

Association of Single Nucleotide Polymorphisms (SNPS) to Black Spot Resistance in Roses

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Abstract. Rose black spot caused by *Diplocarpon rosae* is the most severe and global disease of garden roses. Breeding of disease resistant varieties is one of the most important goals of modern garden rose breeding. Single nucleotide polymorphisms (SNPs) are the most abundant type of polymorphism found in eukaryotic genomes. SNP markers can be used in a wide variety of applications, including association studies, genetic diversity analysis, and marker-assisted selection in plant breeding programs. The aim of this project was to analyze black spot resistance in a rose association panel through leaf inoculation assays with single isolate pathotypes as well as a field mixture of *D. rosae* isolates and to establish a relationship between resistance and the available SNP markers. In this study, 96 diverse cultivars of roses were evaluated phenotypically for resistance against black spot through artificial inoculations. 63000 SNPs that were developed in previous studies were used to genetically analyze the cultivars and find associations with resistance against black spot disease using mixed linear model in TASSEL 3.0. Differences in the phenotypic reaction to field mixture of isolates and the Ab13 single conidial isolate of this pathogen were observed between genotypes. One hundred and forty-nine SNPs were found to be significantly associated with resistance against field mixtures of black spot. For the Ab13 single isolate, only one significant SNP was found. These SNPs were mapped on the rose chromosomes, and found on chromosome one, three and four. These associated SNP and Rdr1 markers can be used for marker-assisted selection in breeding for black spot resistance in rose.

Keywords: black spot; *Diplocarpon rosae*; *rosa*; single nucleotide polymorphism

INTRODUCTION

Rose is one of the most economically important ornamental horticultural plants cultivated worldwide. The genus, *Rosa* L., belongs to the family *Rosaceae* of the order Rosales and the genus *Rosa* (Hummer & Janick, 2009). The genus *Rosa* is divided into four subgenera; *Hulthemia*, *Platyrhodon*, *Hesperhodos*, and *Rosa*. Since ancient times, the rose has been recognized for its beauty and fragrance (Nybom, 2009). In addition to this rose has several uses as for example oil production, culinary and medicinal uses, as a source of vitamin C, as garden plants, cut plants, and pot plants (Hummer & Janick, 2009). Rose species are found throughout temperate regions of the Northern hemisphere (Hummer & Janick, 2009) with about 200 species (Nybom, 2009). Due to the problem of transporting soil, rose production for landscape use is usually local. However, the cut flower production industries have continuous progress and expansion to south and Central America and Africa (Hummer & Janick, 2009).

Rose production is limited by a number of biotic factors. In any part of the world, disease and insect infestations are the major risks for rose cultivation. Diseases like black spot, downy mildew, powdery mildew, rust, anthracnose, and crown gall rot, and pests like aphids, mites, thrips, and nematodes are dangers for roses. Among these rose black spot caused by *Diplocarpon rosae* is the most severe and global disease of garden roses (Blechert, and Debener, 2005). To control this disease, most commonly people use pesticides in private and public gardens as well as in commercial production. However, it is more and more limited by legal restrictions and public concerns (Gachomo & Kotchoni, 2016). Due to this reason, scientists and breeders increase their interest to exploit disease resistant species (Nybom, 2009). Using host resistant cultivars is the best option of controlling plant diseases (Pandit et al., 2020).

Therefore, in order to protect human health and to reduce environmental pollution, it is essential to develop resistant genotypes against black spot and to reduce

the use of pesticides (Rispaill et al., 2007). Breeding of disease resistant varieties is one of the most important goals of modern garden rose breeding (Schulz et al., 2009). Wild species are major sources of resistance to important diseases of rose like black spot and powdery mildew. For example, *R. majalis* (93/09-01) is among the 44 wild rose accessions resistant to the black spot identified when tested with 12 single spore isolates of *D. rosae*. *R. majalis* (93/09-01) was also found to be resistant to powdery and downy mildew (Schulz et al., 2009).

The first resistance gene *Rdr1* was identified from a *R. multiflora* hybrid, which shows a “gene-for-gene” interaction (Von Malek & Debener, 1998). Gene identification is important to improve diseases resistance of the crop by transferring the gene that provides the desired trait (Hummer & Janick, 2009). Although the *Rdr1* gene confers a broad spectrum of resistance to most of the *D. rosae* isolates, there exist isolates that overcome the *Rdr1* gene. Therefore, characterization of further rose genotypes needed in order to identify additional resistance gene sources. By genetic characterization of the resistant parent (93/09-01), Ameha Yaekob Gebreyesus, (2009) reported that the gene, which shows resistance against F004, is a dominant gene found in simplex configuration (Rrrr). This gene is found in the same cluster but not confirmed whether it is similar to or different from the known black spot resistance gene *Rdr1*.

During the interaction between the pathogen and the host plant, the genes may show race specific or non-race specific resistance. These genes could be single or multiple genes and the alleles may be dominant or recessive. To exploit the potential of rose genotypes resistant to black spot, genetic information is the first requirement for a breeding program (Basaki, et al., 2008). Single nucleotide polymorphisms (SNPs) are genome-wide abundant markers that detect allelic variation

between homologous loci of the same sub-genome ((Mammadov et al., 2012; Semagn et al., 2006). In plants, SNP markers are tools for mapping, marker-assisted breeding, and map-based cloning. Recently, Schulz et al. (2016) identified 17 and 351 SNPs associated with the accumulation of anthocyanin and carotenoids content, respectively, that determines the phenotypic characteristics of rose petal color. Furthermore, Nguyen et al. (2017) identified 88 SNPs associated with shoot regeneration on the same rose association panel using the same approach. Therefore, the aim of this project was to analyze black spot resistance in a rose association panel through leaf inoculation assays with single isolate pathotypes as well as a field mixture of *D. rosae* isolates and to establish a relationship between resistance and the available SNP markers. The nature of resistance obtained from the 96 rose genotypes will also be associated with the known black spot resistance gene *Rdr1*.

METHODS

Plant Materials

The materials used in this study were 96 rose genotypes, AB13 isolate and mixture of *Diplocarpon rosae*. The rose genotypes used in this study were collected from the greenhouse of the Bundessortenamt in Hannover, which are part of the genotype collection of the Institute of Plant Genetics of Hannover University. All genotypes were randomly assigned to three groups and maintained in standard conditions. They include an association panel of 96 rose cultivars with code numbers from 1 to 141 (87 tetraploid, 8 triploid, and 1 diploid, (Schulz et al., 2016). The genotypes differ in their type or habit, origins, year of breeding and breeder. They comprise five different horticultural types; shrub, hybrid tea, bedding, climber and ground cover. Most of them originated from Germany (60), six from the USA, 12 from the UK and the rest eight from six different countries. They have eight major different colors (19 red, 15

yellow, 23 pink and light pink, 9 white, 6 violet, 6 orange, 3 apricot, 1 magenta, and 14 mixed colors). Most of the cultivars are commercially available or provided by German rose-breeding companies (Appendix 1 Schulz et al., 2016).

***Diplocarpon rosae* Isolates and Mixtures used for Inoculation Experiments:**

The inoculations of rose leaves were made with the isolate Ab13 collected from Ahrensburg (Menz et al., 2018). The conidia suspensions of *D. rosae* collected from naturally infected rose leaves sampled from genotypes on selection fields of the Rosen Tantau KG Infected leaves collected at two different time points from selected fields of the Rosen Tantau KG, August 2014 (used for Tantau I and Tantau II) and December 2017 (Tantau III), and were stored at -20°C. Inoculation assays on the 96 genotypes were carried out on excised leaves under laboratory conditions. The inoculation procedures in this study followed the established inoculation assay protocol of the working group at the Department of Molecular Plant Breeding, Institute of Plant Genetics, Leibniz Universität Hannover.

The fungal isolates both Ab13 and field mixtures propagated and maintained on detached leaves of susceptible cultivar "Pariser Charme" as described ((Blechert, and Debener, 2005; Von Malek & Debener, 1998). The concentrations of the inoculums were adjusted to 1×10^5 for single isolate Ab13 and Tantau III and 0.5×10^5 conidia mL^{-1} for field mixture of Tantau I and Tantau II. For each rose genotype, one leaf was sampled from each of the three clones planted in from the greenhouse of the Bundessortenamt. Each of the detached leaves was inoculated with 10 droplets of 10 μL of spore suspension. The inoculation boxes were placed in an air-conditioned laboratory at 20°C. After 2 days, the excess of inoculation droplets was carefully removed with tissue paper and the leaves were examined at 12 and 21 days for field

mixtures and at 10 and 15 days for Ab13 isolates after inoculation. The evaluation of the disease symptoms was done using a stereoscope according to the scoring system by Whitaker et al. (2010). The analyses were done based on the mean of the three independent biological replications.

Single nucleotide polymorphisms (SNPs) Genotyping

The SNP data set from the 96 garden rose cultivars was generated using an Affymetrix DNA Microarray. SNPs among tetraploid roses were selected for constructing a genotyping array from cut roses and from garden roses. The genotyping was done with two primers for each SNP read from 3 to 5 end and Vis versa. This set consisted of a total of 68,893 SNPs on the WagRhSNP Axiom array (Smulders et al., 2016). Detailed information of the SNP array can be found in (Koning-Boucoiran et al., 2015). The tetraploid SNP dosage score (AAAA, AAAB, AABB, AB BB, and BBBB) was used to calculate the statistics required for the association study.

Trait marker association SNPs

Trait-marker associations were evaluated using the MLM model in TASSEL 3.0 (Trait Analysis by Association, Evolution, and Linkage; (Bradbury et al., 2007). Sixty-three thousand (63000) SNPs were used to calculate the trait marker associations. Markers with a minor allele frequency below 0.1 with more than 10% missing data were excluded from further analysis. The associations were estimated using mixed linear model with both the Q-matrix for population effects based on the output from STRUCTURE 2.3.4

The population structure was modelled with a subset of 400 AFLP- and 175/427 SSR-marker fragments using STRUCTURE 2.3.4 ((Pritchard et al., 2000; Falush, et al. 2007) and the kinship matrix (K) calculated with SPAGeDi Pairwise kinship coefficients

were estimated using the program SPAGeDi (Hardy & Vekemans, 2002) based on the method of (Hardy, 2003) as described in Schulz et al. 2016 using 10,000 filtered SNP markers at the tetraploid dosage state. Bonferroni adjustments of the p-values were made to correct for the number of

independent tests and to establish a threshold (Johnson et al., 2010). A SNP-marker was considered associated if its log₁₀ p-value was larger than 5.89. The data on the population structure Q and kinship data for the relationship of rose cultivars provided by Dr. Dietmar Schulz.

RESULTS AND DISCUSSION

Phenotypic Characterization of Resistance to Black Spot on the Association Panel

Quantitative Assay

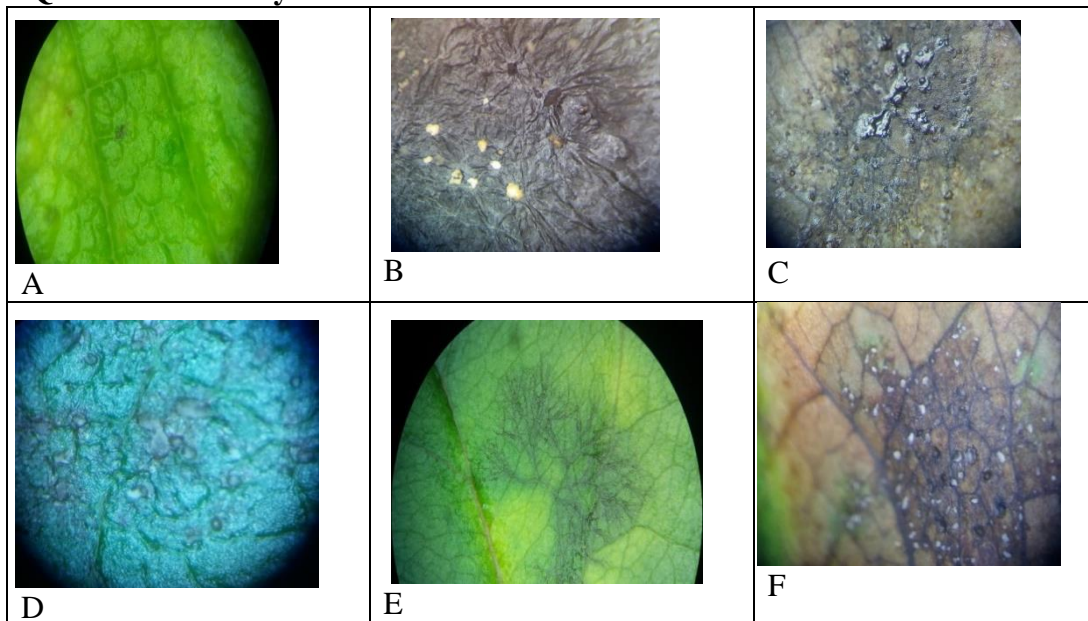


Figure 1. Symptoms on the site of infection on six rose cultivars. A. Mrs. Doreen Pike (0), B. Parole (1), C. Dortmund (2), D. Elfe (3), E. Rose Gaujard (4), F. Small Maidens Blush (4)

The detached leaf assay is more applicable for phenotyping a large number of cultivars to avoid environmental effects. To characterize the resistance against black spot disease phenotyping was done through artificial inoculation (Von Malek & Debener, 1998; Xue and Davidson, 1998). Artificial inoculations using field mixtures sampled at Rosen Tantau KG and the Ab13 single spore isolate were done on the 96 genotypes of association panel. The symptoms in both field mixtures and the Ab13 single conidial isolate showed the same characteristics. On the site of infection, irregular black-brownish spots appeared on the upper side of the leaves, which did not

spread from the area of inoculation, and brown acervuli could be observed with a stereomicroscope. After 21 days post inoculation, chlorosis surrounding the lesion appeared when the tissue started to degrade (Figure 1.) The presence of symptoms such as the appearance of brown spots and formation of acervuli due to the penetration of subcuticular hyphae at the site of infection indicated the susceptibility of the cultivars to the pathogen (Drewes-Alvarez, 2003). Necrotic spots could also be observed at different time points for the different cultivars. The disease ratings for the Tantau I inoculation followed a normal distribution while for Tantau II data displayed a left

skewed distribution at 12 dpi. For both spore mixtures, most of the genotypes had a

disease rate of zero (0) and one (1) at 12 days post inoculation (Figure 2.).

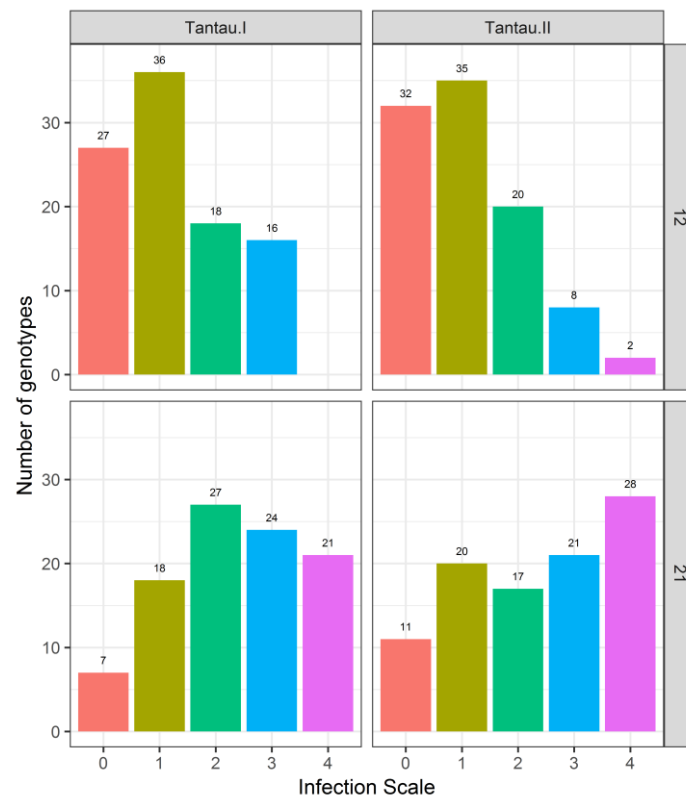


Figure 2. Result from the quantitative assay performed on the association panel using Tantau I and II *D. rosae* field mixtures (frozen leaf). Top left Frequency distribution of Tantau I at 12dpi, Top right Frequency distribution of Tantau II at 12 dpi, bottom left Frequency distribution of Tantau I at 21dpi, bottom right Frequency distribution of Tantau II at 21 dpi.

For Tantau I and Tantau.II spore mixtures 34 and 28 of the genotypes are in the disease scale two (2) and three (3) at 12 days post inoculation respectively. However, the disease score of genotypes increased at 21 days post inoculation, with 45 genotypes having a disease score of 3 and 4 for Tantau I and 49 for Tantau II. Some genotypes showed a delay of infection indicated in Appendix 2. This is due to the fact that the fungus is hemibiotrophic and able to grow, for a limited time, on dead plant tissue(Blechert, and Debener, 2005). In addition, differences on the responses to the pathogen were observed between cultivars (Table 1) (Whitaker et al., 2010).

In the experiments using the Tantau isolate mixtures first at 12 dpi, that 27(32) cultivars presented a disease index zero (0)

indicating the high levels of resistance against field mixture within the association panel. Also, 36(35) cultivars presented scorings one (1) which indicated a low susceptibility with the production of a very low number of acervuli. A limited growth of fungal structures, no formation of acervuli or a hypersensitive response can be seen on resistant cultivars when the pathogen triggers the defence mechanisms of the plant(Agrios, 2005). Some of the other cultivars (18 and 20 cultivars) showed intermediate levels of susceptibility (2) and 16 and eight cultivars were susceptible (3) in Tantau one and two respectively. No cultivar showed high susceptibility at Tantau I at 12 days but there were two cultivars in Tantau II with a disease rate of four. When the results at 12 dpi were compared to the

results at 21 dpi, increased susceptibility of the cultivars was noted for the later time points. Some genotypes still present high resistance against field mixture in both time points (seven and eleven cultivars) respectively with the exception of some cultivars (Table 1). The exception could be due to any technical error such as washing of leaves with water that can damage the tissue and create wound leaves could result in susceptible response even the genotype is resistant.

Furthermore, some cultivars showed low infection level at 21 dpi (Appendix 2). Strong resistance is a race specific which is determined by mono-gene (Agrios, 2005). The field mixtures were collected from many genotypes, but multiplied in “Pariser sharme”. Therefore, some isolates that do not grow well on “Pariser sharme” due to their low abundance in the original mix are lost after several rounds of multiplication (personal communication Prof. Debener). Due to these inconsistencies, several replications of fungal inoculation were needed to get highly reliable phenotypic results. The combined data of the two Tantau mixtures, only four cultivars (“Beverly”, “Cute Haze”, “Mrs Doreen Pike”, “New Dawn”) were completely resistance at 21dpi. In the site of penetration localized cell death initiated to restrict the parasite further invasion. This result is in line with Blechert and Debener (2005). So 14 cultivars showed low level of infection at 21 dpi (“Arabia”, “Focus”, “Hansestadt Rostock”, “Heidtraum”, “Herzogin Friederike”, “Juanita”, “König Stanislaus”, “Kronjuwel”, “Lipstick”, “Stadt Rom”, “Sterntaler”, “Tornella”, “Venice”, “Windrose”).

The genotypes in this study showed different reactions for all field mixtures. Few genotypes showed strong resistance in which macroscopic symptoms were detected. This could be limited growth and penetration of spores after germination. The presence of different types and numbers of resistance

genes within each cultivar could be the reason for the variation in disease resistance among genotypes (Agrios, 2005). The other field mixture (Tantau III) which was used to inoculate the 96 genotypes collected from Tantau at the end of 2017. The cultivars inoculated with this mixture showed the skewed distribution of the genotypes towards the susceptibility disease rates (Figure 3.). After 12 and 21 dpi eight cultivars showed complete resistance against this mixture (zero disease scale) So that, no host reaction was seen in these cultivars (Table 1). However, most of the cultivars, (38) at 12 dpi and (28) at 21 dpi were susceptible.

There were also cultivars (21) and (49) that showed a high level of susceptibility (4) at 12 and 21 dpi-respectively. As expected, there was also an increase of the disease rating for the genotypes at 21 dpi as compared to 12 dpi. Three cultivars had only a lower level of infection at 21 dpi (Appendix 2). *Diplocarpon rosae* is a hemibiotrophic fungal parasite (Von Malek & Debener, 1998; Drewes-Alvarez, 2003) that can grow on live tissue and dead plant parts. In addition to macroscopically visible brownish or black spots development of macroscopic necrotic spots were noticed on the site of inoculation. Most genotypes were highly susceptible for field mixture. This could be increasing the age of plants, which collect the infected leaves, could enhance the pathogen development (Dong, 2014) and increase disease pressure in artificial inoculation.

The cultivars inoculated with the isolate Ab13 showed a more equal distribution of the genotypes within all the disease rates. Twenty six cultivars (26) and 19 cultivars showed a high resistance against this race (zero disease scale) at 10 dpi and at 15 dpi respectively (Table 1). This reaction is similar to the result of the experiment by Blechert and Debener (2005) classified under Interaction Type 8 which no host reaction was seen at all.

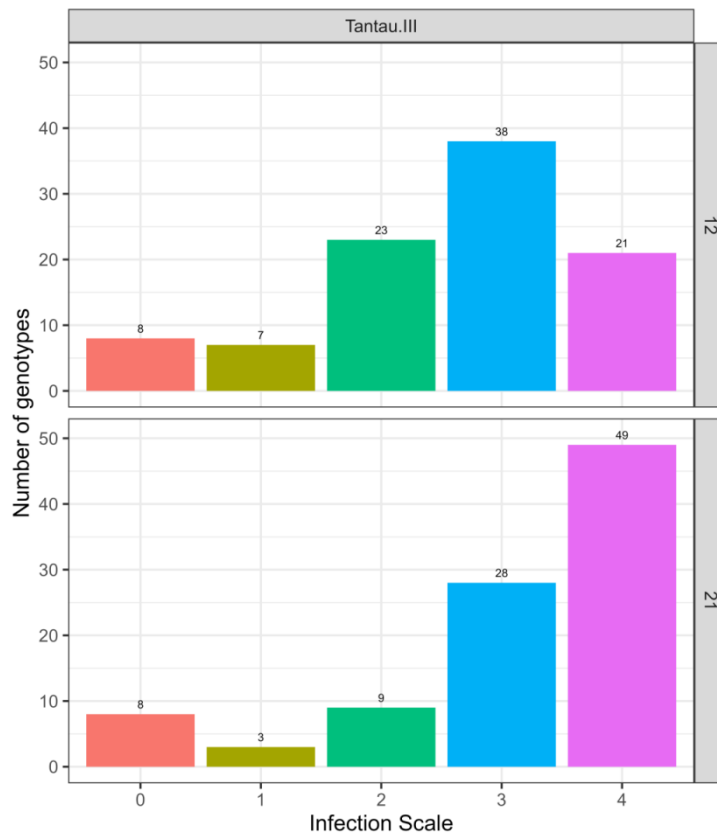


Figure 3. Results from the quantitative assay performed on the association panel using Tantau III from 2017 field isolate mixtures of *D. rosae*. , Top: Frequency distribution of Tantau III at 12 dpi, bottom Frequency distribution of Tantau III at 21 dpi

Most of the cultivars, (28) at 10 dpi and (18) at 15 dpi showed low levels of susceptibility. This type of interaction indicates that partial resistance which is complicated due to hemibiotrophic nature of the fungus (Blechert, and Debener, 2005). One cultivar at 10 dpi and 15 cultivars at 15 dpi were highly susceptible (4). There are also cultivars (42) and (45) at 10 and 15 evaluation days that were moderately susceptible and susceptible (2-3) respectively. The host-pathotypes interaction of these cultivars was compatible (Blechert, and Debener, 2005). There is a slight increase of susceptibility of the genotypes at 15 dpi as compared to 10 dpi (Figure 4). Some genotypes had lower levels of infection at 10 and 15 dpi.

In general, most of the varieties were susceptible to all mixtures and Ab 13 isolate only a few species were resistant to all mixtures and Ab 13 isolate (Malek and

Debener, 1998; Schulz, et al. 2009). In table 1, we can see the interaction and response of genotypes for different field mixtures and Ab13 single isolates. Cute Haze, New Down and Beverly were resistant for both Tantau I and II at both time points. Further Sterntaler, Tornella, Simply and Nostalgie were resistant for Tantau II but not for other mixtures and Ab13 isolate.

Juanita, Heidtraum, and StadtRom also showed resistant for Tantau I. In addition, Juanita and Heidtraum showed resistant for Tantau II at 12dpi respectively but they were susceptible in the later time point. Six genotypes namely Focus Arabia, Chippendale, Westerland, Climbing All gold, Friesia were resistant in Tantau III, and Ab13 isolate. There were also three cultivars (George Vancouver, Jasmina, and Lipstick) which were resistant only for Ab13, isolate. However, Lipstick showed susceptibility in the later time point; it was resistant at 12dpi

for both Tantau I and II. On the other hand, Herzogin Friederike, Kronjuwel, and Hansestadt Rostock were resistant for Tantau II furthermore, Herzogin Friederike

and Kronjuwel for Ab13 isolate and Hansestadt Rostock for Tantau III showed resistant but not Tantau I.

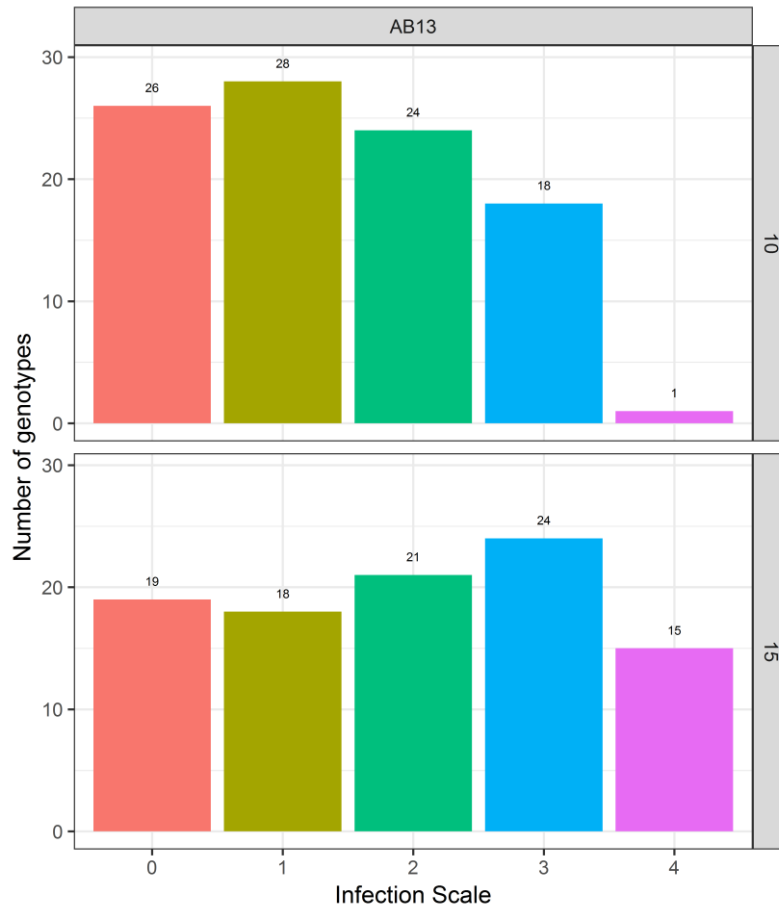


Figure 4. Results from the quantitative assay performed on the association panel using a single spore isolate Ab13. Top: Frequency distribution of Ab13 isolate at 10dpi, (Bottom) Frequency distribution of Ab13 isolate at 15 dpi

Trait Marker Association

Genetic association analysis is a popular approach for identifying genetic variation that correlates with phenotypic variation, such as susceptibility to complex disease. Recently, Schulz et.al (2016) conducted genome wide association study on the accumulation of anthocyanin and carotenoids content, that determine the phenotypic characteristics of rose petal color. Nguyen et al., (2017) also used on the same rose association panel using the same approach on shoot regeneration. To identify markers associated with black spot

resistance, the obtained phenotyping data were associated with 630000 SNP markers. Dr. Dietmar Schulz (Schulz et al., 2016) provided the data on the population structure Q and kinship for the relationship of rose cultivars in mixed linear model (MLM) in TASSEL 3.0. The analysis based on the combined mean of all Tantau isolate mixtures identified 149 significant SNPs in several chromosomal locations associated with black spot resistance/susceptibility to these mixtures. Figure 12 shows the distribution of SNPs on the rose genome from chromosome one to seven. Only SNPs

with a p-value $\leq E-13$ were used to draw the graph.

Significant associated SNPs were located on chromosome one, three, and four based on the Manhattan plot (Figure 5).

Different loci found on different chromosomes have different effects. This indicates that quantitative resistance against black spot is a multi-locus trait with multi-genes.

Table 1. Susceptibility (+) and resistance (-) of different field mixture and Ab13 isolate of *Diplocarpon rosae*

Cultivars	Tantau.I		Tantau.II		Tantau .III		Ab13 isolate	
	12 dpi	21 dpi	12 dpi	21 dpi	12 dpi	21 dpi	10 dpi	15 dpi
Mrs Doreen Pike	-	-	-	-	-	-	-	-
Cute Haze	-	-	-	-	+	+	+	+
New Dawn	-	-	-	-	+	+	+	+
Beverly	-	-	-	-	+	+	+	+
Juanita	-	-	-	+	+	+	+	+
StadtRom	-	-	+	+	+	+	+	+
Heidtraum	-	-	+	+	+	+	+	+
Sterntaler	+	+	-	-	+	+	+	+
Kronjuwel	+	+	-	-	+	+	-	-
Tornella	+	+	-	-	+	+	+	+
Simply	+	+	-	-	+	+	+	+
Nostalgie	+	+	-	-	+	+	+	+
Herzogin Friederike	+	+	-	-	+	+	-	-
Hansestadt Rostock	+	+	-	-	-	-	+	+
Focus	+	+	+	+	-	-	-	-
Arabia	+	+	+	+	-	-	-	-
Chippendale	+	+	+	+	-	-	-	-
Westerland	+	+	+	+	-	-	-	-
Climbing All gold	+	+	+	+	-	-	-	-
Friesia	+	+	+	+	-	-	-	-
George-Vancouver	+	+	+	+	+	+	-	-
Jasmina	+	+	+	+	+	+	-	-
Lipstick	-	+	-	+	+	+	-	-

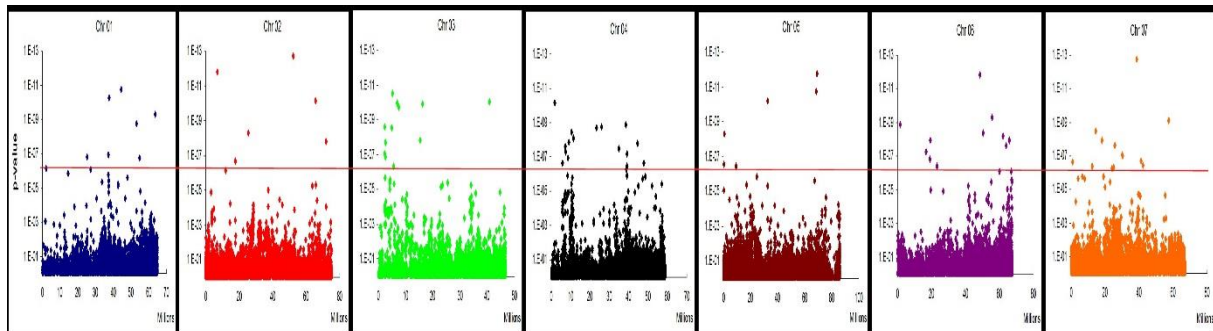
The boxplot for a selection of markers showed the three SNPs which are in the clusters of chromosome one and four (Appendix 3) using untransformed data and also the effect of SNPs depending on allele dosage (Figure 6). In the boxplots, we can see an effect of the allele dosages of the different SNPs on the resistance to *D. rosae* although the data were combined from three different experiments and the cultivars had a different response to each field mixture. SNP marker RHMCRND_2017_1455Q

showed a strong effect on the resistance to *D. rosae*. Genotypes with an allele dosage of zero had a mean number of 22.4 acervuli whereas the homozygous genotypes with a dosage of 4 had a mean acervuli number of 44.6. Heterozygous genotypes showed acervuli numbers in between, increasing with the allele dosage. Even if the effect is less, most SNPs are shown in the homozygous group of allele configuration (Figure 6). This may be because the identified markers are close to a gene, which

has a smaller effect, contributing to the resistance against black spot.

The analysis based on the inoculation of the Ab13 isolate returned only one significant SNP located on chromosome six.

This could be due to the transformation of the tetraploid data into the diploid state for using in TASSEL 3.0, which caused the loss of valuable information.



Chromosome position

Figure 5. Manhattan plot of SNP association on the chromosome of rose genome

The presence of this SNP marker showed a good effect in the heterozygous configuration on the resistance of the genotypes (Figure7). There are some additional SNPs a little above the threshold of $1.78E_6$ (Bonferroni corrected alpha with the number of contigs). These SNPs (Rh12GR_997_289P, RhK5_82_2014P, RhK5_1583_315P, and RhK5_1583_315Q) were identified on the chromosome four nearly on the same position. These SNPs found at 56242983 to 56658787 base pairs. The boxplots (Figure 7) shows significant effects of the two SNPs depending on the allele configuration. The SNP marker *RHK5_82_2014Q* showed a good effect in the heterozygous allele configuration (AB) the distribution of the mean value of the acervuli reduce within 62 cultivars (Figure 7). Therefore, if cultivars can be detected with the homozygous allele it will have a chance to be resistant.

Association of Rdr1 Markers and Black Spot Resistance

There is also an association between resistance to black spot resistance and markers directly generated from Rdr1-family members via a PCR reaction that targets an SSR in the coding region of the gene family (Diro Terefe and T. Debener, 2010). An

association analysis of the Rdr1 markers with the resistance to the field mixture of Tantau was performed and the boxplots were constructed to show the effect on the susceptibility of cultivars for black spot.

The results association between resistance to black spot resistance and Rdr1-markers showed that significant markers associated with field mixture Tantau II and I than Tantau III inoculations. The cultivars, which show the presence of some of these Rdr1-markers (Table 2), are more resistant to the Tantau I and Tantau II mixtures as compared to the cultivars with the absence of this particular marker. None of the 96 genotypes of the association panel carries the Rdr1 gene that confers strong race-specific resistance to a number of races as described in (Menz et al., 2018). However, the gene is part of a large gene family located on chromosome 1 of roses. The fact that some markers derived from family members or relatives of Rdr1 seem to cause a small but significant reduction of black spot infection by the isolates Tantau I, II and III indicates that other members of the gene family might serve as minor resistance loci. However, it cannot be excluded that they are only linked to the true causal genes on chromosome one which might not be related to Rdr1.

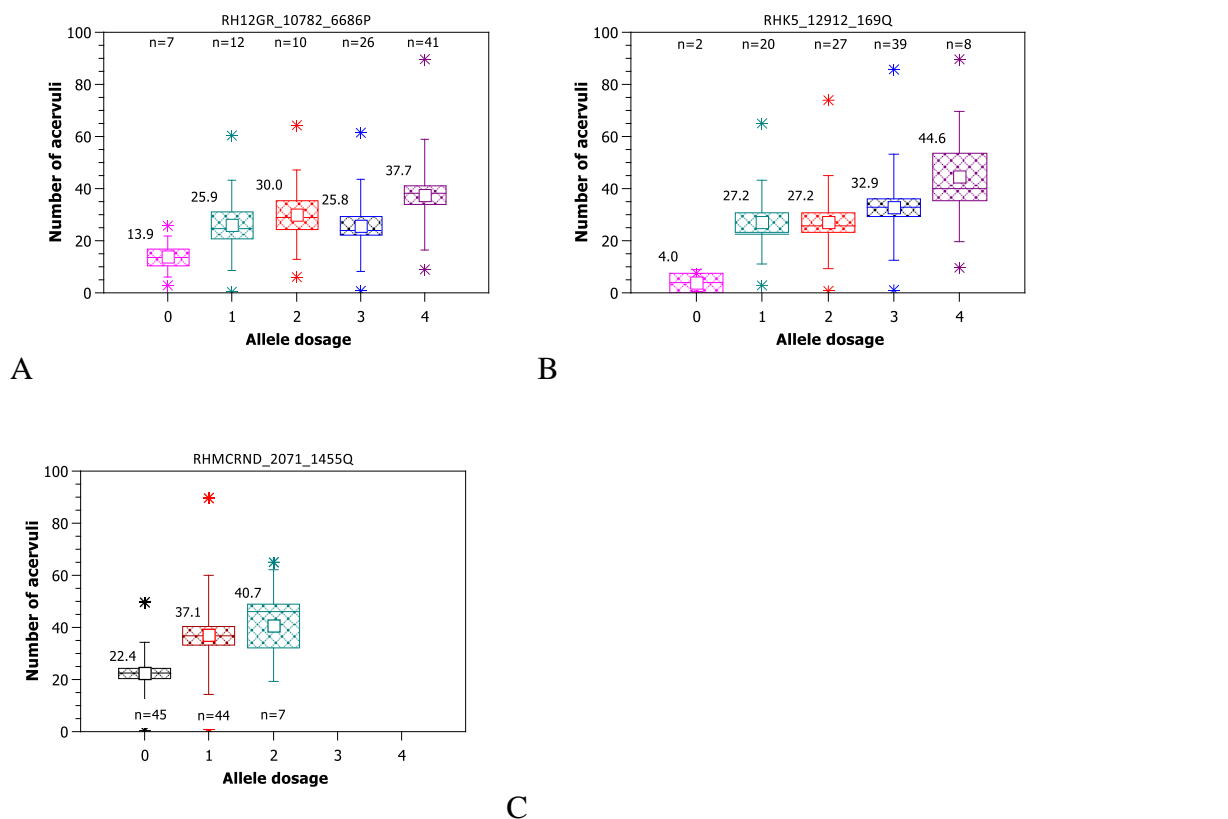


Figure 6. Boxplots displaying the effect of SNP markers in the allele configuration of genotypes against field mixtures of black spot resistance A).RH12_10782_6686P, B). RHK5_12912_169Q C). RHMCRRND_2071_1455Q allele dosages are coded from 0 to 4

Boxplots showed the distribution of the mean value of artificial inoculation with the presence or absence of Rdr1 markers associated with black spot resistance (Figure 8). The boxplots showed only significant markers associated with Tantau.I, Tantau.II and Tantau.III have a certain degree of resistance against Tantau I and Tantau II. This may be because the identified markers are close to a gene which contributes in a small amount to the resistance against this isolate.

The Rdr1 marker M700_5 associated to Tantau I at 12 and 21dpi, M700_7 associated to Tantau I and II at 12dpi and R800_4 were associated to resistance against Tantau II 12 and 21 dpi and U8a with Tantau II 12dpi

field mixture. On the other hand, only one marker (R800_4) was identified to be highly associated with resistance against Tantau III 12 dpi field mixture. However, there is no association between Rdr1 and Tantau III at 21 dpi. Therefore, the Rdr1 gene, pyramiding with only a few other R genes by sexual crosses, might be useful for breeding roses that are resistant to black spot because the spread of new pathogenic races of the fungus appears to be slow. On the contrary, there is no association between the resistance against the isolated Ab13 and Rdr1-markers. This indicates that although Rdr1 confers a broad-spectrum resistance to *D. rosae* this is overcome by the isolate Ab13 (Menz et al., 2018).

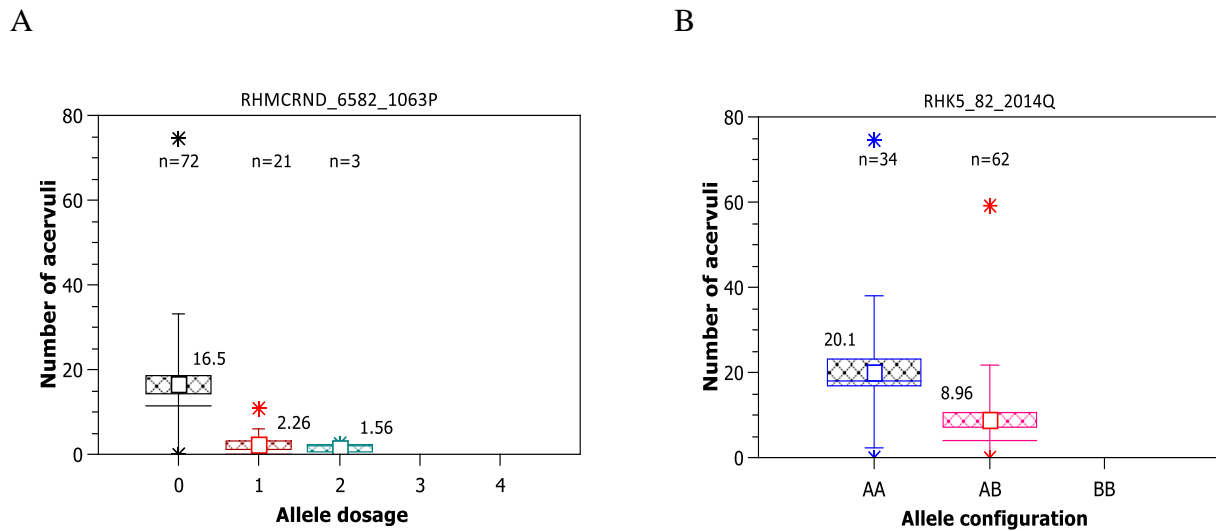


Figure 7. Boxplots displaying the effect of SNP markers in the allele configuration of genotypes against the Ab13 single isolate of black spot resistance A).RHMCRND_6582_1063P, B). RHK5_82_2014Q allele dosages are coded from 0 to 4

Table2. Rdr1 markers associated with resistant on black spot

Tantau.I 12 dpi	U6	R700_5	M700_5	M700_7		
Tantau.I 21 dpi	U6	R700_5	M700_5			
Tantau.II 12 dpi	U5	R800_3	R800_4	M700_7	M800_1	U8a
Tantau.II 21 dpi	U5	R800_3	R800_4		M800_1	R800_1
Tantau.III 12 dpi			R800_4		M800_1	R800_1
Tantau.III 21 dpi	U8a					

Where U: Undigested; R: RSA and M: MboI restriction enzymes respectively; 700/800: nm fluorescent labeled dye; numbers (1, 3, 4, 5, 6, 7,8a): fragment number

Rdr1 provides resistance to different single-spore isolates and broad field mixtures of conidia (Von Malek & Debener, 1998, Menz et al., 2018). However, before using the SNP and Rdr1 markers in breeding programs the highly significant markers

should be tested with a larger number of plants from an independent population to separate the true from the false positive associations and to provide less biased estimates of the allelic effects proposed in our analyses.

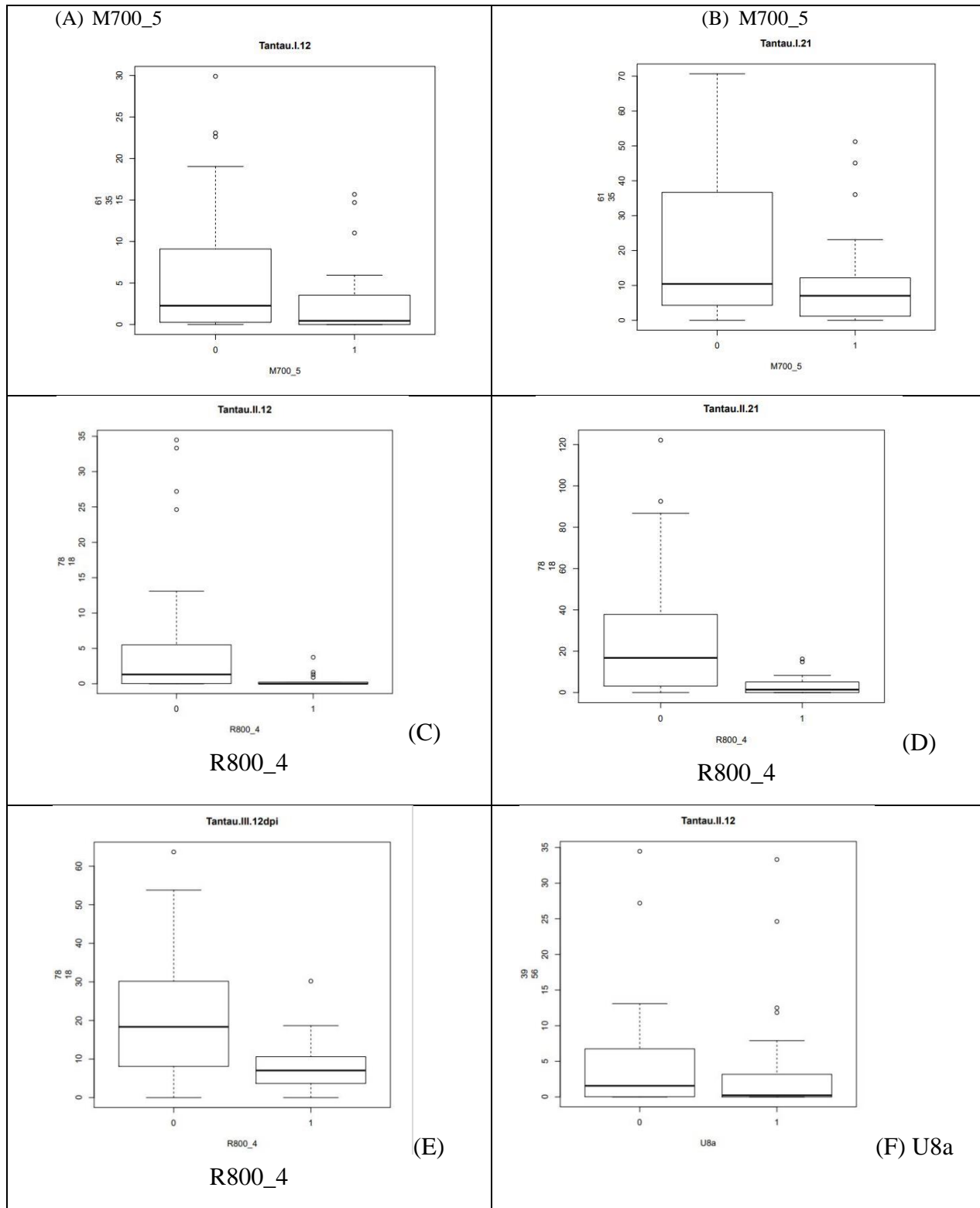


Figure8. Boxplots displaying Rdr1 markers associations with resistance on Tantau I at 12dpi (A), at 21dpi (B), Tantau II at 12dpi (C and F), at 21dpi (D) and Tantau III at 12dpi (E) black spot field mixture black spot

CONCLUSION

In the present study, an association mapping analysis was done to identify SNP markers linked to genes, which are responsible for the variation of black spot resistance using the Affymetrix array. The association was conducted in a panel of 96 diverse commercial tetraploid cultivars. These cultivars were characterized phenotypically in artificial inoculation experiments for black spot resistance using field mixtures and Ab13 single isolate. In this study, some cultivars were identified as resistant to field mixtures but not to the Ab13 single isolate. The cultivars, which were resistant for Ab13 isolate, were not resistant to the field mixtures. Marker-trait associations were estimated using different statistical tools. Several SNP markers were identified significantly associated with black spot resistance. They were located on the chromosome one, three and four in the rose genome. For the Ab13 isolate, which is one of the two races that overcome the resistance gene Rdr1 one significant SNP marker and one marker just below the significance threshold were found on chromosome six. Although there was no association between Rdr1 and resistance to Tantau III field mixtures, a few Rdr1 markers were found which have significant association with resistance against Tantau I and II. These associated SNP and Rdr1 markers can be used for marker-assisted selection of parental rose genotypes, which are used for crosses.

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