Resistance properties of new fungus-resistant grapevine cultivars against *Plasmopara viticola* and the impact of their deployment on fungicide use in viticulture

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1 Introduction

The high susceptibility of European grape varieties (*Vitis vinifera*) to downy mildew (*Plasmopara viticola)* leads to intensive use of fungicides in viticulture. To reduce this use, resistance loci from wild *Vitis* species have been crossed into *Vitis vinifera* in breeding programs. The emerging new fungus-resistant grapevine cultivars (FRCs) represent an important tool for the reduction of pesticide applications in the sense of integrated pest management in viticulture. However, due to varietal differences in resistance, little is known about how different resistance loci contribute to resistance and thus, how much chemical protection can be reduced when FRCs are planted. To ensure durable and sustainable resistance management and breeding, detailed knowledge of the different defense mechanisms conferred by the respective *Rpv*-loci (resistance to *P. viticola*) is essential. The aim of the project is therefore 1. to characterize the degree of resistance, 2. to specify resistance mechanisms of these new varieties and, 3. to evaluate the capability of fungicide reduction in the vineyard. In this context, FRCs will be tested in the field under practical conditions in comparison to traditional varieties at reduced plant protection management. Some of these results have already been published in Wingerter et al., 2021.

2 Differences in the defense responses mediated by the *Rpv3*- and *Rpv12*-loci in response to *P. viticola* infection

In plant-pathogen interactions with biotrophic pathogens, PCD is linked to plant resistance and is a powerful reaction to deprive the pathogen of its food source. As described in several studies, PCD is visible as macroscopic lesions within few days post inoculation (dpi) (Pezet et al., 2004b; Bellin et al., 2009; Venuti et al., 2013; Possamai et al., 2020). However, no macroscopic differences in type and timing of the plant defense response in *Rpv3*- and *Rpv12*-genotypes are reported.
The comparison of the initiation and development of grapevine defense responses against *P. viticola* infection in FRCs containing different resistance loci revealed clear temporal differences in the onset of programmed cell death (PCD) in response to *P. viticola* in *Rpv3*- and *Rpv12*-genotypes. An early onset of PCD was observed in *Rpv12*-genotypes (8 hpi)**,** but was markedly delayed in *Rpv3*-genotypes (28 hpi) (Figure). The rapid induction of PCD within 8 hpi in *Rpv12*-genotypes results in an early arrest of the pathogen as indicated by a lack of mycelial growth (Figure 1). In *Rpv*3-genotypes, PCD was not observed until 28 hpi, allowing a moderate development of the pathogen (Figure 1). These results confirm the observations reported by Eisenmann et al., 2019, who found PCD was not induced in the *Rpv3*-genotype within the first 32 hpi. It can only be speculated that beside the distinct defense mechanisms activated after an infection and also the exact timing of PCD is linked to the respective *Rpv*-locus. A different time scheme is maybe based on the rapidness of elicitor recognition that seems to be caused by different types of NB-LRR receptors encoded by the *Rpv*-locus.

Effector-triggered immunity (ETI) is not only characterized by the induction of PCD but also a number of other cellular events including the rapid production of reactive oxygen species (ROS) and the release of antimicrobial compounds, such as phytoalexins, which are involved in successful limitation of pathogen development (Greenberg, 1997; Jones and Dangl, 2006; Mukhtar et al., 2016). Furthermore, ROS such as hydrogen peroxide have been proposed to act as signalling molecules for activation of defense genes and the HR (Levine et al., 1994; Tenhaken et al., 1995).

To obtain further insights into the time schedules mediated by different *Rpv*-loci, the accumulation of hydrogen peroxide and the phytoalexin *trans*-resveratrol was analyzed in genotypes containing the *Rpv12*-locus or the *Rpv3*-locus respectively. Our results indicate that hydrogen peroxide was produced within 8 hpi at the infection site in *Rpv12*-genotypes which co-incided with the appearance of PCD (Figure 1). In contrast, hydrogen peroxide was not detected in *Rpv3*-genotypes until 24 hpi, which was shortly before PCD and was first observed in leaf cells of this genotype (Figure 1). *Trans*-resveratrol has also been proposed as signal molecule for PCD (Chang et al., 2011). Chitarrini et al., 2020 evaluated *trans*-resveratrol accumulation in *Rpv12*-genotypes but the first sampling point was at 12 hpi, which is surely after the first appearance of PCD in *Rpv12*-genotypes. Our results revealed the first increase of *trans*-resveratrol at 6 hpi, which is prior to the observed production of ROS and the appearance of PCD at 8 hpi (Figure 1). As the accumulation of *trans*-resveratrol precedes the first occurrence of ROS and PCD in *Rpv12*-genotypes, it is tempting to speculate that *trans*-resveratrol, or another stilbene derived from *trans*-resveratrol, may act as an inducer of ROS production and PCD.
Recently Eisenmann et al., 2019 also described elevated *trans*-resveratrol and ε-viniferin levels during *Rpv3*-mediated defense response shortly before the onset of PCD at 32 hpi. Our results confirm this observation, with no significant

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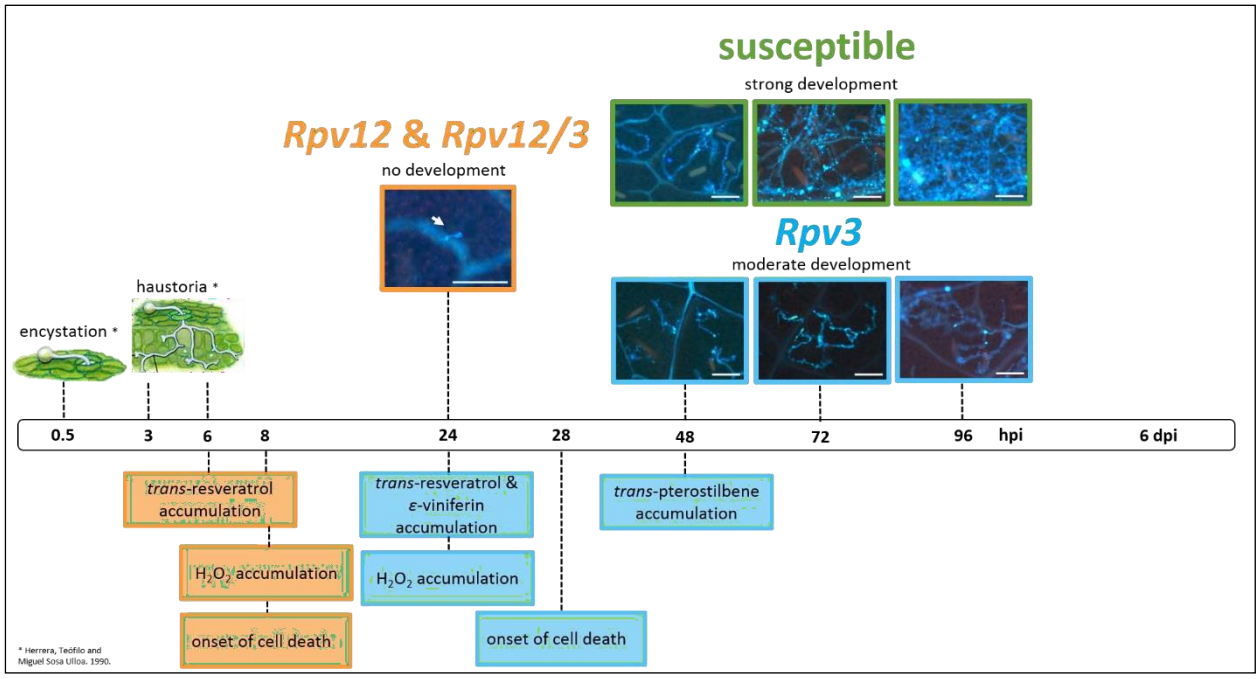

Figure 1: Schematic representation of the differences in *Rpv3*- and *Rpv12*-mediated defense. The *Rpv12*- and *Rpv12/Rpv3*- genotype is shown in orange, the *Rpv3*-genotype in blue and susceptible genotype in green. *P. viticola* development within the leaves after 24, 48, 72 and 96 hpi is shown (aniline blue staining), framed for each genotype in the corresponding colour. A timeline in the middle of the image serves to mark the defence reactions that occur differently over time depending on the genotype. Here, the successive defence reactions for the *Rpv12*- and *Rpv12/Rpv3*-genotypes are marked in orange and those for the *Rpv3*-genotype in blue under the timeline. Here, the first appearance of hydrogen peroxide, PCD as well as increased *trans*-resveratrol, *ε*-viniferin and *trans*-pterostilbene accumulation are indicated in time. Some of these results were published in detail in Wingerter et al., 2021.

increase of *trans*-resveratrol and ε-viniferin production in the *Rpv3*-genotype until 24 hpi (Figure 1). In addition, the increased accumulation of *trans*-pterostilbene in *Rpv3*-genotypes after 48 hpi reported by Eisenmann et al., 2019 could also be confirmed (Figure 1). Besides the possible role of *trans*-resveratrol as a signalling molecule, it has also been proposed to play a role in the *Rpv3*-mediated defense (Malacarne et al., 2011; Eisenmann et al., 2019), by acting as a precursor for the synthesis of the fungi-toxic stilbenes *ε*-viniferin and *trans*-pterostilbene, which suppress the growth and development of *P. viticola* (Pezet et al., 2004a). Even though the results presented here do not provide any further insights into the role of *Rpv*-mediated stilbene production in suppressing the growth of *P. viticola* in a direct manner, they confirm the hypothesis that *trans*-resveratrol may act as a signalling molecule for ROS induction and PCD in *Rpv12*- and *Rpv3*-mediated resistance.

3 Different resistance levels mediated by the *Rpv3*-, *Rpv10*- and *Rpv12*-locus

The results demonstrate clear differences in the level of resistance conferred by the *Rpv3*- relative to the *Rpv12*- or *Rpv10*-locus (Figure 2). Sporulation on *Rpv3*-genotypes was significantly higher compared to *Rpv10*-, *Rpv12*-genotypes (Figure 2). These findings are also in line with previous studies showing a higher resistance level of *Rpv10*- and *Rpv12*- compared to *Rpv3*-mediated resistance (Venuti et al., 2013; Vezzulli et al., 2018; Zini et al., 2019). However, these results, in terms of the level of resistance mediated by the respective loci, are somewhat contradictory. Possamai et al., 2020 reported that the *Rpv10*-mediated resistance was weaker

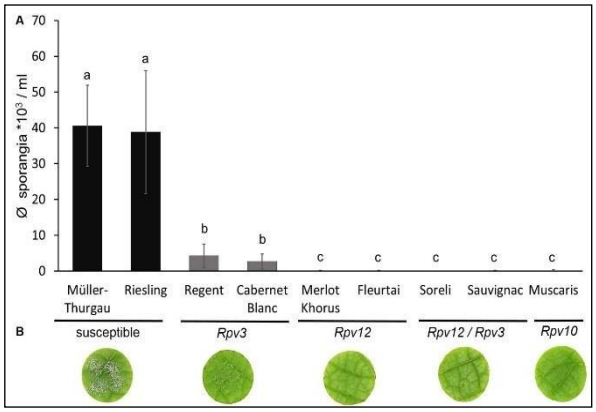

Figure 2: Sporulation of P. viticola isolate avrRpv+ on susceptible and resistant genotypes. Leaf discs of susceptible (‘Müller-Thurgau’ and ‘Riesling’), Rpv3- (‘Cabernet Blanc’ and ‘Regent’), Rpv12- (‘Merlot Khorus’ and ‘Fleurtai’), Rpv12/Rpv3- (‘Soreli’ and ‘Sauvignac’) and Rpv10-genotype (‘Muscaris’) were inoculated with the avrRpv+ P. viticola isolate. (A) Evaluation of P. viticola sporulation on leaf discs. Counted sporangia after 6 dpi were shown. Bars represent the average of three independent experiments (n=63). Error bars show standard deviation. Kruskal-Wallis and Steel-Dwass- Critchlow-Fligner test were used to analyse data and perform multiple pairwise comparison. Means with different letters (a,b,c) are significantly different ($p<0.05$). (B) Representative images of leaf discs (susceptible (´Riesling´); Rpv3- (´Cabernet Blanc´); Rpv12 (´Fleurtai´); Rpv12/Rpv3- (´Sauvignac´) and Rpv10-genotype (´Muscaris´) were taken at 6 dpi.

than that conferred by *Rpv12* and *Rpv3*. Bove and Rossi, 2020, on the other hand, found comparable levels of downy mildew resistance in *Rpv3*-, *Rpv10*- and *Rpv12*-genotypes. One possible explanation for these conflicting results could be the different methods used to evaluate levels of resistance. In the present study, resistance was assessed using the measurement of sporulation on individual leaf discs, whereas Possamai et al., 2020 used a visual scoring system based on the Office International Organisation of Vine and Wine (OIV) scale and Bove and Rossi, 2020 determined the amount of sporangia of specific lesions.

4 Emergence of a new field isolate that overcomes both *Rpv12*- and *Rpv3*-mediated resistance

P. viticola isolates able to overcome *Rpv3*-mediated resistance have been described in previous studies, revealing that durability of resistance conferred by a single resistance locus is limited (Casagrande et al., 2011; Delmotte et al., 2014; Eisenmann et al., 2019). It seems that an *avrRpv3*$^{-}$ isolate acquired further mutations and was thus selected to overcome *Rpv12*-mediated resistance (Figure 3).

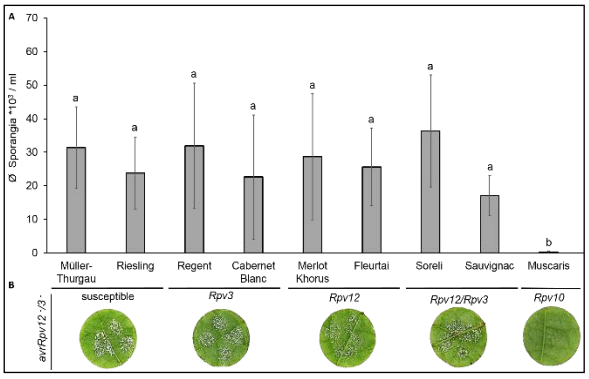

Figure 3: Sporulation of the *avrRpv12*$^{-}$*/3*$^{-}$ *P. viticola* isolate on susceptible and resistant genotypes. Leaf discs of susceptible (‘Müller-Thurgau’ and ‘Riesling’), *Rpv3*- (‘Cabernet blanc’ and ‘Regent’), *Rpv12*- (‘Merlot Khorus’ and ‘Fleurtai’), *Rpv12/Rpv3*- (‘Soreli’ and ‘Sauvignac’) and the *Rpv10*-genotype (‘Muscaris’) were inoculated with *P. viticola* isolates. (A) Quantitative evaluation of sporulation of *P. viticola* isolate *avrRpv12*$^{-}$*/3*$^{-}$ on leaf discs. Sporangia were counted 6 dpi. Bars represent the average of three independent experiments (n= 63). Error bars show standard deviation. Kruskal-Wallis and Steel-Dwass-Critchlow-Fligner test was used for multiple pairwise comparison. Means with the different letters (a, b, c) show significant differences for infection ($p < 0.05$). (B) Pictures of representative leaf discs of susceptible (´Riesling´); *Rpv3*- (´Cabernet Blanc´); *Rpv12*- (´Fleurtai´); *Rpv12/Rpv3*- (´Soreli´) and *Rpv10*-genotype (´Muscaris´) were taken at 6 dpi.

This isolate was detected in a vineyard in which inadequate phytosanitary treatments were performed over the last three decades, which contributes to an enhanced probability of pathogen mutation. An *Rpv3*-cultivar was first planted leading to the selection of the respective *Rpv3*-breaking isolate. As an *Rpv12*-cultivar was planted in addition, further selection took place allowing the development of this isolate designated as *avrRpv12*$^{-}$*/3*$^{-}$. Fortunately, the *avrRpv12*$^{-}$*/3*$^{-}$ isolate was not able to overcome the resistance mediated by the *Rpv10*-locus (Figure 3). This observation clearly indicates with growing apprehension that if cultivars with just one individual resistance locus are grown at the same location with inadequate or no phytosanitary treatments, also cultivars possessing a more durable resistance based on the pyramidization of separate resistance loci can be affected.

5 The capability of resistant grapevine cultivars to reduce the number of fungicide applications depends on both, genetically based resistance levels and climatic factors

On-farm experiments performed 2016-2022 revealed that the variety ’Sauvignac’ (*Rpv12/Rpv3*) was not affected by *P. viticola*, whereas ’Cabernet Blanc’ (*Rpv3*) suffers to a low degree from *P. viticola* infections depending on the infection pressure given in each year (Figure 4).
These difference in the resistance levels of the *Rpv3*- and *Rpv12/Rpv3*-genotypes observed in the field correlates, on the one hand, with the results from the leaf disc assay (Figure 2) and on the other hand, with results from other studies (Venuti et al., 2013; Vezzulli et al., 2018; Zini et al., 2019). The year 2021 was characterized by high precipitation, leading to favourable conditions for downy mildew and thus to subsequent infection events (Figure 4). As a result, the *P. viticola* disease incidence and disease severity on grapes in 2021 in the untreated control is comparable to those observed in 2016 (Wingerter et al., 2021) (Figure 4).
Even though weather conditions favour growth and propagation of *P. viticola*, no infection was detected on grapes of ’Sauvignac’ (*Rpv12/Rpv3*) and ’Muscaris’ (*Rpv10*) (**Errore. L'origine riferimento non è stata trovata.**). On the other hand, varieties such as ’Calardis Blanc’ (*Rpv3-1/Rpv3-2*) and ’Satin Noir’ (*Rpv3*), required at least two fungicide treatments to reduce disease severity levels to or below 10 % (Figure 5). Since susceptible cultivars such as ’Riesling’, ’Sauvignon Blanc’ and ’Muskateller’ had to be sprayed at least 14 times to even reach the 10 % disease severity level, with the exception of ’Cabernet Sauvignon’ that expressed higher disease severity levels, a reduction of just two applications correspond to a fungicide saving potential of about 85 % for ’Calardis Blanc’ (*Rpv3-1/Rpv3-2*) and ’Satin Noir’ (*Rpv3*) (Figure 5). In case for ’Cabernet Blanc’ (*Rpv3*), four treatments were necessary to achieve the 10 % disease severity level (Figure 5).
In conclusion, not only the distinct resistance level based on single resistance locus or combined resistance loci has to be considered but also weather conditions have to be kept in mind. Thus, plant protection strategies have to be adapted to both, the genetically based resistance and the disease pressure. In case of low infection pressure, as seen in 2018 for instance, two to four treatments are sufficient for FRCs with medium resistance to protect grapes (Wingerter et al., 2021). If weather condition are in favour for disease

development, of course more treatments are needed. However, even under a high disease risk with regard to traditional cultivars FRCs with high level of resistance need less than four or just four treatments which represents a fungicide reduction of about 75 % compared to traditional cultivars.

6 Conclusions

The results shown revealed an early defense response in *Rpv12*- and *Rpv12/Rpv3*-genotypes, which was delayed in *Rpv3*-genotypes. Differences in the timeline regarding the defense response seems to be based on the different levels of disease resistance generated by the respective *Rpv*-locus/loci as seen by lab and on-farm experiments. The use of FRCs in combination with reduced plant protection management strategies offers the possibility to significantly reduce the amount of fungicides required for the production of high quality grapes. Furthermore, results obtained from the on-farm studies demonstrated that some FRCs, disposing a very high level of disease resistance, may save up to 100 % of fungicide applications in field. However, the omission of chemical protection can ultimately lead to negative effects on yield, quality and even resistance durability. Further studies are needed to concrete the consequences of reduced fungicide applications when cultivars with combinded *Rpv*-loci are planted close to those cultivars just having one single locus that was also used in pyramidization, but also to validate the putative emergence of other fungal diseases, such as black rot or *Phomopsis viticola* cane and leaf spot.

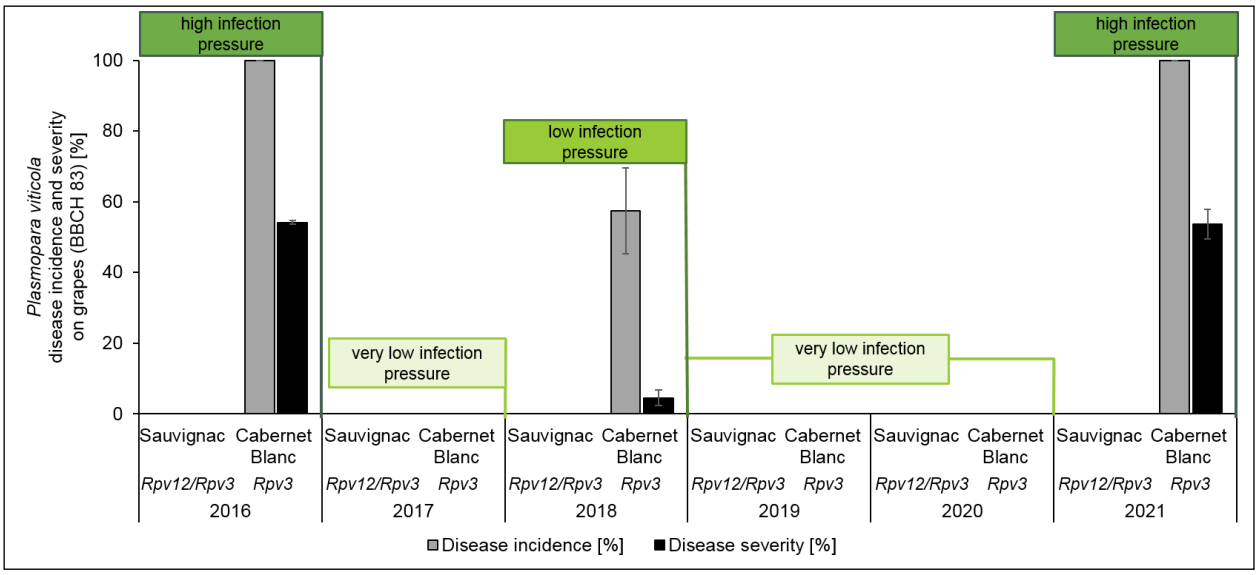

Figure 4: *Plasmopara viticola* disease incidence and severity on grapes (BBCH 75) during the experimental years of 2016-2021. Disease incidence (dark grey) and severity (black) are shown in the untreated control variants of the grapevine cultivar 'Sauvignac' (*Rpv12/Rpv3*) and 'Cabernet Blanc' (*Rpv3*). The bars represent the mean value of *P. viticola* disease incidence and severity on grapes from three different locations (n(grapes)=300). Error bars show standard error (SEM).

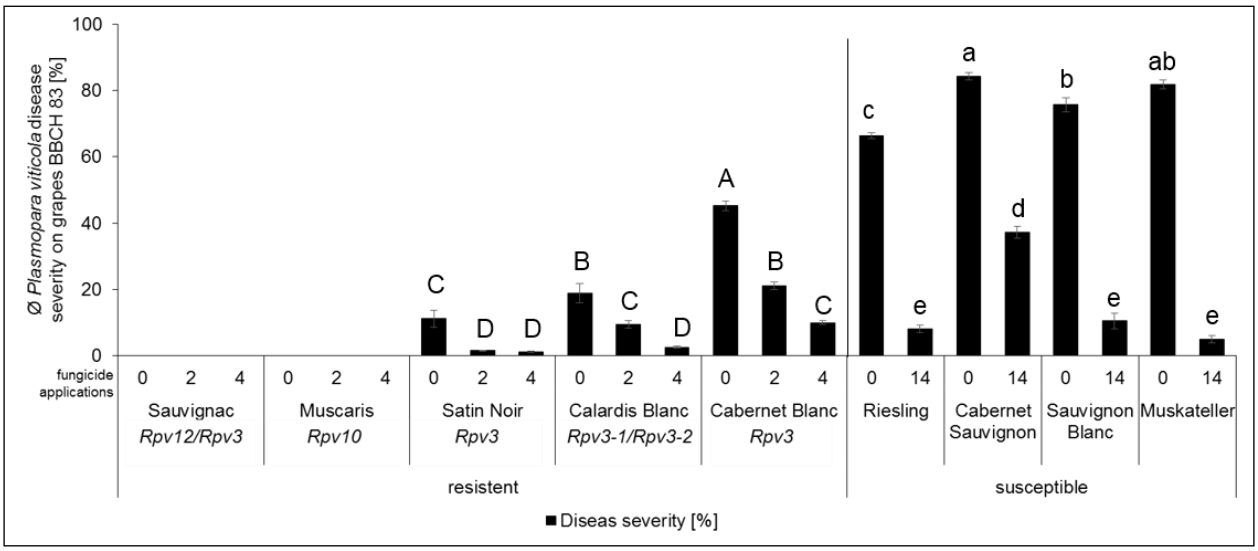

Figure 5: *P. viticola* disease severity on grapes (BBCH 83) of resistant and susceptible grapevine cultivars in 2021. Shown is the

disease severity (black bars) in the resistant cultivars 'Sauvignac' (*Rpv12/Rpv3*), 'Muscaris' (*Rpv10*), 'Satin Noir' (*Rpv3*), 'Calardis Blanc' (*Rpv3-1/Rpv3-2*), and 'Cabernet Blanc' (*Rpv3*) and in the susceptible cultivars 'Riesling', 'Cabernet Sauvignon', 'Sauvignon Blanc', and 'Muskateller', differing respectively in the number of fungicide treatments (0, 2, 4, 14) during a season. The mean values of the disease severity at BBCH 83 (2021) on grapes (n(grapes)=100) per variety and number of fungicide application are given. The results were taken from a vineyard where these varieties were planted randomly at the same location. Error bars show standard error (SEM). For statistical data analysis, the disease severity were analyzed separately from the susceptible cultivars. ANOVA and Tukey's HSD (Honestly Significant Difference) test were used to compare the disease severity (A, B, C, D) at different fungicide treatments within the resistant cultivars and susceptible cultivars (a, b, c, d, e).

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