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Apulian Autochthonous Olive Germplasm: A Promising Resource to Restore Cultivation in *Xylella fastidiosa*-Infected Areas

Michele Antonio Savoia [1,†], Valentina Fanelli [1,†], Monica Marilena Miazzi [1,*], Francesca Taranto [2], Silvia Procino [1,2], Leonardo Susca [1], Vito Montilon [1], Oriana Potere [1], Franco Nigro [1,3] and Cinzia Montemurro [1,4]

[1] Department of Soil: Plant and Food Sciences (DiSSPA), University of Bari Aldo Moro, 70126 Bari, Italy; michele.savoia@uniba.it (M.A.S.); valentina.fanelli@uniba.it (V.F.); silvia.procino@uniba.it (S.P.); leonardo.susca@uniba.it (L.S.); vito.montilon@uniba.it (V.M.); oriana.potere@uniba.it (O.P.); franco.nigro@uniba.it (F.N.); cinzia.montemurro@uniba.it (C.M.)
[2] Institute of Biosciences and Bioresources (CNR-IBBR), 70126 Bari, Italy; francesca.taranto@ibbr.cnr.it
[3] Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile Caramia" (CRSFA), Locorotondo, 70010 Bari, Italy
[4] SINAGRI S.r.l.—Spin Off of the University of Bari Aldo Moro, Via Giovanni Amendola 65/a, 70126 Bari, Italy
* Correspondence: monicamarilena.miazzi@uniba.it; Tel.: +39-3393721987
† These authors contributed equally to this work.

Abstract: The olive tree (*Olea europaea* subsp. *europaea* var. *europaea*) represents the cornerstone crop of Apulian agriculture, which is based on the production of oil and table olives. The high genetic variability of the Apulian olive germplasm is at risk of genetic erosion due to social, economic, and climatic changes. Furthermore, since 2013, the spread of the Gram-negative bacterium *Xylella fastidiosa* subsp. *pauca* responsible for the olive quick decline syndrome (OQDS) has been threatening olive biodiversity in Apulia, damaging the regional economy and landscape heritage. The aim of this study was to investigate the differential response to *X. fastidiosa* infection in a collection of 100 autochthonous Apulian olive genotypes, including minor varieties, F1 genotypes, and reference cultivars. They were genotyped using 10 SSR markers and grown for 5 years in an experimental field; then, they were inoculated with the bacterium. Symptom assessments and the quantification of bacterium using a qPCR assay and colony forming units (CFUs) were carried out three and five years after inoculation. The study allowed the identification of nine putatively resistant genotypes that represent a first panel of olive germplasm resources that are useful both for studying the mechanisms of response to the pathogen and as a reserve for replanting in infected areas.

Keywords: Apulian germplasm; genetic diversity; olive breeding; symptomatology

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1. Introduction

Xylella fastidiosa (*X.f.*) is a globally distributed Gram-negative bacterium hosted by a wide range of plant species, including olive trees, grapes, almonds, fig, citrus, ornamentals (oleander, elm, periwinkle, and oak), and some wild plants [1]. In olives, it causes olive quick decline syndrome (OQDS), which consists of leaf scorch and the desiccation of terminal branches that rapidly spread to the rest of the canopy, even leading to tree death [2] as the xylem vessels are obstructed by the accumulation of bacterial biofilms [3]. The global spread of this pathogen continues to increase via the transport of commodities and plant material (EPPO Global Database). In 2013, *X. fastidiosa* subspecies *pauca* strain ST53 was identified for the first time in the Apulia region (southern Italy) as an agent of the severe epidemic that caused widespread desiccation and tree mortality in the olive groves of Salento [2,4,5]. The meadow spittlebug *Philaenus spumarius* was identified as the vector of the bacterium and is responsible for the rapid spread of the pathogen from its original *foci* area to the Apulia region [6–9].

The difficulty in determining the origin and actual impact of the damage caused by the disease slowed down sanitary efforts, resulting in the spread of the bacterium, which was also promoted by favorable climatic conditions [10]. Therefore, the spread of the epidemic has led to the loss of millions of olive trees, causing severe damage to the Apulian economy and a dramatic change in the landscape [11,12]. So far, various efforts have been made to prevent the disease and to control the pathogen, but these have only led to a slowing down of the epidemic and not to the containment of the disease or the eradication of the bacterium [1]. According to European and Italian legislation, the containment of *X.f.* consists of monitoring the infection status of plants in the buffer zone and the life stage of the meadow spittlebug in order to plan agronomical and chemical control measures [1,13].

The rapid spread of *X.f.* was favored by several co-factors related to biological, social, and climatic aspects. Indeed, olive tree cultivation plays an important social and symbolic role in the Apulia region, as ancient trees characterize the landscape of the region (e.g., the Valley of Millenary's olive trees in the area of Ostuni, Monopoli, and Fasano 40°43′ N; 17°34′ E), and the area is recognized as a World Heritage Site by UNESCO. Among the most commonly cultivated olive cultivars, Cellina di Nardò and Ogliarola Salentina proved to be the most susceptible, while Leccino and FS17 (also called Favolosa) are resistant to *X.f.* [14,15].

Some studies have attempted to decipher the mechanisms underlying the different responses of Leccino and FS17 compared to susceptible cultivars [16–18]. However, a comprehensive elucidation of the resistance mechanisms is still pending.

Current legislation restricts the conversion of infected areas with respect to Leccino and FS17 varieties only (Commission Implementing Decision (EU) 2015/789; https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32015D0789, accessed on 28 July 2023). However, the selection of accessions that combine resistance or tolerance to the bacterium with other economically relevant traits could be a valuable source for the conversion of infected areas into resilient agroecosystems [19]. In this scenario, the use of large olive germplasm collections, including modern and ancient genetic material, is a useful approach for identifying new sources of resistance [20–22].

There are more than 900 olive cultivars in the Mediterranean basin [23] and a large number of ecotypes, local genotypes, and wild trees (*Olea europaea* subsp. *europaea* var. *sylvestris*) [24]. However, the olive germplasm is characterized by high morphological and genetic variability and has many synonyms and homonyms due to misnaming by local farmers. To exploit olive biodiversity for the selection of agronomic traits and pathogen resistance, the accurate identification of available genotypes is required. Recently, the use of molecular markers and the development of next-generation sequencing (NGS) methods, which allow more reliable identification of cultivars, have replaced morphological characterization, which is strongly influenced by environmental factors [25–27]. Among molecular markers, simple sequence repeats (SSRs) are the tools of choice in olives due to their codominant nature, high reproducibility, ease of use, and low cost [28–31].

The identification of new putatively resistant genotypes among the local olive germplasm would limit the loss of cultivars with interesting agronomic traits, contributing to maintaining a high genetic diversity and helping to preserve the Apulian olive growing tradition by allowing replanting in infected areas. Moreover, these genotypes could play an important role in the studies of the mechanisms involved in tolerance relative to *X.f.* The aim of this study was to search for new genotypes that are tolerant or resistant to *X.f.* in addition to the Leccino and FS17 cultivars. Therefore, a collection of 100 olive genotypes established in 2017 within the RedOXy regional project was molecularly characterized using a set of 10 SSR markers and evaluated for resistance to the pathogen.

2. Materials and Methods

2.1. Plant Material and Infection with the Bacterium X.f. subsp. Pauca

A collection of 100 olive genotypes from rural areas of Apulia was screened for resistance to *X.f.*, including 81 local cultivars and 19 F_1 genotypes derived from the open

pollination of the Simone cultivar as the maternal parent, and selected for their interesting agronomic traits (Table 1). The varieties Leccino and Cellina di Nardò were also included as resistant and susceptible reference varieties, respectively. The trial was conducted in an experimental field in Parabita (Lecce, Italy), where disease pressure was high and the vector *P. spumarius* was abundant. Two-year-old plants grafted on olive seedlings were planted in autumn 2018 and arranged according to an experimental randomized block design with three replicates of four plants, each variety being represented by twelve plants. To promote infection with *X.f.*, in summer 2019, each plant was caged with ten infectious *P. spumarius* individuals; fine-mesh nets were used to cover the entire canopy for one month, and then they were removed. To acquire the bacterium, insects were collected using an entomological net by mowing the canopies of different host plant species in orchards in the OQDS infection area; they were confined for four days on symptomatic olive trees that tested positive for *X.f.* by qPCR and then transferred to the experimental field for pathogen transmission.

Table 1. List of genotypes tested in this study. For each accession, the site of origin, province and preferred (√) purpose were indicated.

Genotypes	Origin	Provinces	Purpose	
			Table	Oil
Ac'lin	Castellana Grotte	Bari	√	√
Bambina	Gravina In Puglia	Bari	√	√
Bella Di Cerignola	Ascoli Satriano	Foggia	√	
Bianca	Ceglie Messapica	Brindisi		√
Biancolilla	Valenzano (screen house)	Bari		√
Butirra Di Melpignano	Melpignano	Lecce	√	√
Caduta Morta	Terlizzi	Bari	√	√
Canna	Polignano A Mare	Bari	√	√
Carmelitana	San Severo	Foggia	√	√
Carolea	Valenzano (screen house)	Bari	√	√
Cazzinicchio	Bari	Bari		√
Cellina Di Nardo'	Carpignano Salentino	Lecce		√
Cerasuola	Valenzano (screen house)	Bari	√	√
Cima Di Bitonto	Bitonto	Bari		√
Cima Di Mola	Monopoli	Bari		√
Cipressino	Castellana Grotte	Bari	√	√
Colmona	Ginosa	Taranto	√	√
Coratina	Andria	Bari		√
Corna	Ceglie Messapica	Brindisi	√	√
Crogiola	Ceglie Messapica	Brindisi	√	√
Dolce Di Cassano	Cassano Delle Murge	Bari	√	√
Dolce Tonda	Sannicandro	Bari	√	√
Donna Francesca	Modugno	Bari		√
Donna Giulietta	Modugno	Bari		√
Fragolino	Chieuti	Foggia	√	√
Genotype_F10P1	Valenzano (screen house)	Bari		√
Genotype_F10P5	Valenzano (screen house)	Bari		√
Genotype_F3P1	Valenzano (screen house)	Bari		√
Genotype_F3P2	Valenzano (screen house)	Bari		√
Genotype_F4P1	Valenzano (screen house)	Bari		√
Genotype_F4P2	Valenzano (screen house)	Bari		√
Genotype_F5P2	Valenzano (screen house)	Bari		√
Genotype_F5P3	Valenzano (screen house)	Bari		√
Genotype_F5P4	Valenzano (screen house)	Bari		√
Genotype_F5P5	Valenzano (screen house)	Bari		√
Genotype_F6P2	Valenzano (screen house)	Bari		√
Genotype_F6P5	Valenzano (screen house)	Bari		√
Genotype_F7P2	Valenzano (screen house)	Bari		√

Table 1. *Cont.*

Genotypes	Origin	Provinces	Purpose	
			Table	Oil
Genotype_F7P3	Valenzano (screen house)	Bari		√
Genotype_F7P5	Valenzano (screen house)	Bari		√
Genotype_F8P2	Valenzano (screen house)	Bari		√
Genotype_F8P5	Valenzano (screen house)	Bari		√
Genotype_F9P1	Valenzano (screen house)	Bari		√
Genotype_F9P4	Valenzano (screen house)	Bari		√
Gniastra	Valenzano (screen house)	Bari		√
Grappa	Ostuni	Brindisi	√	√
Grappolo	Fasano	Brindisi		√
Gulliver	Chieuti	Foggia		√
Inchiastra Di Locorotondo	Locorotondo	Bari		√
Leccino Lazio	Valenzano (screen house)	Bari		√
Leccino_Ref	Valenzano (screen house)	Bari		√
Leccio Del Corno	Valenzano (screen house)	Bari		√
Lecciuolo	Valenzano (screen house)	Bari		√
Leucocarpa	Ascoli Satriano	Foggia		√
Lezze	Ceglie Messapica	Brindisi		√
Limongella	Polignano A Mare	Bari	√	√
Maggiorata	Bitetto	Bari	√	√
Marinese	Cerignola	Foggia		√
Matarrese	Turi	Bari		√
Mennella	Ceglie Messapica	Brindisi		√
Morosino	Torremaggiore	Foggia	√	√
Nocella	Santa Cesarea Terme	Lecce	√	√
Nolca	Bitonto	Bari	√	√
Ogliarola Garganica	Biccari	Foggia		√
Oliva Rossa	Locorotondo	Bari		√
Oliva Uva	Turi	Bari		√
Pasola Di Andria	Andria	Bari	√	√
Pepperinella 1	Chieuti	Foggia	√	√
Pepperinella 2	Chieuti	Foggia	√	√
Peppino Leo	Cassano Delle Murge	Bari	√	
Peranzana	San Severo	Foggia	√	√
Permezzana	San Giovanni Rotondo	Foggia	√	√
Pizzuta Della Daunia	Volturino	Foggia		√
Pizzuta Di Ginosa	Ginosa	Taranto		√
Provenzale	Chieuti	Foggia	√	√
Provenzale Di Serracapriola	Serracapriola	Foggia	√	√
Racioppa	Adelfia	Bari		√
Ravece	Orsara Di Puglia	Foggia		√
Ravece Guidacci	Orsara Di Puglia	Foggia		√
Rosciola	Chieuti	Foggia		√
Rosciola Gentile	Serracapriola	Foggia		√
Rosciolone	Serracapriola	Foggia	√	√
Rotondella	Cerignola	Foggia	√	√
Rumanella	San Marco La Catola	Foggia	√	√
Secolare Di Chieuti	Chieuti	Foggia	√	√
Seppunisi	Ceglie Messapica	Brindisi	√	√
Sessana	Ostuni	Brindisi		√
Silletta	Rutigliano	Bari		√
Simone	Castellana Grotte	Bari	√	√
Spina	Ceglie Messapica	Brindisi	√	√
Stingi Ieronimo	Volturino	Foggia		√
Termite Del Medico	Modugno	Bari	√	√
Termite Di Bitetto	Bitetto	Bari	√	√

Table 1. *Cont.*

Genotypes	Origin	Provinces	Purpose	
			Table	Oil
Tondina	San Severo	Foggia	√	√
Torremaggiorese	Torremaggiore	Foggia		√
Tunnella	Chieuti	Foggia		√
Uccellina	San Paolo Di Civitate	Foggia	√	√
Uggiana	Carpignano Salentino	Lecce	√	
Uovo Di Piccione	Massafra	Taranto	√	√
Zibimbolo	San Severo	Foggia		√

2.2. Olive Genotyping

Young leaves were collected from each plant, and genomic DNA was extracted according to [32]. DNA quality and concentration were checked via 0.8% agarose gel electrophoresis and a NanoDrop TM ND2000c (Thermo Scientific, Waltham, MA, USA) spectrophotometer. All concentrations were normalized to 50 ng/µL with 0.1 X TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

Genotyping was carried out by using a set of 10 highly informative SSR markers for the study of genetic variability in olives [33–35]. These markers were selected for their clear amplification, high polymorphism, and reproducibility [29]. PCR reactions were performed in a final volume of 12.5 µL and contained 1X Dream Taq buffer, 0.15 mM dNTP, 0.25 µM primer mix, 0.3 U Dream Taq, and 50 ng genomic DNA. PCR products were prepared as described in [32] and separated using an automated capillary sequencer ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the GeneScan 600 LIZ as an internal size standard (Applied Biosystems, Foster City, CA, USA). The allele size of each amplification product was estimated using GeneMapper v.5.0 software (Applied Biosystems, Foster City, CA, USA).

2.3. Analysis of the Data

Pairwise relatedness analysis (LRM) [36] was performed using GenAlEx v.6.502 software [37] to check the degree of allelic similarity between the analyzed genotypes and to identify the synonymies. In addition, the simple matching dissimilarity index was used to assess genetic relatedness.

Paternity analysis implemented in Cervus v.3.0 software was performed to identify the paternal parent of unknown genotypes using the 81 cultivars and autochthonous Apulian genotypes analyzed in this study, including the maternal parent Simone, and 94 Italian olive genotypes available from previous studies [21,22].

An unweighted neighbor-joining tree [38] was constructed using Darwin5 v.6.0.010 software (http://darwin.cirad.fr, accessed on 28 July 2023). The robustness of the branches was tested with 1000 bootstraps [39]. The molecular profiles of the resistant cultivar FS17® and the susceptible cultivar Ogliarola Salentina, obtained from a previous study by [21], were included in the phylogenetic analysis. A similarity/dissimilarity matrix was then generated using GenAlEx v.6.502 software to perform principal coordinate analysis (PCoA) [40].

2.4. Evaluation of the X.f. Symptoms

Each plant was visually inspected for symptoms from June to October and individually tested for *X.f.* using qPCR in the third and fifth year after the vector-mediated infection. Disease severity indicates the proportion of the foliar area affected by the disease of the plant unit [41]. It can be determined visually using qualitative assessments or by means of quantitative assessments, which are used for diseases in which the symptoms may be expressed on the entire plant [42]. The quantitative ordinal scale for phenotyping *X.f.* resistance is better suited for expressing disease severity at various stages of development in a given area where, due to high disease incidence, yield reduction or variations in growth

cannot be evaluated. In addition, the use of descriptive keys for disease severity class values allows for more accurate measurements and the interpretation of the proportions of the total symptomatic area of the crown. Assessments of the proportion of symptomatic plants are widely used in the selection of *X.f.*-resistant genotypes for different crops, including olive trees [19], and lend themselves to the standardization of the results obtained in different areas of the world [43].

Disease severity was rated on an empirical scale from 0 to 5, where 0 = no visible symptoms, 1 = symptoms confined to one or a few isolated twigs of the plant crown (less than 10% of the canopy showing symptoms), 2 = plant with symptoms on several twigs or on an entire branch (11 to 40% of canopy showing symptoms), 3 = plant with symptoms on several branches (41 to 60% of the tree crown with symptoms), 4 = plant with extensive symptoms (61 to 85% of the tree crown with symptoms), and 5 = severe symptoms with death of branches and tree decline (over 86% of canopy showing symptoms) (Figure 1).

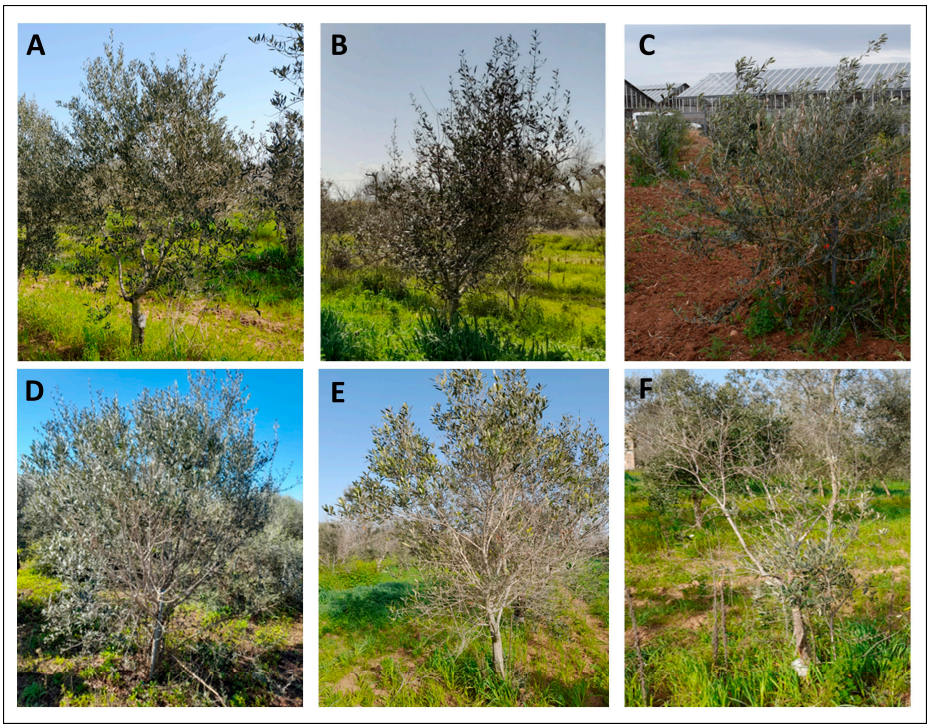

Figure 1. Disease severity rating scale used to assess olive quick decline syndrome (OQDS) symptoms: (**A**) no symptoms (0), (**B**) up to 10% (1), (**C**) 11–40% (2), (**D**) 41–60% (3), (**E**) 61–85% (4), and (**F**) over 86% (5) of disease incidence.

2.5. Quantification of X.f. in Plant

Mature leaves were randomly harvested from the canopy of each tree and pooled. Samples were kept refrigerated at 4 °C until further processing for quantitative PCR analysis (qPCR), which was performed within a few days after the collection. To quantify *X.f. in planta*, starting at the first leaf showing OQDS symptoms, 500 mg of petioles and small stem sections was homogenized for each sample using a Homex mechanical homogenizer (Bioreba, Switzerland) in extraction bags (BioReba, Basel, Switzerland) in the presence of 5 mL of CTAB extraction buffer (2% hexadecyl trimethyl-ammonium bromide, 0.1 M Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, and 1% PVP-40). Total nucleic acids were extracted using a modified CTAB protocol [2]. One mL of the plant extract was transferred to a 2.0 mL microcentrifuge tube and incubated at 65 °C for 30 min. Then, an equal volume (1 mL) of chloroform: isoamyl alcohol = 24:1 v/v was added and mixed well with the vortex for a few seconds. The solution was centrifugated at 16,000× g for 10 min, and 750 µL of the supernatant was transferred to a new 1.5 mL microcentrifuge tube containing

450 µL of pre-cooled isopropanol. After careful twirling, the solution was incubated at −20 °C for 20 min and then centrifuged at 16,000× *g* for 10 min; the supernatant was aspirated and discarded. The pellet was washed in 1 mL of pre-cooled 70% ethanol and centrifuged at 16,000× *g* for 10 min. The supernatant was discarded; then, the pellet was dried in a vacuum centrifuge for 10 min and dissolved in 120 µL of TE. DNA concentration and quality were checked using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, the concentration of each DNA extract was normalized to 100 ng/µL.

Amplification reactions were performed in a C1000TM Thermal Cycler with a CFX96 Real-Time System fluorescence detector (BioRad Laboratories, Hercules, CA, USA) using cycling conditions and specific primers for *X.f.* according to the protocol developed by [44] and using specific primers targeting the 16S rRNA processing protein gene (im) of *X.f.* The primer and probe sequences were as follows: 5′-CAC GGC TGG TAA CGG AAG A-3′; 5′-GGG TTG CGT GGT GAA ATC AAG-3′; 5′ 6FAM -TCG CAT CCC GTG GCT CAG TCC-BHQ-1- 3′. The reactions were performed in a final volume of 11 µL containing the following reagents: 3.7 µL H20; 5.5 µL Taq Man Fast Advanced Master Mix 2X (Thermo Fisher Scientific, Waltham, MA, USA); 0.3 µM of each primer; 0.1 µM of the probe; 1 µL (100 ng/µL) of extracted DNA.

The absolute quantification of *X.f.* cells (CFU/mL) was determined by extrapolating the mean CT (Cycle Threshold) for each test sample into standard curves obtained by plotting the CT values of the decimal dilutions of genomic *X.f.* DNA obtained from 1×10^{-4} to 1×10^{-8} CFU/mL cell suspensions of the bacterium. This was cultured on a Buffered Charcoal Yeast Extract (BCYE) medium at 28 °C for 8–10 days prior to DNA extraction. Known DNA samples from healthy and infected olive trees were included as negative and positive controls, respectively, in all amplification reactions. All samples were tested in duplicate, and the data were subsequently averaged.

3. Results

3.1. Olive Genotyping

The SSR fingerprinting of 100 genotypes resulted in clear allele profiles for all samples. To investigate the genetic relationship between the analyzed genotypes and to show the possible presence of synonyms in the collection, LRM analysis was performed, setting 0.50 as the value for identical genetic profiles. The results showed two cases of synonymy; namely, a genetic identity was found for Pepperinnella1/Ravece Guidacci and Morosino/Pizzuta della Daunia (LRM value = 0.50). The other genotypes were unique, although high allelic similarity was found for the varieties Lezze/Racioppa, Grappa/Pizzuta di Ginosa, and Rosciola/Rotondella (Table 2).

Table 2. List of genotypes with LRM values > 0.40.

Genotypes with 0.40 < LRM < 0.50		
Pepperinella1	Ravece Guidacci	0.50
Morosino	Pizzuta della Daunia	0.50
Lezze	Racioppa	0.45
Grappa	Pizzuta di Ginosa	0.42
Rosciola	Rotondella	0.41

The paternity test performed on the F_1 individuals of the collection made it possible to identify a parent candidate for six F_1 genotypes (Table 3), while both candidate parents were identified only for the F_5P_2 genotype. None were of the Simone variety (Table 3).

Genetic relationships among olive genotypes were elucidated using an unweighted neighbor-joining phylogenetic tree and principal coordinate analysis (PCoA). The phylogenetic analysis divided the collection into two main clusters: cluster A included most of the genotypes (76) and the two resistant references, and cluster B included the remaining

22 genotypes with the two susceptible references Cellina di Nardò and Ogliarola Salentina (Figure 2).

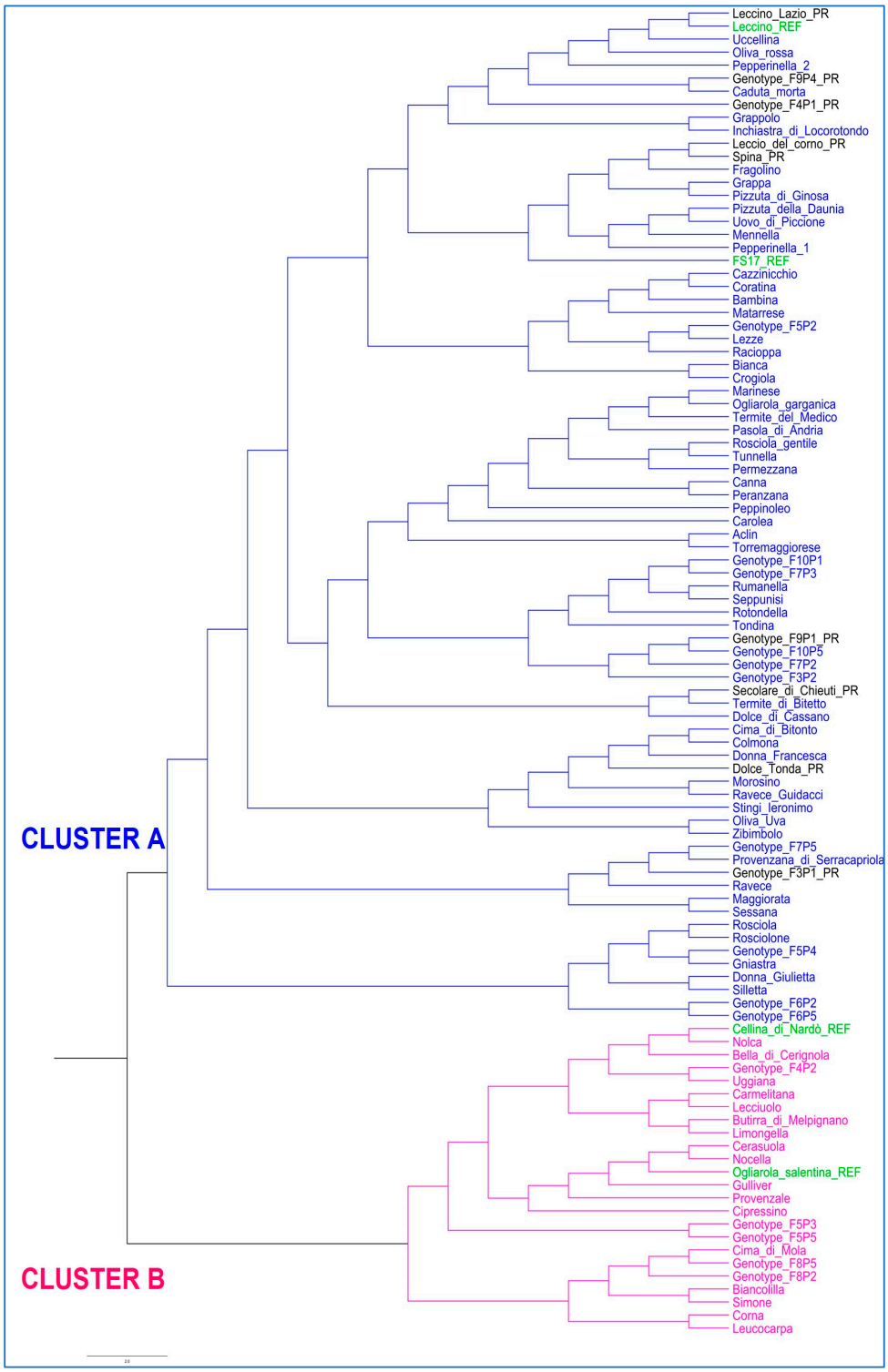

Figure 2. Dendrogram of the 100 genotypes analyzed in our study; the molecular profiles of the cultivars FS17 and Ogliarola Salentina, from a previous study by [21], were also included. The names of the genotypes are colored according to their cluster: blue for genotypes of cluster A and pink for genotypes of cluster B. The reference varieties are shown in green, while putatively resistant (PR) genotypes are marked in black.

Table 3. Putative parent of the F_1 genotypes obtained from the open pollination of the Simone variety determined by the paternity test.

F_1 Genotype	First Candidate	Pair Loci Mismatching	Second Candidate	Pair Loci Mismatching
F10P1	Dolce di Sannicandro	1	-	-
F4P1	Leccino REF	0	-	-
F5P2	Lezze	0	Racioppa	0
F6P5	Framichele	1	-	-
F8P5	Sivigliana	0	-	-
F9P1	Caduta morta	1	-	-

PCoA confirmed our evidence and grouped most of the varieties included in cluster A of the tree close to the two resistant Leccino and FS17 reference varieties and the two susceptible Cellina di Nardò and Ogliarola Salentina reference varieties, which clearly stand out from the main group (Figure 3).

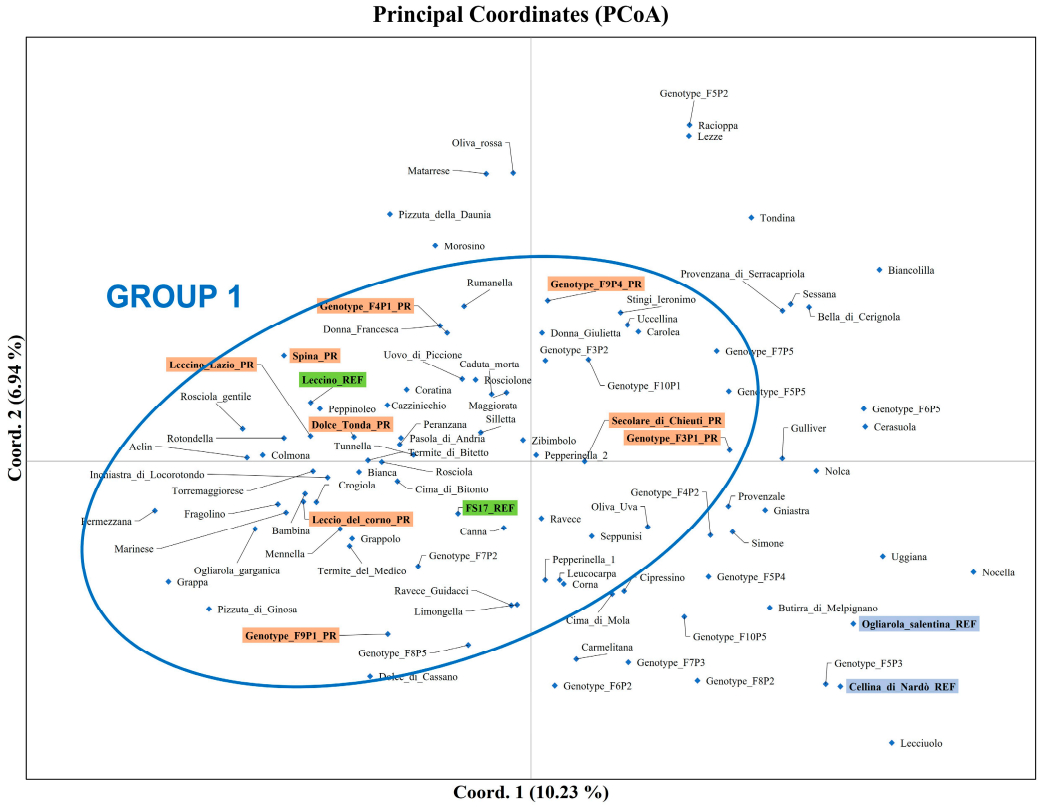

Figure 3. Principal coordinate analysis (PCoA) plot of olive genotypes. The resistant and susceptible references are shown in green and blue, respectively. Putatively resistant (PR) genotypes are indicated in orange.

3.2. In Planta Assessment of Susceptibility to X.f. and the Quantification of the Bacterium

At the first symptom assessment three years after infection, all genotypes were asymptomatic except for the Gulliver and Rosciola Gentile varieties, which had symptom scores of 1 and 3, respectively (Table 4). As the disease progressed, symptoms increased in all cultivars, reaching the highest score of 5 in Torremaggiorese and a score of 4 in varieties Leucocarpa and Bianca five years after the vector-mediated inoculation.

Table 4. OQDS symptoms (SYM), Cq values, and CFU/mL measured for all genotypes three and five years after inoculation with the *X.f* bacterium. The values for the reference varieties and putative resistant (PR) genotypes (Cq > 27) are indicated in bold.

Genotypes	Spring 2021					Spring 2023				
	SYM (0–5)	Cq	SD	CFU/mL	SD	SYM (0–5)	Cq	SD	CFU/mL	SD
Bella Di Cerignola	0	24.17	4.48	7.22×10^5	3.73×10^5	3	22.32	0.55	6.72×10^5	2.36×10^5
Bianca	0	22.44	2.41	1.05×10^6	8.72×10^5	4	21.77	2.78	2.32×10^6	2.54×10^6
Butirra Di Melpignano	0	24.19	0.05	1.74×10^5	4.76×10^3	1	24.45	1.87	2.36×10^5	2.04×10^5
Carmelitana	0	25.24	2.44	2.28×10^5	1.74×10^5	2	22.73	2.7	4.01×10^5	3.47×10^5
Cerasuola	0	NA	NA	NA	NA	1	26.95	7.14	2.14×10^5	4.28×10^5
Cima Di Mola	0	21.22	1.44	1.72×10^6	1.05×10^6	1	22.63	1.40	9.98×10^5	7.40×10^5
Cipressino	0	26.11	5.33	3.18×10^5	3.14×10^5	2	26.55	2.78	7.95×10^4	7.64×10^4
Corna	0	26.90	1.84	3.82×10^4	2.74×10^4	1	23.33	2.24	5.24×10^5	3.30×10^5
Genotype_F4P2	0	28.85	0.69	7.50×10^3	3.95×10^3	2	23.64	0.51	2.67×10^5	9.68×10^4
Genotype_F5P3	0	22.65	0.37	5.17×10^5	7.18×10^4	2	22.52	1.91	6.47×10^5	7.54×10^5
Genotype_F5P5	0	23.06	1.01	4.30×10^5	1.97×10^5	2	21.93	1.22	6.62×10^5	7.82×10^5
Genotype_F8P2	0	29.08	4.33	2.48×10^4	3.41×10^4	1	23.43	1.46	3.00×10^5	3.38×10^5
Genotype_F8P5	0	27.77	5.43	2.75×10^5	2.71×10^5	3	20.75	0.85	2.12×10^6	1.16×10^6
Gulliver	1	28.67	4.47	3.54×10^4	3.45×10^4	1	25.54	3.57	2.26×10^5	2.60×10^5
Lecciuolo	0	NA	NA	NA	NA	1	23.70	6.15	1.25×10^6	2.50×10^6
Leucocarpa	0	21.94	0.67	8.95×10^5	2.58×10^5	4	24.07	2.64	4.53×10^5	4.93×10^5
Limongella	0	26.94	4.18	1.96×10^5	1.87×10^5	2	23.03	1.93	6.82×10^5	6.59×10^5
Nocella	0	23.78	3.22	5.87×10^5	3.24×10^5	1	25.57	3.51	1.46×10^5	1.87×10^5
Nolca	0	27.19	6.23	2.32×10^5	2.31×10^5	1	22.39	1.73	8.99×10^5	8.01×10^5
Provenzale	0	27.38	0.00	19075.58	0.00	1	23.99	2.61	3.83×10^5	2.77×10^5
Simone	0	25.08	6.06	9.25×10^5	1.30×10^6	3	22.08	0.41	7.77×10^5	2.25×10^5
Cellina Di Nardo'	**0**	**23.43**	**2.77**	**6.16×105**	**3.03×10^5**	**2**	**21.45**	**1.84**	**1.79×10^6**	**1.41×10^6**
Uggiana	0	22.83	0.00	4.48×10^5	0.00	2	25.58	2.39	8.92×10^4	9.80×10^4
Ac'lin	0	30.30	2.23	4.21×10^3	3.36×10^3	2	22.95	1.44	6.12×10^5	6.58×10^5
Bambina	0	27.70	0.12	1.53×10^4	1.24×10^3	1	21.45	3.63	1.62×10^6	1.81×10^6
Biancolilla	0	28.17	0.92	1.32×10^4	5.20×10^3	2	23.06	1.15	4.73×10^5	3.13×10^5
Caduta Morta	0	30.80	0.00	1.78×10^3	0.00	1	23.48	2.94	5.14×10^5	6.95×10^5
Canna	0	28.89	0.00	6.71×10^3	0.00	1	22.67	2.00	9.22×10^5	1.08×10^6
Carolea	0	23.24	0.00	3.36×10^5	0.00	1	22.97	2.97	5.20×10^5	4.26×10^5
Cazzinicchio	0	28.27	4.47	9.95×10^4	9.63×10^4	3	23.52	0.28	2.81×10^5	5.72×10^4
Cima Di Bitonto	0	22.10	1.13	9.17×10^5	4.21×10^5	2	22.05	0.06	3.83×10^5	4.43×10^5
Colmona	0	31.11	0.00	1.44×10^3	0.00	1	25.15	2.00	1.05×10^5	1.14×10^5
Coratina	0	23.33	2.53	5.95×10^5	5.04×10^5	1	23.67	2.09	5.07×10^5	6.36×10^5
Crogiola	0	21.88	0.96	9.65×10^5	4.24×10^5	2	21.66	1.05	1.22×10^6	9.74×10^5
Dolce Di Cassano	0	20.64	0.00	2.05×10^6	0.00	1	25.39	3.70	5.18×10^5	9.40×10^5
Dolce Tonda_PR	**0**	**36.30**	**0.00**	**6.44×10^4**	**0.00**	**0**	**30.72**	**0.00**	**6.28×10^2**	**1.09×10^3**
Donna Francesca	0	30.77	1.02	2.06×10^3	9.59×10^2	1	26.23	2.80	6.61×10^4	7.39×10^4
Donna Giulietta	0	NA	NA	NA	NA	2	22.77	3.74	1.79×10^6	1.92×10^6
Fragolino	0	27.71	0.61	1.21×10^4	6.85×10^3	1	25.25	3.38	1.14×10^5	2.18×10^5
Genotype_F10P1	0	23.49	1.87	4.11×10^5	2.98×10^5	2	22.57	2.39	1.49×10^6	2.33×10^6
Genotype_F10P5	0	25.32	4.42	3.54×10^5	3.45×10^5	2	22.87	1.54	5.65×10^5	5.12×10^5
Genotype_F3P1_PR	**0**	**29.51**	**0.00**	**4.37×10^3**	**0.00**	**2**	**30.68**	**4.56**	**4.29×10^4**	**8.44×10^4**
Genotype_F3P2	0	26.38	0.77	4.10×10^4	1.49×10^4	1	24.23	0.73	1.39×10^5	1.26×10^5
Genotype_F4P1_PR	**0**	**23.65**	**0.00**	**2.53×10^5**	**0.00**	**0**	**28.04**	**0.84**	**6.59×10^3**	**8.70×10^3**
Genotype_F5P2	0	27.01	3.52	1.08×10^5	9.59×10^4	1	23.34	2.81	7.91×10^5	9.74×10^5
Genotype_F5P4	0	26.64	5.07	1.94×10^5	2.70×10^5	3	23.89	1.49	2.88×10^5	1.95×10^5
Genotype_F6P2	0	25.67	1.70	9.71×10^4	5.99×10^4	2	22.60	0.24	5.29×10^5	9.15×10^4
Genotype_F6P5	0	26.26	0.00	4.16×10^4	0.00	2	25.36	4.10	2.53×10^5	2.03×10^5
Genotype_F7P2	0	27.01	3.52	1.08×10^5	9.59×10^4	1	23.34	2.81	7.91×10^5	9.74×10^5
Genotype_F7P3	0	30.47	0.00	2.24×10^3	0.00	2	26.56	4.35	2.36×10^5	4.60×10^5
Genotype_F7P5	0	29.72	2.41	6.74×10^3	5.59×10^3	2	21.37	1.37	1.25 X 106	1.51 X 10^6
Genotype_F9P1_PR	**0**	**28.80**	**0.00**	**7.12×10^3**	**0.00**	**0**	**31.67**	**0.00**	**9.75×10^2**	**0.00**
Genotype_F9P4_PR	**0**	**30.40**	**0.00**	**2.92×10^5**	**0.00**	**2**	**28.09**	**2.29**	**2.48×10^4**	**3.24×10^4**
Gniastra	0	25.27	2.16	1.59×10^5	1.11×10^5	1	26.72	4.42	6.67×10^4	1.31×10^5
Grappa	0	25.80	0.87	2.26×10^5	0.00	1	22.14	0.07	3.60×10^5	4.17×10^5
Grappolo	0	23.06	1.45	4.82×10^5	2.96×10^5	2	21.77	2.24	1.92×10^6	2.08×10^6
Inchiastra Di Locorotondo	0	26.20	0.00	4.34×10^4	0.00	3	22.56	1.10	6.58×10^5	4.28×10^5

Table 4. *Cont.*

Genotypes	Spring 2021					Spring 2023				
	SYM (0–5)	Cq	SD	CFU/mL	SD	SYM (0–5)	Cq	SD	CFU/mL	SD
Leccino Lazio_PR	**0**	**33.49**	**0.72**	**3.02×10^2**	**1.62×10^2**	**1**	**32.33**	**0.00**	**0.00**	**0.00**
Leccino_Ref	**0**	**-**	**-**	**-**	**-**	**0**	**32.93**	**0.00**	**9.67×10^3**	**1.32×10^4**
Leccio Del Corno_PR	**0**	**33.66**	**5.10**	**1.51×10^3**	**2.10×10^3**	**1**	**28.66**	**5.32**	**2.67×10^4**	**5.34×10^4**
Lezze	0	21.27	1.65	1.78×10^6	1.19×10^6	1	20.57	1.27	2.81×10^6	2.11×10^6
Maggiorata	0	27.04	0.00	3.41×10^3	0.00	3	20.97	1.06	2.02×10^6	1.64×10^6
Marinese	0	25.40	1.73	1.05×10^5	7.23×10^4	2	23.77	0.41	1.80×10^5	1.34×10^5
Matarrese	0	24.66	0.00	1.25×10^5	0.00	2	21.44	1.44	1.25×10^6	1.63×10^6
Mennella	0	23.50	0.00	2.82×10^5	0.00	2	23.02	1.24	5.44×10^5	5.60×10^5
Morosino	0	22.55	0.66	5.81×10^5	1.35 x10^5	1	22.72	1.64	5.18×10^5	5.96×10^5
Ogliarola Garganica	0	25.27	0.00	8.23×10^4	0.00	1	24.04	0.98	1.08×10^5	1.48×10^5
Oliva Rossa	0	23.33	1.49	4.57×10^5	2.73×10^5	2	21.70	1.17	1.22×10^6	8.56×10^5
Oliva Uva	0	23.80	0.00	2.28×10^5	0.00	3	22.20	1.03	3.94×10^5	5.39×10^5
Pasola Di Andria	0	21.43	0.83	1.28×10^6	4.94×10^5	2	21.48	1.05	1.40×10^6	1.05×10^6
Pepperinella 1	0	27.30	8.02	5.17×10^5	5.16×10^5	2	23.49	2.84	8.34×10^5	1.21×10^6
Pepperinella 2	0	26.93	4.55	1.23×10^5	1.20×10^5	1	26.22	0.90	3.67×10^4	3.61×10^4
Peppino Leo	0	23.98	0.00	2.02×10^5	0.00	0	26.69	3.81	7.78×10^4	1.05×10^5
Peranzana	0	21.90	2.40	1.51×10^6	1.25×10^6	2	20.80	0.33	1.85×10^6	4.28×10^5
Permezzana	0	26.27	0.00	4.12×10^4	0.00	0	25.62	2.28	5.48×10^4	9.60×10^4
Pizzuta Della Daunia	0	26.90	0.00	2.67×10^4	0.00	3	24.60	2.68	3.65×10^5	4.67×10^5
Pizzuta Di Ginosa	0	25.35	3.51	2.14×10^5	1.06×10^5	2	22.58	1.44	7.49×10^5	6.10×10^5
Provenzale Di Serracapriola	0	28.38	0.00	9.57×10^3	0.00	1	24.71	0.00	3.04×10^4	6.07×10^4
Racioppa	0	22.20	0.05	6.93×10^5	1.86×10^4	2	22.64	3.65	1.45×10^6	1.26×10^6
Ravece	0	29.92	0.00	3.29×10^3	0.00	1	22.74	0.15	4.77×10^5	5.18×10^4
Ravece Guidacci	0	23.98	0.00	2.02×10^5	0.00	2	22.47	1.31	7.29×10^5	4.61×10^5
Rosciola	0	22.43	0.00	5.92×10^5	0.00	3	21.25	1.06	1.64×10^6	1.22×10^6
Rosciola Gentile	3	20.70	0.00	1.96×10^6	0.00	2	24.01	0.00	1.97×10^5	0.00
Rosciolone	0	22.69	2.20	8.36×10^5	3.98×10^5	2	21.27	1.39	1.91×10^6	1.97×10^6
Rotondella	0	25.08	4.94	5.29×10^5	5.57×10^5	3	20.93	0.53	1.76×10^6	6.55×10^5
Rumanella	0	23.20	1.98	6.32×10^5	7.40×10^5	2	21.89	0.86	9.96×10^5	6.90×10^5
Secolare Di Chieuti_PR	**0**	**30.72**	**6.93**	**1.30×10^5**	**2.24×10^5**	**1**	**27.11**	**2.98**	**1.33×10^5**	**2.49×10^5**
Seppunisi	0	25.59	0.00	6.62×10^4	0.00	0	24.08	0.10	1.25×10^5	1.09×10^5
Sessana	0	30.40	0.00	2.35×10^3	0.00	0	26.70	2.79	1.23×10^5	2.16×10^5
Silletta	0	24.66	0.00	1.26×10^5	0.00	1	21.47	2.23	2.38×10^6	2.87×10^6
Spina_PR	**0**	**25.55**	**0.00**	**6.78×10^4**	**0.00**	**2**	**27.37**	**5.70**	**1.05×10^5**	**1.82×10^5**
Stingi Ieronimo	0	24.31	1.83	2.30×10^5	2.33×10^5	3	21.18	0.79	1.59×10^6	9.53×10^5
Termite Del Medico	0	27.62	0.00	1.62×10^4	0.00	2	23.79	2.64	5.17×10^5	9.28×10^5
Termite Di Bitetto	0	24.40	0.00	1.51×10^5	0.00	1	25.03	1.66	1.52×10^5	1.47×10^5
Tondina	0	25.49	1.43	1.00×10^5	6.03×10^4	1	24.21	1.86	2.90×10^5	2.68×10^5
Torremaggiorese	0	24.96	1.13	1.76×10^6	8.14×10^5	5	21.25	1.14	1.20×10^6	1.17×10^6
Tunnella	0	25.25	0.00	8.36×10^4	0.00	1	23.63	1.57	3.52×10^5	2.28×10^5
Uccellina	0	26.66	4.96	1.80×10^5	2.51×10^5	2	22.23	0.73	7.34×10^5	3.32×10^5
Uovo Di Piccione	0	22.20	0.00	6.94×10^5	0.00	1	23.37	0.21	1.54×10^5	1.80×10^5
Zibimbolo	0	26.25	0.00	4.18×10^4	0.00	3	25.84	0.00	5.57×10^4	0.00

The results of *X.f.* quantification carried out 3 and 5 years after infection are presented in Table 4 and Figure 4, together with disease symptomatology. A wide range of responses to the pathogen was observed. Positive qPCR reactions were obtained in the majority of plants caged with infected specimens of the vector *P. spumarius*. At the first time point, Cq values ranged from 20.64 for Dolce di Cassano to 33.66 for Leccio del Corno, with values > 23.43 for most varieties as for the susceptible Cellina di Nardò, while a very high Cq value was found in Leccino (34.10).

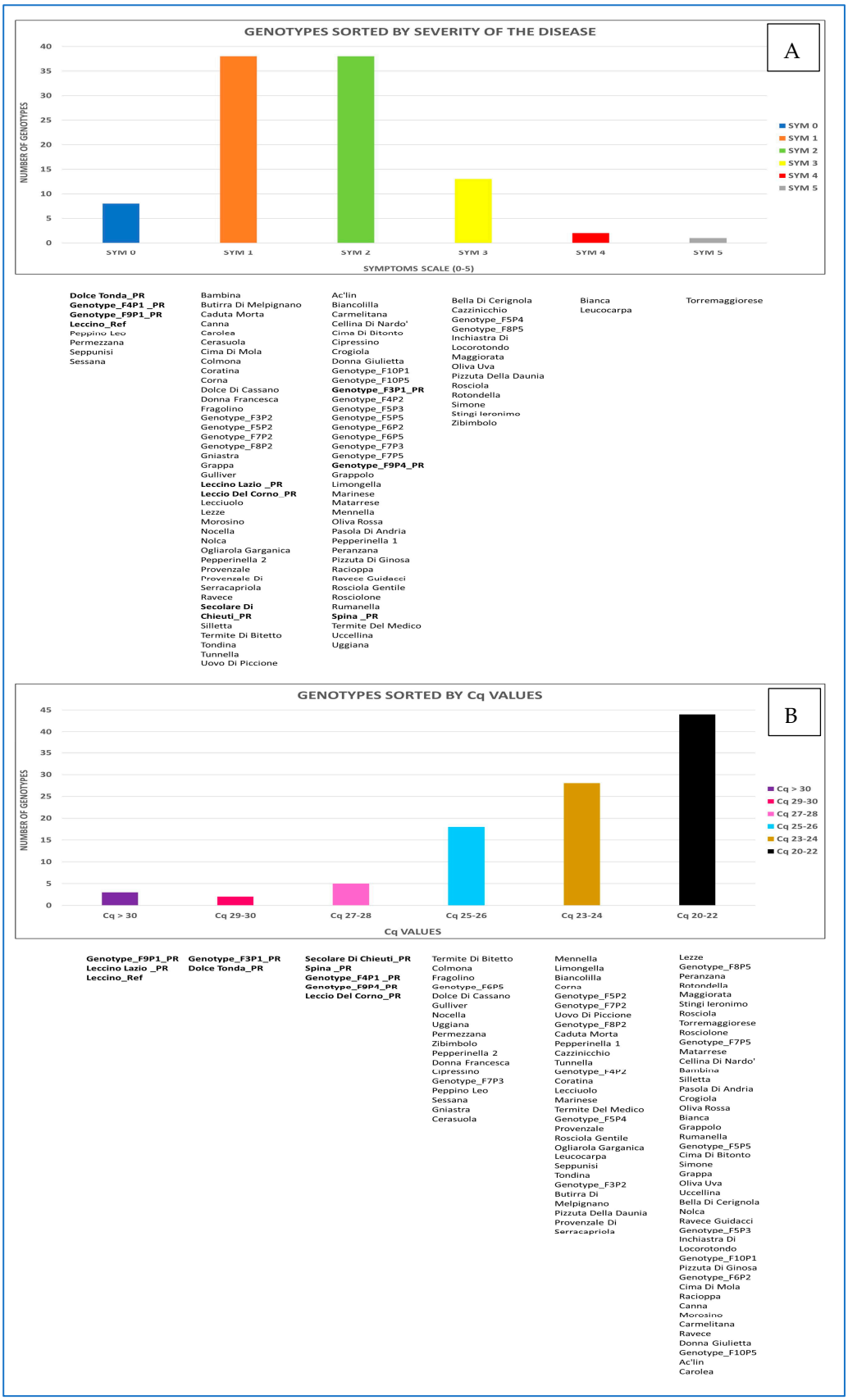

Figure 4. Histograms representing the symptoms (**A**) and Cq values (**B**) observed for the 100 genotypes analyzed in our study.

At the second assessment, most varieties, including the reference-resistant cultivar Leccino (32.93), showed a decrease in Cq values. However, Cq values did not change in 11 genotypes and increased in nine samples. The CFU/mL values were consistent with the Cq values (Table 5).

Table 5. List of genotypes showing a constant or an increasing Cq in the two evaluation periods.

Varieties				
Constant Cq	**Spring 2021**		**Spring 2023**	
	Cq	**SD**	**Cq**	**SD**
Pasola Di Andria	21.43	±0.83	21.48	±1.05
Crogiola	21.88	±0.96	21.66	±1.05
Cima Di Bitonto	22.10	±1.13	22.05	±0.06
Genotype_F_5P_3	22.65	±0.37	22.52	±1.91
Racioppa	22.20	±0.05	22.64	±3.65
Morosino	22.55	±0.66	22.72	±1.64
Mennella	23.50	0.0	23.02	±1.24
Coratina	23.33	±2.53	23.67	±2.09
Butirra Di Melpignano	24.19	±0.05	24.45	±1.87
Pepperinella 2	26.93	±4.55	26.22	±0.90
Cipressino	26.11	±5.33	26.55	±2.78
Genotype_F_3P_1_PR	29.51	0.0	30.68	±4.56
Increasing Cq				
Rosciola Gentile	20.70	0.0	24.01	0.00
Leucocarpa	21.94	±0.67	24.07	±2.64
Dolce Di Cassano	20.64	0.0	25.39	±3.70
Nocella	23.78	±3.22	25.57	±3.51
Uggiana	22.83	0.0	25.58	±2.39
Peppino Leo	23.98	0.0	26.69	±3.81
Spina _PR	25.55	0.0	27.37	±5.70
Genotype_F_4P_1 _PR	23.65	0.0	28.04	±0.84
Genotype_F_9P_1_PR	28.80	0.0	31.67	0.00

Based on the Cq value and the symptoms observed five years after infection, we decided to consider genotypes with Cq values > 27 and symptom values ≤ 2 as putatively resistant (PR). This threshold was used to select the most promising varieties found in Secolare di Chieuti, Spina, Leccio del Corno, Dolce Tonda, and Leccino Lazio and in the F_3P_1, F_4P_1, F_9P_1, and F_9P_4 genotypes. The remaining 90 samples had a Cq value between 20 and 26, with Lezze having the lowest Cq value (20.57). To further characterize the PR genotypes, a paternity test was performed, identifying the putative parents for Dolce Tonda, Leccino Lazio, Secolare di Chieuti, and Spina (Table 6).

Table 6. Results of the paternity test indicating the presumptive parents of the putative resistant (PR) genotypes.

PR Genotypes	**First Candidate**	**Pair Loci Mismatching**	**Second Candidate**	**Pair Loci Mismatching**
Dolce_Tonda_PR	Coratina	0	Nocellara_del_Belice	0
Leccino_Lazio_PR	Leccino_REF	0	Frantoiana	0
Secolare_di_Chieuti_PR	Gulliver	1	Ciddina_FG	0
Spina_PR	-	-	Grappa	1

Symptoms associated with *X.f.* infection did not reflect Cq values in some cases. The most symptomatic cultivars (score > 3), which were Torremaggiorese, Bianca, and Leucocarpa, had Cq values between 21.25 and 24.07. Among the nine genotypes with a Cq value > 27, varieties Leccino, Dolce Tonda, F_4P_1, and F_9P_1 were asymptomatic. The remaining genotypes had a score of 1 or 2. Interestingly, the accession Lezze had a score of

1 on the symptom scale despite having a Cq value of 21.27 and 20.57 in the two evaluations. Varieties Seppunisi, Permezzana, Peppino Leo, and Sessana did not show any symptoms despite a Cq value < 27.

In the cluster analysis, the nine putative resistant genotypes fell into cluster A, and five of them, Leccino Lazio, F_9P_4, F_4P_1, Leccio del Corno, and Spina, belonged to the same subcluster as the resistant varieties Leccino and FS17 (Figure 2). Five F_1 genotypes with a low Cq value between 20.75 (F_8P_5) and 23.64 (F_4P_2) and the two cultivars Bianca and Leucocarpa, with a Cq value of 21.77 and 24.07 and a high symptom score, showed high genetic similarity with the two susceptible cultivars Ogliarola Salentina and Cellina di Nardò. In the PCoA analysis, putatively resistant (PR) samples were grouped in group 1 together with the two resistant references. The two susceptible references stand in the lower quadrant on the right of the graph and are separated from the principal group (Figure 3).

4. Discussion

The first outbreak of the quarantine pathogen *Xylella fastidiosa* in the EU was detected on olive trees in Apulia in 2013 [4]. Since then, the disease has spread widely and caused severe landscape and economic damage. So far, only the Leccino and FS17 varieties are considered resistant [14]. However, in recent years, several regional and national projects have been carried out to identify new sources of tolerance/resistance to *X.f.* in order to be used for replanting in infected areas by characterizing local accessions and studying their response to the pathogen [19]. In 2017, an evaluation program was implemented for this purpose by the University of Bari Aldo Moro in the infected area, where one hundred cultivars/accessions were studied in a trial with randomized blocks of three replicates of four plants.

4.1. Genetic Diversity Assessment

The 100 genotypes were genetically characterized with a set of 10 SSR molecular markers routinely used for olive genotyping, and the data were used to study the genetic relationships between them and the resistant and susceptible reference varieties. The only cases of synonymy revealed by the LRM analysis refer to four varieties in the province of Foggia (Northern Apulia): "Pepperinnella 1-Ravece Guidacci" and "Morosino-Pizzuta della Daunia". It is likely that the misnaming is due to differences in morphology and use (Table 1), which led local farmers to consider these varieties as different. In addition, the LRM analysis showed a clear differentiation of most varieties, although some pairs of varieties were strongly related, having pairwise values > 0.40, such as the pairs "Lezze-Racioppa", "Grappa-Pizzuta di Ginosa", and "Rosciola-Rotondella". These results are in line with those of Miazzi et al. (2020) for Apulian varieties. It is likely that during the process of selection, which occurred within the Apulian agroecosystem, the local varieties were derived from crosses among selected trees or pollen coming from feral or wild olive trees [45,46], indicating the importance of the local role in the diversification process [47]. This can be deduced by the results of the parental analysis for the nineteen F_1 genotypes derived from the open pollination of the Simone variety. Any F_1 genotypes appeared to be derived from Simone, thus they probably were derived from crosses with other local varieties. It is likely that the Simone variety used, although certified, was misidentified. This underlines the need to improve the protocols for the certification and marketing of olive varieties [48]. Despite these results, these F_1 genotypes were retained in our analysis as carrying interesting agronomic traits.

4.2. Evaluation of the Response to X.f. Infection

Infection was monitored using quantitative real-time polymerase chain reaction (qPCR), a diagnostic tool that can detect the pathogen in the early stages of the disease even if the infected plant does not yet show symptoms [49]. The first assay was performed in 2021, three years after infection with the pathogen. No symptoms were observed during the visual assessment in the first two years (2018–2020). In the third year of assessment, almost

all samples showed no symptoms, with the exception of Gulliver and Rosciola Gentile. This was expected as OQDS has a slow progression [50]. The evaluation performed on the second date, five years after inoculation, confirmed the resistance of the cultivar Leccino and the susceptibility of the cultivar Cellina di Nardò [15,51,52]. Based on the Cq value and the symptomatology, nine accessions could be classified as putatively resistant (PR) in the second assessment. These were Secolare di Chieuti, Spina, Leccio del Corno, Dolce Tonda, and Leccino Lazio and genotypes F_4P_1, F_9P_4, F_3P_1, and F_9P_1. Among them are the accessions Dolce Tonda, F_4P_1, and F_9P_1, which have Cq values of 30.72, 28.04, and 31.67, respectively, and they do not show symptoms that deserve more attention. The PR accessions will need to be further characterized as they represent a valuable resource for studying the mechanisms involved in the response to the *X.f.* pathogen.

The susceptible reference cultivar Cellina di Nardò did not seem to be the most susceptible cultivar among the studied genotypes. In fact, the lowest Cq value and the highest CFU/mL value were observed in the cultivar Lezze, although Cellina di Nardò had a symptom score of 2 and Lezze had a symptom score of 1. A discrepancy between the Cq value and symptomatology has been noted before. Studies conducted on different plant species, such as plum, coffee, citrus, and grapevine, have shown that sometimes the symptomatology does not reflect pathogen concentrations [53–56]. The intensification of leaf scorch symptoms during *X.f.* pathogenesis has been shown to be due to several factors related to the physiological status of the plant. For example, some growth regulators, such as ethylene, can stimulate and accelerate leaf senescence, which exacerbates the symptomatology associated with OQDS [57,58]. This could partly explain the leaf scorch symptoms in trees with low concentrations of pathogens and the behavior of PR genotypes Spina, F_9P_4, and F_3P_1, which show a Cq > 27 despite the symptom score of 2. These results show that it is necessary to understand in detail the impact of the developmental status of plants on the manifestation of the symptomatology due to *X.f.* infection.

Symptom scores consistent with the Cq values were found for the cultivars Torremaggiorese, Bianca, and Leucocarpa, which had low Cq values and a high incidence of symptoms due to *X.f.* (symptoms scores between 4 and 5). For this reason, these accessions can be considered highly susceptible to the bacterium. At the same time, varieties Secolare di Chieuti, Leccio del Corno, Dolce Tonda, and Leccino Lazio and PR genotypes F_4P_1 and F_9P_1 showed no or only minor symptoms (score of 1). The symptomatology of the cultivar Leccino Lazio, albeit to a lesser extent (symptoms score of 1), is in line with the results of an earlier study [59], confirming that the resistant Leccino cultivar can also show disease symptoms.

According to [60], plants that consistently show positive qPCR results at 6 and 12 months after inoculation can be considered systemically infected. In the study, Cellina di Nardò, Leccino, and FS17 cultivars all had Cq values of less than 22.05 but a completely different symptomatology. The authors concluded that, despite a similar bacterial load, the different host responses to bacterial infections were due to variations in the physiological state of the plant rather than the direct influence of the pathogen's abundance [60].

In most of the genotypes studied, the Cq value decreases over the years, indicating an increasing bacterial load over time. However, eleven genotypes showed a constant Cq value, and nine genotypes showed an increasing trend. We hypothesized that the increase in Cq values from 2021 to 2023 may depend on the sampling of the plant material, which may influence the qPCR assay's result; indeed, the different concentrations and spatial variability of the pathogen in the plant could be due to the irregular distribution of xylem vessels [61]. Thus, future studies need to be performed by sampling larger portions of the canopy in order to increase the reliability of the detection method [56].

4.3. Comparison between Genetic Data and the Response to X.f.

A comparison between genetic information from the analyzed accessions and their response to the pathogen can provide information on the role of the genetic background with respect to susceptibility/resistance to *X.f.*

LRM analysis highlighted the genetic similarity of five pairs of accessions (Table 2). In these samples, such as the Rosciola and Rotondella varieties, we found a similar response to the bacterium (Cq of 21.25 and 20.93 and a symptomatic score of 3) (Table 4), indicating a possible correlation between the genetic background and the response to the infection.

The genetic relationships between the analyzed genotypes, investigated using phylogenetic analysis and PCoA, showed similar results, separating the two resistant references Leccino and FS17 and five PR accessions from the two susceptible varieties Cellina di Nardò and Ogliarola Salentina. Among PR accessions, F_1 genotypes F_4P_1 and F_9P_1 were highly resistant to *X.f.* infection. Interestingly, a putative parent of F_4P_1 was identified as Leccino, which is presumed to be responsible for its tolerance, while the putative parent of F_9P_1 was the susceptible Caduta Morta variety (Cq value of 23.48 and a symptom score of 1 at the second time point of evaluation). The great variability in the response to bacterial infection shown by F_1 genotypes could be the result of the genetic recombinations in the progenies. Likewise, the promising PR accession Dolce Tonda had the highly susceptible Coratina variety as a putative parent. This is not surprising because the resistant FS17 cultivar also has the partially susceptible Frantoio as a parent [62,63]. Both F_4P_1 and F_9P_1 will require further investigation in order to identify both parents and to elucidate the mechanisms involved in response to OQDS.

For the remaining PR genotypes (Leccino Lazio, Secolare di Chieuti, and Spina), the putative parents were identified in the autochthonous Apulian germplasm, highlighting the importance of minor neglected accessions in the identification of new sources of tolerance.

Both phylogenetic analysis and PCoA grouped the PR genotypes with the two resistant references (Leccino and FS17). In particular, five of them (Leccino Lazio, Leccio del Corno, Spina, F_9P_4, and F_4P_1) were in the same subcluster of the two references. Similarly, five highly susceptible F_1 genotypes (F_4P_2, F_5P_3, F_5P_5, F_8P_2, and F_8P_5) and cultivars Bianca and Leucocarpa exhibited high genetic similarity with respect to the two susceptible cultivars Ogliarola Salentina and Cellina di Nardò (Figure 2). These results suggest a possible share of genetic background and indicate the need to further characterize the mechanisms of responses to the pathogen.

5. Conclusions

In the last decade, several multidisciplinary approaches have been adopted to limit the spread of *X.f.* in Apulia. However, to date, no complete understanding of the pathogenicity of the bacterium, the infection process, and the defense mechanisms of host plants has been achieved. In olive trees, which are of great importance for Apulia's economy and tradition, only the Leccino and FS17 varieties exhibit high tolerance to the bacterium. In our work, we molecularly characterized and evaluated a collection of 100 local olive genotypes after infection with *X. fastidiosa* for bacterial load and symptomatology. We identified nine putatively resistant genotypes, of which genotypes Dolce Tonda, F_4P_1, and F_9P_1 proved to be of particular interest due to their low bacterial load and the absence of symptoms. The further characterization of these genotypes will allow the identification of new sources of tolerance among the local autochthonous Apulian germplasm and the dissection of the mechanisms involved in plant responses to *X.f.* infection.

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