RESEARCH

Breeding for black rot resistance in grapevine: advanced approaches for germplasm screening

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Abstract Crop improvement by means of traditional or molecular breeding is a key strategy to accomplish the European Green Deal target of reducing pesticides by 50% by 2030. Regarding viticulture, this is exacerbated by the massive use of chemicals to control pathogen infections. Black rot is an emergent disease caused by the ascomycete *Phyllosticta ampelicida*, and its destructiveness is alarming vine growers. Implementing and improving effective phenotyping strategies are fundamental preliminary steps to breed disease resistant varieties and this work suggests good practices adopted for this purpose. Primarily, the pedigree of black rot resistance donors was reconstructed based on the collection of phenotypic historical data, highlighting unexplored sources of black rot resistance. Strains used for artificial infections were isolated, genetically characterized and mixed to avoid race-specific resistance selection. A new inoculation protocol based on the use of leaf mature lesions was developed. Ex vivo inoculation on detached leaves was effective for the evaluation of conidia germination and hyphal growth, but not for disease progression. Finally, the pedigree was used for the identification of 23 genotypes to be tested. Two breeding selections (NY39 and NY24) resulted symptomless in all assessments and a third one (F25P52) also showed very high resistance, although with a greater variability. Other two genotypes (F12P19 and 'Charvir') fell within the medium resistance category, making them good candidates in a regime of well-timed preventive treatments. In conclusion, this work was effective to a comprehensive parental line characterization and preparatory towards grapevine breeding programs for black rot resistance.

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Introduction

Crop improvement has always been an intrinsic activity in the history of agriculture. Through natural selection, traditional breeding or with the most recent molecular techniques, the main targets have always been augmented productivity, abiotic stress

resilience and disease resistance. Susceptibility to biotic stresses is an urgent problem that afflicts all cultures and causes significant economic losses in terms of both yield and pecuniary investments in plant protection strategies, the latter also having an additive cost in terms of human health and environmental impact. For this reason, the European Green Deal, the ambitious policy initiative of the European Union to achieve carbon neutrality by 2050, allocated 30% of the budget for research and innovation activities in the fields of agriculture, forestry, and rural areas towards organic sector related issues such as genetic biodiversity with the goal of 50% pesticides reduction by 2030 (European Commission 2020). With this perspective, the exploitation of resistance traits is a promising complementary strategy to control plant disease. Unfortunately, these traits are more commonly controlled by a complex interplay of different biochemical mechanisms. Consequently, the resistance behavior is expressed in a quantitative manner as well as strongly affected by the interaction with the environment. The identification of the genomic region (locus) associated with a phenotypic trait is called quantitative trait locus (QTL) analysis, and feasibility of this kind of studies depends on a fundamental prerequisite: the high quality of both genetic and phenotypic data. While the advances in molecular biology and sequencing technology enable today a deep characterization of the genome, adding multiple layers of complexity, the bottleneck is now represented by the development of optimized and standardized approaches for the acquisition of robust phenotypic data, made difficult by the high dimensionality of the phenome, namely the full set of phenotypes of an individual (Houle et al. 2010).

While for herbaceous plants as well as for well-known diseases the technological improvements are more advanced (high throughput) at the expense of low resolution, the applied research for woody plants or emergent pathogens is more focused on low-throughput and high-resolution approaches. The grapevine is one of the most important fruit crops cultivated worldwide, and many agricultural communities depend on its production. However, the Eurasian species *Vitis vinifera* L. is both the most widespread and the most sensitive to several fungal diseases. Besides the more common mildews – powdery (PM) and downy (DM), caused respectively by *Erysiphe necator* and *Plasmopara viticola* – newly emerging

diseases are threatening viticulture, as in the case of black rot (BR). The causal agent of BR is the asco-mycete *Phyllosticta ampelicida* (Engelm.) Van der Aa (syn. *Guignardia bidwellii*, Rossman et al. 2015) and was introduced in Europe already in 1885 from the USA (Ramsdell and Milholland 1988). This fungus was indirectly controlled by the massive treatments to protect the vineyards from mildews imported from the USA some years earlier (Pirrello et al. 2019). Toward the end of the last century, the increasing interest for a sustainable viticulture encouraged many winegrowers to reduce fungicide treatments and to introduce mildew-tolerant varieties, known as "PIWI", a German abbreviation for *pilzwiderstandsfähig*/ fungus-resistant (https://piwi-international.de/en/). Worldwide the hectarage of PIWI varieties of the main wine-growing countries accounts for 0.6% in France and Italy, 2.5% in Germany, 1.8% in the USA, about 10% in Romania and Hungary, almost 20% in Moldova, Ukraine and Russia and up to 67% in Brazil (Sartori 2016). However, most of those mildew-tolerant varieties are susceptible to BR. This situation, together with the advent of more favorable condition of higher temperature in humid continental areas, fostered a new spread of the disease through France and Switzerland (Jermini and Gessler 1996), Romania, Hungary, Bulgaria, Austria, Italy, and Spain (Roznik 2018), and in Germany where severe outbreaks of BR have occurred in the Moselle valley and other wine-growing regions since 2002 (Harms et al. 2005). Because of this situation, the breeders are now facing the challenge of introducing BR resistance in their programs to produce new resistant varieties, possibly combining it with mildews resistance (R)-loci.

Phyllosticta ampelicida is a non-obligate fungus that can be grown on a cultural medium and produces spores after approximately two weeks. After inoculation by water suspension spray on the leaves, the hemi-biotrophic pathogen penetrates the cuticle and starts to grow subcutaneously creating a 2D-hyphal net and acquiring nutrients from the host tissue indirectly (Ullrich et al. 2009). After eight to ten days, it switches to necrotrophy, and immature lesions appear on leaves as discolored spots. With the disease progression, lesions dry up, turn brown with darker-reddish borders, and then develop black dots growths, that are the fruiting bodies (*pycnidia*) containing the new asexual spores (*conidia*) (Pirrello et al. 2019). Unfortunately, when population studies

or germplasm screening are done, the production of spores from cultured mycelium is exceptionally space and time consuming, since one plate per plant is needed.

The aim of this work was to optimize propagation and inoculation protocols for future high-throughput screening, as well as efficiently evaluate the BR resistance of a subset of parental lines and selections of the breeding program for biotic stresses at Edmund Mach Foundation (FEM, Italy).

Materials and methods

Genetic material and pedigree reconstruction

A panel of 23 genotypes were selected within the plant material available at the FEM breeding program as putative BR resistance donors and grown in the greenhouse as potted grafted plants on Kober 5BB rootstock (Table S1). The plant material, that already carried mildew resistance traits, was divided into two classes: (i) parental lines, comprehending commercially available varieties and two wild genotypes from FEM collection, and (ii) breeding selections of FEM grapevine breeding program for biotic stresses. The genotypes were selected based on their relationship with BR resistant donors, after a deep study of available online resources (Table S2) – scientific literature and reports – about BR resistance evaluation and pedigree information, the last taken principally from *Vitis* International Variety Catalog (*V*IVC, Maul and Töpfer 2022). To focus the dataset on BR resistance, the pedigree of all susceptible *V. vinifera* varieties was excluded and the prefix 'VV_' was added, except for two Georgian *V. vinifera* accessions with documented BR resistance (Töpfer and Maul 2017). Resistance level records were converted (Table S3) to the rating proposed to be included in the OIV descriptors (OIV 2009) by Rex et al. (2014), based on the commonly recognized five-step ordinal scale: (1) very low, (3) low, (5) medium, (7) high, (9) very high resistant. The overall median for each genotype was then calculated, together with the minimum, the maximum, the standard deviation, and also the median for each organ (bunch or leaf) and for the assesments made in USA or Europe regions. All the collected information was then employed for the pedigree reconstruction of the historical origin of the BR resistance trait

and visualized through the software Helium (Shaw et al. 2014). Making use of the 'size based on pedigree contribution' tool implemented in Helium settings, the species that are more recurrent within the BR resistance gene pool were highlighted. The software Pedimap (Voorrips et al. 2012) was then used to extract the subpopulation of multiple genotypes through the tool 'select relatives', to have a snapshot of specific subgroups.

Pathogen isolation and characterization

Black rot outbreaks were documented in two experimental vineyards (Telve and Tenna) of the Valsugana valley in the Trentino region (Italy), where the high humidity caused by the nearby Caldonazzo lake favored BR infection, respectively on two grapevine varieties, 'Prior' and 'Civit6'. Infected leaves and bunches were collected at the end of the season 2018, but *P. ampelicida* isolation was effective only from mummified berries (mummies), because on leaves many other fungi were concomitantly colonizing the tissue. The procedure consists of the sterilization of small fragments of BR lesions (ca. 5×5 mm) with 1% NaOH for one minute, then rinsed three times in sterile water for five minutes and, after air drying, placed on ½ PDA (potato dextrose agar) Petri dishes (Ø 92 mm) with chloramphenicol (0.1 g/l). Monoconidic cultures of the samples from the two regions (named TN1 and TN2) together with three isolates (18.1, 80.88 and 10.133) collected in Germany and provided by Julius Kühn Institute (JKI)–Institute for Grapevine Breeding Geilweilerhof (Siebeldingen, Germany) were transferred and grown on new ½ PDA plates covered with sterilized micropore cellophane film (Disco Cell) to facilitate mycelium collection with a spatula in 2 ml Eppendorf tubes. The mycelium was stored at -80 °C, lyophilized until completely dried and ground with the addition of a steel bead using the TissueLyzer (QIAGEN, Hilden, Germany). Genomic DNA was extracted with Nucleospin Plant II kit (Macherey-Nagel, Oensingen, Switzerland). Internal transcribed spacer 1 (ITS1) region of the two samples isolated in Italy was amplified with ITS5 and ITS4 primers (White et al. 1990), sequenced and blasted (GenBank KF851314.1, KF851310.1, KF851306.1, KF851304.1, KF851296.1, KF851295.1, KF851294.1, KF851293.1, KF851292.1, KF851291.1, KF015265.1) to confirm the isolation of pure cultures of *P. ampelicida*.

The five cultures were genetically characterized following the guidelines published by Narduzzi-Wicht et al. (2014) based on 11 microsatellite markers. The protocol was slightly modified, with regards to the usage of different fluorochromes 5' labels (Table S4). Dual colony assays were carried to support genetic data, as suggested by Roznik (2019): briefly, strains TN1, TN2 and 18.1 were plated together to evaluate if they can grow without forming any barrier or if the genetic diversity inhibits the growth at the interface.

Inoculum propagation

The maintenance of *P. ampelicida* strains was carried on organic oatmeal agar (OMA) medium according to Rex et al. (2014), since there was evidence that this fungus grows faster on this substrate compared to ½ PDA (Hausmann L. personal communication). The protocol was followed with slight modification i.e., the medium was supplemented with 0.1 g/l of chloramphenicol, and no blacklight was used. Briefly, the studied strains were incubated in a growing chamber under continuous light at 24 °C and refreshed every 14 days by transferring a distal piece of mycelium (ca. 5×5 mm) on a fresh plate. After 14 days of growth, the mycelium was collected to produce inoculum for the resistance tests. Ten ml of sterile distilled water was added to the plate and the lightly hydrophobic mycelium was gently rubbed with a spatula to facilitate direct contact with the water. The condition of 100% humidity for 10 min leads to the release of the spores. The suspension was filtered, the concentration was defined using a BRAND® counting chamber BLAUBRAND® Thoma pattern (Merck, Sigma-Aldrich, Burlington, MA, United States) and then the concentration adjusted to 10^4 conidia/ml. This primary inoculum derived from the mycelium grown on plates was then propagated *in planta* as follows. In a greenhouse chamber, the inoculum was sprayed until saturation of the adaxial leaf face (dripping) of leaves of young growing shoots of potted grafted cuttings of the susceptible *V. vinifera* variety 'Teroldego', previously treated to prevent PM infection (CIDELY, Syngenta, Basel, Switzerland), and insects (EPIK SL, Sipcam Italia S.p.A., Pero, Italy) and thrip infestation (Neemilk, Serbios, Badia Polesine, Italy). Inoculated plants were kept overnight at 100% relative humidity (RH) through the live-controlled humidifier of the chamber avoiding direct UV irradiation, and then

maintained at 50% RH at 24 °C, with an artificial light cycle of 12 hours. Approximately 14 days post inoculation (dpi) the leaves started presenting the typical mature brown BR lesions. Pycnidia development was induced with 24 hours 100% RH treatment. During the fall, the development of the disease is slower, taking approximately a week longer for lesion onset.

To optimize artificial infection for large-scale experiments, a new protocol was developed to avoid the necessity to produce one plate per plant, by using fresh symptomatic leaves (14 dpi) directly as inoculum source by soaking them in distilled water, in continuous shaking with a stirring bar. To estimate the conidia release curve over time and therefore determine the optimal leaf soaking time, simple linear regression 'lm()' function from Rstudio environment (RStudio Team, 2022) – which use as test static the *t* value from a two-sided *t* test – was applied to the dataset of three independent experiments, which were conducted as follow: after placing leaf tissue with mature lesions in 15 ml of distilled water, the conidia concentration was measured as previously described at 5, 10, 15, 30, 60 and 120 min. Since the concentrations of conidia strictly depend on the number of mature pycnidia present on the infected leaves used for the experiment, the data were normalized by subtracting the mean of the experiment at each time point. After dipping the leaves, the suspension was filtered, adjusted to a concentration of 10^4 conidia/ml, and readily used for its reiterative propagation *in planta* on *V. vinifera* or for inoculation experiments. Since the project was oriented to breeding purposes, the strains producing active inoculum were combined into a mix for resistance assessment experiments, to avoid the detection of race-specific responses.

In vivo inoculation experiments and macroscopic inspection

For each genotype, three biological replicates (potted plants) per experiment were tested. Ten inoculations were carried out during 2020 and 2021 to obtain three evaluations per each genotype. For the artificial infection, the same protocol as described for inoculum propagation *in planta* was followed. Resistance evaluation was performed through visual inspection of the first five inoculated leaves from apex per each biological replicate, following Rex et al. (2014). To ensure the homogeneity of the data within and among

experiments, the *V. vinifera* susceptible variety 'Teroldego' was included as a reference in each inoculation experiment for data normalization.

Ex vivo inoculation assays and microscopic symptom evaluation

Sterile Linfaboxes (Micropoli, Cesano Boscone, Italy) were prepared with 0.1% water agar medium (0.1 g/l chloramphenicol) covered with sterilized filter paper. The third to fifth growing leaves of the genotypes of interest ('Teroldego' = S, 'Merzling' = MR, 'Seyval' = HR) were collected with the full petiole and sterilized in 1% NaOH for 1 min, then rinse for three times in sterile distilled water for five minutes and left on sterile filter paper under a vertical flux biological hood until dried. Leaves were subsequently placed in the boxes adaxial face-up by cutting the filter paper to introduce the petiole in the medium. After preparing nine leaves per genotype (3×3 time points), 4 to 9 drops of 10 µl suspension (10^4 conidia/ml) depending on the leaf size were placed on one half of the leaves in proximity of the main veins; on the second half the same number of sterile distilled water drops were placed as mock inoculation. The boxes were left overnight in the dark, then the drops dried with sterile filter paper and samples incubated in a growing chamber with continuous light at 24 °C for 14 days. The inoculated spots were made traceable by a mark on the lid of the plates. Microscopic evaluation was carried out at 1, 3 and 7 dpi on three inoculated fragments per leaf after clearing with KOH for one hour at 62 °C, rinsed with sterile distilled water three times for five minutes, stained with aniline blue (0.1% in glycine buffer pH 9.4) (Vanacker et al. 2000) and viewed under bright field and fluorescent light (argon laser excitation at 405 nm) through a confocal microscope (Leica DM 6000 CS) equipped with a CCD camera (DFC310 FX, Leica).

Results

The pedigree reconstruction reveals a large pool of black rot resistance donors

The review of the overall looked-up resources allowed to find 20 BR resistance reports (Table S2), for a total of 498 scores of 263 entities, 246 varieties (*V. vinifera* or hybrids) and 18 species screened, of which 148 and 14 respectively resulted resistant in at least one survey (Table S3). Some of those (78 varieties and 13 species) have been tested more than once, to a maximum of nine times. All the collected data regarding the 148 resistant varieties are summarized in Fig. 1, and all species in Fig. 2, listed based on median resistance. The dataset has then been integrated with the 23 genotypes phenotyped in this work. Finally, including their relatives, a list of 586 entries (Table S5 and S6) was used for the graphical depiction of the overall pedigree of BR resistance donors (Fig. S1).

Three works that screened different wild species accessions were found and included in this study (Soursac 1908 and Viala 1889, reviewed by Galet 1977; and Hausmann et al. 2017), along with a fourth one describing the screening of pure *V. rotundifolia* varieties (Jabco et al. 1985). Those works suggested that very high (median = 9) level of resistance is present in different species: *V. mustangensis* and *V. vulpina*. Sources of high resistance ($7 \leq$ median < 9) are present within *V. acerifolia*, *V. aestivalis*, *V. aestivalis* var. *aestivalis* (syn. *lincecumii*), *V. cinerea*, *V. cinerea* var. *helleri (*syn. *berlandieri)*, *V. monticola, V. riparia*, *V. rupestris* and *V. rotundifolia* and *V. shuttleworthii*, while medium ($5 \leq$ median < 7) resistance has been detected in *V. amurensis* and *V. palmata* (syn. *rubra*) (Soursac 1908) (Fig. 1). Conversely, *V. arizonica*, *V. californica* and *V. labrusca* have been described to be susceptible. Even if *V. vinifera* is generally consider highly susceptible (Fig. 2), two Georgian varieties, 'Muradouli' and 'Ojaleshi', have been described to be medium resistant (Roznik et al. 2017). Upon pedigree contribution analysis of the genotypes considered in this study, the species that resulted most often as BR resistance donor was *V. rotundifolia*, represented by nine resistant cultivars evaluated by Jabco et al. (1985). To evaluate FEM breeding material, five seedlings originating from this species were included in the screening test: F13P86, NY24, NY30, NY39 and NY42; in particular, they have VRH3082-1-42 as a grandparent, namely a *V. rotundifolia*-derived back cross line called by antonomasia BC4 worldwide. The second most prominent species was *V. labrusca*, whose accessions evaluated to date have been found to be susceptible (Soursac 1908). However, by inspecting its subpopulation (Fig. S2) nine varieties shown from 5 to 9 resistance level, and the three
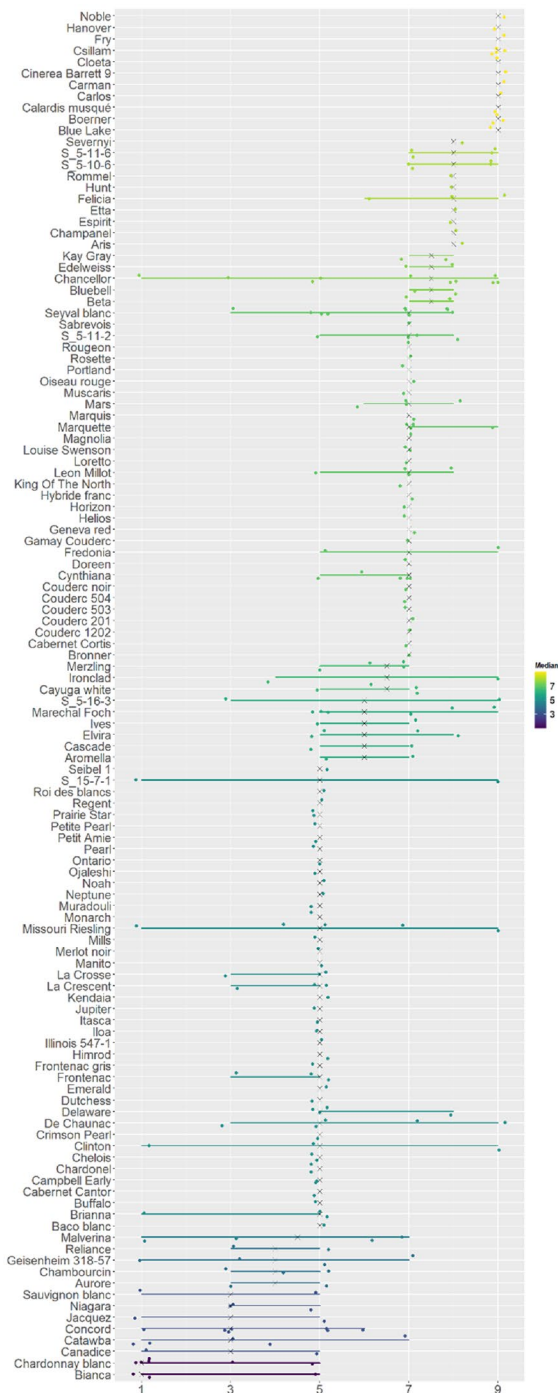
Fig. 1 Graphical representation of historical phenotypic data of black rot resistance evaluation of *Vitis* species accessions (whole plant) collected in this work. Genotypes (y-axes) are sorted and colored by the median (x-axes), represented by a mark (×), from very low resistance (1, violet) to very high (9, yellow)

varieties 'Carman', Fredonia' and 'Triumph' (Fig. 3), showed only *V. labrusca* as wild ancestor, without any unknown ('UNK') or open pollinated ('OP') parent reported in the pedigree. To consider the possibility of a *V. labrusca*-derived resistance trait, two FEM wild parental lines 'Coia 9' and 'Corella 2' – carrying evident *labrusca*-like phenotypic characteristics – were tested for BR resistance.

Focusing on varieties data (Fig. 2, Table S3), cases of strong discrepancy between evaluations were inspected. For instance, 'Bianca' is a complex hybrid widely exploited in Hungary, where it is considered very susceptible to BR (Roznik et al. 2017). On the contrary, its evaluation with *P. ampelicida* strains collected in Germany (Hausmann et al. 2017) classified this variety as medium resistant, and the same difference was detected also for its parental donor 'Villard blanc' (Table S3). A lower resistance have also been detected in North American assessments compared to Europe for 'Catawba', 'Clinton', 'Concord', 'Jacquez', 'Ironclad', 'Missouri Riesling' and 'Triumph', 'De Chaunac', 'Chancellor', 'Fredonia' and 'Seyval blanc' (Fig. 4a). Moreover, a consistent lower resistance of bunches compared to leaves evaluations was highlighted by Roznik et al. (2017) and Töpfer and Maul (2017) (Table S3), and confirmed in the overall dataset (Fig. 4b). The only exception was given by 'Felicia', which nevertheless showed high resistance of all organs. In other cases, a significant difference among USA states or years of evaluations was found, as for 'La Crosse', medium resistant in Minnesota (MGGA 1991, 2016) but susceptible in Nebraska (Read and Gamet 2022), or 'La Crescent', medium resistant in Nebraska (Read and Gamet year 2022) and Minnesota (MGGA 2016) but recently found to be susceptible in Minnesota (MGGA 2018) (Table S3).

Within the varieties, considering the comprehensive dataset, the most frequent genotype determined by pedigree contribution was 'Seyval blanc' with 24 resistant descendants documented (Fig. 5), while it was 'Merzling' for the current studied subset (Fig. S3). The high contribution of 'Merzling' to the pedigree of the studied panel was determined by its extensive use in FEM breeding, giving the possibility to evaluate the inheritance of the BR resistant trait coming from its parent, 'Seyval blanc', a French hybrid well-known for its BR resistance as confirmed by ten evaluations in previous reports (Table S3). The remaining genotypes studied

Fig. 2 Graphical representation of historical black rot resistance phenotypic data collected in this work of grapevine varieties evaluated at least once as resistant (maximum ≥ 5, bunch and leaf). Genotypes (y-axes) are sorted by the median (x-axes), represented with a mark (×), from very low resistance (1, violet) to very high (9, yellow)
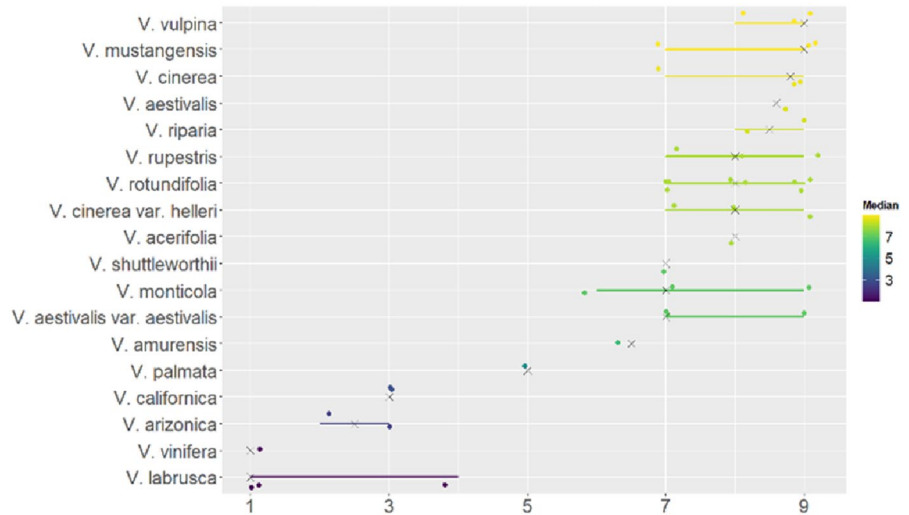
in this work were therefore chosen based on kinship degree with 'Seyval blanc'.

Strains isolated in the Valsugana valley are genetically identical

The samples isolated in Italy were confirmed as *P. ampelicida* by the ITS analysis. Following the molecular characterization of the five studied cultures, the most polymorphic SSRs resulted to be GBMS10, 08 and 11 with three alleles, and GBMS06 and 07 with two. All samples were monomorphic at GBMS01 to 04 and at GBMS09 SSR loci. Upon three assays, no amplification occurred with GBMS05 primers, suggesting a common monomorphic null allele. The three strains provided by JKI resulted to be different among each other and compared to the samples isolated in Trentino (genotype B, C, D). Instead, TN1 and TN2 isolates resulted to be identical (genotype A) (Table 1). Dual colony assays did not show any inhibition between the isolates, in contrast with the outcomes by Roznik (2019). Furthermore, no statistical difference in performance was detected among TN1, TN2, and 18.1 strains. By contrast, it was not possible to produce the primary inoculum from strains 80.88 and 10.133, probably due to loss of virulence caused by long term propagation on plates without re-isolation from infected material.

Based on the estimated standard error predicted by the linear model, conidia concentration changed significantly from 5 to ten minutes, reaching a plateau
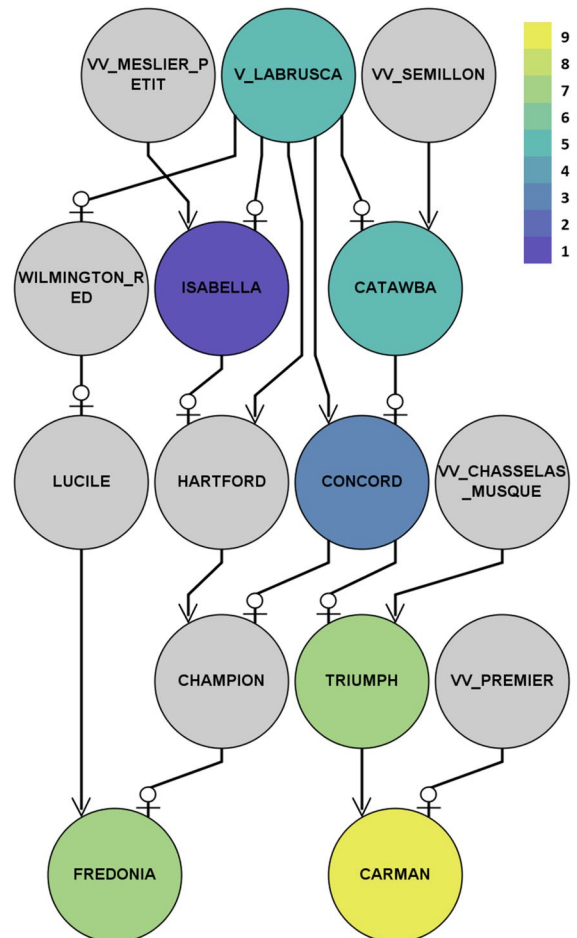

Fig. 3 Pedigree of black rot resistant V. labrusca-derived hybrids 'Triumph', 'Fredonia' and 'Carman'. Color code from very low resistance (1, violet) to very high (9, yellow). Graphical representation by Helium (Shaw et al. 2014)
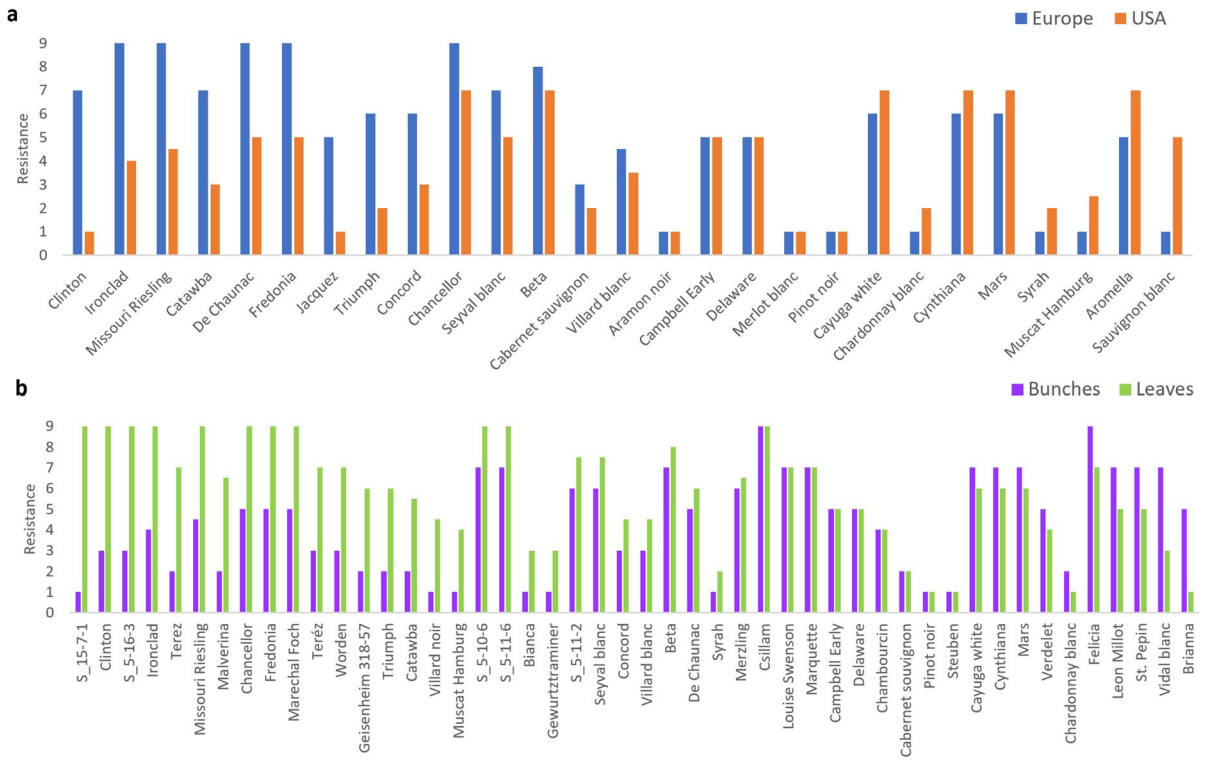
Fig. 4 Contrasting phenotypic evaluation between **a** world regions (Europe and USA) and **b** organs (bunches and leaves). Median resistance from very low (1) to very high (9)

thereafter (Fig. S4). Therefore, the optimal leaf soaking time was settled at ten minutes, consistently with the timing guidelines provided for inoculum production from plates (Hoffman et al. 2002, Wicht et al. 2012, Rex et al. 2014).
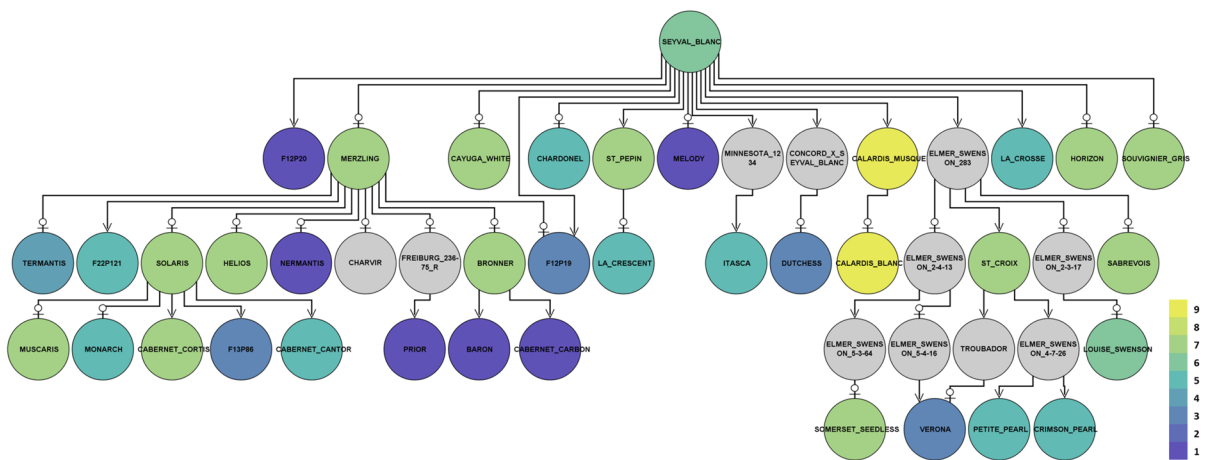

Fig. 5 Progeny of black rot resistant French hybrids 'Seyval blanc'. Color code from very low resistance (1, violet) to very high (9, yellow). Graphical representation by Helium (Shaw et al. 2014)

Both mildew and black rot resistances are combined into the FEM breeding

The foliar artificial inoculations led to the identification of 12 resistant genotypes (mean ≥ 5), which were grouped based on the median end the minimum resistance level (Fig. 6). Three genotypes resulted symptomless in all assessments (mean and minimum = 9). One was 'Seyval blanc', the most exploited resistance donor as highlighted by its pedigree contribution (Figure S1), which already carry two R-loci against PM and two haplotypes of an R-locus against DM. The other two genotypes were NY24 and NY39, two seedlings of the population Eger 99-11.01 × NY 95.0308.02 that were selected in the FEM breeding program for their high resistance to both DM and PM. 'Calardis musqué' and 'Calardis blanc', deriving from 'Seyval blanc' and carrying the same mentioned R-loci, showed a median resistance of 9 and a minimum of 7. The same for F25P52, a breeding line derived from the variety 'Bianca' and descendant of 'Villard blanc', originally selected for its resistance to DM. 'Felicia', 'Merzling' and its offspring 'Solaris', cultivars known for their resistance to DM, also exhibited very high resistance to BR (median = 9), but with an increased variability of the response (minimum = 5). Only 'Villard blanc' showed a median resistance of 7, but unlike the previous group it never showed minimum resistance lower than 7, and it carries an R-locus for DM. Finally, two genotypes exhibited medium resistance (mean = 5). One of them, the breeding line F12P19, was characterized by a resistance distribution that tended to 9 (minimum = 5). 'Charvir' instead, an already patented cultivar due to its resistance to both DM and PM, showed a variability from 3 to 7. All the other genotypes of the studied panel revealed from low to very low resistance (median < 5).

Veins and trichomes are hotspots for conidia germination and hyphal growth

The objective of this experiment was to follow disease progression in genotypes with different degrees of resistance through microscopic assessment of spore germination (1 dpi) and mycelium growth (3, 7 and 14 dpi) on detached leaves. However, no evidence of different germination or hyphal production was detectable between the genotypes, and the

Table 1 Molecular profiling of *Phyllosticta ampelicida* isolates

Marker	TN1	TN2	18.1	80.88	101.33
GBMS01	134	134	134	134	134
GBMS02	173	173	173	173	173
GBMS03	193	193	193	193	193
GBMS04	214	214	214	214	214
GBMS05	–	–	–	–	–
GBMS06	266	266	–	266	–
GBMS07	280	280	280	280	289
GBMS08	318	318	321	321	–
GBMS09	344	344	344	344	344
GBMS10	390	390	378	390	369
GBMS11	390	390	387	387	368
Genotype	A	A	B	C	D

GBMS simple sequence repeat (SSR) markers were used for isolates profiling, as proposed by Narduzzi-Wicht et al. (2014). Null alleles are represented with a dash (-). Markers from 01 to 05 and GBMS09 were monomorphic. GBMS08, 10 and 11 presented three alleles, while markers 06 and 07 showed two alleles. The resulting genetic profiles defined four genotypes (A to D)

progression of disease did not occur, which also happens on leaf discs (Hausmann L. personal communication). Nonetheless, general considerations were made about shared features across the different genotypes. First, at 1 dpi germinated conidia were detectable through the formation of the melanized appressoria that preferentially adhered near the veins (asterisks, Fig. 7A and B). Second, increased concentration of conidia (1 dpi) and hyphal growth was observed around the trichomes that appeared to trap the spores (Fig. 7C and D).

Discussion

Gathered phenotypic and pedigree information to support decision-making process in breeding

Although phenotypic and pedigree data are often publicly available, their use and association are not straightforward since they are dispersed and often recorded without following official standard references and ontologies. Therefore, the value of that information strictly depends on their level of correspondence to FAIR (findability, accessibility, interoperability, and reusability) principles (Wilkinson

Fig. 6 Black rot resistance evaluation on parental lines and breeding selections. Resistance performance of 24 genotypes was assessed by artificial leaf infection under controlled conditions of grafted potted plants, by means of ten inoculations, three evaluation per genotype (three biological replicates). The resistance scale goes from very low (1) to very high (9). Genotypes (yaxes) are sorted by the median (x-axes), represented by a dot (●)
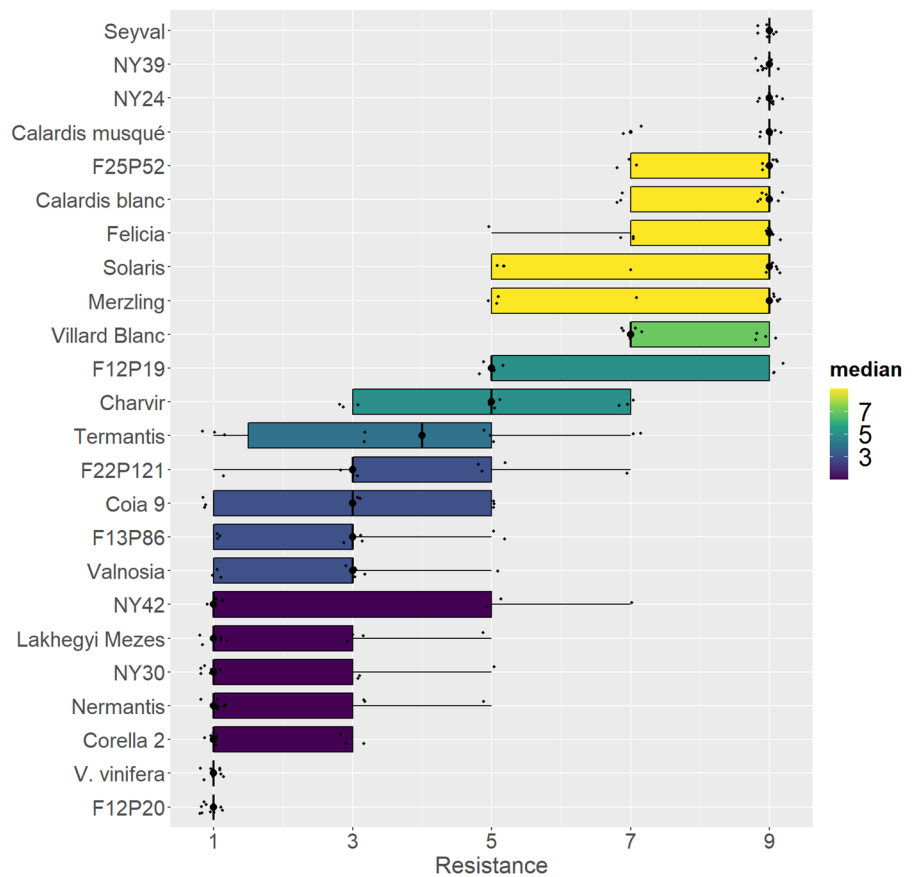
et al. 2016). In fact, breeders' choices are commonly based on their know-how, but with the lack of a systematic screening of available resources. For grapevine, *VIVC* is a very comprehensive database of pedigree information, also specifying variety prime names and synonyms, not a trivial issue in viticulture (Maul et al. 2022). When available, universal marker profiles are also included, to support varietal and parentage identification. However, despite the efforts of *VIVC*, phenotypic data are scattered in a multitude of publications and reports. In this work all available BR resistance data were collected (Table S2 and S3) to give an overview of the available trait donors (Figure S1) and the instruments for the exploration of their pedigree through the viewer Helium (Shaw et al. 2014) (Table S5 and S6). In fact, digitalization and visualization through dedicated software provide a powerful tool for the association and interoperability of pedigree and phenotypic information to support decision-making process in breeding. A comprehensive view of the

trait of interest allows to highlight interconnections and inheritance patterns otherwise difficult to recognize within complex datasets. For this purpose, the graphic representation and navigation of the BR resistance pedigree reconstructed in this work will also be freely available through the user-friendly web application PERSEUS (Jurado-Ruiz et al. 2022).

The availability of a comprehensive database of genetic and phenotypic data is the ultimate challenge. Effective examples are given for instance by the effort of the Rice Research and Development institute of Sri Lanka (Rathnayake et al. 2020) that organized the pedigree, genetic and phenotypic information of its breeding program to support accurate breeding decisions. The same approach was followed by the Crop Genetics Department of John Innes Centre for the historical datasets of national variety performance trials (NVPT) of winter wheat grown in the United Kingdom between 2002 and 2017 (Shorinola et al. 2022), and many other application are given regarding both
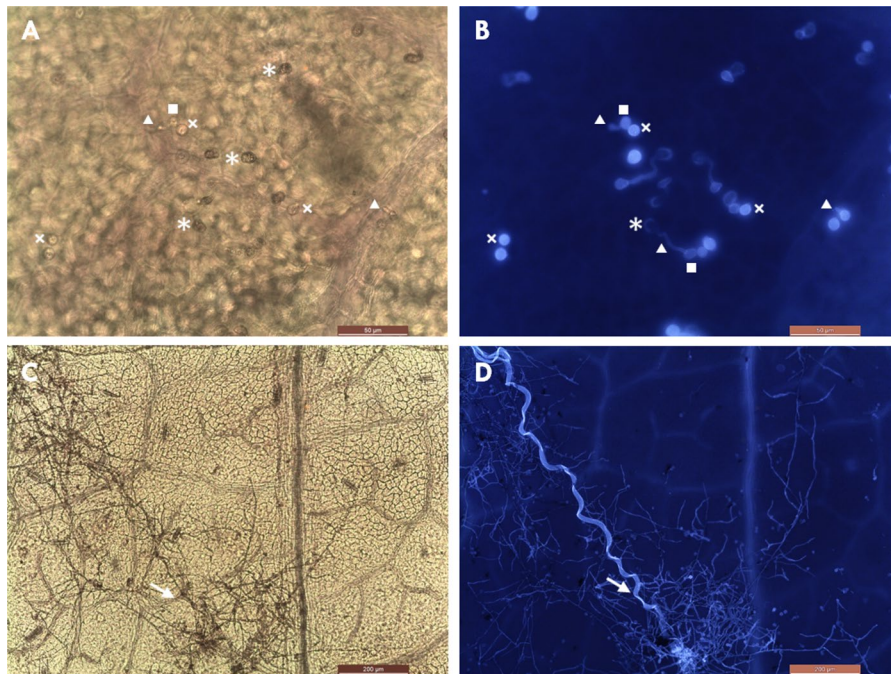
Fig. 7 Microscopic visualization of spore germination and hyphal growth on leaf tissue from the susceptible variety 'Teroldego'. Disease progression assessment through bright field (A and C) and fluorescence (B and D) confocal microscopy at 1 (A and B, 50 μm) and 4 dpi (C and D, 200 μm). Appressoria were clearly detectable at 1 dpi (asterisks) for the characteristic dark brown color due to melanization, and different stages of germinating tube development were visible (triangles). Ungerminated conidia keep a translucent aspect (cross mark), while after germination they become visible just through fluorescence microscopy (squares). Conidia were preferentially trapped by trichomes, where the hyphal growth was concentrated (arrows). Leica DM 6000 CS

herbaceous (i.e., white yam, Norman et al. 2020; maize, White et al. 2020; strawberry, Pincot et al. 2021; red clover, Egan et al. 2019, 2021; soybean, Lee et al. 2015; wheat, Fradgley et al. 2019) and woody (i.e., grapevine, Van Heerden et al. 2018; pear, Montanari et al. 2020; peach, Fresnedo-Ramírez et al. 2016) crops. In this regard, FEM is actively working on the creation of a database for FAIR management and integration of its data relating to the grapevine (FEMVitisDB, Vezzulli et al. 2022).

Exploration of black rot resistance resources for pyramiding strategy

The review of the available information on BR resistance revealed a broad spectrum of *Vitis* species that have genotypes that could be used as BR resistance donors. Pedigree reconstruction gives an important overview of both founders and interesting hybrids to be used in grapevine breeding. Thus, assuming that BR resistant genotypes with unlinked pedigrees will carry different types of resistance mechanisms, there is a concrete possibility to combine them with a pyramiding strategy for durable protection against *P. ampelicida* (Pilet-Nayel et al. 2017). Of course, the validation of the historical pedigrees by means of molecular markers is a fundamental step to support this hypothesis. Pyramiding different resistance loci against the same pathogen is an efficient strategy to increase resistance durability. Recently it has been estimated that four pyramided R-genes would be theoretically impossible to overcome for a pathogen population of cereal PM, since the occurrence of a quadruple mutant capable of eluding four different resistance mechanisms is extremely unlikely (Dry et al. 2019). The importance of the identification of different sources of resistance is even exacerbated for woody perennial plants species. This is because the plants remain in the vineyard for dozens of years, while the fast life cycle of pathogens facilitates the selection of

new strains able to overcome a single specific resistance mechanism. The utilization of donors that have undergone selection for fruit quality is also an efficient strategy, far more desirable compared to the use of wild accessions or first-generation hybrids that often carry undesirable traits (Roznik et al. 2017). In this regard, it is of interest that also some Georgian *V. vinifera* demonstrated BR resistance evidence (Töpfer and Maul 2017). This result is supported by the discovery of PM (Hoffmann et al. 2008; Possamai et al. 2021) and DM (Toffolatti et al. 2018) R-loci in *V. vinifera* varieties of the same region, with the one identified in the variety 'Kishmish vatkana' (Hoffmann et al. 2008) being already widely used in grapevine breeding programs (Katula-Debreceni et al. 2010; Agurto et al. 2017; Fresnedo-Ramírez et al. 2017; Zini et al. 2019). In addition, it is known that there are *V. vinifera* with lower susceptibility to BR (Soursac 1908; Jabco et al. 1985), and it was already noted that varieties with large juicy grains are more susceptible (Galet 1977). As for botrytis (Possamai et al. 2021), it can be presumed that there are specific physiological traits that influence the degree of resistance. This hypothesis is also supported by evidence of an effect of the *V. vinifera* background on DM and PM resistant seedlings with the same R-loci assessment (Bettinelli et al. 2021). We also found cases of acquisition of increased BR resistance in crosses between a hybrid with a *V. vinifera* variety, i.e., 'Calardis musqué' and 'Carman'. Regarding this peculiar occurrence, some hypotheses on the possible causes can be made, such as the segregation of an antagonistic or susceptibility trait in the resistant parent, or epistatic effects determined by the *V. vinifera* background (Bettinelli et al. 2021). Nonetheless, different susceptibility degrees to the strain used for the screening must also be considered. Another interesting case is given by *V. labrusca*, mostly described as susceptible (Fig. 1). Nevertheless, some *V. labrusca* crosses with *V. vinifera* i.e., 'Catawba', have been described as resistant to BR (Fig. 3) (Hausmann et al. 2017). If their pedigree is confirmed by means of molecular markers, it will open a new perspective for breeders, especially in the field of grape juice varieties. Finally, concerning the possible inheritance of the resistant trait from *V. rotundifolia* for BC4 derived genotypes, this is extremely unlikely (Kozma personal communication). In fact, so far, no BR resistance has been described within the 99–1 *V. rotundifolia* hybrids (Fig. S1) extensively used at the breeding institute of Pécs University (Hungary), where BR is heavily present and studied (Roznik et al. 2017).

Interesting varieties that show a high difference between organ evaluations were found and specific trends were highlighted. Primarily, it was evident a divergent resistance of bunches (lower) compared to leaves (higher). The issue of unpaired susceptibility between fruits and leaves had already been raised by Arnaud in 1931 (as reported by Galet 1977) and confirmed by the results of Töpfer and Maul (2017) and Roznik et al. (2017) (Table S3). In general, analyzing the overall collected data (Table S3), within the 48 varieties with both leaf and bunch evaluations, there were only ten cases of higher fruit resistance. Of those, just three of them, 'Brianna', 'Vidal blanc' and 'Verdelet', actually showed resistant bunches and susceptible leaves, all the others were resistant at both organs. However, it must be noticed that leaf assessments were evaluated with high-pressure greenhouse artificial infection experiments, while bunch response was derived from field observations of natural infection, certainly influenced by environmental conditions and seasonality. Nevertheless these outcomes, supported by the cited previous works, suggest the importance to focus also on bunch resistance i.e., for QTL detection and identification of associated molecular markers, as demonstrated in our recent work (Bettinelli et al. 2023). Moreover, numerous cases of increased resistance were also detected in experiments carried out in Europe compared to North America. This may be due to the occurrence in the USA of strains that overcame specific resistance mechanisms due to the selective pressure caused by long-standing coexistence of the resistant varieties with the pathogen, while these strains have not yet been imported or evolved in the EU. Therefore, some specific R-loci remain still effective vs. European *P. ampelicida* population, and this is what we presume for the possible *V. labrusca* derived resistance trait carried by 'Catawba', 'Clinton', 'Concord' and 'Triumph', and maybe 'Ironclad' and 'Missouri Riesling' (*V. labrusca* × *V. riparia*). The same hypothesis can be done about the lower resistance displayed by 'Bianca' and 'Villard blanc' in Hungary. In fact, the extensive use of resistant hybrids in that region and the consequent reduction in treatments may

have led to the selection of strains that overcome 'Villard blanc' derived resistance mechanism, and apparently those strains are not present in Germany and, as suggested by the current study, also in Trentino region. Similarly, strains diversity has also to be considered regarding differences within years of assessment or USA regions. Those evidences suggested that particular attention should be given to the topic of strain genetic variability, selection and diffusion, and therefore genetic characterization when resistance assessments are made. Finally, where the discrepancy was very large and with no apparent justification, the identity of the evaluated genetic material was questioned, since cases of misnomers have already been reported (Maul and Töpfer 2015). This was the case of the questionable susceptibility of 'Seyval blanc' in Nebraska (Read and Gamet 2022), or the resistance of 'Chardonnay blanc' in Kentucky (Ward and Kaiser 2012).

Good practices for pathogen maintenance and inoculation

Genetic characterization of pathogen strains is a fundamental step for the harmonization of phenotypic data, as expressed by the International Union for the Protection of New Varieties of Plants (UPOV) in the European project "Harmorescoll" for the harmonization of reference collections of isolates for resistance DUS-testing (Distinctness, Uniformity and Stability). In modern breeding programs, this should be a mandatory step to take trace of the strain that have been used for selection, and a mix of strains should be recommended to avoid the selection of race-specific resistance mechanisms. Furthermore, during the process of breeding for biotic stresses, it is also very important to propagate the pathogen just on susceptible (*V. vinifera*) plants, avoiding selecting for new mutation overcoming the genetic barriers that are being introgressed.

The optimization of propagation and inoculation protocols is also crucial for an efficient phenotyping process when a relevant number of seedlings need to be screened. Being independent from plate propagation made the workflow much more rapid and efficient. Actually, despite the positive aspect of the possibility to grow *P. ampelicida* on cultural medium, the principle of 'one plate-one plant' generally adopted for artificial infection is not easily feasible because of the need of a lot of space in growing chambers, while a single infected leaf produces enough inoculum for many plants. Moreover, the routinary inoculation of *V. vinifera* permitted continuous feedback on infection dynamics during different seasons. In fact, according to our experience – even the controlled conditions of the greenhouse – the development of the disease was influenced by seasonal factors such as the cycle of natural light and the different rate of growth of the plants, suggesting spring and the first half of summer as the best period for BR inoculation. This introduced an additional level of complexity in the phenotyping procedures in terms of synchronization of pathogen and host life cycles. Lastly, this new approach permits to maintain a highly active and virulent inoculum, while there is evidence of a loss of these important characteristics producing the inoculum from the fungus propagated on the cultural medium.

Deployment of parental selection into disease resistance breeding programs

Although ex vivo assay experiments were useful for visualizing the early stages of conidia adhesion and hypha growth, assessment of disease progression was probably not feasible because the pathogen did not perceive adequate signals on detached leaves. According to in vivo assessments on leaves, 'Seyval blanc' was a symptom-free BR resistant genotype. It originated from the cross between 'Seibel 5656' and 'Rayon d'or' (Fig. S5), the latest being also the BR resistance donor of the fruit symptomless variety 'Csillam' (Kiss et al. 2017). Furthermore, 'Seibel 5656', brother of 'Subereux', is most probably the BR resistance donor of 'Villard blanc', as its mother 'Seibel 6468' was documented susceptible by Soursac (1908). Accordingly, excluding 'Seibel 5656' from the donors, we can conclude that both the resistance trait carried by 'Seyval blanc' and 'Villard blanc' originated from *V. rupestris* and *V. aestivalis* var. *lincecumii* as wild ancestors, respectively (Fig. 4). NY39 and NY24 were also found to be very high resistant to BR, and both share a complex genealogical tree combining *V. aestivalis*, *V. cinerea*, *V. labrusca*, *V. rotundifolia*, *V. rupestris* and *V. riparia*. 'Merzling' and 'Solaris' are respectively one and two

steps forward in the pedigree (Fig. S3) in respect to 'Seyval blanc'. Even if they still have a high degree of resistance, their response is characterized by an increased variability (Fig. 6). The decrease of resistance toward 'Seyval blanc' pedigree is evident also inspecting the different scores of the other 'Merzling' offspring (Fig. S3). This could be indirect evidence that the resistance of 'Seyval blanc' is based on several QTL, some of them not inherited by the 'Merzling' offspring. The breeding selection F25P52 resulted also very resistant and since it is descendant of 'Villard blanc', thus again suggested for the segregation of negative epistatic traits through 'Villard blanc' pedigree, as for 'Seyval blanc'. Finally, the genotypes that showed medium resistance in the high pressure of greenhouse experiments, as 'Charvir' and the breeding selection F12P19, are also promising candidates for the fight against BR under a well-timed organic farming management.

Conclusion

This work outlined good practices for black rot resistance evaluation for breeding, developing a new propagation and inoculation protocol based on fresh infected leaves. Moreover, it gave a comprehensive overview of black rot resistance resources to the grapevine community, and it allowed the spotlight on a subset of genotypes to be evaluated to identify new BR resistance donors within FEM parental lines and breeding selections that were readily introduced in the breeding pipeline. Those genotypes in fact have been already crossed both with *V. vinifera* varieties or with hybrids for the introgression of the trait into DM and PM resistance genotypes, for the generation of disease resistance super-donors.

Declarations

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