = b L W + a	1.27**	-5.84	0.96	13.29	>0.05
		Calardis Blanc			
LA = b L + a	20.93**	-75.34**	0.93	15.30	>0.05
LA = b L^2 + a	1.20**	4.77**	0.96	11.00	>0.05
LA = b W + a	16.29**	-81.69**	0.96	11.00	>0.05
LA = b W^2 + a	0.75**	-0.25NS	0.98	7.15	>0.05
LA = b aLV + a	24.82**	-80.52**	0.94	13.60	>0.05
LA = b aLV^2 + a	1.71**	0.65NS	0.97	9.69	>0.05
LA = b L W + a	1.00**	-0.52NS	0.98	7.70	>0.05
		Felicia			
LA = b L + a	18.07**	-82.77**	0.91	14.20	>0.05

LA = *b* L^2 + *a*	0.89^{**}	2.22^{NS}	0.94	12.20	>0.05
LA = *b* W + *a*	17.98^{**}	-98.21^{**}	0.97	8.93	>0.05
LA = *b* W^2 + *a*	0.78^{**}	-1.91^{*}	0.98	6.56	>0.05
LA = *b* aLV + *a*	26.08^{**}	-94.55^{**}	0.95	11.00	>0.05
LA = *b* aLV^2 + *a*	1.74^{**}	-2.66^{*}	0.97	8.29	>0.05
LA = *b* L W + *a*	0.88^{**}	-3.01^{**}	0.98	7.42	>0.05
----- ABS15 -----					
LA = *b* L + *a*	24.09^{**}	-92.95^{**}	0.88	21.50	>0.05
LA = *b* L^2 + *a*	1.48^{**}	-3.13^{NS}	0.92	18.30	>0.05
LA = *b* W + *a*	17.30^{**}	-89.03^{**}	0.95	14.30	>0.05
LA = *b* W^2 + *a*	0.73^{**}	3.88^{**}	0.98	9.33	>0.05
LA = *b* aLV + *a*	27.98^{**}	-100.28^{**}	0.95	13.90	>0.05
LA = *b* aLV^2 + *a*	1.80^{**}	-0.48^{NS}	0.98	9.19	>0.05
LA = *b* L W + *a*	1.08^{**}	-3.33^{*}	0.97	11.10	>0.05
----- ABS24 -----					
LA = *b* L + *a*	20.72^{**}	-93.80^{**}	0.92	19.60	>0.05
LA = *b* L^2 + *a*	1.04^{**}	-0.16^{NS}	0.95	15.90	>0.05
LA = *b* W + *a*	16.51^{**}	-90.42^{**}	0.95	15.10	>0.05
LA = *b* W^2 + *a*	0.66^{**}	2.96^{*}	0.98	9.98	>0.05
LA = *b* aLV + *a*	23.81^{**}	-96.09^{**}	0.93	18.20	>0.05
LA = *b* aLV^2 + *a*	1.33^{**}	0.05^{NS}	0.96	13.70	>0.05
LA = *b* L W + *a*	0.85^{**}	-0.42^{NS}	0.97	11.60	>0.05
----- Helios -----					
LA = *b* L + *a*	20.78^{**}	-64.95^{**}	0.92	12.10	>0.05
LA = *b* L^2 + *a*	1.41^{**}	5.68^{**}	0.97	7.70	>0.05
LA = *b* W + *a*	15.80^{**}	-73.68^{**}	0.94	10.80	>0.05
LA = *b* W^2 + *a*	0.75^{**}	4.40^{**}	0.95	10.20	>0.05
LA = *b* aLV + *a*	25.10^{**}	-79.87^{**}	0.94	10.60	>0.05
LA = *b* aLV^2 + *a*	1.90^{**}	-1.33^{NS}	0.96	8.78	>0.05
LA = *b* L W + *a*	1.06^{**}	3.07^{**}	0.97	7.63	>0.05
----- Prior -----					
LA = *b* L + *a*	20.89^{**}	-78.65^{**}	0.90	16.90	>0.05
LA = *b* L^2 + *a*	1.18^{**}	5.80^{**}	0.93	13.40	>0.05
LA = *b* W + *a*	17.83^{**}	-79.35^{**}	0.95	12.30	>0.05
LA = *b* W^2 + *a*	0.84^{**}	6.71^{**}	0.98	8.43	>0.05
LA = *b* aLV + *a*	25.22^{**}	-86.49^{**}	0.94	12.80	>0.05
LA = *b* aLV^2 + *a*	1.65^{**}	2.63^{*}	0.97	9.35	>0.05
LA = *b* L W + *a*	1.05^{**}	3.60^{**}	0.97	8.83	>0.05
----- Regent -----					
LA = *b* L + *a*	21.52^{**}	-75.17^{**}	0.81	21.70	>0.05
LA = *b* L^2 + *a*	1.35^{**}	2.60^{NS}	0.87	18.00	>0.05
LA = *b* W + *a*	15.90^{**}	-77.19^{**}	0.95	10.40	>0.05
LA = *b* W^2 + *a*	0.73^{**}	3.38^{*}	0.96	9.75	>0.05
LA = *b* aLV + *a*	24.78^{**}	-86.05^{**}	0.92	14.10	>0.05
LA = *b* aLV^2 + *a*	1.67^{**}	-1.13^{NS}	0.95	10.70	>0.05
LA = *b* L W + *a*	1.05^{**}	-1.75^{NS}	0.96	10.30	>0.05

[1]Square Standard Error (cm^2); [2]p value obtained in residual distribution using Shapiro-Wilk normality test. *Significate different from zero at 5% probability of α error. ** Significate different from zero at 1% probability of α error.

Table S2_B. Coefficients of multiple linear regression to fit the model of leaf area estimated according to the square of leaf length (L^2), leaf width (W^2), and average of lateral veins (aLV^2) for eleven fungal resistant genotypes cultivated in five locations of Santa Catarina state, Brazil.

Cultivares	L^2	W^2	aLV^2	Intercept
'Aromera'	0.314**	0.315**	0.555**	1.591
'Baron'	0.237**	0.324**	0.465**	1.852
'Bronner'	0.440**	0.297**	0.463**	0.116
'Calandro'	-0.140**	0.469**	0.994**	-4.240
'Calardis Blanc'	0.192**	0.480**	0.325**	1.847
'Felicia'	0.109**	0.434**	0.577**	-2.793
ABS15	0.139**	0.336**	0.803**	2.770
ABS24	0.279**	0.285**	0.414**	-0.167
'Helios'	0.522**	0.248**	0.587**	1.205
'Prior'	0.211**	0.378**	0.669**	4.997
'Regent'	0.106*	0.395**	0.769**	-4.151

*Statistically significant at α=0.05; **Statistically significant at α=0.01; NSNot statistically significant.

Table S3. Parameters of model validation for leaf area predict in eleven resistance genotypes of grapevine (*Vitis vinifera*)

	L	L^2	W	W^2	aVL	aVL^2	L·W	Buttaro et al.	mLR
					Aromera				
r[2]	0.94**	0.96**	0.94**	0.96**	0.96**	0.98**	0.98**	0.98**	0.98**
RMSE[3]	16.10	14.35	15.33	13.05	14.37	11.63	9.88	16.78	16.78
BIAS (%)[4]	- 6.68	- 7.61	- 0.67	- 1.77	- 3.98	- 5.44	- 3.68	- 14.36	-1.84
EF[5]	0.85	0.88	0.89	0.94	0.90	0.94	0.96	0.87	0.96
t-test[6]	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05
					Baron				
r	0.95**	0.94**	0.98**	0.97**	0.97**	0.97**	0.98**	0.97**	0.98**
RMSE	23.45	15.47	18.06	10.27	18.33	11.34	9.09	9.25	3.98
BIAS (%)	- 14.43	- 7.37	- 10.98	0.40	- 10.63	- 0.21	- 0.85	0.91	-0.34
EF	0.70	0.84	0.82	0.93	0.80	0.94	0.95	0.95	0.95
t-test	< 0.01	< 0.05	< 0.01	> 0.05	< 0.01	> 0.05	> 0.05	> 0.05	> 0.05
					Bronner				
r	0.96**	0.97**	0.96**	0.98**	0.97**	0.98**	0.98**	0.98**	0.98**
RMSE	16.49	13.99	15.43	14.15	20.76	19.3	10.63	15.43	5.82
BIAS (%)	- 2.20	- 2.86	3.20	4.54	0.73	- 3.13	0.32	- 9.72	0.20
EF	0.90	0.93	0.91	0.95	0.94	0.97	0.96	0.92	0.96
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.05	> 0.05
					Calandro				
r	0.97**	0.98**	0.97**	0.99**	0.97**	0.99**	0.99**	0.99**	0.97**
RMSE	21.39	18.29	20.12	16.35	20.09	16.57	15.34	29.11	6.51
BIAS (%)	6.42	3.98	5.41	- 1.32	4.45	- 3.55	1.90	- 23.32	3.41
EF	0.89	0.94	0.91	0.98	0.91	0.98	0.97	0.80	0.94
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05
					Calardis Blanc				
r	0.96**	0.96**	0.96**	0.97**	0.96**	0.97**	0.97**	0.97**	0.97**
RMSE	14.99	14.15	12.48	11.99	13.84	13.16	11.41	13.76	5.85
BIAS (%)	1.41	- 3.92	1.07	- 0.02	1.59	- 0.72	0.69	- 7.74	0.09
EF	0.92	0.93	0.92	0.93	0.91	0.94	0.94	0.91	0.95
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.05	> 0.05
					Felicia				
r	0.95**	0.96**	0.98**	0.98**	0.97**	0.98**	0.99**	0.99**	0.98**
RMSE	15.61	13.65	11.79	9.90	15.58	15.44	10.91	16.14	12.74
BIAS (%)	3.86	3.52	3.37	0.75	4.02	2.78	1.98	8.76	1.95
EF	0.90	0.91	0.96	0.97	0.93	0.94	0.96	0.90	0.96
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
					ABS15				
r	0.93**	0.96**	0.94**	0.96**	0.96**	0.98**	0.98**	0.98**	0.96**

RMSE	35.35	28.47	31.67	25.87	29.57	24.59	23.56	33.4	10.55
BIAS (%)	- 5.09	- 3.23	- 5.93	- 3.72	- 3.99	- 2.82	- 1.90	- 15.68	-0.84
EF	0.83	0.92	0.86	0.92	0.90	0.96	0.95	0.86	0.93
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05
					ABS24				
r	0.95**	0.97**	0.96**	0.98**	0.96**	0.98**	0.98**	0.98**	0.98**
RMSE	28.62	21.36	25.99	18.66	25.6	19.16	16.83	21.7	8.98
BIAS (%)	- 1.86	- 1.51	- 0.84	- 0.68	1.68	2.03	0.09	9.23	0.83
EF	0.86	0.94	0.89	0.95	0.89	0.95	0.96	0.93	0.95
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
					Helios				
r	0.95**	0.96**	0.95**	0.96**	0.94**	0.96**	0.97**	0.97**	0.96**
RMSE	20.28	16.27	20.68	18.62	19.4	16.82	14.57	24.83	11.34
BIAS (%)	- 1.62	0.05	- 2.02	0.08	- 1.91	2.54	1.50	- 14.99	0.87
EF	0.90	0.92	0.88	0.93	0.89	0.92	0.95	0.81	0.93
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05
					Prior				
r	0.94**	0.95**	0.95**	0.96**	0.96**	0.97**	0.97**	0.97**	0.96**
RMSE	27.24	24.46	21.5	22.27	24.23	20.48	18.51	31.52	9.22
BIAS (%)	- 7.45	- 6.78	- 3.21	- 2.19	- 7.11	- 5.78	- 2.21	- 16.79	-0.78
EF	0.84	0.87	0.90	0.92	0.88	0.92	0.95	0.78	0.92
t-test	< 0.05	> 0.05	> 0.05	> 0.05	< 0.05	> 0.05	> 0.05	< 0.01	> 0.05
					Regent				
r	0.95**	0.97**	0.95**	0.98**	0.96**	0.98**	0.99**	0.99**	0.98**
RMSE	22.21	18.86	18.79	13.07	19.57	15.58	12.3	22.81	2.83
BIAS (%)	- 4.98	- 7.21	- 3.88	- 2.49	- 5.12	- 7.05	- 9.28	- 20.11	-1.70
EF	0.85	0.91	0.89	0.95	0.90	0.94	0.97	0.87	0.97
t-test	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05

[1]Model suggested by Buttaro et al. (2015).
[2]Coefficient of determination between predict and observed leaf area.
[3]Root Mean Square Error (cm^2).
[4]model bias.
[5]Model efficiency.
[6]p-value to t-test between the means about predict and observed leaf area.
[7]p-value to Shapiro-Wilk normality test from equity residual.
*Significant to 5% probability of α error. ** Significant to 1% probability of α error.

Table S4. Models ranking to estimative of the LA, obtained from leaf traits as length (L), width (W), average lateral veins (aLV) and multiple linear regression in eleven PIWI cultivar (*Vitis vinifera*), as well as the Buttaro generalistic model to *V. vinifera* LA estimation. higlight in bold the better model and in italic the better model considering only one measure for each cultivar

Statistic	L	L^2	W	W^2	aVL	aVL^2	L·W	Buttaro et al.[1]	mLR
					Aromera				
r	7	4	7	*4*	4	1	**1**		1
RMSE	7	4	6	*3*	5	2	**1**		8
BIAS (%)	7	8	1	*2*	5	6	**4**	No validated model	3
EF	8	7	6	*3*	5	3	**1**		1
Slope	8	5	6	*2*	7	4	**2**		1
Ranking	7.4 (8)	5.6 (7)	5.2 (5)	*2.8 (2)*	5.2 (5)	3.2 (4)	**1.8 (1)**		2.8 (2)
					Baron				
r				*3*		3	1	3	**1**
RMSE				*4*		5	2	3	**1**
BIAS (%)	No validated model	No validated model	No validated model	*3*	No validated model	1	4	5	**2**
EF				*5*		4	1	1	**1**
Slope				*2*		1	3	4	**5**
Ranking				*3.4 (5)*		2.8 (3)	2.2 (2)	3.2 (4)	**2 (1)**
					Bronner				
r	3	2	3	*1*	2	1	1		**1**
RMSE	6	3	5	*4*	8	7	2		**1**
BIAS (%)	4	5	7	*8*	3	6	2	No validated model	**1**
EF	7	5	6	*3*	4	1	2		**2**
Slope	8	5	7	*2*	5	3	4		**1**
Ranking	5.6 (7)	4 (5)	5.6 (7)	*3.6 (3)*	4.4 (6)	3.6 (3)	2.2 (2)		**1.2 (1)**
					Calandro				
r	3	2	3	***1***	3	1	1		3
RMSE	8	5	7	***3***	6	4	2		1
BIAS (%)	8	5	7	***1***	6	4	2	No validated model	3
EF	5	3	4	***1***	4	1	2		3
Slope	3	1	5	***4***	8	7	5		1
Ranking	5.4 (7)	3.2 (4)	5.2 (6)	***2 (1)***	5.4 (7)	3.4 (5)	2.4 (3)		2.2 (2)
					Calardis Blanc				
r	2	2	2	***1***	2	1	1		1
RMSE	8	7	4	***3***	6	5	2		1
BIAS (%)	6	8	5	***1***	7	4	3	No validated model	2
EF	4	3	4	***3***	5	2	2		1
Slope	6	4	7	***1***	8	1	3		5
Ranking	5.2 (7)	4.8 (6)	4.4 (5)	***1.8 (1)***	5.6 (8)	2.6 (4)	2.2 (3)		2 (2)
					Felicia				
r	5	4	2	***2***	3	2	1	1	2
RMSE	8	5	3	***1***	7	6	2	9	4
BIAS (%)	7	6	5	***1***	8	4	3	9	2

EF	6	5	2	***1***	4	3	2	6	2
Slope	8	2	4	***1***	4	8	6	6	3
Ranking	6.8 (9)	4.4 (5)	3.2 (4)	***1.2 (1)***	5.2 (7)	4.6 (6)	2.8 (3)	6.2 (8)	2.6 (2)
ABS15									
r	4	2	3	*2*	2	1	**1**	No validated model	2
RMSE	8	5	7	*4*	6	3	**2**		1
BIAS (%)	7	4	8	*5*	6	3	**2**		1
EF	7	4	6	*4*	5	1	**2**		3
Slope	8	5	6	*2*	6	2	**1**		4
Ranking	6.8 (8)	4 (5)	6 (7)	*3.4 (4)*	5 (6)	2 (2)	**1.6 (1)**		2.2 (3)
ABS24									
r	4	2	3	*1*	3	1	**1**	1	1
RMSE	9	5	8	*3*	7	4	**2**	6	1
BIAS (%)	7	5	4	*2*	6	8	**1**	9	3
EF	6	3	5	*2*	5	2	**1**	4	2
Slope	8	5	7	*3*	6	1	**3**	9	2
Ranking	6.8 (9)	4 (5)	5.4 (6)	*2.2 (3)*	5.4 (6)	3.2 (4)	**1.6 (1)**	5.8 (8)	1.8 (2)
Helios									
r	3	*2*	3	2	4	2	**1**	No validated model	2
RMSE	7	*3*	8	5	6	4	**2**		1
BIAS (%)	5	*1*	7	2	6	8	**4**		3
EF	4	*3*	6	2	5	3	**1**		2
Slope	8	*4*	7	5	6	2	**1**		2
Ranking	5.4 (6)	*2.6 (3)*	6.2 (8)	3.2 (4)	5.4 (6)	3.8 (5)	**1.8 (1)**		2 (2)
Prior									
r	No validated model	3	3	*2*	No validated model	1	**1**	No validated model	2
RMSE		6	4	*5*		3	**2**		1
BIAS (%)		6	4	*2*		5	**3**		1
EF		4	3	*2*		2	**1**		2
Slope		4	6	*1*		4	**1**		3
Ranking		4.6 (6)	4 (5)	*2.4 (3)*		3 (4)	**1.6 (1)**		1.8 (2)
Regent									
r	5	3	5	*2*	4	2	1	No validated model	**2**
RMSE	8	6	5	*3*	7	4	2		**1**
BIAS (%)	4	7	3	*2*	5	6	8		**1**
EF	7	4	6	*2*	5	3	1		**1**
Slope	8	6	5	*1*	7	4	3		**1**
Ranking	6.4 (8)	5.2 (6)	4.8 (5)	*2 (2)*	5.6 (7)	3.8 (4)	3 (3)		**1.2 (1)**

[1]Model suggested by Buttaro et al. (2015).

[2]Root Mean Square Error.

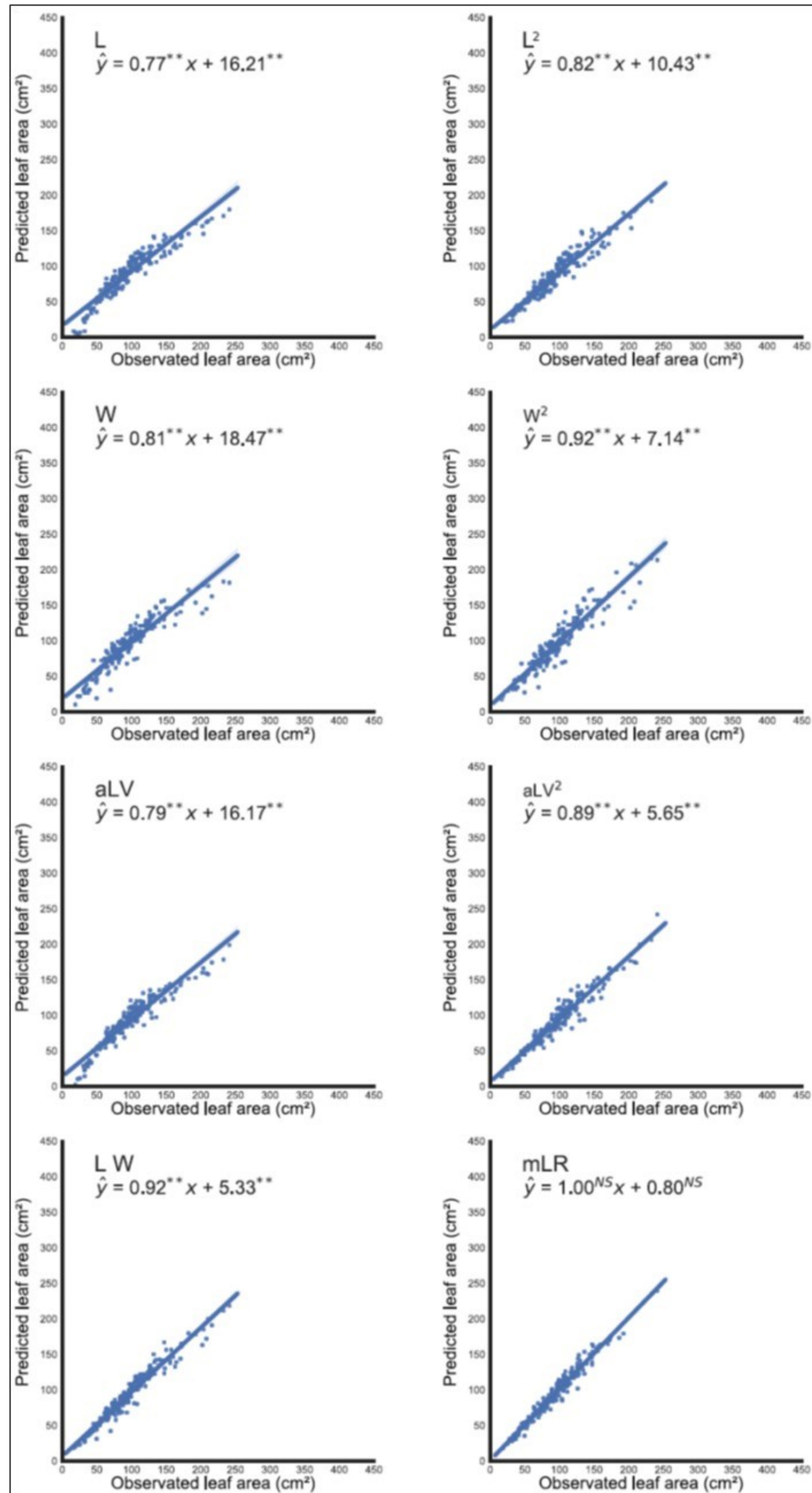


Figure S1A. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Aromera for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR).

[*]Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
[**]Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
[NS] No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

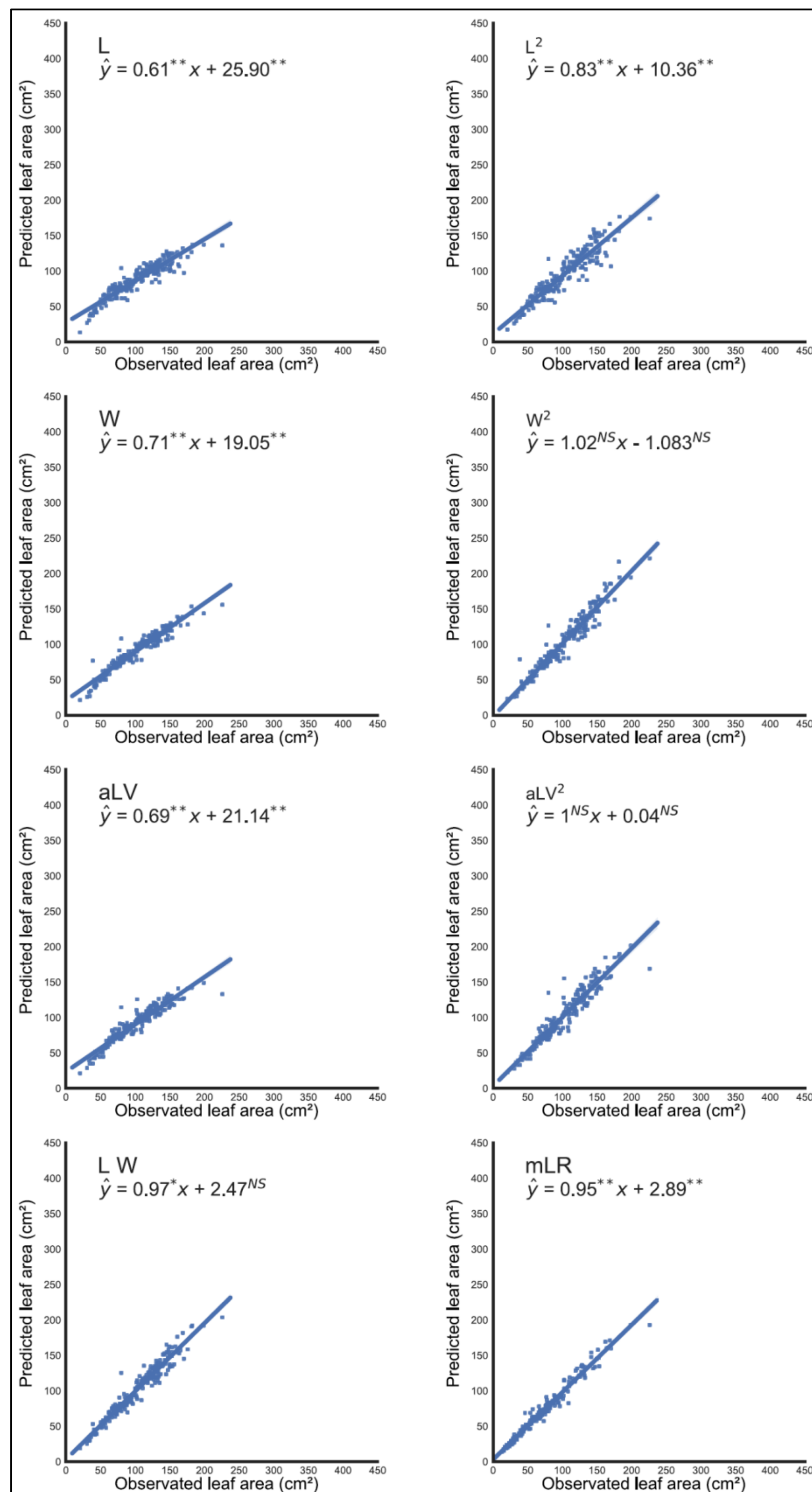


Figure S1B. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Baron for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.

**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.

NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

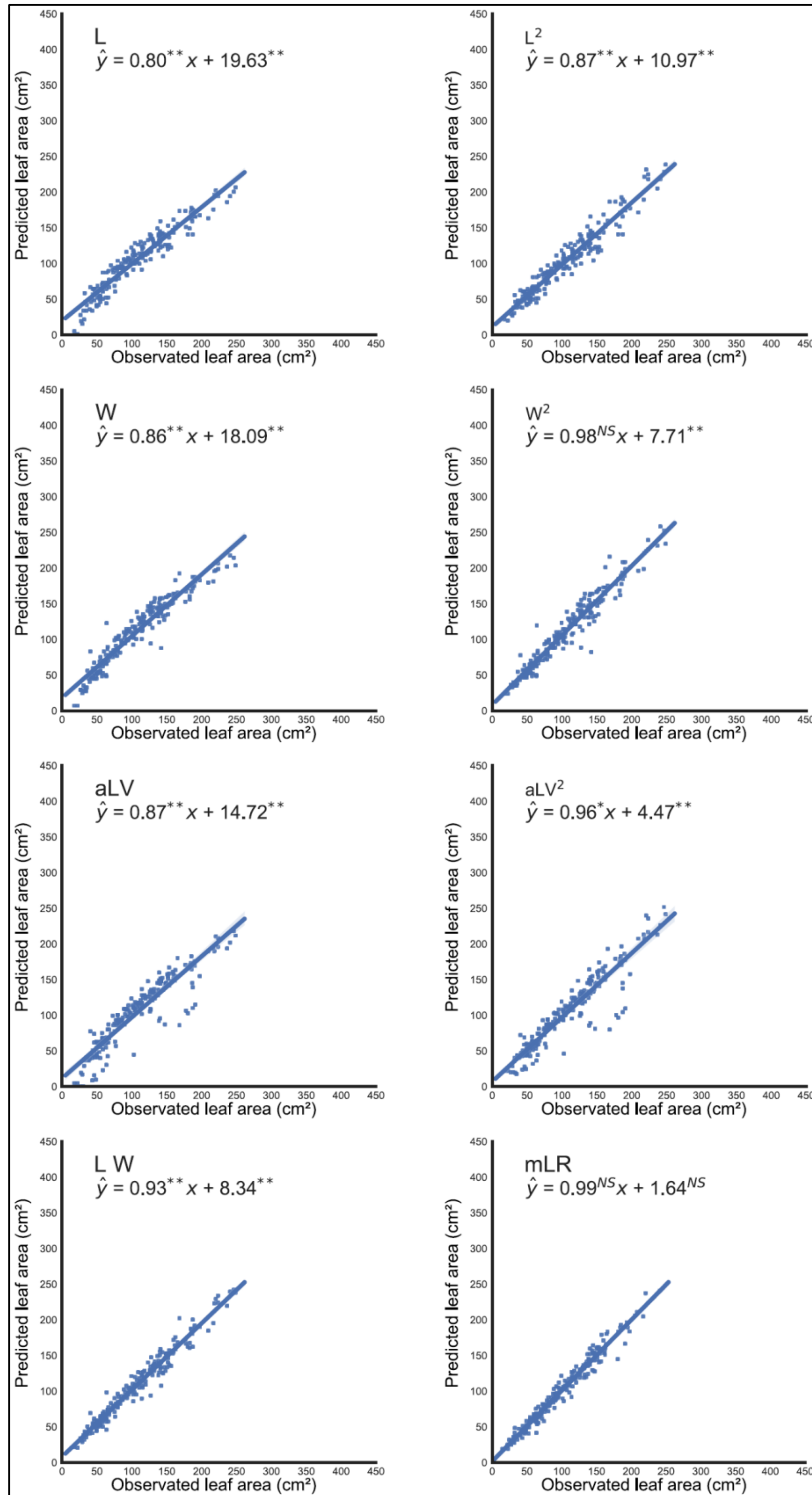


Figure S1C. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Bronner for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
^{NS} No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

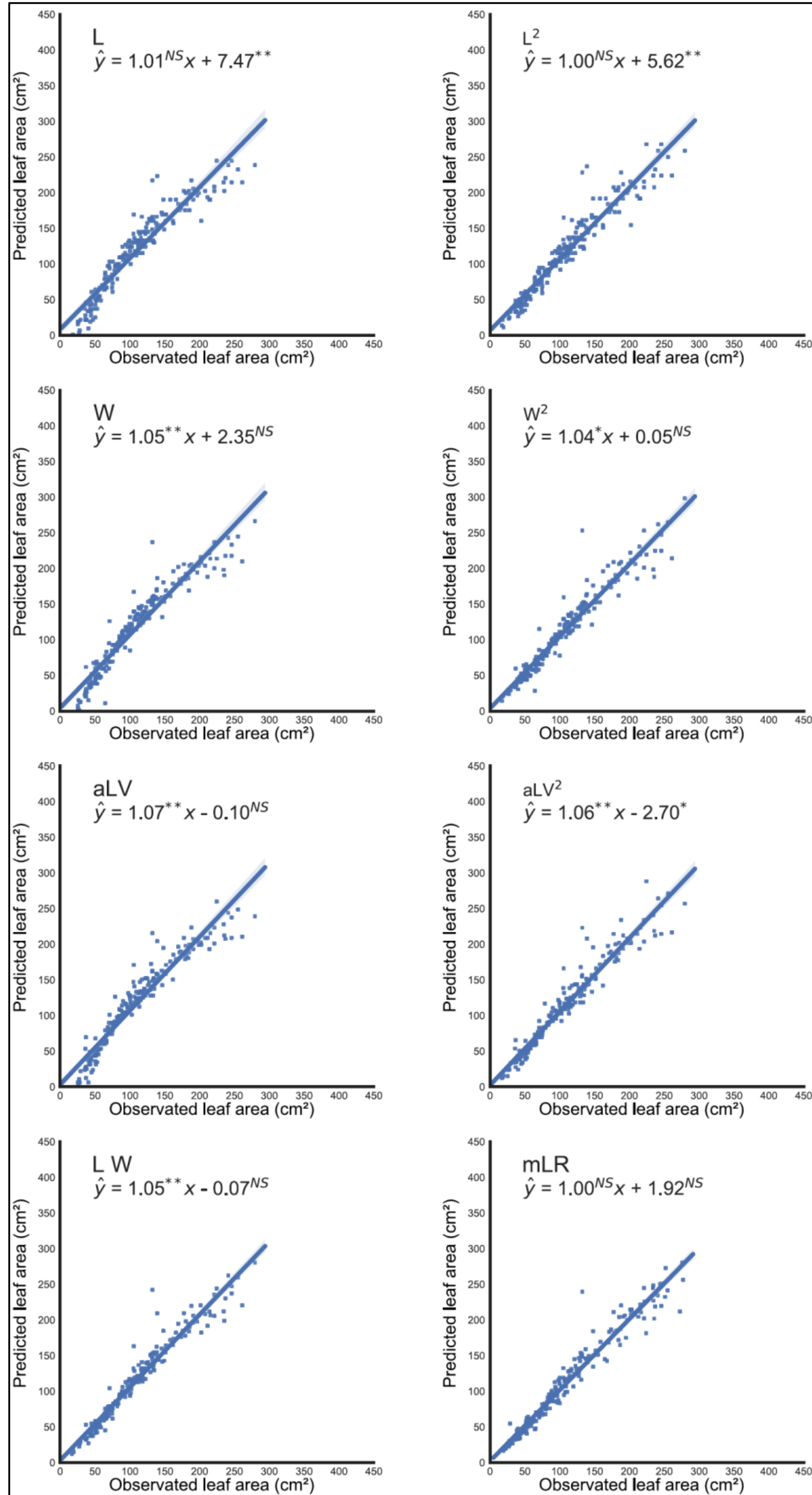


Figure S1D. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Calandro for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

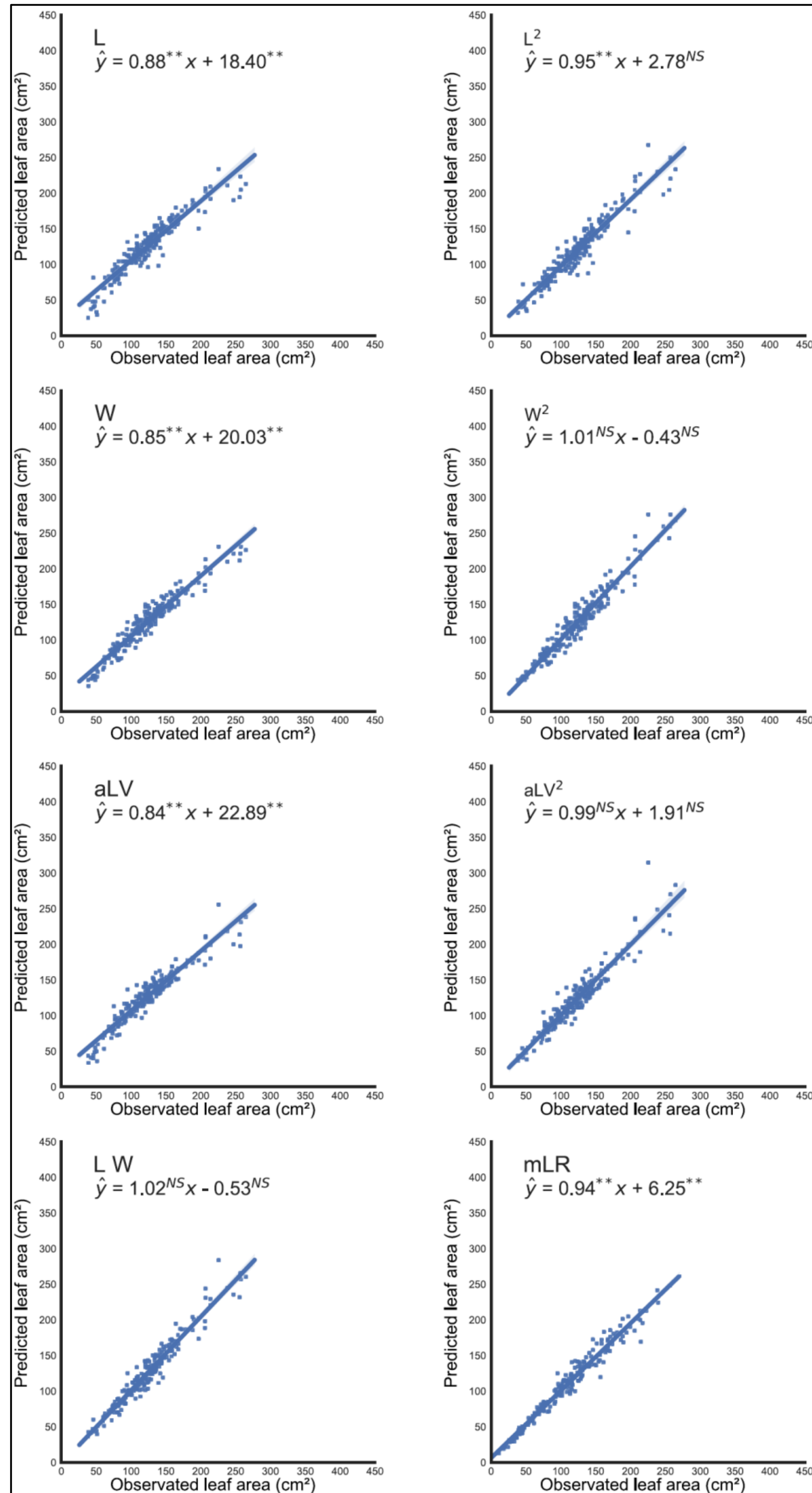


Figure S1 E. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Calardis Blanc for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.

**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.

NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

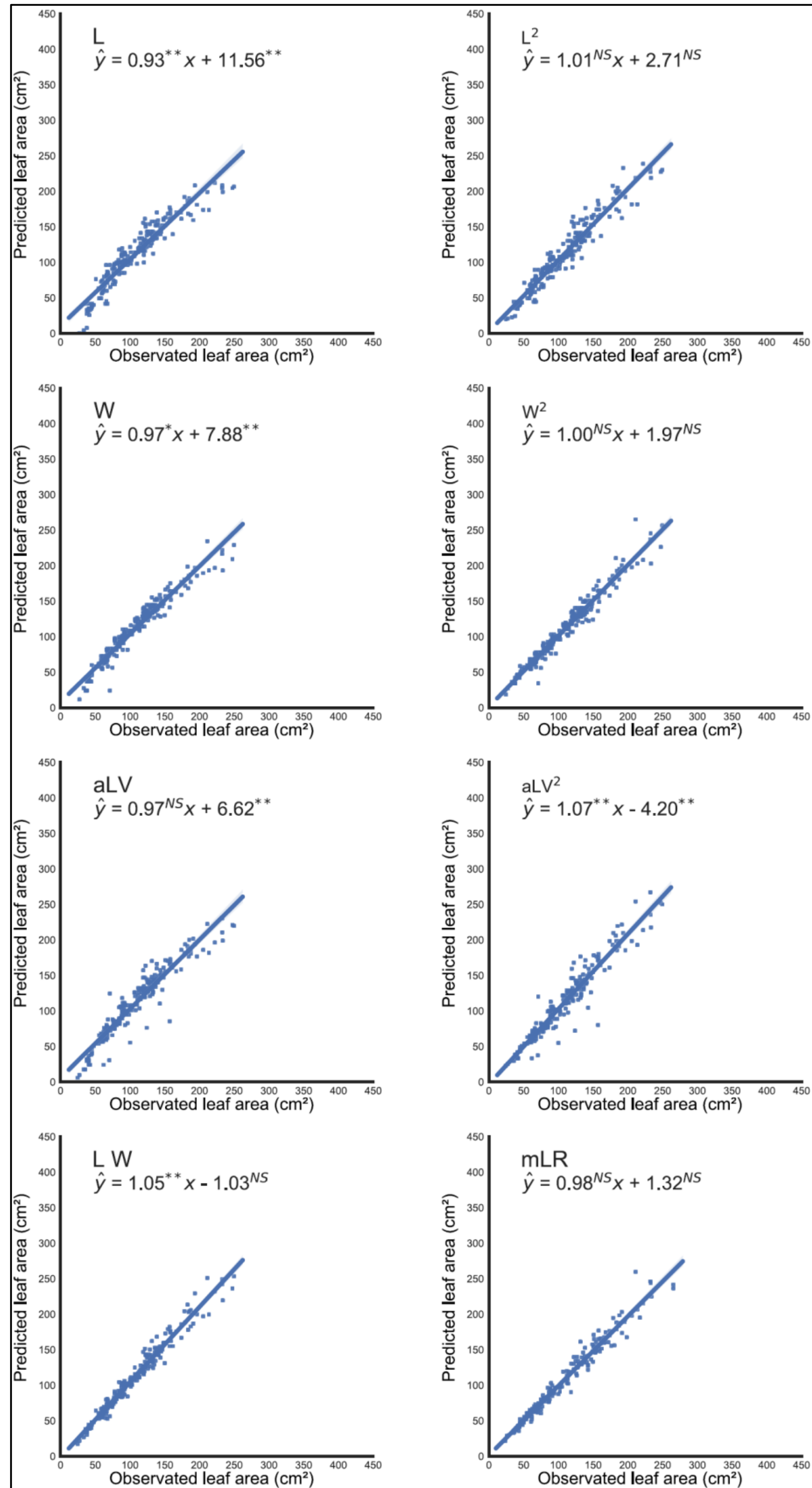


Figure S1F. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Felicia for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
^{NS} No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

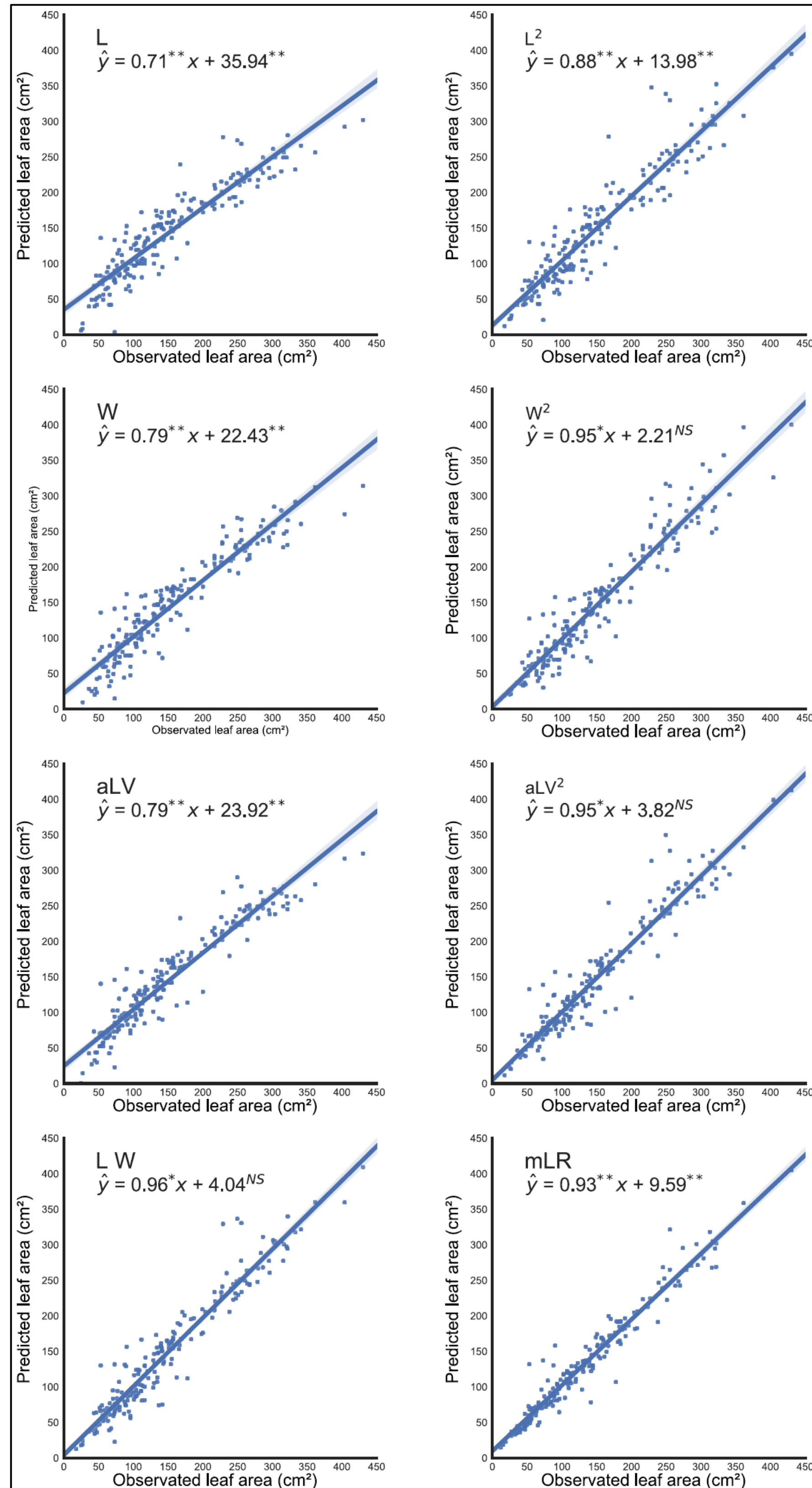


Figure S1G. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes ABS15 for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.

**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.

NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

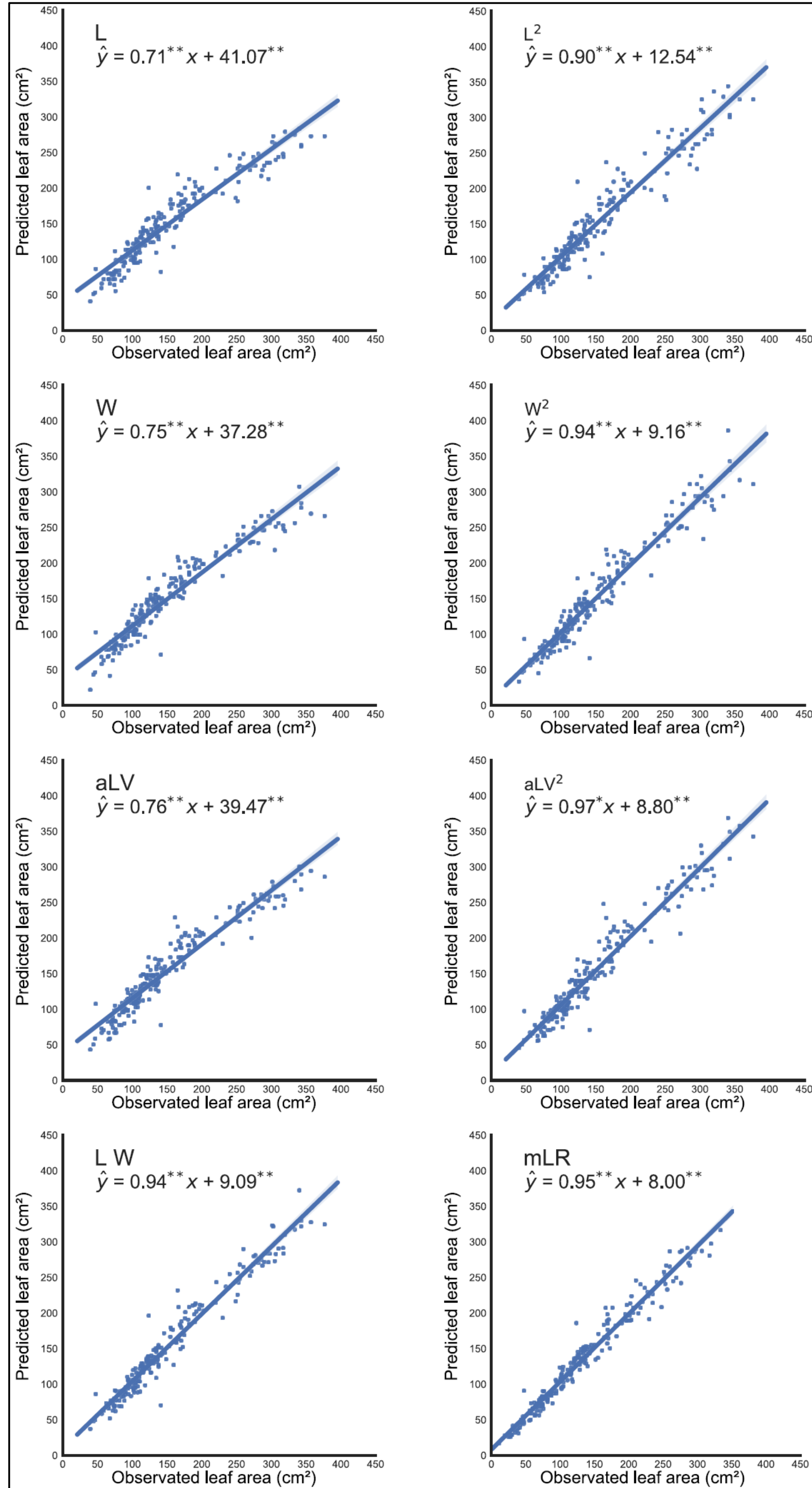


Figure S1H. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes ABS24 for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

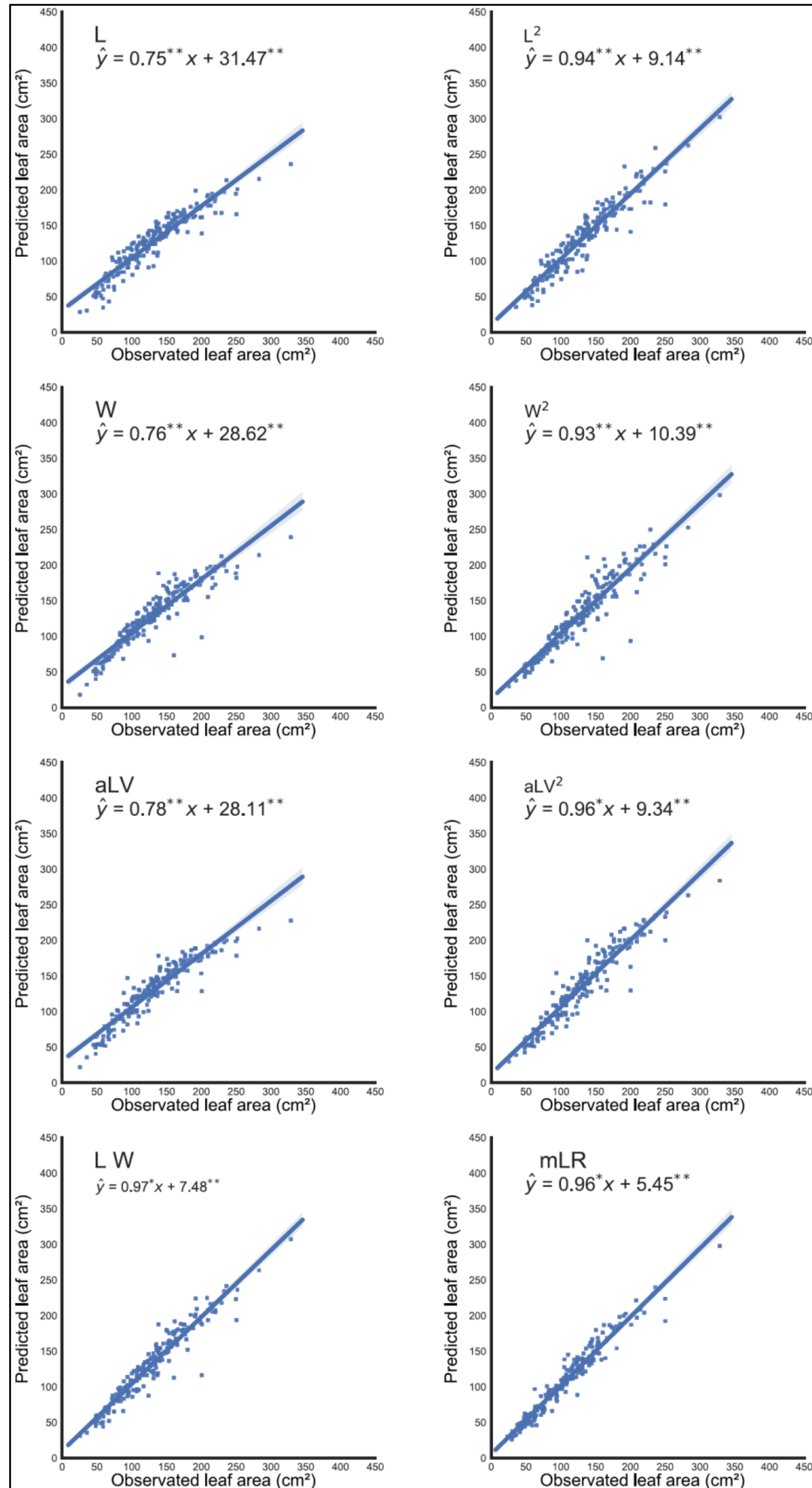


Figure S1I. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Helios for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

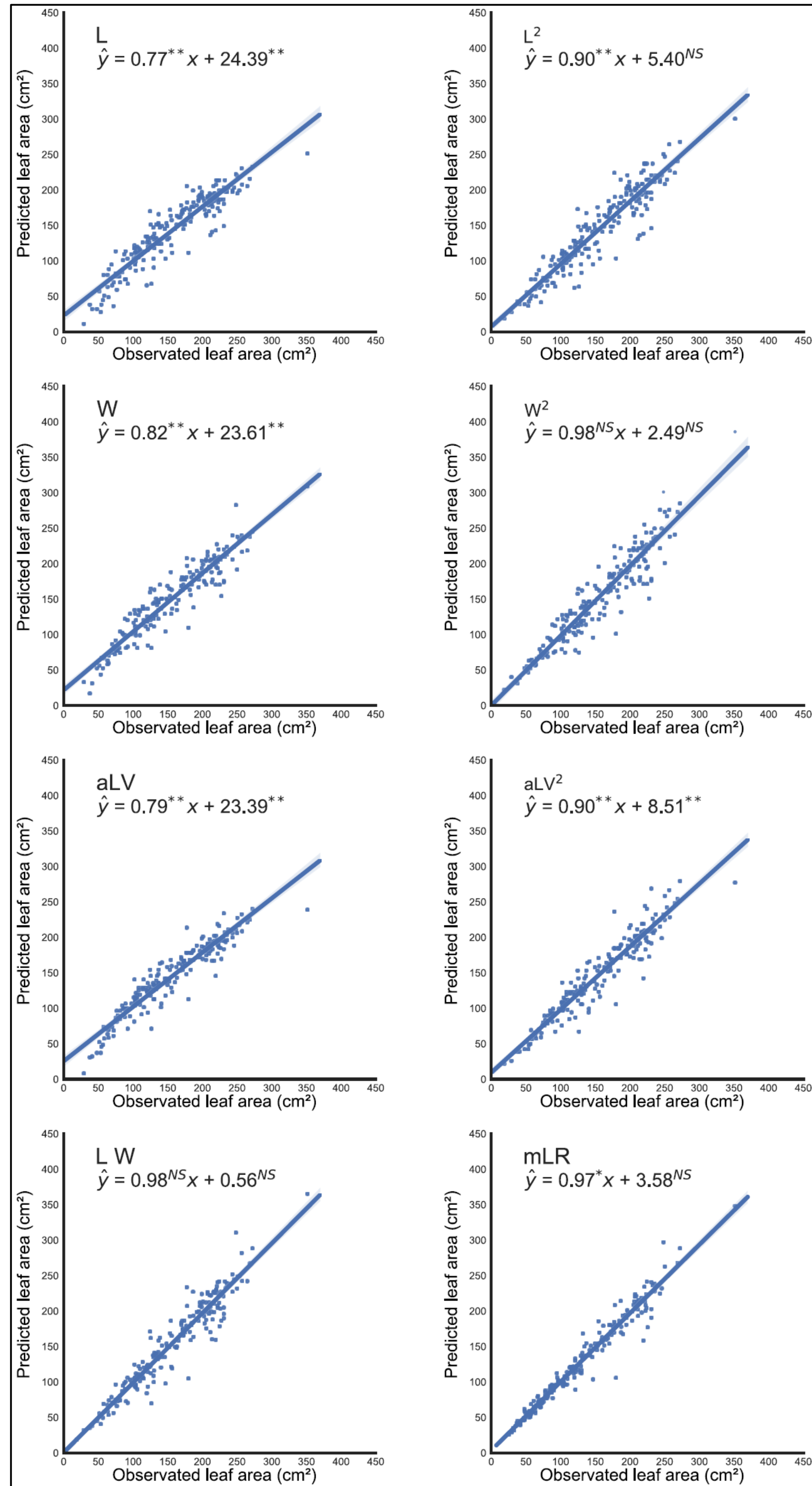


Figure S1J. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Prior for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

[*]Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.
[**]Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.
[NS] No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

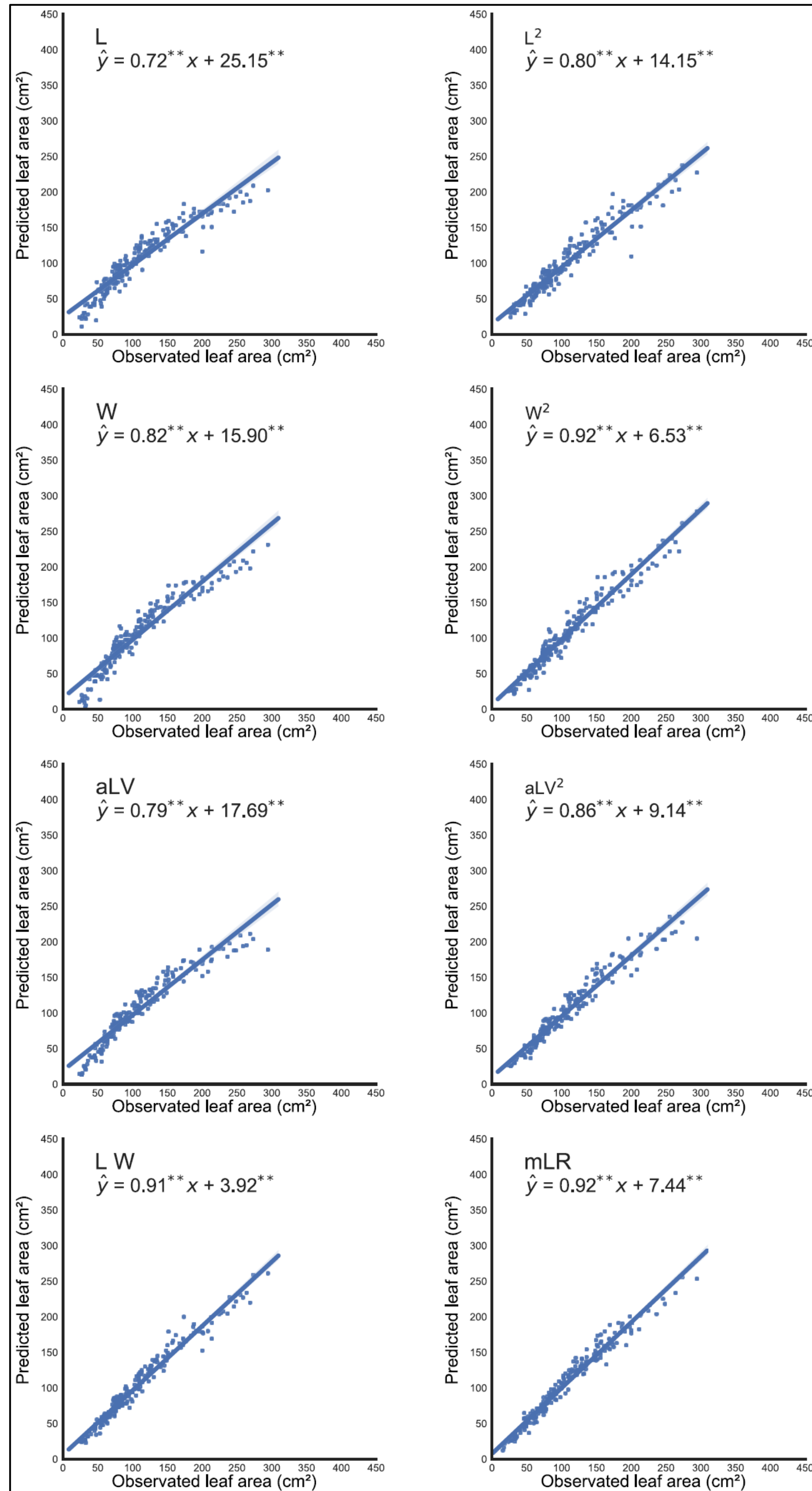


Figure S1K. Relationship between predict and observed leaf area for the *Vitis* resistance genotypes Regent for the models generate by leaf length (L), width (W), average of the lateral veins (aLV) and the multiple linear regression (mLR)**.**

*Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.05.

**Different from the correspondent value (one for angular coefficient and zero for intercept) considering α=0.01.

NS No significative diference from the correspondent value (one for angular coefficient and zero for intercept).

7 CAPÍTULO V - MELHORAMENTO GENÉTICO DA VIDEIRA (*Vitis vinifera*) NO CONTEXTO ATUAL E O EMPREGO DA SELEÇÃO ASSISTIDA POR MARCADORES MOLECULARES PARA *R-LOCOS*

Este capítulo consolida os trabalhos realizados durante o período de doutorado sanduiche na Fondazione Edmund Mach – Trento, Itália

Melhoramento Genético da Videira (*Vitis vinifera*) no contexto atual e o emprego da seleção assistida por marcadores moleculares para R-locos

Atividades de pesquisa relacionadas a tese realizadas durante o período de estágio sanduiche na Fondazione Edmund Mach (FEM)

A Fondazione Edmund Mach (FEM), com sede na comune di San Michele all'Adige, província autônoma di Trento, região do Trentino-Alto Adige, ao Norte da Itália. A FEM, iniciou sua história como um instituto agrário, conhecido pelo nome de Istituto Agragrio di San Michele all'Adige (IASMA), sua fundação ocorreu em 12 de janeiro de 1874, momento na história em que a região fazia parte do Império Austro-Húngaro do Tirol, também conhecido como Südtirol, ou Tirol do Sul. O IASMA foi fundado, pela Dieta Regional Tirolesa de Innsbruck, a partir de uma demanda pelo avanço no conhecimento de técnicas agrícolas, para fortalecer a agricultura do Tirol.

As atividades do IASMA, que iniciaram no outono de 1874, seguiram desde então o princípio sabiamente instituído pelo seu primeiro diretor, Edmund Mach, no qual foi estabelecido que a pesquisa e o ensino não devem prosseguir separadamente, mas devem constituir um binômio indissolúvel do qual extrair as bases para o crescimento do setor. Mach era pesquisador na área de química agrícola e enologia, proveniente da estação experimental de Klosterneuburg, desempenhou papel importante nos primeiros anos do IASMA, atingindo com sucesso objetivos que visavam avançar no conhecimento e nas técnicas agrícolas para a região, motivo principal da sua fundação. As linhas operacionais definidas por Mach foram seguidas pelos sucessivos diretores da instituição, com resultados positivos para o setor e a sociedade tirolesa até o ano de 1919.

Com o advento do fim do império Austro-Hungaro, devido a derrota na primeira guerra mundial. Em 1919, o instituto passou para a província de Trento e em 1926, foi ativado o consórcio com o estado italiano para sua gestão. Apesar das mudanças administrativas, do cenário geopolítico no qual estava inserido, o instituto foi mantido, assim como sua finalidade, de seguir dando suporte para a geração de conhecimentos agrícolas e formação de pessoas capacitadas para o setor.

Dentre os diretores que se seguiram, no período entre guerras, destacou-se historicamente o papel do prof. Enrico Avanzi, que conseguiu elevar o nível de excelência em pesquisa da estação agrícola experimental ao pé de igualdade com as universidades italianas. Sua gestão foi marcada por avanços científicos consideráveis no setor de cereais, frutas e, principalmente enológico, marcando um ponto de virada na matriz produtiva local, promovendo a produção vitivinícola no território. Feito este que se deve em muito ao apoio do pesquisador do IASMA Rebo Rigotti, responsável pela pesquisa em vários campos da viticultura, destacando-se como melhorista genético da videira, gerando novos cruzamentos,

um deles que atualmente leva seu nome, como homenagem, a variedade Rebo (VIVC: 9961), obtida a partir do cruzamento Merlot x Teroldego.

Com a segunda Guerra Mundial, a região foi parcialmente devastada pela Guerra e as atividades de ensino e pesquisa necessitaram de um novo recomeço após 1945. Neste período, destacou-se o papel de Bruno Kessler, que foi ao mesmo tempo presidente da província autônoma de Trento e presidente do IASMA. Durante a sua gestão, nos anos 1960's o IASMA passou a protagonizar papeis de colaborações internacionais com outras instituições de pesquisa europeias. Estes acordos foram fundamentais para incorporar conhecimentos e colaborações que prepararam a instituição para os novos tempos.

No contexto atual, o IASMA tem sua estrutura organizacional definida pela lei provincial trentina n°. 28 de 1990, bem como, pelas suas modificações posteriores. A Lei estabeleceu o IASMA com base nos polos de ensino, pesquisa e extensão. A partir de 2008, com a reestruturação organizacional os polos passaram a ser centros, assim, o IASMA foi transformado em uma fundação, nomeada como Fondazione Edmund Mach, em homenagem ao seu primeiro diretor. A FEM é atualmente subdividida em três centros, antigos polos, Centro Istruzione e Formazione (CIF) dedicado ao ensino, Centro Trasferimento Tecnologico (CTT) dedicado a divulgação do conhecimento e Centro Ricerca e Innovazione (CRI) dedicado a pesquisas cientificas.

A FEM, assim como foi o IASMA, é protagonista no cenário mundial de pesquisas em viticultura. Estando na vanguarda da história em diversos avanços nesta área. Destacando-se em estudos envolvendo o melhoramento genético, o sequenciamento do genoma da videira (VELASCO et al., 2007), a descrição genética de bancos de germoplasma (ZINI et al., 2019), descoberta de novas fontes de resistência genética (VEZZULLI et al., 2019a, 2019b; VEZZULLI; DOLIGEZ; BELLIN, 2019), desenvolvimento de variedades PIWI, entre outros. Gerando sustentabilidade da cadeia produtiva local, porém com impacto mundial, bem como, inúmeros trabalhos científicos em ciência básica e aplicada, que mantem a FEM como protagonista no campo da ciência em viticultura e da genética da videira.

O doutorado sanduiche foi desenvolvido na *Unità di Genetica e miglioramento Genetico della Vite* (UGMGV), grupo sobre o comando do pesquisador Dr. Marco Stefanini, responsável pelos avanços atuais na pesquisa e desenvolvimento de novas variedades de videira pela FEM. Que está inserido no *Dipartimento di Genomica e Biologia Piante da Frutto* (DGBPF), dirigido pelo Dr. Claudio Moser e inserido no CRI. O período no qual foi

desenvolvido as atividades na referida instituição compreendeu oito meses (novembro/18 a junho/19) e as atividades, que foram supervisionadas pela Drª Silvia Vezzulli estão listadas abaixo.

7.1 IDENTIFICAÇÃO VARIETAL VIA LOCOS MICROSSATÉLITES (*TRUE-TO-TYPE*)

Com o advento da colonização italiana no sul do Brasil, instituições governamentais importaram materiais de videira híbridos europeus para serem difundidos entre os colonos, durante a segunda metade do século XIX. Muitos destes materiais formam abandonados para uso comercial por diversos motivos, alguns destes permaneceram sendo cultivados por alguns produtores para consumo familiar, que mantiveram estes híbridos, principalmente pela rusticidade do cultivo. Dentre estes materiais, um híbrido destaca-se pela expressão da resistência a doenças, em especial, ao *P. viticola*. Este, encontra-se cultivado e difundido entre diversos agricultores, principalmente para consumo domestico, pouco explorado para fins comerciais, principalmente em regiões de colonização italiana, todavia, não há um nome registrado para este hibrido e muitos sinônimos são utilizados, dificultando a correta nomenclatura, dentre os nomes populares mais comuns para referir-se a este material, destaca-se "Mar de mina", "Vermont" e "Jaques", no entanto, estes são possivelmente homônimos, pois os dados moleculares, como apresentados no decorrer deste capitulo demonstraram não se tratar destes genótipos. Assim, está variedade será, neste documento, doravante referenciada como VDPR1 (Variedade Desconhecida Portadora de Resistência).

Assim, desenvolveu-se um estudo para identificação da referida variedade, utilizando o perfil de nove microssatélites para identificação varietal, pelo painel *True-To-Type* (TTT). Para isto, uma planta VDPR1, cultivada em Uruguaiana-RS foi utilizada como doadora do material genético. Foram coletadas folhas jovens, que foram transportadas em baixa temperatura (~6 °C) até o LFDGV, onde foram liofilizadas e transportadas em temperatura ambiente para a FEM.

Este estudo foi então desenvolvido na *Unità di Genetica e Miglioramento Genetico della Vite* (UGMGV). O DNA foi extraído utilizando o PureLink Genomic Plant DNA Purification Kit (ThermoFischer scientific). Seguido de confirmação da extração e quantificação do material extraído utilizando o nanodrop® 8.000. A amostra de DNA foi diluída para 10 ng μL^{-1} utilizando água proveniente do kit de extração comercial, em seguida, uma nova diluição foi realizada, para 1 ng μL^{-1}.

Em seguida, reações de PCR foram realizadas em triplicata, em arranjo multiplex, utilizando o mastermix comercial Qiagen multiplex PCR kit (Qiagen), conforme protocolo desenvolvido e mantido pelo UGMGV. Os locos microssatélites utilizados foram VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62 e VrZAG79. A confirmação da amplificação dos multiplex foi realizada via eletroforese em gel de agarose [1.5%], com a adição de um *ladder* com concentração de bandas conhecidas, possibilitando a estimativa da quantificação da concentração dos produtos da PCR.

O produto das PCRs foi diluído em água destilada esterilizada na proporção de 1:1 ou 1:25, conforme o nível de concentração da fluorescência observado na eletroforese horizontal. Após diluído, foi utilizado 1 µL da amostra que foi adicionada em 8.75 µL de formamida e 0.25 µL de marcador de peso molecular para eletroforese capilar GeneScan® LIZ. As amostras foram então desnaturadas a 95 °C por 5 min e em seguida mantidas em gelo por ao menos 3 min. A genotipagem foi realizada em eletroforese capilar, utilizando capilares de 50 cm, preenchidos com polímero POP7® com detecção de fragmentos nos canais FAM, HEX e NED do analisador genético Applied Biosystems™ 3500xL. A leitura dos alelos para cada um dos locos SSR estudados foi realizada com o auxílio do Software GeneMapper™ v. 4.0.

Após a confirmação do perfil de microssatélites, os alelos observados foram cruzados com os perfis TTT disponíveis no banco de dados público disponível no VIVC (MAUL et al. 2020). Adicionalmente, a etapa de avaliação do pedigree foi realizada em colaboração com o Dr. Daniele Migliaro pesquisador do Consiglio per la Ricerca in Agricoltura e l'analisi dell'Economia Agraria (CREA) Centro di Ricerca Viticoltura ed Enologia (Conegliano TV – Itália).

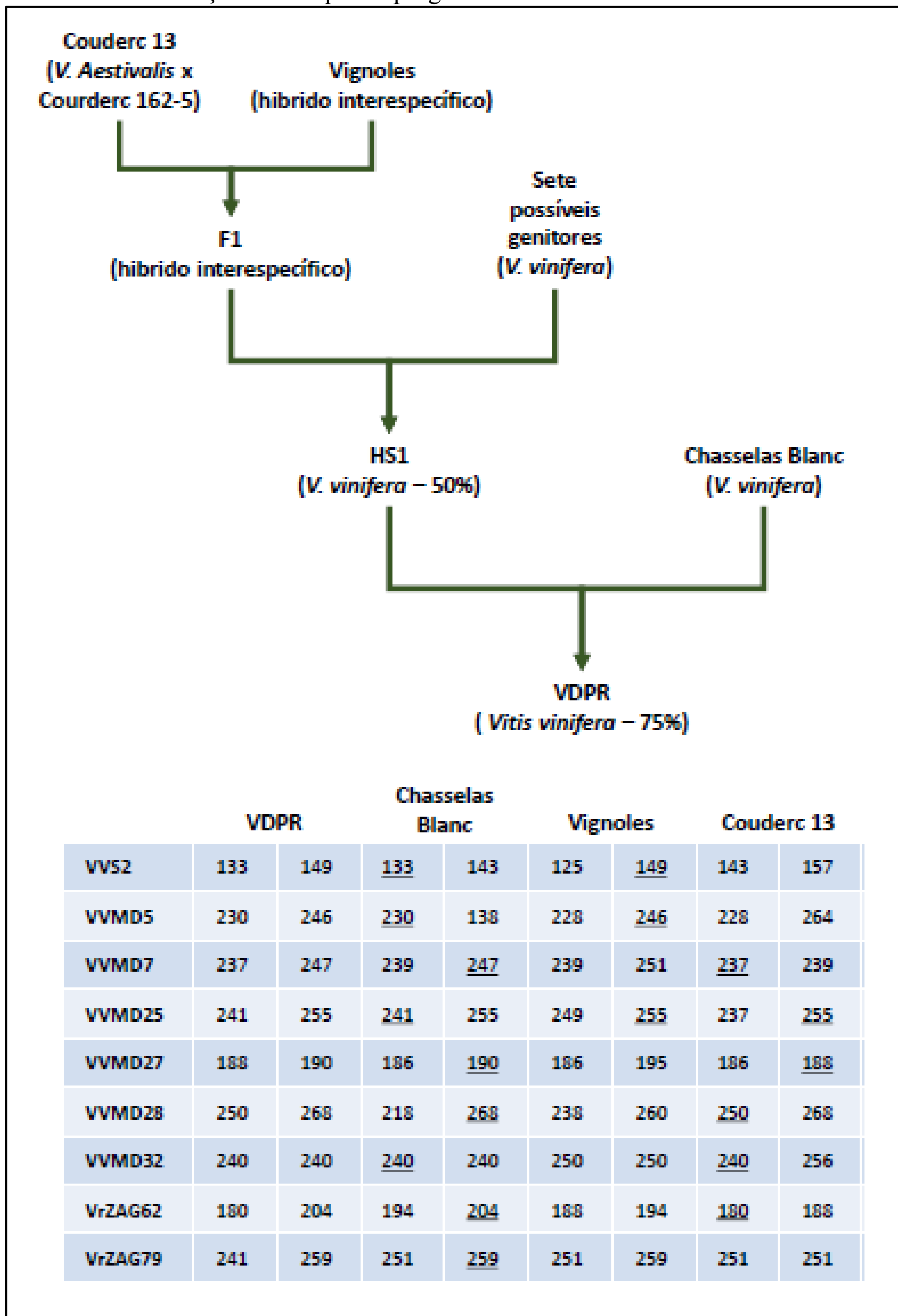
O perfil TTT encontrado para a VDPR1 sugere que a variedade é uma progênie oriunda do cruzamento de Chasselas Blanc (VIVC 2473) com um híbrido sugerido (HS1). O perfil de microssatélites do segundo genitor não foi compatível com nenhum acesso depositado no VIVC ou na base de dados particular do Dr. Migliaro, assim foi gerado um perfil complementar àquele da Chasselas Blanc, buscando por possíveis genitores para a formação deste. Conforme demonstrado na Tabela 1.

Tabela 1. Perfil dos nove locos microssatélites utilizados para identificação varietal em videira, para a progênie, variedade VDPR1 e os possíveis genitores, variedade Chasselas Blanc e o híbrido suposto F1. Alelos em negrito, nos progenitores indicam que foram herdados na progênie.

	Chasselas Blanc		**HS1**		**VDPR1**	
Microssatélite	**Alelo 1**	**Alelo 2**	**Alelo 1**	**Alelo 2**	**Alelo 1**	**Alelo 2**
VVS2	***133***	*143*	***149***	*?*	*133*	*149*
VVMD5	***230***	*238*	***246***	*?*	*230*	*246*
VVMD7	*239*	***247***	***237***	*?*	*237*	*247*
VVMD25	***241***	***255***	***241****/?*	*?/***255***	*241*	*255*
VVMD27	*186*	***190***	***188***	*?*	*188*	*190*
VVMD28	*218*	***268***	***250***	*?*	*250*	*268*
VVMD32	***240***	*240*	***240***	*?*	*240*	*240*
VrZAG62	*194*	***204***	***180***	*?*	*180*	*204*
VrZAG79	*251*	***259***	***241***	*?*	*241*	*259*

O perfil do HS1, possui elevada similaridade com o perfil alélico de outras duas variedades, contudo, estes perfis são complementares entre si, indicando que não são os genitores diretos do HS1, mas estão em sua genealogia. Este ponto é também evidenciado pela ausência do alelo 241 para o loco *VrZAG79*, este alelo é pouco comum e, portanto, apresenta um elevado poder de exclusão. Foram observados somente 11 acessos nos bancos de dados que compartilham este alelo, destes, quatro podem ser descartados devido ao ano de cruzamento, ou ao país de origem, restando assim sete conhecidas possíveis fontes deste alelo (Tabela 2). Todos os possíveis genitores portadores do alelo 241 para o loco *VrZAG79* são *V. vinifera*. Assim, o Pedigree proposto para o VDPR1 pode ser descrito conforme a Figura 1.

Figura 1. Pedigree sugerido para a VDPR, baseado nos nove microssatélites para a identificação varietal para o teste *True-To-Type*, e dados VIVC. Alelos sublinhados indicam a herança do alelo para a progênie.



	VDPR		Chasselas Blanc		Vignoles		Couderc 13	
VVS2	133	149	133	143	125	149	143	157
VVMD5	230	246	230	138	228	246	228	264
VVMD7	237	247	239	247	239	251	237	239
VVMD25	241	255	241	255	249	255	237	255
VVMD27	188	190	186	190	186	195	186	188
VVMD28	250	268	218	268	238	260	250	268
VVMD32	240	240	240	240	250	250	240	256
VrZAG62	180	204	194	204	188	194	180	188
VrZAG79	241	259	251	259	251	259	251	251

Fonte: Tomazetti, TC. (2020)

Tabela 2. Possíveis parentais doadores do alelo 241 para o loco *VrZAG79* para HS1. Em itálico os possíveis genitores, motivo do descarte descrito em observações. Informação faltante no vivc.de (S/I).

Nome (Cod VIVC)	País de origem	Ano de cruzamento	Observação
Alzano (25112)	Itália	S/I	
Belair (10657)	África do Sul	Pós 1900	Descartado devido ao país de origem e ano do cruzamento
Dolga Petla (17675)	Eslovênia	S/I	
Drenak Beli (16564)	Sérvia	S/I	
Gesher Tahton (25093)	Israel	S/I	
Nuragus Moscadeddu (24551)	Itália	S/I	
Rubinia (17748)	Itália	Pós 1911	Descartado devido ao ano do cruzamento
San Pietro E Paolo -Ex Trunzo (26505)	Itália	S/I	
Suosuo Putao (16247)	China	S/I	Descartado devido ao país de origem
Trunzo (12674)	Itália	S/I	
Viola (13107)	Itália	1931	Descartado devido ao ano do cruzamento

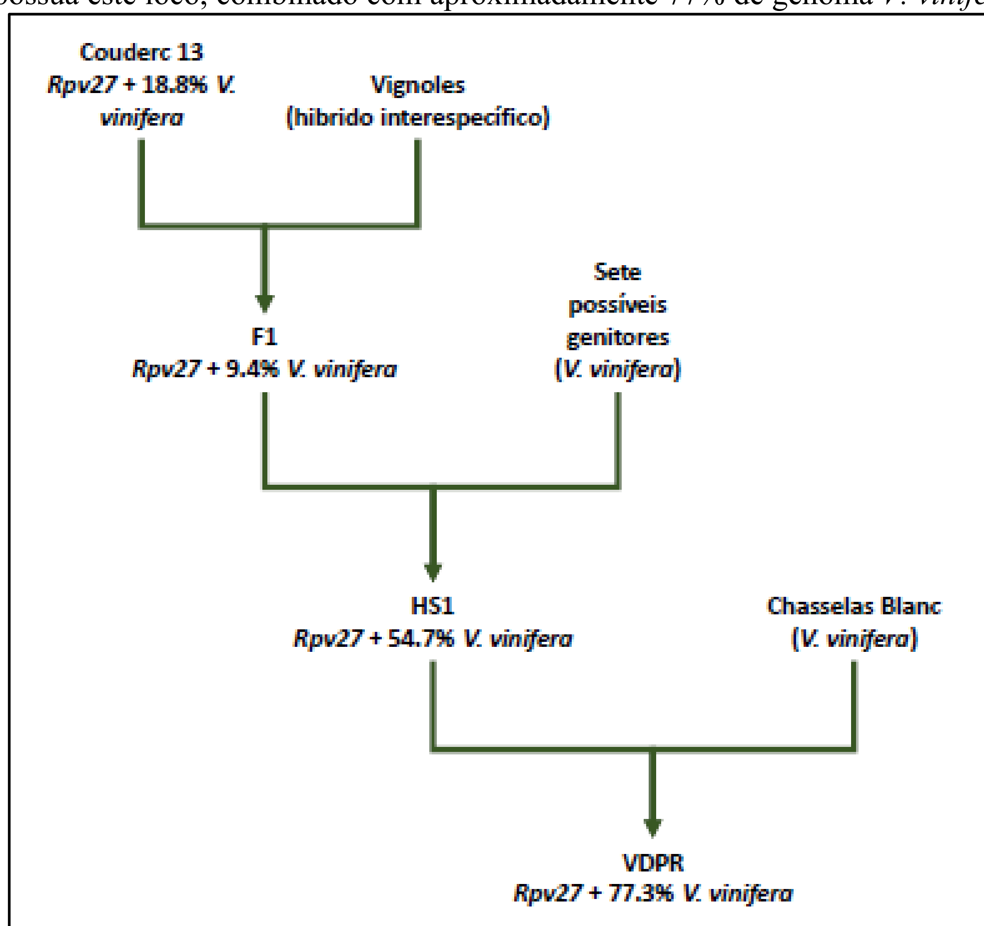
7.2 ANÁLISE DE QTL PARA RESISTÊNCIA AO *Plasmopara viticola*

A partir da variedade Cynthiana (VIVC 3304), popularmente conhecida como Norton, foi mapeado o R-loco *Rpv27*, este loco de resistência é herdado a partir da espécie *V. aestivalis* e está localizado no braço curto do cromossomo 18, flanqueado pelos locos SSR’s *VVCS1H077H16R1-1* e *UDV737* (SAPKOTA et al., 2019), que correspondem respectivamente a posição de 24,636,885 pb e 26,050,244 pb, no genoma de referência da videira PN40024 (JAILLON et al., 2007; VELASCO et al., 2007). A localização genica deste R-loco é próxima ao *Rpv3*, todavia, é relatado que em Norton, portadora do *Rpv27*, não são observados alelos para quaisquer dos haplótipos do *Rpv3*, bem como, na população segregante é relatado polimorfismo para os alelos dos locos *UDV305*, *UDV108*, *UDV112* e *VMC7F2*, que estão ligados ao QTL do *Rpv3* (DI GASPERO et al., 2012).

Devido a presença de resistência genética observada a partir do VDPR, para o míldio, bem como, devido a presença de *V. aestivalis* no pedigree desta variedade, a partir da Couderc 13. Foi realizado o teste para identificação da presença do *Rpv27* na variedade. Para isto, foram utilizados os marcadores para os locos SSR’s *VVCS1H077H16R1-1* e *UDV737*. Adicionalmente, foi também genotipado a variedade Cynthiana, para confirmar a presença dos alelos ligados ao QTL em questão.

As reações de PCR e eletroforese para identificação dos fragmentos foram realizados conforme descrito anteriormente e a presença do *Rpv27* foi confirmada na variedade VDPR, indicando que este R-loco foi herdado a partir de Couderc 13, corroborando a informação da presença desta variedade na genealogia da VDPR (Figura 2). Adicionalmente, este material foi também genotipados para os locos SSR's ligados ao *Rpv3*, onde foi observado a ausência de alelos ligados a qualquer dos haplótipos deste R-loco (DI GASPERO et al., 2012), corroborando a informação de que tratam-se de locos distintos (SAPKOTA et al., 2019).

Figura 2. Pedigree sugerido para a VDPR, baseado nos nove microssatélites, demonstrando a origem do Rpv27, herdado a partir do parental Couderc 13, de modo que a VDPR possua este loco, combinado com aproximadamente 77% de genoma *V. vinifera*.



Fonte: Tomazetti, TC. (2020)

7.3 RESISTÊNCIA A DOENÇAS EM POPULAÇÃO SEGREGANTE

Devido a presença de resistência ao míldio observada na VDPR, uma população foi gerada a partir da autofecundação desta variedade. Para isto, as inflorescências foram ensacadas pouco antes da antese, garantindo a autofecundação das flores. A colheita foi realizada no ponto ideal de maturação das bagas, momento em que as sementes possuíam completo desenvolvimento fisiológico. Após a colheita, as bagas foram armazenadas em 4 °C

até a extração das sementes que ocorreu em até 24 h. Para isto, as sementes foram retiradas e lavadas com água corrente, até a retirada completa da polpa que a envolvia.

Em seguida, foram armazenadas em placas de petri com areia autoclavada, umedecida em água destilada esterilizada até o ponto de capacidade de campo (aprox. – 0.3 atm) em temperatura de 4 °C, por 2500 horas. Após este período as sementes foram lavadas em água corrente e passaram por quebra de dormência com tratamento de ácido nítrico [0.7%] e postas a germinar em bandejas de 128 células, com substrato comercial em câmara úmida.

A semeadura foi realizada na UGMGV-FEM, utilizando bandejas dispostas em câmara úmida. Após apresentarem três folhas verdadeiras, as plantas foram transplantadas para embalagens contendo 500 cm^{-3} de substrato vermiculita e mantidos sobre constante ferti-irrigação, em casa de vegetação com controle de temperatura entre 20 e 30 °C e fotoperíodo em ao menos 16 h de luz.

Foi gerada uma população com 118 indivíduos, dos quais 36 foram utilizados para desafio contra míldio (*P. viticola*), 16 desafiados com o oídio (*Erysiphe necator*) e 16 desafiados com o black rot (*Guignardia bidwellii*). Os demais indivíduos não foram testados para resistência a nenhum dos patógenos devido ao reduzido porte da planta até o momento do encerramento das atividades do doutorado sanduiche, dificultando a avaliação precisa dos mesmos. O reduzido crescimento foi verificado em diversos indivíduos devido, provavelmente a depressão por endogamia, esperado em populações geradas a partir da autofecundação dos indivíduos.

7.3.1 Fenotipagem para resistência ao míldio

Foram coletadas folhas jovens, entre a terceira e a quinta folha. Primeiramente a superfície foliar foi desinfestada com hipoclorito de sódio [1%] por 2 minutos, seguido por tríplice lavagem em água destilada esterilizada, por 1 minuto em cada etapa da lavagem. Em seguida a superfície foliar foi seca em papel toalha e imediatamente os discos foliares foram excisados com auxílio de um furador de rolha de corte liso, com 14 mm de diâmetro interno. Em seguida os discos foram depositados com a face abaxial voltada para cima em placas de Petri previamente preparadas; foram acondicionados cinco discos em cada placa.

Foram utilizadas placas de Petri de poliestireno descartáveis, com 15 cm de diâmetro, o papel filtro utilizado como base para os discos foliares foi previamente cortado em discos de 14.5 cm de diâmetro. Foram dispostos quatro discos de papel filtro em cada placa de Petri e

adicionado 6 ml de água destilada esterilizada. As placas foram então vedadas até o momento do uso.

O patógeno foi obtido a partir de esporulações jovens em folhas de 'Teroldego', gentilmente cedidas pelo Dr. Daniele Prodorutti. A coleta dos esporângios foi realizada utilizando água destilada esterilizada (ADE) gelada (4 °C). A solução obtida foi quantificada utilizando câmara de contagem de células e ajustada para 10^5 esporângios mL^{-1} utilizando ADE para diluição. A inoculação do patógeno foi realizada a partir da pulverização de 150 µL desta solução em cada placa de Petri (30 µL $disco^{-1}$).

As placas foram então depositadas na ausência de luminosidade por 24 h a 22 °C. Em seguida as gotículas resultantes da inoculação foram removidas dos discos foliares com auxílio de papel filtro esterilizado. A partir deste momento as placas foram mantidas em B.O.D com temperatura de 22 °C em fotoperíodo de 16 h de luz. Foram tomadas notas de acordo com o nível de resistência, baseado na escala OIV 452-1, com adaptações (VEZZULLI et al., 2018), estas avaliações foram realizadas em 5 e 7 DPI.

7.3.2 Fenotipagem para resistência ao oídio

As fenotipagens para o oídio, foram a colaboração da dott.ssa Monica Colombo. Foram coletadas folhas jovens, pouco após a abertura, entre a primeira e a segunda do ápice de crescimento. As folhas foram higienizadas conforme protocolo descrito na fenotipagem para resistência ao míldio e acondicionadas em placas descartáveis de poliestireno estéreis, com 400 cm^2 de área. Nestas, foi adicionado 50 mL de meio de cultura agar água [1%], recoberto com papel filtro. As folhas foram então acondicionadas sobre o papel filtro e mantidas em ambiente estéril até a inoculação com o patógeno.

Os esporos do *E. nacator* foram coletados a partir de folhas apresentando abundante esporulação do patógeno oriundas de uma população segregante, sem R-locos. A coleta e aplicação dos esporos foi realizada utilizando um fluxo de ar contínuo sobre as folhas inoculadas, utilizando-se aproximadamente 15 cm^2 de esporulação para cada folha inoculada. As placas foram então fechadas e mantidas em câmara de crescimento com fotoperíodo de 18 h e temperatura média de 25 °C por 15 dias. Ao 15° dia, as folhas foram avaliadas quanto ao surgimento de esporulação do patógeno, utilizando também uma escala de resistência entre 1 (esporulação abundante e sem restrições) a 9 (ausência de esporulação).

7.3.3 Fenotipagem para resistência ao black rot

A fenotipagem para resistência ao black rot foi a única realizada *ex-vitro*. A solução de inoculo foi gentilmente cedida pela pesquisadora Paola Bettinelli. Foi utilizado a esporulação de uma placa de *G. bidwellii*, diluído em 10 mL de água para cada planta testada. As plantas para o ensaio, continham ao menos 10 folhas verdadeiras e foram alocadas em uma câmara de isolamento na casa de vegetação, com temperatura média de 25 °C e umidade relativa entre 80 a 85%. A aplicação do inoculo se deu através da aspersão da solução inoculante aplicada pela parte superior e laterais de cada planta.

As plantas foram mantidas na câmara de crescimento, nas mesmas condições pelos próximos 15 dias. Após este período as avaliações foram visuais, a partir das folhas jovens, porém desconsiderando àquelas brotadas a partir do momento da inoculação. A escala de avaliação da resistência foi entre 1 e 9. Sendo atribuído um, para genótipos que apresentaram lesões com abundância de esporulação e mancha da doença com crescimento sem restrição, três para genótipos apresentando lesões com abundância de esporulação, porém com bordas irregulares, cinco para genótipos que apresentam lesões com restrições na esporulação e bordas irregulares, sete para lesões que apresentam ausência ou escassa esporulação, com bordas irregulares e nove para ausência de lesões.

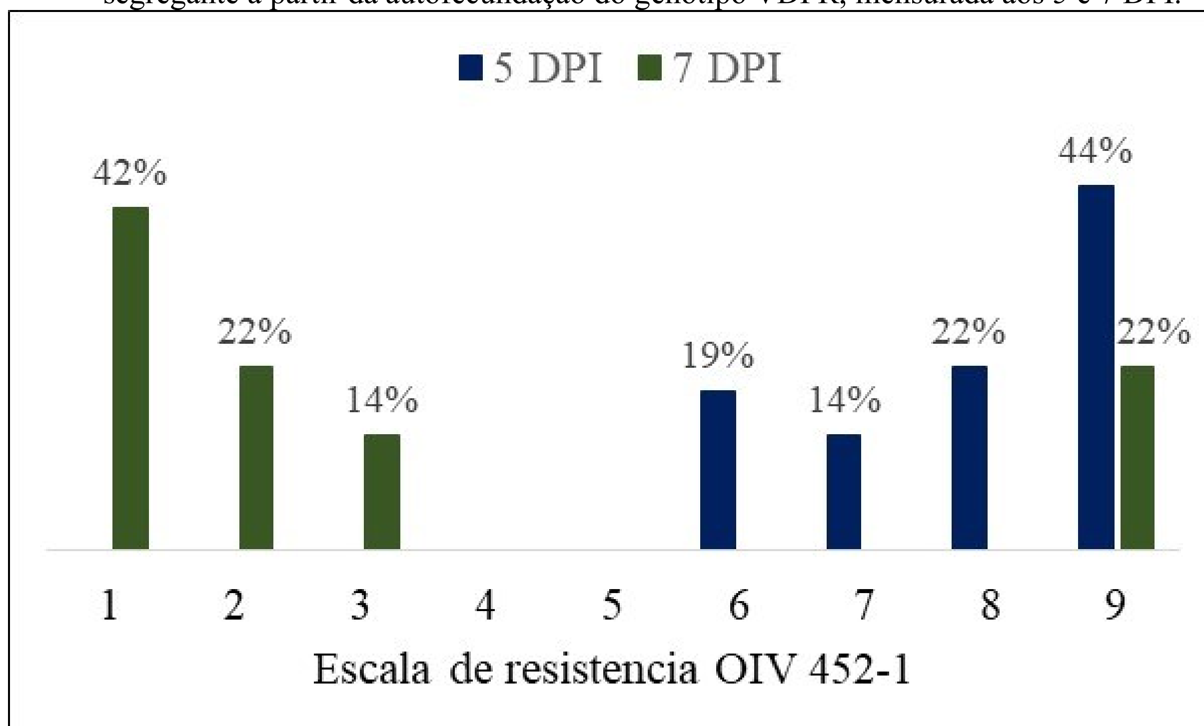
7.3.4 Resultados da resistência dos genótipos

Dos indivíduos avaliados, 44% apresentavam nota nove, representando a máxima resistência, para o míldio aos 5 DPI. Contudo, este valor foi reduzido para 22% aos 7 DPI. A distribuição da resistência dos genótipos avaliados aos 5 DPI estava entre seis e 9, todavia, aos 7 DPI, 42% dos genótipos concentravam-se na classe de menor resistência e, com exceção dos genótipos com maior resistência, os demais (78%) agruparam-se nas três classes de menor resistência (Figura 3). Considerando a população oriunda de uma autofecundação, este comportamento distributivo seria esperado para um gene de resistência atuando em recessividade. Todavia, como o Rpv27 é dominante, existe ainda a hipótese de uma interação epistática com um ou mais genes de susceptibilidade a doença. Todavia, cabe ressaltar que o n amostral é reduzido, prejudicando a inferência de informações precisas para afirmar uma hipótese, contudo, novos estudos podem avançar no entendimento deste comportamento.

A resposta de resistência ao oídio, apresentou uma distribuição mais horizontal, com uma tendência para as notas três e cinco, onde encontraram-se 63% dos genótipos. Os demais, distribuíram-se homogeneamente nas demais notas de resistência. Assim, ressalta-se que 38%

dos genótipos avaliados apresentaram entre alta a muito elevada resistência ao oídio (Figura 4).

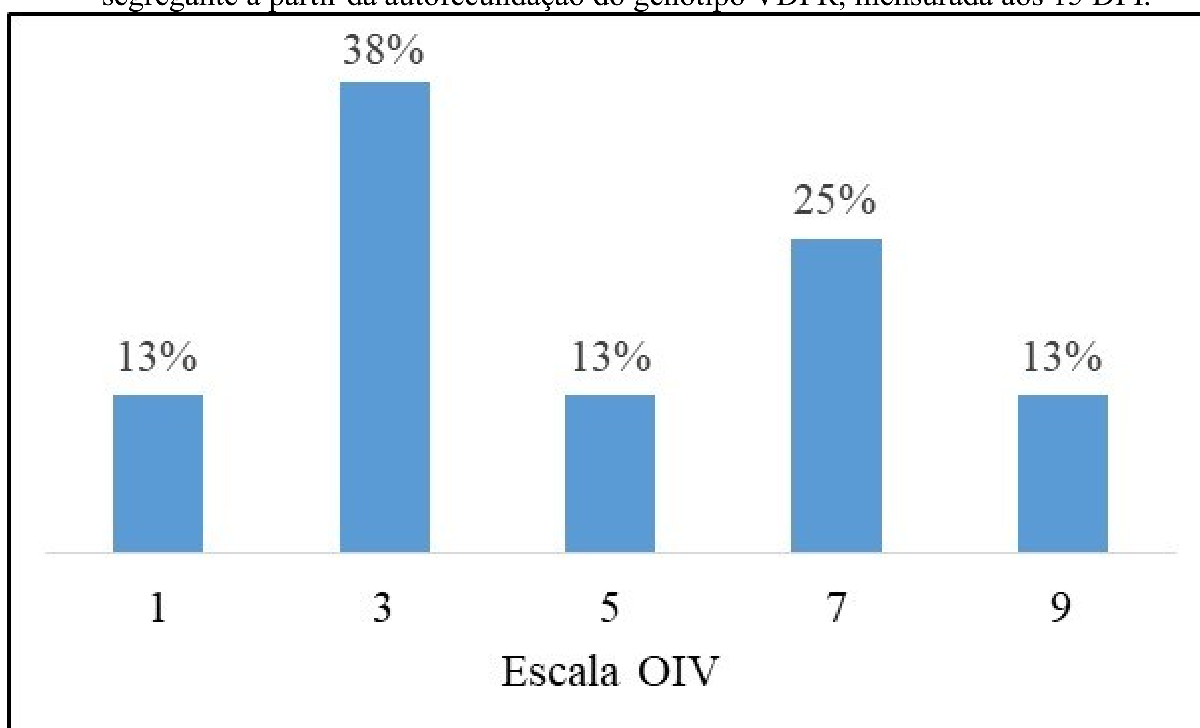
Figura 3. Distribuição da resposta de resistência ao míldio (*P. viticola*) em uma população segregante a partir da autofecundação do genótipo VDPR, mensurada aos 5 e 7 DPI.



Fonte: Tomazetti, TC. (2020)

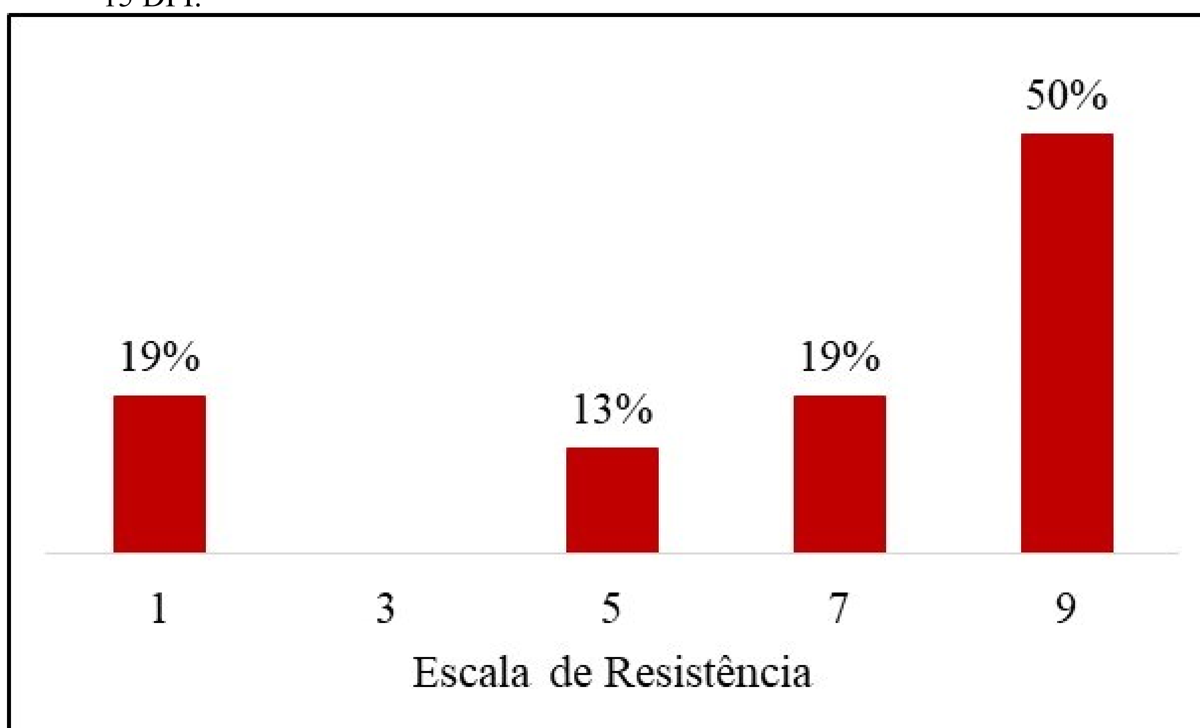
A resposta para o black rot, aponta para a possível atuação de um gene de resistência dominante governando esta característica. Pois 50% dos genótipos não apresentaram as lesões da doença. Somados aos genótipos com elevada resistência ao patógeno, esta taxa é de 69%. Na outra ponta, somente 19% dos genótipos apresentaram abundante esporulação desta doença (Figura 5).

Figura 4. Distribuição da resposta de resistência ao oídio (*E. necator*) em uma população segregante a partir da autofecundação do genótipo VDPR, mensurada aos 15 DPI.



Fonte: Tomazetti, TC. (2020)

Figura 5. Distribuição da resposta de resistência ao black rot (*G. bidwellii*) em uma população segregante a partir da autofecundação do genótipo VDPR, mensurada aos 15 DPI.



Fonte: Tomazetti, TC. (2020)

Cabe ressaltar que na literatura não há relatos de QTL's associados a resistência ao oídio ou ao black rot a partir de material da *V. aestivalis*. Indicando que novos estudos, mais aprofundados podem ser realizados buscando mapear a origem genética desta resistência e selecioná-las para introgressão em programas de melhoramento genético da videira, via mapeamento de QTL's e posteriormente pela MAS.

7.4 REVISÃO DE LITERATURA – ASOMICETOS EMERGENTES NA VITICULTURA

Durante o período de doutorado sanduiche, foi também escrito parte de uma revisão de literatura que buscou demonstrar o estado da arte atual, de maneira interdisciplinar, de novas doenças que possam causar danos às videiras, no cenário atual e futuro. A revisão foi coordenada pela Drª Silvia Vezzulli e compreende o estado da arte atual de três doenças, das quais, a participação escrita limitou-se principalmente a antracnose. A referida revisão encontra-se publicada no periódico Frontiers in Plant Science, na edição de novembro de 2019.

Emergent Ascomycetes in viticulture: an interdisciplinary overview

Abstract

The reduction of pesticide usage is a current imperative and the implementation of sustainable viticulture is an urgent necessity. A potential solution, which is being increasingly adopted, is offered by the use of grapevine cultivars resistant to its main pathogenic threats. This, however, has contributed to changes in defense strategies resulting in the occurrence of secondary diseases, which were previously controlled. Concomitantly, the ongoing climate crisis is contributing to destabilizing the increasingly dynamic viticultural context. In this review, we explore the available knowledge on three Ascomycetes which are considered emergent and causal agents of powdery mildew, black rot and anthracnose. We also aim to provide a survey on methods for phenotyping disease symptoms in fields, greenhouse and lab conditions, and for disease control underlying the insurgence of pathogen resistance to fungicide. Thus, we discuss fungal genetic variability, highlighting the usage and development of molecular markers and barcoding, coupled with genome sequencing. Moreover, we extensively report on the current knowledge available on grapevine-ascomycete interactions, as well as the mechanisms developed by the host to counteract the attack. Indeed, to better understand these resistance mechanisms, it is relevant to identify pathogen effectors

which are involved in the infection process and how grapevine resistance genes function and impact the downstream cascade. Dealing with such a wealth of information on both pathogens and the host, the horizon is now represented by multidisciplinary approaches, combining traditional and innovative methods of cultivation. This will support the translation from theory to practice, to understand biology very deeply and manage the spread of these Ascomycetes.

Keywords
Anthracnose, black rot, disease symptom phenotyping, genetic diversity, grapevine, powdery mildew, resistance loci, transcriptomics, *Vitis* spp.

Introduction

The earliest evidence of viticulture, namely grapevine cultivation and winemaking, was found in Iran, dating back to 7,400-7,000 B.C. (McGovern, 2004). Among the 60 hybridizing species ($2n$ = 38) belonging to the *Vitis* genus, the Eurasian grapevine (*Vitis vinifera* L.) is the most extensively cultivated and of renowned worldwide economic importance, being used for the production of high-quality wines, table grapes and raisins (Olmo, 1979). With the exception on a few recently explored *V. vinifera* accessions coming from the Caucasian cradle of grapevine domestication (e.g. Toffolatti et al., 2018), *V. vinifera* cultivars are generally highly susceptible to most fungal diseases, such as downy mildew, grey mold, powdery mildew (PM), black rot (BR), and anthracnose (AN) (Olmo, 1971). For this reason, the main strategy to prevent yield losses due to biotic adversities is the application of fungicide which is necessary to control the causal agents with inevitable negative impact on humans, animals and environment. Around 68,000 tons of fungicides per year are used in Europe to manage grapevine diseases, i.e., 65% of all fungicides used in agriculture, though viticulture encompasses only 4% of the arable land available in the EU (Eurostat report., 2007). Forecast prospects large increases in this trend, especially in viticulture, consistently with the worldwide data available (FAO, 2016).

A useful strategy to reduce the impact of pesticides towards a sustainable viticulture relies on breeding, by introducing resistance traits from wild species into domesticated varieties. Therefore, the change to these current varieties (cultivars) is now strongly advised. After initial difficulties, due to considerations regarding the quality of the wine produced with these varieties, resistant cultivars have recently been allowed by the EU Commission for the production of *PDO* (*Protected Denomination of Origin*) Dons wines in Denmark

(Implementing Regulation 2018/606). In the last decade, it has been reported that the cultivation of new varieties resistant to downy mildew and PM, whose management needs less copper and sulphur-based treatments, favored BR diffusion (Harms et al., 2005; Töpfer et al., 2011). In fact, most cultivars which exhibit adequate resistance against mildews are highly susceptible to BR (Harms et al., 2005). In addition, considering BR as originally native in northern America, the appearance of BR symptoms is ongoing in previously BR-free area (CABI, Crop Protection Compendium, 2018) (**Figure 1A**). Analogously, AN is sometimes problematic on highly susceptible interspecific cultivars, which typically receive only modest fungicide programs to control other diseases (Wilcox et al., 2017).

The transition to production of resistant cultivars however needs to be framed within the current climatic challenges. The two main players involved in the climate crises are temperature (global warming) and precipitation (including extreme phenomena) (Higgins and Scheiter, 2012). Besides having already extremely impacted natural phenology by limiting yields through drought and spring-frost damages, dramatic changes in climate will result in the accelerated reproductive cycles of biological organisms, destabilizing even further the precarious equilibrium among pathogens, pests and hosts (Caffarra et al., 2012; Salinari et al., 2006). Global surveys to identify the most relevant diseases and pests in many grape-growing regions worldwide provided preliminary results which allowed to determine the distribution of diseases by 2050 as function of agroclimatic indicators. Upon these recent investigation, PM derived from northern America (**Figure 1B**), and AN which originated in Europe (**Figure 1C**) were also recently discovered in extremely diverse climatic conditions, including temperate regions with high rainfall, especially during spring months (Bois et al., 2017; Bregaglio et al., 2013; Wilcox et al., 2017).

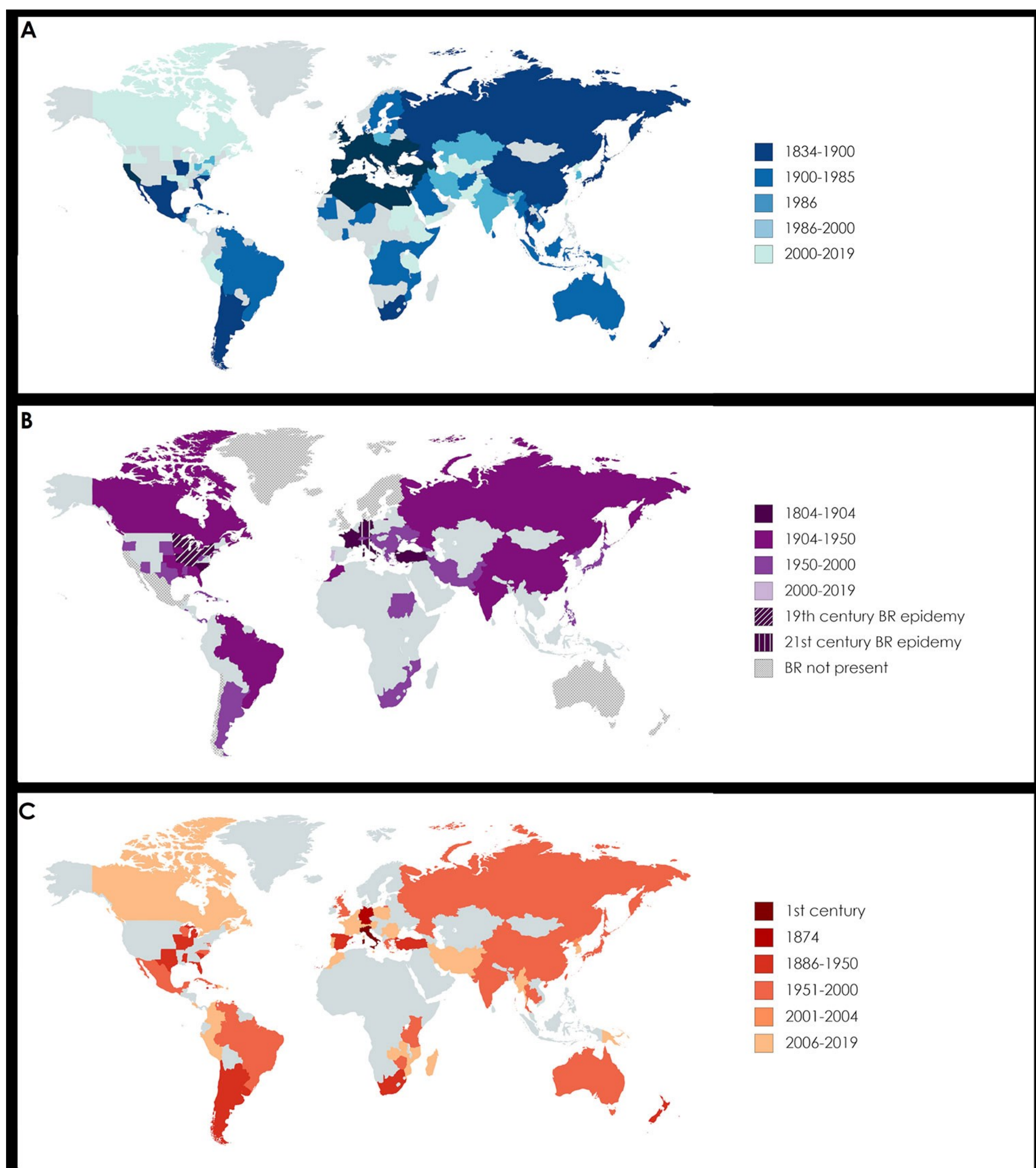


Figure 1 Worldwide diffusion of powdery mildew (PM, panel A), black rot (BR, panel B), and anthracnose (AN, panel C). The relative bibliographic sources are reported in Table S1. **(A)** PM was first reported in northeastern America in 1834 by Schweinitz. In 1845 it was introduced in Europe and less than 10 years later was affecting all the wine producing country of the Mediterranean region. In 1986, Amano published an outstanding review listing the countries all over the world where fungi causing PM were present at that time in relation with their specific plant hosts, including grapevine. Today PM can be considered a "worldwide grapevine disease", since it afflicts vineyards all over the world. **(B)** BR is native of northeastern America. In 1804 it was noticed in Dufour's vineyard (Kentucky) and it became epidemic in the second half of the 19th century in all the Great Lake Region, where the entire yield in many fields was lost. The first occurrence in Europe was recorded in 1885 by Viala and Ravaz in Southern France, then it spread all around the world, although without a huge

economic impact. In 1989, BR showed an increasing presence in Switzerland, but it was in the 21th century that a second outbreak afflicted Europe, starting from Germany, where the economic losses were severe, to the Alps area (Ticino, Switzerland; Friuli and Veneto, Italy), Hungary and Romania. Regions with an unfavourable climate, as Scandinavia for cold and Mexico for dry weather, are considered BR “free” today. Interestingly, also in Australasia and Chile BR remains absent. **(C)** AN is considered one of the oldest known plant disease, since reference to it were reported in ancient Rome by Theophrastus (in *De causis plantarum*) and by Pliny the Elder (in *Naturalis historia*), dating back to the first century of the Christian era. Its European origin was also confirmed by the first report of the modern era in 1874 by De Bary, in Germany. AN diffusion was not alarming until its arrival in Tropical areas, such as South America. Nowadays AN is again becoming a threat in Europe.

Finally, in this challenging and dynamic viticultural context, we are witnessing the emergence of fungal diseases caused by Ascomycetes. The term "emergent disease" in this case is not to be interpreted in the strict sense of phytosanitary emergencies, but refers to diseases whose causative agents are already known, and for which control plans exist. These diseases however have manifested themselves or already conquered new regions, due to the recovery or the onset of favorable conditions, representing a real threat to worldwide viticulture.

Disease description

Disease symptom assessment

Powdery mildew

The causative agent of PM is the biotroph (obligated parasite) *Erysiphe necator* Schw. (asexual morph *Oidium tuckeri* Berk.) (**Figure 2**). PM, is recognized by the appearance of a whitish-gray dusty layer on the grape which is caused by the spreading of mycelia and conidia onto green tissues (Pearson and Gadoury, 1992). Biological assays for the assessment of disease symptoms are fundamental to shed light onto host-pathogen interactions; conveniently these can be carried out by observations in the field (e.g. Li, 1993; Pap et al., 2016; Wang et al., 1995) in greenhouses (e.g. Amrine et al., 2015; Li, 1993; Pap et al., 2016; Pessina et al., 2016) and *ex vivo* (e.g. Li, 1993; Pap et al., 2016; Pessina et al., 2016; Staudt, 1997; Wang et al., 1995).

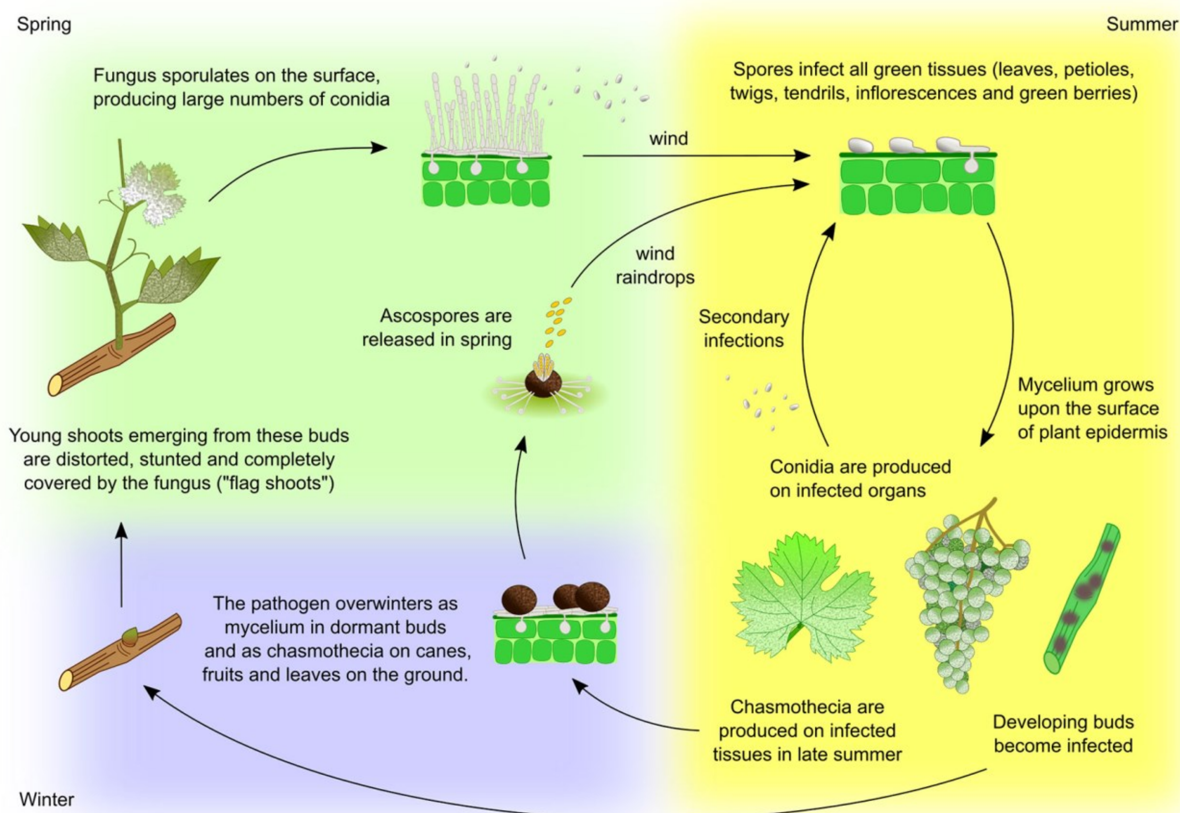


Figure 2 *Erysiphe necator* life cycle. Two overwintering strategies have been observed in *E. necator*. In areas with relatively mild winters, the fungus commonly overwinters as mycelium on leaf primordia within dormant buds. In the following spring mycelium activity resumes, resulting in the production of heavily infected and deformed shoots, called 'flag shoots'. The fungus sporulates on these shoots, producing a large number of conidia that are carried by the wind to healthy plant tissues. Alternatively, the fungus can overwinter as chasmothecium (syn. cleistothecium, a former term for this structure that is still widely used) in bark, on canes, leftover fruit, and on leaves on the ground. Chasmothecia form on the surface of heavily diseased tissues from mid-summer to autumn. During spring rainfall the chasmothecia open and release ascospores, which are spread by wind or raindrops to infect the lower leaves near where the chasmothecia have overwintered. Although free water is necessary to release ascospores, continued wetness is not required for subsequent spore germination and infection. At each new infection site, conidia and ascospores germinate and form an appressorium. From its lower surface a penetration peg develops, piercing the cuticle and entering through an epidermal cell where a haustorium is formed. Mycelium grows upon the surface of the plant epidermis and new conidia are produced within a few days, completing the cycle. Repetition of this cycle continues throughout the growing season resulting in a rapid increase in disease incidence (Wilcox et al., 2017).

In the field, observations are carried out in mid-summer, when the symptoms are more evident and it is possible to estimate the occurrence of the pathogen in vineyards treated and not-treated with fungicide (Li, 1993; Wang et al., 1995). Symptoms can be monitored studying artificial infections (inoculations) using amplified conidia (Pap et al., 2016). To

standardize results, the age of the leaf must be recorded, since older leaves display ontogenic resistance (Gadoury et al., 2012). In a greenhouse setting, different strategies can be adopted: for instance Li (1993) and Pap et al. (2016) inoculated potted plants by spraying them with a suspension of *E. necator* conidia in aqueous and Tween solution using 5×10^5 conidia/ml and 0.7×10^5 conidia/ml respectively. Pessina et al. (2016) brushed the adaxial surface of older leaves with young leaves carrying *E. necator* sporulation. Under these growing conditions, disease severity can be assessed within 3 to 21 days post-inoculation (dpi). *Ex vivo* pathogenesis assays are conducted using detached leaves or leaf disks and different inoculation strategies have been used. Péros et al. (2006) tested three different spots of inoculum on detached leaves, using a glass needle to transport 20-60 conidia a time. Alternatively, Staudt (1997) brushed leaf disks with conidia from the mycelium of *E. necator*, while Miclot et al. (2012) developed a ventilation method. Wang et al. (1995) and Pap et al. (2016) successfully sprayed conidia suspension on detached leaves and foliar disks (**Figure 3A-C**).

Figure 3 Fungal morphological characteristics and symptoms of powdery mildew (PM, panel **A-C**), black rot (BR, panel **D-G**), and anthracnose (AN, panel **H-K**). **(A)** PM on grapes (field). **(B)** PM on leaves (greenhouse). **(C)** PM leaf disc infection under

different magnification (above); conidiophores and conidia on a leaf surface (below-left); mature (black) and immature (yellow) chasmothecia (below-right). **(D)** BR field symptoms on leaves and grape cluster. **(E)** *G. bidwellii* pycnidia on petiole and leaf (above), on berry with detail of cirri development under humid conditions (below). **(F)** Detail of *G. bidwellii* pycnidia and conidia under different magnification. **(G)** *G. bidwellii* isolate growing on culture media (above) and leaf symptoms after artificial infection (below). **(H)** AN field symptoms on a grape cluster, leaf and young shoot, along with detail about the typical "shot-hole" lesions on old infected leaf. **(I)** AN symptom details on berries. **(J)** *E. ampelina* colony on culture media. **(K)** *E. ampelina* acervulus releasing conidia and detail of conidia.

In these studies, symptom assessment was valued qualitatively – counting the ratio of organs infected over the healthy ones – and quantitatively – by measuring the percentage of organ surface affected by symptoms with respect to the total surface. Miclot et al. (2012) compared three different inoculation techniques to assess the best method for quantitative analysis of PM resistance in grapevine. Starting from dry (Cartolaro and Steva, 1990), wet (Yamamoto et al., 2000) and drop (Moyer et al., 2010) inoculations they determined a semi-quantitative index, which integrates pathogen sporulation and mycelium growth values. To normalize their results, data were converted into values according to the OIV 455 descriptor (Organisation Internationale de la Vigne et du Vin, 2009). This system relies on a rating of 1 to 9, where 1 represents the highest sporulated surface area (total susceptibility), 3 represents a strong infection, 5 indicates a medium infection, 7 a weak infection and 9 indicate that no symptoms are recognized (completely resistant). Within the EPPO (European and Mediterranean Plant Protection Organization, 2001) code, the two indicated parameters of quality (disease incidence) and quantity (disease severity) are available for symptom assessment. Improved accuracy in symptom assessment can be achieved through histochemical staining and microscopic analysis. Light microscopy can distinguish hyphae, appressoria, conidia and conidiophore, while haustoria cannot be monitored (Cadle-Davidson et al., 2010). Also confocal scanning electron microscopy (SEM) and low-temperature scanning electron microscopy (LTSEM) have been used to study *E. necator* (Cadle-Davidson et al., 2010; Carver et al., 1994; Gadoury et al., 2012; Ramming et al., 2012) (Cadle-Davidson et al., 2010; Carver et al., 1994; Gadoury et al., 2012; Gao et al., 2016; Ramming et al., 2012). Stainings successfully employed are: i) Coomassie blue, to monitor conidium germination and infiltration into host cells (Ramming et al., 2011, 2012), ii) Trypan blue, to label the plant dead cells, following conidium germination, hyphae growth, and conidiophore emergence (Gao et al., 2016); iii) Aniline blue, to follow the infection using bright microscopy (Fekete et

al., 2009; Gao et al., 2012a) as well as to localise the spores using fluorescence (Pessina et al., 2016; Vanacker et al., 2000).

Black rot

The causal agent, *Guignardia bidwellii* (Ellis) Viala & Ravaz, is a hemibiotrophic pathogen [asexual morph *Phyllosticta ampelicida* (Engelm.) Aa]. BR can attack all the herbaceous expanding organs of the plant (leaves, shoots, tendrils, petioles and berries), with young shoots and fruits being extremely sensitive (Kuo and Hoch, 1996). The infection is characterized by a first symptomless phase and a second necrotic and damaging phase (Kuo and Hoch, 1996; Luttrell, 1974). On the adaxial surface of the leaves, the fungus causes the appearance of small circular spots that evolve into light brown lesions with darker borders. The central portion of the spot turns necrotic and pycnidia become visible as small black dots. On the fruits, the first occurrence is the appearance of small whitish dots that rapidly expand concentrically around the berry, forming a brown patch. Later, darker pycnidia develop as the berries rot and shrink, turning into black mummies (Ramsdell and Milholland, 1988).

BR is a polycyclic disease with repeated cycles of primary and secondary infections (**Figure 4**). As reviewed by Onesti (2015), the fruiting bodies bearing the ascospores (ascocarps) have been referred to as either perithecia or pseudothecia, since the wall of stromal tissue of the pseudothecium can be confused with the wall formed by the peridium in a simple perithecium; hence the term pseudothecia will be used throughout the manuscript. Ascospores and conidia are both released during precipitation events: Ferrin and Ramsdell (1978) found a positive correlation between BR infections and the magnitude of rainfall, the number of events and their duration, and the persistence of water on leaves. These observations were also confirmed recently (Onesti et al., 2018). As ascospores and conidia are both sensitive to desiccation, BR is not a prevalent disease in dry climates (Ferrin, 1976; Ferrin and Ramsdell, 1977; Spotts, 1976, 1977), however, in the field, spores can germinate even after dry summer periods (Besselat and Bouchet., 1984; Ferrin and Ramsdell, 1978; Hoffman et al., 2004) and pycnidia can produce conidia even after three months of low humidity (Onesti et al. 2017). Field experiments demonstrated that release dynamics of both types of spores are conserved: conidia are released approximatively at budburst stage, while for ascospores it occurs two weeks later; in the course of the fruiting season, when berries are

pea-sized, both types reach their maximum (Onesti et al., 2018). For the ascospores, the peak is registered between flower pre-blooming to anthesis (Ferrin and Ramsdell, 1977).

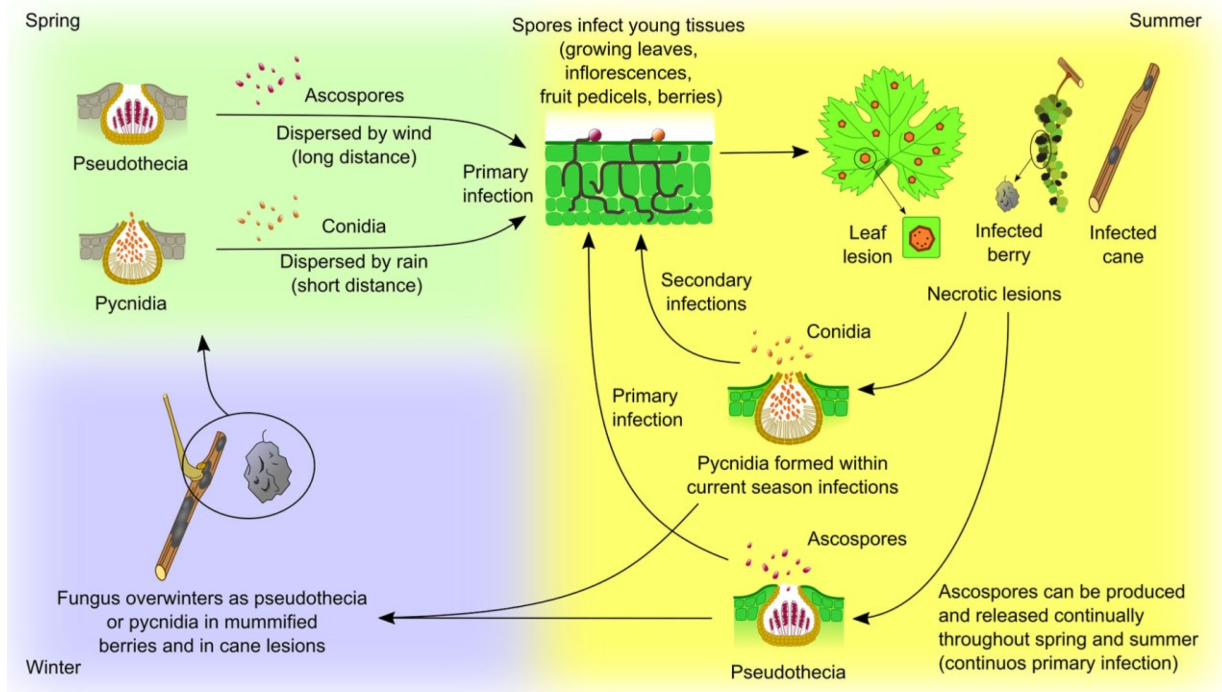


Figure 4 *Guignardia bidwellii* life cycle. The fungus overwinters in mummified berries, retained on the vine or fallen to the ground, and on infected canes. Berry mummies host both pseudothecia, containing asci with ascospores, and pycnidia, with conidia, while canes and tendrils host pycnidia. Lesions capable of producing conidia can persist in the wood for at least two years. In spring, ascospores and conidia are released when pseudothecia and pycnidia become thoroughly wet; infection is therefore favored by frequent rainfall as the spores need water to be released and to germinate. Ascospores released from mummified berries are the most common form of primary inoculum. They are ejected actively from the asci during rainfall and are dispersed by wind currents (long distance dispersion). On the contrary, conidia are exuded from the pycnidium in a white, mucilaginous cirrus from which they can be splashed away by rain (short distance dispersion). Primary infection from ascospores or conidia takes place on young, rapidly growing green tissues (growing leaves, inflorescences, fruit pedicels, berries). Adult leaves and ripe fruits, that have become fully expanded, are not susceptible to infection. Pycnidia are produced rapidly within the necrotic lesions found on leaves, shoots and berries, and, once mature and dampened by rain, they release the conidia which serve as secondary inoculum throughout the season. When the weather is moist, ascospores may be produced and released continually throughout spring and summer from mummies retained in the canopy, providing continuous primary infection, although most of them are discharged in the spring. In late summer, the sexual cycle initiates on infected berries and pseudothecia are formed (Wilcox et al., 2017).

The most sensitive period for direct infection on berries is after flowering, from fruit onset to the beginning of bunch closure. A field-trial of artificial infection in a *V. labrusca* ‘Concord’ vineyard revealed that plants infected between mid-bloom and fruit onset present

the highest lesion number on leaves and the greatest berry infection (Ferrin and Ramsdell, 1978). These data were confirmed also in greenhouse conditions (Kuo and Hoch, 1996) (**Figure 3D-G**). Moreover berries can be susceptible for longer to *G. bidwellii* compared to other relevant pathogens, for istance *E. necator* (Gee et al., 2008). Since the duration of phenological stages can differ among cultivars, windows of susceptibility (Hoffman et al., 2002) and the number of days after infection for symptom appearance (Roznik et al., 2017) are cultivar-specific.

Ontogenic resistance of plant hosts to fungi is widely documented (Populer, 1978), and for *G. bidwellii* it might be a defense mechanism able to counteract the pathogen, rather than a reduction in the germination ability of the fungus in older tissues (Kuo and Hoch, 1996; Molitor and Berkelmann-Löhnertz, 2011). Notably, the extent of the infection negatively correlates with leaf size, since smaller leaves display greater infected surface, than larger, older leaves (Jabco et al., 1985; Luttrell, 1948). Kuo and Hoch (1996) suggest “expanding” and “non-expanding” organs to better describe the resistance displayed by aging tissue. With regards the duration of pathogen incubation, Molitor et al. (2012) do not report differences on leaves of different ages. In contrast, at grape level ontogenic resistance under field conditions is responsible for the decrease of the number of infected berries and the increases of the incubation time (Hoffman et al., 2002; Hoffman and Wilcox, 2002). Roznik et al. (2017) suggested that the developmental stage of plant tissue is crucial for the results of the artificial tests, which could explain the incongruence of research group results. Finally, this issue is tightly linked to different inoculation conditions since constant temperature shortens the incubation period rather than fluctuations (Spotts, 1980), the required incubation time on leaves (Molitor et al., 2012; Spotts, 1977) and shoots (Northover, 2008), and the release of ascospores are temperature dependent (Rossi et al., 2015).

Cross-inoculation experiments between fungi collected from different species (*Parthenocissus tricuspidata*, *P. quinquefolia*, *Muscadinia rotundifolia*, *V. labrusca, V. bourquina, V. vinifera*) demonstrated that *G. bidwellii* includes three *formae speciales* (*f.sp.*), named *parthenocissi, muscadinii* and *euvitis*, with different degree of pathogenicity on the different hosts (Luttrell, 1946, 1948). Greenhouse assays were later performed (Jabco et al., 1985) with *f.sp euvitis* and *muscadinii* exploring leaf and petiole infection to assess the specific resistance response of four grapevine classes: vinifera, french and american hybrids, rotundifolia. The latter class showed a medium to high susceptibility to *f.sp. muscadinii*, while it resulted high resistant to *f.sp. euvitis*; in contrast, the other three classes displayed high to

medium resistance to *f.sp. muscadinii*, but medium to high susceptibility to *f.sp. euvitis*, with vinifera class developping larger lesions. *In vitro* cultures were also used to clarify the life cycle of the homotallic *G. bidwellii* (Jailloux, 1992). Light triggers pseudothecia maturation and differentiation, but during the first phase of mycelial growth, it inhibits pseudothecial growth and it induces pycnidia production. The optimal temperature for mycelial growth and pycnidia development (25°C) is also adequate for the first phase of pseudothecial growth, however lower temperatures are necessary for the maturation and differentiation of the ascospores. Temperature was also found to influence the dynamic and the number of pycnidia production and conidial germination (Onesti et al., 2017b).

Anthracnose

The causal agent of AN is commonly attributed to the hemibiotrophic *Elsinoë ampelina* Shear, whose asexual morph is *Sphaceloma ampelinum* de Bary (**Figure 5**). Moreover, some authors reported also *Colletotrichum* spp., another Ascomycete, associated with the disease symptoms, such as *C. nymphaeae*, *C. fructicola* and *C. gloeosporioides* (Guginski-Piva et al., 2018; Liu et al., 2016; Sawant et al., 2012), *C. goditiae* (Baroncelli et al., 2014; Zapparata et al., 2017) and a complex of species that were grouped as *C. viniferum* (Yan et al., 2015). However, according to another author, *Colletotrichum* spp. is the causal agent of “ripe rot” in grapevine (Huang, 2016; Wilcox et al., 2017). Therefore, this association between pathogen and disease is still controversial.

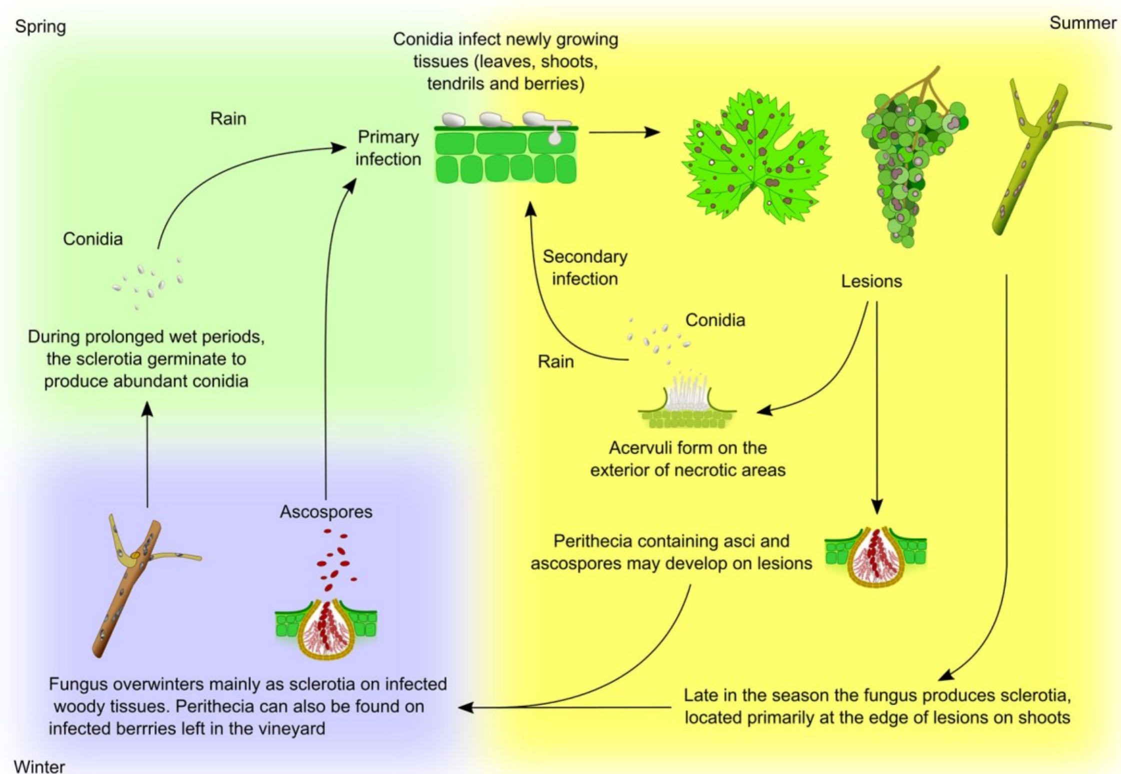


Figure 5 *Elsinoë ampelina* life cycle. The fungus overwinters mainly in infected canes as sclerotia, dense mycelial masses which are formed in autumn at the edge of lesions on shoots. In spring, sclerotia develop stromata on which, under humid conditions, produce abundant conidia. These conidia are then carried by rain or free water to young, rapidly growing green tissues (leaves, shoots, tendrils and young berries) where they germinate causing the primary infection. Conidia are by far the most important source of primary inoculum in spring. The fungus can also overwinter on infected berries, left on the vine or vineyard floor, as conidia or ascospores, which may also cause primary infections. Once the disease is established, the pathogen produces lesions upon which acervuli form and produce conidia which, dispersed by raindrops, serve as secondary source of inoculum for the rest of the growing season. During periods of humid weather, conidia can be released the entire spring and summer providing continuous infection. Heavy rainfall and warm temperatures are ideal for disease development and spread. At times pseudothecia, containing asci and ascospores, develop on the lesions (Wilcox et al., 2017).

The symptoms reported for the disease are similar, regardless of the causal agent. The pathogen attacks all aerial green parts of the plant, including fruit stems, leaves, petioles, tendrils, young shoots, and berries, however lesions of the pathogen are more common and distinctive on young shoots and berries (Magarey et al., 1993a) (**Figure 3H-K**). Structures of the pathogen are also found in dead tissues of the host, such as branches and fruits, making it difficult to eliminate the initial inoculum source of the pathogen (Magarey et al., 1993b). Infection on branches is recognizable by small circular reddish spots, afterwards the spots enlarge, forming a depression with gray center and rounded or angular edges, and eventually becomes surrounded by reddish brown or violet edges. Later on, the lesions may coalesce,

killing the infected tissues. In some cases, slightly raised edges surrounding the lesions are also visible (Ellis et al., 2008; Magarey et al., 1993a). On berries, the symptoms appear similar, initially, reddish circular spots appear, which evolve in size, and normally become slightly sunken. As spots grow, the center of the lesion turns whitish-gray, while edges assume a reddish-brown to black color. At this stage the lesion resembles a “bird's eye”, hence the popular name for the disease (Jang et al., 2011; Magarey et al., 1993a). Young leaves are more susceptible to infection than older leaves. The initial lesions are small, circular and chlorotic. The lesions become larger with gray centers and brown to black margins with round or angular edges. The center of the lesions becomes dry and ash, and often drops out, forming a “shot hole” appearance (Magarey et al., 1993a). The size of the lesions may vary with the degree of resistance of the host genotype. While bigger and circular spots are reported for susceptible cultivars, smaller and with irregular shape lesions were observed in resistant cultivars (Kono et al., 2012) (**Figure 3H-K**).

The identification of resistance sources to AN has been a major task for grapevine breeders, especially in Brazil and China. Screenings for AN resistance in fields with ongoing natural infections have effectively been applied for genetic improvement goals (Fennell, 1948; Li et al., 2008; Mortensen, 1981; Wang et al., 1998). However, in the field the disease is highly influenced by the climatic conditions, requiring some years of evaluation to produce robust data. This, in addition to the perennial nature of the host, makes the analysis time consuming and costly.

Therefore, many authors focused their efforts on establishing alternative methodologies based on artificial infection with conidia on potted plants in greenhouses and on detached cane, detached leaf or leaf piece assays. For these purposes, *in vitro* production of conidia of *E. ampelina* proved to be a major challenge, due to its poor and unstable sporulation in culture. Even though Hopkins and Harris (2000) produced conidia from 3- to 4-week-old cultures of *E. ampelina* maintained on potato dextrose agar (PDA), other researchers were unable to reproduce these results (Santos et al., 2018a, 2018b). In order to improve the yield of conidia, Yun et al. (2006) transferred the pathogen in Fries liquid medium and incubated it in a shaking incubator (140 rpm) at 28ºC for 10 days. Cultures were then transferred to a V-8 juice agar medium and incubated at 28ºC under a near ultraviolet lamp for two days to stimulate spore production. Kono et al. (2009) took a step forward and was the first to test the effect of culture conditions on conidia formation of *E. ampelina*. The study revealed the following three indispensable conditions for conidial production: (i) the

density of colonies in pre-culture should be lesser than 2.5 colonies per cm^2; (ii) depending on colony density, colonies should grow for 4 to 9 days on PDA; and (iii) grown colonies should be cultured in water with shaking (150 rpm) for 8 to 10 h in the dark at 24ºC. Santos et al. (2018a) used a similar protocol. Conidial suspensions were obtained by placing *E. ampelina* cultures in rainwater and shaking at 200 rpm in darkness for 7 days to induce sporulation. Mortensen (1981) set up greenhouse screenings for resistance to *E. ampelina*, however the protocol was deemed not reliable; later Hopkins and Harris (2000) optimized greenhouse screening by misting the seedlings with a suspension containing 10^6 conidia mL^{-1} and placing the inoculated seedlings in a moist chamber at 24°C for 48 hours, followed by 8 days on a greenhouse bench. However, consistent results were obtained at temperatures ranging from 20 to 28°C. Inoculations using conidia suspension were also performed on detached canes (Santos et al., 2018a), on detached leaves (Kono et al., 2012; Tharapreuksapong et al., 2009) and on leaf pieces (Poolsawat et al., 2012). After the inoculation, the tissues were maintained under high humidity at a 25-27°C. The symptoms were assessed in variable times of incubation. The results obtained by these assays were consistent and were applied for pathogenicity analysis (Santos et al., 2018a; Tharapreuksapong et al., 2009), germplasm characterization (Kono et al., 2012) and genetic analysis (Poolsawat et al., 2013; Tantasawat et al., 2012).

As an alternative to artificial infection with conidia, Yun et al. (2006) et al. (2006) developed a bioassay based on fungal cell-free culture filtrate (CFCF). In this case, the pathogen was cultivated in Fries medium for 21 days at 28 °C, after then the CFCF was obtained by centrifugation and sterilized through ultra-filtration (0.2 µm). The CFCF was applied on wounded young leaves. The results demonstrated that the bioassay with culture filtrates of the pathogen were consistent with those from pathogen inoculation in the greenhouse and screening in the vineyard. The methodology was further validated by Yun et al. (2007) and applied for germplasm characterization (Jang et al., 2011; Louime et al., 2011) and genetic analysis (Kim et al., 2008).

Powdery mildew, black rot and anthracnose control measures

Effective disease control encompasses the combination of sanitary as well as cultivation practices, the use of resistant or at least less susceptible grapevine varieties, the application of fungicides and decision support systems (e.g. Hoffman et al., 2004; Molitor and Beyer, 2014; Wilcox, 2003). Cultivation and sanitation practices are essential to manage PM,

BR and AN; for instance, adequate pruning and removal of leaves covering clusters provide conditions to reduce infections of PM. The removal of mummified bunches, infected canes and tendrils from the vineyard allows to reduce the primary inoculum of BR and AN, as well as to cover mummies or infected berries on the ground by soil cultivation or mulching (Wilcox et al., 2017). Especially for BR, abandoned vineyards should be cleared since BR can build up high amounts of inoculum at these places that can escape under favorable weather conditions and cause unexpected serious damages in the neighboring vineyards (Ullrich et al., 2009).

Due to the susceptibility of *V. vinifera* cultivars to PM, BR and AN, fungicide applications are necessary to control these diseases. In particular, thallus of *E. necator* develops almost completely outside of the infected tissues on the leaf and bunch surface, therefore the fungus is susceptible to topical applications of several contact active ingredients (Wilcox et al., 2017). Since 19th century, sulfur remains the most widely used fungicide, due to its low cost and protectant-curative action (Wilcox et al., 2017). The persistence of sulfur efficacy relies on the lack of resistance development, depending on its multi-site mechanism of action by direct contact and vapour phase: respiration inhibition, chelation of heavy metals needed for biochemical pathways and disruption of protein function (Oliver and Hewitt, 2014). It causes the damage of cellular membrane followed by loss of water and therefore death of the fungus by dehydration. Other than sulfur, several single-site synthetic fungicides are effective against PM, including contact, translaminar and systemic products, with specifically targeted mechanisms of action. Among them, mitosis and cell division inhibitors (e.g. benzimidazoles) and cell membrane synthesis alteration via ergosterol biosynthesis inhibitors (e.g. triazoles); different mechanisms concern respiration chain inhibition – via quinon inhibitors (e.g. strobilurines) or succinate dehydrogenase inhibitors – and signal transduction inhibition (e.g. azanaftalenes) (Oliver and Hewitt, 2014; Wilcox et al., 2017). Alternation of active ingredients with different mode of action is important to avoid the development of pathogen resistance, especially towards single-site systemic fungicides. Regular plant protection with chemical fungicides is the strategy of choice to control BR. High efficacy was observed with the fungicide classes of demethylation inhibitors (DMI), quinone outside inhibitors (QoI) and dithiocarbamates (Molitor and Beyer, 2014). Analogously to PM and BR, AN control relies on prophylactic fungicide applications. The first measure against the disease is to reduce the inoculum source by the elimination of cultivation remains and dormant spray of lime sulfur (Magarey et al., 1993b). The initial

development of the fungus occurs outside the green tissues, resembling the conditions described for PM. For chemical control of the AN disease, multi-site active fungicides based on copper (e.g. copper hydroxide, copper oxychloride, cuprous oxide and copper sulfate) and on the chemical groups dithiocarbamate, phthalimide, phthalonitrile and anthraquinon may be employed. Single-site synthetic fungicides are also effective acting on cell division (e.g. thiophanate), on extracellular inhibitor of quinone (e.g methoxy-acrylate) and on sterol biosynthesis (e.g. triazoles) (FRAC, 2019).

Due to the potential negative impacts of fungicide application, non-synthetic chemicals and organic control measures are also used to regulate these three diseases. For organic viticulture, applications of copper and sulfur are recommended, but generally they are less effective in comparison to the synthetic active compounds (e.g. Loskill et al., 2009; Wilcox et al., 2017). Nowadays, organic management against PM can rely, other than sulfur, on non-toxic substances such as botanical oils and inorganic salts, acting by contact with the fungal thallus (Wilcox et al., 2017). The application of *Ampelomyces quisqualis* (hyperparasite fungus) at the time of chasmothecia formation can help reducing the overwintering inoculum of *E. necator* (Pertot et al., 2017). Concerning organic BR control, the application of copper is insufficient but in combination with sulfur showed a clearly higher effect to reduce the disease impact (Loskill et al., 2009). Moreover, first attempts with plant extracts from *Primula* roots and *Hedera helix,* containing saponine as active compound, showed clear effects in greenhouse experiments. However, under field conditions the activity was low due to the high water solubility (Koch et al., 2013). Consequently, in organic farming, it is crucial to integrate adequate sanitation, new varieties with genetic resistance or high tolerance, application of forecast models and web-based decision support systems (Molitor et al., 2016; Onesti et al., 2016) for the optimization of the fungicide spray regime. Regarding AN, plant extracts from *Chaetomium cupreum* and *C. globosum*, antagonistic microorganisms such as *Trichoderma harzianum*, *T. hamatum*, *Penicillium chrysogenum* and the natural antibiotic substances Rotiorinol, Chaetoglobosin-C and Trichotoxin A50 have been tested and proved to reduce AN incidence on leaves, shoots and grapes (Soytong et al., 2005). Bacterial antagonists are also cited for organic control, like *Bacillus* species strains TS-204 and TL-171 (Sawant et al., 2016).

Ascomycete resistance to fungicide

To date more than 50 modes of action have been identified for fungicides (FRAC, 2019), those ones that have a single-site mode of action are more problematic since resistance can rapidly evolve by a single mutation (Brent and Hollomon, 2007). Pathogens show cross resistance to compounds with the same mode of action but not to the other ones (Hollomon, 2015).

Among the widely employed fungicides used to control PM are the sterol DMI and QoI. *E. necator* resistance to DMI was reported in the 80s from California, Portugal and Australia (Gubler et al., 1996; Ogawa et al., 1988; Savocchia et al., 1999; Steva and Cazenave, 1996; Ypema et al., 1997). The DMI resistance is a multigenic trait, but with one major mechanism involving a single mutation in the gene *CYP51* coding for the cytochrome P450 lanosterol C-14α demethylase. Studies on DMI fungicide resistance revealed several possibilities to confer reduced sensitivity: (i) mutation of *CYP51*; (ii) overexpression of *CYP51*; (iii) overexpression of transporter coding for efflux pumps and (iv) other unknown mechanisms able to confer weak resistance (Cannon et al., 2009; Cools and Fraaije, 2013; Corio-Costet et al., 2003; De Waard et al., 2006; Délye et al., 1997a, 1998; Frenkel et al., 2015; Hamamoto et al., 2000; Hayashi et al., 2002; Kretschmer et al., 2009; Leroux et al., 2007; Leroux and Walker, 2011; Luo et al., 2008; Lupetti et al., 2002; Ma et al., 2006; Schnabel and Jones, 2001; Sombardier et al., 2010; Stergiopoulos et al., 2003; Wyand and Brown, 2005). *E. necator* is one of the first fungi for which it was demonstrated that a point mutation in *CYP51* is associated with DMI resistance. A mutation in codon 136 converts tyrosine (Y) to phenylalanine (F), reducing the sensitivity to the fungicide (Délye et al., 1997a). Moreover, a nucleotide substitution in position 1119 (A1119C) increases the *CYP51* expression causing a comparable lower sensitivity to the fungicide (Frenkel et al., 2015). QoI fungicides inhibit mitochondrial respiration by binding to the cytochrome bc1 enzyme complex (complex III) at the Qo site, blocking the electron transfer to cytochrome c1, and preventing the synthesis of adenosine-5′-triphosphate (ATP). Several point mutations in the *cytochrome b* (*CYTB*) gene confer QoI resistance (Gisi et al., 2002). *E. necator* resistance to QoI was initially described in the United States (Baudoin et al., 2008; Miles et al., 2012; Wilcox, 2005) and it is mainly associated with a point mutation in the codon 143 of *CYTB* that converts glycine (G) to alanine (A) (Bartlett et al., 2002; Dufour et al., 2011; Ma and Michailides, 2005). Recently, the emergence of *E. necator* resistance to other fungicides was

reported, such as metrafenone, a benzophenone of which mode of action is still not known, and boscalid, a fungicide that inhibits the activity of the enzyme succinate dehydrogenease (Cherrad et al., 2018; Kunova et al., 2016).

G. bidwellii resistance to fungicide is not well studied even if the first report of DMI fungicide reduced sensitivities dates back to 1986 in France (Thind et al., 1986). Different field experiments demonstrated that DMI and QoI are almost 100% efficient in the BR control (Ellis, 1986; Harms et al., 2005; Lafon et al., 1984; Loskill et al., 2009; Tomoiaga and Comsa, 2010; Wilcox, 2000; Wilcox et al., 1999; Wilcox and Riegel, 1996, 1997). Moreover, sequence analysis pinpoints that *G. bidwellii* has a low risk to generate QoI resistance (Miessner et al., 2011).

Fungicide resistance in *E. ampelina* has not yet been characterized. So far just one report describes the reduced sensitivity of the pathogen to carbendazim, a methyl benzimidazole carbamate fungicide, that inhibits microtubule assembly during mitosis (Deokate et al., 2002).

Genetic variability

Molecular marker development

Erysiphe necator

Variations in the overwintering strategies have been observed in *E. necator* specimens in correlation to their geographic location. Given the scarcity of information about PM epidemiology, in the 90's Délye et al. (Délye et al., 1997b; Délye and Corio-Costet, 1998) carried out a number of studies based on the use of RAPD (Random Amplified Polymorphic DNA) molecular markers, as well as mutagenized *CYP51* which encodes a eburicol 14α-demethylase, a highly conserved cytochrome P-450 enzyme essential for sterol biosynthesis (Délye et al., 1999). Analyzing the genetic variation among populations of *E. necator* from Europe, Asia, North-Africa and Australia, the existence of two main genetic groups was identified: the flag-shoot (A) and the ascospores (B) biotypes. In contrast, Cortesi et al. (2004) using the Inter-Simple Sequence Repeat (ISSR) markers did not observe any correlation

between overwintering strategies and genetic groups. To better understand the distribution of the two biotypes, more in-depth research was devised. Some studies employed tagging of specific sequences by PCR (e.g. Brewer et al., 2011; Oliveira and Cunha, 2015), while others reported the use of dominant markers such Amplified Fragment Length Polymorphisms (AFLPs) (Núñez et al., 2006) and Random Amplification of Polymorphic DNA (RAPDs) (Péros et al., 2005). Codominant markers, i.e. Sequence Characterized Amplified Regions (SCARs), Random Fragment Length Polymorphisms (RFLPs), and Single Nucleotide Polymorphisms (SNPs), have been adopted on *CYP51* and *Entub* genes with the same aim (Amrani and Corio-Costet, 2006; Evans et al., 1997; Hajjeh et al., 2005; Miazzi et al., 2008; Stummer et al., 2000). More recently, Single Sequence Repeats (SSRs) were proven to be most effective markers because of their high polymorphism, co-dominance and reproducibility. SSRs are widely used but their application in fungi was limited due to scarce sources of genetic diversity; moreover microsatellites are less abundant and present a reduced number of repeats in fungi (Dutech et al., 2007). One of the first attempts to use SSR markers to distinguish genetic groups of *E. necator* was made by Péros et al., 2006a). Wakefield et al. (2011) used cDNA-AFLPs to investigate the transcriptional changes during pathogenesis stages. Currently, the leading study on this topic is Frenkel et al. (2012) which developed microsatellite markers to investigate population structure of the causal agent of grapevine PM in North America, using 11 transcriptome-based microsatellites. According to these studies − despite a higher amount of genetic group A isolates is usually found early in the growing season slightly giving away to group B over the course of the epidemics (Amrani and Corio-Costet, 2006; Miazzi et al., 2008) − the separation between the two biotypes is not dependent on the primary source of inoculum (Núñez et al., 2006). Moreover, in Europe the distribution of the two biotypes seems to be linked to the geographic location and host cultivar though in some cases both groups were detected in the same vineyard or even on the same plant (Amrani and Corio-Costet, 2006; Oliveira and Cunha, 2015).

Guignardia bidwellii

G. bidwellii-specific SSR markers appeared almost ten years later than *E. necator* markers. Only recently, Narduzzi-Wicht et al. (2014) developed 11 specific SSR markers, which were used to evaluate more than 1300 specimens, albeit without finding a relation between the species/cultivar of *Vitis* host, and the genotype of the infecting *G. bidwellii*. These 11 SSR markers were also used to analyze 37 strains of *G. bidwellii* and to assess the

genetic variability of the fungus. This analysis revealed the presence of 56 haplotypes from 421 analyzed berries divided into four main subpopulations, pinpointing that the sexual reproduction is a crucial step in the progression of the epidemic in the vineyard (Rinaldi et al., 2017).

Elsinoë ampelina

The genus *Elsinoë* includes at least 75 species causing diseases on many plant hosts, including economically important crops. Hyun et al. (2001) performed a molecular characterization of several *Elsinoë* strains using RAPD markers, dominant markers with a limited application in genetic diversity studies. More recently, genome sequencing approaches allowed the revision of the fungus taxonomy, using both DNA sequences and published morphological data (Fan et al., 2017). As of today, codominant markers (SSRs or SNPs) are not available for genetic studies. Very little is known about *E. ampelina* genetic variability. First evidences of the pathogen variability were based on morphological characterization, which revealed polymorphism of colony size, colony color/appearance and conidial morphology (Mathukorn Sompong, 2012; Poolsawat et al., 2009). However, the morphological characteristics are too variable and do not necessarily reflect the genetic diversity (Poolsawat et al., 2009, 2010). Therefore, molecular characterization is required to precisely differentiate the isolates. The use of the dominant RAPD markers confirmed the high pathogen diversity in Thailand (Poolsawat et al., 2010; Tharapreuksapong et al., 2009). It should be noted that, in this instance, genetically divergent isolates have shown different levels of pathogenicity. For example, Poolsawat et al. (2010) analysed five isolates, representing four genetically different groups, testing nine genotypes of grapevine. All isolates were pathogenic to susceptible genotypes: in particular, the host genotypes Wilcox321 and Illinois547-1 were highly resistant to all isolates. NY65.0550.04 was highly resistant to most isolates except Nk4-1. NY88.0517.01 and NY88.0507.01 were resistant only to some isolates and NY65.0551.05 was susceptible to most isolates except Nk5-1. These results suggest the presence of different resistance genes in the host and corresponding avirulent genes of the pathogen.

Within *Colletotrichum* spp., only *C. gloeosporioides* has 39 SSR markers identified (Penet et al., 2017) with a range of 2-29 alleles, enabling genetic diversity studies without the need of previous sequencing. However, there are no studies reporting the extensive use of

these markers for isolate characterization. So far, the publications exploring *Colletotrichum* diversity and phylogeny involved the sequencing of genomic regions (Baroncelli et al., 2014; Guginski-Piva et al., 2018).

Barcoding and genome sequencing

Erysiphe necator

The first internal transcribed spacer (ITS) of ribosomal gene sequence from *E. necator* was released in 1999 (Saenz and Taylor, 1999): this enabled the identification of primers unique to *E. necator* to differentiate this fungus from other causal agents of PMs (Falacy et al., 2007). Since then several sequences have been deposited and nowadays there are 3380 nucleotide accessions available at the NCBI (National Center for Biotechnology Information), whereas 6681 genes are reported in the Ensembl Fungi database. However, since control of PM infection in the field is based on the use of chemical fungicides and canopy management, a deep focus on '-omics' approaches is fundamental to get a broad picture of the pathogen profile. Given the wealth of know-how and technologies becoming available, in the last decade this resulted timely and feasible and the genome of five *E. necator* isolates has been release in 2014 (Jones et al., 2014).

Causal agents of PMs (Ascomycota) are the most important biotrophic fungi together with rusts (Basidiomycota). In the last decade the genome of the most impacting pathogens belonging to Erysiphales were deciphered (Hacquard, 2014; Jones et al., 2014; Spanu et al., 2010; Wicker et al., 2013). Surprisingly the genome-size of these biotrophic pathogens (≈125Mb in *E. necator*) is 3-4 times larger than other Ascomycetes. Sanger sequencing of *Blumeria graminis* (Spanu et al., 2010) and shotgun approaches on *E. necator* (Jones et al., 2014) uncovered that genome expansion is a consequence of transposable element (TEs) and microsatellite accumulation, constituting more than the 60% of the total genome. Moreover, genes encoding enzymes involved in repeat-induced point mutations (RIPs), a natural mechanism to prevent TE accumulation, were lost. Genes, whose products participate in the synthesis of primary and secondary metabolites not required for biotrophy, have been lost too (Hacquard, 2014). This suggests the high capacity of fungal genome to adapt to different environmental conditions is closely linked to their biotrophic life cycle (Bindschedler et al.,

2016). Following the sequencing of five wild strains of *E. necator*, the data comparison confirmed the large number of repetitive elements and the difference in their copy number variations (CNVs) among isolates (Jones et al. 2014). This study demonstrated the adaptive role of CNVs in the establishment of resistance to DMI (sterol demethylase inhibitors) fungicides since structural variations are related to their target protein EnCYP51 (cytochrome P450 lanosterol C-14α-demethylase).

Guignardia bidwellii

The identification of *G. bidwellii* commonly relied on morphological analyses and on the observation of the symptoms on the affected plants (Kong, 2009), however these parameters are not sufficient for specific pathogen identification. Currently, a few hundred *G. bidwellii* reference sequences are available in public databases, most of them related to the ITS1 and ITS2 of ribosomal RNA genes (*18S* rRNA and *28S* rRNA), calmodulin and beta-tubulin genes. Some of these sequences (ITS1-2 and calmodulin genes) were used to confirm that Boston ivy infections were due to *G. bidwellii* (Kwon et al., 2015) and to analyze *G. bidwellii* samples collected from several grapevine cultivars and ornamental plants suggesting a specificity at the host genus level (Wicht et al., 2012) (**Figure S1A**). Detailed phylogenetic trees were generated based on ITS, actin, TEF1A and GAPDH sequences (Zhang et al., 2013). The genetic relationship of *G. bidwellii* (asexual morph *P. ampelicida*) to other *Phyllosticta* species suggested to consider the *Parthenocissi* form as a new species.

Currently, full genome sequencing of *G. bidwellii* is not yet available. This would greatly advance the wealth of biological information of this pathogen and it will certainly enhance the potential development of tools aimed to control infections.

Elsinoë ampelina

The recognition of AN mostly relies on the observation of symptoms on the plants and/or of fungal morphology by *in vitro* culture, as well as characterization of conidia (Fan et al., 2017; Mortensen, 1981). Recent studies have employed molecular tools to identify isolates using sequences of fungal barcode regions (Schoch et al., 2012; Seifert, 2009), which aim at the characterization and differentiation within the fungal species (Guginski-Piva et al., 2018; Santos et al., 2018a). Sequencing analysis of a selection of genes from *E. ampelina* is

also quite recent; the sequencing of ITS, TEF1A and HIS3 in 39 *E. ampelina* isolates collected in 38 vineyards in southern and south-eastern Brazil revealed low genetic variability (Santos et al., 2018b). HIS3 sequence resulted to be the most informative, enabling the grouping of isolates into five haplotypes. Using the same genes, Santos et al. (2018a) compared the sequences of 18 isolates from Brazil and 17 isolates from Australia, where low levels of genetic variability were also detected. Remarkably, ITS and TEF sequences obtained from 35 isolates were identical. Polymorphism was observed only for HIS3 sequences, showing four distinct haplotypes. One of them was most predominant (82.9%) and was observed in both countries, other two were found exclusively in Brazil and one uniquely in Australia. The authors also found cultivation and conidial variation among the isolates, however no relationship was observed between the haplotype network structure and morphological characteristics of the isolates. Different levels of pathogenicity were observed, but this was not related to the origin of the isolates. Currently, there are no reports of complete sequencing, or scaffolding of the genome of *E. ampelina* in public databases. On NCBI 913 nucleotide sequences are available for this fungus, however 676 ESTs of them are related to the expression of host genes when this is challenged by the pathogen. Thus, only 236 deposited sequences are fragments of the genome of *E. ampelina*. Among these, ITS1 and ITS2 are the most representative with 78 and 79 deposited sequences, respectively, as well as the sequence for the 18S rRNA small subunit (SSU rRNA) with 36 sequences deposited and 42 sequences deposited as ssRNA, while for 5.8S and 28S, there are 78 and 36 deposited sequences respectively (**Figure S1B**).

Sequencing of the barcode regions was also used to identify *C. nymphaeae* as the causal agent of AN in China (Liu et al., 2016) and *C. godetiae* as the causal agent of AN in Italy (Zapparata et al., 2017) and the United Kingdom (Baroncelli et al., 2014). Among the various *Colletotrichum* spp. reported as causing AN, only *C. nymphaeae* and *C. fructicola* have scaffold sequences deposited on NCBI. With regards to *C. nymphaea* (access: JEMN01000491.1) 14,404 genes have been found within a 49.96 Mb genomic region, with 52.7% GC content. While for *C. fructicola* three sequencing projects are reported, one in the contig assembly phase and two with the scaffold of the genome; the bio design with greater detail of the genome (access: ANPB00000000.1) has a sequencing coverage of 55.61 Mb, with 53.5% GC content, where 15,469 genes and 88 pseudogenes were annotated, totaling 15,381 deduced proteins.

Grapevine-ascomycete interaction

Resistance loci identification

Powdery mildew

Conidia of *E. necator* germinate on green tissues of living grapevine plants producing infection vesicle that penetrates the surface and allows the mycelium to grow between the cells. To cope with the many microorganisms and potential pathogens on their surface, plants have evolved a system of basal immunity. This non-host resistance is based on the recognition of a pathogen-associated molecular pattern (PAMP), probably including chitin in PM (Qiu et al., 2015), sensed by an extracellular receptor-like kinase (RLK) (Dry et al., 2010). The response consists of the production of extracellular antimicrobial compounds and accumulation of molecules to reinforce cell-wall as callose papillae. In grapevine-*E. necator* interaction, PAMP-triggered immunity (PTI) can be overcome by the secretion of fungal effectors, detected by the plant and able to trigger effector-triggered immunity (ETI) mediated by resistance (*R*) genes to reach programmed cell death (PCD) (Gadoury et al., 2012).

Table 1. *R*-loci associated with powdery mildew (*Run/Ren*) and black rot (*Rgb*) resistance (improved based on VIVC web source and Merdinoglu et al., 2018).

Locus	Chr	Origin of resistence	Genotype of origin	Resistence level	Associated marker	Reference
Ren1	13	*V. vinifera*	Kishmish vatkana	Partial	UDV-020	Hoffmann et al. 2008
					VMC9h4-2	Hoffmann et al. 2008
					VMCNg4e10.1	Hoffmann et al. 2008
Ren2	14	*V. cinerea*	Illinois 547-1	Partial	CS25	Dalbó et al. 2001
Ren3	15	American *Vitis*	Regent	Partial	UDV-015b	Welter et al. 2007
					VViv67	Welter et al. 2007
					ScORA7-760	Akkurt et al. 2007
					VChr15CenGen02	van Heerden et al. 2014
					GF15-28 / VViv67	Zyprian et al. 2016
					ScORGF 15-32	Zyprian et al. 2016
					GF15-42	Zendler et al. 2017
Ren4	18	*V. romanetii*	C166-043	Partial	VMC7f2	Riaz et al. 2011
			C87-41		SNPs	Mahanil et al. 2012
Ren5	14	*M. rotundifolia*	Regale	Total	VMC9c1	Blanc et al. 2012

Ren6	9	*V. piasezkii*	DVIT2027	Total	PN9-057 PN9-068	Pap et al. 2016 Pap et al. 2016
Ren7	19	*V. piasezkii*	DVIT2027	Partial	VVIp17.1 VMC9a2.1 Ren6 minor QTL	Pap et al. 2016 Pap et al. 2016
Ren8	18	American *Vitis*	Regent	Partial	Ren3 minor QTL	Zyprian et al. 2016
Ren9	15	American *Vitis*	Regent	Partial	CenGen6	Zendler et al. 2017
Ren10	2	American *Vitis*	Seyval blanc		S2_17854965 Haploblock validation	Teh et al. 2017 Teh et al. 2017
Run1	12	*M. rotundifolia*	VRH3082-1-42	Total	VMC4f3.1 VMC8g9 49MRP1.P2 CB53.54	Barker et al. 2005 Barker et al. 2005 Feechan et al. 2013b Feechan et al. 2013b
Run2.1	18	*M. rotundifolia*	Magnolia	Partial	VMC7f2 VMCNg1e3 VVIn16	Riaz et al. 2011 Riaz et al. 2011 Riaz et al. 2011
Run2.2	18	*M. rotundifolia*	Trayshed	Partial	VMC7f2	Riaz et al. 2011
Rgb1	14	*V. cinerea*	Boerner	Partial	Gf14-42	Rex et al. 2014
Rgb2	16	*V. cinerea*	Boerner	Partial	VChr16c	Rex et al. 2014

With regards to PM resistance, in the last decades, it emerged that American and Asian *Vitis* represent a valuable source of *R* genes, which are localized within *R*-loci or genomic intervals (**Table 1**). *Run1* (Resistance to *Uncinula necator* 1) is a single dominant locus on chromosome 12 known to confer high resistance to *E. necator* detected in *M. rotundifolia* (Barker et al., 2005; Bouquet, 1986). Introgressed into a *V. vinifera* background through marker-assisted selection (MAS) (Pauquet et al., 2001), it was found to co-segregate with the *Rpv1* (Resistance to *Plasmopara viticola* 1) locus and to encode full-length and truncated TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide-binding site-leucine-rich repeat) resistance proteins (Feechan et al., 2013a). Surveys on resistant cultivars showed that this locus is involved in the induction of PCD within penetrated cells at 24 and 48 hours post-inoculation (hpi) (Dry et al., 2010). Subsequently, the *Run2.1* and *Run2.2* loci variants (haplotypes) were identified on chromosome 18 in *M. rotundifolia* 'Magnolia' (Riaz et al., 2011), while *Ren5* (misnamed, actually *Run3*) was mapped on chromosome 14 in *M. rotundifolia* 'Regale' (Blanc et al., 2012). Resistance to *E. necator* due to PCD was also observed in 'Kishmish vatkana' and 'Dzhandzhal kara'. These related cultivars share the *Ren1*

(Resistance to *E. necator* 1) locus carried on the chromosome 13 (Coleman et al., 2009; Hoffmann et al., 2008) and are an exception among the PM resistance donors since they belong to the *V. vinifera proles orientalis.* Very recently, a genome-wide characterization revealed role of NBS-LRR genes during PM infection in *V. vinifera* (Goyal et al., 2019).

In the same years, several Quantitative Trait Loci (QTL) analyses were carried out with the aim to identify new PM resistance loci. Partial resistance is conferred by major QTLs found on different chromosomes. *Ren2* on chromosome 14 confers race-specific resistance in *V. cinerea cinerea* (Cadle-Davidson et al., 2016; Dalbó et al., 2001). *Ren*3 on chromosome 15 – derived from an undetermined American *Vitis* species – was localized in the hybrid 'Regent' (Welter et al., 2007) and recently found to determine race-specific hypersensitive response by two different regions on that chromosome; in fact, Zendler et al. (2017) defined the *Ren3* limit and identified *ex novo* the distal *Ren9* locus. In addition, *Ren8* was mapped on chromosome 18 although with an uncertain origin (Zyprian et al., 2016). Besides the American sources, the wild Chinese species *V. romanetii* is donor of a non-race-specific and tissue-independent resistance conferred by the dominant locus *Ren4* on chromosome 18 (Mahanil et al., 2012; Riaz et al., 2011); this was introgressed into *V. vinifera* background to obtain vines able to prevent hyphal emergence from the PM agent (Ramming et al., 2011). Moreover, two major *R*-loci against *E. necator* were discovered in another Chinese species, *V. piasezkii*, *Ren6* and *Ren7* which are respectively localized on chromosome 9 and 19, and they both act in the post-infection stage bringing to PCD. The highest strength of (total) resistance is conferred by *Ren6*, even higher than *Run1*, while *Ren7* is responsible of a weak partial resistance to the pathogen (Pap et al., 2016). Finally, Teh et al. (2017) identified the new *Ren10* locus on chromosome 2 acting moderately against PM sporulation (**Table 1**).

Given the reported advances also on the molecular characterization of the studied Ascomycetes, we can assert that nowadays pathogen genetics can inform host genetics and host-pathogen interaction mechanisms. For instance, in the Eastern US, where the pathogen co-evolved with many mapped PM resistance genes, the *Ren2* locus has recently fully broken down and is no longer detectable in vineyard (Cadle-Davidson, 2018). Actually, in North America naturally occurring isolates displaying virulence on vines carrying the *Run* loci were already observed demonstrating that qualitative (vertical) resistance is strong, but since it is race-specific can be easily overcome (Feechan et al., 2015). By contrast, partial (horizontal) resistance – which typically is controlled by at least 4-5 QTLs – is usually more durable, particularly when it involves morphological or developmental changes in the plant, although

might be prone to gradual loss (erosion) in the long term (Stuthman et al., 2007). Therefore, to achieve long lasting resistance, the combination of both types is needed; this process, named *R* gene pyramiding, relies on genetics built into vines.

Pyramiding entails stacking multiple genes leading to the simultaneous expression of more than one gene in a cultivar to develop durable resistance expression. Gene pyramiding is gaining considerable importance as it would improve the efficiency of plant (including grapevine) breeding, leading to the development of genetic stocks and precise development of broad spectrum resistance capabilities (Joshi and Nayak, 2010). Both wine and table grape breeding programs for PM resistance around the world are using this approach, providing endurance both in term of absolute disease resistance degree (e.g. Feechan et al., 2015) and of the degree fluctuation reduction during different years (e.g. Zini et al., 2019). According to the most updated information on MAS applications at European level, in France "ResDur" varieties presenting assorted combinations of *Rpv1, Rpv3, Rpv10* associated with DM and of *Run1, Ren3* and *Ren3.2* associated with PM resistance were obtained by breeding "Bouquet" varieties with American, Asian and wild *Vitis* backgrounds (Delmotte et al., 2018). In Italy, Vezzulli et al. (2019) were able to obtain pyramided genotypes carrying two or three *Run/Ren* loci, up to seven *R*-loci in total, while Foria et al. (2018) developed resistant genotypes derived from "élite" cultivars carrying *Rpv1, Rpv12* coupled with *Run1* and *Ren3.* Finally, besides increasing host diversity and complexity, to achieve higher durability, populations of biotrophic pathogens should be regularly monitored for their virulence frequencies and virulence combinations (Miedaner, 2016).

In order to indirectly dissect PM resistance, an alternative approach relies on the biological candidacy of susceptibility (*S*) genes. Unlike *R* genes, *S* genes are required for successful pathogen infection, and thus are considered essential for compatible plant-pathogen interactions. A state of the art about the investigation and the future application of grapevine *S* genes associated with PM susceptibility is reported in the **Supplementary Text**.

Black rot

In contrast to PM, for which several *R*-loci have already been discovered in various grapevine cultivars, very little is known about the genetic loci involved in BR resistance (**Table 1**). It has been observed almost one century ago that North American and French hybrids are more resistant to *G. bidwellii* infection, while *V. vinifera* is extremely susceptible

(Barrett, 1955; Demaree et al., 1937; Hausmann et al., 2017). The American origin of this ascomycete explains the occurrence of genetic resistance in American *Vitis* species, due to their coevolution (Hausmann et al., 2017; Ramsdell and Milholland, 1988). Hints about the genetic segregation pattern of BR resistance were suggested in a 'Blue Lake' progeny by Mortensen (1977), but the first QTL analysis was performed much later by Dalbó et al. (2000). They studied the inheritance of BR resistance in the cross 'Horizon' ('Seyval' × 'Schuyler') × 'Illinois 547-1' (*V. rupestris* × *V. cinerea*) and constructed a genetic map based on RAPD markers for each parent. This analysis identified one QTL on chromosome 14 associated with the CS25b marker (Dalbó et al., 2000, 2001). Interestingly, the latter map interval was also associated with PM resistance though to different degrees (Dalbó et al., 2000).

Later Rex et al. (2014) carried out a QTL analysis using a cross between a cultivar that shows high resistance to BR (cultivar 'Börner', an inter-specific hybrid of *V. riparia* × *V. cinerea*) and a susceptible breeding line (V3125, 'Schiava grossa' × 'Riesling'). They performed six independent resistance tests in a climate chamber and one disease evaluation in the field, and identified two *R*-loci; the major QTL on chromosome 14, called *Resistance to G. bidwellii1* (*Rgb1*) co-mapped with the QTL identified by Dalbó et al. (2000). A second QTL on chromosome 16 (named *Rgb2*) was detected both in climate chamber and in field analysis, while the results on other minor QTLs were not reproducible. All these data suggest a polygenic nature of the resistance (Rex et al., 2014). The analysis of Sequence-Tagged Sites (STS) markers did not allow a more accurate delineation of the *Rgb2* locus position. For *Rgb1* locus, the fine mapping on chromosome 14 restricted the region of QTL to about 2.4 Mb in the reference grapevine genome and allowed the authors the development of a new marker, GF14-42, that showed a strong association with *Rgb1*. Some genes with a putative defense function were annotated in the genomic region of *Rgb1* (i.e. chitinase, RIN4-like protein, MAP kinase and F-box domain protein; Rex et al., 2014). The QTL site on chromosome 14 was also detected in another biparental mapping population derived from a cross between 'GF.GA-47-42' × 'Villard Blanc' with both parents being resistant to BR. This study revealed the presence of seven *R*-loci distributed over the genome. The major QTL on chromosome 14 in this mapping population was tightly linked to the SSR markers GF14-04 and UDV-095, both are located in the vicinity of the above mentioned markers CS25b and GF14-42 (Hausmann et al., 2017) (**Table 1**). All these data suggest that the major *R*-locus on chromosome 14, identified in three different biparental population, plays a leading role in

conferring BR resistance. Finally, from the application point of view, nowadays some new bred varieties with a good field resistance to BR − carrying *Rgb* loci − are available (e.g. Töpfer et al., 2018; Töpfer and Eibach, 2016).

Anthracnose

In contrast to *E. necator* and *G. bidwellii* no QTL analysis has been published for *E. ampelina*. This is perhaps due to the fact that AN is not a major disease in the main grapevine production regions, such as Europe. However, the use of resistant cultivars, which reduces the amount of fungicide associated with the climatic break-down expected, might increase the incidence of this disease also in these regions. Therefore, the development of new varieties resistant to AN will be a major task worldwide in the near future and it is essential to develop molecular markers linked to AN resistance genes.

Resistance sources to *E. ampelina* have been identified through natural infection in field screenings (Fennell, 1948; Li et al., 2008; Louime et al., 2011; Mortensen, 1981; Poolsawat et al., 2012; Wang et al., 1998; Yun et al., 2006), by artificial infection in greenhouse (Hopkins and Harris, 2000), by detached-leaf assays (Kono et al., 2012; Poolsawat et al., 2012) and filtrate culture (Jang et al., 2011; Kim et al., 2008; Louime et al., 2011; Yun et al., 2006). In the American genepool, variable levels of resistance to AN were found in different *Vitis* spp. Based on natural infections, Fennell (1948) reported that the majority of the American tropical grapevine species show moderate to good resistance to AN. Accessions of *V. gigas* (Fen), *V. rufotomentosa* (Small) and *V. smalliana* (Bailey) were classified as highly resistant. Similarly, Mortensen (1981) reported as sources of resistance to AN accessions of *M. rotundifolia* Michx., *V. aestivalis* ssp. *simpsonii*, *V. aestivalis* ssp. *smalliana, V. caribeae* DC (syn. *V. tiliafolia* Humb. & Bonpl.)*, V. champini* Planch., *V. labrusca* L., *V. munsoniana* Simps, *V. rupestris* Scheele, *V. shuttleworthii* House and *V. vulpina* L. Field evaluations under natural infection of *E. ampelina* revealed that all the Asian *Vitis* species tested were classified as highly resistant or resistant to AN (Li et al., 2008; Wang et al., 1998). The species evaluated were *V. adstricta*, *V. amurensis*, *V. bashanica*, *V. betulifolia*, *V. bryoniifolia*, *V. davidii*, *V. flexuosa*, *V. hancockii*, *V. liubanensis*, *V. piazezkii*, *V. pseudoreticulata*, *V. qinlingensis*, *V. quinquangularis*, *V. romanetii*, *V. sinocinerea*, *V. wilsonae* and *V. yenshanensis*. In the same fields, the *V. vinifera* cvs. Cabernet Sauvignon,

Carignane and Chardonnay were classified as susceptible, highly susceptible and highly susceptible, respectively.

Under natural conditions the *V. vinifera* cultivars evaluated (e.g. Alexandria, Cabernet Sauvignon, Cardinal, Carignane, Chardonnay, Chasselas Golden, Exotic, Lignan Blanc, Malaga, Muscat of Alexandria, Perlette, Red Muscat, Sultanina, Thompson Seedless) were classified as susceptible or highly susceptible to the disease (Fennell, 1948; Li et al., 2008; Mortensen, 1981; Wang et al., 1998). In contrast, a detached-leaf assay Exotic and Perlette cultivars possess moderate resistance (Kono et al., 2012). Fennell (1948) reported that, when tropical species were crossed to the highly susceptible *V. vinifera* cultivars, all the F_1 progenies were susceptible. The susceptibility was conditioned by two or three dominant genes, and the resistance transmitted as a recessive locus. A more detailed investigation to elucidate the inheritance of AN resistance of American *Vitis* species was performed by Mortensen (1981). His genetic strategy pinpointed that AN susceptibility/resistance is controlled by three genes, two dominants for susceptibility (*An1* and *An2*), and one dominant (*An3*) conferring resistance. The three genes segregate independently. When both dominant susceptibility alleles (*An1* and *An2*) are present, the phenotype is susceptible regardless of whether *An3* is present or no. If either *An1* or *An2* or both are not present, than *An3* conditions resistance and *an3* susceptibility. According to Mortensen (1981), most *V. vinifera* cultivars are homozygous dominant for both *An1* and *An2* (e.g. Cabernet Sauvignon; *An1An1*/*An2An2*/*an3an3*), complicating the introgression of AN resistance genes from American species into *V. vinifera* background. On the other hand, F_1 progenies derived from crosses between hybrids with variable levels of genetic composition from several American species (*V. cinerea*, *V. riparia*, *V. rupestris*, *V. labrusca*, and *V. lincecumii*, along with *V. vinifera*) and *V. vinifera* segregated for the resistance to AN (Kim et al., 2008; Poolsawat et al., 2013). The proportion of resistant and susceptible plants in the progenies obtained by (Kim et al. (2008) suggests that the resistance is conferred by a single dominant gene. One example is the crossing between Concord (resistant) and Neomuscat (susceptible), for which a proportion of 1 resistant: 1 susceptible was obtained. The disease phenotyping was done by culture filtrates. In accordance, Poolsawat et al. (2013) reported high narrow sense heritability for AN resistance, suggesting the prevalence of additive over non-additive gene action. On the other hand, the crossing of a susceptible hybrid with a susceptible *V. vinifera* cultivar also segregated for AN resistance, suggesting the presence of susceptibility genes (Poolsawat et al., 2013).

The resistance present in the Asian species seems to be monogenic and dominantly inherited, without the presence of susceptibility genes. Wang et al. (1998) reported that all the progenies derived from the crosses between the Asian *Vitis* species *V. amurensis*, *V. davidii*, *V. piasezkii*, *V. pseudoreticulata*, *V. quinquangularis* and *V. romanetii* with *V. vinifera* were resistant to the disease, like their native parents used as resistant donor. The absence of susceptibility genes renders the Asian germplasm very interesting for breeding, since the segregation of resistance in the first generation saves time in the process of gene introgression. Finally, it is important to mention that in all progenies evaluated, a continuum variation of the AN symptoms were observed, suggesting also a quantitative inheritance of the disease resistance (e.g. Fennell, 1948; Mortensen, 1981; Yun et al., 2006). Taking all the data together, it may indicate that major genes – which explain the main variation observed – together with minor genes – confer the resistance to AN, as observed in other pathosystems. So far only one work describes the genetic mapping of genes conferring resistance to AN (Kim et al., 2008). A RAPD marker closely linked to an AN-resistant gene was identified using bulked-segregant analysis (BSA) and was converted into a SCAR marker. The SCAR amplifies a specific band in resistant cultivars/hybrids with different American backgrounds (*V. berlandierii, V. champinii*, *V. labrusca, V. riparia, V. rupestris*) and no band in susceptible ones, mainly *V. vinifera* cultivars. However, no information was found about the use of the SCAR in routine breeding programs to assist the introgression of the resistant gene into *V. vinifera* germplasm. Recently, also RGA-SSCP markers have been found associated with AN resistance (Tantasawat et al., 2012).

Finally, AN resistance was also pursuit in an *in vitro* study carried out to select resistant cv. Chardonnay (*V. vinifera*) plants using *E. ampelina* culture filtrates (Jayasankar et al., 2000).

Transcript, protein and metabolite exploration

Erysiphe necator

To date, several studies aimed to decipher the interaction between different *Vitis* genetic backgrounds and *E. necator* at different molecular levels, i.e. by analyzing transcripts, proteins and metabolites

As an obligate biotroph, one of the most challenging steps for transcriptional characterization of *E. necator* is the harvest of fungal tissue free of contaminating grapevine material. The technique for the isolation of fungal RNA developed by Cadle-Davidson et al. (2010) constituted a turning point. Taking advantage of it, Wakefield et al. (2011) investigated the genetic regulation of conidiation in *E. necator*. They identified new genes involved in conidiation, never found before in other filamentous fungi, probably related to the obligate biotrophic lifestyle. With the advent of next generation sequencing technologies additional progresses have been made. *De novo* transcriptomes were used to detect polymorphisms between different isolates of *E. necator* (Myers, 2012) as well as to identify candidate effector proteins specific to haustoria (Barnett, 2015). Transcriptome analyses, in addition to assisting in genome annotation (Jones et al., 2014), revealed the presence of several transcripts derived from transposable elements, indicating that they are transcriptionally active and can account for the observed genome expansion (Jones et al., 2014; Snyder, 2018).

Similarly, a large number of studies have been carried out to determine the genetic basis of resistance in *Vitis* species (**Table 2A, Table S1A**). Plants react to pathogen attack with multiple common defense responses, such as cell wall reinforcements through callose and lignin synthesis at the site of attempted penetration and accumulation of antimicrobial secondary metabolites (phytoalexins) and of pathogenesis-related (PR) proteins (e.g. chitinases and glucanases, able to degrade fungal and oomycete cell walls). Pathogen recognition can also be followed by a burst of reactive oxygen species (ROS) culminating in a PCD at the site of invasion (Wilkinson et al., 2019). Moreover, plant defense responses are tightly regulated by hormone-mediated signaling pathways, with jasmonate (JA) and salicylic acid (SA) playing the main role (Berens et al., 2017). Genes implicated in PM resistance can be identified thanks to their activation or increased transcription during infection and/or different expression levels between resistant and susceptible species. First significant data on grapevine resistance mechanisms were showed by Jacobs et al. (1999), which observed the specific and local induction of some PR genes in susceptible *V. vinifera* cultivars in response to PM infection. Godfrey et al. (2007) characterized the *V. vinifera Germin-like proteins* (*GLP*) gene family, ubiquitous plant proteins known to be involved in the response to various stress conditions including plant defense against pathogens (Bernier and Berna, 2001), and found that *VvGLP3* was specifically and locally induced in the epidermal cells in response to

pathogen attack, suggesting a role in the penetration-based defense response against PM infection.

Table 2. Transcriptomics, proteomics and metabolomics of grapevine-Ascomycete interaction. A) *Erysiphe necator*; B) *Elsinoë ampelina*.

A)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
PR proteins, e.g. chitinases (PR-2), glucanases (PR-3) and thaumatin-like (TL) proteins (PR-5)	Pathogenesis-Related protein	*V. vinifera* spp. *sativa*	Sultana, Cabernet Sauvignon	Susceptible	Leaves, berries	Jacobs et al. 1999
VvGLP3	Germin-like proteins 3	*V. vinifera* spp. *sativa*	Chardonnay	Susceptible	Leaves, berries	Godfrey et al. 2007
VvGLP4	Germin-like proteins 4					
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Fung et al. 2007
		V. aestivalis	Norton	Resistant		
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Fung et al. 2008
		V. aestivalis	Norton	Resistant		
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Fekete et al. 2009
		V. pseudoreticulata	Baihe-35-1	Resistant	Leaves	Xu et al. 2009
VaEDS1, VvEDS1	Orthologs of *Arabidopsis thaliana ENHANCED DISEASE SUSCEPTIBILITY1*	*V. aestivalis*	Norton	Resistant		Gao et al. 2010
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible		
VpWRKY1, VpWRKY2	WRKY domain transcription factor	*V. pseudoreticulata*	Baihe-35-1, Baihe-13, Baihe-13-1, 6-12-6, Guangxi-1	Resistant	Leaves	Li et al. 2010
		V. pseudoreticulata	Guangxi-2, Hunan-1, 6-12-2, Shangnan-2,	Susceptible		

			Baihe-35-2			
		V. vinifera spp. *sativa*	Carignane	Susceptible		
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Marsh et al. 2010
VpSTS	Stilbene synthase	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Xu et al. 2010a
VpPR10	Pathogenesis-Related protein 10	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Xu et al. 2010b
VpGLOX	Glyoxal oxidase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Guan et al. 2011
		V. pseudoreticulata	Guangxi-2	Susceptible		
VpRFP1, VvRFP1	C4C4-type RING finger protein	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Yu et al. 2011
		V. vinifera spp. *sativa*	Carignane	Susceptible		
STS	Stilbene synthase	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Xu et al. 2011
		V. vinifera spp. *sativa*	Thompson Seedless	Susceptible		
		V. vinifera spp. *sativa*	Carignane	Susceptible		
STS8, STS13, STS16, STS17, STS22, STS23, STS27, STS31	Stilbene synthase	*V. aestivalis*	Norton	Resistant	Leaves, berries	Dai et al. 2012
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible		
		V. quinquangularis	Shang-24	Resistant	Leaves	Gao et al. 2012a
		V. pseudoreticulata	Hunan-1	Susceptible		
VpALDH2B4	Aldehyde dehydrogenase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Wang et al. 2007 Wen et al. 2012

VpWRKY3	WRKY domain transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Zhu et al. 2012a
VpNAC1	NAC transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Zhu et al. 2012b
		V. vinifera spp. *sativa*	Touriga Nacional	Susceptible	Leaves	Borges et al. 2013
VpRFP1, VvRFP1	C4C4-type RING finger protein	*V. pseudoreticulata*	Baihe-35-1	Resistant		Yu et al. 2013a
		V. vinifera spp. *sativa*	Carignane	Susceptible		
VpEIRP1	E3 ubiquitin ligase *E. necator*-induced C3HC4-type Really Interesting New Gene (RING) finger protein 1	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Yu et al. 2013b
VpWRKY11	WRKY domain transcription factor	*V. pseudoreticulata*	Baihe-35-1	Resistant		
VpERF genes	Ethylene response factor	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Zhu et al. 2013
VvWRKY family	WRKY domain transcription factor	*V. aestivalis*	Norton	Resistant	Leaves	Wang et al. 2014
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible		
		V. pseudoreticulata	Baihe-35-1	Resistant	Leaves	Wang et al. 2014
VvNPF3.2	NITRATE TRANSPORTER1 / PEPTIDE TRANSPORTER FAMILY	*V. vinifera* spp. *sativa*	Cabernet Sauvignon	Susceptible		Pike et al. 2014
		V. aestivalis	Norton	Resistant		

trans-Resveratrol		*V. quinquangularis* Rehd.	Danfeng-2, Taishan-12, 83-4-96, Shangnan-24		Berries	Shi et al. 2014
		V. ficifolia Bunge	Weinan-3			
		V. amurensis Rupr.	Shuangyou, Zuoshan-1, Zuoshan-2			
		V. piasezkii Maxim.	Liuba-7			
		V. pseudoreticulata W.T.Wang	Hunan-1, Guangxi-1			
		V. thunbergii Sieb.et Zucc	Anlin-3			
		V. yeshanensis J.X.Chen	Yanshan-1			
		V. vinifera spp. *sativa*	Ugni Blanc, Pinot Noir, Carignane, Cabernet Sauvignon			
STS family; *trans*-Resveratrol		*V. quinquangularis*	Danfeng-2	Resistant	Leaves, berries	
		V. vinifera spp. *sativa*	Pinot Noir	Susceptible		
VpPR10-1	Pathogenesis-Related protein 10	*V. pseudoreticulata*	Baihe-35-1	Resistant		Xu et al. 2014
REN1-associated genes		*V. vinifera* spp. *sativa*	Carignane	Susceptible	Leaves	Amrine et al. 2015
		V. vinifera spp. *sativa*	Late Vavilov	Mid-Susceptible		
		V. vinifera spp. *sativa*	Husseine, Khalchili, Sochal	Mid-Resistant		
		V. vinifera spp. *sativa*	Karadzhandal	Resistant		
		V. vinifera spp.	O34-16, DVIT3351.27	Resistant		

		sylvestris				
		V. pseudoreticulata	Baihe-13-1	Resistant	Leaves	Jiao et al. 2015
		V. pseudoreticulata	Hunan-1	Susceptible		
		V. quinquangularis	Shang-24	Resistant		
VpCN	Disease resistance protein RxCC-like-NB-ARC	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wen et al, 2015
VqSTS6	Stilbene synthase	*V. quinquangularis*	Danfeng-2	Resistant	Leaves	Cheng et al. 2016
VpPR4-1	Pathogenesis-Related protein 4	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Dai et al. 2016
vvi-NewmiR2118 (*MIR2118*)	miRNA	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Han et al. 2016
VfMlo-like gene family	Powdery-mildew resistance locus o-like	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016a
VpSTS, VvSTS	Stilbene synthase	*V. pseudoreticulata*	Baihe-35-1	Resistant		Jiao et al. 2016
		V. vinifera spp. *sativa*	Carignane	Susceptible		
		V. vinifera spp. *sativa*	Cabernet Sauvignon	Susceptible	Leaves	Toth et al. 2016
VqDUF642	Domain of Unknown Function 642	*V. quinquangularis*	Danfeng-2	Resistant	Leaves, berries	Xie and Wang, 2016
VpUR9	RING-type ubiquitin ligase gene	*Vitis pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Dai et al. 2017
VqMAPKKK38	Raf-like Mitogen-activated protein	*V. quinquangularis*	Danfeng-2	Resistant	Leaves	Jiao et al. 2017

	kinase kinase kinase					
VpEIFP1	F-box/Kelch-repeat protein	*Vitis pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wang et al. 2017a
VpRH2	RING-H2-type ubiquitin ligase	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wang et al. 2017b
		V. vinifera spp. sativa	Thompson seedless	Susceptible		
VqWRKY52	WRKY domain transcription factor	*V. quinquangularis*	Shang-24	Resistant	Leaves	Wang et al. 2017c
VaSTS19	Stilbene synthase	*V. amurensis* Rupr.	Tonghua-3	Resistant	Leaves	Wang et al. 2017d
VpTNL1	TIR-NB-ARC-LRR R protein	*V. pseudoreticulata*	Baihe-35-1	Resistant	Leaves	Wen et al. 2017
*VvTLP*gene family	Thaumatin-like protein (TLP)	*V. vinifera*	Red Globe	Susceptible	Leaves	Yan et al. 2017
VqTLP29		*V. quinquangularis*	Shang-24	Resistant		
		V. pseudoreticulata	Hunan-1	Susceptible		
VqJAZ7	Jasmonate ZIM-domain (JAZ) transcriptional repressor	*V. quinquangularis*	Shang-24	Resistant	Leaves	Hanif et al. 2018
*NBS-LRR*family	Nucleotide Binding Sites-Leucine Rich Repeats proteins	*V. vinifera* spp. *sativa*	DVIT3351.27, Husseine, O34–16, Karadzhandal, Khalchili, Late vavilov, Sochal	From mid-susceptible to resistant		Goyal et al. 2019
			Carignane, Thompson	Susceptible		

	seedless

B)

Gene or Metabolite	Description	Taxon	Genotype	Defense Response	Organ	Reference
		Vitis interspecific crossing (Florida hybrids)	Lake Emerald, Blue Lake	Tolerant	Leaves	Vasanthaiah et al. 2009
		Vitis interspecific crossing (Florida hybrids)	Blanc du Bois, Suwannee	Susceptible		
		Muscadinia (or Vitis) rotundifolia (muscadine grape)	cv. Carlos	Tolerant		
ATP Synthase, GS	Adenosine Triphosphate Synthase beta subunit, Glutamine Synthetase					
Rubisco	Ribulose 1-5 bisphosphate-carboxylase					
		Vitis interspecific crossing (Florida hybrids)	Lake Emerald, Blue Lake	Tolerant	Leaves	Vasanthaiah et al. 2010
		Vitis interspecific crossing (Florida hybrids)	Blanc du Bois, Suwannee	Susceptible		
CHI	Chitinase					
STS	Stilbene Synthase					
	Transcription factor, Protein kinase, Sugar kinase genes					

		M. rotundifolia	54 cultivars, ranging from tolerant to highly-susceptible		Leaves	Louime et al. 2011
STS, CHS, CHI, PGIP, LIP	Stilbene Synthase, Chalcone Synthase (CHS), Chitinase (CHI), Polygalacturonase Inhibiting Protein (PGIP), Lipid Transfer Protein (LIP)		Tolerant and susceptible cultivars randomly selected from the 54 previously observed	from susceptible to tolerant	Leaves	
		V. coignetiae		Resistant	Leaves	Choi et al. 2011
		V. quinquangularis	Shang-24	Resistant	Leaves	Gao et al. 2012b
		V. vinifera	Red Globe	Susceptible		
		V. flexuosa	VISKO001	Resistant	Leaves	Ahn et al. 2014a
VfGlu gene family	β-1,3-glucanase	*V. flexuosa*	VISKO001	Resistant	Leaves	Ahn et al. 2014b
VfRPS5-like gene family	Resistance to *Pseudomonas syringae* 5 (*RPS5*), member of the NBS-LRR family	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam et al. 2015a
VfRLK gene family	Receptor-like protein kinase	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam et al. 2015b
VfMlo-like gene family	Powdery-mildew resistance locus o-like	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016a
VfCXE gene family	Carboxylesterase	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2016b
VfGST gene family	Glutathione S-transferase	*V. flexuosa*	VISKO001	Resistant	Leaves	Ahn et al. 2016
VfEDL1, VfEDL2, VfEDL3	Enhanced Disease Susceptibility 1 (EDS1)-like1	*V. flexuosa*	VISKO001	Resistant	Leaves	Islam and Yun 2017
*VvTLP*gene	Thaumatin-like	*V. vinifera*	Red	Susceptible	Leaves	Yan et al. 2017

family	protein (TLP)		Globe	
VqTLP29		*V. quinquangularis*	Shang-24	Resistant
		V. pseudoreticulata	Hunan-1	Susceptible

A more comprehensive view of the networks and mechanisms mediating plant defense was obtained through high-throughput methods, able to monitor transcriptome and proteome-wide changes. Comparative transcriptomic studies revealed substantial variation in gene expression between *V. vinifera* and the resistant *V. aestivalis*, which showed a constitutively elevated expression of several PR genes, as well as higher endogenous SA levels (Fung et al., 2007, 2008). Moreover, the very weak PM-induced response occurring in *V. aestivalis*, unlike the major transcriptome remodelling observed in *V. vinifera*, suggests that the constitutive transcriptomic profile of the resistant genotype is already defense-oriented (Fung et al., 2008). Transcriptome and proteome changes observed in Cabernet Sauvignon during infection (Fekete et al., 2009; Marsh et al., 2010) demonstrated that the susceptible plant is able to initiate a basal defense response, since several defense-related transcripts and proteins, as well as SA, accumulate in infected leaves. This response however turns out to be insufficient to restrict fungal growth, thanks to the ability of the pathogen effectors to suppress the host defense system, as already observed in the barley (*Hordeum vulgare*)-PM agent (*Blumeria graminis* f. sp. *hordei*) interaction (Caldo et al., 2004, 2006). Indeed, the expression levels of many grapevine defense-related genes reached a maximum at 12 hpi and then declined as the fungal infection became established (Fung et al., 2008).

In addition to differential transcriptional regulation, species- or cultivar-specific genes may contribute substantially to determine variable disease susceptibility among *Vitis* species (Da Silva et al., 2013; Venturini et al., 2013). Xu et al. (2009) identified several ESTs potentially associated with plant defense responses in *V. pseudoreticulata*. Gao et al. (2012) focused on PM-induced gene expression changes in *V. quinquangularis*. Among the genes differentially expressed, a large part encoded for resistance and stress-related proteins, and some of them showed specific PM-induction only in the resistant genotype.

High-throughput transcriptomic analyses were performed more recently by next-generation sequencing (RNA-seq). Weng et al. (2014) investigated how global gene expression profile changes in response to PM infection in *V. pseudoreticulata*. They identified

several genes and pathways that may contribute to PM resistance and found that the enhancement of JA pathway, SAR and ROS-dependent hypersensitive responses as well as the accumulation of phytoalexins play a key role in the defense response mechanism. Jiao et al. (2015) *de novo* assembled the transcriptomes of three wild Chinese *Vitis* accessions and studied the differences with the reference genome. A large number of distinct transcripts were identified in the resistant accessions, which resulted to be highly enriched in genes involved in plant secondary metabolisms, such as biosynthesis of phenolic compounds, and defense responses. These studies, together with other research done in grapevine (e.g. Borges et al., 2013) and in several other plant-PM agent interactions (e.g. Xu et al., 2011; Fu et al., 2016; Tian et al., 2019; Zhu et al., 2018; Polonio et al., 2019), further highlight the contribution of secondary metabolites to the plant defense mechanisms. Amrine et al. (2015) studied wild and cultivated Central Asian *V. vinifera* accessions carrying a common *Ren1* locus but displaying significant variations in disease susceptibility. They identified several genes potentially involved in the *Ren1*-dependent resistance mechanisms, as well as genes whose expression levels correlated with the different levels of resistance observed among accessions. These transcriptomic data have recently been used by Goyal et al. (2019) to investigate the role played by the *NBS-LRR* genes. They identified a total of 63 PM-responsive *NBS-LRR* genes in different *V. vinifera* accessions, ranging from susceptible to partially resistant. Gene expression levels in response to PM infection changed greatly between cultivars. Some genes were either up-regulated or expressed only in partially resistant cultivars. Moreover, at 5 dpi most of the genes were up-regulated in all the accessions, indicating a putative role in later stages of infection.

Since 1999, in different genetic backgrounds several genes that may contribute to PM resistance have been identified, such as *VpGLOX* (Guan et al., 2011); *VpPR-10.1* (Xu et al., 2010b, 2014); *VpRFP1* (Yu et al., 2011); *VpALDH2B4* (Wen et al., 2012); *VpNAC1* (Zhu et al., 2012b); *VpPR4-1* (Dai et al., 2016) (**Table 2A, Table S1A**). Many of them are transcription factors (Zhu et al., 2012a, 2012b), as expected since they are master regulators in controlling plant response to biotic stress (Amorim et al., 2017; Ng et al., 2018). In particular, *WRKY* transcription factors play an important role in plant defense (Pandey and Somssich, 2009) and WRKYs conferring resistance towards bacterial or fungal agents have been identified in several plants (Phukan et al., 2016). *WRKY* genes involved in response to fungal pathogens had been identified also in *V. vinifera*, i.e. *VvWRKY1* (Marchive et al., 2007) and *VvWRKY2* (Mzid et al., 2007). The entire *V. vinifera WRKY* gene family was later

characterized by Wang et al. (2014). Interestingly, a significant induction of many *VvWRKY* genes occurred in the susceptible cv. Cabernet Sauvignon in response to PM infection, unlike the resistant cultivar Norton which however showed a constitutive higher level of *WRKY* genes expression, potentially confirming their role in defense even in resistant cultivars. Other *WRKY* genes implicated in PM resistance were identified in *V. pseudoreticulata* (Li et al., 2010; Yu et al., 2013b; Zhu et al., 2012a) and *V. quinquangularis* (Wang et al., 2017c).

Stilbenes, the grapevine phytoalexins, play a critical role in plant defense (Schnee et al., 2008). In grapevine stilbene synthase (*STS*) genes, the key enzyme in the biosynthesis of stilbenic compounds, are organized in an unusually large family (Parage et al., 2012; Vannozzi et al., 2012). Expression analyses performed in *V. vinifera* and *V. aestivalis* showed that individual *STS* genes are differentially expressed during leaf and berry development as well as in response to PM attack (Dai et al., 2012). PM-induction of *STS* genes was observed mainly in *V. vinifera*, whereas *V. aestivalis* showed constitutively higher transcript levels of some *STS* genes (Dai et al., 2012; Fung et al., 2008). Chinese wild grapevines are an important source of resistance genes related to stilbene production since several of them have a significantly higher resveratrol content than most European *V. vinifera* cultivars, and this accumulation correlates with *STS* genes expression. Indeed, both the endogenous and the PM-induced *STS* genes expression levels were much higher in the resistant *V. quinquangularis* than in *V. vinifera* (Shi et al., 2014). Comparative analyses of *STS* genomic regions and promoter activities in resistant and susceptible species revealed that the differential responsiveness of the *STS* genes is associated with differences in their promoter sequences, whereas the coding regions are highly conserved (Jiao et al., 2016; Xu et al., 2010a, 2011b). Jiao et al. (2017) identified in *V. quinquangularis VqMAPKKK38*, a PM-inducible Raf-like MAPKKK gene involved in the very complex network controlling activation of *STS* transcription.

Unlike the majority of the studies seen so far, which focus mainly on the early or midterm stages of infection, Borges et al. (2013) investigated the specific long term remodeling of the grapevine transcriptome operated by the pathogen to promote its survival, which requires uptake of nutrients and suppression of host defense responses. Interestingly none of the genes differentially expressed identified had correspondence to the ones detected by Fekete et al. (2009) in the early stages of infection. The mechanisms used by the pathogen to modulate host physiology were further investigated by Pike et al. (2014) through the functional characterization of *VvNPF3.2,* a putative pathogen-inducible transporter in *V.*

vinifera (Fung et al., 2008), and more recently by Toth et al. (2016), who identified genes induced by PM colonization but not by SA treatment.

Plant microRNAs (miRNAs) play pivotal roles in plant defense processes (reviewed in Islam et al., 2018; Niu et al., 2015). Han et al. (2016) used high-throughput sequencing of small RNAs to identify miRNAs potentially involved in PM resistance in *V. pseudoreticulata*. They showed that the expression of the highly accumulated vvi-NewmiR2118 miRNA, whose predicted targets are *NBS-LRR* type *R* genes, strongly and rapidly decreases following infection.

Guignardia bidwellii

Regarding the interaction between grapevine and *G. bidwellii* or only on the ascomycete itself there are still no transcriptomic, proteomic or metabolomic data available. A possible reason of this delay could be attributed to the fact that research activities and investments from funding agencies mainly address major diseases (e.g. PM as well as downy mildew). Filling this gap of knowledge in the near future will be very helpful to understand not only the causal agent of BR, but also the molecular events which occur during the infection process.

Elsinoë ampelina

Unlike PM, few transcriptomic and proteomic studies have been conducted to elucidate the molecular interactions between *Vitis* species and *E. ampelina* **(Table 2B, Table S1B)**. A proteomic investigation identified several proteins differentially expressed between tolerant (mid-resistant) and susceptible cultivars, and between uninfected and infected leaves (Vasanthaiah et al., 2009). Two of them, the mitochondrial adenosine triphosphate synthase and glutamine synthetase, were newly expressed in tolerant cultivars upon *E. ampelina* inoculation. Conversely, several proteins, including ribulose-1,5-bisphosphate carboxylase (Rubisco) involved in photosynthesis, were suppressed in infected susceptible genotypes. These results highlighted the ability of the pathogen to significantly affect host physiology, especially in the susceptible cultivars. The tolerant genotypes had the ability to up-regulate and to induce new proteins in order to defend themselves from pathogen invasion and to maintain the normal physiological processes. In tolerant genotypes, a rapid and specific

induction of various defense-related genes, including chitinase, stilbene synthase, chalcone synthase, putative proline-rich cell wall protein, thaumatin-like protein, pathogenesis-related (PR) protein 10 and other signal related genes were observed upon *E. ampelina* inoculation (Choi et al., 2011; Gao et al., 2012b; Louime et al., 2011; Vasanthaiah et al., 2010). Most of these genes were strongly induced by the pathogen only in the resistant accessions.

Ahn et al. (2014) *de novo* assembled the transcriptome of the resistant Korean species *V. flexuosa* inoculated with the pathogen. This analysis led to the identification and subsequent characterization of many resistance related responsive genes to *E. ampelina* inoculation, such as β-1,3-glucanase genes (Ahn et al., 2014b), RPS5-like genes (Islam et al., 2015a), receptor-like protein kinase (RLK) genes (Islam et al., 2015b), Mlo-like genes (Islam and Yun, 2016a), carboxylesterase genes (Islam and Yun, 2016b), glutathione S-transferase genes (Ahn et al., 2016) and EDS1-like genes (Islam and Yun, 2017). Several *V. vinifera* thaumatin-like proteins (*VvTLP*) genes responsive to anthracnose were also recently identified (Yan et al., 2017).

Discussion

Considering the combination of the three studied diseases, we have concluded that grapevine cultivars resistant to PM- and downy mildew are mostly susceptible to BR and AN. This result could suggest a potential compromise of resistance to diseases, but instead can be attributed to the use of a limited source of genetic variation that confers resistance in breeding, particularly when compared with the genetic variability commonly found in grapevine germplasm collections. Secondly, positive selection of resistance to BR and AN is often overlooked, even when parental lines are resistant to one of these diseases in addition to PM. This implies the future proper use of multi-disease resistance sources, considering that potential interactions are not excluded. Indeed, it is currently unknown whether a common resistance mechanism is present in the genus *Vitis*, and if so at what biological scale, in light of the fact that the causal agents are all Ascomycetes. Indeed, *R*-loci are polymorphic among species, and the upstream recognition phase is specific while a number of downstream mechanisms are common, highlighting that variability is present in the regulation, rather than

the mechanisms themselves. Indeed, heterologous expression of pathogen-responsive genes mostly confers broad-spectrum resistance, including in fungi of the same family, as well as bacteria. Focusing on single diseases, studies show that the pyramiding of *R*-loci provides an equal or higher degree of disease resistance, which confers long-term ability of overcoming pathogenic invasions.

Ultimately, it will be of paramount importance to characterize genes and pathways underpinning the quantitative PM, BR and AN resistance traits displayed by certain wild *Vitis* species. In order to prove conclusively that these genes do contribute to disease resistance, it will be necessary to demonstrate that the segregation of resistance is genetically linked to the inheritance of these candidate genes. This will also elucidate whether these genes are able to function in different genetic backgrounds i.e. in *V. vinifera*, which will be essential if they are to constitute the basis for any gene pyramiding strategy. Even nowadays, detailed insights about mechanisms underlying gene pyramids are missing – such as redundancy, additive effects or synergistic mechanisms (epistatic effects) – and expression and functional studies on pyramidized materials are very rare or lacking. The adoption of integrated strategies might facilitate the closing of the circle among grapevine/host disciplines (genomics, transcriptomics, and metabolomics).

Multidisciplinary approaches are also crucial and needed for a more comprehensive characterization of the Ascomycete/pathogen. The identification of the described Ascomycetes commonly relied on the analysis of their morphological characters and on the observation of the affected plant symptoms, however these parameters are not sufficient for specific pathogen discrimination. The recent advances in high-throughput sequencing technologies and in fungal genomics are opening new possibilities for fungi barcoding. Despite the economic importance of these pathogens, many aspects of their biology have not yet been fully explored because some (i.e. *E. necator*) are impossible to cultivate and propagate *in vitro* due to their obligate biotrophy lifestyle. In this respect, genomic studies, although complicated and challenging, are strongly advised to contribute to identify candidate effector genes involved in the pathogenicity mechanisms. On the other hand, more efforts and funding should be addressed to deepen our understanding of the mechanisms of biotrophy, shedding light onto its molecular and evolutionary basis. So far, to develop artificial media which resembles the host, only empiric strategies could be attempted to design media supplemented with (most probably several) plant molecules able to energetically support the fungus as well as to stimulate its growth.

To face the overall challenge, the vision is that each host discipline will inform the corresponding pathogen discipline, and vice versa, based on the practice of multidisciplinary culture.

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8 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

O desenvolvimento e a avaliação de genótipos contendo R-locos piramidados em diferentes combinações permitiu conhecer o efeito aditivo para a maioria das combinações genicas encontradas, sendo estas *Rpv1+Rpv3.3*, *Rpv1+Rpv3.1*, *Rpv3.1+Rpv10* e *Rpv1+Rpv3.1+Rpv10*. Através deste estudo foi relatado as maiores resistências genéticas para os genótipos piramidados *Rpv1+Rpv3.1+Rpv10* e *Rpv3.1+Rpv10*. Foi também possível descrever a o baixo nível de resistência a partir de genótipos portadores dos R-locos *Rpv3.3* e *Rpv1* não piramidados. Bem como, o efeito de maior resistência nos genótipos portadores do *Rpv10*.

Da parte do patógeno, através do estudo envolvendo a avaliação da genética de populações de *Plasmopara viticola* hospedados em genótipos com diferentes combinações de R-locos, implantados em diferentes ambientes, foi possível conhecer os efeitos do hospedeiro e do ambiente na estruturação polulacional do patógeno. Este estudo demonstrou que populações do *P. viticola* hospedadas em genótipos *srpv* possuem maior diversidade genética, provavelmente devido a ausência, ou escassez de barreiras para o patógeno completar seu ciclo de vida. Quando considerando somente os genótipos portadores de R-locos, foi ainda observado que genótipos contendo *Rpv3*, principalmente o genótipo piramidando os haplótipos *Rpv3.1+Rpv3.3*, demonstrando ausência de efeito aditivo neste genótipo. Esta informação está de acordo com o verificado para a menor resistência deste loco no estudo de população segregante para diferentes R-locos. Assim como relatado anteriormente, em genótipos contendo diferentes R-locos piramidados, o efeito de resistência foi maior, isto pode ser confirmado pela restrição da diversidade genética das populações do patógeno nestes genótipos, independente do vinhedo avaliado. Ainda quanto ao ambiente, foi observado a interação genótipo ambiente, refletindo na diversidade genética do patógeno, provavelmente devendo-se a relação do hospedeiro com o ambiente em que é cultivado, todavia, o efeito dos hospedeiros foi superior ao efeito ambiental.

Para entender melhor as relações entre os genótipos portadores de genes de resistência e o ambiente, o estudo avaliando o comportamento ecofisiológico de quatro genótipos PIWI demonstrou que todos estes genótipos avaliados apresentavam exigência térmica compatível para o cultivo no clima avaliado, Região dos Vales da Uva Goethe. Dentre estes, a cv. Felicia apresentou a menor exigência térmica para completar seu ciclo (1260 °C dia), contrastando com a cv. Aromera que apresentou a maior exigência térmica (1454 °C dia). Foi ainda demonstrado que, para todos os genótipos, a fase fenológica com maior demanda térmica é

entre o florescimento e o *véraison*. Adicionalmente, foi acompanhado o plastocrono de outros genótipos PIWI, avaliando a exigência térmica para emissão de novas folhas em 5 cultivares, implantadas em dois ambientes distindos. Novamente foi observado interação entre genótipos e ambiente e foi demonstrado que, o ambiente de maior altitude resultou em aumento da necessidade de acúmulo térmico para emissão de novas folhas no genótipo GF.2004.043.0015 e na cv. Regent. O comportamento oposto foi verificado na cv. Bronner. Alterações significativas não foram observadas para o genótipo GF.2004.043.0024, assim como, para a cv. Calardis blanc.

Assim como a emissão de novas folhas, a área foliar é também outro fator ecofisiológico importante para o desempenho viticola, portanto, um estudo foi realizado utilizando modelos lineares para descrever a área foliar de 11 genótipos PIWI, cultivados em cinco ambientes distintos. Sendo assim verificado que a área foliar pode ser estimada com somente uma medida da folha, preferencialmente a largura, todavia, modelos desenvolvidos a partir de mais de uma medida apresentam uma estimativa melhor da área foliar real. A área foliar coletada foi também testada para a existência de interação entre genótipo ambiente, demonstrando que, existe interação entre estes fatores, além de demonstrar que o ambiente de cultivo possui impacto na área da folha gerada pela planta.

O período de sanduiche realizado na Fondazione Edmund Mach (FEM, Trento-IT) teve importante papel, principalmente para o desenvolvimento de estudos com um genótipo de origem genética desconhecida, porém, portador de resistência a algumas doenças. No âmbito da *Unità di Genetica e Miglioramento Genetico della Vite* (UGMGV) foi desenvolvido trabalho de identificação varietal deste material genético e com os resultados dos nove SSR's foi desenhado a sua provável genealogia. A origem da resistência vem da espécie *V. aestivalis*, de onde porta o R-loco *Rpv27*. A presença deste R-loco foi confirmada no genótipo via amplificação de marcadores SSR's ligados ao loco. Todavia, a população segregante obtida a partir da autofecundação deste genótipo apresentou segregação não condizente com a atuação de somente este gene governando a resistência ao míldio, uma hipótese é a existência de uma relação epistática com um possível gene de sucetibilidade a doença. Contudo, uma avaliação com n amostral maior deve ser realizada em trabalhos futuros para confirmar a distribuição da resposta encontrada neste estudo. A população a partir deste genótipo segregou também para a resistência a oídio e black rot, contudo, não foi observado nenhum gene de resistência a estas doenças, já descritos na literatura em seu

genoma, portanto, novo estudo de mapeamento de QTL's pode ser desenhado para atingir este objetivo.

Ainda durante o estágio sanduiche na FEM, ressalta-se a participação em revisão de literatura, publicada no periódico "*Frontiers in Plant Science*" que demonstra o estado da arte atual de três novas doenças fungicas emergentes na viticultura, oídio, black rot e antracnose, sendo explorados aspectos do ciclo de vida dos patógenos, genética e biologia molecular, relações planta patógeno bem como descrição de recursos genéticos portadores de resistência possíveis de serem empregados em programas de melhoramento genéticos da videira.

Com base em tudo o que foi posto, evidencia-se que o desenvolvimento das pesquisas mencionadas teve importante papel para o avanço do conhecimento científico na área, assim como, gerou conhecimentos a serem aplicados, principalmente no programa de melhoramento genético da videira.

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