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ORIGINAL RESEARCH ARTICLE

A muscadine locus confers resistance to predominant species of grapevine root-knot nematodes (*Meloidogyne* spp.) including virulent populations

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ABSTRACT

Root-knot nematodes (RKNs) *Meloidogyne* spp. are extremely polyphagous pests and four species severely affect grapevines throughout the world: *M. arenaria*, *M. incognita*, *M. javanica* and *M. ethiopica*. Californian populations of *M. arenaria* and *M. incognita* are reported to be virulent to widely used rootstocks and to the rootstock 'Harmony' in particular. Breeding RKNs-resistant grape rootstocks is a promising alternative to highly toxic nematicides. Muscadine (*Vitis rotundifolia* syn. *Muscadinia rotundifolia*) is a resistance (R) source with undercharacterised genetics. To this end, we used a segregating progeny between the RKN-resistant *Vitis* x *Muscadinia* accession 'VRH8771' from the muscadine source 'NC184-4' and the RKN-susceptible *V. vinifera* cv. Cabernet-Sauvignon. We first phenotyped its resistance to isolates of the i) *M. arenaria*, ii) *M. incognita* and iii) *M. javanica* species, and then to iv) two mixed Harmony-virulent Californian populations of *M. arenaria* and *M. incognita*. Finally, we created an isolate of *M. arenaria* and *M. incognita* from these Harmony populations and phenotyped the progeny to each of them [v) and vi)], and to vii) an isolate of *M. ethiopica*. The resistance phenotype of all the progeny's individuals was independent of the RKN isolates or populations used. Resistance was mapped in a region of chromosome 18 in VRH8771, supporting the hypothesis that it is conferred by a single gene with an unprecedented wide spectrum in grapevine, including Harmony-virulent isolates. This dominant gene, referred to as *MsppR1*, is linked to the telomeric QTL *XiR4* for *X. index* resistance from the same source. Additionally, plant mortality data showed that *MsppR1*-resistant material expressed a high-level resistance to the Harmony-virulent isolates. Our results are a first step towards the development of marker-assisted breeding using SSR and SNP markers for resistance to RKNs in accession VRH8771.

KEYWORDS: Grapevine, gene, mapping, muscadine, nematode, resistance, rootstock

INTRODUCTION

Root-knot nematodes (RKNs) *Meloidogyne* spp. are extremely polyphagous pests that develop on most crops and weeds. Among them, four species are considered to severely affect grapevines throughout the world: *M. arenaria*, *M. incognita*, *M. javanica* and *M. ethiopica* (Saucet *et al*., 2016). Those mitotic species are mainly located south of the Mediterranean and in hot climates, but their distribution area is extending to temperate climates because of global warming. Another RKN, the meiotic species *M. hapla*, has a temperate distribution and appears to be less destructive (Howland *et al*., 2015; Zasada *et al.*, 2019). *Meloidogyne* spp. are sedentary endoparasitic nematodes with the second-stage juvenile (J2s) phase as the motile infecting stage. Juveniles hatch from an egg mass into the soil, penetrate root tips and move intercellularly to the vascular cylinder to induce a feeding site composed of multinucleated specialised cells designated as 'giant cells' (Abad and Williamson, 2010). The vermiform juveniles develop into third- and fourth-stage juveniles, and into female adults, all stages being swollen, fixed and imbedded within a characteristic gall. Under optimal climatic conditions, the complete cycle is 4 to 5-weeks long and several generations per season may develop (Saucet *et al*., 2016).

Snyder (1936) looked for resistance (R) to RKN species within *Vitis* accessions in California, and the American species *V. champinii*, *V. longii*, *V. doaniana* and *V. cinerea* have been shown to be potential sources (Lider, 1954). A wider spectrum of resistance (including to *M. arenaria, M. incognita* and *M. javanica*) has been detected in diverse accessions of muscadine (*Vitis rotundifolia* syn. *Muscadinia rotundifolia,* referred to as *M. rotundifolia* in our manuscript) (Bloodworth *et al*., 1980; Firoozabadi and Olmo, 1982; Lider, 1954; Nesbitt, 1974; Walker *et al*., 1994). In the US, severe damage and nematicide removal have driven plant breeders to collaborate closely with nematologists in order to focus efforts on the development of hybrid rootstocks resistant to RKN species jointly with desirable agronomical traits. *Vitis champinii* was used as the main source in the first resistant rootstocks bred with this objective. Among them, the Harmony and Freedom rootstocks, both hybrids of *V. champinii*, were resistant in many RKN-infected locations (Loubser and Meyer, 1987; McKenry and Anwar, 2006). However, cases of severe local attacks by *M. incognita* have been reported in California since 1954 on accessions of *V. champinii* and *V. longii* (Cain *et al*., 1984; Lider, 1954; Lider, 1960), and on Harmony and Freedom in particular (McKenry, 1992; McKenry and Kretsch, 1995). These later authors also noticed severe damage by a virulent population of *M. arenaria*. Breeding efforts of Californian teams for resistance to this *M. arenaria* population and to other resistance-breaking biotypes then led to the release of accessions such as the RS (Ramsey x Schwarzmann) hybrids [*V. champinii x (V. riparia* x *V. rupestris*)] (Anwar *et al.,* 2002), the complex hybrid accessions 6-19B, 10-23B and Demko 10-17A [this later accession is a cross between Edna (an interspecific *Vitis* crossing) and *V. simpsoni*] (Anwar and McKenry, 2002a, 2002b; Anwar *et al*., 2000; Anonymous, 2012), and the UCD GRN series (Ferris *et al*., 2012). The rootstock UCD GRN1, an F1 hybrid involving the muscadine accession 'Cowart', was shown to be resistant to two populations of *M. arenaria* and *M. incognita* that were virulent to Harmony (Ferris *et al*., 2012).

Resistance to RKN species is commonly controlled by major genes either within annuals, such as the *Mi* genes from tomato (Williamson, 1998) and the *Me* genes from pepper (Djian-Caporalino *et al*., 2001), or within perennials (Saucet *et al*., 2016), such as the *Ma* gene from plum (Claverie *et al*., 2004, 2011), the *RMia* gene from peach (Duval *et al*., 2014) and the *RMja* gene from almond (Van Ghelder *et al*., 2010, 2018). A single dominant gene has been shown to confer resistance to *M. incognita* in *V. champinii* (Lider, 1954). This monogenic hypothesis has been confirmed for the Harmony and Freedom sources for a non-virulent *M. incognita* population (Cousins and Walker, 2002). In a *V. mustangensis* accession, a single dominant allele controlled this non-virulent population together with two virulent *Meloidogyne* sp. populations (Cousins *et al*., 2003). More recently a single dominant gene for resistance to *M. javanica* - designated *MjR1* - has been identified in a *V. cinerea* accession and mapped on chromosome 18 (Smith *et al*., 2018)**.** By contrast to *Vitis* sources, RKN resistance conferred by *Muscadinia* material has been poorly studied, presumably because high numbers of backcross 1 (BC1) individuals putatively segregating for the character of interest are difficult to obtain from most muscadine accessions. Using *Vitis* x *Muscadinia* hybrids, Bloodworth *et al*. (1980) observed that the resistance of *M. rotundifolia* to *M. incognita*, *M. arenaria* and *M. javanica* was most often dominant.

We report here results related to the genetics of resistance to RKN species in plant material derived from the muscadine accession 'NC184-4' (Esmenjaud *et al*. 2010). Our study aimed at identifying and mapping *R* factor(s) carried by 'VRH8771', which is a '*V. vinifera* x NC184-4' hybrid accession already shown to carry the QTLs *XiR2, XiR3* and *XiR4* for resistance to *X. index* (Rubio *et al.* 2020). We evaluated segregating BC1 material derived from 'NC184-4' for its resistance to an isolate of the four grapevine RKN species, *M. incognita*, *M. arenaria*, *M. javanica* and *M. ethiopica*, predominant on a worldwide scale. We also evaluated the resistance of this material to a mixture of two populations of *M. arenaria* and *M. incognita* that are virulent to the Harmony rootstock, as well as its resistance to a pure isolate of each RKN species obtained from these mixed populations. After the complete RKN resistance phenotyping of the BC1 progeny, a single major dominant gene was proposed and located on the map of accession VRH8771.

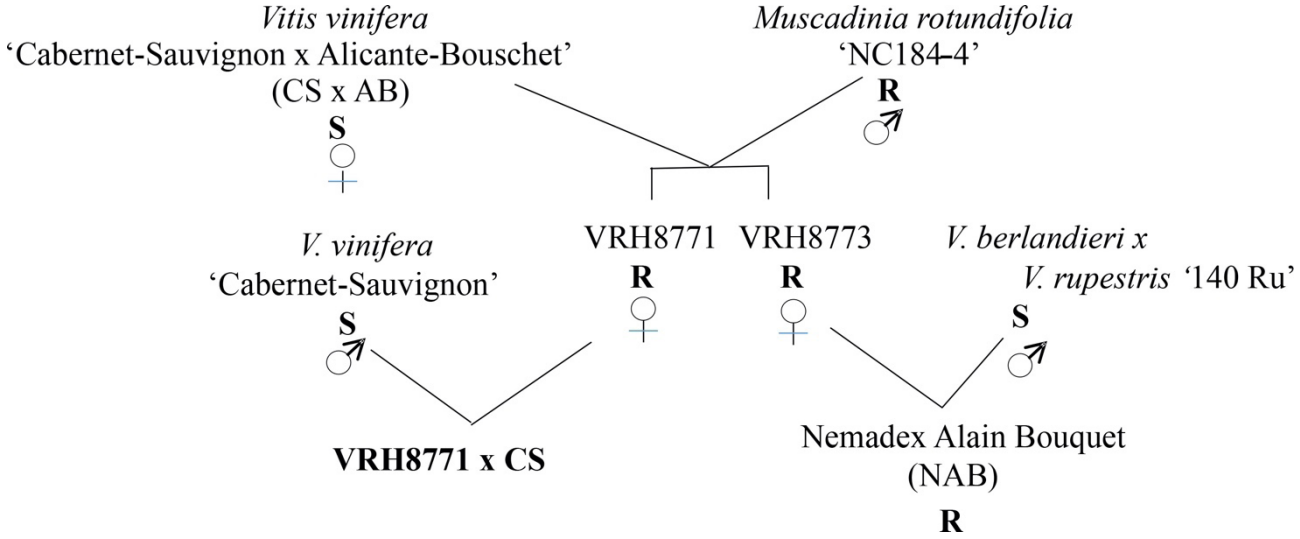


FIGURE 1. Pedigree of the plant material used in the study (R resistant, S susceptible).

MATERIAL AND METHODS

1. Plant material

The pseudo-testcross (referred to hereafter as a backcross 1 (BC1)) between the hybrid VRH8771 (*V. vinifera x M. rotundifolia*; female parent) and *V. vinifera* cv. Cabernet-Sauvignon (CS; male parent) was used (Figure 1). This cross was initiated by Alain Bouquet at INRAE Montpellier in 2005 and was continued at INRAE Bordeaux (UMR EGFV) after 2008; it has been used in a previous study on the genetics and mapping of resistance to *X. index* in muscadine (Rubio *et al.,* 2020). VRH8771 is a hybrid between the *V. vinifera* accession 'Cabernet-Sauvignon x Alicante-Bouschet' and the *M. rotundifolia* accession 'NC184-4'. VRH8771 is resistant to all tested RKN species, whereas CS is susceptible. Three reference genotypes were also tested: i) 'Nemadex Alain Bouquet' (NAB), a *X. index*-resistant rootstock from the cross VRH8773 (a brother clone of VRH8771) x 140 Ru (*V. berlandieri x V. rupestris*) (Figure 1); ii) VRH8624, an F1 hybrid accession between *V. vinifera* 'Carignan x CS' and *M. rotundifolia* 'Trayshed', which is moderately susceptible to *X. index (*Esmenjaud *et al*., 2010); and iii) *V. rupestris* cv. du Lot (RL), an *X. index*-susceptible accession. In the experiments on the two created and evaluated Harmony-virulent RKN isolates (see 'Nematode material section' hereafter), the Harmony rootstock accession was also included. All plant material (parental and reference genotypes, BC1 progeny) originated from the INRAE germplasm conservatory in UMR EGFV (Villenave d'Ornon, France).

2. Nematode material

An isolate of each of the RKN species *M. arenaria* (MA), *M. incognita* (MI), *M. javanica* (MJ) and *M. ethiopica* (MEth), (Sophia Antipolis, France) was used: *M. arenaria* 'Six-Fours' (Provence, France), *M. incognita* 'Morelos' (Mexico DF, Mexico), *M. javanica* 'Higuera' (Catalonia, Spain) and *M. ethiopica* 'Nancagua' (VIth Region, Chile) respectively. Those isolates were kept on plants from the tomato cv. St Pierre in the nematode collection of INRAE at UMR ISA (Table 1). All isolates were reared from a single egg mass and maintained on the tomato (*Lycopersicon esculentum* Mill.) cv. St. Pierre. The identity of the isolates, at the species level, was checked once a year, by determining their isoesterase phenotype (Janati *et al*., 1982).

A mixture of two field RKN populations from the vineyard that were each virulent in the rootstock 'Harmony' was introduced from UC Davis (University of California). These two mixed samples were identified as *M. arenaria* 'Harmony A' and *M. incognita* 'Harmony C' (Table 1). From these mixed Harmony-virulent populations, pure isolates of each of the *M. arenaria* and *M. incognita* species were created from a single egg mass (Table 1).

3. Experimental design

Twenty-eight to 51 individuals from the cross VRH8771 x CS, depending on the RKN isolate or population tested, were selected out of the 60 individuals previously evaluated for their resistance to *X. index* (Rubio *et al*., 2020). Together with the parental and reference accessions, they were evaluated in five successive annual experiments.

For each annual experiment, homogenous hardwood cuttings of the plant material were rooted in alveolated plates in the nursery at INRAE UMR-EGFV (Bordeaux, France) in February. At the end of April to early May, cuttings were delivered to INRAE UMR-ISA (Sophia-Antipolis, France) and planted individually in a greenhouse in 2-litre containers. For each RKN isolate or population, the pots were arranged in a completely randomised design on glasshouse benches. Evaluations of resistance to the isolates MA (Year 1), MI (Year 2), MJ (Year 3) were conducted with 4 inoculated replicates and a single non-inoculated (NI) replicate per individual. The evaluations of resistance

TABLE 1. List of isolates and populations used in the study.

	Species	Isolate (I) or population (P)	Origin and host	Additional information
RKN representative isolates	*M. arenaria*	Six-Fours (I)	Six-Fours, Provence, France. Tomato	Isolate previously used in Duval *et al.* (2019)
	M. incognita	Morelos (I)	Morelos, Mexico DF, Mexico. Tomato	Isolate used for the genome sequencing of *M. incognita* (Abad *et al.*, 2008)
	M. javanica	Higuera (I)	Cataluna, Spain. Fig	Isolate previously used in Duval *et al.* (2019)
	M. ethiopica	Nancagua (I)	Nancagua, VIth Region, Chile Grapevine	Idem
Harmony-virulent RKNs	*M. arenaria* + *M. incognita*	*M. arenaria* 'Harmony A' (P) + *M. incognita* 'Harmony C' (P)	California, USA	Mixture of 2 field populations virulent on Harmony
	M. arenaria	*M. arenaria* 'Harmony A' (I)	Idem	Isolate created from the virulent Harmony population
	M. incognita	*M. incognita* 'Harmony C' (I)	Idem	Isolate created from the virulent Harmony population

to the mixed Harmony-virulent populations of *M. arenaria* and *M. incognita* (Year 4) were conducted with 4 to 5 inoculated replicates and a single non-inoculated (NI) replicate per individual. The evaluations of resistance to the Harmony-virulent isolates of *M. arenaria* and *M. incognita* and the isolate *M. ethiopica* (Year 5) were conducted with 3 inoculated replicates and a single non-inoculated (NI) replicate per individual.

The nematode inoculum was produced by multiplying each isolate or population on the susceptible tomato cultivar 'St Pierre'. Two months after tomato inoculation, galled roots were recovered, cut into 5-mm pieces, mixed with the soil from their containers and an amount of approx. 150 ml of the infected soil+roots mixture was deposited onto the upper part of each container. The pots were inoculated at least three weeks after planting (end of May to early June). The number of J2s released by this inoculum was evaluated by placing three control glasses at random among the pots at the time of inoculation. The control samples were then placed on a filter paper retained by a coarse sieve over a bowl in a mist chamber and the J2s, having migrated through the paper, were counted after 7 and 14 days. The total number of J2s released from the soil+roots mixture was high (always over 5,000 J2s per pot).

During the experiments, the plants were watered as needed with a drip irrigation system. The glasshouse temperature was controlled, and a cooling system maintained the plants under the threshold of 30°C in Years 1 to 4. In the evaluation conducted in Year 5, this threshold was surpassed, the plants being submitted to peak temperatures of up to 35 °C from mid-June to early August, which induced high plant mortality as from September.

4. Phenotyping and statistical analysis of the data

At harvest, i.e., 4.5 to 5 months after inoculation (~ 3-4 nematode life cycles), the aerial parts of each plant were cut, and each pot was hermitically placed in a plastic bag and stored in a climatic chamber at 6°C until the final ratings. At final ratings, the root systems were cautiously washed free of soil under tap water and a gall index (GI) rating was attributed to each plant on a 0-5 scale (0 = no gall; 1 = 1-10 %; 2 = 11-30 %; 3 = 31-70 %; 4 = 71-90 %; 5 > 90 % of root system galled) completed by 0.5 steps when galling was intermediate between two categories (Barker, 1985). An individual exhibiting a single gall was rated 0.5. Plants were classified as resistant when their mean gall value was lower than 1 (GI < 1) and as susceptible when their mean gall index value was superior or equal to 1 (GI ≥ 1).

In the Year-5 experiment, the combination of RKN attacks and high temperatures arrested the development of a part of the plants and those plants progressively died from September to early December, the date on which all the plants were harvested for final ratings. As a result, as from September, the dead/alive status of the plants was recorded twice a month and the recently dead plants were attributed a GI rating.

The GI rating was considered for the R/S plant classification and the plant mortality data was used for the evaluation of the aggressiveness of the Harmony-virulent isolates of *M. arenaria* and *M. incognita*, and the isolate *M. ethiopica*. For the Harmony-virulent isolate of *M. arenaria* that induced the highest mortality, we estimated the number of J2s collected at harvest using a few parental/reference accessions and R/S segregating individuals for which all 3 replicates were still alive. Total fresh roots from each root system were recovered and weighed. From these, a 10-g sample of feeder rootlets was randomly selected and placed in a mist chamber. The J2s that had migrated from the roots were recovered and counted under a low magnification microscope after 3 and 5 days and totalled to evaluate their number per gram of roots (Nbr/g).

The analysed rating criteria were the gall index (GI) rating and the number of J2s per gram of roots (Nbr/g). Nbr/g values were $\log_{10}(x+1)$ transformed for normality (Noe, 1985). Original data (GI) and transformed data (Nbr/g) were submitted to a one-way ANOVA using XLSTAT software (version 2022.3.1; Addinsoft, Paris, France). Mean GIs were then compared using the Fisher LSD multiple range test at $P \leq 0.05$.

5. Genetic mapping

A preliminary mapping of resistance was performed at the chromosome level applying an *in silico* BSA approach and using 15 phenotyped individuals and two parental maps constructed with simple-sequence repeats (SSR) markers. Then, in order to land more precisely on the chromosome, we used thirty-eight common individuals evaluated for resistance to the isolates of *M. arenaria* (MA), *M. incognita* (MI) and *M. javanica* (MJ), as well as a first version of the two parental genotyping-by-sequencing (GBS) maps (G. Lalanne-Tisné, unpublished) based on the PN40024 12X. v1 grapevine reference genome.

Finally, a second version of the GBS high-density parental maps (Rubio *et al*., 2020) based on the PN40024 12X.v2 grapevine reference genome (Canaguier *et al*., 2017) was used in a QTL analysis. Both versions of the maps had been constructed using SSR markers and single nucleotide polymorphism (SNP) markers from the same genotyping-by-sequencing (GBS) data set; both versions of the reference genome can explain the difference in SNP markers. GBS data can be retrieved from BioProject PRJNA553991 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA553991). For the second version of the VRH8771 maternal map, 2271 SNP and 135 SSR markers had been assembled, resulting in 19 linkage groups (LGs). As reported by Rubio *et al*. (2020), genetic maps and marker order had been determined using the maximum likelihood (ML) algorithm with Haldane function and default parameters of JoinMap®4.1 software. LGs had been designed with a minimum threshold logarithm of odds (LOD) score of 6.0. They had been grouped and numbered based on their corresponding physical chromosome numbers (Jaillon *et al*., 2007). A QTL analysis (Rqtl) was performed using phenotyping data from 48 individuals evaluated for resistance to *M. javanica* and using the second version of the GBS map with one-dimension scan function, scanone, of Rqtl software and the argument model = 'binary'.

RESULTS

1. Phenotype of VRH8771 x CS progeny resistance to the isolates of *M. arenaria*, *M. incognita* and *M. javanica* (Years 1 to 3)

Regardless of the RKN species, parental accessions VRH8771 and CS were completely free of galls and highly galled respectively, as illustrated for *M. arenaria* (Table 2), *M. incognita* (Table S1) and *M. javanica* (Table S2). As expected, the reference muscadine-derived material (i.e., the BC1 accession NAB and the F1 accession VRH8624) were also resistant to each of the three RKN isolates, while the *Vitis* accession RL was severely galled by each of them.

The individuals from the VRH8771 x CS progeny that were evaluated for resistance to *M. arenaria* (44 individuals: Table 2), *M. incognita* (45 individuals; Table S1) and *M. javanica* (51 individuals; Table S2) had segregated for resistance. The GI ratings (0-5) clearly fell into two statistically different classes ($P < 0.05$), regardless of the isolate considered: resistant individuals (R) with a GI rating of < 1 and susceptible individuals (S) with a GI rating of ≥ 1.0. Among the S individuals evaluated for resistance to the same RKN species, variable levels of host suitability were observed. These differences in host suitability were not considered in our work, which treated the susceptible class as a whole. To illustrate this result, the GI ratings for *M. arenaria* and *M. incognita* are reported in Table 3 for a subset of 16 R/S segregating individuals. Each of the 44 common individuals tested for resistance to all three isolates expressed the same resistance behaviour (R or S), regardless of the RKN species (Table S3). In other words, all the individuals that were classified as resistant to *M. arenaria* were also classified as resistant to *M. incognita* and *M. javanica*. Thus, a complete match between the R/S classifications for all three RKN species was observed.

2. Phenotype of VRH8771 x CS progeny resistance to the mixture of the two Harmony-virulent populations of *M. arenaria* and *M. incognita* (Year 4)

For the mixture of the Harmony-virulent populations (Table 1), the parental accessions VRH8771 and CS were completely free of galls and highly galled respectively. The reference accessions NAB and VRH8624 were also resistant (Table S4). The 32 individuals from the VRH8771 x CS progeny had segregated for resistance and fell into one of the two statistically different ($P \leq 0.05$) resistant (R) and

TABLE 2. Gall index (GI: 0-5) ratings in the segregating progeny VRH8771 x CS for resistance to an isolate of *M. arenaria* (MA). Data are means of 4 replicates. Values with different letters significantly differ according to Fisher LSD test ($P \leq 0.05$). The horizontal line separates the susceptible and resistant plant individuals.

Part 1/2

Plant material	Gall index	Groups
48	4.5	A
CS	4.5	A
240	4.4	A B
207	4.3	A B C
82	4.3	A B C
262	4.1	A B C D
271	4.1	A B C D
279	4.0	A B C D E
4	4.0	A B C D E
236	3.9	B C D E F
225	3.8	C D E F G
246	3.6	D E F G H
267	3.6	D E F G H
233	3.5	E F G H I
247	3.4	F G H I J
RL	3.3	G H I J
237	3.3	G H I J K
244	3.3	G H I J K
238	3.2	H I J K
235	3.2	H I J K L
37	3.0	I J K L M
31	2.9	J K L M
68	2.9	J K L M
232	2.8	K L M
14	2.6	L M
21	2.5	M
248	2.5	M
1	0.0	N
17	0.0	N
227	0.0	N
228	0.0	N
234	0.0	N
239	0.0	N
250	0.0	N
251	0.0	N

Part 2/2

Plant material	Gall index	Groups
263	0.0	N
266	0.0	N
270	0.0	N
273	0.0	N
277	0.0	N
28	0.0	N
281	0.0	N
306	0.0	N
33	0.0	N
42	0.0	N
93	0.0	N
NAB	0.0	N
VRH8624	0.0	N
VRH8771	0.0	N

TABLE 3. Gall index (GI) ratings and resistance classification of a sample of 16 individuals from the cross VRH8771 x CS evaluated to isolates of *M. arenaria* (MA) and *M. incognita* (MI). Data are based on four replicates. GI values followed by the same uppercase letter do not differ according to the Fischer multiple range test at $P \leq 0.05$. R resistant, S susceptible.

Nematode	Parents		Segregating individuals															
	VRH8771	CS	1	17	28	33	42	227	228	21	14	232	68	31	37	4	82	48
M. arenaria	0.0n	4.5a	0.0n	0.0n	0.0n	0.0n	0.0n	0.0n	0.0n	2.5m	2.6lm	2.8klm	2.9jklm	2.9jklm	3.0ijklm	4.0abcde	4.3abc	4.5a
	R	S	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S
M. incognita	0.0j	4.5a	0.0j	0.0j	0.5j	0.0j	0.1j	0.0j	0.0j	3.0efghi	2.4hi	2.3i	3.1defgh	3.8abcde	2.6ghi	3.5bcdef	2.6ghi	4.0abc
	R	S	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S

susceptible (S**)** classes (Table S4). As for previous RKN isolates, variable levels of host suitability were observed among the S individuals. All 32 individuals had been previously evaluated for resistance to the MA, MI and MJ isolates. Those that had been classified as resistant were also classified as resistant to the mixed populations, and those that had been classified as susceptible were also classified as susceptible to the mixed populations. Therefore, we observed a complete match between the R/S classifications of all segregating individuals when evaluated for resistance to the mixture of the two Harmony-virulent populations and to each of the three isolates of *M. arenaria*, *M. incognita* and *M. javanica*.

3. Phenotype of VRH8771 x CS progeny resistance to each of the Harmony-virulent isolates of *M. arenaria* and *M. incognita* and to the *M. ethiopica* isolate (Year 5)

The Harmony-virulent isolates of *M. arenaria* and *M. incognita* (Table 1) were created and multiplied between Years 4 and 5 to confirm and enhance the results obtained in Year 4 from the mixed populations. As expected, both isolates produced galls on the Harmony rootstock accession that had been used as a positive control, even though its galling by the *M. arenaria* isolate was much higher (GI = 4.0) than by the *M. incognita* isolate (GI = 1.5) (Tables S5 and S6 respectively). For each isolate, the parental accessions VRH8771 and CS were completely free of galls and highly galled respectively. Of the reference accessions, NAB and VRH8624 were resistant to each Harmony-virulent isolate, while the accession RL was susceptible (Tables S5 and S6 respectively). The 28 hybrid individuals that were evaluated for resistance to each isolate segregated and fell into one of the two statistically different ($P \leq 0.05$) resistant (R) and susceptible (S**)** classes (Tables S5 and S6 respectively). The phenotype of all 28 individuals was the same as in their previous evaluations with the other isolates (Tables S3) or the mixed populations from which they originated (Table S4).

TABLE 4. Evaluation of the plant mortality in the Harmony-virulent isolates of *M. arenaria* and *M. incognita* in comparison with the isolate of *M. ethiopica*. R resistant, S susceptible.

Plant material	Isolate	Total plants	Total dead plants	Plant mortality	Mortality in R/S phenotype		GI rating in R/S phenotype	
					R plants	S plants	R plants	S plants
Parental and reference material								
	M. arenaria Harmony	18	6	33%	0/9	6/9		
	M. incognita Harmony	18	5	28%	0/9	5/9		
	M. ethiopica	18	1	6%	0/12	1/6		
Segregating material								
	M. arenaria Harmony	84	24	29%	0/27	24/57	0	3.4
	M. incognita Harmony	84	20	24%	0/27	20/57	0	3.2
	M. ethiopica	84	10	12%	0/27	10/57	0	3.1

TABLE 5. Evaluation of J2s numbers per gram of roots (Nbr/g) for the Harmony-virulent isolate of *M. arenaria* in parental/reference accessions and in R/S segregating individuals. Data are means of three replicates. Data are real numbers, but Nbr/g values were log10(x+1) transformed for normality. Values with different letters significantly differ according to Fisher LSD test ($P \leq 0.05$). The horizontal line separates the susceptible and resistant plant individuals.

Accession or hybrid	Nbr/g	Groups
Harmony	1278.2	A
225	683.1	A
14	509.2	A
236	497.4	A
NAB	131.2	B
28	46.2	B
VRH8771	3.9	C

The evaluation of resistance to the *M. ethiopica* isolate showed that the parental accessions VRH8771 and CS were completely free of galls and highly galled respectively, and that, among the reference accessions, NAB, VRH8624 and Harmony were resistant, while RL was susceptible (Table S7). The 28 hybrid individuals evaluated for resistance to this isolate segregated and fell into one of the two statistically different ($P \leq 0.05$) resistant (R) and susceptible (S) classes (Table S7). The phenotype of all the individuals was identical to that of their previous evaluations with the other isolates or populations (Tables S3 to S6). The phenotype of all the individuals evaluated for resistance to all tested isolates and populations over the 5 years of our study is summarised in Table S8.

4. Comparison of the aggressiveness of the Harmony-virulent isolates of *M. arenaria* and *M. incognita*, and the *M. ethiopica* isolate, using plant mortality data

Twenty-eight individuals were evaluated for resistance to the three isolates under high temperature conditions in Year 5; this allowed us to compare their respective aggressiveness using the final numbers of dead plants and GI ratings. Among parental and reference accessions, the percentage of dead plants was 33% for *M. arenaria*, 28 % for *M. incognita* and only 6 % for *M. ethiopica* (Table 4). All the dead plants belonged to susceptible accessions; i.e., the parent CS and the reference accession RL; meanwhile, none of the plants from the resistant accessions (VRH8771, VRH8624 and NAB) died (Table S9). All the Harmony rootstock plants were galled by the isolates of *M. arenaria* and *M. incognita,* but they survived. This rootstock was resistant to the *M. ethiopica* isolate (Tables S7 and S9). Regarding the segregating individuals of the VRH8771 x CS progeny, the highest mortality percentage (29%) was associated with *M. arenaria*, followed by *M. incognita* (24%) then *M. ethiopica* (12%). As for parental/reference accessions, all the dead plants belonged to S individuals and no R plants died (Table S9). Of the S individuals, *M. arenaria* induced a higher GI than *M. incognita* and *M. ethiopica,* but the differences were small (Table 4). Altogether, our data on mortality rates and, to a lesser extent, on GI ratings, illustrate that the Harmony

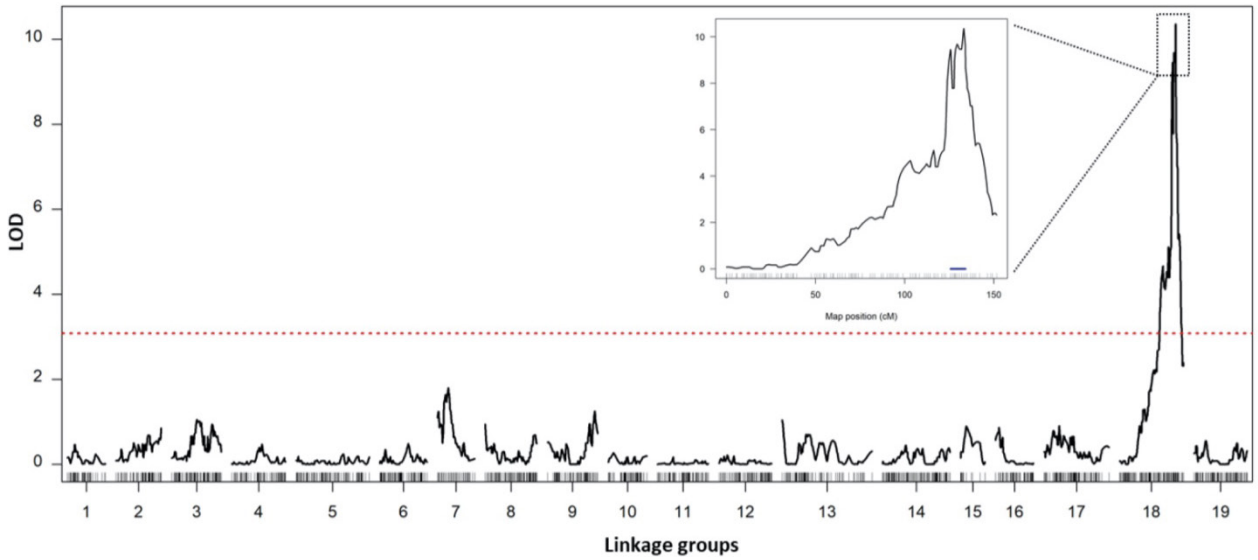


FIGURE 2. QTL analysis of the location of resistance to *M. javanica* (MJ) performed on 48 segregating individuals. The curves in the plot indicate the genetic coordinate in the 19 linkage groups of the VRH8771 map (x-axis) and the LOD score obtained by the binary mapping (y-axis). The horizontal dotted line shows the LOD significant threshold estimated with 1000 permutations ($P \leq 0.05$). The box is the zoom-in image of the peak on chromosome 18. The horizontal line at the basis of the peak shows the confidence interval detailed in Figure 3.

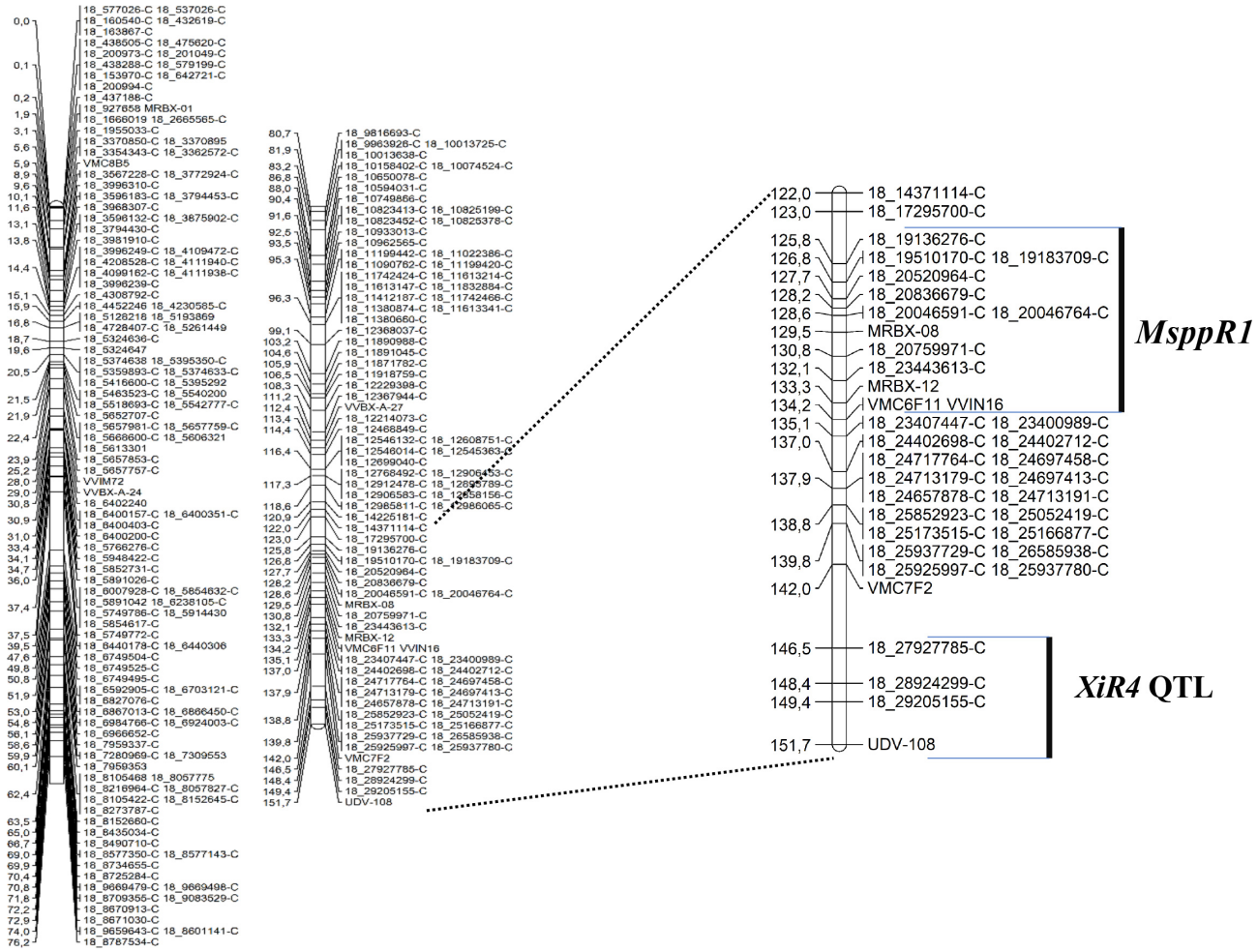


FIGURE 3. Location of the RKN *MsppR1* locus on the chromosome 18 (shown in two parts) of VRH8771. Genetic distances in cM are displayed on the left-hand side and marker names (SNP and SSR markers) are displayed on the right-hand side. The detailed map shows the region encompassing *MsppR1* (this study) and the *XiR4* QTL for resistance to *X. index* (Rubio *et al.*, 2020).

M. arenaria isolate had the highest aggressiveness, the Harmony *M. incognita* isolate intermediate aggressiveness, and the *M. ethiopica* isolate the lowest aggressiveness.

5. Evaluation of J2 numbers from the *M. arenaria* Harmony isolate in surviving parental/reference accessions and R/S segregating individuals

Because the Harmony *M. arenaria* isolate induced the highest mortality, we aimed to confirm its effect on the R/S status of the different accessions and segregating individuals. To this end, we retained the three replicates of representative genotypes with no mortality at harvest in Year 5 (Table S9) and we estimated the number of infective J2s recovered from a 10-g sample of total plant roots. Regarding the S accessions, this only concerned the Harmony rootstock of which all replicates survived, given that both CS and RL had no surviving plants. Regarding the R accessions, we retained VRH8771 and NAB. Regarding the segregating material, we randomly retained three S (#14, #225 and #236) out of the six individuals that comprised all three surviving replicates and one R (#28) individual. The Harmony S accession and the three S segregating individuals were significantly more infected than the resistant plant material; i.e., NAB, the R segregating individual and VRH8771 (Table 5). Out of the R materials, VRH8771 had significantly the lowest numbers of infective J2s. Of the S materials, Harmony contained the highest number of nematodes (Table 5). Consequently, nematode number per gram of roots in segregating individuals and in parental and reference accessions fell into the two statistically different ($P \leq 0.05$) classes expected from their GI rating (Table S5).

6. Location of resistance on the map of VRH8771

A preliminary mapping of resistance was performed applying an *in silico* BSA approach with 10 S individuals and 5 R individuals and the two parental SSR maps. Four microsatellite markers (MRBX-08, VVIN16, VMC7F2 and UDV-108) that were linked to resistance were thereby detected in a region of chromosome 18 (Figure. S1). Then, using 38 common individuals phenotyped for their resistance to *M. arenaria*, *M. incognita* and *M. javanica* and the first version of the high-density VRH8771 GBS/SSR map (Lalanne-Tisné, unpublished)), it was possible to identify a putative resistance factor in an interval of ~5.4 Mb of the VRH8771 map between SNP markers 18_18034398 and 18_23400980 (Figure S2). The heterozygous status of SNP markers of the R individuals (and the cognate homozygous status of the S individuals) in the region of this R factor is in line with the hypothesis that resistance is dominantly inherited from the muscadine-derived resistant parent VRH8771 (Figure S2).

Leaning on the hypothesis that there is a single and common R factor for all four RKN species considered in our study, we used the 48 individuals genotyped and phenotyped for resistance to a representative RKN, the species *M. javanica* (the highest number), to complete our results; to this end, we carried out a QTL analysis based on the second version of the high-density VRH8771 GBS/SSR map (Rubio *et al*., 2020). The binary mapping detected a strong QTL that peaked near marker MRBX-12 on linkage group 18, between 125.8 cM and 134.2 cM. This QTL was supported by a LOD score of 10.35, explaining 77.8 % of the phenotypic variance (Figure 2). The confidence interval of the R factor and the SNP and SSR markers that it contains are shown in Figure 3.

DISCUSSION

This study aimed to identify and map the *R* factor(s) carried by 'VRH8771' relative to the globally predominant grapevine RKN species (McKenry *et al*., 2001; Nicol *et al*., 1999; Saucet *et al.,* 2016). Using an isolate of each of the *M. arenaria, M. incognita, M. javanica,* and *M. ethiopica* species, together with Harmony-virulent RKN material, we showed that accession VRH8771, derived from the muscadine source NC184-4, has a wide resistance spectrum (Table S8). Our genetic study based on the VRH8771 x CS progeny found a clear R/S segregation, which indicates that VRH8771 is heterozygous for resistance. The independence of this segregation from the different RKN species that were evaluated shows that VRH8771 carries (an) R factor(s) that control(s) all tested RKN species. For the highest number of individuals evaluated for resistance to *M. javanica* (n = 51, Table S8), the segregation ratio was 22R:29S. To determine whether this ratio was statistically different from the 1R:1S segregation expected for monogenic resistance, we performed a Chi-2 test (one degree of freedom). The Chi-2 value (0.483) corresponded to a p-value of 0.487, a probability that is much higher than the threshold value p = 0.05 (3.841). Consequently, our data support the hypothesis that resistance is conferred by a single major dominant factor at the heterozygous state in the accession VRH8771. Nevertheless, a distorted segregation ratio in favour of S individuals was observed, which might be due to a higher survival of S seedlings. Indeed, the overall survival rate of the seedlings of the VRH8771 x CS progeny was only 37%, and after eliminating the plants which did not root well from the analysis, only 31% could be phenotyped. No genetic information is available for the 63 % of dead seedlings.

Only a low-resolution location of the *R* factor was obtained due to the small number of phenotyped individuals (38 in common for the different nematode species); the analysis of a larger population would have resulted in higher resolution data. The missing 13 individuals needed to reach a total of 51 available individuals are under evaluation at the time of writing to confirm these results. Despite these limitations, using the high-density map from VRH8771 (Rubio *et al.,* 2020), we were able to clearly locate the R factor in a single region of chromosome 18. We named it the *MsppR1* gene (for '*Meloidogyne* spp. Resistance 1' including *M. arenaria, M. incognita, M. javanica* and *M. ethiopica*), given that the different mapping steps support the hypothesis of a single factor controlling all tested RKN species. *MsppR1* is located within a ~5.4-Mb interval that contains the microsatellite markers MRBX-08, MRBX-12, VMC6F11 and VVIN16,

which could be interesting tools for marker-assisted breeding (Figure 3). Few major RKN R genes have been identified and mapped in perennial plants (Saucet *et al*., 2016), and only one, the *MjR1* gene, has been mapped in grapevine (Smith *et al*., 2018); this gene from *V. cinerea* (accession C2-50) controls *M. javanica* and is also located on chromosome 18, but using another map, it was located in a more telomeric position between the SNP flanking markers, S18_31160355 and S18_33954011. In VRH8771, this telomeric region also contains *XiR4* QTL for resistance to *X. index* (Rubio *et al*., 2020) (Figure 3, Figure S2). In *M. rotundifolia*, chromosome 18 has also been shown to contain other R genes for resistance to downy mildew (*Rpv2/Plasmopara viticola*) and powdery mildew (*Run2.1, Run2.2 /Erysiphe necator*) (Delrot *et al*., 2020; Dry *et al*., 2019; Merdinoglu *et al*., 2018).

We consider the isolates of the four species and the two Harmony isolates to be representative of the different grapevine RKNs in nature. Nevertheless, they do not guarantee that the resistance inherited from NC184-4 will be active against each of their specific populations or a mixture of them. For example, despite its resistance in separate tests to a *M. incognita* population, a *M. javanica* isolate and a Harmony-virulent *M. arenaria* isolate, the *X. index*-resistant rootstock VR 039-16 (*V. vinifera x M. rotundifolia*) (Walker *et al*., 1991) was found to be susceptible to a field mixture of populations of *M. incognita, M. arenaria* and *M. javanica* (McKenry and Anwar, 2006). However, our results are in line with those that have been obtained since 1954 for diverse muscadine-derived materials that have all been shown to be resistant to the various RKN species (Bloodworth *et al*., 1980; Ferris *et al.*, 2012; Firoozabadi and Olmo, 1982; Lider, 1954; Nesbitt, 1974).

In addition to the four main species parasitising grapevine, a particular focus of our study was the 'Harmony' populations. We phenotyped the resistance of segregating progeny to a mixture of the two populations *M. arenari*a Harmony A and *M. incognita* Harmony C, and then to an isolate obtained from each of these populations. The data obtained from the mixed Harmony populations suggest a high level of resistance conferred by the *MsppR1* gene. Testing each of the obtained isolates separately then allowed us to assess their aggressiveness more accurately. The aggressiveness of the Harmony isolate of *M. arenaria* towards the reference Harmony rootstock was confirmed by the marked galling that was induced and the high number of J2s recovered on this accession (Table S5, Table 5). By contrast, the *M. incognita* isolate was less aggressive towards this rootstock (Table S6). This particular difference in aggressiveness is in line with our data, illustrating that the *M. incognita* isolate induces lower plant mortality than the *M. arenaria* one. Nevertheless, regardless of the Harmony isolate considered, we observed a complete survival of the resistant individuals (heterozygous state: *MsppR1/msppR1)* in contrast to the elevated mortality of the susceptible individuals (homozygous recessive state: *msppr1/msppr1*); this indicates that the *MsppR1* allele inherited from VRH8771 might be associated with a mechanism of high resistance to RKNs.

Besides the parental accession VRH8771, we also evaluated other muscadine or muscadine-derived reference accessions. Accession VRH8624 displayed the same complete-spectrum resistance to the isolates and tested populations as VRH8771. This might signify that VRH8624 has inherited (from its muscadine parent 'Trayshed') an *MsppR1* R allele expressing a similar efficiency to the VRH8771 allele. The BC1 rootstock NAB (Figure 1) was also found to have wide-spectrum resistance. Nevertheless, it often showed a very low, but non-null gall index rating (Tables S1, S4 to S7), although its pedigree should make it express the same gall-free phenotype as the R segregating individuals (i.e., carrying the *MsppR1* R allele of VRH8771), with which it shares the same resistant grandparent NC184-4. Given that NC184-4 is presumably homozygous for resistance, a plausible hypothesis for the slightly weaker phenotypic expression of resistance in NAB is that its parent VRH8773 (Figure 1) inherited the other R allele of the *MsppR1* gene, and that this latter allele would confer a slightly lower protection.

The histological mechanisms underlying resistance in muscadine NC184-4 are unknown. Nevertheless, studies conducted on a *M. arenaria* population breaking the resistance carried by Harmony found that the resistant accession Demko 10-17A expressed an early hypersensitive response (Anwar and McKenry, 2002b) with no root galling, a mechanism that might be shared by NC184-4 and other muscadine accessions. Within *Vitis* spp., an equivalent hypersensitive response has been observed for the gene *MjR1* in *V. cinerea* (Smith *et al*., 2018); meanwhile, in the rootstock RS9 (a *Vitis* hybrid of *V. champinii*), a late resistance mechanism has been found to occur, inducing slight galling with undersized adult females and little or no egg production (Anwar and McKenry, 2002a).

CONCLUSION

Resistance from the 'NC184-4' muscadine source in the *Vitis x Muscadinia* accession VRH8771 controls an isolate of each of the RKNs *M. arenaria, M. incognita, M. javanica* and *M. ethiopica,* together with *M. arenaria* and *M. incognita's* Californian populations virulent to the Harmony rootstock. This wide-spectrum resistance is presumably conferred by a dominant gene designated as *MsppR1* and mapped on grapevine chromosome 18. In the ~5.4-Mb interval encompassing *MsppR1*, SNP and SSR markers (MRBX-08, MRBX-12, VMC6F11 and VVIN16 in particular) can be used for marker-assisted breeding.

The wide spectrum resistance conferred by *MsppR1* meets the needs of worldwide viticulture - in particular in California for the control of *M. arenaria, M. incognita, M. javanica* and the Harmony-virulent populations (Saucet *et al*. 2016), in South America against the predominant *M. ethiopica* species (Meza *et al.,* 2016) and in Australia against the prevalent *M. javanica* species (Smith *et al.*, 2017, 2018). In the genetic background of VRH8771, this gene also appears to confer

high-level resistance, a feature which is worth promoting in the context of increased RKN pressure due to global warming. In VRH8771, marker-assisted breeding of *MsppR1* together with the *X. index* R QTLs *XiR2, XiR3* and *XiR4* should allow us to select highly performing grapevine rootstocks derived from muscadine sources.

The similar wide-spectrum resistance inherited from another muscadine source in VRH8624 suggests that many muscadine accessions carry other highly beneficial R alleles of the *MsppR1* gene. Nevertheless, as *V. vinifera* x *M. rotundifolia* hybrids may be sensitive to Phylloxera (Bouquet, 1983; Rubio *et al*., 2020), the interest of such genotypes as genitors of rootstocks should be carefully assessed through marker-assisted selection and *in planta* tests for the soil-borne aphid *Daktulosphaera vitifoliae.*

ABBREVIATIONS

ANOVA: Analysis of variance; BSA: Bulked segregant analysis; GBS: Genotyping-by-sequencing; GI: Gall index; J2: Second-stage juvenile; LG: Linkage group; LOD: Logarithm of odds ratio; LSD: Least significant difference; ML: maximum likelihood; QTL: Quantitative trait locus; SNP: Single nucleotide polymorphism; SSR: Simple sequence repeats

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DECLARATIONS

Ethics approval and consent to participate

The plants used in this study are available in the germplasm conservatory in UMR EGFV (Villenave d'Ornon, France). Experimental research and plant studies, including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Availability of data

All the data analysed during this study are included in the article and in its supplementary information (data files S1 to S4).

GBS data obtained using the PN40024 12X.v2 grapevine reference genome can be retrieved from BioProject PRJNA553991 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA553991)

Competing interests

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

DE, MYB, CVG and NO contributed to the design of the study. JPT and ML obtained the progenies and multiplied the plant material. AW collected and provided the Californian 'Harmony' populations. UP performed nematode identification and multiplication. DE and MYB collected and analysed the phenotypic data. DE and BR analysed the mapping data. DE and MYB wrote the manuscript. All the authors read and approved the final manuscript.

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