



Molecular detection of powdery mildew resistance in indigenous and exotic *Vitis* genotypes

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ABSTRACT

The obligate biotrophic ascomycete fungus *Erysiphe necator* belongs to the family Erysiphaceae (Leotiomycetes; Erysiphales) causes powdery mildew (PM), a most destructive and widespread fungal disease in grapevine. Exploring the potential genetic resistance in grape genotypes and the introgression of this efficient genetic resistance into cultivated grapevines could be a sustainable alternative for managing powdery mildew disease. Marker-assisted selection by using 21 known SSR markers associated with different PM-resistant loci combined with controlled (artificial) inoculations was utilized in this study to screen the wide range of *Vitis* germplasm to identify resistance to powdery mildew. The genotype with strong resistance to PM was further used to determine response alterations in biochemical parameters compared to the susceptible genotypes. Amongst 40 *Vitis* genotypes, Male Hybrid, Pusa Navrang, Pearl-of-Csaba, Pusa Swarnika, Dog Ridge, Salt Creek, 110 Richter, St. George, 1103 Paulsen, Couderc 1613, *V. parviflora* and *V. jacquemontii* were identified as resistant sources. Two SSR markers, VMC1A5 and VMC3d12 were the most informative for identifying genotypes with effective powdery mildew disease resistance. The total phenolic content increased significantly in both the resistant and susceptible genotypes compared to the non-inoculated vine leaves (6.29 and 9.53%). After artificial inoculation, the leaves of the susceptible genotype showed 31.36% higher malondialdehyde accumulation compared to the resistant genotype. The artificial inoculated leaves of the resistant genotype, *V. parviflora* had 58.96% enhancement in phenylalanine ammonia-lyase antioxidant activity compared to non-inoculated leaves (23.70%) in the susceptible genotype.

Key words: Antioxidant activities, *Erysiphe necator*, Ren 1, resistant loci, SSR markers.

INTRODUCTION

Vitis is a genus of Order Vitales that is widely dispersed having diverse taxonomy. However, most of the world's commercial grape production is concentrated mainly on *V. vinifera* L., originating from Europe and Asia Minor. This species is susceptible to several pathogens, such as powdery mildew (PM) caused by the ascomycete biotrophic pathogen *Erysiphe necator* [(syn. *Uncinula necator* (Schw.) Burrill)]. Powdery mildew is an economically important disease originating in the Eastern North America, causing substantial yearly losses (up to 45% reduced yield) (Kunova *et al.*, 14). It has become a more severe disease than grape downy mildew in the changing climatic scenarios causing huge losses (Bendek *et al.*, 3). *Erysiphe necator* infects all the green tissues of the grapevine (host) and appears as a dusty whitish-grey layer formed by the spread of its mycelia and conidia across the surface. Young colonies are whitish and metallic in colour before sporulation and highly affected grapevine leaves develop necrotic blotches, senesce and drop prematurely. Inflorescence and young berries are

highly susceptible and often become fully coated with whitish mildew growth (Gadoury *et al.*, 11).

Grape is a crop that demands high pesticide application for managing pests and diseases. Currently, the main strategy for controlling PM in the grape vineyards largely depends on preventive fungicides with 10-20 applications under favourable conditions for epidemic development (Kunova *et al.*, 14). Worldwide, the average viticulture pesticide consumption is 35% of the total pesticide production (Essling *et al.*, 9). Synthetic fungicides are the most preferred option for controlling PM, but their effectiveness may be hindered due to the development of resistance by the pathogen. Geographical regions where pathogens and host plants have evolved tend to harbour natural sources of disease resistance (Dry *et al.*, 7). The most sustainable alternative to grape PM management is exploiting host genetic resistance. The innate resistance of wild American grapevines, such as *Vitis rotundifolia* (also known as *Muscadinia rotundifolia*), *V. rupestris*, *V. aestivalis* and *V. riparia* from USA, *V. vinifera* subsp. *silvestris*, *V. vinifera* subsp. *vinifera*, *V. romanetii*, *V. yenshanensis*, *V. lanata*; and *V. vinifera* cvs. Kishmish vatkana & Karadzhandal Kara representing

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the Asian cultivars etc., against the majority of *E. necator* isolates found globally is a consequence of their coevolution. Alternative resources are needed to increase resistance durability in breeding efforts since common sources of PM resistance, such as *V. rotundifolia*, are threatened species even before they are used for commercial purposes (Barba *et al.*, 2).

In the past thirty years, several researchers have made progress in evaluating grapevine genotypes aiming to identify the resistance source to powdery mildew, which serves as a valuable donor parent genotype. The most common evaluation method is field observation of infected leaves in the natural environment. Few researchers utilized a single isolate for inoculating detached leaves in sealed Petri plates to assess the disease reaction to PM fungus. Presently, molecular genetic techniques are more frequently employed in preliminary screening and breeding efforts. Novel alleles associated with resistance at specific loci were present in several grapevine genotypes resistant to powdery mildew. Of them, *Run1* (Resistance to *Uncinula necator* 1), which originates from *V. rotundifolia*, has a resistance locus linked to grapevine powdery mildew (Pauquet *et al.*, 15), *Ren1* (Resistance to *Erysiphe necator* 1) belongs to 'Kishmish Vatkana' and 'Dzhandzhak Kara', two Central Asian *V. vinifera* cultivars *Run2*, *Run2.1* and *Run2.2*, *Ren2* (Feechan *et al.*, 10), *Ren3* (Welter *et al.*, 19), *Ren4* (Ramming *et al.*, 16), *Ren5* (Blanc *et al.*, 4) etc. In natural populations, the genetic resources of *Vitis* species are significant sources of variability that are worth conserving for introgression of such gene(s) in cultivated types.

Conversely, the induced antioxidant responses due to the host-pathogen interaction have received little attention. The leaf epidermal tissue response alterations due to pathogen presence in resistant and susceptible genotypes must be exploited. Indigenous Himalayan *Vitis* sp., namely, *V. parviflora* is reported to have multiple disease resistance, i.e., anthracnose, downy mildew, rust and powdery mildew (Gurjar *et al.*, 12). Hence, we have compared *V. parviflora* as a resistant source against the susceptible Pusa Trishar. The ROS scavenging enzymes include phenylalanine ammonia-lyase (PAL) and phenols concentration are two additional parameters to assess the altered oxidative status of plant tissue under both stresses (abiotic and biotic) to maintain homeostasis. The present study aimed to evaluate PM-resistant *Vitis* sp. using simple sequence repeat (SSR) markers linked to the PM resistance loci and also by examining the response alterations in biochemical changes in the presence (inoculated) and absence of pathogen (non-inoculated) in both the resistant and susceptible genotypes.

MATERIALS AND METHODS

Forty *Vitis* genotypes maintained in the Grapevine Field Gene Bank at the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, India were selected for the experiment. Out of these 40 grape genotypes, 34 represented *V. vinifera* types, two were *V. champini*, one *V. rupestris*, three inter-specific hybrids of *Vitis* species and two indigenous Himalayan genotypes (*V. parviflora* & *V. Jacquemontii*) were evaluated. The genotypes investigated in this study are referred to by their short names as listed in Table 1.

Small sections (about 1 cm²) of newly emerged healthy leaves were excised from the field-grown vines, quickly sealed in plastic bags (to prevent drying out), and cooled using ice packs. The collected leaves were frozen in liquid nitrogen and brought to the lab. Total DNA was isolated from the selected 40 genotypes using the CTAB method (Rogers and Bendich, 18). The quality of DNA was ascertained using a Nanodrop™ (ThermoFisher Scientific, USA) and gel electrophoresis on 0.8% (w/v) agarose gel.

Sequence length polymorphisms of simple sequence repeats (i.e., microsatellites) were used to validate 40 grape genotypes. Twenty-one SSR marker sets from the VMC (*Vitis* Microsatellite Consortium), GF, UDV and SC series reported to be associated with PM resistance were analysed. About 10 µl volume reaction mixtures containing 1 µl of template DNA, 1 µl of SSR primers (forward + reverse), 5 µl of PCR master mix, and 3 µl of sterile double-distilled water were used for PCR amplification. The PCR was performed using a thermo-cycler (S1000™, Bio-Rad, Hercules, California) with a pre-denaturation at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min., annealing for 30 s, extension at 72°C for 30 s and final extension at 72°C for 12 min. using 35 cycles. The amplified product was then separated on a 3% (w/v) agarose gel using 1X TAE buffer at 90 volts and SSR allele size was determined based on the position of the bands relative to a DNA ladder (FastGene™ 100 bp DNA marker).

The malondialdehyde (MDA) level of the inoculated leaves at 11-day post inoculation (dpi) and non-inoculated leaves of both resistant (*V. parviflora*) and susceptible cultivar (Pusa Trishar) was estimated. Results were expressed as MDA content (nmol MDA g⁻¹ FW), obtained by oxidative degradation of lipid-fatty acids (Hodges *et al.*, 13). Total phenolic compounds were assessed by the Folin-Ciocalteu colorimetric method with minor modifications and were determined as mg gallic acid equivalents (GAE) µg 100 g⁻¹ FW. The phenylalanine ammonia-lyase (PAL) enzyme activity was measured

Table 1. Genetic polymorphism of 42 grape genotypes with three SSR markers linked to the *Ren1* locus on chromosome 13.

Genotype	SC47-18		VMC1A5		VMC3d12	
Anab-E-Shahi (AES)	260	260	100	100	230	230
Alumwick (AW)	260	260	100	100	230	230
Banqui Abyad (BA)	260	260	100	100	230	230
Bharat Early (BE)	260	260	90	100	230	230
Black Muscat (BM)	260	260	100	100	240	240
Black Prince (BP)	-	-	80	80	240	240
Beauty Seedless (BS)	260	260	90	90	230	230
Cardinal (CARD)	260	260	90	90	230	230
Centennial Seedless (CES)	260	260	90	90	230	230
Cabernet Sauvignon (CAS)	260	260	90	90	230	230
Chardonnay (CH)	280	280	90	90	230	230
Fakhri (FK)	-	-	90	90	230	230
Flame Seedless (FS)	260	260	90	90	230	230
Hur (HUR)	260	260	90	90	240	240
Julesky Muscat (JM)	260	260	90	90	230	230
MACS Punjab Purple (MACS)	260	260	100	100	-	-
MA × BS (MB)	260	260	100	100	230	230
Male Hybrid (MH)	230	260	80	80	230	230
R ₂ P ₁₉ (P19)	260	260	100	100	230	240
R ₂ P ₃₆ (P36)	260	260	100	100	230	240
Pusa Aditi (PA)	-	-	90	90	240	240
Perlette (PER)	260	260	90	90	230	240
Pusa Navrang (PN)	230	260	80	90	200	200
Pearl-of-Csaba (POC)	230	230	80	90	200	200
Pusa Purple Seedless (PPS)	260	260	90	90	230	230
Pusa Seedless (PS)	260	260	100	100	230	230
Pusa Swarnika (PSW)	230	260	100	100	230	230
Pusa Trishar (PT)	260	260	100	100	230	240
Pusa Urvashi (PU)	260	260	100	100	230	230
Tas-A-Ganesh (TAG)	260	260	100	100	230	240
Tempranillo (TEMP)	260	260	100	100	230	240
H-70-56 (70-56)	260	260	100	100	230	230
Dog Ridge (DR)	230	260	90	90	200	230
Salt Creek (SC)	260	260	90	90	200	200
110 Richter (110R)	230	230	80	80	200	200
St. George (STG)	230	260	80	80	200	200
1103 Paulsen (1103P)	230	230	80	80	230	230
Couderc 1613 (C 1613)	230	230	-	-	200	200
<i>V. parviflora</i> (VP)	260	260	80	90	200	200
Jacquemontii (VJ)	260	260	90	90	200	200

Genotypes carrying R-alleles are represented in bold (rows). Allele 230 at marker SC47-18, allele 80 at marker VMC1A5 and allele 200 at marker VMC3d12 were linked to powdery mildew resistance.

as the conversion rate of L-phenylalanine to cinnamic acid (Gurjar *et al.*, 12).

The disease severity was assessed by the visual scoring of mycelial growth on the leaf surface. The leaf rating scores were taken in four replicates and the mean value per genotype (each year) was represented as a single direct score in all the genotypes for *in-vitro* evaluation performed each year. Antioxidant assays and phenolic levels were analysed using a completely randomized experimental design comprising two variables (two contrasting genotypes × two stages of disease infection). R.4.3 version of the RStudio program was used for two-way ANOVA (p -value ≤ 0.05). Principal coordinate analysis (PCoA) and Neighbor-joining dendrogram were carried out with the PowerMarker program (version 3.2). DARwin software (version 5.0.158) was employed for the genetic diversity analysis.

RESULTS AND DISCUSSION

To identify whether the resistant genotypes genetically correspond to the resistant locus (*Ren* 1), we analysed 40 genotypes using 21 SSR markers, *i.e.*, UDV124, VMC9h4.2, VMCNG4E10-1, UDV-020a, SC8-0071-014, Sc47-20, SC47-18, VMC1A5, VMC3d12, VMC8B5, VVC62, GF15-42, GF15-28, GF15-30, VCh15CenGen06, PN9-057, PN9-068, VMC4f3.1, VMC8g9, VMC7f2 and VMCNg2f12.1, of which amplification was noted concerning the resistant locus of three markers, SC47-18, VMC1A5 and VMC3d12. These microsatellite loci were noted closely linked to DNA markers, correlating with the presence of the *Ren* locus as reported by Coleman *et al.* (5) and Riaz *et al.* (16). The previous findings highlighted that the SC47-18 marker detected the PCR product of 239 bp size (Fig. 1A), 70 bp size by the VMC1A5 marker (Fig. 1B) and 201 bp by the VMC3d12 marker (Fig. 1C) due to the presence of the *Ren*1 locus allele, which regulates PM resistance in the majority of the grapevine genotypes. The above results confirmed that the target gene *Ren*1 identifies genotypes with PM resistance. The target fragment was identified in 13 analysed genotypes; namely, Male Hybrid, Pusa Navrang, Pearl-of-Csaba, Pusa Swarnika, Dog Ridge, Salt Creek, 110 Richter, St. George, 1103 Paulsen, Couderc 1613, *V. parviflora* and *V. Jacquemontii* had the R-allele with three SSR markers. The target alleles at SC47-18, VMC1A5, and VMC3d12 correlated with resistant alleles at the *Ren*1 locus on chromosome 13 (Table 1). Our results found that gel electrophoresis patterns with the allelic size differences, *i.e.*, single base length variation, between the *Vitis* sp. did not vary much, which may be more accurate if capillary electrophoresis

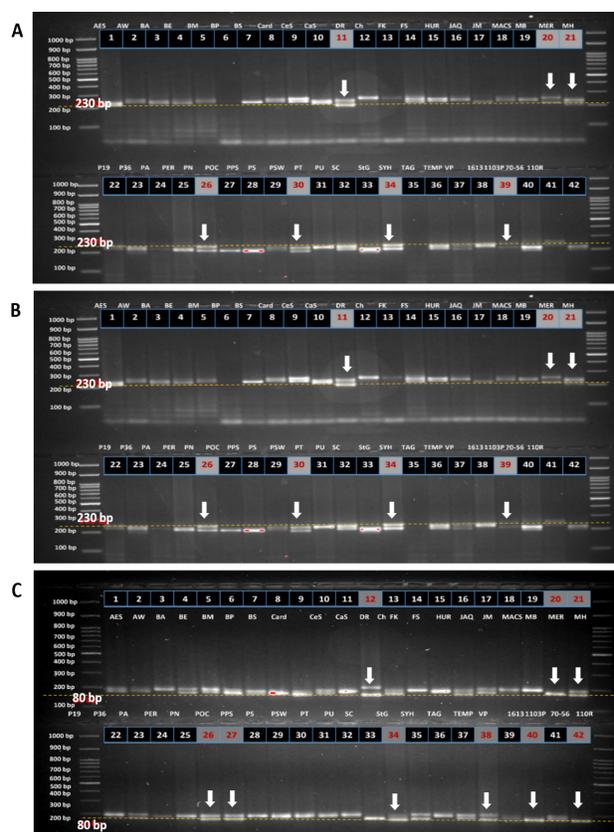


Fig. 1. PCR products generated by three SSR markers, *i.e.* SC47-18 (A), VMC1A5 (B) and VMC3d12 (C) associated with powdery mildew resistance.

was used to detect more alleles. The SC47-18 and VMC3d12 loci with a span of ~12 cM genetic distance on chromosome 13, which was also reported to be polymorphic in an earlier study (Riaz *et al.*, 17).

The set of 40 diverse grape genotypes was subjected to Principal Coordinate Analysis (PCoA) and Neighbor-Joining (NJ) clustering to observe the similarity among them based on SSR allelic profiles (Fig. 2 & 3). According to our findings, these 40 genotypes were scattered over the phylogenetic tree. Nine main branches belonged to three distinct populations: resistant, intermediate (Moderate resistance to moderate susceptible) and susceptible genotypes. Population 1 (P1) contains two groups (Groups 4 and 5) belonging to *V. vinifera*, which are closely related. Most of the individuals in the P1 group (Bharat Early, Pusa Swarnika, Tas-A-Ganesh, hybrid R2P36, Tempranillo, R2P19, Anab-E-Shahi and MACS Punjab Purple) had moderately resistant to moderately susceptible reactions to disease except Pusa Trishar and Alumwick that are susceptible. Groups 6 and 9 had the genotypes (P2) with resistant reactions, which includes the resistant

Table 2. Genetic diversity of *Vitis* species at three SSR markers linked to PM-resistant *Ren1* locus. The alleles highlighted in bold are associated with resistance at the *Ren1* locus.

Marker	Na	H _o	H _e	PIC	p-value	HW	PD	r	Allele	Allele frequency	Absolute count
SC47-18	3	0.17	0.49	0.44	0.65	ND	0.49	0.48	230	0.18	15
									260	0.69	58
									280	0.03	3
VMC1A5	3	0.07	0.64	0.57	0.47	**	0.64	0.79	80	0.17	14
									90	0.44	37
									100	0.36	31
VMC3d12	3	0.19	0.61	0.54	0.59	***	0.60	0.51	200	0.27	23
									230	0.55	46
									240	0.15	13
Mean	3	0.14	0.58	0.54	0.57	-	0.58	0.59	-	-	-

The observed number of different alleles (Na), Observed heterozygosity (H_o), Expected heterozygosity (H_e), Polymorphic Information Content (PIC), Hardy-Weinberg Equilibrium (HW), discrimination power (PD) and null allele frequency estimate (r).

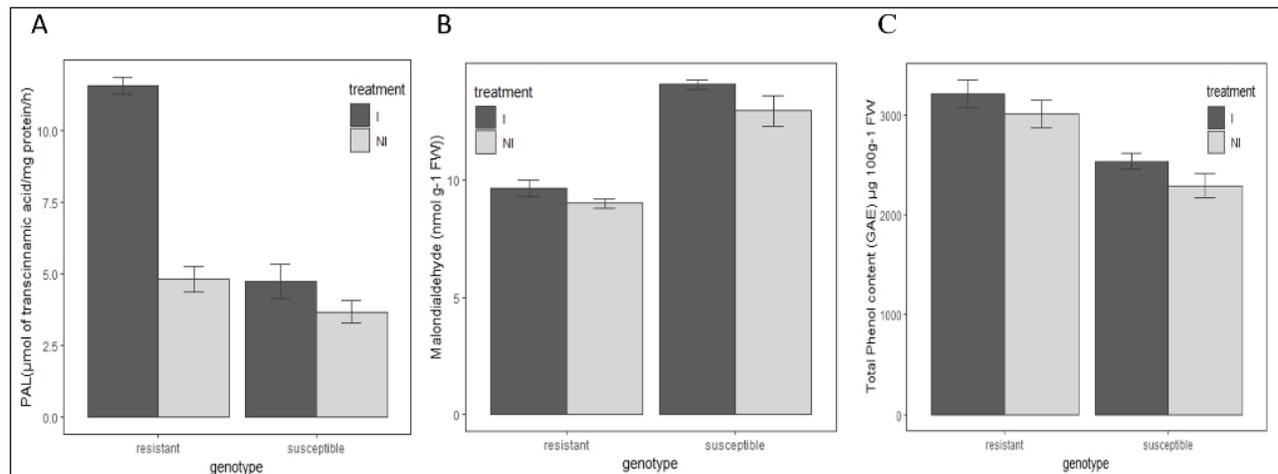


Fig. 4. Evaluation of antioxidant capacity in resistant and susceptible plants. (A) Phenylalanine ammonia lyase (PAL) µmol of trans-cinnamic acid/ mg protein/ h, (B) malondialdehyde (nmol g⁻¹ FW) and (C) phenol content (µg GAE 100 g⁻¹ FW).

the leaves of host plants. The presence of lipids in plants is purely based on genotype. Consequently, the varying levels of lipids among diverse genotypes tested can be ascribed to the inoculation with *E. necator* (Della Corte *et al.*, 6).

Total phenolic content increased significantly in both genotypes compared to leaves of non-inoculated genotypes (6.29 and 9.53%) at 11 dpi, respectively (Fig. 4C). The antioxidant activity (PAL), was 23.70% higher in leaves of the non-inoculated resistant genotype than the non-inoculated susceptible genotype. The inoculated leaves of the susceptible genotypes had no significant changes in antioxidant activities, while the resistant genotype had an increment of 58.96% (Fig. 4A). An increase

in antioxidant activity and phenolic compounds was observed after PM infection in most of the *Vitis* genotypes, suggesting a close relationship between the two (Yilmaz *et al.*, 20). The phenylalanine amino acids lead to p-cinnamic acid catalyzed by PAL, and this activity was observed in plant defence. Furthermore, it has been shown that p-cinnamic acid is involved in the pathway leading to the generation of hydroxyphenolic acid (El-Seedi *et al.*, 8). Earlier studies have also demonstrated similar results, elucidating a weakened PM defensive response in susceptible plants (Agurto *et al.*, 1).

The findings of the present investigation led to the identification of resistance levels of diverse *Vitis* genotypes, which can be used in breeding

for the introgression of resistant allele(s). Thus, incorporating the inherent genetic factors into commercial grapevine varieties represents a viable strategy for developing resistant genotypes or hybrids that would reduce the need to apply chemical fungicides for crop protection and ultimately support sustainable grapevine production.

AUTHORS' CONTRIBUTION

Conceptualization of research (SKS, JP, CK); Designing of the experiment (SKS); Contribution of experimental materials (SKS, JP, CK); Execution of field/lab experiments (RSM, MR); Analysis of data and interpretation (RSM, MR); Preparation of the manuscript (RSM, SKS).

DECLARATION

The authors declare that there is no conflict of interest.

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