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ARTICLE

Behavior of grape breeding lines with distinct resistance alleles to downy mildew (*Plasmopara viticola*)

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Abstract: *Downy mildew (Plasmopara viticola) is the main grapevine disease in humid regions. In the present investigation, marker-assisted selection (MAS) was used to develop grapevine lines homozygous in loci Rpv1 and Rpv3 for resistance against P. viticola. The experimental populations UFSC-2013-1 (n = 420) and UFSC-2013-2 (n = 237) were obtained by self-pollination of two F_1 full-sib plants, originated from a cross between two distinct breeding lines containing the downy mildew resistance loci Rpv1 and Rpv3 in heterozygosity. The two experimental populations were genotyped with four microsatellite markers flanking the two downy mildew resistance loci. Among 637 genotyped plants, 300 (48.2%) were homozygous for at least one resistance locus and 10 (1.57%) were homozygous for both Rpv1 and Rpv3 loci. These 10 plants challenged with P. viticola inoculum showed a clearly enhanced level of resistance. These plants have a great potential as resistance donors in grapevine breeding.*

Key words: *Vitis, disease resistance, gene pyramiding, distorted segregation.*

INTRODUCTION

The diploid obligate biotrophic oomycete *Plasmopara viticola* (Berk & Kurt) causes downy mildew, one of the main diseases that reduce grape (*Vitis vinifera*) production (Caffi et al. 2010). This yield loss is not confined to one season; early leaf death and defoliation caused by the disease weakens the vine and leads to poor harvests in subsequent seasons (Matasci et al. 2008). The pathogen was found originally in North America on wild *Vitis* (Olmo 1986); however, it has since spread across the globe to many countries, including Brazil, by introduction of American vines contaminated with the pathogen (Santos-Neto 1955). Currently, the pathogen is responsible for extreme damages to vineyards without effective crop protection in South Brazil, where the humid climate is especially amenable for the pathogen's life cycle and proliferation (Hamada et al. 2008, Peruch and Bruna 2008).

For more than two centuries, *V. vinifera* and *Muscadinia rotundifolia*, a donor of resistance genes, have been bred with the objective of combining high-quality fruit production with effective disease resistance (Olmo 1986, Alleweldt and Possingham 1988). Nevertheless, high levels of distorted segregation in plants

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from interspecific crosses led to low survival rates in the progeny, thus hindering breeding efforts (Bouquet 1986, Lu et al. 1998); however, some successful hybridizations were obtained when *M. rotundifolia* was used as the male parent (Lu et al. 1998).

Grapevine improvement, similar to all perennial species, takes a considerably long time to develop new varieties because maturity often requires several growing seasons before breeding success can be evaluated. However, indirect selection with the assistance of molecular markers, which can be used at any developmental stage of the plant, can reduce this interim time considerably (Töpfer et al. 2011a).

More recently, these markers have allowed the construction of genetic maps, QTL analyses, and implementation of marker-assisted selection (MAS), particularly applied for pyramiding resistance alleles in grapevine (Alzate-Marin et al. 2005, Eibach et al. 2007, Töpfer et al. 2011b). In addition to the pyramiding of resistance genes the development of inbred lines has a crucial role in gene introgression, because it ensures that all progeny will inherit the alleles of interest. Moreover, vines homozygous for downy mildew resistance allele *Rpv12* (Venuti et al. 2013) and for seedlessness (Sdl) [according to VIVC table of loci] locus responsible for apyrenic grape fruits have been reported (Doligez et al. 2002, Akkurt et al. 2012).

The locus *Rpv1* is located on chromosome 12 of *M. rotundifolia*, and it is associated to locus *Run1*, which confers resistance to downy mildew (Barker et al. 2005, Merdinoglu et al. 2003). *Rpv1/Run1* alleles were introduced in *V. vinifera* by backcrossing (Pauquet et al. 2001). The locus *Rpv3* was identified on chromosome 18 in the grape variety 'Regent' (Fischer et al. 2004, Welter et al. 2007) and conferred significant resistance to downy mildew on both leaves and berries in all evaluated years (Welter et al. 2007). In grapevine, this *Rpv3* locus was responsible for causing the hypersensitive response (HR) at infection sites within two days after the inoculation (dpi) (Bellin et al. 2009, Casagrande et al. 2011).

The resistance alleles *Rpv1* and *Rpv3* revealed complete dominance at both of their loci. Through pyramiding *Rpv1* and *Rpv2*, Eibach et al. (2007) obtained heterozigous plants that exhibited resistance to downy mildew. The effect of either *Rpv1* or *Rpv3* in single plants showed significantly higher resistance to downy mildew; however, genotypes with both resistance alleles showed no infection (Eibach et al. 2007). In other studies, the combination of multiple resistance alleles, such as *Rpv1* and *Rpv3* (Calonnec et al. 2013); *Rpv3* is equal to QTLRgD as already described in Bellin et al. (2009), Di Gaspero et al. (2012), Zyprian et al. (2016), *Rpv3* and *Rpv10* (Schwander et al. 2012), *Rpv3* and *Rpv12* (Venuti et al. 2013), likewise conferred significantly enhanced resistance to pathogen infection in comparison to single resistance alleles. Thus, the objective of this study was to develop, characterize, and select breeding lines homozygotic for the pyramided resistance alleles *Rpv1* and *Rpv3* for future breeding purposes.

MATERIAL AND METHODS

Plant materials

A total of 637 plants from two segregating population were used in this study. The first, UFSC-2013-1 (n=420), was obtained by self-pollination of the F_1 plant 2000-305-97 (red grapes). The second, UFSC-2013-2 (n=217), was obtained by the self-pollination of the F_1 plant 2000-305-134 (white grapes). The two self-pollinated F_1 plants are full sib obtained by the cross between VRH 3082-1-42 x 'Regent' and are heterozygous for both *Rpv1* and *Rpv3* pyramided loci (Eibach 2007).

Genotyping

DNA extraction

DNA was extracted from approximately 100 mg of leaf samples dissected on silica gel. The samples were macerated using four 2.8 mm ceramic beads placed in 2 ml self-standing micro-tubes (Precellys® 24 homogenizer, Bertin Technologies) during two 30 s agitation cycles at 5000 rpm. Subsequently, the DNA was isolated using a NucleoSpin® Plant Kit as described by the supplier (Macherey-Nagel). The DNA samples were quantified with a NanoDrop 1000 Spectrophotometer (Thermo Scientific). The DNA was diluted to a concentration of 1 ng μL^{-1} for further use in PCR reactions.

Amplification of microsatellite markers by PCR

Microsatellite markers Sc34_8 and Sc35_2 (NCBI STS database accession: GF111545 and GF111546), flanking the *Rpv1* locus located on chromosome 12, and the markers GF18-06 (Schwander et al. 2012) and GF18-08 (Zyprian et al. 2016), located on chromosome 18, which flank the *Rpv3* locus, were used to genotype the plants. The four pairs of primers were added in the same polymerase chain reaction (PCR) to obtain all amplicons at once.

The PCR reactions were performed in a total volume of 5 µL, using the KAPA 2G Kit (Kapa Biosystems, Inc. Boston, USA). The mix contains 1x of KAPA Fast Multiplex Mix and four pairs of primers (0.01 µM). The forward primer (5') was labeled with 6-FAM, VIC or PET fluorophore. The PCR reactions were carried out in a C-1000TM Thermal Cycler (Biorad), with the following thermal profile: 94 °C for 3 min, followed by 30 touchdown cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final elongation of 40 min at 72 °C. To confirm amplification, 5 uL of PCR product was used for electrophoresis in 1.5% agarose gel and visualized under UV transilluminator.

Capilar electrophoresis

Sequencing reaction was prepared to a final volume of 10 µL of diluted PCR product (1.0 µl PCR reaction in 27 µl autoclaved ultrapure water), 0.5 µl of size ladder Gene Scan 600 LIZ (Life Technologies) and 8.5 µL of Formamide (Life Technologies). The alleles were separated by capillary electrophoresis done in a 3500 XL Applied Biosystems with the polymer POP-7. Allele sizes were determined using a Gene Mapper Version 4.1 software (Applied Biosystems).

Resistance phenotyping on artificially inoculated leaf discs

To validate the genotyping data, 142 randomly-selected plants in variable proportions from the nine obtained allelic combinations were evaluated for resistance to *P. viticola* (Table 1). Inoculations were performed on leaf discs according to Schwander et al. (2012). All materials used during the inoculations, except the inoculum, were autoclaved at 120 °C and 1.1 atm for 19 min.

The fourth leaf from the apex to the base of all plants were collected. The leaves were sterilized with sodium hypochlorite (1% of active chlorine) for 30 s, and, subsequently, triple-rinsed in ultrapure water (Milli-Q®) for 30 s, 30 s and 60 s, respectively. In a laminar flow hood, six leaf disks 12 mm in diameter were excised from each plant and placed abaxial-side-up on moist filter paper in glass petri dishes to produce a moist chamber for pathogen proliferation. Then, a droplet of 30 µL of the sporangia suspension, at a concentration of 50,000 spores mL^{-1}, was applied to each leaf disk. Three replicates were performed for each plant.

The Petri dishes were sealed and incubated in a growth chamber. In the first 10 h the disks were kept in the dark at 22 °C. Subsequently, conditions were changed to a photoperiod of 14 h light and a constant temperature of 25 °C. After 15 h, the droplet was removed from each disc, and left incubated for seven days to evaluate the sporulation of downy mildew. The degree of infection was rated in each genotypic class on the basis of sporangia intensity formed by leaf disc.

Table 1. Number of plants and phenotypic mean evaluated for each genotypic class

Genotypic Classes	Number of phenotyped plants	Phenotypic mean**
Rpv1*/Rpv1, Rpv3/Rpv3	10	8.2
Rpv1/Rpv1, rpv3/rpv3	7	3.3
Rpv1/Rpv1, Rpv3/rpv3	12	6.3
Rpv1/rpv1, Rpv3/Rpv3	19	7.3
Rpv1/rpv1, Rpv3/rpv3	40	7.2
Rpv1/rpv1, rpv3/rpv3	16	4.4
rpv1/rpv1, Rpv3/rpv3	13	4.7
rpv1/rpv1, Rpv3/Rpv3	11	5.2
rpv1/rpv1, rpv3/rpv3	14	2.1
	142	

* = The Capital letter identifies the resistance allele; ** = Based on Scale OIV 452

Statistical analysis

Frequency distribution and chi-square tests were used to analyze the obtained segregations in genotypic data from the expected ones. When distorted segregation was statistically significant, the Lorieux et al. (1995) methodology was used to determine the type of selection acting in the analyzed populations. Confidence intervals (CIs) of 95% were used for comparison of means of phenotypic evaluations of the downy mildew. The degree of resistance of other genotypes to *P. viticola* was calculated based on the susceptible genotypes *rpv1*/*rpv1* and *rpv3*/*rpv3* that showed 100% of mildew infection.

RESULTS AND DISCUSSION

Genotyping

Genotyping with four microsatellite markers linked to resistance alleles *Rpv1* and *Rpv3* (with two pairs of primers flanking each of the loci) classified plants according to the number of resistance alleles present in each plant. Plants were considered to have the resistance loci when the two microsatellite markers for each locus presented alleles originally linked to the resistance alleles. Out of 637 genotyped plants, 15 showed genetic recombination (2.3%) between the pairs of microsatellite markers and were excluded from the analysis. This recombination rate of 2.3% rate is quite acceptable to breeders in terms of indirect selection efficiency.

Of the remaining 622 plants, 36 (5.8%) did not show any of the resistance alleles, 92 (14.8%) had only the *Rpv1* allele, 194 (31.2%) carried only the *Rpv3* allele, and 300 (48.2%) exhibited both resistance alleles *Rpv1* and *Rpv3* (Figure 1). Out of these 300 plants, 10 of them (1.6% of total plants) showed resistance alleles in homozygous state at both loci (*Rpv1*/*Rpv1*, *Rpv3*/*Rpv3*). A similar frequency of genotypic classes was observed in both segregating populations (Figure 1).

The analysis of the genotypic classes of individually loci clearly showed a distorted segregation from the expected Mendelian proportion, especially for the Sc34_8 and Sc35_2 markers linked to *Rpv1* (Table 2). In the UFSC-2013-1 population, both markers segregated in the proportion of 23 *Rpv1/Rpv1*, 229 *Rpv1/rpv1* and 161 *rpv1/rpv1* [x^2 = 97.12 (P=2.2^{e-16})], equivalent to 1: 10.5: 6.7. A similar result was showed by the UFSC-2013-2 population, being 11 *Rpv1/Rpv1*, 129 *Rpv1/rpv1* and 69 *rpv1/rpv1* [x^2 = 43.68 (P=3.274^{e-10})] or 1: 11.6: 2.2 (Table 2). In both F_2 populations, a deficit of homozygous resistant plants to downy mildew was verified with the markers Sc34_8 and Sc35_2. The significant deviation revealed by the x^2 test could be due to the occurrence of zygotic selection in this locus, resulting changes in allele and genotypic frequencies in both populations.

The segregation of the markers GF18-06 and GF18-08, linked to the *Rpv3* resistance locus, was Mendelian in one population and distorted in the other (Table 2). The Mendelian segregation occurred in the UFSC-2013-2 population: 53 *Rpv3/Rpv3*, 104 *Rpv3/rpv3* and 52 *rpv3/rpv3* [x^2 = 0.014 (P = 0.99)]. In the UFSC-2013-1 population, both markers segregated to proportion of 132 *Rpv3/Rpv3*, 205 *Rpv3/rpv3* and 76 *rpv3/rpv3* [x^2 = 15.20 (P= 0.0005)], therefore, distorted. Significant deviations in the value of χ^2 in that population were partly influenced by the death of 51 sensitive plants.

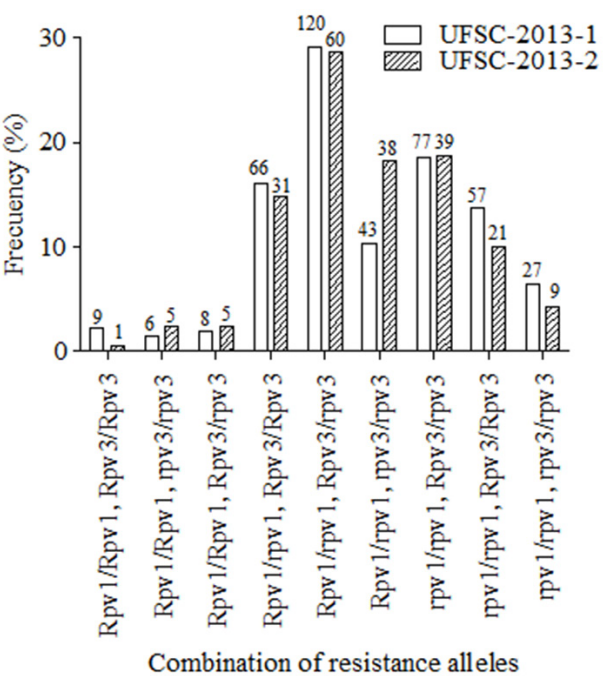

Figure 1. Genotypic frequency distribution (in percentage) of two loci conferring resistance to downy mildew in 622 plants of UFSC-2013-1 and UFSC-2013-2 populations. Numbers above the bars indicate quantity of plants.

Table 2. Result of segregation the two populations and chi-square test revealed by SSR markers

Locus	Populations	
	UFSC-2013-1 (Plants)	UFSC-2013-2 (Plants)
Chromosome 12		
Rpv1/Rpv1	23	11
Rpv1/rpv1	229	129
rpv1/rpv1	161	69
x^2 (1:2:1)	97.1***	43.7***
Distortion [1/]	Zygotic	Zygotic
x^2 [p (Rpv1) = q (rpv1)]	95.5***	32.8***
x^2 = [p^2:^2pq:q^2]	25.7***	23.9***
Chromosome 18		
Rpv3/Rpv3	132	53
Rpv3/rpv3	205	104
rpv3/rpv3	76	52
x^2 (1:2:1)	15.2***	0.014
Distortion [1]	Gametic	None
x^2 [p (Rpv3) = q (rpv3)]	16.2***	0
x^2 = [p^2:^2pq:q^2]	0.1	0.01
TOTAL PLANTS	413	209

[1]According to the methodology employed by Lorieux et al. (1995).
*** P<0.001

The deviation from the expected Mendelian segregation ratio has often been observed in the offspring of intra- and inter-species hybrids (Harushima et al. 1996). Thus, segregation distortion comprises a group of systems of genic drive found in a wide range of organisms, usually involving a small number of interacting primary loci (Lyttle 1991).

Because the parental donor VRH 3082-1-42 had been obtained from interspecific cross between *Muscadinia rotundifolia* G52 x *Malaga seedling* No.1 (*V. vinifera*), followed by four backcrosses to *V. vinifera* varieties (Pauquet et al. 2001), it is possible that the plants inherited, in addition to resistance alleles of locus *Run1* (that is linked to *Rpv1*), some factor that cause the deviations of segregation on the region where the allele of resistance *Rpv1* is located. In this same cross, Bouquet (1986) observed a high distorted segregation ratio due to chromosome disequilibrium and plant mortality. Likewise, Pauquet et al. (2001), studying populations of the fourth and fifth backcross to *V. vinifera* from this same cross (*M. rotundifolia* x *M. seedling*), also found two populations with an excess of susceptible genotypes that caused a distorted segregation ratio. The authors hypothesized that this was the result of the influence of genetic background from which the gene was taken. A comparison of physical and genetic mapping data indicates that recombination is severely repressed in the vicinity of *Run1* (chromosome 12), possibly due to divergent sequence contained within the introgressed fragment from *M. rotundifolia* that carries the locus *Run1* (Backer et al. 2005), which is linked to *Rpv1*.

Segregation distortions were also found in other interspecific crosses when one of the homozygotes was favored, which may suggest the involvement of gametophytic and zygotic factors. In grapes, segregation distortions were found in the crosses *V. rupestres x V. arizonica/girdiana* (Riaz et al. 2006), and *V. vinifera x V. arizonica/candicans* (Riaz et al. 2008). In the chromosomal region where the allele *Run1*/*Rpv1* is located, the zygotic selection acted in favor of susceptible homozygous or heterozygous genotypes in the two studied populations, which can prevent the development of a larger number of plants homozygous with resistance alleles. Thus, the deficit of homozygote genotypes found in the present work for the resistance alleles suggests the occurrence of zygotic selection.

Phenotyping

One-fourth of the genotyped plants were tested phenotypically for resistance to downy mildew, whereby all nine obtained allelic combinations for the two populations are represented (Table 1). The analysis of the average number of sporangia per leaf disc resulted in the formation of three general groups: the first group showed the highest incidences of downy mildew infection and were associated with absence of resistance alleles; the second group showed moderate infection and was associated with resistance alleles in one locus, regardless if the alleles were homozygous or heterozygous

(*Rpv1/rpv1, rpv3/rpv3; Rpv1/Rpv1, rpv3/rpv3; rpv1/rpv1, Rpv3/rpv3 and rpv1/rpv1, Rpv3/Rpv3*); and the third group showed less incidence of the disease and exhibited the resistance alleles pyramided (*Rpv1/rpv1, Rpv3/rpv3; Rpv1/Rpv1, Rpv3/rpv3; Rpv1/rpv1, Rpv3/Rpv3 and Rpv1/Rpv1, Rpv3/Rpv3*). Genotypes with at least one resistance allele in each loci demonstrated better performance in the disease control compared to plants with one or neither resistance allele in both loci (Figure 2). The lowest average value of sporangiophores/disc (4.9) was observed in plants homozygous for both loci carrying the resistance alleles.

Table 1 includes also the phenotypic mean for downy mildew resistance, in which the number of sporangiophores was converted to 9 to 1 Scale, according to OIV 452. Again, it was possible to verify that the resistance alleles of the locus *Rpv3* have higher contribution to resistance to downy mildew when compared to *Rpv1*.

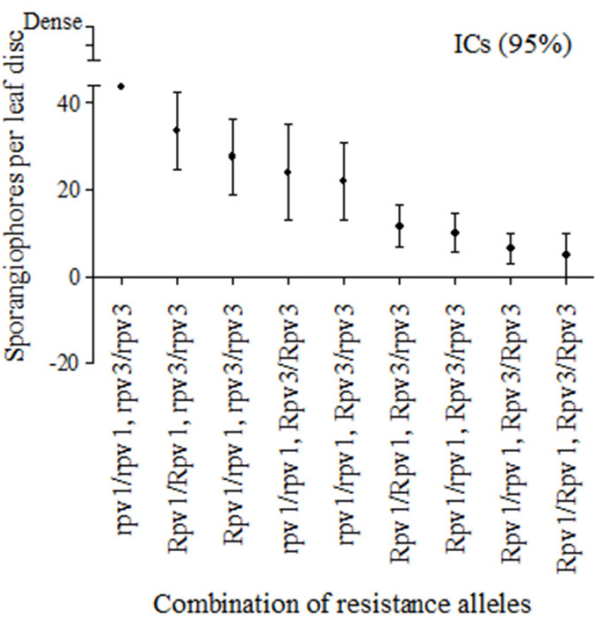

Figure 2. Average values of the number of sporangiophores per leaf of nine genotypic combinations of the loci *Rpv1* and *Rpv3*. Leaf discs of plants with genotype rpv1/rpv1, rpv3/rpv3 could have 50 or more sporangiophores. Bars represent a 95% confidence interval.

Resistance to *P viticola* in grape is greatly increased through pyramiding resistance alleles (Figure 3). Plants with absence of resistance alleles showed the highest level of infection (μ = 2.1; s=1.7). Intermediate resistance was shown by genotypes with resistance alleles in one of the loci (Rpv1, μ =4.0; s=2.1 and Rpv3, μ =4.9; s=2.2), and the highest level of resistance was obtained with the presence of resistance alleles in both loci (μ =7.2; s= 2.0). However, the presence of a specific resistance allele was not enough to consistently be associated with a specific phenoype (Figure 3). The frequency distribution indicated that, in addition to the resistance alleles, the genetic background of the plant could be responsible for the variation of phenotypes associated with plants with the same alleles. In this study, plants with the same genotypic class showed varying response to the pathogen in each inoculation (Figure 2). In addition, the effect of single locus or combination of resistance genes was associated with degrees of resistance. Thus, when the plants carried resistance alleles from only one locus, regardless whether it homozygous or heterozygous, the disease control was less than or equal to 45.1%. However, when the plants carried a combination of resistance alleles of two loci the infections control by *P. viticola* was between 76.8 to 88.9%.

The inoculation technique on leaf discs is widely used in genetic resistance studies against *P. viticola*. In grape, this strategy is effective for distinguishing phenotypic differentiation between resistant and susceptible plants. With the use of first bioassays in grape leaves, it was possible to select genotypes with reasonable accuracy. This approach could possibly be used as a diagnostic marker for selecting which individual plants with disease resistance are most suitable to be planted in the field (Calonnec et al. 2013). The combination of alleles at loci *Rpv1* and *Rpv3* showed better disease control when compared to the intermediate resistance of individual loci. These results suggest that each locus has a partial and additive effect on grape resistance to downy mildew and the dominance of the resistance allele within a locus.

Phenotype variations in plants carrying the same resistance alleles might be partially due to the methodology and the different genetic backgrounds of each resistance alleles. It is also possible that some minor QTLs or other coding sequences, which also participate in plant pathogen response, segregated in the studied populations. Therefore, different genotypes with the same major resistance alleles can show different degrees of disease resistance, as based on observed average confidence levels for disease resistance in each genotype in this study.

Previously, Eibach et al. (2007) demonstrated that the combination of alleles from both genes *Rpv1* and *Rpv3* in the F_1 progeny of the cross VRH3082-1-42 x Regent, resulted in an additive effect in disease resistance. Likewise, Schwander et al. (2012) observed an additive effect on resistance to downy mildew by combining the *Rpv3* and *Rpv10* resistance

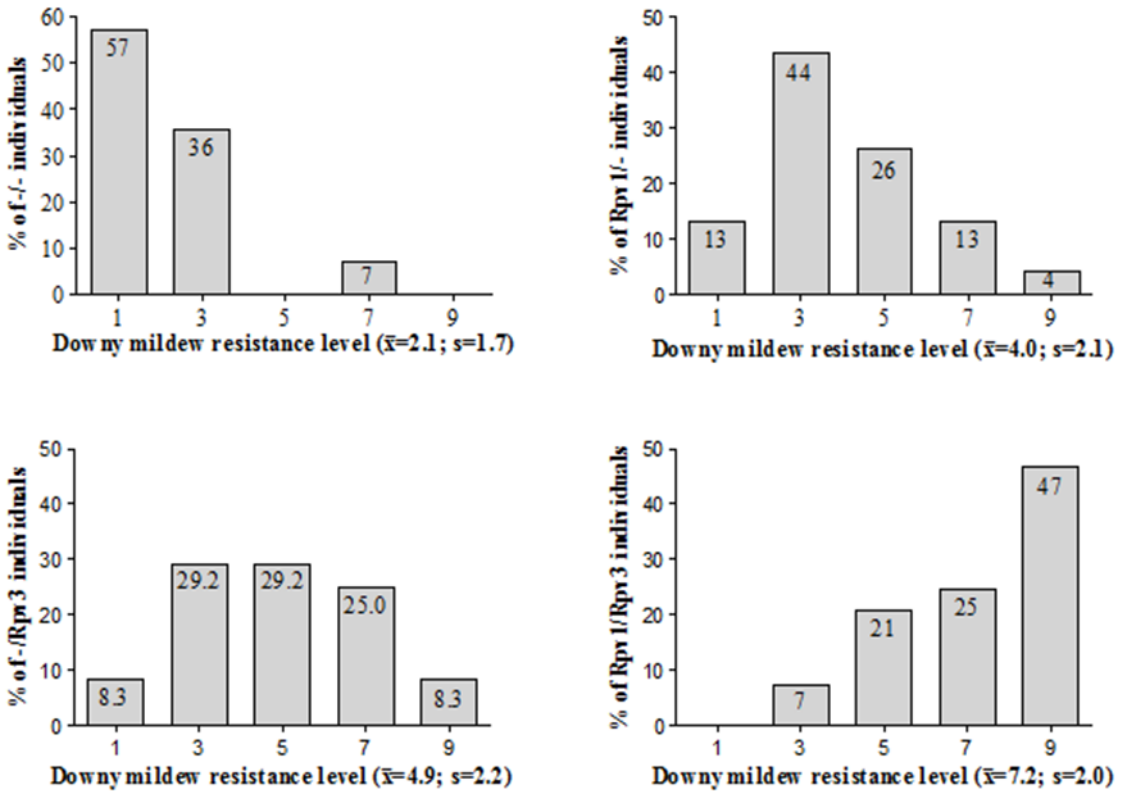

Figure 3. Frequency distribution (%) for the degree of infection of *P. viticola* in 142 plants of UFSC-2013-1 and UFSC-2013-2 populations. The average values number of sporangiophores per leaf of genotypic combinations of the loci *Rpv1* and *Rpv3* were classified according to the OIV descriptor 452 from susceptible (1) to resistant (9). The intensity of sporangiophore formation was rated (9: zero to three, 7: four to 10, 5: ten to thirty, 3: thirty to fifty, 1: more than fifty), according the methodology described by Schwander et al. (2012). Y axis is label for the presence (+) or absence (-) of the resistance correlating alleles Rpv1 and Rpv3. Mean of the downy mildew resistance level in each group with standard deviation (s) in brackets is given in the X axis label.

loci, indicating that they are applicable for pyramiding of resistance pathway MAS. Introgression of resistance alleles *Rpv3* and *Rpv12* from *V. amurensis* to *V. vinifera* also showed the positive effects of pyramiding resistance genes; plants heterozygous for both alleles restricted pathogen growth much more effectively than plants with only one or neither of the resistance alleles (Venuti et al. 2013).

The positive effect of resistance gene pyramiding in controlling pathogens extends to other plant species: *Exserohilum turcicum* and *Sphacelotheca reiliana* in maize (Min et al. 2012); *Pyricularia grisea* Sacc in rice (Hittalmani et al. 2000); *Phakopsora pachyrhizi* in soybean (Maphosa et al. 2012).

The analyzed plants in the current study containing resistance alleles in homozygous state at the two loci showed the best results in terms of disease control. However, these genotypes did not react significantly different to *P. viticola* from other genotypic classes that have pyramided resistance alleles at the two loci, suggesting other type of gene action than additive effect between distinct alleles.

The results of the phenotypic evaluations showed that the loci *Rpv1* and *Rpv3* contribute significantly to disease control against downy mildew. The creation of plants homozygous with resistance alleles *Rpv1* and *Rpv3* is greatly useful to breeders and may contribute to creation of new elite cultivars. These selected plants can be crossed with other varieties of economic interest, ensuring that the F_1 plants will carry at least one resistant allele in each locus and ensuring some level of disease resistance. In addition, they can speed up the breeding programs due to the reduction

of time in getting resistant varieties. Moreover, the selected resistant plants could be used to introduce resistant alleles in currently used tolerant cultivars, such as BRS Vitoria, which is tolerant to downy mildew (Maia et al. 2014), since it is of the interest of a local viticulture industry, especially those ones focused on the fruit export.

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