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Scarcity of major resistance genes against Verticillium wilt caused by *Verticillium dahliae*

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Abstract

Verticillium dahliae is a soil-borne fungal pathogen that causes vascular wilt disease in numerous plant species. The only described qualitative resistances against *V. dahliae* are the *Ve1* gene and the *V2* locus in tomato. These resistances have been overcome by virulent strains. We tried to identify additional resistances. Out of the methods we tested, comparing the canopy area of *V. dahliae*-inoculated plants with mock-inoculated plants yielded the best discriminative power in resistance tests. Out of six wild tomato accessions that were previously reported to possess some resistance, *Solanum pimpinellifolium* G1.1596 and *Solanum cheesmanii* G1.1615 displayed the lowest stunting and the least colonization by *V. dahliae*. Recombinant inbred line (RIL) populations were developed of both populations. No QTLs were identified in the G1.1596 RIL population. In the G1.1615 population, four small-effect QTLs were associated with reduced stunting. Many studies in other hosts also failed to discover major resistance genes against *V. dahliae*. We hypothesize that the scarcity of major resistance genes against *V. dahliae* is caused by its endophytic behaviour in nature. The limited damage in nature would not lead to evolutionary pressure to evolve major resistances. However, in agriculture, *V. dahliae* can behave more pathogenic, leading to serious damage.

KEYWORDS
disease resistance test, resistance, endophyte, QTLs, resistance, tomato, *Verticillium dahliae*

1 | INTRODUCTION

Verticillium dahliae is a soil-borne fungus that invades roots of its hosts, where it attempts to enter the xylem. Once in the xylem, it produces conidiospores that spread upwards throughout the plant with the sap stream (Fradin & Thomma, 2006). On tomato, common symptoms may include stunting, wilting, yellowing and necrosis of the leaves. Once the fungus has entered the plant and when it resides in the vascular system, it has become unreachable for most fungicides, rendering these fungicides ineffective. Once host tissues become necrotic and start to die, the fungus produces resting structures called microsclerotia which are released in the soil upon tissue decomposition and that can remain viable in the soil for many years (Wilhelm, 1955). Combined with its broad host range, which comprises hundreds of plant species, this explains why *V. dahliae* remains a challenging pathogen to control. Host resistance would therefore be a very welcome solution.

Already in the 1950s, the *Ve*-locus was introgressed into tomato, providing dominantly inherited, major resistance to *V. dahliae* (Deseret

news and telegram, 1955; Schaible et al., 1951). Further fine-mapping and cloning showed that the *Ve* locus consists of two genes, encoding the receptor-like proteins (RLPs) Ve1 and Ve2 (Kawchuk et al., 2001), of which *Ve1* was determined to be the only causal resistance gene (Fradin et al. 2009). Although the introduction of the *Ve*-locus into commercial tomato cultivars was welcomed by farmers, *V. dahliae* strains emerged that were able to overcome the resistance of *Ve* plants and cause disease only a few years after its introduction (Alexander, 1962; Robinson, 1957). Since then, resistance-overcoming *V. dahliae* strains have been assigned to race 2, whereas strains that remain to be contained by the *Ve*-locus have been assigned to race 1.

Considerable efforts have been made to identify race 2 resistance in tomato. Whereas most of these efforts have remained unsuccessful, the Japanese rootstock accession *Solanum lycopersicum* 'Aibou' was reported to display qualitative resistance against race 2 strains. This resistance was found to be based on a single dominantly inherited locus, *V2* (Usami et al., 2017). However, no *R* gene has been identified yet for this resistance. Importantly, the resistance governed by *V2* appears to be effective to only a subset of race 2 strains, as several strains were found to be able to cause severe foliar and vascular symptoms on these plants. Therefore, *V. dahliae* is presently classified into three races. According to this latest classification, strains which have overcome *Ve1* or *V2* resistance are classified as race 2 and race 3, respectively, while strains which are contained by *Ve1* and *V2* are classified as race 1. Whereas the *V. dahliae* avirulence effector that is recognized in Ve1 tomato plants was identified nearly a decade ago as VdAve1 (de Jonge et al., 2012), also the effector that corresponds to *V2* has recently been cloned as Av2 (Chavarro-Carrero et al., 2020), making that *V. dahliae* race assignment can presently be based on molecular markers.

To control race 3 strains and also to provide further resistance against *V. dahliae* in general, novel resistance sources are desirable. Except for *Ve1*-homologues in some crop species (Song et al., 2017), studies on *V. dahliae*-resistance in crops other than tomato typically report quantitative resistance based on multiple QTLs (Antanaviciute et al., 2015; Bolek et al., 2005; Rygulla et al., 2008; Toppino & Barchi, 2016; Wang et al., 2008; Zhao et al., 2014).

Previously, a wide collection of 57 wild tomato germplasm was screened for resistance against race 2 of *V. dahliae* (Yadeta, 2012). Some of these wild accessions displayed a reduction in *V. dahliae*-associated stunting symptoms (Yadeta, 2012). Here we report careful quantification of some of these resistances to a panel of *V. dahliae* strains, including race 3 strains. In view of this phenotyping and in view of the QTL analysis, we evaluated different parameters of the plant's response to the infection, searching the parameter that provided the highest statistical discriminative power between the accessions. Out of the evaluated parameters, relative canopy area appeared to show the highest discriminative power. The two accessions that showed the clearest levels and widest spectra of resistance were selected for further genetic analysis of the resistances, by means of development of recombinant inbred lines (RILs), followed by phenotyping, genotyping and QTL analysis. For one accession, no QTLs were found but for the other accession we mapped four QTLs. No major resistance genes were detected. We discuss possible causes for this scarcity of major resistance genes against *V. dahliae* in tomato and other host species.

2 | MATERIALS AND METHODS

2.1 | Tomato accessions

All tomato accessions (Table 1) were grown in potting soil (Potgrond 4, Horticoop, Katwijk, The Netherlands) in a greenhouse (Unifarm, Wageningen University & Research, the Netherlands) at 25°C/19°C (day/night) with 60% relative humidity and a minimal light intensity of 100 W/m^2. Day length was set at 16 h, night length at 8 h.

2.2 | Preparation of inoculum and inoculation procedures

For production of the inoculum for testing the 10 wild accessions with different strains (Table 2), two petri dishes with potato dextrose agar (PDA) were inoculated per *V. dahliae* strain from −80°C glycerol stocks. The fungus was grown on plates at 24°C for at least 5 days. Using these cultures, three fresh petri dishes with PDA were inoculated per *V. dahliae* strain and kept at 24°C for 5–7 days. Then, conidiospores were harvested from the PDA plates, washed and resuspended in tap water to a final concentration of 10^6 conidiospores per ml. Inoculations were carried out with the root dipping method as described by Fradin et al. (2009). Ten-day-old seedlings were carefully uprooted, and roots were washed in tap water. On average, for each tomato accession, 10 plants were root-dipped for 10 min in water and 10 other plants were root-dipped for 10 min in

TABLE 1 Tomato accessions used in this study

Species	Accession	Accession numbers as used by Yadeta (2022)
Solanum lycopersicum	Moneymaker	
Solanum lycopersicum	Moneymaker 35S:Ve1	
Solanum pimpinellifolium	G1.1554	VG-3
Solanum cheesmanii	G1.1615	VG-20
Solanum pimpinellifolium	G1.1596	VG-21
Solanum pimpinellifolium	G1.1310	VG-22
Solanum pimpinellifolium	G1.1594	VG-55
Solanum pimpinellifolium	G1.1914	VG-63
*Solanum lycopersicum*cv. Moneymaker × *Solanum pimpinellifolium* VG-3	RIL660 (F6)	
Solanum lycopersicum cv. Moneymaker × *Solanum pimpinellifolium* VG-3	RIL708 (F6)	

TABLE 2 Overview of all *Verticillium* strains used in this study

Species	Strain	Race	Country of origin	Originally isolated from	Genome sequenced?
Verticillium dahliae	JR2	1	Canada	*Solanum lycopersicum*	Yes
Verticillium dahliae	2009–650	1	Ukraine	*Capsicum annuum*	Yes
Verticillium dahliae	CBS38166	1	Canada	*Solanum lycopersicum*	Yes
Verticillium dahliae	Vd52	1	Austria	*Capsicum annuum*	Yes
Verticillium dahliae	St.1401	1	USA	*Pistacia vera*	Yes
Verticillium dahliae	CBS321.91	2	Netherlands	*Solanum lycopersicum* cv Criterium	No
Verticillium dahliae	DVDS29	3	Canada	*Soil*	Yes
Verticillium dahliae	VdLs17	3	California	*Lactuca sativa*	Yes
Verticillium dahliae	VPRI 42079	2	Australia	*Solanum lycopersicum*	No
Verticillium dahliae	DVD161	3	Canada	*Solanum tuberosum*	Yes
Verticillium dahliae	DVDS26	2	Canada	*Soil*	Yes
Verticillium dahliae	DVD3	3	Canada	*Solanum tuberosum*	Yes
Verticillium nonalfalfa	CBS451.88	2	Belgium	*Unknown*	No
Verticillium dahliae	GF1207	2	Japan	*Solanum lycopersicum* 'Momotaro Sunny'	Yes
Verticillium dahliae	HOMCF	3	Japan	*Solanum lycopersicum* 'Momotaro CF'	Yes

the inoculum. After inoculation, seedlings were placed in 14 cm pots and randomly distributed across the greenhouse compartment.

To produce the inoculum for screening the RIL populations, five petri dishes with PDA were inoculated with the strain from a −80°C glycerol stock. Agar blocks of ~1 cm^2 were cut from the five petri dishes with the fungal culture and transferred to freshly made potato sucrose broth that was produced by boiling 400 g of pre-peeled potato dices in 1 L of water for 15 min. The water in which the potatoes were boiled was then filtered through cheese cloth. Per L of broth, 1 L of water and 40 g of sucrose were added, followed by autoclaving for 30 min at 120°C. After inoculating the broth with the agar blocks, it was kept in an incubator for at least 5 days at 24°C at 100 rpm. Using Mira cloth, mycelium was filtered out of the broth. Subsequently, the inoculated broth was centrifuged at 3500 rpm for 30 min and the supernatant was removed. Next, the conidiospores were resuspended in tap water to a final concentration of 10^7 conidiospores/ml.

To inoculate the RIL populations, 12-day-old seedlings were carefully uprooted, and the roots were rinsed in tap water. Approximately six plants were mock-inoculated by root-dipping in water for 10 min, whereas six other plants were root-dipped for 10 min in the inoculum. After inoculation, seedlings were potted into trays of small cups containing sowing soil. From these trays, the cups were transferred to 14 cm pots according to a randomized scheme (Figure S2).

2.3 | Phenotyping

Different phenotypic traits were compared as parameter for the resistance levels: (1) The stem diameter (cm) just above the cotyledons measured with a digital calliper at 14- and 21-day postinoculation (dpi); (2) Plant height from the cotyledons upwards (cm); (3) Canopy diameter (cm); (4) Canopy area (cm^2) (Figure S1). The combined data from two separate experiments were used. In each experiment, at least four mock treated and 10 *V. dahliae*-treated plants were used. A total of 10 genotypes were included in the analysis. The individual plants were photographed using a Nikon D3200 DSLR camera that was fixed on a tripod. For image analysis, we used ImageJ version 1.51f (Schneider et al., 2012). For plant height, we used side view pictures, and for canopy diameter and canopy area, top view pictures were taken (Schneider et al., 2012); (5) *V. dahliae* biomass in infected plants, determined as described below.

Stunting was calculated as follows:

$$Stunting(\%) = \left(1 - \frac{canopy\ area\ of\ V.\ dahliae\ inoculated\ plant\ of\ genotype\ x}{average\ canopy\ area\ of\ mock\ treated\ plants\ of\ genotype\ x}\right) \times 100\%.$$

2.4 | Determination of *V. dahliae* biomass in infected plants

To determine the colonization of *V. dahliae* in inoculated plants, a stem fragment of 2–3 cm was harvested from the stem directly above the cotyledons at 22–23 dpi. Stem fragments were placed in 15-cm-long plastic tubes, which were stored in liquid nitrogen directly after harvest. The stem fragments were then freeze-dried for at least 24 h. After freeze-drying, stems were manually ground to a powder with a metal rod. Next, DNA was isolated from the stems with a modified version of the procedure from Pak et al. (1997). To determine the *V. dahliae* biomass, real-time PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad,

Veenendaal, The Netherlands) with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Veenendaal, The Netherlands). The real-time PCR programme consisted of an initial denaturation step of 10 min at 98°C followed by 41 cycles of 98°C for 15 s and 60°C for 1 min. After the 41 cycles, a stepwise melting curve analysis was performed from 65°C to 95°C with steps of 0.5°C and 5 s per step. The fungus-specific primer ITS1-F, which is based on the internal transcribed spacer region of the ribosomal DNA from Lievens et al. (2006), was used in combination with *V. dahliae*-specific reverse primer ST-Ve1-R to determine the amount of *V. dahliae* DNA in the DNA samples. To determine the amount of tomato DNA, primers designed on the tomato Elongation Factor 1α (Appiano, 2016) were used. Of each stem sample, two technical replicates were tested with the real-time PCR. Stem samples of which the two technical replicates displayed a difference greater than 1 Ct for the tomato amplicons were not further analysed. Relative levels of fungal biomass compared with the amount of host DNA were then determined with the 2-ΔΔCt method (Livak & Schmittgen, 2001). Statistical differences were determined based on the ΔΔCt values with a one-way ANOVA, followed by a Fishers' Least Significant Difference (LSD) test. Data points with studentized residuals below −2.5 or above 2.5 were considered as outliers and removed.

2.5 | Estimating the discriminative power of the resistance test

To estimate the discriminative power of the *V. dahliae*-associated symptoms of the 10 wild tomato accessions, a one-way ANOVA was performed on these measurements coming from two separate experiments. In this ANOVA, we tested for each symptom, per genotype, for significant differences between mock-inoculated and *V. dahliae*-inoculated plants. The experiment number was included in the analysis as a blocking factor. Where necessary, we performed a square-root or log10-transformation to guarantee the data met the normality and equality of variance assumptions. Outliers were detected based on the studentized residuals from the ANOVA analysis. Data points with studentized residuals below −2.5 or above 2.5 were considered as outliers and removed. To estimate the discriminative power of each *V. dahliae*-associated symptom, the *F*-value from the ANOVA of the interaction between genotype * treatment was used. This *F*-value reflects the significance of the difference of the effect of the *V. dahliae* inoculation on plant size between genotypes.

2.6 | Development of RIL-populations and genotyping

For the two out of the 10 wild accessions that showed the most promising levels of resistance, RIL populations were produced, aiming at unravelling the genetic bases of these resistances. To produce these RIL-populations, *Solanum pimpinellifolium* G1.1596 and *Solanum cheesmanii* G1.1615 were crossed with *S. lycopersicum* cv. 'Moneymaker' with the latter as the mother plant. After selfing of F_1 plants, seeds of the two F_2 populations were further selfed by single seed descent until the F_6 was reached. To genotype the RIL populations, leaves were harvested from the F_5 plants from which the F_6 seeds were harvested. To select SNP markers for the genotyping of the RIL populations, all markers from Sim et al. (2012) were tested on G1.1596, G1.1615 and 'Moneymaker'. Then, based on the tomato EXPMIMP 2009 genetic map (Ashrafi et al., 2009), the genetic positions of the markers were interpolated onto this map. Out of all 7688 SolCap-SNPs, 288 SNP markers from Sim et al. (2012) were selected for genotyping the G1.1615 RIL population and 192 SNPs for genotyping the G1.1596 population. The selected SNPs are distributed across the genome with intervals of on average 4 cM for the G1.1615 population and 6 cM for the G1.1596 population.

2.7 | Marker validation and construction of the genetic maps

Before constructing the genetic map, all markers were first inspected. Markers of which the genotyping resulted in no data or only heterozygous data for all genotypes were removed. Next, using the R-packages R/QTL and AsMAP (Taylor & Butler, 2017), markers were validated by checking for (1) unusually skewed segregation, (2) >10% of missing data, (3) identical genetic positions but very different physical positions and (4) unusually high crossover rates between physically closely located markers. Markers which were considered as unreliable based on these criteria, 25 in total, were removed. Based on the 263 and 188 remaining markers for G1.1615 and G1.1596, respectively, a genetic map was made. Using the 'MSTmap' function of the R package ASMap (Taylor & Butler, 2017), linkage groups were constructed for each chromosome with a *p*-value of 10^{-4} with the options 'anchor' and 'byChr' enabled. The physical order and genetic order were evaluated. If the genetic order was not in agreement with the physical order, the likelihood of both marker orders was estimated using the 'ripple' function of the package R/QTL version 1.45-6 (Broman et al., 2003). If the genetic order and physical order of the markers were equally likely, we favoured the physical order of the markers.

For the mapping, we used of the G1.1596 RIL population 96 RILs in the first experiment and 92 RILs in the second experiment. Of the G1.1615 population, we used 90 RILs in the first experiment and 87 RILs in the second experiment.

2.8 | Randomizing RILs in the greenhouse

Each RIL population was screened twice for resistance in consecutive experiments, leading to two experiments. For each RIL, we used per experiment on average six inoculated plants and six mock-treated plants. The replicate plants of each RIL were randomized across the greenhouse compartment in six blocks. The randomization was performed in such a way that in each block, every genotype was

represented by one replicate mock-inoculated and one replicate *V. dahliae*-inoculated plant immediately adjacent to each other (Figure S2). The parental plants and other control plants were randomly assigned to the blocks.

2.9 | Phenotyping of the RIL populations

Plants of the RIL populations were phenotyped at 24 or 25 days after inoculation. Experimental blocks 1 to 3 were phenotyped at 24 dpi and blocks 4 to 6 at 25 dpi. To detect outliers, a one-way ANOVA was performed for differences in stunting. Data points with studentized residuals below −2.5 or above 2.5 were considered as outliers and removed.

The broad-sense heritability was estimated from the output of a one-way ANOVA, using:

$$\textit{Estimated broad sense heritability}\left(H^2\right)=\frac{\sigma_g^2}{\sigma_g^2+\sigma_e^2}$$

whereas

$$\sigma_e^2=\textit{Mean Squares}_{residual}$$

and

$$\sigma_g^2=\frac{\textit{Mean Squares}_{genotype}-\sigma_e^2}{\textit{average number of replicates per genotype}}$$

2.10 | QTL analysis in R

We performed QTL analyses with version 1.45-6 of the R package R/QTL (Broman et al., 2003). The QTL analysis consisted of three steps. First, an interval mapping analysis was performed using the 'em' algorithm in the function 'scanone'. A permutation test with 1000 permutations was then performed with the 'scanone' function to determine the significance threshold ($p < .05$). For the QTL analysis, two covariates were added. We used experiment as first covariate. As a second covariate, the average canopy area of mock-inoculated plants of each RIL was included. After the 'scanone' analysis, a two-dimensional QTL scan called 'scantwo' was performed, again using the 'em' algorithm. Also, in this case, a permutation test was performed. Due to the high amount of computational time required for this permutation test, the permutations were divided into 10 batches using the 'n.batch' option and were performed on a Linux-based server cluster. As third step, all putative QTLs found with 'scanone' and 'scantwo' were combined into a multiple QTL model using the function 'makeqtl', followed by 'fitqtl', and refined with the 'refineqtl' function. Using the function 'stepwiseqtl', the most likely multiple QTL models were evaluated. Based on this evaluation, a final multiple QTL model was constructed using 'makeQTL', 'fitQTL' and 'refineqtl', which was then plotted with the function 'plotLODprofile'. The significance threshold for the stepwiseqtl analysis was determined using the permutation test of 'scantwo' with the 'imp' algorithm. Supporting information S1 contains the R-scripts.

3 | RESULTS

3.1 | Canopy area at three weeks after inoculation provides the highest discriminative power to distinguish resistance level differences

To explore tomato germplasm for novel sources of resistance against race 2 and 3 *V. dahliae* strains, we compared phenotyping methods that have been used by others to score Verticillium wilt disease. This phenotyping method should provide the best discriminative power to optimally detect differences in *V. dahliae* symptoms between host genotypes. This discriminative power can be estimated with a one-way ANOVA. Using a panel of 10 tomato genotypes (Table 1), we compared the discriminative power of multiple phenotyping methods. Based on our earlier observations in greenhouse trials with *V. dahliae*-inoculated tomato plants, for instance, the trials described by Fradin et al. (2009) and Yadeta (2012), we know that symptoms start to develop around 10 dpi, after which they aggravate.

Taking the practicalities of large-scale screenings in mind, in which we prefer to terminate experiments three weeks after inoculation, we decided to focus the phenotyping efforts on 14 and 21 dpi. Initially, we aimed to assess both the scoring of *V. dahliae*-induced foliar yellowing symptoms and plant size-related Verticillium wilt symptoms. Unfortunately, some of the tomato genotypes, especially *S. cheesmanii* G1.1615, developed spontaneous yellowing of the lower leaves in mock-inoculated plants. Simultaneously, several plants of the susceptible 'Moneymaker' did not develop apparent yellowing symptoms upon inoculation with *V. dahliae* strain DVDS26 (Figure S1). Therefore, we concluded that yellowing could not be used as a symptom that is consistently associated with Verticillium wilt disease under our experimental conditions. Therefore, we continued with measurements of the stem diameter, canopy diameter, canopy area and plant height on mock-inoculated and *V. dahliae*-inoculated plants. Of these parameters, the canopy area of inoculated plants relatively to mock-treated plants displayed the highest discriminative power both at 14- and 21-day postinoculation. Moreover, the discriminative power of the canopy area at 21 dpi was higher than at 14 dpi (Figure 1).

Given the typically relatively large degree of variation in *V. dahliae* symptoms among plants of the same genotype, we also assessed the effect of the removal of outliers on the discriminative power of each of the symptoms. Removal of outliers with studentized residuals below −2.5 or above 2.5 (Table S4) yielded a clear effect on the canopy area and canopy diameter, as it increased the discriminative power both at 14 and 21 dpi. At 14 dpi, outlier removal also resulted in a slight increase in the discriminative power of plant height and stem diameter. Overall, our analysis demonstrates that canopy area

FIGURE 1 Estimated discriminative power of canopy area, canopy diameter, plant height and stem diameter at 14 and 21 days after inoculation with *Verticillium dahliae* DVDS26, based on the complete dataset and on the dataset from which outliers were or were not removed based on the studentized residuals (Table S4). The complete dataset consisted of two separate experiments, consisting of10 genotypes. In each experiment, at least four mock-inoculated and 10 *V. dahliae*-inoculated plants per genotype were tested. Discriminative power was estimated with a one-way ANOVA, of which the *F*-value of the interaction genotype * treatment was used as the value for discriminative power.

measurements at 21 dpi provided the highest discriminative power to distinguish resistant from susceptible genotypes and that outlier removal helped to further improve the discriminative power of the resistance test. Therefore, we choose stunting, based on measurements of canopy area, as parameter for our subsequent resistance tests. The equation for stunting is shown in the paragraph Phenotyping in the Section 2.

3.2 | Optimization of the inoculation procedure

To assess whether the scoring of canopy area at 21 dpi as a phenotyping measure could be optimized further, an attempt was made to optimize the inoculation method. To do this, the two wild tomato accessions *S. pimpinellifolium* G1.1596 and *S. cheesmanii* G1.1615 that were previously found to display a relatively high degree of resistance against *V. dahliae* race 2 by Yadeta (2012) were selected together with the susceptible control 'Moneymaker'. The effects of three modifications to the standard root-dipping inoculation method described by Yadeta (2012) on the discriminative power of the disease test were evaluated. First, the effect of the inoculum concentration was tested by increasing the conidiospore concentration from 10^6 to 10^7 conidiospores/ml (Fradin et al., 2009; Jiménez-Díaz et al., 2017; Parisi et al., 2016; Tsolakidou et al., 2019). Secondly, the addition of nutrients twice a week after the second week postsowing was assessed. Finally, trimming of the roots before inoculation was tested as this has been suggested to promote *V. dahliae* infection (Papadaki et al., 2017; Parisi et al., 2016).

Compared with the large differences in discriminative power between tests with different phenotyping methods (Figure 1), smaller differences in discriminative power were detected when different inoculation methods were compared (Figure 2). The discriminating power did not improve clearly when increasing the conidiospore concentration (Figure 2). Furthermore, while the addition of nutrients significantly increased the canopy area of mock-treated plants of all

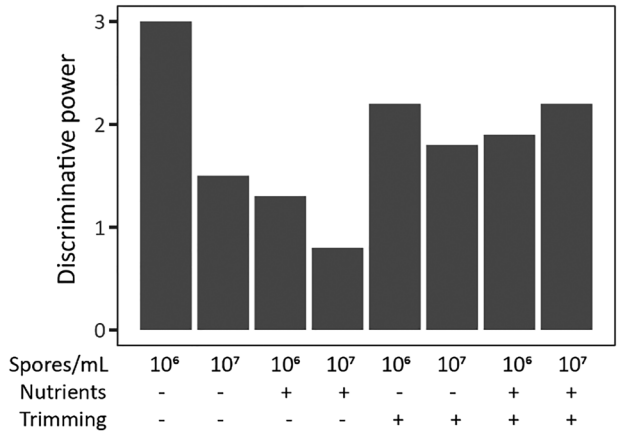

FIGURE 2 Estimated discriminative power of canopy area at 21 days after inoculation with *Verticillium dahliae* DVDS26. Outliers were removed based on the studentized residuals (Table S5). Discriminative power was estimated with a one-way ANOVA, of which the *F*-value of the interaction between the genotype and the treatment.

genotypes, we found that the addition of nutrients had no significant effect on *V. dahliae*-induced stunting for any of the genotypes (Figure S4). Trimming had variable effects on the discriminative power (Figure 2). The highest discriminative power was obtained when using a spore concentration of 10^6 conidiospores/ml, without adding nutrients and without trimming (Figure 2).

3.3 | Wild tomato accessions displayed reduced stunting upon inoculation with two *V. dahliae* race 2 strains

To confirm and further study the previously described resistances (Yadeta, 2012), we selected six wild tomato accessions (Table 1) which

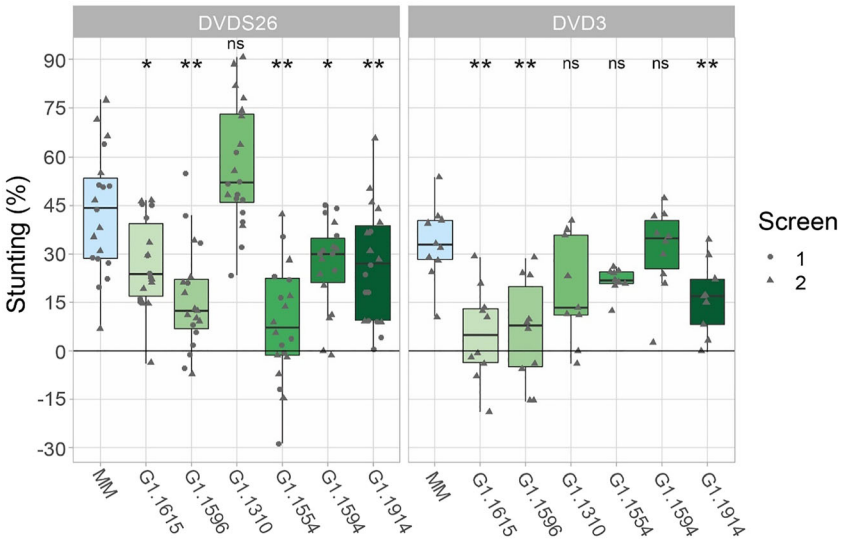

FIGURE 3 Stunting of wild tomato accessions challenged with *Verticillium dahliae* race 3 and 2 strains DVD3 and DVDS26 at 21 days postinoculation. 'MM' represents the susceptible control *Solanum lycopersicum* 'Moneymaker'. G1.1615 is of the species *Solanum cheesmanii*, the remaining GI-numbers refer to wild accessions of the species *Solanum pimpinellifolium*. Asterisks indicate significant differences in stunting compared with 'Moneymaker'; *$p < .01$, **$p < .005$. Significant differences were determined with a one-way ANOVA followed by a fishers LSD test.

previously displayed a relatively low level of *V. dahliae*-induced wilting and chlorosis and challenged these accessions with the race 2 *V. dahliae* strain DVDS26 and race 3 strain DVD3. At 21-day postinoculation, we measured the stunting of *V. dahliae*-inoculated plants. Compared with the susceptible control 'Moneymaker', all six wild tomato accessions, except for accession G1.1310, displayed a reduction in stunting symptoms when challenged with *V. dahliae* DVDS26 (Figure 3). When challenged with *V. dahliae* DVD3, only G1.1615, G1.1596 and G1.1914 displayed reduced stunting. Overall, G1.1615 and G1.1596 showed the best resistance levels.

3.4 | Wild tomato accessions G1.1596 and G1.1615 displayed reduced stunting upon inoculation with a phylogenetically diverse set of *V. dahliae* strains

In view of the promising results of G1.1596 and G1.1615 (Figure 3), these accessions were selected for assessment of their resistance spectra when being confronted with a diverse set of *V. dahliae* strains. To be able to make a phylogenetically diverse selection of strains, we mostly selected strains of which the genome was sequenced and for which a phylogenetic tree was available. Where possible, at least one tomato-infecting race 2 or 3 strain was selected from each major branch of the phylogenetic tree (Figure S3). As none of the strains in the lowest major branch of the tree are pathogenic on tomato, no strains were selected from this branch. An additional *V. dahliae* strain from Australia and the Netherlands, and one *Verticillium nonalfalfa* strain from Belgium was included (Table 2).

Compared with 'Moneymaker', both wild accessions displayed reduced stunting symptoms when challenged with the *V. dahliae* strains (Figure 4a). Similarly, when challenged with the *V. nonalfalfa* race 2 strain CBS451.88, the accessions displayed a reduction in symptoms. To further test the response of the wild accessions to *V. dahliae* race 1 strains, we selected four *V. dahliae* race 1 strains from different branches of the phylogenetic tree (Figure S3). Compared with 'Moneymaker', the two wild accessions displayed reduced stunting when challenged with these four race 1 strains, except for G1.1615, which displayed no reduced stunting when challenged with *V. dahliae* strain Vd52 (Figure 4b). Finally, we challenged the wild accessions with a race 2 and a race 3 strain of *V. dahliae* from Japan (Usami et al., 2017), further demonstrating that the resistances are also effective against these race 3 strains of *V. dahliae* (Figure 4c). Overall, our analysis demonstrates that, compared with 'Moneymaker', the wild accessions G1.1615 and G1.1596 displayed reduced susceptibility towards a diversity of *V. dahliae* strains.

3.5 | G1.1596 and G1.1615 reduced the colonization of a diverse set of *V. dahliae* strains

To confirm the findings of Yadeta (2012) that G1.1596 and G1.1615 indeed restrict the colonization of *V. dahliae* and not solely reduce the damage from the *V. dahliae* infection, the colonization of *V. dahliae* was measured with real-time PCR. Analogous to the reduced stunting of G1.1596 and G1.1615, these accessions also displayed reduced colonization of the race 1 strain JR2, the race 2 strain DVDS26 and the race 3 strains DVDS29, DVD3 and HOMCF (Figure 5). This confirms that these accessions indeed possess broad-spectrum, race non-specific resistance against *V. dahliae*.

3.6 | No QTLs could be identified for the resistance from *S. pimpinellifolium* G1.1596

To study the genetics of the *V. dahliae* wilt resistance of *S. pimpinellifolium* G1.1596, we developed an F_6 RIL-population from a cross with the susceptible *S. lycopersicum* cultivar 'Moneymaker'. The SNP marker data for this RIL population are shown in supporting information S2 and S3. Based on Figure 4, we selected the *V. dahliae* strain to which (1) the accession displayed both a reduction in stunting symptoms and

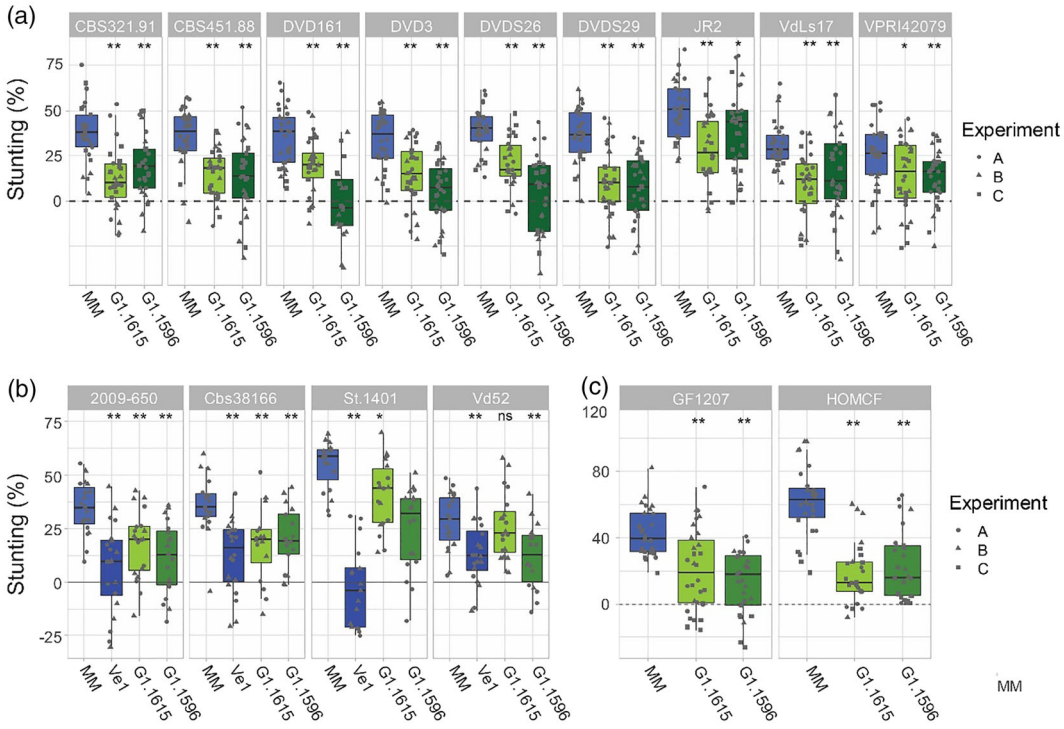

FIGURE 4 Infection screens with a phylogenetically diverse set of *Verticillium* strains. 'MM' refers to *Solanum lycopersicum* 'Moneymaker'. G1.1615 is of the species *Solanum cheesmanii*, G1.1596 belongs to *Solanum pimpinellifolium*. CBS451.88 is *V. nonalfalfa*; the other strains belong to *V. dahliae*. JR2, 2009-650, CBS38166, Vd52 and St.1401 are race 1 strains, HOMCF, DVD3, DVDS29, DVD161 and VdLs17 are race 3 strains, while the remaining strains are race 2 strains. The *Y*-axis indicates the stunting (%) of inoculated plants at 21 dpi. Panels a–c display results from three separate experiments where different shapes of the scattered dots represent separate experiments. Asterisks indicate significant differences in stunting when compared with the stunting of 'Moneymaker'; $*p < .05$, $**p < .005$. Significances were determined in R using a one-way ANOVA followed by a Fishers' LSD test.

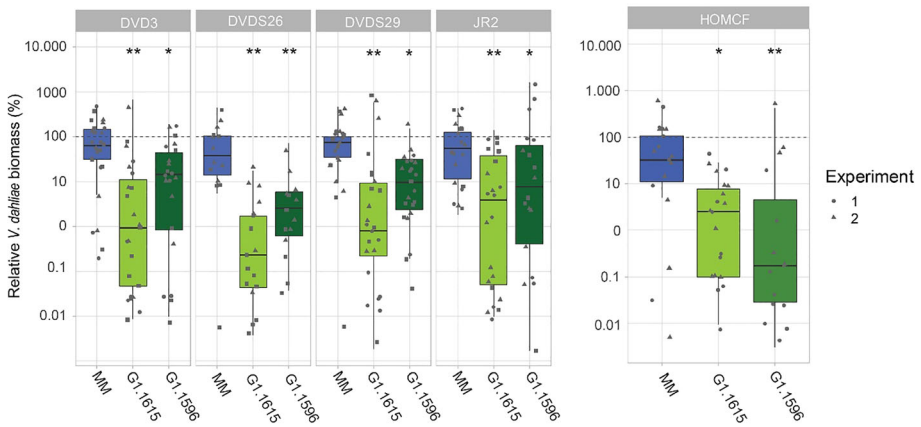

FIGURE 5 Biomass of *Verticillium dahliae* in wild tomato accessions G1.1615 and G1.1596 relative to the biomass of *V. dahliae* in *Solanum lycopersicum* 'Moneymaker' (MM). G1.1615 is of the species *Solanum cheesmanii*, G1.1596 of the species *Solanum pimpinellifolium*. Biomass was determined in lower parts of stems of the plants of which the stunting symptoms are displayed in Figure 3a,c. Biomass of *V. dahliae* DVD3 and DVDS29 was determined in all three repeat screenings, while the biomass of *V. dahliae* DVDS26, JR2, HOMCF and VdLs17 was determined in two repeat screenings. Asterisks indicate significant differences when compared with 'Moneymaker'. Significance levels were determined by a one-way ANOVA followed by a fishers LSD; $*p < .05$, $**p < .005$. Numbers under each boxplot indicate the average relative *V. dahliae* biomass when compared with 'Moneymaker'.

a reduction in pathogen biomass and (2) that provided a clear contrast in stunting symptoms between the susceptible and resistant parent. For G1.1596, this was *V. dahliae* strain DVDS29. The RILs were phenotyped in a climate-controlled greenhouse. To increase the accuracy of phenotyping, we used on average 12 plants for each genotype, half of which were *V. dahliae*-inoculated while the other half were

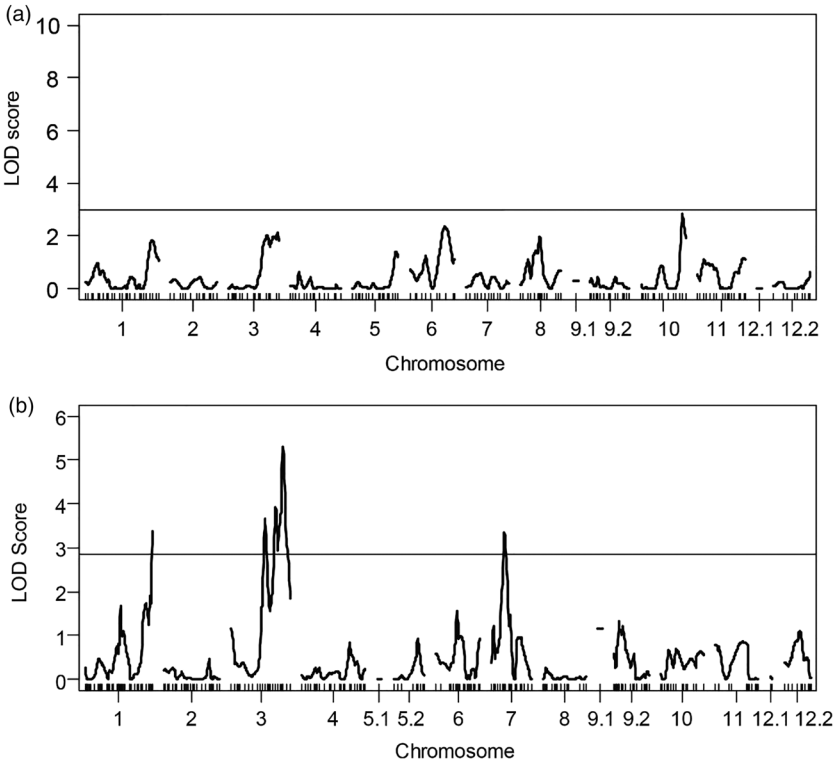

FIGURE 6 LOD-profiles from the interval mapping based on the stunting phenotype in (a) the *Solanum lycopersicum* 'Moneymaker' × *Solanum pimpinellifolium* G1.1596 RIL population (96 and 92 RILs) and (b) the 'Moneymaker' × *Solanum cheesmanii* G1.1615 RIL population (90 and 87 RILs). QTLs are based on the phenotypes of on average 12 pairs of inoculated and mock-treated plants, merged from two consecutive experiments. The horizontal line represents the significance threshold ($p < .05$), determined with a permutation test (1000 permutations).

mock-inoculated. The phenotyping was performed during two experiments, harbouring on average six mock-inoculated and six *V. dahliae*-inoculated replicates per experiment. To furthermore attempt to reduce phenotypic variation, plants were randomized across six blocks in pairs of mock-inoculated and *V. dahliae*-inoculated plants.

The *V. dahliae*-induced stunting in the 'Moneymaker' × G1.1596 RIL population did not show a clear segregation into separated groups (Figure S5). In the two experiments, 'Moneymaker' grouped with 38.2% and 43.6% stunting among the RILs with the highest degree of stunting and G1.1596 grouped with 7.9% stunting and 16.7% stunting among the RILs with the lowest degree of stunting. The F1 displayed an intermediate level of stunting.

The reproducibility of the symptoms was low. Several RILs displayed large differences in symptoms between the two experiments. Removal of these RILs (Figure S6) from the dataset resulted in an increase of the reproducibility of $R^2 = 0.08$ to $R^2 = 0.17$. The estimated heritability of the stunting of *V. dahliae*-inoculated plants in this population was $H^2 = 0.10$ (Table S2), indicating that most of the variance in stunting was based on non-genetic factors. For this RIL population, we did not find any evidence that variation in canopy area of mock-inoculated plants affected the degree of stunting (Figure S7). We therefore did not include canopy area of mock-inoculated plants as a covariate in our analysis. No QTLs for stunting of *V. dahliae*-inoculated plants could be identified in this population, neither based on the individual screens nor on the phenotypes of both screens combined (Figure 6a). Very likely, *S. pimpinellifolium* G1.1596 does not harbour genes with a major effect on resistance to the used *V. dahliae* strain, in spite of its promising resistance level (Figures 3–5). The low heritability (Table S2) indicates that the genetic resistance is obscured by the environmental variation, in spite of the high number of replicates per RIL aiming at more precise estimations of the resistances of the individual RILs, the controlled glasshouse environment, the careful randomization approach, the paired positioning of inoculated and mock-treated plants of the same RIL within a block and the accurate canopy area measurements. This is underlined by the low reproducibility between the two consecutive experiments (Figure S6).

3.7 | G1.1615 possesses QTLs for resistance against *V. dahliae* race 3 strain DVDS29

Similarly as for *S. pimpinellifolium* G1.1596, RIL populations were developed for *S. cheesmanii* G1.1615. The phenotyping was performed in a similar fashion. Significant differences were observed in the canopy area between the blocks in both experiments (Figure S8a, c). However, no differences in the degree of stunting, that is, the canopy area ratios for pairs of mock-inoculated and *V. dahliae*-inoculated plants, were observed between the blocks (Figure S8b,d). This suggests that the paired randomization design likely reduced variability in stunting between the experimental blocks.

'Moneymaker' grouped among the most susceptible genotypes that displayed the highest degree of stunting in both phenotyping screens (45.8% and 47.45%), while the F_1 and G1.1615 grouped among the resistant genotypes with the lowest degree of stunting (9.18%, 16.5% stunting for G1.1615, 0% and 9.5% stunting for F_1) (Figure S9), confirming the previously found resistance of G1.1615. The low degree of stunting of the F_1 in both screens suggests a dominant inheritance of the resistance. In the G1.1615 population, we also

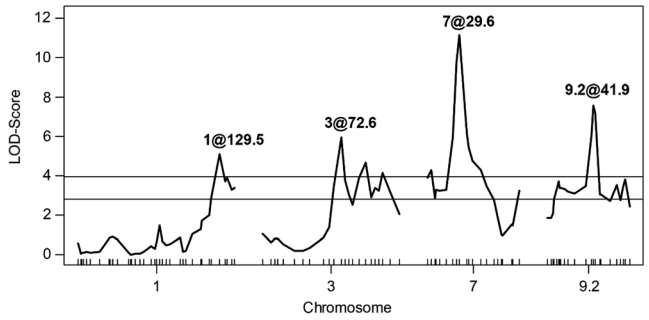

FIGURE 7 The four most likely QTLs for *Verticillium dahliae* resistance in the 'Moneymaker' × G1.1615 RIL population. The most likely QTLs were determined with a QTL analysis in R using the package R/QTL. The positions in this figure were estimated using 'fitqtl' with the inclusion of the canopy area of mock-inoculated plants as a covariate. The *Y*-axis shows the LOD-scores, the *X*-axis the position of the LOD-profiles. The lower horizontal black line represents the main-effect penalized LOD-threshold, the upper horizontal black line represents the strict interaction penalized LOD-threshold, determined with a two-dimensional permutation test (1000 permutations). The red horizontal line at ch01 indicates the position of the QTL for mock canopy area.

observed a continuum of stunting levels between the most susceptible and the most resistant plants without a clear separation of RILs in susceptible and resistant groups. Removing eight RILs with large differences between the two experiments (Figure S10) increased the reproducibility from 0.10 to $R^2 = 0.26$. Therefore, these eight RILs were not included in the QTL analysis. Besides the low reproducibility, also, the estimated broad-sense heritability was low, ranging between $H^2 = 0.21$ and $H^2 = 0.16$ (Table S3) for the first and second experiment, respectively. This indicates that a large degree of the variation in stunting of *V. dahliae*-inoculated plants was due to non-genetic factors. Removal of the eight previously mentioned RILs did not considerably improve the estimated heritability.

For detection of QTLs for resistance against *V. dahliae* DVDS29 in the G1.1615 RIL population, we used both interval mapping and a two-dimensional two-QTL scan from the R package R/QTL. However, based on the phenotypes of the two individual experiments in the greenhouse, we could not identify significant or reproducible QTLs for stunting of *V. dahliae*-inoculated plants. Therefore, we combined the phenotypic observations from both experiments in one overall QTL analysis, indicating the two experiments with a covariate. Based on the correlation between the canopy area of mock-treated plants and stunting of inoculated plants (Figure S11), we included the canopy area of mock-inoculated plants as a second covariate. First, a simple interval mapping analysis was performed, which provided evidence for QTLs on ch01, ch03 and ch07 (Figure 6b). To further search for QTLs, also taking into account interactions between QTLs, and to determine which combination of QTLs is best supported by our data, we performed several rounds of a stepwise selection method called 'stepwiseqtl' from the package R/QTL. Based on this analysis, we determined that a model consisting of the QTLs on ch01, ch03, ch07 and ch09 is best supported by the data (Figure 7 and Table 3).

The QTLs on ch01 and ch07 were provided by G1.1615, together explaining 26.6% of the phenotypic variance (Table 3). Interestingly, the other two QTLs were contributed by 'Moneymaker', collectively explaining 21.5% of the phenotypic variance. The included covariates together explained more of the phenotypic variance than the QTLs from 'Moneymaker', namely, 16.1%, with 11.1% for mock canopy area and 5% for the experiment number.

3.8 | Two QTLs from G1.1615 and two QTLs from moneymaker caused reduced stunting symptoms

To assess how well the QTLs on ch01 and ch07 explain the resistance of G1.1615 to *V. dahliae* DVDS29, we evaluated their effects on the stunting of *V. dahliae*-inoculated plants. As expected, RILs with the G1.1615 allele of either the QTL on ch01 or ch07 displayed a reduction in stunting compared with RILs with the 'Moneymaker' allele for both QTLs (Figure 8a). Although RILs with the G1.1615 allele of the QTLs on ch01 and ch07 were less stunted than RILs with the MM alleles, they were still more stunted than the G1.1615 parent itself (Figure 8a). This suggests that these QTLs do not fully account for the resistance of G1.1615. Similarly, RILs which possessed of the QTLs on ch01 and ch07 the 'Moneymaker' allele were less stunted than 'Moneymaker' (27.9% stunting versus 45% stunting) (Figure 8a).

Of the QTLs from 'Moneymaker', the QTL on ch03 and the QTL on ch09 appear to act additively, as RILs which possess of both QTLs the MM allele displayed the lowest level of stunting, whereas RILs which possessed of both QTLs the G1.1615 allele displayed the highest level of stunting (Figure 8b). Overall, we thus conclude that two QTLs from G1.1615 and two QTLs from 'Moneymaker' reduce the stunting of RILs challenged with the *V. dahliae* race 3 strain DVDS29.

4 | DISCUSSION

From a series of wild tomato accessions with resistance to *V. dahliae* (Yadeta, 2012), we selected two of the most promising genotypes, showing resistance to a broad spectrum of resistance against race 2 and 3 strains, using a carefully optimized resistance test, based on the canopy area of plants and image analysis. These two rather resistant genotypes were crossed with the susceptible reference 'Moneymaker', and RIL populations were developed. In order to minimize 'environmental noise', we phenotyped the two RIL populations in a climate-controlled greenhouse, using per RIL on average 12 pairs of inoculated and mock-treated plants, separated among two consecutive experiments. We regard this as a high number of replicates for the RIL populations. Further, we used the race 3 strain that provided the highest contrast in stunting symptoms between the resistant parents *S. pimpinellifolium* G1.1596 and *S. cheesmanii* G1.1615 at the one hand and the susceptible cultivar 'Moneymaker' at the other hand. In spite of these efforts, we did not detect any QTL for the G1.1596 RIL population. For the G1.1615 population, we did not find QTLs in the two separate experiments with six replicates per experiments. Only

TABLE 3 The most likely QTL models for resistance against *Verticillium dahliae* DVDS29 in the 'Moneymaker' × G1.1615 RIL population, based on the QTL analysis performed in R

Ch	Position (cM)	LOD score	% explained variance	Donor of resistance/ tolerance	LOD 1.5 interval (cM)[1]	LOD 1.5 interval (Mb)[1]	Number of annotated genes in interval
1	129.5	5.7	7.9	G1.1615	122.3–129.5	93.5–95	~220
3	72.6	5.9	9.4	Moneymaker	65.2–116.9	55.5–65.9	~870
7	29.6	11.1	18.7	G1.1615	23.5–36.4	3.8–56.6	~870
9.2	41.9	7.5	12.1	Moneymaker	40.6–48.7	66.7–68.37	~220

Note: The QTL analysis was performed with the combined data from two experiments. The QTLs for resistance (reduced stunting) were estimated using 'stepwiseqtl' the inclusion of the canopy area of mock-inoculated plants and the experiment number as a cofactor. For estimation of the number of genes, we used the physical positions of the flanking markers of the QTLs (LOD-1.5 interval) on the reference genome of *Solanum lycopersicum* 'Heinz' SL2.50. Gene annotations were based on the ITAG annotations version 2.5.

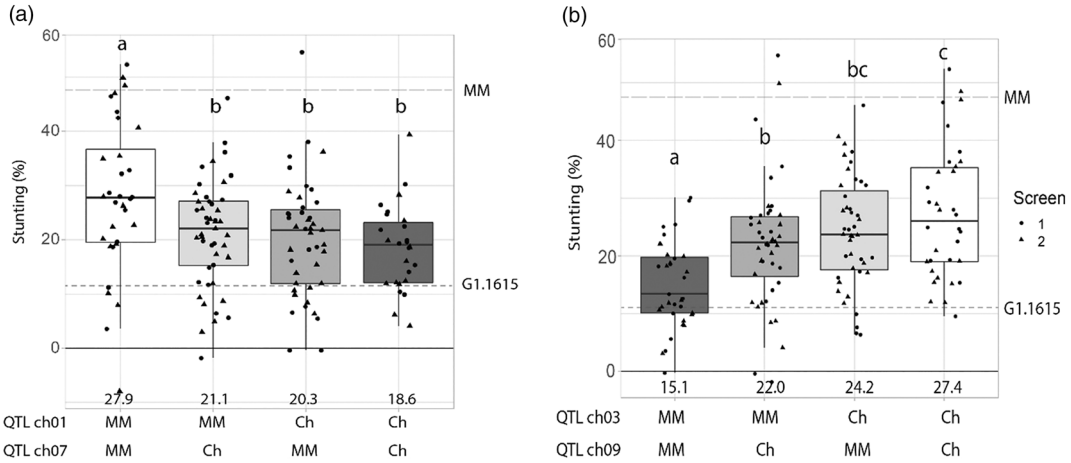

FIGURE 8 Effects of the QTLs contributed by *Solanum cheesmanii* G1.1615 (Ch) (a) and *Solanum lycopersicum* 'Moneymaker' (MM) (b) on the average stunting of *Verticillium dahliae*-inoculated plants (*y*-axis). To estimate this effect, all RILs were grouped based on their alleles of either the QTLs on ch01 and ch07 (a) or the QTLs on ch03 and ch09 (b). (a) Effects of the QTLs from by *S. cheesmanii* G1.1615 on ch01 and ch07 on stunting. The stunting of G1.1615 itself (dashed line) is lower than the stunting for the combined QTLs; (b) effects of the QTLs from *S. lycopersicum* 'Moneymaker' (MM) on ch03 and ch09 on stunting. The horizontal dashed line with large dashes represents the average stunting of 'Moneymaker' (MM). Numbers under each boxplot indicate the average stunting of each group of RILs. Boxplots with different letter labels are significantly different according to a one-way ANOVA, followed by a Fisher's LSD test ($p < .01$).

after combining the two experiments, leading to 12 replicates per RIL, and including two co-factors, we were able to find two rather weak QTLs for resistance from G1.1615 and two other QTLs for some resistance, contributed by the susceptible reference 'Moneymaker'. This may indicate that 'Moneymaker' also possesses some basal defence against *V. dahliae*, which has a different genetic basis than the resistance of G1.1615. However, we were not able to prove reproducibility of these QTLs among the two experiments.

From these observations, we draw two main conclusions: The first main conclusion is that although 'Moneymaker' has been regarded as the susceptible control in numerous experiments, it still harbours some loci for weak resistance or tolerance against *V. dahliae*. The second, and even more important conclusion is that although we selected from a larger set of accessions the most promising genotypes, we still did not find major genes for resistance, nor strong-effect QTLs. This indicates a serious scarcity of major resistance QTLs against *V. dahliae* in tomato. This observation is not limited to tomato. A series of QTL studies in other hosts of *Verticillium* did not result in the identification of major dominant genes either (Antanaviciute et al., 2015; Rygulla et al., 2008; Toppino & Barchi, 2016; Wang et al., 2008; Zhao et al., 2014). Apparently, this is a common pattern for this pathogen. Only two major resistance genes against *V. dahliae* have been found in tomato thus far, that is, *Ve1* (Fradin et al., 2009; Kawchuk et al., 2001) and *V2* (Usami et al., 2017), both in tomato. All the major resistance genes found in other crops are homologues of *Ve1*, such *SlVe1* in *Solanum lycopersicoides*, *StVe1* in potato, *StVe1* and *StoVe1* in *Solanum torvum* and *Vr1* in lettuce (Song et al., 2017). Finally, it is not unlikely that the majority of the studies for genetic mapping of resistance to *V. dahliae* have not been published, due to poor results and absence of major effect QTLs. As already in 1919, van der Lek advocated to search and breed for *V. dahliae* resistance; the question emerges why so few major resistances have been found in over a century, in spite of numerous studies in many crops by breeding companies and research organizations.

Soilborne *Verticillium* fungi collectively cause disease in at least 400 crops (Berlanger & Powelson, 2000). However, reports of *V. dahliae* infestations and disease development in natural ecosystems are rare. Reports of Verticillium wilts are mainly confined to agricultural settings. Given the observation that even in agricultural settings *V. dahliae* is encountered not only in pathogenic but also in endophytic populations (Wheeler et al., 2019), it is tempting to speculate that *V. dahliae* in natural ecosystems mainly occurs as endophyte in root systems of numerous plant species (Malcolm et al., 2013). In such endophytic interactions, it may occur in the xylem of host plants, without causing serious damage, whereas in agricultural settings, lineages may have evolved that specialized on monocultures of a limited set of genotypes where they were happened to cause disease due to more extensive proliferation. In view of a relatively low level of damage in natural vegetations, there may not have been sufficient evolutionary pressure to evolve recognition specificities to direct immune responses and thus evolve resistance genes, against this endophyte. The endophyte hypothesis is supported by the lack of clear specialization of the fungus to particular host species. The fungus has a very wide host range (Berlanger & Powelson, 2000; Fradin & Thomma, 2006) and did not develop clear pathotypes or *formae speciales*, underlining the lack of clear specialization to specific hosts. This contrasts with the vascular wilt fungus *Fusarium oxysporum*, which adopts a highly similar lifestyle as *V. dahliae*. Whereas as a species *F. oxysporum* has a host range that is probably as diverse as that of *V. dahliae*, individual strains are characterized by narrow host ranges and are therefore grouped in *formae speciales*. Intriguingly, several major resistance sources against *F. oxysporum* have been identified and cloned from tomato. Thus, although largely speculative, we hypothesize that a main reason for the scarcity of major resistances against *V. dahliae* may be the relatively low damage of this pathogen to its hosts under natural circumstances, resulting in the absence of evolutionary pressure to direct immune responses against this organism.

However, *V. dahliae* can still have some tendencies for pathogenicity in nature, as supported by the presence of the major resistance genes *Ve1* and *V2* in tomato and corresponding virulence factors (Chavarro-Carrero et al., 2020; Fradin et al., 2009). We hypothesize that the agricultural settings associated with monocultures, a possibly lowered basal immunity against pathogenic microbes and a lowered biodiversity of the microbiome in soils and plants, may lead to more extensive colonization by *V. dahliae* in these plants, leading to adverse effects including symptoms of disease. Thus, in agricultural systems, the fungus may be more aggressive towards host plants, leading to more damage.

We conclude that there is a scarcity of major resistance genes against *V. dahliae*. We hypothesize that this is caused by the endophytic behaviour of this fungus in nature, with very limited levels of damage to its hosts in natural vegetations. Although *V. dahliae* is not very offensive to specific plant species in nature, it can still survive, thanks to its wide host range and lack of clear specialization. The low levels of damage may have prevented the need of development of major resistance genes. However, *V. dahliae* can be pathogenic to some extent, as supported by the presence of two major resistance genes in tomato (*Ve1*, *V2*). Probably, agricultural circumstances can enforce this pathogenic behaviour of the fungus.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

CODE AVAILABILITY

The R script used in our QTL analysis is available in the supporting information S1.

AUTHOR CONTRIBUTIONS

HJS, BT and YB conceived the study. JV and KH designed and performed the experiments and analysed data. JV, HJS, KH, BT and YB wrote the manuscript. All authors read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The SNP marker data of both RIL population are available in the supporting information S1 and S2.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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