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RICCARDO RUSSO

# Traditional and advanced methods to detect genotypes resistant to Mal secco disease

*PhD Thesis*

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# Index

## Abstract

Mal secco is the most severe disease that caused heavy yield losses to the Italian lemon industry. It is a tracheomycotic disease caused by the fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley. Since the first reported disease in the Sicilian lemon orchards, the main goal for breeders and lemon growers was to identify lemon clones resistant to Mal Secco Disease (MSD). Different genetic improvement methods were used to obtain resistant plant material, but none of them had fully solved the problem. Some lemon (*C. limon* L. Burm. *f.*) clones with some degree of tolerance and high fruit quality have been selected (Femminello Zagara Bianca, Femminello Continella), but these selections usually get infected in many growing area with high pressure of the pathogen. Consequently, growers can only use Monachello, a clone having high tolerance to the disease but also low fruit quality. This lemon clone has guaranteed the survival of lemon orchards in many areas afflicted by the disease, but it does not guarantee the Italian lemon industry's competitivity. The project's main goal was identify sources of tolerance and resistance to the disease in the citrus germplasm and to enhance the knowledge about the genes involved in MSD tolerance, resistance, and sensitivity. Two approaches were used, based on traditional techniques and transcriptomic analysis. A phenotypic survey based on the evaluation of the different level of tolerance or resistance to MSD, has been performed in a germplasm field planted at CREA, Italy, in an area of high pathogen pressure and was combined with a molecular screening for the presence of the pathogen based on real-time PCR. The results revealed sources of tolerance in lemon and citron hybrids. The molecular screening identified *P. tracheiphilus* in all lemon clones and clones without apparent symptoms, indicating their ability to tolerate the disease. This project also provided a reliable method for MSD detection by Real time PCR analysis by which a significant correlation with disease symptoms was calculated. Based on the survey result, we selected the most suitable parents to generate two segregating populations to introduce the putative resistance genes in lemon genotypes, which may help to detect possible Quantitative Trait Loci (QTLs) associated with the resistance to MSD.

Using Simple Sequence Repeats (SSRs) molecular markers, these populations were screened to verify the parentage of the progenies and discard the F1 hybrids derived from selfing. A genome-wide mapping of QTLs controlling the resistance to MSD will be a desirable tool to use in the next years, with the final purpose to identify the most valuable molecular markers suitable for marker-assisted selection (MAS) in the breeding programs.

With the main aim of identifying candidate genes involved in the defence response of citrus plants to MSD, we performed a *de novo* transcriptome analysis of rough lemon (*Citrus jambiri* Lush.) seedlings subjected to artificial inoculations of *P. tracheiphilus* in comparison with plants inoculated with water. Under fungus challenge, the rough lemon seedlings significantly down-regulated the genes involved in the light-harvesting and the photosynthetic electron flow, thus probably inducing a shortage of energy for cellular functions. Moreover, the systemic acquired resistance (SAR) is activated through the induced salicylic acid cascade, probably preparing the plants to a successive pathogen attack.

Interestingly, RPM1 interacting protein 4, an essential positive regulator of plant defence, and BIR2, which is a negative regulator of the basal level of immunity (namely PTI, pathogen-associated molecular patterns triggered immunity) have been identified thus representing useful targets genes for future breeding. The identification of candidate genes involved in plant -pathogen interaction could be useful for future biotechnological approaches. In particular, the application of new plant breeding techniques (NPBTs), specifically genome editing and cisgenesis, can offer an alternative to conventional breeding strategies to modify resistance or susceptibility genes in high-quality, susceptible lemon varieties.

## Sintesi

Il Mal secco degli agrumi è la più grave micopatia che colpisce il limone (*C. limon* L. Burm. *f.*) ed è causa di ingenti perdite di produzioni alla limonicoltura italiana. L'agente patogeno della malattia è il fungo *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley. Il principale obiettivo per i breeder e per i limonicoltori, fin dalla prima segnalazione della malattia nei limoneti siciliani, è stato quello di identificare cloni di limone resistenti al Mal secco degli agrumi (MSD). Differenti metodi di miglioramento genetico sono stati utilizzati, ma nessuno di essi fino ad oggi ha permesso di raggiugere i risultati sperati. Infatti, sebbene qualche varietà di limone meno suscettibile alla malattia e con buone caratteristiche produttive sia stata selezionata (Femminello Zagara Bianca, Femminello Continella), in alcuni areali l'alta pressione del patogeno ha consentito agli agrumicoltori il solo utilizzo del Monachello, un clone di limone che ha garantito la sopravvivenza della limonicoltura nelle aree severamente afflitte dalla malattia ma che non ha assicurato, per le sue mediocri caratteristiche qualitative, competitività alla limonicoltura italiana. Il principale obiettivo del progetto è stato identificare sorgenti di tolleranza e resistenza alla malattia in germoplasma di agrumi ed implementare le conoscenze sui geni coinvolti nella resistenza, tolleranza o sensibilità al MSD. Sono stati utilizzati due approcci, basati su tecniche tradizionali di miglioramento genetico e sull'analisi trascrittomica. L'indagine è stata effettuata nel campo di germoplasma del CREA di Acireale, Italia, in un'area con un'alta pressione del patogeno. I genotipi in collezione sono stati sottoposti ad analisi di fenotipizzazione dei sintomi della malattia, e alla diagnosi molecolare, tramite Real-time PCR, per la valutazione della presenza del patogeno. L'analisi molecolare ha permesso di diagnosticare il patogeno in tutti i cloni di limone, anche in quei cloni senza chiari sintomi, indicando la loro attitudine a tollerare la malattia stessa. Inoltre, questo lavoro, combinando due tecniche analitiche, fenotipizzazione e diagnosi molecolare, fornisce un valido metodo per la valutazione della presenza del MSD, infatti la correlazione calcolata tra le due variabili ottenute è risultata significativa. In base ai risultati dell'indagine fenotipica, sono stati selezionati i parentali più adatti a generare due popolazioni segreganti, con la possibilità di indentificare dei QTLs (Quantitative Trait Locus) relativi alla resistenza al patogeno. Queste popolazioni, grazie all'utilizzo di marcatori molecolari SSRs (Simple Sequence Repeats), sono state analizzate in modo da eliminare tutti gli ibridi F1 derivanti dall'autoimpollinazione del parentale femminile. La mappatura dei QTLs che controlla la resistenza al MSD sarà uno strumento utile, da utilizzare nei prossimi anni, con lo scopo di identificare quei marcatori molecolari adatti ad essere utilizzati nella selezione assistita da marcatori (MAS) per i programmi di breeding. Con l'obiettivo di identificare i principali geni candidati coinvolti nella riposta difensiva al MSD, è stata effettuata un'analisi *de novo* del trascrittoma di semenzali di limone rugoso (*Citrus jambiri* Lush.) confrontando piante inoculate con *P. tracheiphilus* e piante controllo inoculate con acqua. I principali risultati di questo studio hanno evidenziato che, a causa del patogeno, il limone rugoso ha significativamente sottoespresso i geni coinvolti nella fase luminosa della fotosintesi, probabilmente inducendo una carenza di energia per le funzioni cellulari. In aggiunta, la resistenza sistemica acquisita (SAR) è stata indotta attraverso la cascata di reazioni attivate dall' acido salicilico, probabilmente nel tentativo di preparare le piante ad un successivo attacco del patogeno.
È interessante notare, come la RPM1 interacting protein 4, un regolatore essenziale che favorisce la difesa delle piante, e BIR2, un altro regolatore che modifica negativamente il livello basale di immunità (chiamato PTI, pathogen-associated molecular patterns triggered immunity) siano stati identificati, rappresentando obiettivi utili per il miglioramento genetico del limone. L'identificazione di geni candidati coinvolti nell'interazione ospite-patogeno potrebbe essere utile in futuri approcci biotecnologici. In particolare, l'applicazione di nuove *New Plant Breeding Techniques* (NPBTs), *genome editing* e *cisgenesis*, possono offrire dei metodi alternativi per l'ottenimento di genotipi di limone resistenti al MSD.

## Keywords:

*Breeding; Citrus;* de novo assembly*; lemon; phenotyping; mapping populations;* Plenodomus tracheiphilus*; polyploids; real-time PCR; RNAseq; rough lemon; SAR; SSR.*

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# INTRODUCTION

## 1. Mal secco disease

### 1.1 History, geographical distribution, and economic impact

The name of the disease comes from two Italian words: “Male” (disease) and “Secco” (dry) (Nigro et al., 2011). The term Mal secco refers to nonspecific symptoms that were initially used in a broad sense to denote citrus diseases of various origins (Savastano, 1925). In 1929 Petri used the term “mal secco disease of citrus” to indicate the tracheomycotic disease that was spreading in lemon orchards in Sicily, and he described the fungus causing Mal secco disease as a new species and named it *Deuterophoma tracheiphila*, which he proposed as the type-species of the new genus *Deuterophoma* (Petri, 1929). Later the species was transferred to the genus *Phoma* by Kantschaveli and Gikachvili in 1948 (Boerema et al., 2004), and in 1969 Ciccarone and Russo improved the description of the fungus, and they confirmed this binomial as the correct name (Ciccarone and Russo, 1969; Ciccarone, 1971).

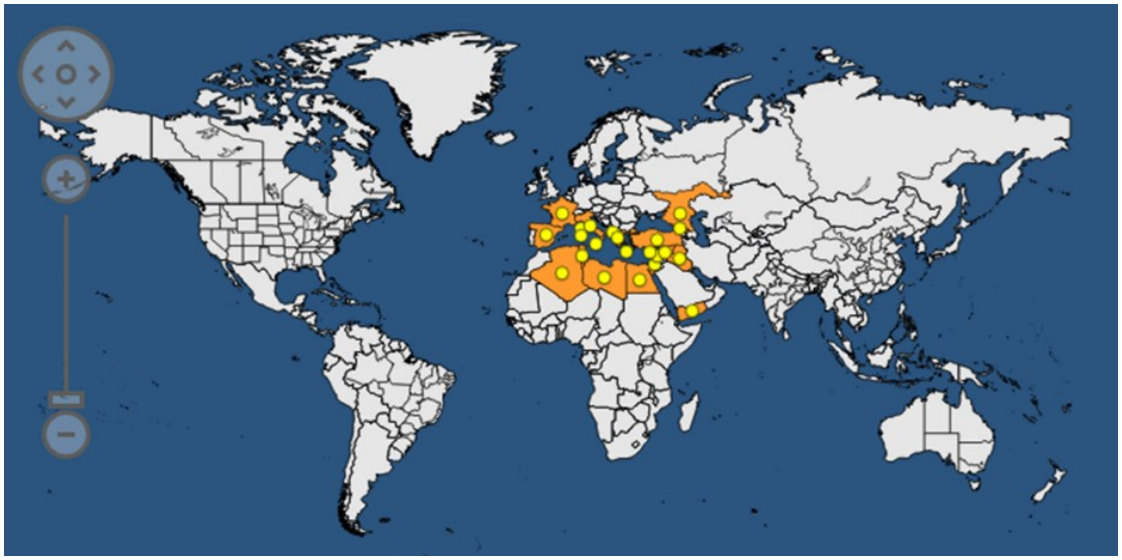

Figure 1. The picture represents the spread of MSD into the different countries. The yellow point shows in which country MSD was reported (EPPO Global Database, 2020).

Recently, based on phylogeny studies, determined by analysis of sequence data of the large subunit 28S nrDNA (LSU) and Internal Transcribed Spacer regions 1 & 2 and 5.8S nrDNA (ITS) the fungus was reclassified in the genus *Plenodomus* (De Gruyter et al., 2013) as *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley (ex *Phoma tracheiphila* (Petri) Kantschaveli & Gikachvili). Mal secco disease (MSD) was discovered for the first time in 1894 in two Aegean Greek islands: Chios and Poros, and later it spread to the other Mediterranean and the Black Sea countries. It was recently found in Spain, while it is not present in Portugal, Morocco, Maltha, and Croatia (EPPO Global Database, 2020) (Figure 1).

MSD was reported for the first time in Italy in 1918 (Sarejanni, 1935, 1939; Ruggieri, 1948) and, it still has a significant impact on the Italian lemon industry. Lemon (*C. limon* L. Burm. *f.*) is one of the most sensitive species to this pathogen. It has been estimated that about 3,000 ha of lemon orchards in Sicily (the region that accounts for more than 90% of Italian lemon production) were destroyed 15 years after its first detection (Savastano, 1923; Casella, 1935). While later, Ruggieri reported that in the years from 1918 to 1953, MSD has destroyed in Sicily no less than 12,000 ha of

lemon groves (Ruggieri, 1953). According to Salerno and Cutuli (Salerno and Cutuli, 1981), the mean yield production of lemon orchards in Sicily was about 20 tons/ha in the presence of MSD, whereas in lemon orchards not affected by MSD yield could reach 60 to 80 tons/ha. In the other Mediterranean countries, the situation was not different; for example, in the district of Mersin (Turkey), at least 20.000 lemon plants were destroyed by the disease in 15 years (Karel, 1956) and other two Turkish researchers reported an average annual yield loss of 12,3% (Akteke and Karaca, 1977). In Greece, the situation was critical; in fact, the annual yield loss estimated was 70, 45, 54, and 53 % in Patras, Temeni, Alissos, and Chania respectively (Thanassoulopoulos and Manos, 1992). The disease is very damaging for lemon, but also citron (*C. medica L.*), bergamot (*C. bergamia* Risso and Poit.), and chinotto (*C. myrtifolia* Raf.) (Catara and Cutuli, 1972; Nigro et al., 2011; Migheli et al., 2009). Regarding the susceptibility to MSD of other citrus species, Cutuli et al., (1984) reported the most comprehensive list regarding the behavior of citrus species and accessions in response to *Plenodomus* infection. However, the information of the listed accessions regards phenotypic observation of plants in different developmental stages (young seedlings or adult plants), or in response to different sources of inoculum (artificial or natural) so it is not possible to perform an appropriate comparison among them and define a clear ranking of susceptibility. Additional information regarding species susceptibility is reported in alemow (*C. macrophylla* Wester), Yuzu orange (*C. junos* Sieb. ex Tan.), common sour orange (*C. aurantium* L.), S. Marina selection (*C. aurantium* L.), siamelo (*C. paradisi* Macf. x *C. sinensis* (L.) Osbeck) and, Nansho Daidai sour orange (*C. taiwanica* [Tan. and Shim]). No additional information has been reported in the last one decades. Table 1 summarizes the known degree of susceptibility of 134 citrus species and accessions based on the above cited references.

| Latin binomial and common name | Susceptibility (1) | References |
|---|---|---|
| *Citrus jambiri* Lush., Rough lemon | +++ | (Ruggieri, 1948; Reichert and Chorin, 1956; Russo, 1977; Reforgiato Recupero, 1979) |
| | ++ (2) | (Crescimano et al., 1973) |
| *C. volkameriana* Ten. et Pasq., Volkamer lemon | + | (Russo, 1956) |
| | ++ | (Russo, 1977) |
| | +++ | (Salerno et al., 1967; Catara and Cutuli, 1972) |
| *Citrus meyeri* Y. Tan., Meyer lemon | ++ | (Ruggieri, 1948; Russo, 1977) |
| | + | (Hohryakov, 1952; Egorova, 1958; Dzhanelidze, V.S., Razmadze, 1960) |
| *C. limonimedica* Lush., Citron lemon | + | (Russo, 1977; Catara and Cutuli, 1972) |
| *Citrus limonia* Osbeck, lemon CRC. 2322 | +++ | (Russo, 1977) |
| *Citrus limonia* Osbeck, lemon CRC. 2323 | +++ | (Russo, 1977) |
| *C. ichangensis* Swing. x *C. maxima* (Burm.) Merr., Ichang lemon CRC. 1215 | + | (Russo, 1977) |
| [*C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr. × *C. medica* (L.)], Cardinale lemon | + | (Russo, 1977) |
| Citronnier Japonnais | +++ | (Russo, 1977) |
| *Citrus limonia* Osbeck, Borneo lemon CES 2424 | +++ | (Ruggieri, 1948) |
| Lemon real (Filippine CES 2317 | +++ | (Ruggieri, 1948) |
| *C. webberii* West., Kalpi | +++ (2) | (Ruggieri, 1948) |
| *C. webberii* West., Kalpi CES 767 | +++ | (Ruggieri, 1948) |
| *C. webberii* West., Kalpi CEC 2092 | +++ | (Russo, 1977) |
| *Poncirus trifoliata* (L.) Raf. Barnes trifoliata CES 2554 | +++ | (Ruggieri, 1948) |
| *Citrus spp*. (Filippine) CES 760 | +++ | (Ruggieri, 1948) |
| *Citrus spp*. (Filippine) CES 643 | +++ | (Ruggieri, 1948) |
| *Citrus s*pp. (Cina) CES 1213 | +++ | (Ruggieri, 1948) |
| *Citrus Karna Raf.*, Karna lemon | +++ | (Ruggieri, 1948; Catara and Cutuli, 1972) |
| Italian lemon Lyallpur | +++ | (Ruggieri, 1948) |
| Id lemon or Id limbu (Poena) | +++ | (Ruggieri, 1948) |
| *C. medica* L., Diamante citron | +++ | (Petri, 1930; Ruggieri, 1948; Catara and Cutuli, 1972) |

| | | |
|---|---|---|
| *C. medica* L., Etrog citron | +++ | (Reichert and Fawcett, 1930; Catara and Cutuli, 1972; Solel and Oren, 1975) |
| *C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr., Vozza Vozza citron | + | (Russo, 1977) |
| *C. limettoides* Tanaka, sweet lime of Palestine | ++ | (Ruggieri, 1948; Russo, 1977) |
| | +++ | (Reichert and Chorin, 1956) |
| | + | (Catara and Cutuli, 1972; Solel and Oren, 1975) |
| *C. limonia Osbeck*, Rangpur lime | +++ | (Ruggieri, 1948; Chapot, 1963; Russo, 1977) |
| | ++ | (Catara and Cutuli, 1972) |
| *C. aurantifolia* (Christm.) Swing., Mexican lime | +++ | (Ruggieri, 1948; Catara and Cutuli, 1972) |
| | +++ (2) | (Crescimano et al., 1973) |
| *C. limetta* Risso, Romana lime | +++ | (Ruggieri, 1948) |
| Zuccherina lime | +++ | (Ruggieri, 1948) |
| Rough lime | +++ | (Ruggieri, 1948) |
| *C. aurantifolia* (Christm.) Swing. Egiziana lime | +++ | (Ruggieri, 1948) |
| *C. latifolia* Tanaka, Bearss seedless lime | +++ | (Ruggieri, 1948) |
| *Microcitrus australasica* (F. Muell.) Swing., Australian red lime CRC 2319 | +++ | (Russo, 1977) |
| *Citrus limonia* Osbeck, Phylippine red lime CRC 2318 | +++ | (Russo, 1977) |
| | ++ (3) | (Reforgiato Recupero, 1979) |
| *Citrus limonia* Osbeck, Santa Barbara red lime CRC 712 | +++ | (Russo, 1977) |
| *Citrus limonia* (India) CRC 2476 | +++ | |
| *C. bergamia* Risso, bergamot | + | (Ruggieri, 1948) |
| | +++ | (Catara and Cutuli, 1972; Terranova and Cutuli, 1975) |
| *C. deliciosa* Ten., common mandarin | + | (Ruggieri, 1948) |
| *C. deliciosa* Ten., Bonaccorsi mandarin | + | (Ruggieri, 1948) |
| *C. reticulata* Blanco, Wilking mandarin | ++ | (Catara and Cutuli, 1972; Solel and Oren, 1975) |
| *C. reticulata* Blanco, Michal mandarin | ++ | (Solel and Oren, 1975) |
| *C. reticulata* Blanco, Ponkan mandarin | + | (Russo, 1977) |
| *C. oleocarpa* hort. ex. Tanaka, Tim-Kat mandarin | + | (Russo, 1977) |
| *C. Kinokuni* hort. ex. Tanaka, Kinokuni mandarin | + | (Russo, 1977) |

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|---|---|---|
| *C. reticulata* Blanco, hung Kat mandarin | + | (Russo, 1977) |
| *C. reticulata* Blanco, Kinnow mandarin | +++ (3) | (Russo, 1977) |
| *Citrus nobilis Lour.,* King mandarin | + | (Battiato, C., 1948) |
| *Citrus nobilis Lour., hybr.* Japanese hybrid mandarin | + | (Ruggieri, 1948) |
| *C. clementina* Tan., common clementine | ++ | (Catara and Cutuli, 1972; Grasso S., 1973; Solel and Oren, 1975; Cutuli, G., Salerno, M., 1980) |
| *C. clementina* Tan., Nules clementine | ++ | (Catara and Cutuli, 1972) |
| *C. clementina* Tan., Oroval clementine | ++ | (Catara and Cutuli, 1972) |
| *C. reshni* Hort. ex. Tan., Cleopatra mandarin | + | (Ruggieri, 1953; Russo, 1977; Reforgiato Recupero, 1979) |
| *Citrus unshiu* Marcovitch, Satsuma mandarim | ++ | (Donadze, 1970) |
| *C. macrophylla* Wester, alemow | +++ | (Catara and Cutuli, 1972; Crescimano et al., 1973; Russo, 1977; Reforgiato Recupero, 1979; Protopapadakis and Zambettakis, 1982; E. Traversa, 1991; Nigro et al., 2000; 2011, 2015) |
| *C. junos* Sieb. ex. Tan., Yuzu orange C. | +++ | (Ruggieri, 1948; Catara and Cutuli, 1972; Russo, 1977; Reforgiato Recupero, 1979; Nigro et al., 1996; 2008; 2011, 2015) |
| | ++ (2) | (Crescimano et a., 1973) |
| *C. sinensis* (L.) Osbeck, sweet orange | + | (Ruggieri, 1948) |
| | ++ | (Donadze, 1966) |
| *C. sinensis* (L.) Osbeck, Valencia sweet orange | ++ | (Solel and Oren, 1975) |
| *C. sinensis* (L.) Osbeck, Washington Navel sweet orange | ++ | (Solel and Oren, 1975) |
| *C. aurantium* L., common sour orange | +++ | (Ruggieri, 1940, 1948; Salerno, 1964; Catara and Cutuli, 1972; Nigro et al., 2008; Migheli et al., 2009; Nigro et al., 2011, 2015) |
| *C. aurantium* L., Pennisi | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., African CES 1705 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Florida CES 1690 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Dai Dai CES 656 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Auratium CES 2371 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Brasilian CES 1110 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Paraguay CES 660 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Standard CES 660 | +++ | (Ruggieri, 1948) |

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|---|---|---|
| *C. aurantium* L., Standard CES 628 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L., Bouquetier | + | (Ruggieri, 1948) |
| *C. aurantium* L., Santa Marina 1 | ++ (3) | (Reforgiato Recupero, 1979) |
| *C. aurantium* L., Santa Marina 2 | ++ (3) | (Reforgiato Recupero, 1979; Nigro et al., 2008; 2011, 2015) |
| *C. aurantium* L., Dai Dai | +++ (2) | (Crescimano et a., 1973) |
| *C. aurantium* L., Bittersweet | +++ (2) | (Crescimano et a., 1973) |
| *C. aurantium* L., Sicilian sour | +++ (2) | (Crescimano et a., 1973) |
| *C. aurantium* L., California sour | +++ (2) | (Crescimano et a., 1973) |
| *C. paradisi* Macf., grapefruit | + | (Ruggieri, 1948; Catara and Cutuli, 1972; Solel and Oren, 1975; Russo, 1977) |
| | +++ | (Donadze, 1966) |
| *C. maxima* (Burm.) Merr., Cuban Shaddock CRC 1462 | ++ | (Russo, 1977) |
| *C. myrtifolia* Raf., chinotto | +++ | (Ruggieri, 1948; Catara and Cutuli, 1972) |
| *C. madurensis* Lour., calamondin | ++ | (Ruggieri, 1948) |
| *C. madurensis* Lour., calamondin CRC 643 | + | (Russo, 1977) |
| *C. madurensis* Lour., Calamondin Chi Chiek CRC 2592 | + | (Russo, 1977) |
| *C. ichangensis,* Ichang papeda CES 1219 | +++ | (Ruggieri, 1948) |
| *C. ichangensis,* Ichang papeda CRC 2431 | + | (Russo, 1977) |
| *C. pennivesculata* (Lush.) Tan., gajanimma | +++ | (Catara and Cutuli, 1972; Crescimano et al., 1973; Reforgiato Recupero, 1979) |
| *Citrus moi* CRC 2434 | + | (Russo, 1977) |
| *C. pectinifera* CES 2448 | + | (Ruggieri, 1948) |
| *C. pectinifera* CRC 2710 | +++ | (Russo, 1977) |
| *C. taiwanica* (Tan. and Shim), Nansho Daidai sour orange | +++ | (Crescimano et al., 1973; Russo, 1977; Reforgiato Recupero, 1979; Nigro et al., 1996; 2008; 2011, 2015) |
| *Citrus macroptera* CRC 432 | +++ | (Ruggieri, 1948; Russo, 1977) |
| *C. latipes* (Swing.) Tan., Khasi papeda | + | (Russo, 1977) |
| *Citrus amblycarpa* (Hassk.) Ochse, Nasnaran CRC 2485 | +++ | (Russo, 1977) |
| Citrus species (Java) CRC 2459 | + | (Russo, 1977) |
| Citrus longispina CES 754 | +++ | (Ruggieri, 1948; Russo, 1977) |
| *Citrus celebica* Southwickii CRC 2453 | +++ | (Russo, 1977) |
| *C. reticulata* Blanco, Laranja cravo CRC 2893 | + | (Russo, 1977) |

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|---|---|---|
| *C. reticulata* Blanco, Tien Chiek CRC 2376 | + | (Russo, 1977) |
| Koubs el arsa CRC 2422 | ++ | (Russo, 1977) |
| Krauch Chang Sack | +++ | (Russo, 1977) |
| *C. vulgaris* sour orange hybrid, "Japanese orange" CRC 760 | + | (Russo, 1977) |
| *Citrus excelsa* Wester, Le Nestour | +++ | (Russo, 1977) |
| Citrus spp. CRC 320 | +++ | (Russo, 1977) |
| Bigaraldin CRC 2623 | + | (Russo, 1977) |
| *C. lumia pyriformis* Hassk. (Tanaka), Ponderosa lemon | + | (Russo, 1977) |
| *C. paradisi* Macf. × *C. sinensis* (L.) Osbeck, Siamelo CRC 2586 | + | (Russo, 1977; Reforgiato Recupero, 1979; Ippolito et al., 1991; Nigro et al., 1996; 2008; 2011, 2015) |
| *C. paradisi* Macf. × *C. reticulata* Blanco, Sampson tangelo CRC 2418 | + | (Russo, 1977) |
| *C. paradisi* Macf. × *C. reticulata* Blanco , Orlando tangelo CRC 2790 | +<br>+++ (2) | (Russo, 1977; Reforgiato Recupero, 1979)<br>(Crescimano et al., 1973) |
| *C. paradisi* Macf. × *C. reticulata* Blanco, Orlando tangelo | ++ | (Catara and Cutuli, 1972) |
| Thoruton tangelo | ++ | (Catara and Cutuli, 1972) |
| *C. × tangelo,* J.W. Ingram & H.E. Moore, Sexton tangelo | ++ | (Catara and Cutuli, 1972) |
| Shangyuan | + (2) | (Crescimano et al., 1973) |
| *C. indica* Tanaka, Indian wild orange | +++ (2) | (Crescimano et al., 1973) |
| *C. aurantium L.,* Granitos | +++ (2) | (Crescimano et al., 1973) |
| *P. trifoliata* (L.) Raf., trifoliate orange | + | (Petri, 1930; Ruggieri, 1948) |
| *P. trifoliata* (L.) Raf., Poncirus Rubidoux | +++ (2) | (Crescimano et al., 1973) |
| *P. trifoliata* (L.) Raf., Poncirus English large | +++ (2) | (Crescimano et al., 1973) |
| *P. trifoliata* (L.) Raf., (different selections) | ++ | (Russo, 1977) |
| *Fortunella* sp., kumquat | + | (Hohryakov, 1952; Catara and Cutuli, 1972) |
| *Fortunella crassifolia* Swingle, Meiwa Kumquat CRC 1471 | + | (Russo, 1977) |
| *Severinia buxifolia* (Poir.) Ten., box orange CRC 1489 | ++ | (Russo, 1977) |
| *Eremocitrus glauca* (Lindl.) Swing. ×*C. limon* (L.) Burm. 'Meyer lemon', Eremolemon CRC 2439 | +<br>+++ (*) | (Russo, 1977)<br>(Reforgiato Recupero, 1979) |

| | | |
|---|---|---|
| *Microcitrus Australasica* var. sanguinea (F.M. Bail.) Swing., Australian finger lime CRC 1484 | + | (Russo, 1977) |
| *C. sinensis*(L.) Osbeck 'Washington Navel' × *P. trifoliata* (L.) Raf., 'Washington Navel', Carrizo Citrange CRC 2863 | ++ | (Russo, 1977) |
| | +++ (3) | (Reforgiato Recupero, 1979) |
| *C. sinensis* (L.) Osbeck 'Washington Navel' × *P. trifoliata* (L.) Raf., Carrizo Citrange | +++ (2) | (Crescimano et al., 1973) |
| *C. sinensis* (L.) Osbeck 'Washington Navel' × *P. trifoliata* (L.) Raf., Troyer Citrange CRC 1459 | ++ | (Russo, 1977) |
| | +++ (3) | (Reforgiato Recupero, 1979) |
| *C. sinensis* (L.) Osbeck 'Washington Navel' × *P. trifoliata* (L.) Raf., Troyer Citrange | +++ (2) | (Crescimano et al., 1973) |
| | ++ | (Catara and Cutuli, 1972; Solel and Oren, 1975) |
| *P. trifoliata* (L.) Raf. × *C. sinensis* (L.) Osbeck, Yuma Citrange | +++ (2) | (Crescimano et al., 1973) |
| *P. trifoliata* (L.) Raf. × *C. sinensis* (L.) Osbeck, Savage Citrange CES 275 | +++ | (Ruggieri, 1948) |
| *C. aurantium* L. × *P. trifoliata* (L.) Raf., Citradia CRC 50917 | +++ | (Russo, 1977) |
| *Fortunella sp.* × (*C. sinensis* (L.) Osbeck × *P. trifoliata* (L.) Raf.), Thomasville citrangequat CRC 1439 | + | (Russo, 1977) |
| *Microcitrus australasica* ( F. Muell.) Swing. × (*Fortunella margerita* (Lour.) Swing. × *C. aurantifolia* ( Christm.) Swing.), Faustrime CRC 2891 | + | (Russo, 1977) |
| *Microcitrus australasica* ( F. Muell.) Swing. × (*Fortunella sp.* × *C. recitulata* Blanco, Calamondin), Faustrimedin CRC 1466 | + | (Russo, 1977) |
| *P. trifoliata* (L.) Raf. × *C. limon* (L.) Burm. f., Citremon CRC 1448 | +++ | (Russo, 1977; Reforgiato Recupero, 1979) |
| *P. trifoliata* (L.) Raf. × *C. limon* (L.) Burm. f., Citremon CRC 1449 | + | (Russo, 1977) |
| | ++ (3) | (Reforgiato Recupero, 1979) |
| *P. trifoliata* (L.) Raf. × *C. paradisi* Macf., Citrumelo CRC 3343 | +++ | (Russo, 1977; Reforgiato Recupero, 1979) |

Table 1. Susceptibility to MSD of some Citrus species, allied genera, and hybrids other than lemon under natural pressure of the pathogen[4]. (1) Susceptibility level : + = low; ++ = medium; +++ = high. (2) data obtained from artificial infection. (3) data obtained from natural infections of seedlings. (4) Adapted and updated from (Cutuli et al., 1984; Nigro et al., 2011).

## 1.2 Disease and pathogen

The fungus is a quarantine pathogen included on the list A2 quarantine pests of the European and Mediterranean Plant Protection Organization (EPPO), and the lists of other regional plant protection organization, worldwide, such as the Asia and Pacific Plant Protection Commission (APPPC), Caribbean Plant Protection Commission (CPPC), Comité Regional de Sanidad Vegetal del Cono Sur (COSAVE), North American Plant Protection Organization (NAPPO) and Inter-African Phytosanitary Council (IAPSC) which provide detailed information for avoiding or restraining the spread of the pathogen (Nigro et al., 2011; Pérez-Tornero et al., 2012). The primary source of inoculum is represented by the fungal conidia, pycnoconidia, that differentiate in late autumn and winter at relatively low temperatures, while the second source is represented by phialoconidia (Figure 2), that are

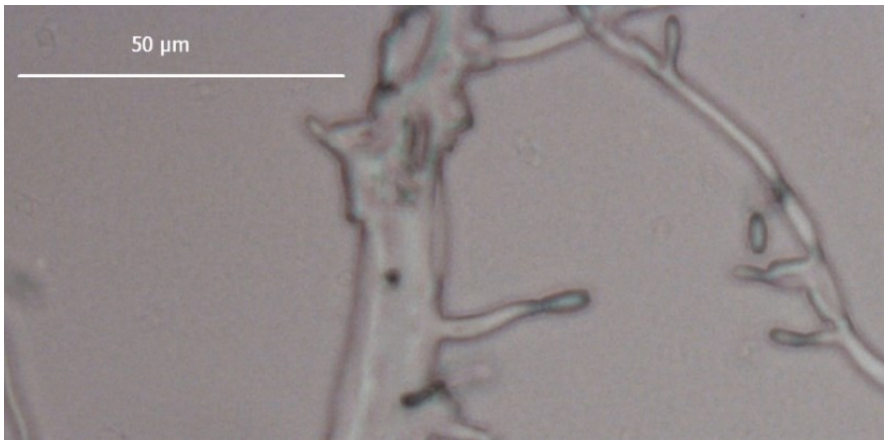


Figure 2. Optical microscope view of hyphae and phialoconidia of *Plenodomus tracheiphilus*.

produced quickly and abundantly on wounded infected shoots, and are responsible for the epidemic explosion of the infections after hail storms and heavy rain with strong wind during late summer-early autumn (Salerno and Cutuli, 1976; Salerno et al., 1976) when there are no longer pycnoconidia able to germinate (Grasso and Perrotta, 1980).

Pathogen dissemination is favoured by wind and rainfalls, strictly correlated with infection rates (Solel, 1976; Tuttobene, 1994). The optimum temperature for pathogen growth and symptom expression is between 20°C and 25 °C. At temperature above 28 °C fungal growth ceases and symptoms are not expressed. According to Ruggieri (Ruggieri, 1956), MSD is a tracheomycotic disease, and symptoms show a seasonal fluctuation. The infection occurs mainly in the foliage and, less frequently, in the rooting system. The first symptoms appear in the apical leaves that show a discolouration, stronger in the new shoots, with significant yellowing of veins, followed by the fall of leaves primarily without the petioles that remain on the shoots (Figure 3); the infected shoots become chlorotic, sometimes only in one side, while the basal part is remaining green (Nigro et al., 2011; Migheli et al., 2009).

Figure 3. Petioles on the shoots after the fall of leaves on infected shoots.

The fungus penetrates through the wounds (Butera et al., 1986; Migheli et al., 2009; Nigro et al., 2011), mostly caused by hail, or leaf abscission zones (Zucker and Catara, 1985). On the affected plants (Figure 4), there is a development of vigorous suckers that are soon reached by the pathogen, forming abundant pycnidia fructifications of the fungus (Figure 5), located, in aggregate or sparse manner, below the epidermis.

Figure 4. Lemon tree (cv. Selinunte) with severe symptoms of MSD located in the germplasm collection of CREA-OFA, Acireale, Italy.

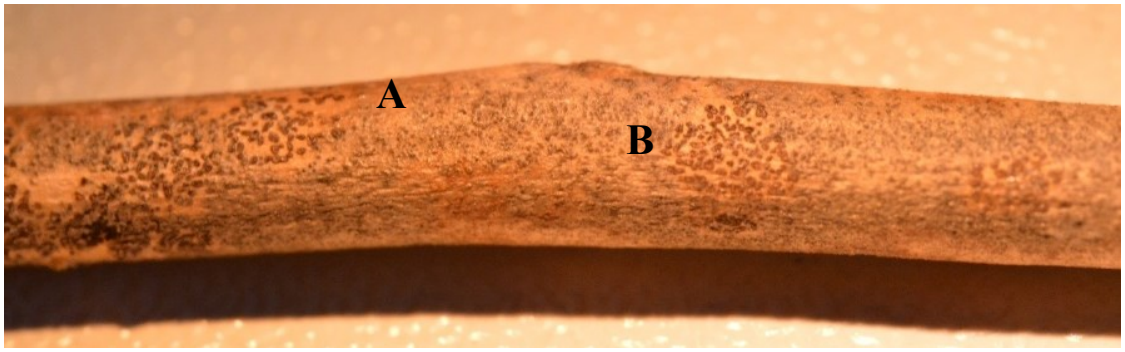


Figure 5. Pycnidia of *Plenodomus tracheiphilus* (A) and acervula of *Colletotrichum gloeosporioides* (B).

Another symptom is the red carrot colouration of the wood (Figure 6) when the infection is just starting. If the pathogen penetrates through the roots, we can have two different faces of the disease. The first one is called “mal nero”, and the pathogen remains in the host’s xylem. When the pathogen reaches the out layer of xylem and the phloem, the apical leaves and the branches show the typical symptoms of the disease until the plant in less than one year died. The name “mal nero” comes from the typical symptom that has the trunk when it is cut. The second face of the disease is called “mal fulminante”. The difference is that in this case, the plant died immediately; in fact, the plant in less than one year died from the infection, and the wilting leaves remain attached into twigs. The disease causes a severe reduction of the lemon orchards’ productivity since it determines plant losses due to the fungus infections, the heavy cuttings of the infected branches, the utilization of low amounts of nitric fertilization to reduce vegetative growth. The other indirect damages to the lemon industry are related to the lower fruit quality of the most tolerant cultivars, representing the only option for lemon growers in areas with high pathogen pressure (Cutuli, 1985).

Figure 6. Pink salmon colouration of the wood in infected lemon tree.

## 1.3 Disease Management

The essential cultural practice that relies on prophylaxis and curative interventions against MSD is represented by pruning infected shoots and branches. MSD has been controlled by the expensive practice of pruning disease twigs since it appears into the lemon orchard (Palm, 1996; Salerno and Cutuli, 1981). This practise consists of removing with attention all the withered shoots, suckers, and further burning of pruned branches to reduce the inoculum of the pathogen. Sanitation pruning should be performed ideally as soon as symptoms are more easily recognized in spring and early summer, preferably before the infected leaves fall to the ground (Traversa and Lima, 1993). Sanitation pruning cuts should be done at least 50 cm under symptoms of wood discolouration. However, this method is often untrustworthy because pruners make cuts only on twigs and branches with pink-salmon discolouration symptoms (Migheli et al., 2009). In older chronically diseased plants that may carry latent infections, the attempts to sanitize through pruning may result in more abundant vegetation and faster colonization of the healthy parts, as the infection can hardly be completely removed. In any case, to achieve this goal in practice.

Figure 7. New plantation of lemon orchard under hail nets in Sicily, Italy.

When symptoms are pervasive, it is preferable to pollard the plant at a level always a bit under the last diseased section, and regraft. With hardened plant, where the disease progresses slowly, it would be advisable to remove just the diseased limbs (Nigro et al., 2011). Another good practice is utilising of clean, healthy plants from certified nurseries that prevent the disease's spread. Furthermore, the usage of windbreaks and hail nets reduce injuries and, consequently, the risk of infection through wounds (Reichert and Chorin, 1956; Cutuli, 1982; Timmer et al., 1988) (Figure 7). So, the control of MSD is mainly focused on preventing the entry of the fungus inside the plant. Also, many authors (Ruggieri, 1948; Raciti et al., 1990) suggested that frequent irrigation in comparison with the high level of nitrogen fertilization would favour the disease, just like severe pruning that produce exaggerated flushing (Cutuli and Salerno, 1977). Tree spacing is also importance since the disease impact is lower in densely spaced plantations (Nigro et al., 2011). Chemical control consists of applying copper-based fungicides and ziram that may decrease infection by *P. tracheiphilus* (Timmer et al., 1988). These fungicides must be used every 2 to 4 weeks in the winter and after particular events, like a hailstorm or strong windy, that are conditions where the infections are favourable. However, many treatments are not cost-effective in commercial lemon groves (Migheli et al., 2009). In contrast, it is suggested to spray in a confined area like greenhouse or screenhouse. Some systemic fungicides, that now are not allowed to use anymore, such as benomyl, carbendazim, and thiophanate methyl have been tried in many experimental trials, but they were useless on mature trees, and they were never used in lemon orchards (Solel, 1977). In the end, the disease is not possible to control with chemical or agronomic technique. The most effective tool to control MSD on a large scale would be to use resistant lemons grafted on resistant rootstocks, but unluckily at present, this strategy is not available. In fact, the base of genetic resistance at molecular and gene levels is not known, and only one work evaluated the response to the disease in segregating populations (Reforgiato Recupero et al., 1997). The authors hypothesized that resistance to the disease is due to three alternative genes (A, B, and C) determining dominant resistance. So, the presence of a single dominant allele can, therefore, confer resistance. Also, they suggested the existence of a fourth dominant gene (D), which can cancel the dominance of the B allele. This hypothesis relies on analysing progenies' segregation obtained from crosses between the monoembryonic species Khasi papeda (*C. latipes* [Swing.] Tan.), as female parent, with polyembryonic sour orange, trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and volkamer lemon (*C. volkameriana* Ten. et Pasq.) as male parents. The pathogen's response was inferred from chitinase production, a pathogenesis-related (PR) protein reported in significant quantities in Mal secco–resistant *Citrus* genotypes (Reforgiato Recupero et al., 1997). However, PR proteins' actual role in cell tolerance to P. tracheiphilus has been questioned (Bas and Koç, 2006; Migheli et al., 2009). So in many lemon growing area of Sicily where the disease is almost endemic, the susceptible lemon cultivar Femminello has been substituted by the cultivar Monachello (Figure 8), that previously was considered a spontaneous hybrid between lemon and citron (Russo, 1977) and now is considered a true lemon based on genetic analyses (Barry et al., 2020). Monachello is resistant to MSD, but present other less desirable traits such as reduce yield and low quality of the fruits. Another Sicilian lemon selected from Femminello Ovale is called Santa Teresa Riva (Figure 9.), this clone is less susceptible than other clones of Femminello but produces low- quality fruits outside the area where it was developed (Ruggieri, 1937b, 1956). Interestingly, the tree where Santa Teresa Riva was selected, was an old tree, without any symptoms of MSD, found in a Femminello lemon orchard that had almost been destroyed by MSD (Russo, 1955). The search for lemon lines resistant to MSD has been one of the most crucial goals prosecuted since the first report

of the disease and is still on the way. Although the results so far obtained have not been entirely satisfactory, they have permitted the survival of lemon groves in many areas affected by MSD (Migheli et al., 2009).

Figure 8. A young plant of Monachello lemon of 5 years old in Sicily, Italy.

Figure 9. An old plant of Santa Teresa lemon of 45 years old in Sicily, Italy.

# 2. The lemon

## 2.1. Origin and diffusion

The lemon's origin is unknown, Tanaka (1954) reported its presence at 1000 m in Ketah, the northeastern corner of Myanmar and Dudya in central India. Moreover, Singh (1981) suggests that lemons are native to Eastern Himalaya because many high-quality lemons growing in a wild state were collected in Assam, Sikkim, and nearby regions. Furthermore, no such varieties have been found elsewhere (Singh, 1981). Later on, different authors confirmed this hypothesis and assumed that it appeared in the Himalayas' foothills, in India (Swingle and Reece, 1967; Bonavia, 1888). In the end, it is believed that lemon appears millennia years ago under cultivation between the foothills of the Himalayas and the Middle East (in Media of ancient Persia, now Northwestern Iran) (Barry et al., 2020).

According to Langgut (2017), citron was the first citrus brought into the Western Mediterranean from Persia during the early Roman period, and lemon was the second introduced citrus, while sour orange, lime (*C. aurantifolia* Christ.), and pummelo (*C. maxima* Burm. Merr.) were introduced in the tenth century AD (Zhong and Nicolosi, 2020). Furthermore, a lots of sculptures, mosaics, and frescoes were regarded as evidence to support the idea that lemon arrived in Europe no later than the second century (Swingle and Reece, 1967), but the pictures of that in ancient Rome (Figure 10) were too vague to be sure and support this opinion (Deng et al., 2020). So, it was Arab, that first used lemon as an ornamental plant in early Islamic gardens (Morton, 1987) and spread lemon between 1000 and 1500 century through the Mediterranean region in connection with the expansion of the Arabian Empire (Morton, 1987). Afterwards, the famous navigator Christopher Columbus brought lemon seeds on his travel and diffused lemon and introduced it to the New World near the 16th century. Coincidently, Spanish conquest throughout the New World helped spread lemon seeds further (Deng et al., 2020).

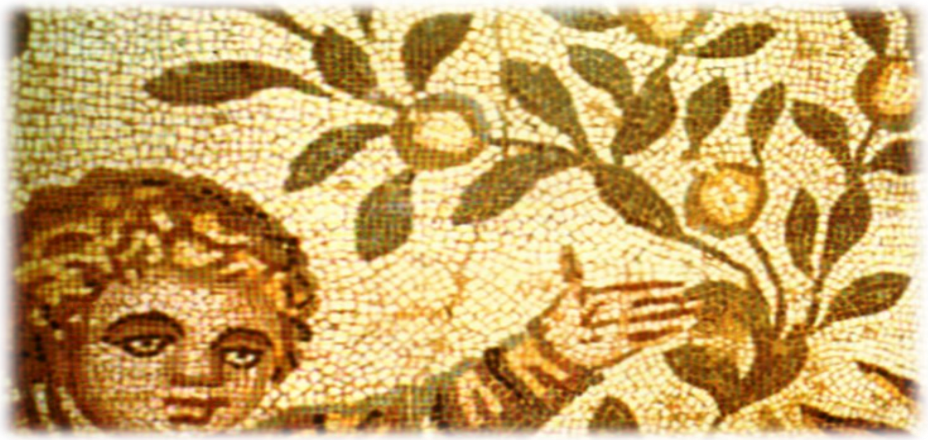

Figure 10. Citrus fruits represent in a mosaic of Villa del Casale in Piazza Armerina (Sicily, Italy).

Nowadays lemon is restricted to the Mediterranean-type climates, for example, the coastal and semi-coastal locations of Southern California, Greece, Spain, Italy, Turkey and South Africa and Argentina. Commercial lemon culture developed first in Italy, where it was the first country for lemons production until a few decades ago. However, today Italy is not anymore even in the first ten countries for lemon and lime production and the first place is taken by Mexico (Spreen et al., 2020). Many causes are the reason for this decline of lemon production. One of these is due to MSD.

## 2.2. Genetic background

Several authors reported the genetic diversity of a large sample of lemon cultivars from a wide range of geographic locations with molecular markers. Different lemon varieties, such as Eureka, Lisbon, Femminello, and Villafranca, could not be distinguished by 77 nSSR (nuclear Simple Sequence Repeats) markers (Yang et al., 2015), which suggested that they originated from a single clone parent via a series of mutations (Gulsen and Roose, 2001). Therefore, different lemons were highly heterozygous and were very similar to each other. Uzun et al. (Uzun et al., 2011) performed the evaluation of genetic diversity in lemons and some of their relatives based on SRAP (Sequence Related Amplified Polymorphism) and SSR (Simple Sequence Repeats) markers. Although nearly all accessions could be distinguished, there was a low level of genetic diversity detected among lemon cultivars (Deng et al., 2020).

Curk et al. (Curk et al., 2016) carried out comprehensive analyses of the diversity, genetic structure, and origin of 133 citrus accessions (limes and lemons) on a large scale by cytoplasmic and nuclear markers. The results showed that all lemon accessions were highly heterozygous, with interspecific admixture of two and three ancestral taxa genomes in *Citrus* (Deng et al., 2020).

From true lemon derivate, between hybridization with another citrus, different hybrids have been reported by Continella (Continella, 2012):

- Citron lemon: utilized for consume direct or in substitute to citron in confectionery (Spadafora and Piretto lemon)
- Lemonange: a cross between lemon and sweet orange, called Meyer lemon, used as ornamental plant
- Lemonime a hybrid with lime called Perrine lemon
- Lemandarin: that came from south of China, a cross with mandarin called red limonia (hong ning-mong) and white limonia (bai ning-mong), used as rootstock.

The lemon has resulted from a cross between sour orange and citron (Yang et al., 2015; Curk et al., 2016). Previously, the lemon was conventionally considered a species by the two most widely cited taxonomic systems (Swingle and Reece, 1967; Tanaka, 1961). However, Nicolosi et al. (Nicolosi et al., 2000) were the first to propose that lemons arose from hybridization between sour orange and citron. By using chloroplast DNA analyses, Carvalho et al. (2005) demonstrated that the lemon displayed a contribution from the sour orange genome. The same authors, using cytogenetic studies, also showed that citron was a true parental species of lemons (Carvalho et al., 2005). Moreover, by comparison of chromosome types between lemon and citron, Carvalho showed that citron was probably a cytogenetically homozygous accession, but the lemon was a cytogenetically heterozygous accession, and all the chromosome types of citron were clearly presented in lemons (Carvalho et al., 2005). Furthermore, an increasing number of studies based on nuclear markers supported the opinion proposed by Nicolosi and colleagues (Nicolosi et al., 2000). The results suggested that lemon was likely to be of hybrid origin, with sour orange being the maternal parent and citron being the paternal parent (Gulsen and Roose, 2001; Garcia-Lor et al., 2012; Ollitrault et al., 2012; Garcia-Lor et al., 2013; Yang et al., 2015; Curk et al., 2016; Deng et al., 2020).

## 2.3. Plant and fruit traits

The lemon fruit is medium size (5–9 cm diameter) having adherent peels and containing numerous segments (Tolkowsky, 1938; Tanaka, 1954; Scora, 1975; Webber, 1943, 1967; Ramon-Laca, 2003; Mabberley, 2004; Swingle, 1943; Zhang and Mabberley 2008); the plant is vigorous, upright-spreading, and open in growth habit. The light green leaves are lanceolate with short, wing-margined petioles (Reuther et al., 1967). The plant produces large flowers (2.5–4.5 cm in diameter), commonly with a pink tinge and is highly sensitive to cold conditions. The new shoot growth is purple-tinted. The main fruit traits are high acidity and a distinctive form with an oval to elliptical shape with a nipple with a low apical papilla. It is constituted with a thick peel that is yellow when the fruit is mature and presents prominent oil glands. It is cultivated for use as fresh fruit, flavouring, and the distinct flavour due to limonin and d-limonene (Barry et al., 2020).

Most of the lemon varieties are more or less ever-flowering due to low floral induction requirements, and consequently more-or-less everbearing depending on cultural practices (Barry et al., 2020). This different bearing produces different fruit typologies with different traits regarding fruit quality and shape (Damigella and Continella, 1970) as indicated in Table 2.

| Harvest | Period of harvest | Acidity (%) | Juice (%) | Number of seeds (n°) |
|---|---|---|---|---|
| Limoni | December to May | 6.8 | 28.4 | 3.7 |
| Bianchetti | April to June | 6.0 | 23.7 | 6.9 |
| Verdelli | June to September | 5.5 | 23.5 | 3.1 |

Table 2. Main fruit quality traits at different harvesting time.

Due to the lemon seed's polyembryonic and heterozygosity nature, it is challenging to breed lemon varieties by hybridization with true lemon characteristics. Most of the current commercial lemon varieties have been developed through natural or induced mutations from the original ancestral form (Barry et al., 2020), and so, even if there are currently no active large-scale conventional breeding projects, in some countries like Italy, Turkey, and Greece, the development of new varieties resistant to MSD is a priority goal (Gentile et al., 2007; Gulsen et al., 2007; Nigro et al., 2015; Polat, 2018; Raveh et al., 2020).

# 3. Traditional and advanced methods of genetic improvement of lemon

In this chapter, we explore the different techniques that were used for the genetic improvement of lemon, except for the last paragraph where is described an innovative technique that we hope can be used for lemon genetic improvement. Many different techniques were utilized with the primary goal to obtain lemon clones resistant to MSD. The great amount of cited bibliography suggests the considerable work made in these years after the disease appears for the first time in the Sicilian lemon orchards. In these last years, the interest in lemon was set aside, and this justifies the old, cited bibliography, sometimes also in Italian, utilized in this thesis.

## 3.1 Hybridization

Obtaining new commercially valuable cultivars from hybridization is a significant challenge for citrus breeders (Caruso et al., 2020). Unlike many other vegetal crops, citrus suffers some limitation in the setting up a breeding program based on sexual hybridization. A first drawback is common to all fruit tree species: the long juvenile phase. Once the cross is made, citrus plants underwent a juvenile period of approximately five years before the first fruits are produced with direct repercussion in terms of costs and time needed to select a novel cultivar. Moreover, sexual hybridization is limited by the genome's high heterozygosity and the genus Citrus's complex reproductive biology property (Cuenca et al., 2018). The high heterozygosity also has a strong impact in developing a novel breeding program since, in a controlled cross, the offspring will not resemble the parental lines due to a significative reshuffling of the genome. The high heterozygosity is partially due to complex reproductive biology hampering sexual hybridization (apomixis, male or female sterility, and self- and cross-incompatibility) (Raveh et al., 2020). So, lemon improvement by hybridization has been limited due to nucellar embryony, extended juvenility, and high heterozygosis level in *Citrus spp*. Nevertheless, hybridization studies have been carried out since 1946 by the Experimental Station of Citriculture of Acireale, Italy (Carrante and Bottari, 1952; Russo and Torrisi, 1952; Russo, 1985; Russo, F., 1990) and the Research Institute for Citrus Genetics of Palermo, Italy (Geraci, 1986; Tusa et al., 1992), without much success. When lines possessing good vigour and fair MSD resistance were obtained, their fruits often had low qualitative characteristics (Russo, 1977; Cutuli et al., 1981; Russo, 1985; Russo, F., 1990). This suggests that resistant to MSD is transmitted with unwanted traits (Russo, F., 1990).

By contrast, hybridization is one of the most used breeding methods to obtain new mandarin varieties. This technique is adopted to increase the mandarin-like species' genetic diversity, even though it comes to the price of a general increase in the seed content (Raveh et al., 2020). After more than 100 years of systematic breeding, there are many examples of successful mandarin varieties diffused worldwide obtained through controlled crosses. The seedlings selection from uncontrolled hybridization events, such as in Clementine (*C. clementina* Hort. ex Tanaka) has also played a role in generating successful hybrids. To conduct an efficient breeding program, a large number of progenies should be generated. It is not easy to estimate the most appropriate number of hybrids that must be evaluated to find something promising. It depends on the breeding program's objectives, the quality of available parents, and what screening can be performed by marker-assisted selection (MAS) or greenhouse phenotyping before the field evaluation. Experience suggests that screening thousands or even tens of thousands of hybrid progenies is needed to select superior cultivars, although there are examples of successful cultivars being selected from tiny populations (Caruso et al., 2020). For intended cross-hybridizations, pollen is collected from selected male plants and female flowers are

emasculated, pollinated, and covered with insect-proof nets to prevent any unintended cross-pollination events. To encourage blooming, fruits from the previous season should be removed from the trees as early as possible, before new crosses are made. Also, girdling of branches or trunks can increase fruit set. Afterwards, seeds are collected from the evolving mature fruit and grown in nurseries to produce viable seedlings. Buds of the new varieties are grafted onto rootstocks, which produce high-quality, flavourful fruit (Benjamin et al., 2013). After vigorous plants have been obtained under greenhouse conditions, the seedlings are planted in the orchard and then screened for various growth characteristics and important fruit-quality traits (Raveh et al., 2020).

### 3.2 Triploid breeding

The principal interest in triploids is to generate sterility. Although triploid plants are sterile, citrus is considered a parthenocarpic crop, so seedless fruit can be obtained. Seedless is one of the essential characteristics for the fresh market, but fruit with reduced numbers of seeds can also be useful for the juice industry. Triploid hybrid can be obtained through the cross between a diploid maternal line and a tetraploid parental line (Aleza et al., 2012). Triploid hybrids obtained using the tetraploid Lisbon lemon and the diploid Trovita sweet orange proved resistance to MSD, but their fruits had a poor commercial quality (Geraci, 1986). In a comparative trial conducted under a controlled condition with different cultivars and inter and intra-specific lemon hybrids grafted on sour orange, Lemox (Figure 11), a triploid obtained by crossing the tetraploid lemon Doppio Lentini and the spontaneous lemon hybrid Femminello × Pera del Commendatore (Reforgiato Recupero et al., 2005), and Femminello Siracusano 2Kr resulted in the most susceptible to MSD, followed by Lunario and Femminello comune. Moreover, triploid hybrid of Femminello × allotetraploid somatic hybrids of Valencia sweet orange × Femminello, were very susceptible, whereas, Milam lemon × Femminello, and Key lime × Valencia sweet orange were moderately resistant, although not as much as Monachello lemon (Cacciola et al., 2010).

Figure 11. Lemon fruits of Lemox, triploid hybrid obtained by crossing the tetraploid lemon Doppio Lentini and the spontaneous lemon hybrid Femminello × Pera del Commendatore.

### 3.3 Mutagenesis

Spontaneous mutations in citrus are relatively frequent, causing the occurrence of new cultivars having a genetic background similar to the parent while presenting some new quality traits (fruit size, flesh colour) or agronomical traits (harvesting period) of interest. The frequency of these naturally occurring mutations can be dramatically increased by exposing the vegetal material to various physical or chemical mutagenesis agents (Raveh et al., 2020). This method's main advantage is preserving the genetic background of the original cultivar and the modification of only one or a small number of agronomical traits. Another advantage of this technology is its simplicity (it is unnecessary to have previous knowledge of gene control traits), rapidity (resulting trees will not display juvenile phase) and inexpensiveness. The main disadvantages are the large populations needed to find desirable stable mutations and the frequent chimeric status mutations. Since 1935, various mutagenesis agents, mainly gamma irradiation, have been used to obtain new cultivars (Aleza, 2015), and Star Ruby grapefruit was the first commercial cultivar obtained by irradiating seeds of Hudson grapefruit; later, Rio Red grapefruit was obtained by irradiation of Ruby Red grapefruit (Hensz, 1971). This technique is mainly used for obtaining diploid low-seeded genotypes, and there are many examples of recently released seedless cultivars like Mor or Spring Sunshine, Murcott low seed, Murina from Murcott tangor (Pablo Aleza Gil, 2020 pers. Comm.) and Orri from Orah mandarin (Vardi et al., 2003); and Tango from Nadorcott tangor (Roose and Williams, 2007; Cuenca et al., 2018). For lemon improvement by mutagenesis, Starrantino and Russo (1977) utilized this technique to irradiated a fruit of Femminello Siracusano. The ovules, obtained from the irradiate fruit, were cultivated *in vitro* (Figure 12) and have formed the nucellar mutant called Femminello Siracusano 2Kr, which reserve a very high fruit quality but it is very susceptible to MSD (Nigro et al., 2011).

Figure 12. Lemon plant regeneration through *in vitro* ovules culture.

### 3.4 Clonal selection

As already stated, citrus are incredibly prone to mutate, and once a new source of variation is identified, the desired trait can be fixed and propagated through clonal selection. Therefore, the new plant is grafted, and the stability of the novel trait is evaluated for 2–3 years. After this first evaluation, plants are micrografted to obtain virus-free plants. Virus-free scions are then grafted on different rootstocks and planted in different environments to test the trait stability to such changes. Once the trait stability has been proven, and the plants are exempt from the virus, the new plant can be vegetatively propagated and commercialized (Raveh et al., 2020). Mutations involving a change in DNA and its frequency are influenced by environment and cultural practice such as pruning. It may occur in single buds or limbs, as a portion of an entire tree or as the entire tree. Most mutations generate undesirable traits such as abnormally thick peels, dry fruits or atypical leaf colour (Figure 13). However, in some cases, these changes have resulted in desirable characters, i.e. fruit size, ripening period, peel and pulp colour. As previously stated, the lemon is cultivated for more than 100 years (Polat, 2018) so many lemon clones were identified during these years. The first author that reports on MSD resistant genotypes is Ruggieri (1935, 1936, 1937a). He described two resistant lemons: Monachello and Interdonato. The same author later described another resistance lemon clone, called Santa Teresa (Ruggieri, 1956). Despite these encouraging field observations, this approach did not give the expected results, although the clonal selection was strongly adopted in Italy (Crescimanno and Sacco, 1955; Damigella and Continella, 1970; Spina, 1975; Granata et al., 1977; 1979; Baratta et al., 1979; Continella and Tribulato, 1979; Cutuli, 1979; Cutuli et al., 1983).

More recently in 2001 Calabrese selected and described five cultivars (Akragas, Erice, Kamarina, Segesta, and Selinunte) that were evaluated, starting from 1980, for the MSD resistance and seedlessness character (Calabrese et al., 2001). Through the clonal selection, in all these years, it was not possible to identify a genotype that combines high-quality production and the resistance to MSD. Russo (1977) believed that only “intermediate resistance” could be obtained through clonal selection (10- 30% of dead plants during 15-20 years of field observations), as shown by data on the Femminello clones (Santa Teresa, Continella, Zagara Bianca) and Lisbon (Rosenberger, Monroe, and Strong) lemon (Nigro et al., 2011).

Figure 13. A common mutation that generate undesirable trait such as atypical leaf colour in plant of Tarocco Gallo nuc.898 (*Citrus sinensis* (L.) Osbeck) in Sicily, Italy.

### 3.5 Nucellar selections

This method utilises polyembryonic varieties to generate embryos and embryogenic calli from somatic tissues of the seed both *in vivo* and *in vitro* (Figure 14).

Figure 14. An example of polyembryonic genotypes, where it is possible to obtain more than one plant from one seed.

Embryos develop from the nucellar tissues surrounding the embryo sac and can be recovered from developed seeds and undeveloped ovules (Frost and Soost, Frost, H.B., Soost, 1968; Esen and Soost, 1977; Kepiro and Roose, 2007; Wang et al., 2017). Many citrus species and varieties' capability to produce plants identical to the mother plant by seeds has been known for centuries (Cook, 1907). In recent years, nucellar selections have been developed with the principal purpose of obtaining virus-free varieties (Bruno, 1962; Navarro and Juarez, 1977; Starrantino and Russo, 1980). Shoot tip grafting has largely replaced nucellar regeneration as a sanitation method since nucellar plants often carry many undesirable traits such as thorniness and reversion to a juvenile state (Navarro et al., 1975; Gmitter and Moore, 1986). Nucellar selection can be considered a genetic improvement method and has led to the release of valuable cultivars. Nucellar selections often differ from the mother plant for few or many characteristics, depending on the original genotype from which they originated. Specifically, this method does not create completely true-to-type plants since the nucellar tissues originate only from the L2 histogenic layer. As a result, if a specific phenotype of a mother plant is determined by a somatic mutation occurred in L1, that characteristic will be lost in the nucellar plant (Cameron et al., 1964; Caruso et al., 2020). The nucellar selections have also been utilized as a genetic improvement method for lemon, without expected results. The old lines of Femminello and Monachello lemons showed lower susceptibility to MSD and a lower and slow disease progression than nucellar clones (Catara and Cutuli, 1972; Perrotta and Tribulato, 1977).

### 3.6 Somaclonal variation

The term somaclonal variation is defined as a genetic variation present in plants regenerated from tissue cultures, either uncovered or induced by a tissue culture process (Larkin and Scowcroft, 1981). Regarding the genetic improvement of lemon, embryogenic culture lines of lemon resistant to Mal secco toxins were obtained, which produced somatic embryos keeping resistance to the toxin (Nadel and Spiegel-Roy, 1987). More recently, *in vitro* selection of Femminello lemon calli with *P. tracheiphilus* toxins provided a cell line named Femminello-S, from which numerous plants tolerant to the toxins were regenerated from protoplasts, and planted in the field (Gentile et al., 1992). The Femminello-S callus and the regenerated plants tolerant to the toxins *in vitro* showed a ten-fold increase in chitinase and glucanase enzymatic activity in extracellular extracts compared to common Femminello. Callus and regenerated plants of Femminello-S behaved as the resistant genotypes (Gentile et al., 1993). Among the different somaclones grafted on sour orange, two (FS01 and FS11) showed mild symptoms of MSD, having the same resistance level as that of Monachello (Gentile et al., 2000). Similarly, two toxin-resistant cell lines were obtained by an embryogenic callus line of Femminello Siracusano lemon, and plants regenerated from each line showed a resistance to the toxins equivalent to that of the resistant Monachello. High chitinase activity was detected among the intra- and extracellular proteins extracted from leaves of regenerated plants (Deng et al., 1995; Nigro et al., 2011).

Bas and co-authors (2006) from callus of Kütdiken lemon, obtained a resistant cells line, called 20b. The filtrate of Kütdiken 20b enormously decreases *P. tracheiphilus's* growth compare to the filtrates from cell suspension cultures of sensitive Kütdiken lemon (Nigro et al., 2011).

### 3.7 Somatic Hybridization

Development of hybrid plants through the fusion of somatic protoplasts of two different plant species/varieties is called somatic hybridization. Somatic hybridization is a useful tool to manipulate ploidy and to increase the genetic variability of plant species. It allows overcoming sexual incompatibility between species and combining nuclear, chloroplastic, and mitochondrial genomes. This technique via symmetric and asymmetric protoplast fusion represents a primary strategy for obtaining improved disease-resistant scion and rootstocks. It allows the production of hybrids that incorporate both parents' genome without recombination, thus avoiding the problem of high heterozygosity that occurs in citrus. In 2000 Tusa and co-authors (2000) got an allotetraploid somatic hybrid Valencia+Femminello and two Femminello lemon cybrids, obtained by symmetrical and asymmetrical protoplast fusion. These plants were evaluated by the stem and leaf inoculation tests and by analysis of propagule number of *P. tracheiphilus* in the xylem of stem-inoculated plants. The somatic hybrid and the cybrids showed a medium degree of resistance to MSD, with slight differences in disease symptoms, compared to the resistant Monachello and the susceptible Femminello, used as controls. An interesting result was the lower mortality in the asymmetrical lemon cybrids hints that specific mechanisms of resistance to MSD could be activated in these lemon genotypes (Nigro et al., 2011).

### 3.8 Genetic transformation

Genetic engineering is a powerful tool for plant improvement and can integrate desirable characteristics into existing genomes (Dönmez et al., 2016). Genetic modification (also called transgenic or genetically engineered plants) as a tool for citrus breeding programs has been gaining popularity as in many plants (Pons et al., 2012). This method helps cases where it is impossible to introduce a particular trait of interest to another elite cultivar using conventional breeding. Recent developments in gene transfer techniques via the classical regeneration method have been applied to the *Citrus* genus and have opened the way to induce a specific genetic change within a shorter period than using the classical genetic selection method (Pons et al., 2012; Donmez et al., 2013). The citrus transformation has now been achieved in several laboratories; for example, *Agrobacterium tumefaciens* and *A. rhizogenes*, particle bombardment, electroporation and RNA interference are used in citrus transformation (Pons et al., 2012; Donmez et al., 2013; Polat, 2018).

After that, *Agrobacterium* has been used as a DNA delivery tool and has been the most utilized citrus transformation method. The chitinase gene (*chit42*) from *T. harzianum* was utilized to induce resistance to pathogenic fungi on lemon plants. All the phase of how the transgenic lemon plans were obtained is described in Figure 15 (La Malfa et al., 2007). The *Chit42* gene was successful introduced into Femminello Siracusano lemon by Agrobacterium-mediated transformation, and the transgenic clones were tested in *vitro* and in *vivo* for resistance to MSD. The transgenic foliar proteins of Femminello Siracusano lemon strongly inhibited *in vitro* the conidia germination and the fungal growth of *P. tracheiphilus*, while no effects were detected on controls (Gentile et al., 2007). Fruits of this transgenic endochitinase Femminello Siracusano showed resistance to different fungi that cause severe losses in post-harvest (Gentile et al., 2007). It was recently demonstrated the substantial equivalence of fruit of transgenic Femminello Siracusano lemon (Muccilli et al., 2020). However, considering the current Italian and EU laws that restrict field experiments with the transgenic plants, and the general unwillingness of consumers for transgenic products, it is difficult to provide further developments and practical transgenic resistance applications.

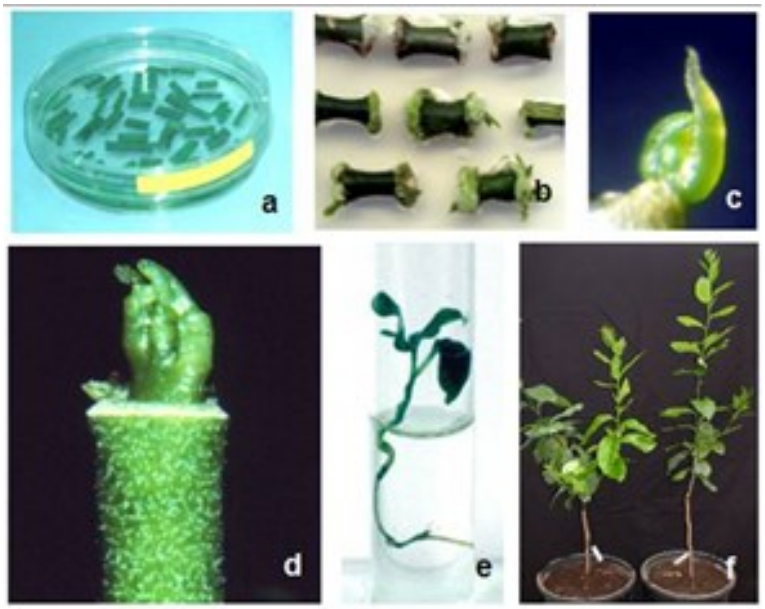


Figure 15. Transgenic Lemon plants obtained by culture *in vitro* of stem internodes: a) infection of stem internodes; b) regeneration and selection; c) putative transgenic bud; d) micrograft; e) development of the micrograft plant; f) two transgenic lemon plants grown in a greenhouse.

### 3.9 New Plant Breeding Techniques (NPBTs)

The development of novel varieties with improved resistance to various pests and pathogens is one of the main aims of citrus breeding programs. Conventional breeding strategy in citrus has demonstrated numerous limitations due to biological characteristics common to woody plants, such as long juvenile period, large size, long generation time, and the lack of knowledge on how the most important horticultural traits are inherited. Also, citrus display other limitations, such as nucellar polyembryony, self-incompatibility, and high heterozygosity, that genetic engineering and New Plant Breeding Techniques (NPBTs) (Eriksson et al., 2018; Limera et al., 2017) can overcome, leading to the development of novel varieties with the incorporation of selected traits, while retaining the unique characteristics of the original cultivar (Poles et al., 2020).

The aim is to produce high quality citrus fruits (in terms of size, sugar and acidity balance, juice yield, and seedlessness), healthy and rich in antioxidant compounds, tolerant or resistant to different abiotic and biotic threats, and with high productivity. Conventional citrus breeding is a long-term and expensive process; long time and resources to obtain progenies and evaluate their traits are needed. Also, sexual breeding is not always feasible because some cultivars used in crosses are incompatible, sterile, or polyembrionic (Talon and Gmitter, 2008). This process can be also longer in the case of rootstock breeding (25 years and more). Despite their relatively low efficiency, traditional breeding methods enabled the release of most of the new varieties and rootstocks in citriculture. Since the 1990s, new biotechnology techniques including molecular markers, genome mapping, sequencing, and *in vitro* culture have been applied to breeding, providing efficient alternatives to traditional methods to improve novel varieties. Many new techniques have been developed and classified as new plant breeding techniques (NPBTs). These include (I) zinc finger nuclease technology, (II) oligonucleotide directed mutagenesis, (III) cisgenesis, (IV) intragenesis, (V) RNA-dependent DNA methylation, (VI) grafting on genetically modified rootstock, (VII) reverse breeding, (VIII) agro-infiltration, and (IX) synthetic genomics (Lusser et al., 2011). However, the efficiency of NPBTs requires knowledge on the genetic control of horticulturally important traits, which remains limited in citrus compared to other major crops. In the last 20 years, the development of different technologies and sequencing platforms has resulted in the publication of genomes from several horticultural species. So far, NPBTs are ruled as genetically modified organisms (GMO) according to the GMO 2001/18 legislation, because they are included among the recombinant DNA technologies. NPBTs produce targeted and minimal modifications to selected genotypes, such as elite cultivars, which are highly valued by consumers for their quality and productivity but can be further improved. Unlike traditional breeding, and similarly to transgenesis approaches, NPBTs do not alter the genetic background, which is particularly important for elite cultivars. NPBTs include different biotechnological tools used to induce DNA modification, such as insertion, deletion, gene replacement, or stable gene silencing. NPBTs provide alternative methods for advancing biotic and abiotic resistance, nutritional quality, and crop performance (Cao et al., 2016; Lassoued et al., 2018); among the others, genome editing and cisgenesis represent two of the most promising strategies to develop genetically improved tree crops. Genome editing, or sequence-specific nuclease technology, involves the production of a permanent and inheritable mutation in a specific DNA sequence that can be inaccurately repaired by the plants' own repair mechanism (leading to gene knock-out), or that can be accurately repaired using a DNA-repair template (leading to target mutation or gene replacement) (Jinek et al., 2012; Bortesi and Fisher, 2015; Liu et al., 2017). The successful application of genome editing in citrus was first based on CsLOB1, the gene responsible for susceptibility to citrus bacterial

canker CBC (Hu et al., 2014; Duan et al., 2018). CBC is a severe quarantine disease caused by the bacterium *Xanthomonas citri* pathovar *citri* (Xcc) and *aurantifolii* (Xca), which are found globally, except for the Mediterranean basin (Gottwald et al., 2001). Genetic engineering is the best approach to induce resistance to CBC (Zhang et al., 2010). Considering the bacterium, *X. citri* strains are characterized by specific pathotypes (PthA4, PthA, PthAw, PthB, and PthC), which are distinguished based on the conservation of repeated variable diresidues (RVDs), encoding transcription activator-like (TAL) effectors. These recognize the corresponding effector binding element (EBE) in the promoter of susceptibility plant genes, such as LOB and Sugar Transport TFs (Hutin et al., 2015). Genome editing of a single EBE allele (type 1) in the promoter of CsLOB1 in 'Duncan' grapefruit (Jia et al., 2016) allowed the generation of transgenic lines resistant to a mutated Xcc strain, but susceptible to wild type-Xcc. Mutations in both EBEs alleles (type I and type II) of CsLOB1 in "Duncan" grapefruit and "Wanjincheng" orange, resulting in reduced symptoms in transformed plants caused by wild-type Xcc infection (Jia et al., 2016; Jia et al., 2017; Peng et al., 2017). Furthermore, to improve resistance through CRISPR/Cas9 approach on the CsLOB1 promoter (Zhou et al., 2017), homozygous mutants have been generated directly from citrus explants decreasing the susceptibility to CBC knocking out the CsWRKY22 a marker gene for pathogen-triggered immunity in 'Wanjincheng' orange (Wang et al., 2019; Salonia et al., 2020). Moreover, the availability of Cas12a has been successfully used in Citrus. The efficiency of CRISPR/Cas12a has been examined for editing CsPDS in 'Duncan' grapefruit via Xcc-facilitated agroinfiltration to modify two alleles of EBEPthA4-CsLOBPs. One of seven transformed 'Duncan' plants has been found to contain the highest mutation rate, demonstrating reduced canker susceptibility (Jia et al., 2019). Zhu et al. (2019) recently used Fortunella hindsii, a wild Citrus species characterized by a juvenile phase of about eight months and a dwarf habit, to observe the effects of a successful CRISPR/Cas9 experiment using Agrobacterium-mediated transformation. Two gRNAs were synthesized to edit the PDS gene, and five transgenic lines exhibited targeted mutagenesis sites, resulting in a global and mosaic albino phenotype (Zhu et al., 2019). These results suggest that editing or cisgenesis to induce early flowering could be a successful strategy to speed up gene characterization in functional genomic studies, especially for characters related to reproductive biology and fruits. The term cisgenesis was first introduced by Schouten et al., in 2006, who defined it as "the genetic modification of plants using genes that originate only from the species itself or from a species that can be crossed conventionally with this species." So, cisgenesis involves the transfer of genes resulting from cross-compatible species. It is based on transformation with genetic material from closely related species capable of sexual hybridization, particularly cisgenesis using a copy of a complete natural gene (Schouten et al., 2006; Lusser et al., 2012). Compared to genome editing approaches, there were fewer applications of cisgenesis in citrus. This was due to technical difficulties and limited knowledge of specific genes and promoters' function. The only example in citrus involved the use of the Ruby gene, which is intronless, flanked by the CaMV 35S promoter and terminator (a classical intragenesis experiment), to produce intending to (Citrus aurantifolia) fruits highly enriched in anthocyanins content (Dutt et al., 2016). According to the above definition, cisgenesis involves the transfer of a gene (introns included) along with its controlling sequences (promoter and terminator, in the sense direction) from one genotype to another of the same or of a sexually compatible species (Schouten et al., 2006; Lusser and Davies, 2013). Cisgenesis can overcome the major bottleneck of traditional breeding, termed the 'linkage drag' (unwanted gene transfer along with the gene of interest), allowing the transfer of the gene of interest without other genetic regions controlling undesirable traits (Jacobsen and Schouten,

2007). Therefore, the gene pool considered by cisgenesis can also be theoretically transferred through classical breeding approaches (Holme et al., 2013). However, cisgenesis has several drawbacks, which limit its more comprehensive application. In particular, the casual insertion of the cisgene in the host genome could induce a negative effect (Vanblaere et al., 2014) and potentially interrupt or modify genic or intergenic relevant sequences. The many deposited genomes give information on genes and related annotations that can be used for the cisgenic approach; however, in many cases, the lack of efficient promoters and selectable markers remain the main bottleneck in the application of this technology (Limera et al., 2017). Moreover, the number of gene copies that will be integrated into the host genome may represent an additional drawback, even if, as for transgenesis and intragenesis, any clear correlation has been reported (Jones et al., 1987; Zanek et al., 2007; Zeng et al., 2009; Joshi et al., 2011). To date, few examples of cisgenic plants have been reported, and these are found almost exclusively in apple and grape and aim to induce resistance to scab (Joshi et al., 2011; Vanblaere et al., 2011; Vanblaere et al., 2014; Gessler et al., 2014) and fire blight in apple (Krens et al., 2015; Würdig et al., 2015), as well as powdery mildew in grape (Dhekney et al., 2011; Salonia et al., 2020).

## 4. Project purpose

Mal secco is a tracheomycotic disease caused by the fungus *Plenodomus tracheiphilus*, that has resulted in severe damage and yield losses in the citrus industry of the Mediterranean area for over a century. The disease can affect different cultivated citrus species, with lemon and citron being the most susceptible. Identifying clones and hybrids resistant to MSD is a major desirable goal for lemon growers and breeders. The existing tolerant varieties, like Monachello, often do not have good quality characteristics. To obtain new lemon genotypes resistant or tolerant to MSD, we carried out a research program based on two different approaches. The first one was based on traditional breeding and a phenotypic survey, finalized to identify resistant or tolerant parents used in a crossing program to introgress resistance genes into true lemons. The second one was based on a transcriptomic analysis of rough lemon (*C. jambhiri* Lush.) seedlings in response to artificial *P. tracheiphilus* inoculation, to identify genes specifically involved in plant-pathogen interaction .

The phenotypic survey was performed in a germplasm field planted at CREA, Italy, in an area of high pathogen pressure. The visual phenotyping was conducted four times for three consecutive years, on a total of 50 accessions, with 2 or 3 replicate trees per accession. This survey was performed in combination with a molecular screening based on real-time PCR, for two consecutive years, analysing DNAs from twigs, young leaves, and mature leaves of all plants. The molecular method was useful to detect the pathogen in the absence of clear visual symptoms. The results of the survey were essential to categorize the clones in different groups based on the different resistance levels to the pathogen. The data produced from this first phase are a valuable resource for identifying the most tolerant lemon varieties, which are suitable for areas with high pathogen pressure. Based on the survey result we selected the contrasting parents, one susceptible and two resistants, to generate two segregating populations to identify QTLs (Quantitative Trait Loci) related to pathogen resistance. A set of microsatellite markers was useful for verifying the hybrid parentage and discarding the F1s derived from selfing. The obtained populations could represent a reference for the identification of genes and QTLs associated with the disease.

With the main goal of identifying candidate genes involved in the response of citrus plants to MSD, we performed a *de novo* transcriptome analysis of rough lemon seedlings subjected to artificial inoculations of *P. tracheiphilus* in comparison with plants inoculated with water. Rough lemon was chosen as a model plant because it is susceptible to the MSD, and its seedlings are fast-growing and showed clear mal secco symptoms after artificial inoculations in controlled conditions. The analysis of Differential Expressed Genes (DEGs) between control and inoculated samples highlighted the molecular response triggered by the pathogen. The analysis of the most significantly enriched pathways indicated which genes play a crucial role in the plant response to the fungus. A set of candidate genes that could be used for future molecular breeding approaches was identified. This study led to obtaining novel information to clarify the host-pathogen interaction, highlighting the molecular and biochemical mechanisms involved in the response of *C. jambhiri* against *P. tracheiphilus*.

In conclusion, the present project represents a solid basis for conventional and molecular lemon breeding programs aimed at the generation of mal secco-resistant cultivars. The creation of two populations of hybrids will allow correlating phenotypic data to genotypic data to identify QTLs, in order to perform marked assisted selections (MAS) for MSD resistance. The identification of candidate genes for MSD resistance by the transcriptome analysis will facilitate genome editing approaches to develop resistant lemon varieties.

# 5. Identification of field tolerance and resistance to mal secco disease in a citrus germplasm collection in Sicily

## 5.1 Introduction

Mal secco is a vascular disease caused by the quarantine fungus *Plenodomus tracheiphilus* Petri Gruyter, Aveskamp & Verkley (De Gruyter et al., 2013). It was previously classified as *Phoma tracheiphila* and is now included in the A2 list of quarantine pests of the European and Mediterranean Plant Protection Organization (Nigro et al, 2011; Pérez-Tornero et al., 2012).This fungus was discovered in 1984 on two Aegean Greek islands, Chios and Poros, and later it spread to other Mediterranean and Black Sea countries. Recently, it was also found in Spain, although it is not present in Portugal, Morocco, Malta, and Croatia (https://gd.eppo.int/taxon/DEUTTR/distribution). Lemon is one of the most susceptible sensitive species to this pathogen. The fungus penetrates through wounds (Butera et al., 1986; Migheli et al., 2009; Nigro et al., 2011; Zucker and Catara, 1985) caused by heavy rains, hail, and wind; these atmospheric conditions favour the spread of the disease. The symptoms usually begin with leaf vein chlorosis and leaf drop. Afterwards, the pathogen reaches twigs and branches and it is possible to observe red discoloured strands in the xylem of stems. This is followed by the dieback of twigs and branches and the eventual death of the tree. The disease severity shows a seasonal fluctuation and varies in different growing areas depending on the climatic conditions. Ruggieri (Ruggieri, 1953) reported that in the years from 1918 to 1953, mal secco disease (MSD) destroyed no less than 12,000 ha of lemon groves in Sicily, Italy. According to Salerno and Cutuli (1981), the mean yield of the production of lemon orchards in Sicily was approximately 20 tons/ha in the presence of MSD, whereas in lemon orchards not affected by MSD, the yield could reach 60 - 80 tons/ha. The pathogenicity of the different isolates collected in different Mediterranean countries was characterized in many studies (Salerno and Perrotta, 1966; Graniti, 1969; Surico and Jacobellis, 1980; Butera et al, 1986; Cacciola et al., 1986; Balmas et al., 2005; Licciardello et al., 2006; Ezra et al., 2007; Kalai et al, 2010; Kalai-Grami et al, 2012; Kroitor-Keren et al., 2013; Ziadi et al., 2014), and efficient protocols were optimized to detect fungal infection in different plant tissues (Licciardello et al., 2006; Demontis et al., 2008).Chemical treatments in commercial orchards can only be used to prevent infections (Salerno and Perrotta, 1978). Therefore, the selection of field-tolerant lemon varieties is the most effective strategy to control the disease (Nigro et al, 2011; Khanchouch et al, 2017).Lemons have a narrow genetic base since most of them are bud sports of a single ancestor, which is a hybrid between sour orange and citron (Curk et al., 2016; Barry et al., 2020). Such a genetic background exposes the species to the threat of the pathogen and hampers the identification of resistant varieties. Although most lemons are susceptible to the disease, some sources of tolerance were observed in field conditions, specifically in Monachello (Ruggieri, 1935, 1936, 1937a), Interdonato (Ruggieri, 1935, 1936, 1937a), Santa Teresa (Ruggieri, 1956), Quattrocchi (Ruggieri, 1940), Zagara Bianca and Continella (Damigella and Continella, 1970, 1971; Salerno, 1992). Unfortunately, none of them combine high fruit quality and productivity with tolerance to the disease. Many citrus and citrus relatives were classified as susceptible or resistant to MSD (Nigro et al, 2011), but the classification was based on a comparison among few citrus species and lemon varieties by visual screening or artificial inoculum (Ruggieri, 1948; Catara and Cutuli, 1972; Crescimano et al., 1973; Solel and Oren, 1975; Russo, 1977; Cutuli et al., 1984). Most of the bibliographic information is based on observations of single or few cultivars grown in the same field,

while phenotypic studies comparing several accessions in the same field block are lacking. Obtaining a lemon cultivar with good qualitative and pomological traits as well as resistance to MSD is a major challenge for the Mediterranean citrus industry (Khanchouch et al, 2017). The use of genetic transformation might be useful to improve the resistance to MSD or other diseases (Gentile et al., 2007; Muccilli et al., 2020), but the use of genetically modified organisms (GMOs) raises public concerns regarding their safety. Consequently, traditional breeding approaches are so far the only means of releasing improved cultivars. To achieve this aim, identifying and characterizing sources of tolerance in lemon germplasm is needed to provide growers with improved varieties that could be grown under high pathogen pressure, reducing the yield losses caused by MSD and achieving acceptable productivity and fruit quality (Migheli et al, 2009). Moreover, identifying sources of resistance within the lemon-like germplasm, and more generally in other citrus species, is essential for the introgression of resistance genes into lemon commercial cultivars as a part of a long-term strategy. In this study, we analysed the behaviour of a germplasm field collection, which is mostly comprised of lemons, in response to *P. tracheiphilus* natural infections by visual observation of symptoms and detection by real-time PCR. The objectives of the present study were (i) the identification of sources of MSD tolerance or resistance in the lemon and lemon-like germplasm by comparison of several clones and hybrids grown in the same field block under the same high pathogen pressure; (ii) the successful application of a fast and reliable method to detect *P. tracheiphilus* in natural infection conditions; and (iii) the identification of sources of resistance in other citrus species that could be used to introgress resistance genes into lemon interspecific hybrids.

## 5.2 Materials and Methods

### 5.2.1 Plant material and phenotyping

Phenotyping started in 2018 at the CREA germplasm collection of Acireale (37° 37' 23" N, 15° 09' 50" E). The original collection was planted in 2002. Plants were grafted onto the sour orange, in which lemon clones were replicated three times, and the other genotypes were replicated two times. The plants were grown with standard cultural practices, allowing comparative evaluation of the MSD symptoms under similar natural pathogen pressure. The studied germplasms included 1 citron clone, 27 lemon clones, 15 lemon and citron hybrids (most of them of unknown parentage), and 7 varieties belonging to other citrus species. The list of analysed accessions and their reported parentage is included in Table 3. Information regarding yield and fruit quality of 18 of the 27 lemon clones was previously reported by Di Vaio et al. (Di Vaio, et al., 2010). Phenotyping was carried out through a visual screening in four different periods for three consecutive years when the symptoms were more pronounced. Field evaluation was always performed by the same personnel. The wood of desiccated or defoliated twigs was examined for pink salmon discolouration, which is typical of MSD infection (Figure 16A), by removing the bark. Phenotyping also included measurement of the canopy volume of each tree because pruning was routinely performed to remove infected branches since the establishment of the collection field, influencing the canopy development of the most susceptible trees. Canopy volume was measured at the end of the last vegetative flush each year and was approximated as one half prolate spheroid with this formula (Turrell, 1946):

$$V=4/6\pi h(d/2)2 \qquad (1)$$

where h is the tree height and d is the tree diameter.

For each survey, symptom severity was scored according to an empirical scale based on the following assigned values:

0 = no symptoms - the plant did not show any twigs or branches with symptoms (Figure 16B);

1 = few symptoms - fewer than 5 twigs had visible symptoms (Figure 16C);

2 = medium symptoms - more than 5 twigs had visible symptoms (Figure 16D);

3 = strong symptoms - all branches had visible symptoms (Figure 16E),

4 = dead plant (Figure 16F).

| Common/cultivar name | Description | Origin | Botanical name | Reference |
|---|---|---|---|---|
| ‘Adamo VCR’ lemon | Shoot tip grafted clonal selection | Italy | *C. limon* (L.) Burm. f. | (Reforgiato Recupero et al., 2010) |
| ‘Akragas’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Calabrese et al., 2001) |
| ‘CNR L58’ lemon | Shoot tip grafted clonal selection | Italy | *C. limon* (L.) Burm. f. | (Abbata et al., 2019) |
| ‘Cerza’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Reforgiato Recupero et al., 2010) |
| ‘Continella M84’ lemon | Shoot tip grafted clonal selection | Italy | *C. limon* (L.) Burm. f. | (Damigella and Continella, 1970) |
| ‘Dosaco M503’ lemon | Shoot tip grafted clonal selection | Italy | *C. limon* (L.) Burm. f. | (Damigella and Continella, 1970) |
| ‘Fino VCR’ lemon | Shoot tip grafted clonal selection | Spain | *C. limon* (L.) Burm. f. | (UCR Citrus Variety Collection, 2020) |
| ‘Erice’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Calabrese et al., 2001) |
| ‘Interdonato’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Barry et al., 2020) |
| ‘Kamarina’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Calabrese et al., 2001) |
| ‘Lo Porto’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (UCR Citrus Variety Collection, 2020) |
| ‘Mascali seedless’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | unknown |
| ‘Ovale di Sorrento’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | unknown |
| ‘Pink Fleshed’ lemon | clonal selection | USA | *C. limon* (L.) Burm. f. | (UCR Citrus Variety Collection, 2020) |
| ‘Quattrocchi’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Ruggieri, 1940) |
| ‘Femminello-S’ lemon | Nucellar callus of 'Femminello' lemon, selected *in vitro* for tolerance to *P. tracheiphilus* toxin | Italy | *C. limon* (L.) Burm. f. | (Gentile et al., 1992) |
| ‘Scandurra’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | unknown |

| | | | | |
|---|---|---|---|---|
| ‘Segesta’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Calabrese et al., 2001) |
| ‘Selinunte’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (Calabrese et al., 2001) |
| ‘Sfusato Amalfitano’ lemon | clonal selection | Italy | *C. limon* (L.) Burm. f. | (UCR Citrus Variety Collection, 2020) |
| ‘Siracusano 2Kr’ lemon | Lemon obtained from nucellus from irradiated fruits by CO60 | Italy | *C. limon* (L.) Burm. f. | (Starrantino and Russo, 1977) |
| ‘Zagara Bianca M79’ lemon | Shoot tip grafted clonal selection | Italy | *C. limon* (L.) Burm. f. | (Damigella and Continella, 1970) |
| ‘46515’ tetraploid lemon | Autotetraploid Femminello lemon | Italy | *C. limon* (L.) Burm. f. | (Carrante and Bottari, 1952) |
| ‘46245’ tetraploid lemon | Autotetraploid Femminello lemon | Italy | *C. limon* (L.) Burm. f. | (Carrante and Bottari, 1952) |
| ‘46321’ tetraploid lemon | Autotetraploid Monachello lemon | Italy | *C. limon* (L.) Burm. f. | (Carrante and Bottari, 1952) |
| ‘Doppio’ tetraploid lemon | Autotetraploid lemon | Italy | *C. limon* (L.) Burm. f. | unknown |
| ‘Doppio Lentini’ tetraploid lemon | Autotetraploid lemon | Italy | *C. limon* (L.) Burm. f. | unknown |
| ‘Vozza Vozza’ | Lemon × pummelo hybrid | Italy | *C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr. | (Deng et al., 1995) |
| ‘India CRC 2476’ rangpur lime | Mandarin × citron hybrid | India | *C. limonia* Osbeck | (UCR Citrus Variety Collection, 2020) |
| ‘India CRC 2322’ lemon hybrid | lemon hybrid of unknown parentage | India | *C. limonia* Osbeck | (UCR Citrus Variety Collection, 2020) |
| Volkamer lemon | Mandarin × citron hybrid * | Italy | *C. volkameriana Ten. et Pasq*. | (UCR Citrus Variety Collection, 2020) |
| ‘Fantastico’ bergamot | Sour orange × lemon hybrid * | Italy | *C. bergamia* Risso and Poit. | (UCR Citrus Variety Collection, 2020) |
| ‘Femminello’ bergamot | Sour orange × lemon hybrid * | Italy | *C. bergamia* Risso and Poit. | (UCR Citrus Variety Collection, 2020) |

| | | | | |
|---|---|---|---|---|
| ‘Cardinale’ | Lemon × pummelo × citron hybrid | Italy | [*C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr. × *C. medica* (L.)] | (Deng et al., 1995) |
| ‘Spatafora’ | Lemon × citron hybrid | Italy | [*C. limon* (L.) Burm. f. × *C. medica* (L.)] | (Deng et al., 1995) |
| ‘Incomparabile’ | Sour orange × citron hybrid | Italy | [*C. aurantium* (L.) × *C. medica* (L.)] | (Deng et al., 1995) |
| ‘Mangiagli lemon | lemon hybrid of unknown parentage | Italy | *C. lumia* | unkown |
| ‘Palestinian’ sweet lime | (Pummelo × mandarin ) × citron * | India | *C. limettiodes* Tan. | (UCR Citrus Variety Collection, 2020) |
| ‘Limetta romana’ sweet lime | Sour orange × citron hybrid * | India | *C. limetta* Risso | unkown |
| ‘Corrugated red lime’ rangpur lime | Mandarin × citron hybrid | India | *C. limonia* Osbeck | (UCR Citrus Variety Collection, 2020) |
| ‘Ponderosa lemon’ | Pummelo × citron hybrid | Italy | [*C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr. × *C. medica* (L.)] | (Deng et al., 1995) |
| Sour Orange | pummelo × mandarin F1 hybrid*, ** | Italy | *C. aurantium* L. | (UCR Citrus Variety Collection, 2020) |
| ‘ISA’ clementine | Clonal selection of clementine (Mandarin x sweet orange **) | Italy | *C. clementina* hort. ex Tanaka | (Ollitrault et al., 2012) |
| ‘Khasi’ papeda | Wild nonedible citrus species | India | *C. latipes* Swing. Tan. | (Singh, 1981; Dutta, 1958) |
| ‘Tachibana’ | Wild nonedible mandarin** | Taiwan | *C. tachibana* (Mak) Tan. | (Wu et al., 2018) |
| ‘Changshou’ kumquat | Considered to be a chance hybrid between two *Fortunella* species | Japan | *Fortunella obovata* hort. ex. Tanaka | (Swingle, 1943) |
| ‘Doppio Sanguigno’ orange | clonal selection of Sweet orange (pummelo × mandarin complex hybrid*) | Italy | *C. sinensis* L. Osbek | (Caruso et al., 2016) |
| ‘Chandler CRC 3224’ pink pummelo | Hybrid of Siamese Pink pummelo and Siamese Sweet pummelo | USA | *C. maxima* (Burm.) Merr. × *C. maxima* (Burm.) Merr. | (UCR Citrus Variety Collection, 2020) |

| | | | | |
|---|---|---|---|---|
| ‘Siamelo CRC 2586’ tangelo | King tangor × grapefruit | USA | *C. paradisi* Macf. × *C. sinensis* (L.) Osbeck | (UCR Citrus Variety Collection, 2020) |
| ‘Diamante’ citron | Citron cultivar | Italy | *C. medica* L. | (Curk et al., 2016; UCR Citrus Variety Collection, 2020) |

Table 3. List of accessions phenotyped for MSD susceptibility at the CREA experimental farm of Acireale, Italy. Asterisks in the description of the citrus species refers to the species parentage as revealed by Curk et al. (Curk et al., 2016)(*) and Wu et al. (Wu et al., 2018)(**).

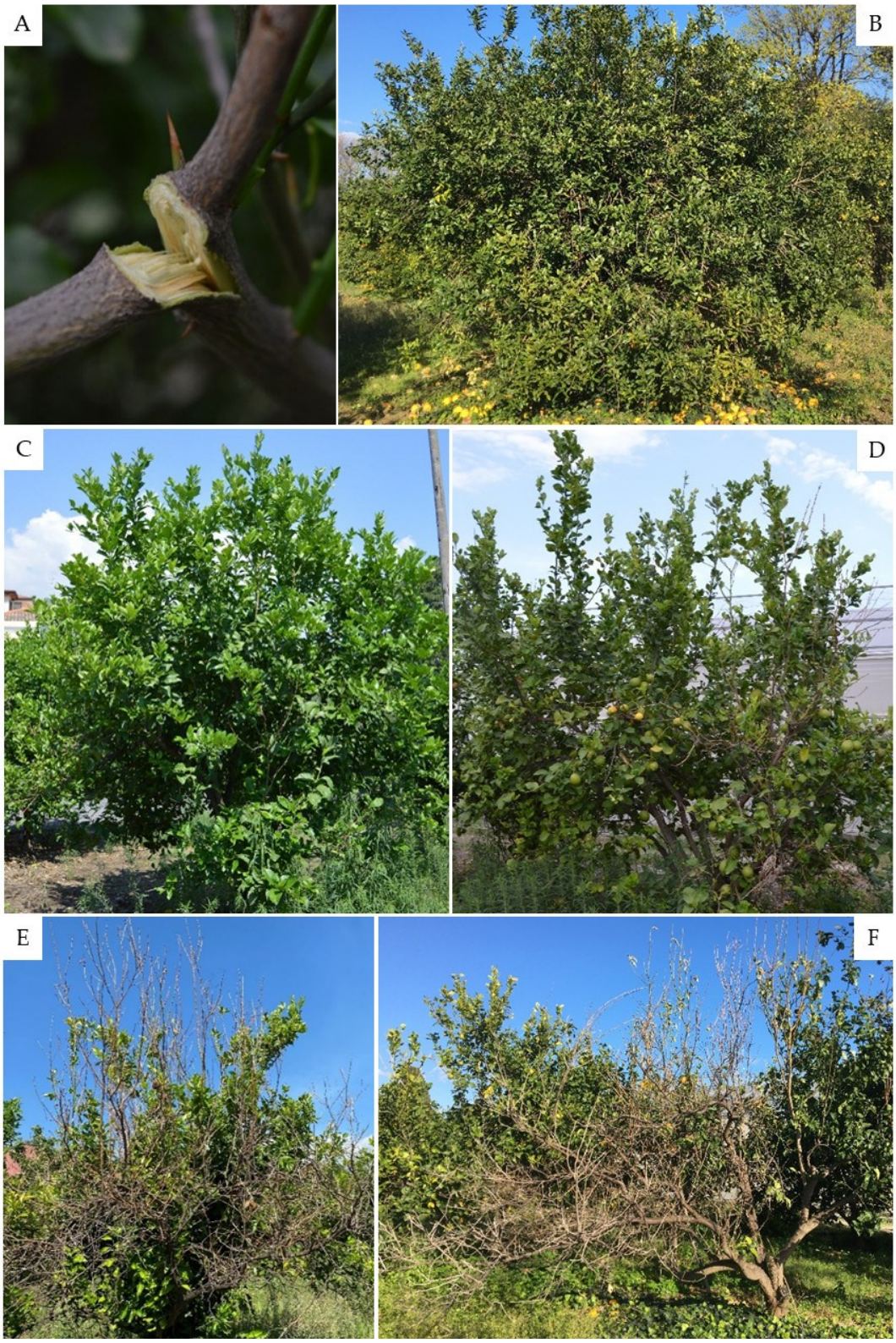

Figure 16. Citrus plants of the CREA germplasm showing different MSD symptoms A: infected shoot shows a yellow or pink-salmon to reddish discoloration of the wood; B: a plant of Khasi papeda that shows no symptoms of MSD scored with 0; C: a plant of Mascali seedless lemon that shows few symptoms of MSD scored as 1; D: a plant of Zagara Bianca M79 lemon that shows medium symptoms of MSD scored as 2; E: a plant Femminello Dosaco M503 lemon that shows strong symptoms of MSD scored as 3; F: a plant of Akragas lemon that died of MSD scored as 4.

### 5.2.2 DNA extraction

Samples of plant tissues for real-time PCR analysis were collected in the four cardinal directions for each plant in July 2018 and July 2019. For each tree, three types of samples were collected: one consisted of bulks of 10 young leaves (less than 6 months), one consisted of bulks of 10 mature leaves (6-12 months), and one consisted of 5 twigs, for a total of 9 samples per accession (3 biological replicates per tissue type). When only two plants per accession were present, the third biological replicate consisted of bulks of tissues from the two plants. For accessions with one or two replicates that were missing due to plant death, the samples were taken from the survivor plant to obtain nine samples from each accession. A total of 828 samples were collected from the 84 surviving plants. All samples were first surface-sterilized in a solution of 2.5% sodium hypochlorite and then washed twice with sterile distilled water. Ten grams of each sample was homogenized using liquid nitrogen, and less than 0.1 g was collected for DNA extraction (Kalai et al., 2010).The *P. tracheiphilus* Pt10 strain (kindly provided by Professor Vittoria Catara, Di3A, University of Catania) was cultured for DNA isolation as a reference for the real-time PCR experiments. The fungus was cultured for 10 days at 21 °C ± 2 °C in Petri dishes containing potato dextrose agar medium. One hundred micrograms of mycelium were harvested with a sterile loop from the surface of the colony, placed into an Eppendorf tube, frozen at -80 °C, and homogenized with a grinder (Tissuelyser - Qiagen, Hilden, Germany). DNA extraction of both plant and fungal tissues was performed by the CTAB method as described in Caruso et al. (2014), with slight modifications. Briefly, tubes containing 0.1 g of powdered plant tissues were mixed with 400 µL of extraction buffer (2% CTAB, 20 mM EDTA, 1.44 mM NaCl, 100 mM Tris HCl, pH 8) and 0.1% β-mercaptoethanol. Samples were vortexed and incubated at 65 °C for 60 min, agitating for the first 5 min. After adding 300 µL of chloroform-isoamyl alcohol (24:1), the vials were vortexed for 15 s and finally centrifuged at 20.800 *g* for 10 min. The supernatant was recovered, 500 µL of 100% ethanol was added and incubated at -20 °C for at least 30 min or at 4 °C overnight, followed by centrifugation at 20.800 *g* for 10 min. The pellet was rinsed with 1000 µL of 70% ethanol, resuspended in 50 µL of sterile distilled water, and stored at 4 °C until analysis. The quality and concentration of the isolated DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific™, Waltham, MA, USA). The 260/280 and 260/230 ratios were approximately 1.80 and 2.20, respectively, and the concentrations ranged from 50 to 300 ng $\mu L^{-1}$. All the samples were diluted at 10 ng $\mu L^{-1}$.

### 5.2.3 Real-time PCR analysis

Real-time PCR amplifications were performed according to the protocol described by Licciardello et al. (2006) using GR70 forward primer (5′-GATCCGTACGCCTTGGGGAC-3′), GL1 reverse primer (5′-AGAAGCGTTTGGAGGAGAGAATG-3′), and the probe PP1 (5′-FAM-CACGCAATCTTGGCGACTGTCGTT-TAMRA-3′). Each sample was amplified using the following mix: 2X real-time PCR master mix (TaqMan™ Fast Advanced Master Mix Applied Biosystems™), 200 nM forward primer, 200 nM reverse primer, 100 nM fluorogenic probe, and 40 ng/µL genomic DNA. Negative controls using water in place of DNA were routinely included. Amplifications were carried out in an ABI 7500 Real-Time PCR System (Applied Biosystems™) using the following program: 50 °C for 2 min, 95 °C for 30 s followed by 40 cycles at 95 °C for 10 s and 62 °C for 30 s. Calibration of the standard curve for fungal DNA quantification by real-time PCR was assessed using *P. tracheiphilus* DNA (100 µg $ml^{-1}$) extracted from the Pt10 strain and serially diluted in sterile distilled water as described in Licciardello et al. (2006).

### 5.2.4 Statistical analysis

To perform the comparison of means, a value of 40 was assigned to all runs where *P. tracheiphilus* was nondetectable. The correlation between the five variables measured (severity symptom scores, Ct value from young leaves, Ct values from old leaves, Ct values from twigs, and canopy volume) was performed using Spearman's method at the 95% confidence level. Statistical analysis and the analysis of correlation among the variables were performed using R software, version 3.6.3 (R Core Team, 2019), using the packages "corrplot" (We and Simko, 2017) and "corrgram" (Wright, 2018).

## 5.3 Results and discussion

### 5.3.1 Field phenotyping

In the present study, we evaluated the disease responses of 50 accessions belonging to the *Citrus* and *Fortunella* genera to natural MSD infections. The main purpose of this work was the identification of sources of tolerance and resistance in lemons and in other citrus species and hybrids. Information is essential to plan lemon breeding programs based on hybridization and somaclonal variation to generate new varieties with improved tolerance to the disease. The field trial was conducted in an area where the environmental conditions are particularly favorable to the disease. The field trial originally planted in 2002 included 50 accessions replicated two or three times, for a total of 123 plants. The accessions initially included in the field are listed in Table 3. Some plants died from MSD within the first years after planting (Reforgiato Recupero et al., 2010), and were replanted in 2009 (Table 4). At the end of the survey (May 2020), just 84 plants belonging to 46 accessions survived. Specifically, 79 plants were the original plants (16 years old) and 5 were the replanted replicates (9 years old). All the trees of the following selections died before the beginning of the survey: Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon, and Diamante citron. Attempts to replant them to re-constitute the original collection were made, but plants died again due to MSD, confirming their high susceptibility. The rest of the missing plants were replicates of other lemon clonal selections or susceptible citrus accessions as indicated in Table 4.

| Cultivar | Ct value young leaves | | Ct value mature leaves | | Ct value twigs | | Symptom severity scores | Average canopy volume ($m^3$) | Number of original plants (2002) | Replanted replicates (2009) | Number of dead plants (May 2020) | Number of surviving plants (May 2020) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2018 | 2019 | 2018 | 2019 | 2018 | 2019 | | | | | | |
| ‘Adamo VCR’ lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| ‘Akragas’ lemon | 23 | 37 | 34 | 36 | 40 | 28 | 2.66 | 16.64 | 3 | 0 | 2 | 1 |
| ‘CNR L58’ lemon | 36 | 38 | 38 | 38 | 28 | 30 | 3.00 | 11.80 | 3 | 0 | 1 | 2 |
| ‘Cerza’ lemon | 35 | 39 | 36 | 38 | 25 | 27 | 3.16 | 8.28 | 3 | 3 | 5 | 1 |
| ‘Continella M84’ lemon | 40 | 40 | 31 | 29 | 34 | 34 | 1.83 | 41.67 | 3 | 0 | 1 | 2 |
| ‘Dosaco M503’ lemon | 35 | 37 | 38 | 36 | 28 | 34 | 2.25 | 14.71 | 3 | 1 | 2 | 2 |
| ‘Fino VCR’ lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| ‘Erice’ lemon | 36 | 38 | 37 | 33 | 26 | 40 | 2.33 | 6.69 | 3 | 3 | 5 | 1 |
| ‘Interdonato’ lemon | 30 | 18 | 34 | 32 | 28 | 30 | 1.12 | 14.03 | 2 | 0 | 1 | 1 |
| ‘Kamarina’ lemon | 34 | 40 | 36 | 38 | 25 | 31 | 2.16 | 22.00 | 3 | 0 | 1 | 2 |
| ‘Lo Porto’ lemon | 40 | 36 | 30 | 32 | 28 | 38 | 0.50 | 8.51 | 2 | 0 | 0 | 2 |
| ‘Mascali seedless’ lemon | 21 | 23 | 23 | 25 | 20 | 30 | 1.50 | 14.90 | 2 | 1 | 1 | 2 |
| ‘Ovale di Sorrento’ lemon | 34 | 32 | 29 | 31 | 22 | 30 | 3.16 | 7.28 | 3 | 0 | 1 | 1 |
| ‘Pink Fleshed’ lemon | 38 | 40 | 37 | 37 | 32 | 34 | 3.00 | 21.75 | 2 | 0 | 1 | 1 |
| ‘Quattrocchi’ lemon | 34 | 40 | 34 | 40 | 32 | 40 | 0 | 11.00 | 2 | 0 | 0 | 2 |
| ‘Femminello-S’ lemon | 40 | 36 | 38 | 34 | 25 | 31 | 3.16 | 2.98 | 3 | 0 | 2 | 1 |
| ‘Scandurra’ lemon | 40 | 34 | 38 | 36 | 23 | 35 | 3.16 | 25.88 | 3 | 0 | 2 | 1 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ‘Segesta’ lemon | 34 | 40 | 28 | 36 | 30 | 34 | 0.66 | 20.74 | 3 | 0 | 0 | 3 |
| ‘Selinunte’ lemon | 28 | 40 | 40 | 36 | 30 | 34 | 2.83 | 75.63 | 3 | 0 | 2 | 1 |
| ‘Sfusato Amalfitano’ lemon | 38 | 40 | 39 | 37 | 30 | 32 | 3.16 | 6.35 | 3 | 0 | 2 | 1 |
| ‘Siracusano 2Kr’ lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| ‘Zagara Bianca M79’ lemon | 31 | 39 | 40 | 38 | 23 | 31 | 2.75 | 16.08 | 3 | 0 | 1 | 2 |
| ‘46515’ tetraploid lemon | 33 | 39 | 34 | 38 | 29 | 27 | 1.00 | 19.31 | 2 | 1 | 1 | 2 |
| ‘46245’ tetraploid lemon | 33 | 35 | 28 | 30 | 26 | 30 | 1.87 | 11.48 | 2 | 1 | 2 | 1 |
| ‘46321’ tetraploid lemon | 38 | 40 | 38 | 40 | 38 | 38 | 0 | 5.65 | 2 | 0 | 0 | 2 |
| ‘Doppio’ tetraploid lemon | 37 | 39 | 36 | 38 | 38 | 38 | 0 | 7.27 | 2 | 0 | 0 | 2 |
| ‘Doppio Lentini’ tetraploid lemon | 37 | 39 | 38 | 40 | 37 | 39 | 0 | 36.82 | 2 | 0 | 0 | 2 |
| ‘Vozza Vozza’ | 32 | 36 | 40 | 40 | 35 | 35 | 0.11 | 5.69 | 3 | 0 | 0 | 3 |
| ‘India CRC 2476’ rangpur lime | 29 | 33 | 36 | 36 | 33 | 35 | 2.87 | 5.03 | 2 | 1 | 2 | 1 |
| ‘India CRC 2322’ lemon | 31 | 33 | 40 | 40 | 33 | 35 | 2.87 | 5.30 | 2 | 0 | 1 | 1 |
| Volkamer lemon | 36 | 38 | 34 | 38 | 31 | 33 | 2.00 | 9.32 | 3 | 0 | 0 | 3 |
| ‘Fantastico’ bergamot | 34 | 36 | 34 | 36 | 39 | 39 | 0.62 | 33.91 | 3 | 0 | 0 | 3 |
| ‘Femminello’ bergamot | 30 | 34 | 32 | 34 | 35 | 37 | 2.12 | 12.00 | 2 | 0 | 1 | 1 |
| ‘Cardinale’ | 36 | 38 | 35 | 35 | 37 | 37 | 0.12 | 7.19 | 2 | 0 | 0 | 2 |
| ‘Spatafora’ | 36 | 36 | 37 | 37 | 36 | 38 | 0.25 | 16.39 | 2 | 0 | 0 | 2 |
| ‘Incomparabile’ | 34 | 36 | 32 | 36 | 37 | 39 | 0 | 1.61 | 2 | 0 | 0 | 2 |
| ‘Mangiagli lemon | 40 | 40 | 40 | 40 | 38 | 40 | 0 | 19.16 | 2 | 0 | 0 | 2 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ‘Palestinian’ sweet lime | 36 | 38 | 37 | 37 | 38 | 38 | 0 | 22.20 | 2 | 0 | 0 | 2 |
| ‘Limetta romana’ sweet lime | 30 | 36 | 33 | 35 | 30 | 34 | 3.00 | 1.34 | 2 | 0 | 1 | 1 |
| ‘Corrugated red lime’ rangpur lime | 40 | 40 | 40 | 40 | 35 | 37 | 2.00 | 17.38 | 2 | 0 | 0 | 2 |
| ‘Ponderosa lemon’ | 37 | 37 | 40 | 40 | 38 | 40 | 0 | 18.85 | 3 | 0 | 0 | 3 |
| Sour Orange | 40 | 40 | 40 | 40 | 37 | 39 | 0 | 7.39 | 3 | 0 | 0 | 3 |
| ‘ISA’ clementine | 40 | 40 | 40 | 40 | 38 | 40 | 0 | 9.29 | 2 | 0 | 0 | 2 |
| ‘Khasi’ papeda | 38 | 38 | 38 | 38 | 38 | 38 | 0 | 33.21 | 2 | 0 | 0 | 2 |
| ‘Tachibana | 40 | 40 | 40 | 40 | 37 | 37 | 0 | 1.64 | 3 | 0 | 0 | 3 |
| ‘Changshou’ kumquat | 40 | 40 | 40 | 40 | 34 | 38 | 0 | 2.32 | 2 | 0 | 0 | 2 |
| ‘Doppio Sanguigno’ orange | 39 | 39 | 40 | 40 | 38 | 38 | 0 | 13.55 | 2 | 0 | 0 | 2 |
| ‘Chandler CRC 3224’ pink pummelo | 33 | 37 | 34 | 38 | 35 | 37 | 0 | 1.75 | 2 | 0 | 0 | 2 |
| ‘Siamelo CRC 2586’ tangelo | 40 | 40 | 40 | 40 | 40 | 40 | 0 | 28.93 | 2 | 0 | 0 | 2 |
| ‘Diamante’ citron | - | | - | | - | | 4.00 | 0.00 | 2 | 2 | 4 | 0 |

Table 4. Results of MSD germplasm phenotyping based on real-time PCR, visual observation of symptoms and canopy volumes. Values of the real-time PCR refer to the mean values of the three replicates sampled in 2018 and the three replicates of 2019 approximated to the nearest integer. Values of symptoms represent the average of four scores recorded between May 2018 and May 2020. The table shows the number of original plants, the number of replicates replanted in 2009 and the number of surviving plants at the end of the survey. The list also includes the accessions that died of MSD before the beginning of the survey.

Visual screening was performed 4 times, starting from May 2018, for 3 consecutive years to check the behavior of each plant in response to 18 years of natural infection and to follow the possible progression of the disease during the 3 years of observations.

The scores used for the estimation of symptom severity ranged between 0 (absence of symptoms) and 4 (plant death). Score 4 was also assigned to the replicates that died of MSD before May 2018 or during the visual screening. The mean scores of symptom severity recorded in the three years are shown in Table 4. Several accessions showed no symptoms and had a score of 0 (Table 5). This group includes only a true lemon, Quattrocchi, a clone very similar to Monachello, already known for its high tolerance to the disease. Other accessions with citron ancestry, such as Palestinian sweet lime (*C. limettiodes* Tan.), Ponderosa lemon [*C. limon* (L.) Burm. f. × *C. maxima* (Burm.) Merr. × *C. medica* (L.)], and Incomparabile [*C. aurantium* (L.) × *C. medica* (L.)], showed no symptoms, indicating that it is theoretically possible to generate mal secco-resistant lemon-like phenotypes through hybridization. In this group, the only accession not included in the genus *Citrus*, namely, Changshou kumquat (*Fortunella obovata* hort. ex. Tanaka), which differs from the most common kumquat (*Fortunella margarita* [Lour.] Swingle) in its rounded shape, is also present. Interestingly, sour orange also had a score of 0. This species is reported as susceptible (Nigro et al., 2011; Migheli et al., 2009) and is often used to evaluate the pathogenicity of the *P. tracheiphilus* strains at the seedling stage (Magnano di San Lio, G., Cacciola, S. O., Pane, A., and Grasso, 1992; Traversa et al., 1992; Raimondo et al., 2007, 2010; Hajlaoui et al., 2010; Kalai-Grami et al., 2014); however, we observed no symptoms during the three years of evaluation.

The genotypes with very few symptoms that had a score less than 1 were Spatafora [*C. limon* (L.) Burm. *f.* × *C. medica* (L.)], Cardinale [*C. limon* (L.) Burm. *f.* × *C. maxima* (Burm.) Merr. × *C. medica* (L.)], Vozza Vozza (*C. limon* (L.) Burm. *f.* × *C. maxima* (Burm.) Merr.), and Fantastico bergamot. These genotypes are all lemon or citron hybrids and show a high tolerance to the disease. The accessions: India CRC 2476' rangpur lime (*C. limonia* Osbeck), 'India CRC 2322' lemon *C. limonia* Osbeck*)*, Volkamer lemon, corrugated red lime rangpur lime (*C. limonia* Osbeck)*,* Femminello bergamot and limetta romana sweet lime (*C. limetta* Risso*)* showed a range of symptoms with a score from 2 to 3. These genotypes are all citron hybrids and showed susceptibility to MSD. Some lemon clones revealed field tolerance, such as Continella M84, Segesta, Interdonato, Zagara Bianca M79, Lo Porto, and Mascali seedless, with scores ranging between 0.6 and 2. Other lemon clones with scores between 2 and 3 were Femminello S, Kamarina, Dosaco M503, Selinunte, Scandurra, Sfusato Amalfitano, Ovale di Sorrento, Pink Fleshed, CNR L58, and Akragas. Higher scores among the two lemon clones were assigned to Erice and Cerza, with 3.3 and 3.4, respectively. Finally, the highest score was assigned to 3 lemons, Siracusano 2Kr, Adamo VCR, and Fino VCR, and citron Diamante, with a 0% survival rate before the beginning of the survey, which can be considered the most susceptible to the disease.

Among the autotetraploid lemon clones (Doppio Lentini, Doppio, 46321, 46245, and 46515), we generally noticed a high tolerance or the absence of symptoms, with some differences. Specifically, no symptoms were found in Doppio Lentini, Doppio, and 46321 (probably Monachello 4x) during the three years of visual monitoring, while few infected branches were observed in 46245 and 46515. In addition to symptom observation, we measured the canopy volume of all surviving plants, not as an indication of plant vigour but as an additional parameter to describe the sensitivity of each accession to MSD. The canopy volume can be drastically reduced by pathogen attack and by pruning infected branches. Indeed, pruning is one of the few effective measures to contain the spread of the

disease in lemon orchards. In our survey, we analyzed many different citrus species, and a lower canopy volume of some accessions was due to the different growth habits and not necessarily to MSD infections (Table 4). Specifically, some citrus species, such as Chandler pink pummelo [*C. maxima* (Burm.) Merr. × *C. maxima* (Burm.) Merr.] or tachibana (*C. tachibana* (Mak) Tan.), showed a very low canopy volume in the absence of MSD symptoms, probably because they are poorly adapted to the growing environment. Consequently, we found no general correlation (r = 0.01) between canopy volume and symptoms when analyzing the whole dataset (Figure 17). However, a higher correlation between canopy volume and symptoms (r = -0.40) was found when the comparison was limited to the lemon clonal selections (Figure 18). The accessions that had lower canopy volumes, such as CNR L58 lemon, Erice lemon, and Cerza lemon, were generally the ones that underwent severe pruning due to the presence of more symptoms. All the most susceptible clones had canopy volumes below 10 $m^3$, whereas many tolerant clones showed values ranging from 11.00 $m^3$ and 75.63 $m^3$ with some exceptions, such as Doppio, 46321, and Lo Porto.

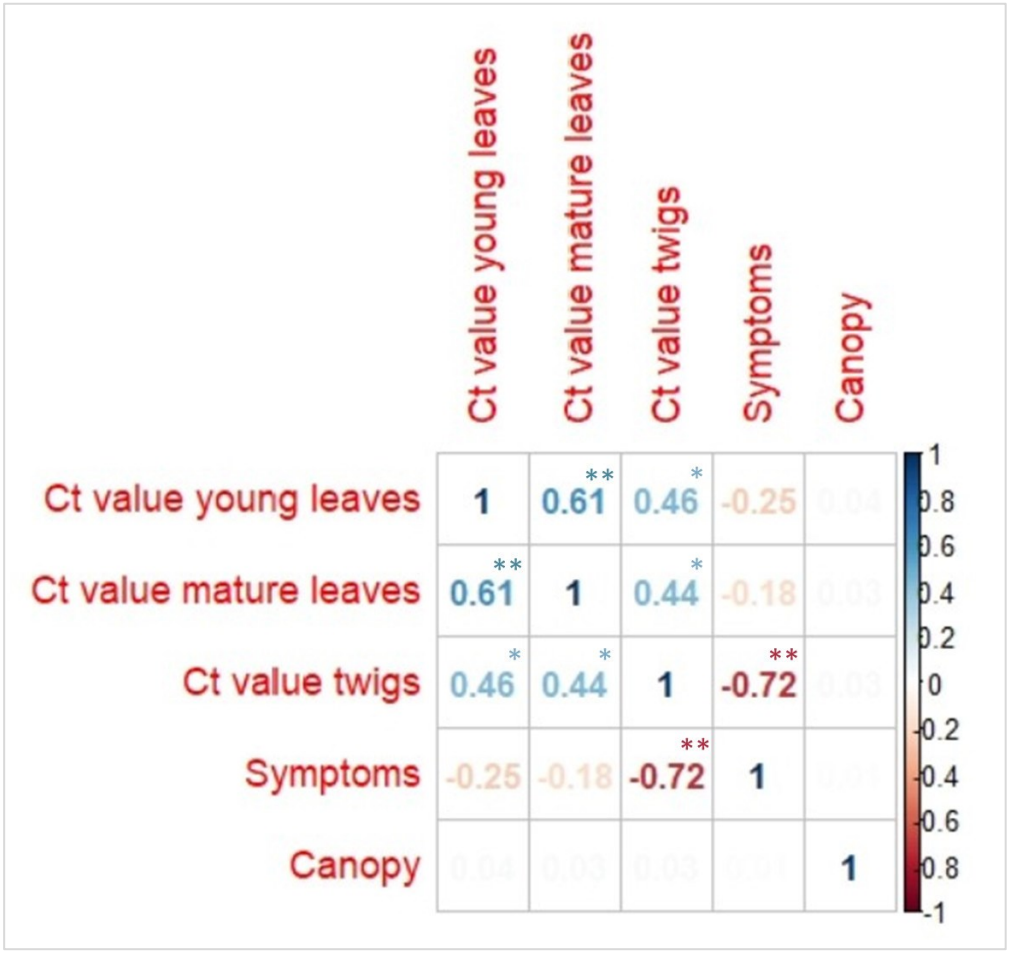


Figure 17. Pairwise correlation matrix of five traits measured in the CREA germplasm. Blue numbers represent positive correlations and red ones represent negative correlations. Faded numbers correspond to very low correlation values (<0.1). Correlation values were statistically significant with the following p-values * < 0.05; ** < 0.001; values without asterisks were not statistically significant.

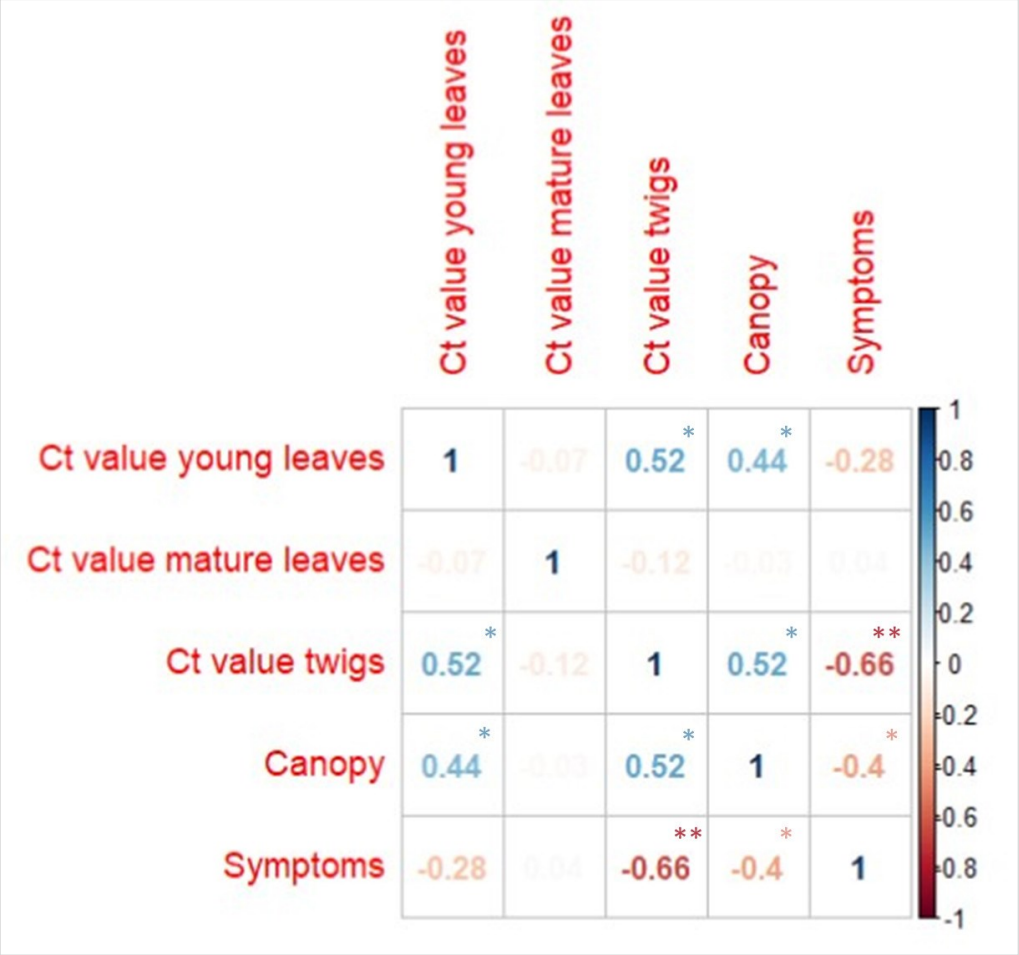


Figure 18. Correlation index between the five variables of the lemon clones in the CREA germplasm. Blue numbers represent positive correlations and red ones represent negative correlations. Faded numbers correspond to very low correlation values (<0.1). Correlation values were statistically significant with the following p-values * < 0.05; ** < 0.001; values without asterisks were not statistically significant.

### 5.3.2 Real-time PCR detection of *P. tracheiphilus*

In addition to visual phenotyping, we performed molecular screening to obtain a more exhaustive and reliable assessment of the *P. tracheiphilus* infection in all replicate trees of the germplasm collection, especially in the absence of clear symptoms. Sample collections for DNA isolation were conducted in the first week of July because symptoms usually appear during spring and early summer (Nigro et al, 2011). Molecular detection was performed using different types of tissues (twigs, young leaves, and mature leaves), amplifying a genomic region of the fungus by real-time PCR as reported by Licciardello et al. (2006). In our experiment, Ct values ranged from 22 to 39 in young leaves, from 24 to 39 in mature leaves, and from 17 to 39 in DNA from twigs. Licciardello et al. (2006) reported that the minimum amount of pathogen DNA that could be quantified accurately using real-time PCR was 1 pg, corresponding to a Ct value of 37.93. Therefore, Ct values above 38 cannot reflect the occurrence of *P. tracheiphilus* infection (Table 3). Leaf samples were included because the pathogen is able to penetrate through leaf wounds, so this survey could be potentially useful to identify early infections. However, the correlation between leaf Ct values and symptom scores was generally weak (Figure 17), and in some cases, it was not useful to detect infections that were clearly visible in parts of the canopy, such as in the lemon clones CNR L58, Kamarina, Sfusato Amalfitano, Pink fleshed and Cerza. DNA samples from twigs were the most effective for *P. tracheiphilus* detection. The real-

time PCR analysis of twig samples confirmed the presence of *P. tracheiphilus* in all genotypes where the symptoms were present. Furthermore, the molecular analysis detected *P. tracheiphilus* in xylem tissues of many accessions where no symptoms were present from any of the phenotyping data. This phenomenon occurred in Quattrocchi lemon and Chandler pink pummelo. These cases may include plants that were infected recently so that symptoms were not yet visible or plants that showed some tolerance and that were able to block the movement of the pathogen and recover from the disease. In many replicates, the pathogen was detected only in the twigs and not in leaves, such as in Vozza Vozza, India CRC 2322, Corrugated red lime, Sour orange, ISA clementine, Tachibana and Changshou Kumquat. Moreover, Ct values from twigs showed a high correlation (r= -0.72; p-values < 0.001) with the symptom scores (Figure 17). In particular, they ranged between 17 and 30 in the susceptible clones showing field symptoms, while they were higher (Ct value > 30) in tolerant clones and hybrids. Figure 19 shows the relationship between symptoms and Ct values in twigs and provides a view of the different degrees of susceptibility to MSD observed in the germplasm. The most susceptible accessions are in the lower right part of the plot, while the field tolerant or resistant accessions are grouped in the upper left part. Specifically, the right side of the plot includes all the lemon clones with the exceptions of Quattrocchi and Segesta, which are in the upper left side, grouped with some tetraploid lemons (Doppio Lentini and Doppio, 46321), different citron and lemon hybrids (Vozza Vozza, Cardinale, Incomparabile, Spatafora, and Palestinian sweet lime) and other citrus species that are resistant to MSD (Khasi papeda, Doppio Sanguigno (*C. sinensis* L. Osbek), Mangiagli lemon, Chandler pink pummelo, and ISA clementine).

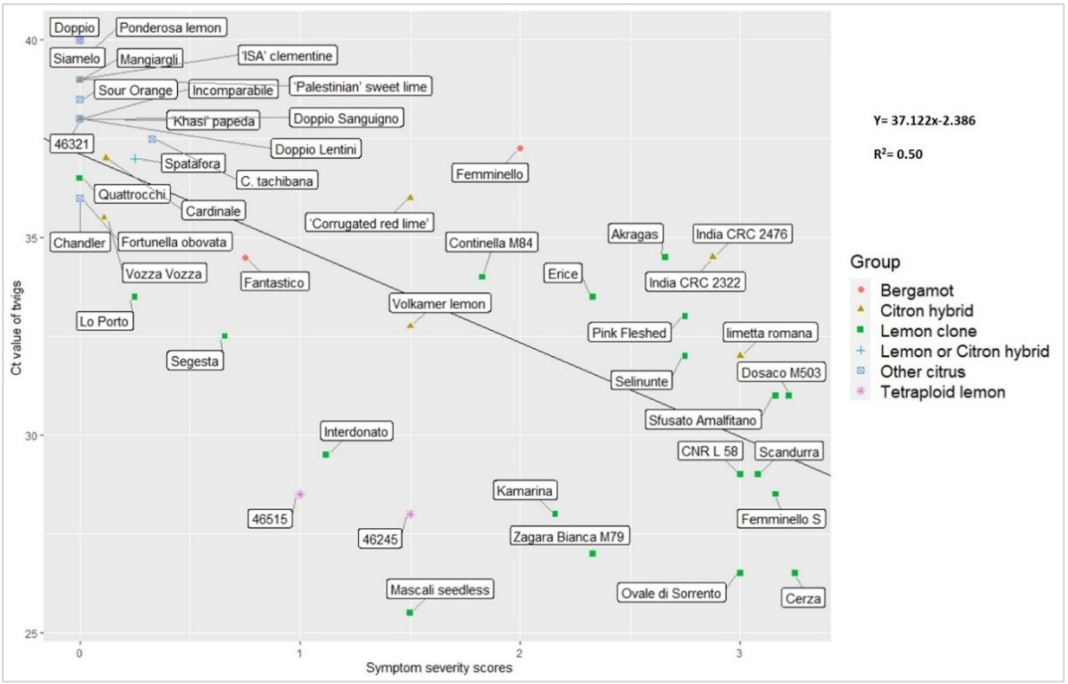

Figure 19. Scatterplot with regression line showing the relationships between Ct value of twigs and symptoms of the germplasm collection of CREA considered in this study.

Significant correlations were also found analyzing the subset of the lemon clonal selections between Ct values of twigs and symptoms (r = -0.66; p-values < 0.001; Figure 18), and between Ct values of twigs and canopy volumes (r = 0.52; p-values < 0.05; Figure 18) . A scatterplot revealing the relationship Ct values of twigs and canopies is shown in Figure 20.

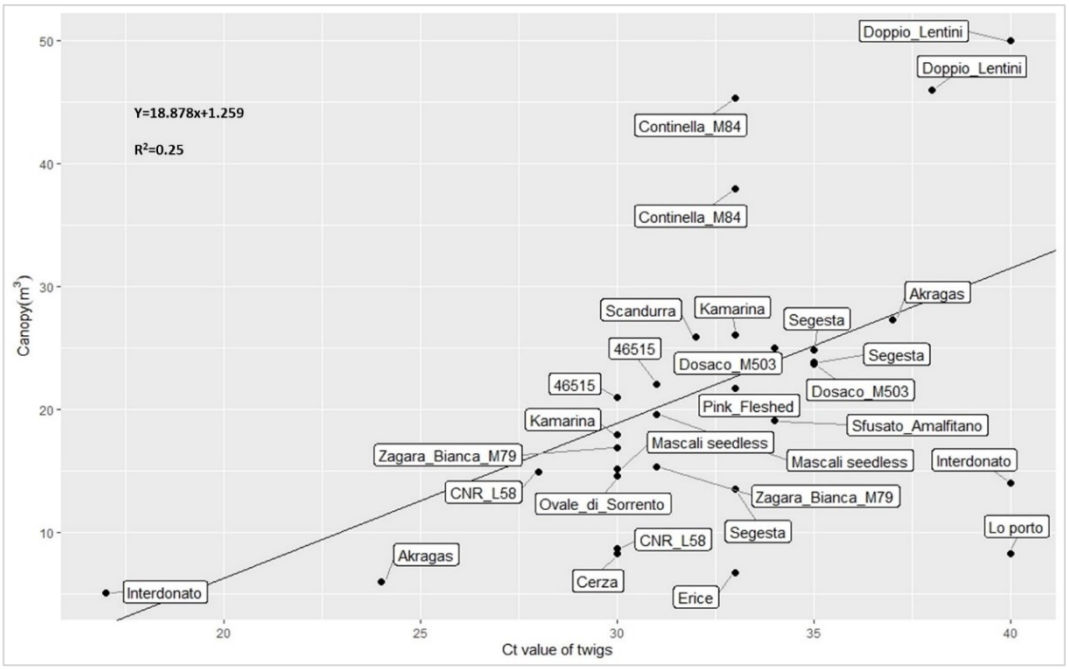


Figure 20. Scatterplot with regression line showing the relationships between canopy of each surviving replicate of the lemon clonal selections and Ct values of twigs.

### 5.3.3 Assignment of the accessions to disease severity groups

Based on the symptom severity scores, canopy volumes, and real time PCR results, we assigned the analyzed accessions to seven different disease severity groups.

For determining disease groups, we considered the complete absence of the pathogen (immunity), the cases of very limited pathogen movement in the xylem with no visible symptoms (field resistance), the presence of very few symptoms with the ability of the plant to recover from infections (field tolerance), and successful colonization of the pathogen leading to clear disease symptom expression and, in some cases, to plant death (susceptibility). The groups and the list of accessions assigned to each group are listed in Table 5. Pictures of plants representative of each severity group were included as supplementary material (Figures 21 to 26).

Figure 21. A representative picture of the disease severity group 2. A plant of Erice lemon.

Figure 22. A representative picture of the disease severity group 3. A plant of Ovale di Sorrento lemon.

Figure 23. A representative picture of the disease severity group 4. A plant of Continella M84 lemon.

Figure 24. A representative picture of the disease severity group 5. A plant of Segesta lemon.

Figure 25: A representative picture of the disease severity group 6. A plant of Cardinale.

Figure 26. A representative picture of the disease severity group 7. A plant of Doppio Lentini tetraploid lemon.

| Disease severity group | Accessions |
|---|---|
| Group 1: the most susceptible accessions, all plants died. | Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon, Diamante citron. |
| Group 2: susceptible to MSD. Very severe symptoms, some replicates died. | Erice and Cerza. |
| Group 3: medium to severe symptoms, two of the three original replicates died of MSD. In some cases, a slight recovery of the plants during the three years of observation was recorded. | Akragas lemon, Femminello S, Selinunte, Dosaco M503, Sfusato Amalfitano, Scandurra, Ovale di Sorrento, Mascali seedless, 46245, Pink Fleshed, Limetta romana India CRC 2476, India CRC 2322, and Femminello bergamot. |
| Group 4: tolerant to the MSD, different range of symptoms from mild to severe, but real-time PCR showed Ct values between 30 and 31. | Continella M84 Lemon, Interdonato lemon, CNR L58 lemon, Zagara Bianca M79 lemon, Kamarina lemon, Lo Porto lemon, 46515 tetraploid lemon, Corrugated red lime, and Volkamer lemon. |
| Group 5: high tolerance to MSD, and very few symptoms were detected during the visual screening. Mean real-time PCR Ct value of 32. All replicates planted in 2002 are still alive. | Segesta. |
| Group 6: very few symptoms during the field phenotyping. Real-time PCR mean Ct values between 35 and 37. | Chandler pink pummelo, Fantastico bergamot, Vozza Vozza, and Cardinale. |
| Group 7: no symptoms in the field, mean Ct value >37. | Doppio Lentini tetraploid lemon, 46321 tetraploid lemon, Doppio tetraploid lemon, Palestinian sweet lime, sour orange, Khasi papeda, ISA Clementine, Doppio Sanguigno orange, Ponderosa lemon, Tachibana, Changshou kumquat, Quattrocchi lemon, Siamelo CRC 2586 tangelo, Spatafora, Incomparabile and Mangiagli lemon. |

Table 5. List of disease severity groups based on visual observations, real-time PCR results of twig samples and canopy volumes, and accessions assigned to each group.

Group 1: in this group, we included the most susceptible accessions. Specifically, the lemon cultivars Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon, and Diamante citron died before the beginning of the survey. Reforgiato Recupero and colleagues (2010) reported that MSD was the cause of death of all replicates of the original field, and later attempts to replace the dead plants were not successful since the new plants died again due to MSD.

Group 2: accessions in this category are susceptible to MSD and were also planted twice. Very severe symptoms were found on all plants, and some replicates died. This group includes the lemon clones Erice and Cerza.

Group 3: the accessions in this group showed a different range of symptoms, from medium to severe, and two of the three original replicates died of MSD. In some cases, a slight recovery of the plants during the three years of observation was recorded. This group includes Akragas lemon,

Femminello S, Selinunte, Dosaco M503, Sfusato Amalfitano, Scandurra, Ovale di Sorrento, Mascali seedless, 46245, Pink Fleshed, Limetta romana India CRC 2476, India CRC 2322, and Femminello bergamot.

Group 4: this group includes accessions that can be considered tolerant to the disease, namely, Continella M84 Lemon, Interdonato lemon, CNR L58 lemon, Zagara Bianca M79 lemon, Kamarina lemon, Lo Porto lemon, 46515 tetraploid lemon, and Corrugated red lime. The plants showed a different range of symptoms from mild to severe, but real-time PCR showed medium levels of the pathogen with Ct values between 30 and 31. Moreover, their canopy volume is generally higher than the accessions included in the previous groups, confirming their ability to tolerate the disease and guarantee canopy growth. This group also includes Volkamer lemon. This species was reported to exhibit a medium level of susceptibility by Russo (Russo, 1977), while other reports described it as highly susceptible (Salerno et al., 1967; Catara and Cutuli, 1972). We also observed different responses among the three replicates, with one healthy plant with very limited symptoms and the other two with severe dieback and reduced canopy volume.

Group 5: in this group, we can find just a lemon clone, Segesta. It showed high tolerance to the disease, and very few symptoms were detected during the visual screening. The plants had a high canopy volume, and real-time PCR confirmed the low level of infections in twigs, with a mean Ct value of 32. All replicates planted in 2002 are still alive.

Group 6: in this group, we were able to detect very few symptoms during the field phenotyping, but a low level of the fungus was detected periodically by real-time PCR (mean Ct values were between 35 and 37). Under field conditions, the pathogen could not establish in these hosts. The accessions are Chandler pink pummelo, Fantastico bergamot, Vozza Vozza, and Cardinale.

Group 7: this group includes all the accessions where the pathogen was detected in very low quantities with a Ct value >37, and the plants did not show any symptoms in the field. Therefore, these accessions showed resistance in the field conditions of natural pathogen pressure. The accessions in this group are Doppio Lentini tetraploid lemon, 46321 tetraploid lemon, Doppio tetraploid lemon, Palestinian sweet lime, sour orange, Khasi papeda, ISA Clementine, Doppio Sanguigno orange, Ponderosa lemon, Tachibana, Changshou kumquat, Quattrocchi lemon, Siamelo CRC 2586 tangelo, Spatafora, Incomparabile and Mangiagli lemon. The possible resistance of these accessions needs confirmation on a larger number of replicates, since some of these genotypes, such as a mandarin hybrid or sweet orange, showed sporadically mild infections; although, the pathogen caused the typical symptoms of “mal nero”, a form of the disease where the fungus enters the plant through the roots (Grasso S., 1973; Hajlaoui *et al*., 2008; Karapapa et al., 2015). Sour orange is reported to be very sensitive to the disease (Ruggieri, 1940, 1948; Salerno, 1964; Catara and Cutuli, 1972; Nigro et al., 1996; Migheli et al., 2009; Nigro et al., 2011), but in our study, no symptoms or pathogens were detected by phenotyping or real-time PCR, respectively. This might be due to different degrees of susceptibility to different clonal selections. Some sour orange clones are reported as resistant to MSD, as already confirmed by Reforgiato Recupero (1979) and Nigro et al (2015). It is also well known that plant age is a determinant of susceptibility since adult plants are more tolerant than young seedlings (Migheli et al, 2009).

## 5.4 Conclusions

This survey was useful to discriminate many citrus accessions belonging to true and derived species based on their field tolerance to MSD. Many accessions were found to be immune or resistant to the disease under natural pathogen pressure, but a broad degree of tolerance was also observed. Several degrees of field tolerance cannot be explained by a single gene involved in the resistance (Russo, 1977). The presence of many genes involved in host-pathogen interaction was also supported by Reforgiato Recupero et al. (1997).

We found that DNA isolation from twigs coupled with real-time PCR detection is a reliable method for field phenotyping. This method could be routinely used to validate phenotyping of mapping populations or germplasm collections to better understand the genetic basis of MSD resistance.

A putative source of resistance was found in Doppio Lentini (autotetraploid lemon) and 46321 (probably a tetraploid Monachello) since no symptoms were found during the three years of visual monitoring and no pathogen was detected by real-time PCR analysis. This resistance seems not to be exclusively related to tetraploidy (Grosser et al., 2015), since other autotetraploids included in the phenotyping, namely 46515 and 46245, showed clear symptoms confirmed by real-time PCR detection.

This survey was also useful to identify sources of resistance among other citrus species that could be used to introgress resistance genes into the lemon genome. Therefore, based on the phenotyping results, two monoembryonic mal secco-resistant species, namely Khasi papeda and Clementine, were chosen as female parents and crossed with Femminello Siracusano 2Kr, a very susceptible lemon clone, to create two populations that might be helpful in the future for studying the segregation of MSD susceptibility and for identifying candidate genes and QTLs associated with the disease.

## 6. Generation of lemon breeding material and mapping populations for the identification of loci associated with MSD resistance

### 6.1 Introduction

Since the first report of MSD in the lemon orchards, the main goal of growers and of breeders was to identify lemon clonal selections resistant to *P. tracheiphilus*. As previously reported, different tolerant clones were identified, but none of them has good fruit quality and high productivity. These tolerant clones have guaranteed the survival of lemon orchard in many areas afflicted by the disease (Nigro et al, 2011), but they do not guarantee the competitivity of the Italian lemon industry.

In addition to clonal selection, hybridization has been extensively used at CREA several years ago to try to obtain resistant lemon hybrids with good qualitative traits. Unfortunately, the strategy was not successful since most of the tolerant hybrids did not show the typical fruit traits of a true lemon (Russo, 1977). However, it is not clear how many hybrids were evaluated in the field, since many of the hybrids died of root rot before field evaluation to MSD (Giuseppe Reforgiato Recupero, personal communication). On the other hand, hybrids with lemon-like fruit traits were generated from previous breeding programs, such as Lemox® (Reforgiato Recupero et al., 2005), indicating that is possible to obtain a fruit like a true lemon using hybridization. It could be reasonable to hypothesize that a hybrid combining lemon-like quality traits and resistance to MSD could be obtained increasing the number of hybrids generated and evaluated in the field. Therefore, the generation of many thousands of hybrids might be considered a potential strategy to obtain new resistant cultivars. To facilitate the evaluation of lemon hybrids for MSD tolerance, breeders can exclusively rely on field phenotyping, which is particularly expensive and time consuming. Marker assisted selection tools are not available for MSD and are needed in a medium to long term strategy to make a breeding program affordable. Reforgiato Recupero and colleagues (1997), analysing populations of the monoembryonic species Khasi papeda, as female parent, with polyembryonic sour orange, trifoliate orange, and volkamer lemon as male parents, hypothesized that the genetic resistance is controlled by several genes, However, the genetic basis of MSD resistance /susceptibility is still unknown. The pre-requisite for the identification of loci linked to MSD susceptibility is the generation of mapping populations from parents showing opposite behaviour in response to *P. tracheiphilus* infections. The choice should also consider the level of diversity between parents, to facilitate the identification of polymorphic markers to generate the linkage maps (Di Guardo, 2017). The phenotypic survey described in section 5 revealed high variability in the CREA germplasm collection regarding MSD susceptibility, and was helpful to identify potential parents for the generation of mapping populations. This phenotypic variability is likely due to the underlying genetic complexity derived from multiple loci interacting together. A genomic region containing one or more genes affecting a quantitative trait is called Quantitative Trait Locus (QTL) (Di Guardo, 2017). A strategy that could be used for identifying loci associated to MSD is bi-parental QTL analysis. This technique is based on the use of populations from two parents showing opposite phenotype for the analyzed trait. This approach is based on the analysis of the phenotypic difference and the genotypic segregation of the offspring to identify which of the parental marker alleles are linked to the phenotype of interest (Di Guardo, 2017). The key factors for this analysis are the quality of the phenotypic data, the size of the population, the marker density and the heritability of the trait. All these aspects have a direct influence on the quality and reliability of the analysis, since the number of individuals reflects the number of meiosis (and therefore the number of examined recombinations) and marker density influence the size of the confidence interval of the QTL (Mauricio, 2001). Here we describe four populations having true

lemons or citron as parents, generated with the following purposes: (I) to generate MSD resistant lemon-like hybrids to be used as breeding or pre-breeding material; (II) to map the loci associated with MSD susceptibility. In a long-term strategy, phenotyping and genotyping of these populations will be helpful to identify resistance or susceptibility genes and to facilitate the introgression of resistance genes into lemon genotypes.

## 6.2 Materials and methods

### 6.2.1 Hybridization

The crosses were made during the spring of 2018 at the CREA experimental farm of Palazzelli (Siracusa, Italy) (37_ 200 N, 14_ 530 E, 48 m a.s.l.), San Salvatore (Acireale, Italy) (37°37'23.90''N 15°09'50.09''E, 201 m a.s.l.) and at the University of Catania experimental farm of Primosole (Catania, Italy)(37°24'32.86''N, 15°03'21.74''E, 5 m a.s.l.).

Parents were selected based on their MSD resistance/susceptibility behaviour previously described (section 5)

The second parameter, that was specifically used for the selections of the female parents was the absence of nucellar embryony. Nucellar embryony characterizes many citrus species and varieties (Cook, 1907) and allows the cells of the nucellus to generate somatic embryos. In fact, in polyembryony genotypes, the seeds present nucellar embryos in addition to zygotic embryos. These nucellar embryos produce seedling identical to mother plants because these embryos are generating from mother tissue, the nucella, while zygotic embryos tend to be weak and variable, and very often do not survive because they have to compete for nutrients and space with nucellar embryos (Frost and Soost, 1968).

A third parameter, i.e. the genetic diversity from the true lemons, was specifically considered to choose the parents for the mapping populations, in order to facilitate the identification of polymorphic loci using a future genotyping by sequencing (GBS) approach.

Based on the above described criteria, the following hybridizations were performed: Clementine (resistant to MSD, monoembryonic) x Femminello Siracusano 2Kr (susceptible to MSD), Khasi papeda (resistant to MSD, monoembryonic) x Femminello Siracusano 2Kr (susceptible to MSD), Interdonato (resistant to MSD, monoembryonic) x Femminello Siracusano 2Kr (susceptible to MSD), Interdonato (resistant to MSD, monoembryonic) x Vozza Vozza citron (resistant to MSD, Diamante Citron (susceptible to MSD, monoembryonic) x *Poncirus trifoliata* 'Rubidoux' (resistant to MSD).

Interdonato x Femminello Siracusano 2Kr (IxS) and Interdonato x Vozza Vozza citron (IxV) crosses were performed to obtain new breeding and pre-breeding material resistant to MSD. The crosses Clementine x Femminello Siracusano 2Kr (CxS), Khasi papeda x Femminello Siracusano 2Kr (KxS) and Diamante citron x *Poncirus trifoliata* 'Rubidoux' (DxR) were specifically performed to generate mapping populations.

### 6.2.2 DNA extraction

To verify the parentage of the hybrids that will be used for mapping, samples of plant tissues were collected from 293 seedlings of KxS and from 30 seedlings of CxS after 6 months of growth in a greenhouse. A higher number of KxS seedlings was sampled because Khasi papeda is self-compatible, consequently, a higher number of hybrids from selfing could be potentially found in the population. On the contrary, Clementine is self-incompatible, so a lower number of hybrids was chosen to exclude cases of open pollinations.

DNA extraction was performed by the CTAB method as described in Caruso et al., (2014), making slight changes. Briefly, tubes containing 0.1 g of powdered plant tissues were mixed with 400 µL of extraction buffer (2% CTAB, 20 mM EDTA, 1.44 mM NaCl, 100 mM Tris HCl, pH 8), 0.1% b-mercaptoethanol. Samples were vortexed and incubated at 65 °C for 60 min, agitating for the first 5 min. After adding 300 µL of chloroform-isoamyl alcohol (24/1), vials were vortexed for 15 s and finally centrifuged at 20.800 *g* for 10 min. The supernatant was recovered and 500 µL of 100% ethanol was added and incubation at -20 °C during at least 30 min or at 4 °C overnight, followed by centrifugation at 20.800 *g* for 10 min. The pellet was rinsed with 1000 µL of 70% ethanol, resuspended in 50 µL of sterile distilled water, and stored at 4°C until analysis.

The quality and concentration of the isolated DNA were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific™, Waltham): The ratio 280/260 and 260/230 were around 1.80 and 2.20, respectively, and the concentrations ranged from 50 to 300 ng/µL. All the samples were diluted at 10 ng/ µL.

### 6.2.3 SSR marker analysis

To verify the hybrid origin and discard the F1s derived from selfing, an analysis with 6 SSR markers was performed. The primer combinations are reported in Table 6. The PCR reaction consisted of 0.5 ng/µL of template DNA, 0.2 mM dNTPs, 0.025–0.05 µM forward and reverse primers, 1× Promega PCR buffer, 2–4 mM magnesium chloride and 0.1 U/µL Promega Taq DNA polymerase. The DNA was amplificated on a ( Gene Amp PCR System 9700 thermocycler) using the following parameters: denaturation a 95 °C for 4 min, 35 cycles at 95 °C for 1 min, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and final extension of 10 min at 72°C.

The amplification products were diluted at 1/20. In a plate of 96 plots, each sample was composed by 0.5 µL of amplification products labelled with FAM and VIC dyes, 13.5 µL of formamide and 0.5 µL of Liz 500 size Standard (Applied Biosystems). After 5 min of denaturation at 95°C and 5 min in ice, samples were subjected to capillary electrophoresis using the ABI3130 Genetic Analyzer (Applied Biosystems). Capillary electrophoresis data was analyzed with GENESCAN 3.1.2. and converted into electropherograms, that permitted to compare the profile between the different hybrids.

| Marker | | Sequence (5'- 3') | Fluorophore | Reference |
|---|---|---|---|---|
| GT03 | Forward | GCCTTCTTGATTTACCGGAC | FAM | (Barkley et al., 2006) |
| | Reverse | TGCTCCGAACTTCATCATTG | | |
| TAA15 | Forward | GAAAGGGTTACTTGACCAGGC | VIC | (Kijas et al., 1997) |
| | Reverse | CTTCCCAGCTGCACAAGC | | |
| CT19 | Forward | CGCCAAGCTTACCACTCACTAC | VIC | (Barkley et al., 2006) |
| | Reverse | GCCACGATTTGTAGGGGATAG | | |
| CAC39 | Forward | AGAAGCCATCTCTCTGCTGC | VIC | (Kijas et al., 1997) |
| | Reverse | AATTCAGTCCCATTCCATTCC | | |
| TAA41 | Forward | AGGTCTACATTGGCATTGTC | FAM | (Kijas et al., 1997) |
| | Reverse | ACATGCAGTGCTATAATGAATG | | |
| AG14 | Forward | AAAGGGAAAGCCCTAATCTCA | FAM | (Barkley et al., 2006) |
| | Reverse | CTTCCTCTTGCGGAGTGTTC | | |

Table 6. The table shows the primer combinations that were used in SSR analysis.

## 6.3 Results and discussion

### 6.3.1 Hybridization

The crosses performed, and the number of seedlings generated from each cross are indicated in Table 7. The total number of pollinated flowers was 1.450. The highest number of pollinations were performed for the cross KxS, with 621 flowers, followed by the crosses CxS with 297 flowers and IxS with 200 flowers. For the other crosses, it is possible to see the result in Table 7.

The rate of fruit set was very different between the crosses. A high percentage of fruit was obtained in the cross CxS with almost 40 %, followed by the crosses (IxS) with 19.5%. The lowest fruit set was found in the crosses (KxS) with 8.3 %. The number of seeds per fruit was the highest in (KxS), with an average of 15 seeds per fruit, followed by (CxS) with 12. We obtained 450 seedlings of (KxS), 400 seedlings of (CxS), 150 seedlings of (IxS) and 120 seedlings of (IxV). We were not able to obtain fruits and seeds from the Diamante citron x *Poncirus trifoliata* Rubidoux.

| **Female Parent** | **Male parent** | **Number of flowers (n°)** | **Number of fruits (n°)** | **Fruit set (%)** | **Number of seeds (n°)** | **Seed/fruit (n°)** | **Number of seedlings (n°)** | **Grafted Hybrids (n°)** |
|---|---|---|---|---|---|---|---|---|
| Khasi Papeda | Femminello Siracusano 2Kr lemon | 621 | 52 | 8.37 | 780 | 15 | 450 | 150 |
| Interdonato lemon | Femminello Siracusano 2Kr lemon | 200 | 39 | 19.5 | 322 | 8.25 | 150 | 120 |
| Diamante Citron | *Poncirus trifoliata* 'Rubidoux' | 57 | / | | / | | / | / |
| Interdonato lemon | Vozza Vozza Citron | 183 | 26 | 14.20 | 144 | 5.53 | 120 | / |
| Clementine Fedele | Femminello Siracusano 2Kr lemon | 297 | 118 | 39.73 | 1298 | 11 | 400 | 140 |

Table 7. The table shows the different crosses that were performed and the number of flower, the number of fruits, the fruit set, the number of seed, the number of seed/fruit, the number of seedlings and the grafted plants that were obtained from each cross.

### 6.3.2 SSR marker analysis

SSR analysis was performed to verify parentage of the hybrids of the mapping populations KxS and CXS. All six SSR markers produced amplification products that were easy to interpret. All loci were polymorphic between the two parents of KxS population. All loci except CAC39 had no shared alleles between parents and were helpful both to exclude F1s from selfing and possible pollen contaminations. Table 8 and Table 9 show the total number of alleles per locus, and the allele size found in the two parents. Also for the CxS population all loci were polymorphic between the two parents, but some loci like as GT03 and CAC39 showed some allele in common between the two parent, but they were also useful to discard F1s from possible pollen contaminations. An example is reported in Figure 27, where the two parents were heterozygous and polymorphic at the AG14 locus. The SSR analysis allowed to discard 22 F1s which were selfed seedlings (Figure 28), and to verify that all seedlings had lemon as male. A total of 150 KxS hybrids, verified as true hybrids by SSRs, were grafted onto Carrizo Citrange in spring 2019, with two replicates per hybrid. The propagated population was planted in the field in summer 2020 (Figure 29). The CxS population, consisting of 140 hybrids, was grafted onto Carrizo Citrange in spring 2020 and will be used as a complementary population for phenotyping and mapping. A higher number of KxS seedlings (150) was analysed because Khasi papeda is self-compatible, consequently, a higher number of hybrids from selfing could be potentially found in the population. On the contrary, Clementine is self-incompatible, so a lower number of hybrids (30) was chosen to discard cases of open pollinations.

Figure 29. The picture shows the Khasi papeda x Femminello Siracusano 2Kr (KxS) hybrids, grafted onto Carrizo Citrange, planted in summer 2020 at the CREA experimental farm of San Salvatore, Acireale, Italy.

| | GT03 F 150 | GT03 F 160 | GT03 F 170 | GT03 F 183 | AG14 F 116 | AG14 F 132 | AG14 F 148 | AG14 F 153 | AG14 F 155 | TAA41 F 121 | TAA41 F 125 | TAA41 F 143 | TAA41 F 145 | TAA41 F 148 | TAA41 F 153 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Femminello Siracusano 2KR | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 |
| Khasi Papeda | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| Clementine | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |

Table 8. Total number of alleles per locus GT03, AG14 and TAA41, and the allele size found in the parents of the KxS and CxS population. The 0 represent the not presence of the allele, while 1 represent the presence of the allele.

| | TAA15 V 164 | TAA15 V 174 | TAA15 V 188 | TAA15 V 191 | CT19 V 138 | CT19 V 140 | CT19 V 141 | CT19 V 145 | CAC39 V 170 | CAC39 V 176 |
|---|---|---|---|---|---|---|---|---|---|---|
| Femminello Siracusano 2KR | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| Khasi Papeda | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| Clementine | 0 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 0 |

Table 9. Total number of alleles per locus TAA15, CT19 and CACA39, and the allele size found in the parents of the KxS and CxS population. The 0 represent the not presence of the allele, while 1 represent the presence of the allele.

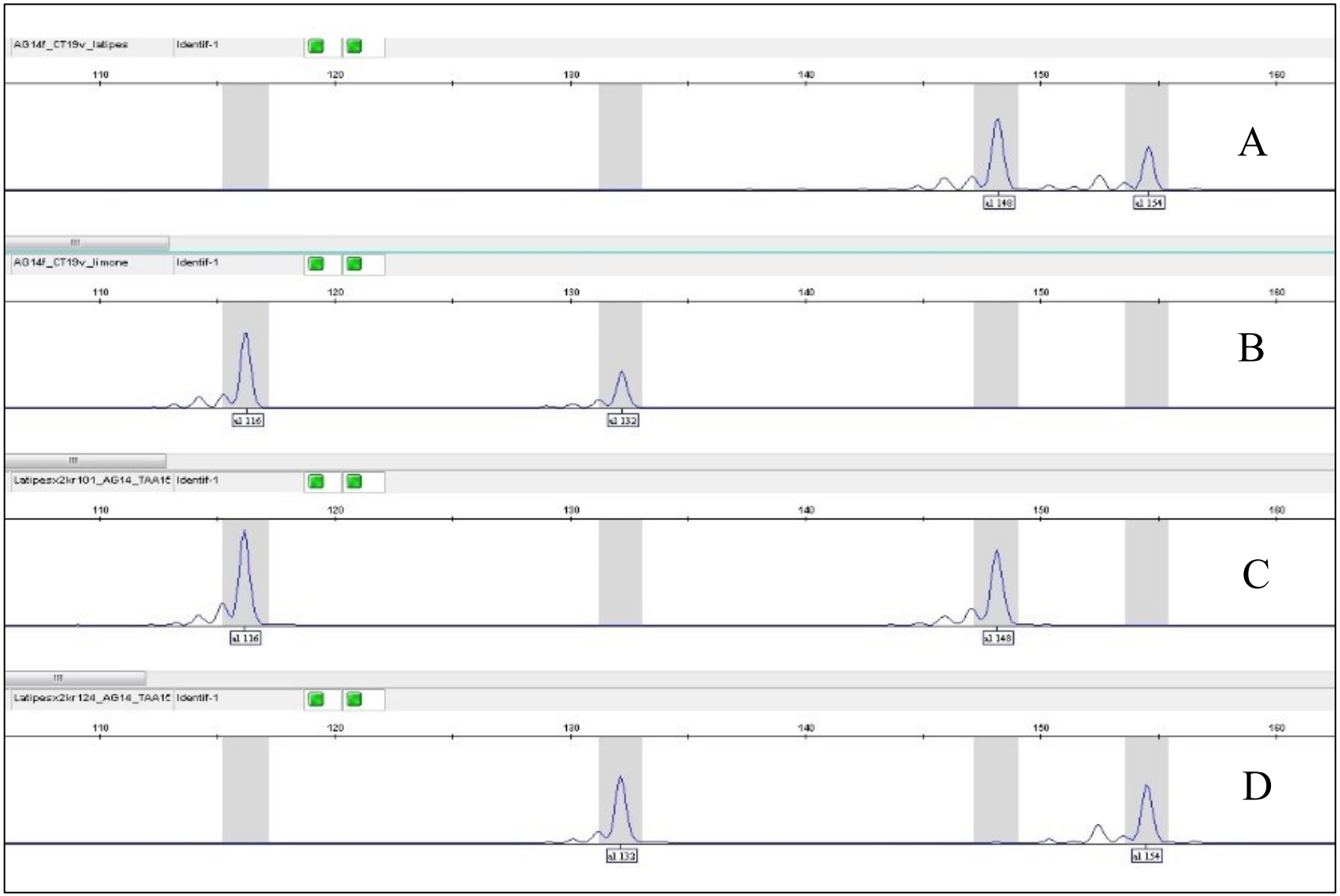


Figure 27. Polymorphic profiles of Khasi papeda (A), lemon (B) and two KxS hybrids (C and D) at the AG14 locus.

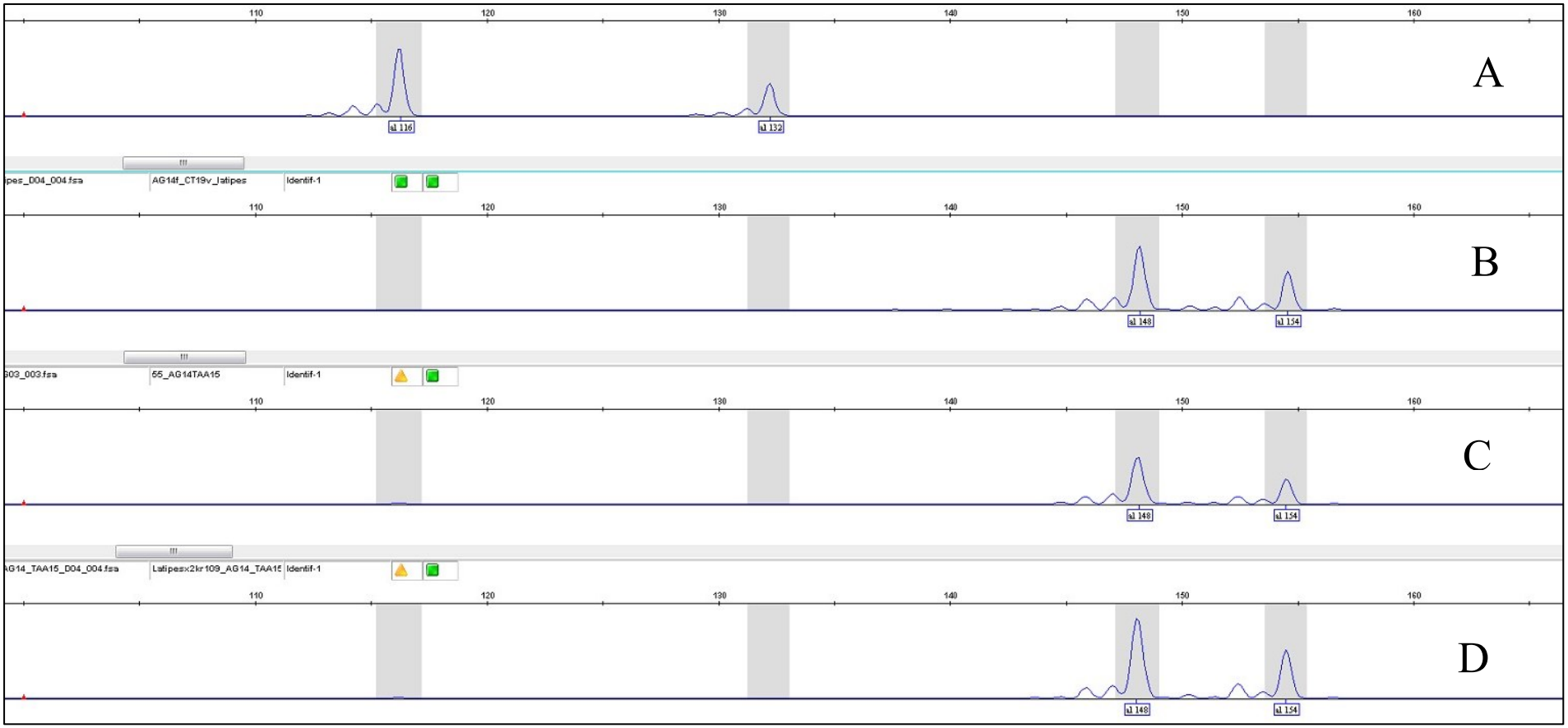


Figure 28. Polymorphic profiles of lemon (A), Khasi papeda (B), and two F1s seedlings derive from self-pollination of Khasi papeda (C and D) at the AG14 locus.

## 6.4 Conclusion

Based on the phenotypic survey to MSD, previously described in section 5, the most suitable parents were selected to create 5 populations that will be used for different purposes. In fact, the 120 hybrids obtained from the cross Interdonato x Femminello Siracusano 2Kr (IxS), grafted on alemow (*C. macrophyilla* Wester) and the 150 seedlings obtained from the cross Interdonato x Vozza Vozza citron (IxV) were performed to generate new breeding and pre-breeding material resistant to MSD, while the crosses Clementine x Femminello Siracusano 2Kr (CxS), Khasi papeda x Femminello Siracusano 2Kr (KxS) and Diamante citron x *Poncirus trifoliata* 'Rubidoux' (DxR) were specifically performed to generate mapping populations. The KxS population consist of 150 hybrids grafted on Carrizo Citrange and it was planted in summer 2020 at CREA experimental farm of San Salvatore, Acireale, Italy. While the CxS populating consist of 140 hybrids that were grafted on Carrizo Citrange in the spring 2020 and will be planted next years. For the last population (DxR) we were not able to obtain fruits and seeds.

In conclusion, this part of the project developed useful breeding and pre-breeding material that could be used in the future in the framework of the CREA and University of Catania lemon breeding programs. The KxS and CxS populations represent the first examples of reference segregating populations specifically developed for understanding the genetic basis of MSD. Due to the complexity of the plant-pathogen interaction, phenotyping for MSD will be performed for several years. GBS of the KxS hybrids for the construction of a genetic map is underway.

# 7. *De novo* transcriptome sequencing and assembly of rough lemon leaves (*Citrus jambhiri* Lush.) in response to *Plenodomus tracheiphilus* infection.

## 7.1 Introduction

Citrus, one of the most important fruit crops in the world, is sensitive to many environmental stresses of both abiotic and biotic nature, often leading to poor tree growth and reductions in fruit yield and quality (Talon and Gmitter, 2008). Mal secco disease (MSD) is a severe vascular disease of citrus caused by the mitosporic fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp and Verkley (syn. *Phoma tracheiphila* (Petri) Kantschaveli and Gikashvili). It appeared in the second half of 19th century (1894) in Chios and Poros, two Greek Aegean islands, from which it derived its first name ("Poros's disease"). In Italy, MSD was first reported in 1918 in the district of Messina (eastern Sicily), probably following the introduction of infected plants from Greece (Ruggieri, 1949). The current geographical distribution of MSD comprises the east coast of the Black Sea (Georgia) and mainly all citrus-growing countries of the Mediterranean Basin, except for Morocco (EPPO Global Database, 2020).

The MSD pathogen infects mainly lemon (Russo et al., 2020). Citron and other citrus species and hybrids having citron or lemon as parent, such as lime, bergamot, Volkamer lemon, Alemow (*C. macrophylla* Wester), and rough lemon are also particularly susceptible to the disease (Russo et al., 2020; Nigro et al., 2011). Rough lemon is counted among the most mal secco susceptible species (EFSA Panel Plant Health, 2014). *C. jambhiri* is native to northeastern India and is a mandarin × citron F1 natural hybrid (Wu et al., 2018). Due to fruit typology, as the name implies, characterized by a very coarse exterior, it is unsuitable as a scion cultivar but it has been widely used in many countries as a rootstock (Bowman and Joubert, 2020). The distinct symptomatology of the disease, characterized by desiccation of twigs, branches, or the whole plant, suggested its extant name mal secco meaning "dry disease" (Savastano, 1925; Catara and Catara, 2019), a denomination ever since adopted internationally (Nigro et al., 2011). The first symptoms of the disease usually appear in spring on the leaves of the uppermost shoots, which display a slight discoloration of the primary and the secondary veins (Migheli et al., 2009; Batuman et al., 2020). The leaves then turn yellow or sometimes brown and fall. Newly infected shoots show a yellow or pink-salmon to reddish discoloration of the wood, which occurs also in the wood of the main and secondary branches, as well as in the trunk, where the pathogen is advancing. A progressive basipetal desiccation of shoots, branches, and trunk follows and, finally, the whole plant may die (Ruggieri, 1956). Glycoproteins of 93 KDa and 60 KDa (called Pt60) belonging to the malseccin complex have been isolated from culture filtrates and host plants infected by P. tracheiphilus (Nachmias et al., 1977; Fogliano et al., 1994; Fogliano et al., 1998). Both were able to reproduce all the symptoms of the disease when injected into different plants (Fogliano et al., 1998). The toxic effects of the malseccin complex on citrus leaves are clearly visible only under illuminated conditions, suggesting that light plays a role in the toxin activity. In light conditions, the induction and formation of reactive oxygen species (ROS) can damage cellular structures as ROS induce lipid membrane peroxidation leading to the loss of membrane integrity, electrolyte leakage, and cell death. Oxidative stress in plant pathology has been a general subject of investigation and its ability to drive the metabolism of both host and pathogen during their interaction has been demonstrated (Fedoroff, N., 2006). It has been shown that the synchronous presence of hydrolytic enzymes, toxic compounds, oxidative stress inducers, and membrane transporters in the fungus, and the differential ability to modulate the lipoperoxidative pathway in the host can play a central function in P. tracheiphilus infection of C. limon (Reverberi et

al., 2008). The knowledge at the molecular level of the mechanisms that occur in plant–pathogen interaction, not only in tolerant but also in susceptible interactions, is the basis for the development of innovative tools for phytosanitary control and that may lead to eco-sustainable interventions to minimize or replace the massive use of agro-pharmaceuticals. Gene expression profiling by RNA-Seq provides an unprecedented high-resolution view of the global transcriptional landscape. A primary objective of many gene expression experiments is to detect transcripts showing differential expression across various conditions. In this context, next-generation high-throughput sequencing techniques have become an increasingly useful tool for exploring whole plant genomes, providing a means for analyzing plant molecular regulatory mechanisms in specific abiotic and biotic stress conditions. The identification of candidate genes is a prerequisite for the application of new genome editing techniques by which targeted genetic modifications can lead to the introduction of precise changes directly into the genome of commercial varieties, offering an alternative to traditional methods of genetic improvement (Gentile et al., 2019; Salonia, F., Ciacciulli, A., Poles, L., Pappalardo, H. D., La Malfa, S., Licciardello, 2020; Poles, L., Licciardello, C., Distefano, G., Nicolosi, E., Gentile, A., La Malfa, 2020). Different authors in the last years conducted transcriptomic analysis to better understand Citrus plants response to biotic stress caused by pathogens (Fan et al., 2011; 2012; Hu et al., 2017; Yu et al., 2017; Naveed et al., 2019; Arce-Leal et al., 2020). Specifically, a study evaluated the transcriptional reprogramming of both rough lemon and sweet orange leaf tissue during the asymptomatic stage of infection caused by Candidatus Liberibacter asiaticus. Functional analysis of the differentially expressed genes (DEGs) indicated that genes involved in the mitogen activated protein kinase (MAPK) signaling pathway involving WRKY transcription factors were highly upregulated in rough lemon. Among the most biologically relevant transcripts in the gene set enrichment analysis were those related to several functional categories suggesting that DEGs with different functions were subjected to reprogramming. Therefore, using global transcriptome analysis approach, both a wide range of candidate genes and information that could be useful for genetic engineering to control Huanglongbing disease have been identified (Yu et al., 2017). Considering the impact of mal secco in the Mediterranean citrus industry, the aim of this work was to unravel the transcriptomic reprogramming of a highly susceptible citrus species subjected to P. tracheiphilus infection by applying a de novo sequencing and assembly RNAseq approach. This is the first report concerning the transcriptome analysis of a susceptible Citrus species challenged by the causal agent of “mal secco” disease.

## 7.2 Materials and methods

### 7.2.1 Plant material and inoculum

Seeds of rough lemon (*C. jambhiri*) were sowed on sterile peat in May 2019. After 6 months of growing in a chamber at 25 °C and 90% humidity, the plants were inoculated with the pathogen *Plenodomus tracheiphilus* PT10 strain (kindly provided by Professor Vittoria Catara, University of Catania). Rough lemon was chosen as plant material for the following reasons: (I) It was previously reported as very susceptible to the disease (EFSA Panel Plant Health, 2014); (II) it has a high degree of polyembryony, higher than true lemons or other citron hybrids (Barrett and Rhodes, 1976), allowing the production of true-to-type seedlings; and (III) it is very vigorous, with seedlings growing faster than those of other citrus species. Moreover, our preliminary inoculation tests indicated that symptoms after artificial inoculations were easier to detect in rough lemon than in *C. limon* seedlings. The inoculum was prepared according to a slight modification of the method described in (Salerno

and Catara, 1967). Briefly, three pieces of young fungus grown at 21 °C ± 2 in Petri dishes containing potato dextrose agar medium (PDA) were placed in 7 different flasks containing 100 mL of carrot broth and incubated for 5 days in a heidolph unimax 2010 shaker at 22 °C. Successively, the growth medium was filtered and centrifugated at 8,000 rpm × 20 min. The pellet was recovered and the phialoconidia were counted with a counting chamber to adjust the inoculum concentration at $10^6$ $mL^{-1}$. The inoculation was performed by depositing 10 µl on wounds obtained by cutting the midvein of three leaves for each plant with a sharp sterile blade. Overall, five plants were inoculated with the pathogen and five plants were inoculated with water as control. Both inoculated and control samples were collected 15 days after inoculation, considering that inoculated plants showed evident symptoms of the disease. Leaves were immediately frozen with liquid nitrogen and stored at −80 °C until both DNA and RNA extractions were performed.

### 7.2.2 DNA and RNA extraction

DNA extraction was performed according to (Springer, 2010). Briefly, samples were powdered using liquid nitrogen in mortar and pestle. Two hundred milligrams of grinded leaves were mixed approximately with 500 µL of CTAB buffer (2% CTAB, 20 mM EDTA, 1.44 mM NaCl, 100 mM Tris HCl, pH 8.0) and 0.2% β-mercaptoethanol. Samples were vortexed and incubated at 65 °C for 30 min, then the CTAB-plant extract mixture was transferred into a microfuge tube. After adding 300 µL of chloroform-isoamyl alcohol (24/1), the tubes were mixed by inversion and centrifuged at 14,000 rpm for 10 min. The supernatant was recovered into a clean microfuge tube and 7.5 M ammonium acetate (50 µL) followed by 1000 µL of ice cold 100% of ethanol were added to each tube. The tubes were mixed by inversion and then centrifuged at 10,000 rpm for 10 min. The pellet was rinsed twice with 1000 µL of ice cold 70% ethanol, resuspended in 50 µL of sterile distilled water and stored at 4 °C until analysis. The DNA concentration and purity were checked by a Nanodrop 2000 spectrophotometer (Thermo Scientific™, Waltham, MA, USA). The RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were checked using the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Before sequencing, sample RNA integrity (RIN) was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

### 7.2.3 Real-time confirmation of infected plants

Taqman Real-time PCR was performed to reveal the presence of the pathogen within the inoculated plants using an ABI 7500 Real-Time PCR System (Applied Biosystems™, Foster City, CA, USA). The analysis was performed according to the method described in (Licciardello et al., 2006), using DNA extracted from both inoculated and control leaves as template. Forward primer GR70 (5′-GATCCGTACGCCTTGGGGAC-3′) and reverse primer, GL1 (5′-AGAAGC GTTTGGAGGAGAGAATG-3′), dual-labeled fluorogenic probe, PP1, (5′-FAM-CACGCAATCTTGGCGACTGTCGTT-TAMRA-3′) were used with the aim to amplify a 84-bp segment of the pathogen DNA. Each reaction contained 200 nM forward primer, 200 nM reverse primer, 100 nM fluorogenic probe, and 4 µL of genomic DNA in a final volume of 15-µL. Negative control contained the same mixture, with sterile water replacing the DNA template. The assay was performed on three biological replicates, each one repeated twice. The thermal cycling conditions for *P. tracheiphilus* DNA template amplification were 50 °C for 2 min (1 cycle), 95 °C for 30 s (1cycle),

95 °C for 10 s, 62 °C for 30 s (40 cycles). Standard curve for fungal DNA quantification was constructed using *P. tracheiphilus* DNA (100 µg $mL^{-1}$) extracted from the Pt10 strain and serially diluted in sterile distilled water as described in (Licciardello et al., 2006).

### 7.2.4 Library preparation and sequencing

After the QC procedures, sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations and as reported in (Sicilia et al., 2019). Briefly, mRNA was enriched using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X), followed by cDNA synthesis using random hexamers and M-MuLV Reverse Transcriptase (RNase H-). After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added containing dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand by nick-translation. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then, 3 µL USER Enzyme by NEB was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Successively, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and then diluted to 1 ng/µL before checking insert size on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Cluster generation and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). After cluster generation, the libraries were sequenced on Illumina HiSeq2000 platform to generate pair-end reads. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads. Sequences putatively belonging to pathogen in inoculated rough lemon samples were removed by filtering out the reads mapped to the fungus genome (https://mycocosm.jgi.doe.gov/Photr1/Photr1.info.html, accessed 18th November 2020). Then Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

### 7.2.5 De novo transcriptome assembling and gene functional annotation

De novo transcriptome assembly was accomplished using Trinity (r20140413p1 version) with min_kmer_cov:2 parameters (k = 25). Then Hierarchical Clustering was performed by Corset (v1.05 version, https://github.com/Oshlack/Corset/wiki) to remove redundancy (parameter -m 10) and the longest transcripts of each cluster were selected as Unigenes. The flow chart of the rough lemon de novo transcriptome assembly is stackable to that reported in (Sicilia et al., 2019). The *Citrus jambhiri* transcriptome was uploaded to NCBI (https://www.ncbi.nlm.nih.gov/geo/, accessed 29th December 2020) accession number GSE164096. Gene function was annotated based on the following databases: National Center for Biotechnology Information (NCBI) non-redundant protein sequences (Nr), NCBI non-redundant nucleotide sequences (Nt), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Ortholog database (KO) and Gene Ontology (GO). A pathway analysis was conducted using MapMan3.6.0RC1

(https://mapman.gabipd.org/, accessed 19th October 2020). All the unigenes were annotated and mapped using Mercator4 V2.0, an on-line tool of MapMan (https://www.plabipd.de/portal/mercator4, accessed 5th November 2020) which accurately assigns hierarchal ontology providing visual representation of genes in different plant processes. The significant DEGs (padj < 0.05), with the corresponding $log_2$FoldChange values, were used as dataset to align with the Mercator map

### 7.2.6 Quantification of gene expression and differential expression analysis

Gene expression levels were estimated by RSEM (v1.2.26 version, http://deweylab.github.io/RSEM/) with bowtie2 mismatch 0 parameters to map the Corset filtered transcriptome. For each sample, clean data were mapped back onto the assembled transcriptome and readcount for each gene was then obtained. Differential expression analysis between control (CK) and infected (*Pt*) samples was performed using the DESeq R package (1.12.0 version, padj < 0.05, https://bioconductor.org/packages/release/bioc/html/DESeq.html). The resulting p-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate (Benjamini and Hochberg, 1995). Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed. A $log_2$FoldChange threshold of 0.58 (1.5 fold change) was adopted. The GO enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages (1.10.0, 2.10.0 version, corrected *p* value < 0.05 based) Wallenius non-central hyper-geometric distribution. Furthermore, all of the unigenes were submitted to the KEGG pathway database for the systematic analysis of gene functions. KOBAS software (v2.0.12 version, corrected *p*-Value < 0.05, kobas.cbi.pku.edu.cn) was used to test the statistical enrichment of differential expression genes in KEGG pathways.

### 7.2.7 Real-time validation of selected DEG candidates using qRT-PCR

Total RNA (2.5 µg) extracted from sample leaves as described above, was reversed transcribed using the SuperScript™ Vilo™ cDNA synthesis kit by ThermoFisher Scientific (Warrington WA1 4SR, UK), according to the manufacturer's instructions. Real-time qRT-PCR was performed for a total of 10 DEGs with PowerUp SYBR Green Master mix by ThermoFisher Scientific and carried out in the Bio-Rad iQ5 Thermal Cycler detection system. All the genes were normalized with *Citrus clementina* actin (LOC18039075). All reactions were performed in triplicate and fold change measurements calculated with the $2^{-\Delta\Delta CT}$ method. The selected DEGs and their corresponding primer sequences are provided in Table 10.

| Pattern | Cluster ID | Annotation | Primer F | Primer R |
|---|---|---|---|---|
| *Pt*_vs_CK /Up | 7300.1 | Peroxisomal membrane protein PMP22 | TGCCATTTTCAAGGGAAGGGAC | TTCTCGGAATTGCTGAGGGACG |
| *Pt*_vs_CK /Up | 20465.1 | LRR receptor-like serine/threonine-protein kinase BIR2 | TGCCAGCCTCCATTTCTGCATC | GCACAACCCCGATTCCATACAC |
| *Pt*_vs_CK /Up | 14701.26429 | Pathogenesis-related protein 1-like | GCGACTGCAATCTTGTGCATTC | TATAGTGCCCACACACCTTGCC |
| *Pt*_vs_CK /Up | 14701.59152 | Chitin elicitor receptor kinase 1 | GCCGAGACATACTATGCCAACC | TCAGGGATGAAAACCAAGCCAC |
| *Pt*_vs_CK /Up | 14701.49196 | Salicylic acid-binding protein 2 | TGCACACATTCCAGGCATACAG | GCCGTCCTCTTTTCCCATCTTC |
| *Pt*_vs_CK /Down | 14701.30701 | Calcium-transporting ATPase 4, plasma membrane-type | AGCTCTGAGAACTCTCTGCCTG | TTTGAACCGCCTCCTTGACTCC |
| *Pt*_vs_CK /Down | 17016.0 | Vegetative cell wall protein gp1 | ATGATGAGTTGCCCCAACAAGG | TCCGTATGACGGATAAGCGGAG |
| *Pt*_vs_CK /Down | 14701.18090 | Pectinesterase 2 | ACTCCAAACACTTCGCCGTC | AGCAATCAGCCCATGCAACC |
| *Pt*_vs_CK /Down | 14701.83847 | Calcium-dependent protein kinase 1 | GGCCAGAATTTGCATGGGACAG | CAAGATAACGCCAGCACTCCAC |
| *Pt*_vs_CK /Down | 14701.23987 | CKI1_ARATH Histidine kinase CKI1 | AGTTCGCGCAGCAGTAAAGAAG | AATCCCCTTGCCCGTATCATCC |
| All | 14701.53432 | Actin | CTCACTGAAGCACCACTCAACC | CACCATCTCCAGAGTCAAGCAC |

Table 10. Primers used to validate the RNAseq experiment by real time PCR.

## 7.3 Results

### 7.3.1 Effect of *Plenodomus tracheiphilus* infection on Citrus jambhiri phenotype and fungus detection

The effectiveness of fungal inoculation was evaluated by both visual inspection of inoculated leaves and by detection of fungus genome by Taqman real time PCR. As shown in Figure 30A, the typical symptoms consisting of the midrib and main vein chlorosis were detected 15 days after inoculation. All the inoculated plants were chlorotic on the adaxial leaf surface (Figure 30B); that chlorosis symptom is different from the aforementioned vein chlorosis and more specifically indicates that a pathogen-induced micronutrient deficiency has occurred. As expected, the untreated plants appeared healthy (Figure 30B). As described in (Licciardello et al., 2006), quantitative detection of P. tracheiphilus was performed by real-time PCR assay. The fungus was detected in inoculated rough lemon plants, whereas no fluorescence emission was detected in the case of DNA extracted from healthy samples as well as from negative control (NTC, inoculated with water) (Table 11). The standard curve for fungal DNA quantification gave a coefficient of determination $R^2 = 0.98$ (data not shown).


Figure 30. Effect of P tracheiphilus on *C. jambhiri* phenotype. A) Inoculation site showing the typical MSD symptoms. B) Picture of the plants after 15 days from inoculation. On the top, control plants of rough lemon on good healthy state; On the bottom, inoculated plants of rough lemon that showed typical symptoms of MSD.

| Sample Name | Ct value | Standard deviation Ct value | Quantification |
|---|---|---|---|
| NTC | Undetermined | | |
| NTC | Undetermined | | |
| T1 | 24.758 | 0.436 | 0.074882 |
| T1 | 24.1411 | 0.436 | 0.107628 |
| T2 | 22.7171 | 0.46 | 0.248684 |
| T2 | 23.3674 | 0.46 | 0.169646 |
| T3 | 23.1696 | 0.0701 | 0.190574 |
| T3 | 23.0705 | 0.0701 | 0.202013 |
| C1 | Undetermined | | |
| C1 | Undetermined | | |
| C2 | Undetermined | | |
| C2 | Undetermined | | |
| C3 | Undetermined | | |
| C3 | Undetermined | | |

Table 11. Real-time detection of P. tracheiphilus in inoculated plants: Ct (threshold cycle), T (treated plant), C (control plant), NTC (negative control, plants inoculated with water).

### 7.3.2 Transcripts assembly and annotation

In this work, a comprehensive identification of the transcriptional response of rough lemon to *P. tracheiphilus* infection was carried out by RNAseq approach (see the experimental design in the "Material and Methods" section). The quality of RNA samples has been assessed before libraries preparation by RIN measurement. The mean RIN value was 8.2 (Table 12) indicating that very low level of RNA degradation occurred and that it was suitable for further downstream analysis. After library construction and sequencing, reads containing adapters or reads of low quality were removed by filtering the raw reads, so that the downstream analyses are based on a total of 228 million clean reads with an average of ~38 million reads (~11.4 G) per sample, the average percentage of Q30 and GC being 92.8% and 44.2%, respectively. De novo assembly of clean reads resulted in 115,100 transcripts and 77,631 unigenes with N50 length of 2372 and 2060, respectively (Table 12), indicating that a good coverage of the transcriptome had been achieved. The assembly consistency was evaluated by mapping back the filtered unique reads to the final assembled leaf transcriptome and the average read mapping rate using the alignment software Bowtie2 was 83.40%. Both transcript and unigene length distributions are reported in Figure 31. These data showed that the throughput and sequencing quality were high enough to warrant further analysis. To achieve comprehensive gene functional annotation, all assembled unigenes were blasted against public databases, including National Center for Biotechnology Information (NCBI), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), SwissProt, Ortholog database (KO) and Gene Ontology (GO) (Figure 32). The 80.89% of the obtained total unigenes were annotated in at least one searched database. Among them, 72.93% and 78.25% assembled unigenes showed identity with sequences in the Nr and Nt databases, respectively. The percentage of assembled unigenes homologous to sequences in KO, KEGG, Swiss-Prot, Pfam, GO and KOG databases were 27.13%, 15.36%, 53.35%, 24.52%, 15.53 and 23.59%, respectively (Figure 32).

| | |
|---|---|
| Average RIN | 8.2 |
| Clean reads | 228 million |
| N° of transcripts | 117,626 |
| N° of Unigenes | 79,821 |
| Average of read mapped rate | 83.40% |
| Transcripts N50 (bp) | 2,353 |
| Unigenes N50 (bp) | 2,039 |
| Q30 (%) | 92.82 |
| GC content (%) | 44.22 |

Table 12. Summary statistics of the RNA quality and sequencing results.

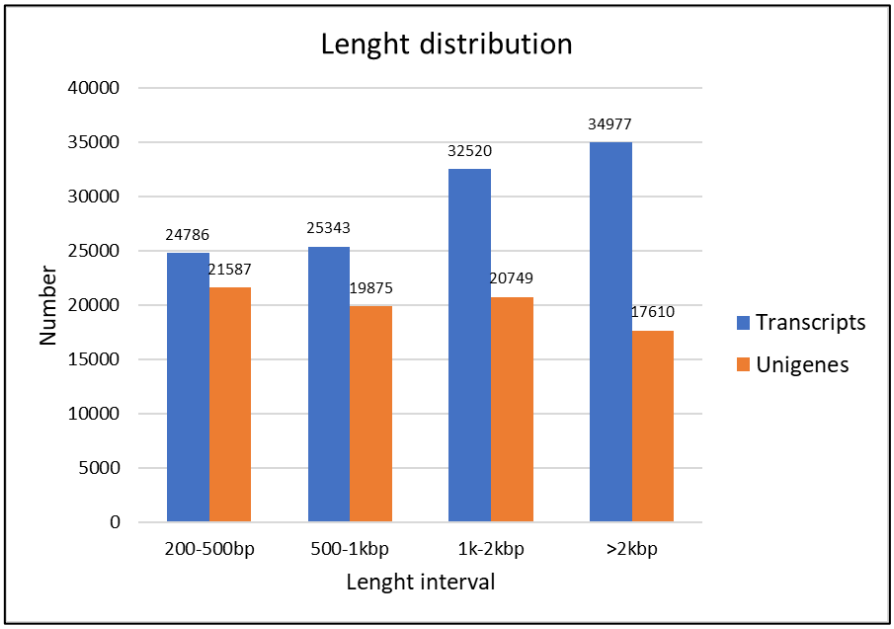


Figure 31. Overview of the number of transcripts and unigenes in different length intervals.

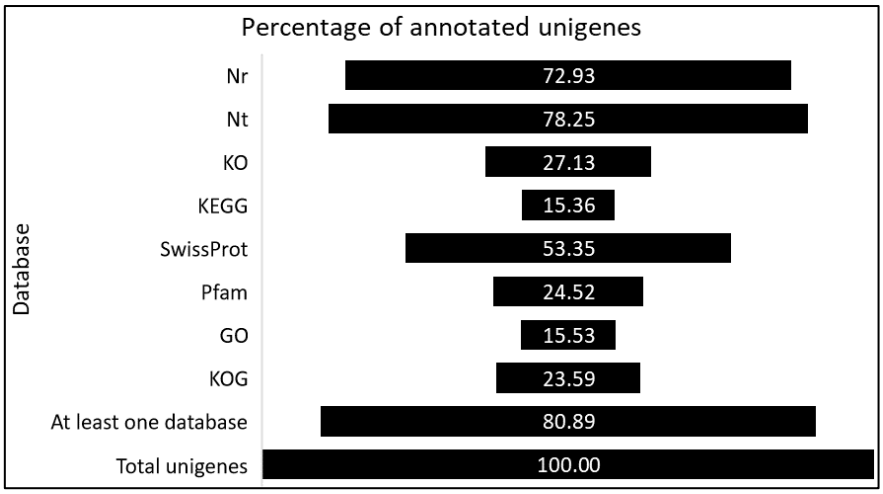


Figure 32. The number and percentage of successful annotated genes.

### 7.3.3 Identification of differentially expressed genes (DEGs)

The unigenes whose expression level changed upon pathogen infection were identified as differentially expressed genes (DEGs) and they were used to characterize the transcriptomic response of *C. jambhiri* to fungal attack. A total of 4,986 differentially expressed genes were identified from the comparison *Pt* vs CK (*P. tracheiphilus* sample set versus control sample set), of which 2,865 up-regulated genes and 2,121 down-regulated genes (Figure 33). Validation of expression levels for ten selected DEG candidates was carried out by quantitative real-time PCR (qRT-PCR). The results show high congruence between RNA-Seq results and qRT-PCR (coefficient of determination $R^2 = 0.92$) indicating the reliability of RNA-Seq quantification of gene expression (Figure 34). Therefore, the selected genes could also constitute useful markers of pathogen infection in rough lemon.

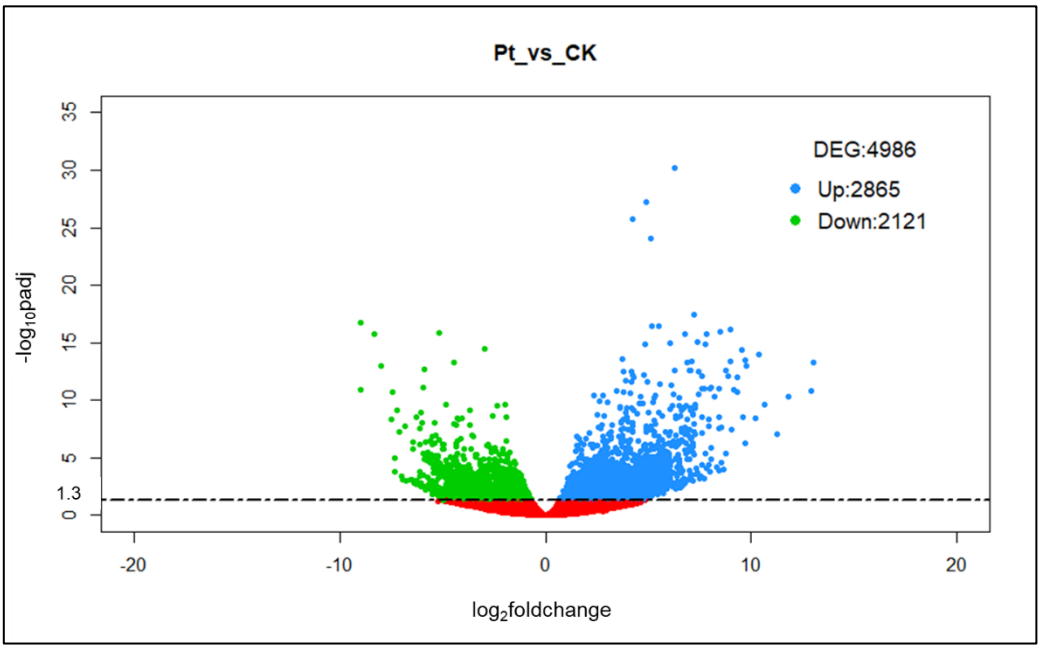


Figure 33. Volcano plot showing the DEGs of Pt vs CK comparison. The up-regulated genes with statistically significance are represented by blue dots, the green dots represent the down-regulated genes and the red dots are DEGs with -log10padj < 1.3, adopting log2FoldChange threshold of 0.58 (1.5 fold change). The X-axis is the gene expression change, and the Y-axis is the pvalue adjusted after normalization.

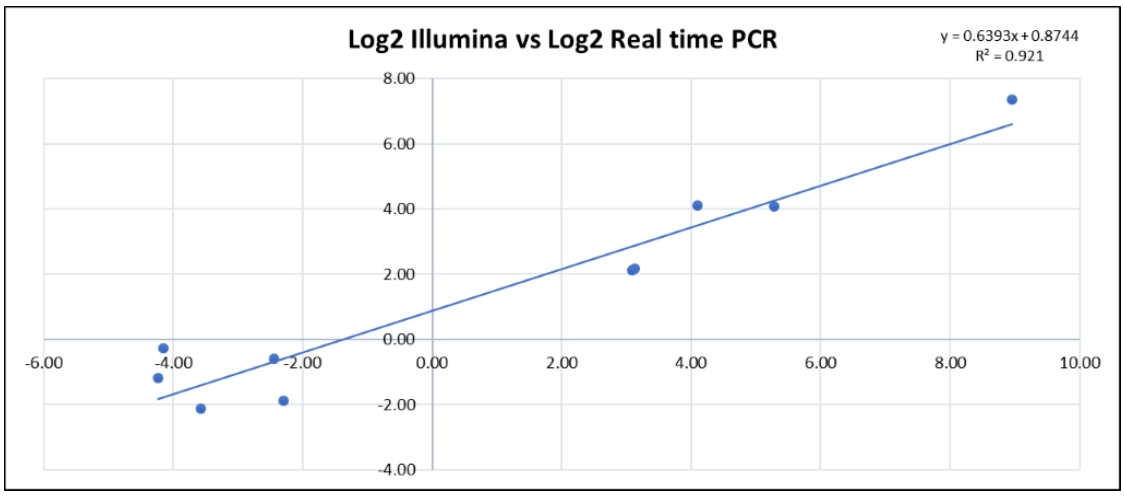


Figure 34. Validation of DEGs in Pt vs CK comparison by Real Time qRT-PCR.

### 7.3.4 Functional classification of DEGs

Gene Ontology (GO) terms, Clusters of Orthologous Groups of proteins (KOG) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichments were performed to identify possible biological processes or pathways involved in the response of plant to pathogen. Considering the GO enrichment, “oxidoreductase activity” (GO:0016491) (104 up-regulated and 65 down-regulated), “transmembrane transporter activity” (GO:0022857) (75 up-regulated and 27 down-regulated) and “DNA-binding transcription factor activity” (GO:0003700) (37 up-regulated and 14 down-regulated) are the three most enriched terms in Molecular Function (MF) category, while “transport” (GO:0006810) (86 up-regulated and 36 down-regulated) and “transmembrane transport” (GO:0055085) (70 up-regulated and 27 down-regulated) are the two most enriched terms in Biological Process (BP) category (Figure 35).

To predict and classify possible functions, all the 77,631 unigenes were aligned to the KOG database and were assigned to the KOG categories (Figure 36). Among the KOG categories, the cluster for “General function prediction only” (15.8%) represented the largest group, followed by “Posttranslational modification, protein turnover, chaperones” (12.9%) and “Signal transduction mechanisms” (9.1%). “Translation, ribosomal structure and biogenesis” (7.3%) and “RNA processing and modification” (6.8%) were the largest next categories (Figure 36).

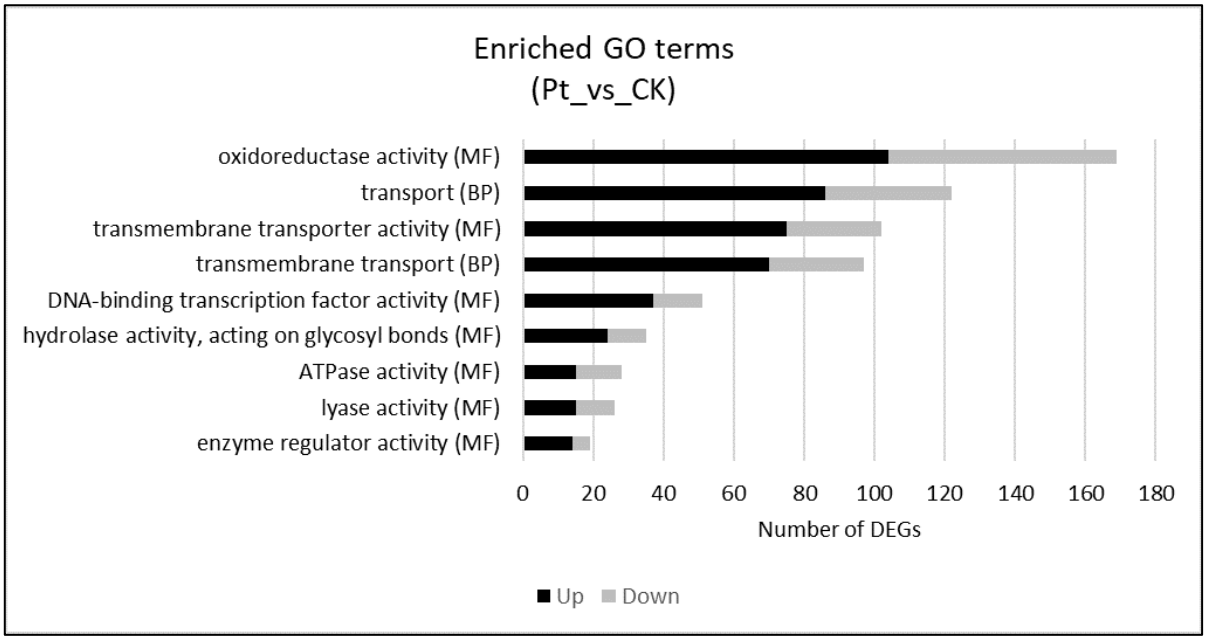


Figure 35. GO enrichment analysis for the DEGs in *C. jambhiri* (*Pt* vs. CK comparison). The X-axis indicates the numbers related to the total number of GO terms, and the Y-axis indicates the subcategories. BP, biological processes; MF, molecular functions.

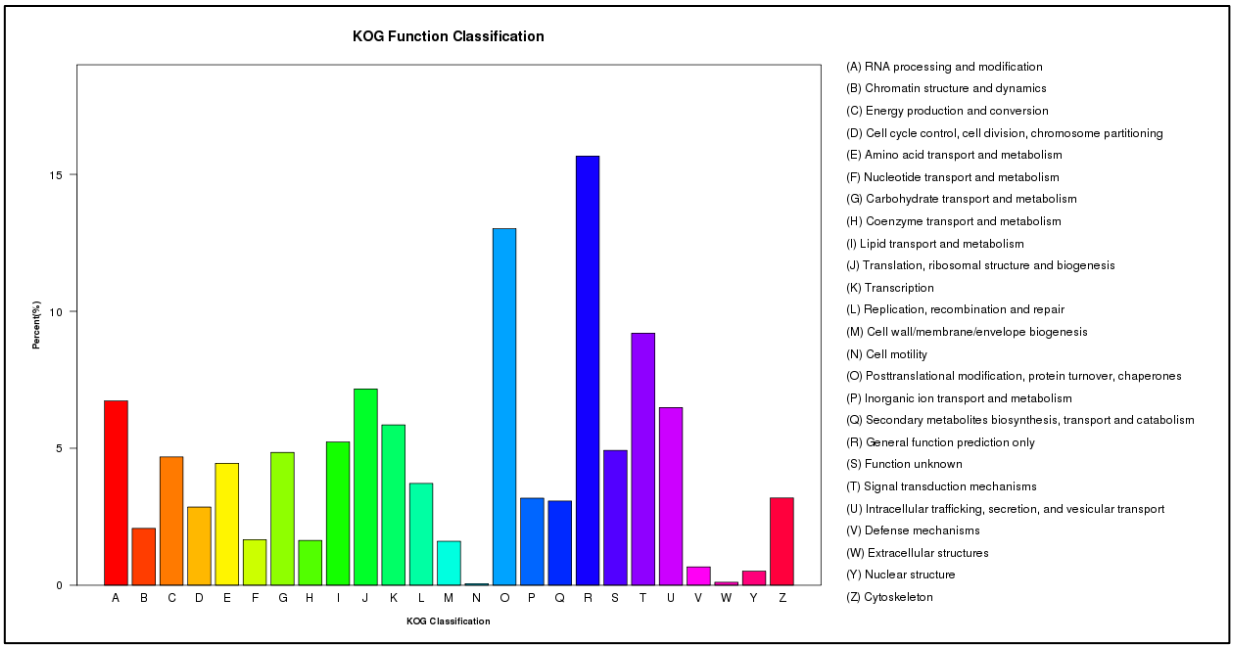


Figure 36. KOG function classification.

The main KEGG pathway terms were in the "Carbon metabolism" and "Phenylpropanoid biosynthesis" categories, followed by and "Biosynthesis of amino acids" indicating that a deep cellular rearrangement occurred in presence of the fungus (Figure 37). The reprogramming activity of the metabolic pathways is supported by the involvement of other important pathways such as "Plant hormone signal transduction" and "Starch and sucrose metabolism". The strong involvement of "Plant hormone" category in the response to pathogen is also indicated by the presence of different pathways involved in amino acid biosynthesis and metabolism such as "Tyrosine metabolism", "Phenylalanine metabolism", "Phenylalanine, tyrosine and tryptophan biosynthesis", and "Arginine biosynthesis", known to be precursors of plant hormones (Figure 37).

Because of their fundamental involvement of "Plant hormone" (Table 13, Figure 38), "Transcription factors" (Figure 39) and "Defense and pathogenesis" related genes (Table 14) in the host-pathogen interaction, we have analyzed them further. The following description of DEGs belonging to the above-mentioned pathways was carried out considering a $\log_2$foldchange threshold of ±2.32 (corresponding to a fold change=±5). In the following tables, the coding sequence of each clusters were identified as orthologs of *A. thaliana* gene genes (http://plantgdb.org/prj/GenomeBrowser, accessed on 23 November 2020). Congruously, tables report clusters whose % of identity was higher than 50 and the e value < 0.05.

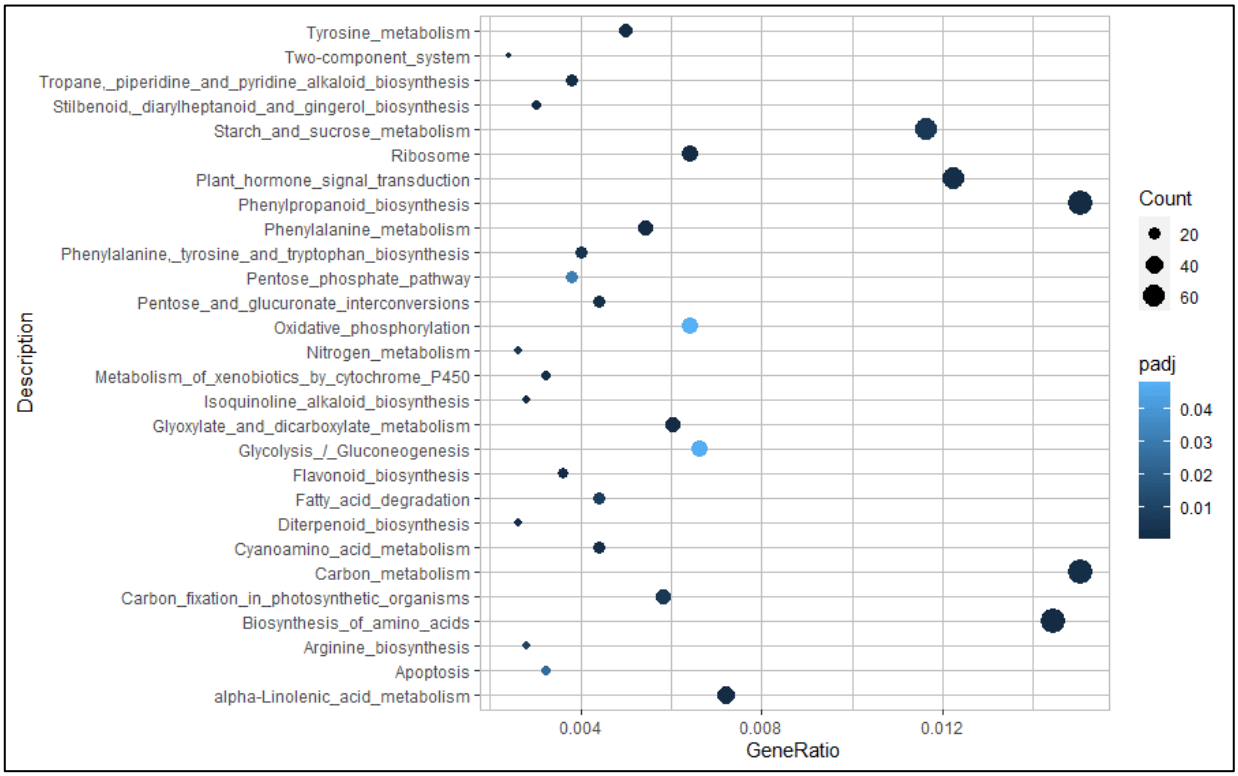

Figure 37. Distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differential expressed genes (DEGs) in the *Pt* vs. CK sample set.

| Cluster | Symbol | Annotation | TAIR code | $Log_2$FoldChange | Identity score | e-value |
|---|---|---|---|---|---|---|
| | | *Auxin* | | | | |
| 5112,0 | YUC6 | Flavin-binding monooxygenase family protein | AT5G25620 | -4.22 | 69% | 4e-69 |
| 15782,1 | AUX1 | Transmembrane amino acid transporter family protein | AT2G38120 | -5.18 | 74% | 0.0 |
| 14701,68946 | TIR1 | F-box/RNI-like superfamily protein | AT3G62980 | -2.62 | 66% | 2e-26 |
| 10078,0 | IAA18 | Indole-3-acetic acid inducible 18 | AT1G51950 | -3.18 | 79% | 7e-09 |
| 16281,1 | IAA4 | AUX/IAA transcriptional regulator family protein | AT5G43700 | -2.91 | 75% | 3e-63 |
| 16862,0 | IAA32 | Indole-3-acetic acid inducible 32 | AT2G01200 | -3.39 | 70% | 3e-20 |
| 14701,19495 | IAA7 | Indole-3-acetic acid 7 | AT3G23050 | -5.42 | 83% | 5e-71 |
| 16281,0 | IAA3 | AUX/IAA transcriptional regulator family protein | AT1G04240 | -3.61 | 81% | 0.001 |
| 14701,30415 | ARF3 | Auxin-responsive factor AUX/IAA-related | AT2G33860 | +4.30 | 73% | 2e-168 |
| 14701,26809 | GH3.3 | Auxin-responsive GH3 family protein | AT2G23170 | +3.42 | 72% | 5e-123 |
| 17976,0 | | SAUR-like auxin-responsive protein family | AT2G36210 | +3.41 | 82% | 3e-04 |
| | | *Ethylene* | | | | |
| 20624,0 | ACS2 | 1-amino-cyclopropane-1-carboxylate synthase 2 | AT1G01480 | +7.94 | 68% | 2e-131 |
| 14701,32226 | ACO1 | ACC oxidase 1 | AT2G19590 | +6.30 | 72% | 9e-133 |
| 17499,2 | ACS6 | 1-aminocyclopropane-1-carboxylic acid (acc) synthase 6 | AT4G11280 | +5.09 | 69% | 4e-139 |
| 14701,57239 | ETR2 | Signal transduction histidine kinase, hybrid-type, ethylene sensor | AT3G23150 | +3.46 | 66% | 2e-170 |
| 14701,58523 | EBF1 | EIN3-binding F box protein 1 | AT2G25490 | +8.86 | 66% | 2e-87 |
| 14701,46599 | EIN3 | Ethylene insensitive 3 family protein | AT3G20770 | +7.72 | 76% | 0.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 6645,0 | ERF1 | Ethylene response factor 1 | AT3G23240 | +3.32 | 72% | 6e-58 |
| 14701,24495 | ERF2 | Ethylene responsive element binding factor 2 | AT5G47220 | +2.49 | 76% | 3e-46 |
| 14701,21798 | ERF13 | Ethylene-responsive element binding factor 13 | AT2G44840 | +2.53 | 78% | 1e-37 |
| 14701,35256 | ERF4 | Ethylene responsive element binding factor 4 | AT3G15210 | +2.42 | 78% | 2e-36 |
| 14701,7830 | ERF110 | Ethylene response factor 110 | AT5G50080 | +4.23 | 77% | 6e-28 |
| | | *Salicylic acid* | | | | |
| 14701,45136 | PAL1 | Phenylalanine ammonia lyase 1 | AT2G37040 | +3.82 | 74% | 0.0 |
| 14701,67897 | 4CL | 4-coumarate--CoA ligase-like 5 | AT1G51680 | +3.89 | 72% | 1e-30 |

Table 13. List of “Plant hormone” related DEGs identified in *Pt* vs. CK comparison.

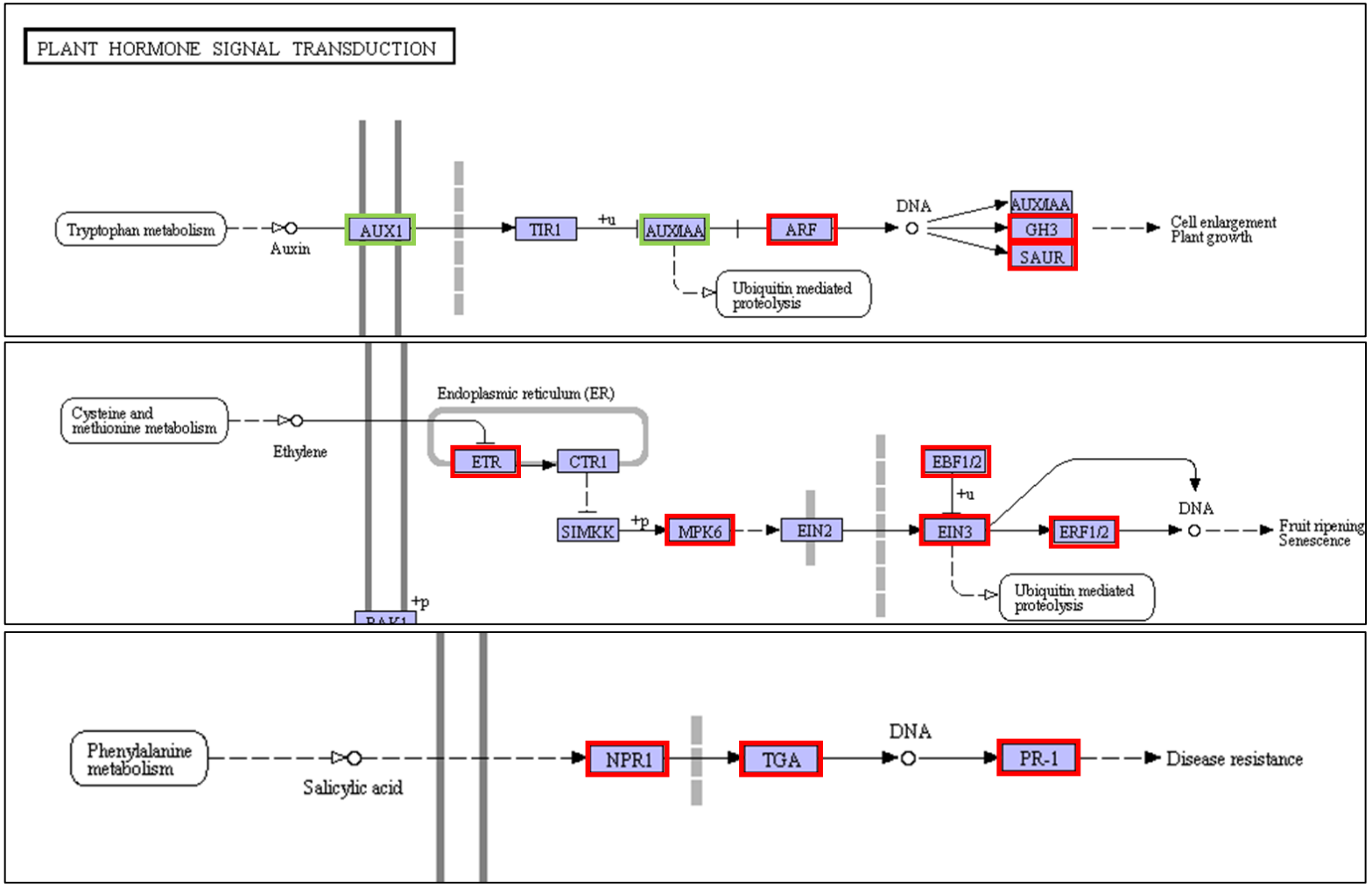


Figure 38. Scheme of the metabolic pathways involved in the "Plant hormone" category (clusters in boxes surrounded by a green line are down regulated, clusters in boxes surrounded by a red line are up- regulated).

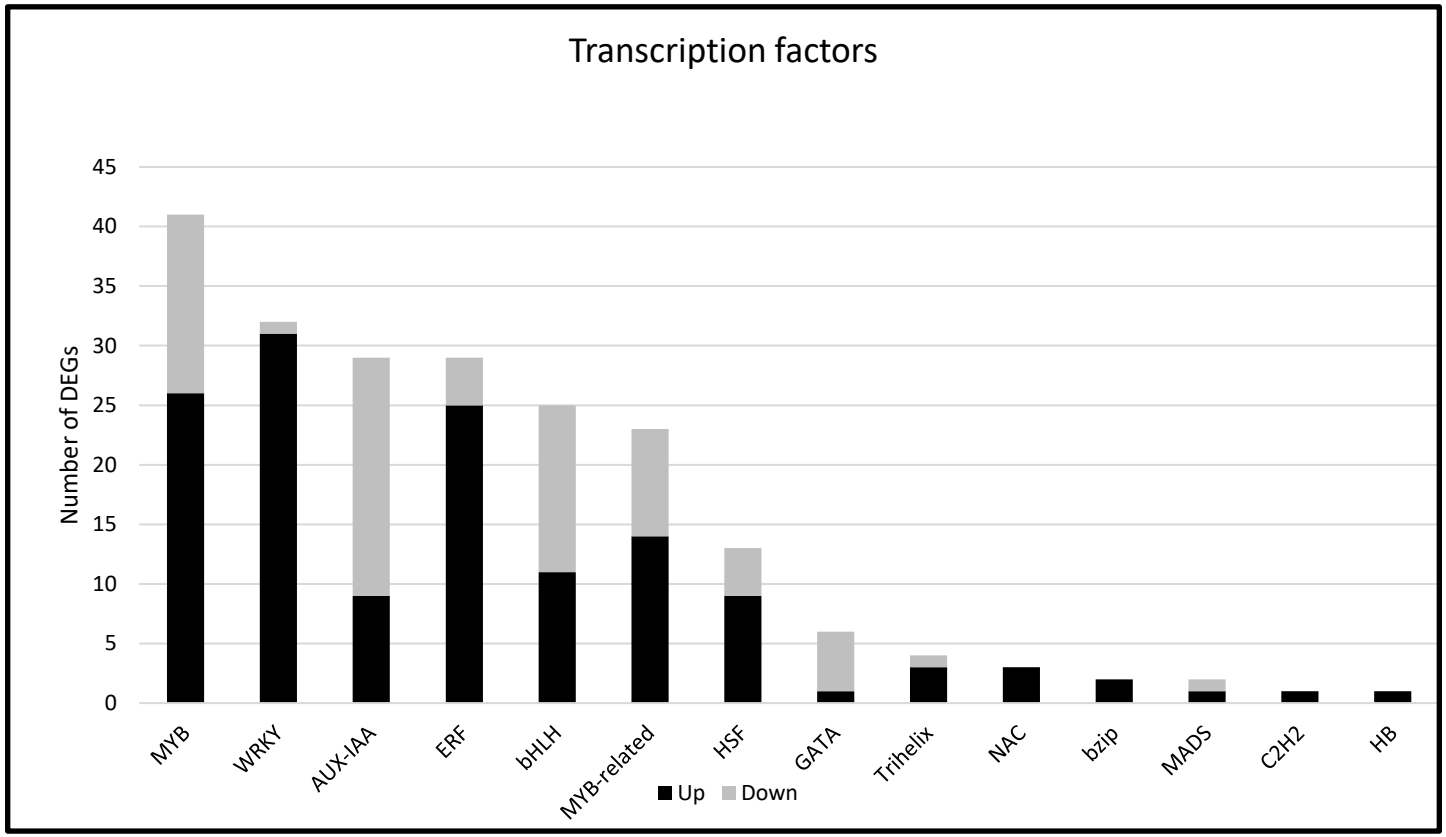


Figure 39. Distribution of rough lemon transcription factors responsive to *P. tracheiphilus* infection. Each bar represents the number of DEGs belonging to a transcription factor family.

| Cluster | Symbol | Annotation | TAIR code | $Log_2$FoldChange | Identity score | e-value |
|---|---|---|---|---|---|---|
| | | *Response to pathogen* | | | | |
| 14701,26919 | CPK33 | calcium-dependent protein kinase 33 | AT1G50700 | +3.13 | 67% | $4 \times 10^{-22}$ |
| 14701,66288 | CRCK3 | calmodulin-binding receptor-like cytoplasmic kinase 3 | AT2G11520 | +2.33 | 71% | $5 \times 10^{-59}$ |
| 17682,2 | CML11 | calmodulin-like 11 | AT3G22930 | +3.33 | 76% | $2 \times 10^{-77}$ |
| 14701,33952 | MPK3 | mitogen-activated protein kinase 3 | AT3G45640 | +5.42 | 76% | $2 \times 10^{-28}$ |
| 14701,16139 | MAPKKK15 | mitogen-activated protein kinase kinase kinase 15 | AT5G55090 | +2.49 | 67% | $1 \times 10^{-58}$ |
| 20990,0 | MAPKKK17 | mitogen-activated protein kinase kinase kinase 17 | AT2G32510 | +3.46 | 64% | $1 \times 10^{-33}$ |
| 14701,65619 | CERK1 | chitin elicitor receptor kinase 1 | AT3G21630 | +5.56 | 75% | $3 \times 10^{-18}$ |
| 8490,0 | PR1 | pathogenesis-related gene 1 | AT2G14610 | +4.09 | 68% | $8 \times 10^{-31}$ |
| 14701,26429 | PRB1 | basic pathogenesis-related protein 1 | AT2G14580 | +8.96 | 68% | $2 \times 10^{-32}$ |
| 16905,0 | NPR1 | regulation of innate immune response | | +5.40 | | |
| 13144,0 | NPR1 | *Citrus sinensis* protein NIM1-INTERACTING 3 (LOC107177379) | | +4.21 | 100% | 0.0 |
| 18290,0 | CF-9 | *Citrus clementina* receptor-like protein 9DC3 (LOC18042467) | | +5.75 | 97% | $3 \times 10^{-121}$ |
| 6996,2 | BIR2 | Inactive LRR receptor-like serine/threonine-protein kinase | AT3G47570 | +5.22 | 98% | 0.0 |
| 14701,81960 | CES101 | lectin protein kinase family protein | AT3G16030 | +5.49 | 77% | $7 \times 10^{-11}$ |
| 14701,15619 | IOS1 | Leucine-rich repeat transmembrane protein kinase protein | AT2G19230 | +5.15 | 68% | $2 \times 10^{-16}$ |
| 14701,38537 | EIX2 | *Citrus sinensis* receptor-like protein EIX2 (LOC102609951) | | +4.79 | 80% | 0.0 |
| 14701,79574 | LECRK3 | *Citrus clementina* G-type lectin S-receptor-like serine/threonine-protein kinase (LOC18049964) | | +4.20 | 99% | 0.0 |
| 14701,84653 | RGS1 | G-protein coupled receptors; GTPase activators | AT3G26090 | +4.76 | 70% | $4 \times 10^{-81}$ |
| 14701,13865 | TGA2 | transcription factor TGA2.3 isoform X1 | AT5G06950 | +3.44 | 70% | $5 \times 10^{-102}$ |
| 14701,40930 | BAD1 | Ankyrin repeat family protein BAD1 | AT1G14500 | +3.18 | 75% | $2 \times 10^{-07}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 19125,1 | RIN4 | RPM1 interacting protein 4 | AT3G25070 | -4.22 | 69% | $2 \times 10^{-14}$ |
| 14701,27598 | RBOHF | respiratory burst oxidase protein F | AT1G64060 | +2.41 | 75% | 0.0 |
| 14701,78394 | RBOHB | respiratory burst oxidase homolog B | AT1G09090 | +6.89 | 72% | $4 \times 10^{-154}$ |
| 14701,77930 | RBOHC | NADPH/respiratory burst oxidase protein D | AT5G51060 | +3.51 | 72% | $1 \times 10^{-08}$ |
| 14701,55000 | RBOHD | respiratory burst oxidase homologue D | AT5G47910 | +2.74 | 72% | 0.0 |
| | | *WRKY transcription factors* | | | | |
| 16089,0 | WRKY35 | WRKY DNA-binding protein 35 | AT2G34830 | +2.39 | 83% | $1 \times 10^{-92}$ |
| 15844,0 | WRKY49 | WRKY DNA-binding protein 49 | AT5G43290 | +2.89 | 77% | $1 \times 10^{-08}$ |
| 14701,6540 | WRKY23 | WRKY DNA-binding protein 23 | AT2G47260 | 2.78 | 76% | $5 \times 10^{-80}$ |
| 14701,12356 | WRKY4 | WRKY DNA-binding protein 4 | AT1G13960 | +2.35 | 77% | $5 \times 10^{-24}$ |
| 21223,0 | WRKY72 | WRKY DNA-binding protein 72 | AT5G15130 | +5.75 | 82% | $7 \times 10^{-87}$ |
| 14701,60912 | WRKY50 | WRKY DNA-binding protein 50 | AT5G26170 | +5.31 | 78% | $3 \times 10^{-36}$ |
| 14701,18458 | WRKY40 | WRKY DNA-binding protein 40 | AT1G80840 | +5.28 | 74% | $5 \times 10^{-27}$ |
| 16962,0 | WRKY75 | WRKY DNA-binding protein 75 | AT5G13080 | +5.07 | 76% | $4 \times 10^{-53}$ |
| 14701,3630 | WRKY71 | WRKY DNA-binding protein 71 | AT1G29860 | +4.71 | 84% | $2 \times 10^{-24}$ |
| 14701,66972 | WRKY18 | WRKY DNA-binding protein 18 | AT4G31800 | +4.34 | 77% | $2 \times 10^{-17}$ |
| 14701,51257 | WRKY70 | WRKY DNA-binding protein 70 | AT3G56400 | +4.03 | 72% | $1 \times 10^{-18}$ |
| 14701,2889 | WRKY44 | WRKY family transcription factor family protein | AT2G37260 | -2.43 | 80% | $2 \times 10^{-43}$ |

Table 14. List of DEGs identified in *Pt* vs. CK comparison.

#### 7.3.4.1 Plant hormone related genes

A significant deviation in the expression of genes involved in "Plant hormone" category was observed between the infected and control samples (Table 13, Figure 38). Considering auxin, known to be required for plant growth, the gene encoding one of the main biosynthetic enzyme, such as flavin-binding monooxygenase family protein YUC6 (Woodward and Bartel, 2005), was downregulated as well as the transmembrane amino acid transporter protein (AUX1) and three auxin-responsive IAA proteins (IAA32, IAA7 and IAA3) indicating that auxin biosynthesis and signaling are impaired in the inoculated plants. However, auxin-responsive transcription factors have been found up regulated suggesting that several pathways might be differently regulated. In this study, transcripts encoding several isoforms of the 1-amino-cyclopropane-1-carboxylate synthase, involved in the ethylene biosynthesis, have been found up-regulated. Moreover, many genes belonging to the ethylene signal transduction pathway and acting downstream of ethylene (signal transduction histidine kinase, hybrid-type, ethylene sensor (ETR2), mitogen-activated protein kinase 1 (MPK1), EIN3-binding F box protein 1 (EBF1/2), Ethylene insensitive 3 family protein (EIN3) and ethylene response factor 1 (ERF1/2) resulted up-regulated (Table 13, Figure 38), clearly indicating an activation of the ethylene signaling which might lead to the inhibition of plant growth and changes in a plant's life cycle. Salicylic acid (SA) is synthesized via the shikimic acid pathway, with chorismic acid serving as an important precursor that can be converted to SA via two distinct branches. In one branch, chorismic acid is converted to SA via phenylalanine and cinnamic acid intermediates by the key enzyme phenylalanine ammonia lyase (PAL). In the other branch, chorismic acid is converted to SA via isochorismic acid by the enzyme isochorismate synthase (ICS1/SID2) (Dempsey et al., 2011). Among the up-regulated transcripts, phenylalanine ammonia-lyase and 4-coumarate-CoA both implicated in one branch of salicylic acid biosynthesis have been found induced in the *Pt* vs. CK comparison. Moreover, genes encoding ICS1 were not among the DEGs suggesting that the main route for salicylic acid biosynthesis under biotic stress in rough lemon is that starting by phenylalanine and catalyzed by PAL.

#### 7.3.4.2 Transcription factors

Reprogramming of gene expression upon *P. tracheiphilus* infection is regulated by many transcription factors. In Figure 39 the most represented transcription factor (TF) families in terms of number of DEGs are reported. The results showed that 41 DEGs belong to MYB family (26 up-regulated and 15 down-regulated), 29 to both auxin responsive protein (AUX/IAA) and ethylene-responsive transcription factor (ERF) families, these latter already cited above ("*Plant hormone related genes*" section) indicating that they play a key role in regulating the transcriptional response induced by the pathogenic fungal infection (Figure 39). In addition, 32 genes encoding WRKY transcription factors have been found among the DEGs, most of which were over-expressed (31 up-regulated and 1 down-regulated). Due to their involvement in plant response to pathogenic fungi infection (Li et al., 2006; Ryu et al., 2006; Libault et al., 2007; Lai et al., 2008; Gao et al., 2011) the analysis of their role will be are included in the following paragraph (Table 14).

### 7.3.4.3 Defense and pathogenesis related genes

In Table 14 differential expressed genes involved in defense mechanisms and pathogenesis are summarized in order to provide a complete picture of the rough lemon response to pathogen attack. A plethora of genes encoding calmodulin-like protein, calcium-dependent protein kinase, mitogen-activated protein kinase 3, mitogen-activated protein kinase kinase kinase 15, mitogen-activated protein kinase kinase kinase 17, and GTPase activators have been found up-regulated in the *Pt* vs. CK sample set. These results clearly indicate that fungal infection triggers a wide reprogramming of the cellular signal transduction. Among the DEGs, several leucine rich repeat (LRR) domains, which might have a role as plant resistance (R) genes (IOS1, EIX2 and LECRK3) have been found up-regulated in the inoculated plants. However, the up-regulation of BIR2, which is negative regulator of basal level of immunity (namely PTI, pathogen-associated molecular patterns triggered immunity) strongly suggests that plant defense is already impaired at this first level (Halter et al., 2014). Nevertheless, some of R genes are also known to activate prolonged resistance by inducing phytohormones and pathogenicity related genes (PR genes) that collectively give rise to broad spectrum systemic acquired resistance (SAR) against future infections (Jones and Dangl, 2006). Indeed, the members of the pathogenesis-related protein 1 (PR-1) family, which are among the most abundantly produced proteins in plants on pathogen attack, have been found up-regulated in rough lemon infected plants (Table 14). Concomitantly, genes encoding the positive regulator protein NPR1, which is involved in the induction of defense gene and PR-1 gene expression, and the TGA transcription factor which NPR1 interacts with in the nucleus, have been found up-regulated in the inoculated plants. These findings suggest that systemic acquired resistance (SAR) mechanism occurred in the rough lemon interaction with the pathogen, probably giving rise to broad-spectrum systemic protection against future infections. According to these results, also other signal component of the SAR pathway such as BAD1, functioning upstream NPR1 to regulate defense responses, has been found induced by pathogen in the *Pt* vs. CK comparison (Table 14). Finally, transcript encoding CERK1 Lysin motif (LysM) receptor kinase that functions as a cell surface receptor in chitin elicitor signaling involved in the resistance to pathogenic fungi (Wan et al., 2008) was up-regulated in the infected plants (Table 14). It probably acts by sensing microbe-associated molecular patterns (MAMP) and pathogen-associated molecular patterns (PAMP) as component of the PTI. Finally, RPM1 interacting protein 4 is an essential regulator of plant defense, which plays a central role in resistance in case of infection; it acts in association with avirulence proteins with which triggers a defense system including the hypersensitive response (HR) limiting the spread of disease. Interestingly, this transcript was found down-regulated in the inoculated plant (Table 14) suggesting that it might have a role in susceptibility of rough lemon which is not able to avoid the pathogen circulation inside the plant. Transcriptional regulation of defense related genes is crucial for defeating pathogens. The involvement of chitin elicitation that is suggested by the up-regulation of CERK1 appears to play a significant role in plant defense to fungal pathogens through the activity of transcription factors belonging to WRKY family (Libault et al., 2007). Different genes encoding for WRKY DNA-binding protein have been found overexpressed in *C. jambhiri* infected plants. In detail, we found the up-regulation of *WRKY14*, *WRKY23*, *WRKY49*, *WRKY72*, *WRKY75*, *and WRKY71*. Moreover, *WRKY4*, that is reported to have a positive role in resistance to necrotrophic pathogens (Lai et al., 2008), *WRKY51*, acting as positive regulator of salicylic acid (SA)-mediated signaling (Gao et al., 2011)), *WRKY40*, *WRKY18* and *WRKY70* specifically responding to chitin (Libault et al., 2007) were also induced by *P. tracheiphilus* attack (Table 14). Finally, in response to pathogen

infection, the induction of the calcium-dependent respiratory burst oxidase homologues (RBOHB, RBOHC, RBOHD and RBOHF), which represent the major sources of ROS production in plants induced by pathogen infection, has been observed in inoculated rough lemon plants (Daudi et al., 2012).

### 7.3.4.4 Main processes or pathways affected by P. tracheiphilus infection

In order to have a comprehensive view of the metabolic changes occurring in rough lemon infected by *P. tracheiphilus*, all the 4,986 significant DEGs were mapped to the MapMan 3.6.0RC1 pathways, and the metabolism overview is showed in the Figure 40. Overall, the analysis indicates that the pathways which are more specifically involved in the response to *P. tracheiphilus* infection are "Reactive oxygen" (both up- and down- regulated genes), "Light reaction" (mostly down-regulated genes), "Nutrient homeostasis" (both up- and down- regulated genes), "Carbohydrate metabolism" ( up-regulated genes), all of these will be singularly analyzed (Table 15).

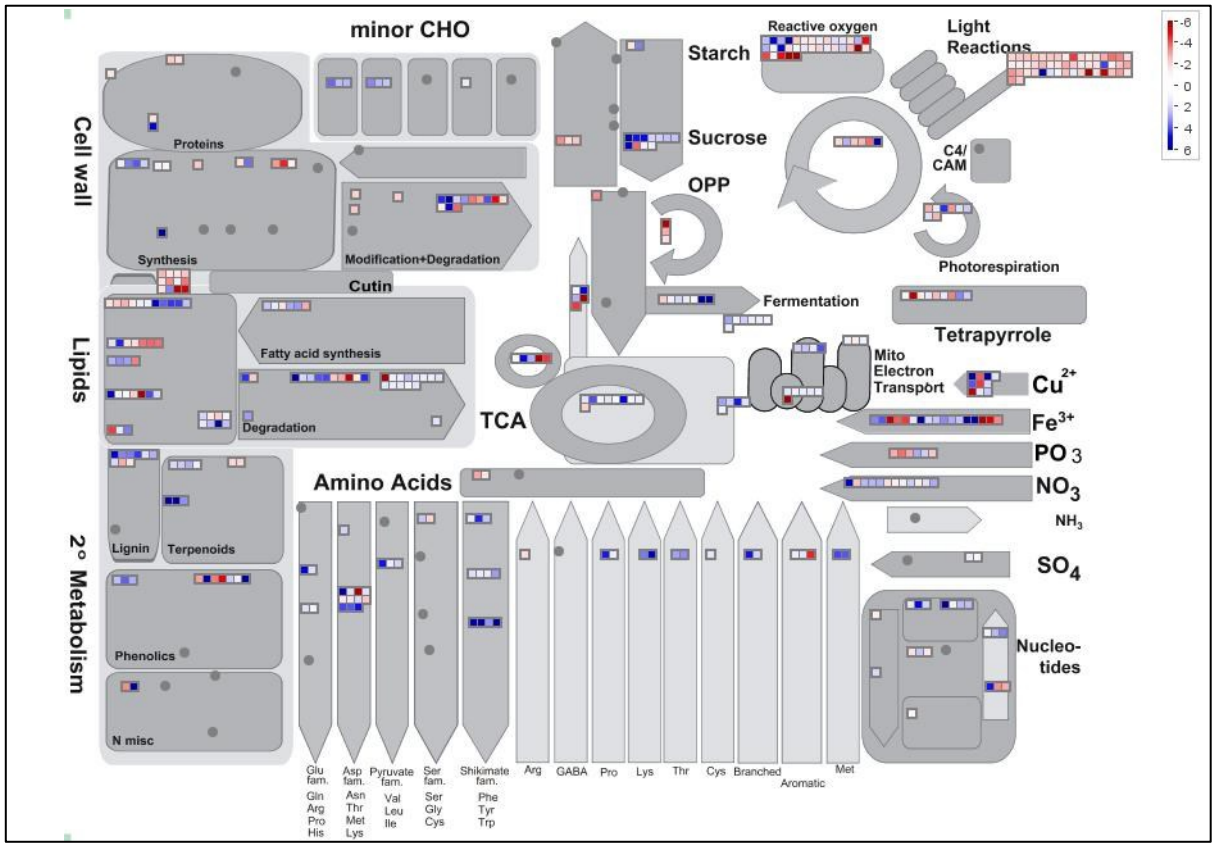


Figure 40. MapMan analysis of differentially expressed genes in *C. jambhiri* affected by *P. tracheiphilus*. Red dots represent up-regulated genes, blue dots represent down-regulated genes in the *Pt* vs. CK comparison.

| Cluster | Symbol | Annotation | TAIR code | $Log_2$FoldChange | Identity score | e-value |
|---|---|---|---|---|---|---|
| | | *Reactive oxygen - Oxidoreductase activity* | | | | |
| 14701,18284 | CSD1 | Copper/zinc superoxide dismutase 1 | AT1G08830 | +9.31 | 69% | $2 \times 10^{-04}$ |
| 14701,15083 | APX2 | Ascorbate peroxidase 2 | AT3G09640 | +5.11 | 78% | $2 \times 10^{-166}$ |
| 14701,14158 | PMP22 | Peroxisomal membrane 22 kDa | AT4G04470 | -4.28 | 80% | $2 \times 10^{-30}$ |
| 14701,8276 | AOS | Allene oxide synthase | AT5G42650 | +9.59 | 67% | $6 \times 10^{-26}$ |
| 14701,29676 | ALDH3H1 | Aldehyde dehydrogenase 3H1 | AT1G44170 | +3.70 | 68% | $3 \times 10^{-06}$ |
| | | *Reactive oxygen - Glutathione metabolism* | | | | |
| 14701,7488 | GSTU19 | Glutathione S-transferase TAU 19 | AT1G78380 | +7.58 | 70% | $1 \times 10^{-56}$ |
| 14701,35413 | GSTU10 | Glutathione S-transferase TAU 10 | AT1G74590 | +5.23 | 73% | $1 \times 10^{-17}$ |
| 14701,48103 | GSTF9 | Glutathione S-transferase PHI 9 | AT2G30860 | +5.22 | 72% | $1 \times 10^{-53}$ |
| 14701,17358 | GSTU7 | Glutathione S-transferase TAU 7 | AT2G29420 | +4.54 | 71% | $1 \times 10^{-33}$ |
| 14701,48102 | GSTF9 | Glutathione S-transferase PHI 9 | AT2G30860 | +4.32 | 69% | $3 \times 10^{-05}$ |
| | | *Light reaction* | | | | |
| 14701,61813 | PSAE-2 | Photosystem I subunit E-2 | AT2G20260 | -2.58 | 77% | $1 \times 10^{-06}$ |
| 14701,4480 | PSBE | Photosystem II reaction center protein E | ATCG00580 | -2.31 | 95% | $9 \times 10^{-110}$ |
| 14701,34255 | ATPD | ATP synthase delta-subunit gene | AT4G09650 | -3.10 | 72% | $2 \times 10^{-59}$ |
| 14701,26690 | PSBS | Chlorophyll A-B binding family protein | AT1G44575 | -3.50 | 76% | $2 \times 10^{-24}$ |
| 14701,72032 | TAP38 | Thylakoid-associated phosphatase 38 | AT4G27800 | -3.42 | 68% | $5 \times 10^{-42}$ |
| 14701,83115 | FKBP16 | FK506-binding protein 16-2 | AT4G39710 | -5.55 | 75% | $2 \times 10^{-90}$ |
| 14701,66882 | NDF4 | NDH-dependent cyclic electron flow 1 complex | AT3G16250 | -7.12 | 74% | $1 \times 10^{-61}$ |
| 14701,65295 | NDHB.2 | NADH-Ubiquinone/plastoquinone (complex I) protein (chloroplastic) | ATCG01250 | -2.44 | 98% | 0.0 |
| 14701,64497 | PPDK | Pyruvate orthophosphate dikinase (chloroplastic) | AT4G15530 | -2.79 | 77% | 0.0 |
| | | *Nutrient homeostasis* | | | | |
| 14701,19268 | | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | AT1G55290 | +7.77 | 68% | $2 \times 10^{-86}$ |
| 14701,28407 | FRO4 | Ferric reduction oxidase 4 | AT5G23980 | +7.14 | 66% | $2 \times 10^{-120}$ |
| 19914,4 | FRO2 | Ferric reduction oxidase 2 | AT1G01580 | +3.94 | 69% | $1 \times 10^{-36}$ |
| 14701,21908 | FRO7 | Ferric reduction oxidase 7 (chloroplastic) | AT5G49740 | -3.93 | 73% | $1 \times 10^{-81}$ |
| 14701,21905 | FRO6 | Ferric reduction oxidase 6 | AT5G49730 | -4.33 | 75% | $3 \times 10^{-105}$ |
| 14701,82040 | FRO8 | Ferric reduction oxidase 8 (mithocondrial) | AT5G50160 | -5.65 | 69% | $9 \times 10^{-106}$ |
| 4412,0 | IREG2 | Iron regulated 2 | AT5G03570 | -5.78 | 79% | $6 \times 10^{-40}$ |
| 14701,68697 | ASP3 | Aspartate aminotransferase 3 (chloroplastic) | AT5G11520 | +2.74 | 80% | 0.0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 14701,45698 | GLN1;1 | Glutamine synthase clone R1 (cytosolic isozyme 1) | AT5G37600 | +2.66 | 77% | 0.0 |
| 20088,0 | NRT2:1 | Nitrate transporter 2:1 | AT1G08090 | -2.41 | 73% | $2 \times 10^{-111}$ |
| 14701,24935 | PHT1;4 | Phosphate transporter 1;4 | AT2G38940 | +2.78 | 73% | 0.0 |
| | | *Carbohydrate metabolism* | | | | |
| 14701,30461 | SUS2 | Sucrose synthase 2 | AT5G49190 | +5.52 | 79% | $7 \times 10^{-63}$ |
| 14701,11795 | SUS6 | Sucrose synthase 6 | AT1G73370 | +2.33 | 71% | 0.0 |
| 14701,60145 | SPS4F | Sucrose-phosphate synthase 4 | AT4G10120 | -2.33 | 82% | $4 \times 10^{-29}$ |
| 14701,28539 | BETAFRUCT4 | Acid beta-fructofuranosidase | AT1G12240 | +8.66 | 70% | 0.0 |
| 14701,71035 | INV-E | Alkaline/neutral invertase (chloroplastic) | AT5G22510 | +2.36 | 72% | $6 \times 10^{-162}$ |
| 14701,25319 | FBA1 | Fructose-bisphosphate aldolase 1 | AT2G21330 | -3.27 | 74% | $8 \times 10^{-10}$ |
| 14701,77303 | HXK1 | Hexokinase 1 | AT4G29130 | -3.81 | 73% | $2 \times 10^{-97}$ |
| | | *Cell wall modification and degradation* | | | | |
| 11195,0 | QRT3 | Pectin lyase-like superfamily protein | AT4G20050 | +6.07 | 67% | $2 \times 10^{-38}$ |
| 14701,45234 | | Pectinacetylesterase family protein | AT4G19420 | +5.45 | 69% | $1 \times 10^{-75}$ |
| 8874,0 | | Pectin lyase-like superfamily protein | AT1G11920 | +3.10 | 72% | $3 \times 10^{-64}$ |
| 13011,0 | | Pectate lyase family protein | AT1G67750 | -3.55 | 77% | 0.0 |
| 14701,76034 | | Pectinacetylesterase family protein | AT3G05910 | -3.75 | 73% | $1 \times 10^{-101}$ |
| 14701,45231 | | Pectinacetylesterase family protein | AT4G19420 | -4.87 | 69% | $1 \times 10^{-75}$ |

Table 15. List of DEGs identified in *Pt* vs. CK comparison.

#### 7.3.4.4.1 Reactive oxygen

Table 15 reports the DEGs related to "reactive oxygen" category. Two main gene sets were found to be strongly up-regulated in the *Pt* vs CK comparison: genes involved in the oxidoreductase activity and glutathione transferases. In particular, genes encoding copper/zinc superoxide dismutase, ascorbate peroxidase were induced by pathogen to overcome the damage induced by ROSs that play a central role during plant–necrotrophic fungus interactions through the stimulation of the plant's defense responses (Barna et al., 2012). The gene encoding allene oxide synthase, involved in the pathway of oxylipin biosynthesis starting from unsaturated fatty acids was found strongly up-regulated. Their chemical nature renders unsaturated fatty acids intrinsic antioxidants; that is, they can directly react with ROS and thus consume them. Their oxidation gives rise to various oxylipins that, in turn, modulates ROS levels and signaling (He et al., 2020). Transcript of aldehyde dehydrogenase 3H1 involved in oxidative stress tolerance by detoxifying reactive aldehydes derived from lipid peroxidation was also found up-regulated in diseased rough lemon plants (Table 15). Interestingly, numerous genes encoding glutathione transferases (GSTs) belonging to different GST classes have been induced by the fungal infection. This gene family can positively contribute to antimicrobial resistance in host plants by mostly unknown mechanisms, although a recognized GST function is their participation in the elimination of ROSs and lipid hydroperoxides that accumulate in infected tissues (Puglisi et al., 2013; Gullner et al., 2018).

#### 7.3.4.4.2 Light reactions

As shown in Table 15 and Figure 41, the light reactions of the photosynthetic pathway were strongly affected by *P. tracheiphilus* inoculation as most of the components of both light harvesting and photosynthetic electron flows (cyclic and non-cyclic) as well as subunits of the CF0F1-ATP synthase were down regulated in inoculated plants (Figure 41). In detail, the PSAE-2 photosystem I subunit E-2 and the PSBE photosystem II reaction center protein as well as thylakoid-associated phosphatase 38 (Table 15) were down regulated in seedlings the diseased plant. This last gene is involved in light-harvesting complex of photosystem II (LHCII) dephosphorylation, facilitating its relocation to photosystem I. The expression of NDH-dependent cyclic electron flow 1 complex, that is involved in the cyclic electron transport by accepting electrons from ferredoxin (Fd), was sharply repressed. Moreover, the expression of the CF1-ATP synthase subunit was downregulated suggesting that the photophosphorylation of ADP leading to the ATP synthesis is strongly impaired because of fungal infection. Considering that photosynthesis is the main metabolic pathway devoted to energy supply in the green part of the plants, these findings clearly indicate that inoculated plants were suffering of energy shortage.

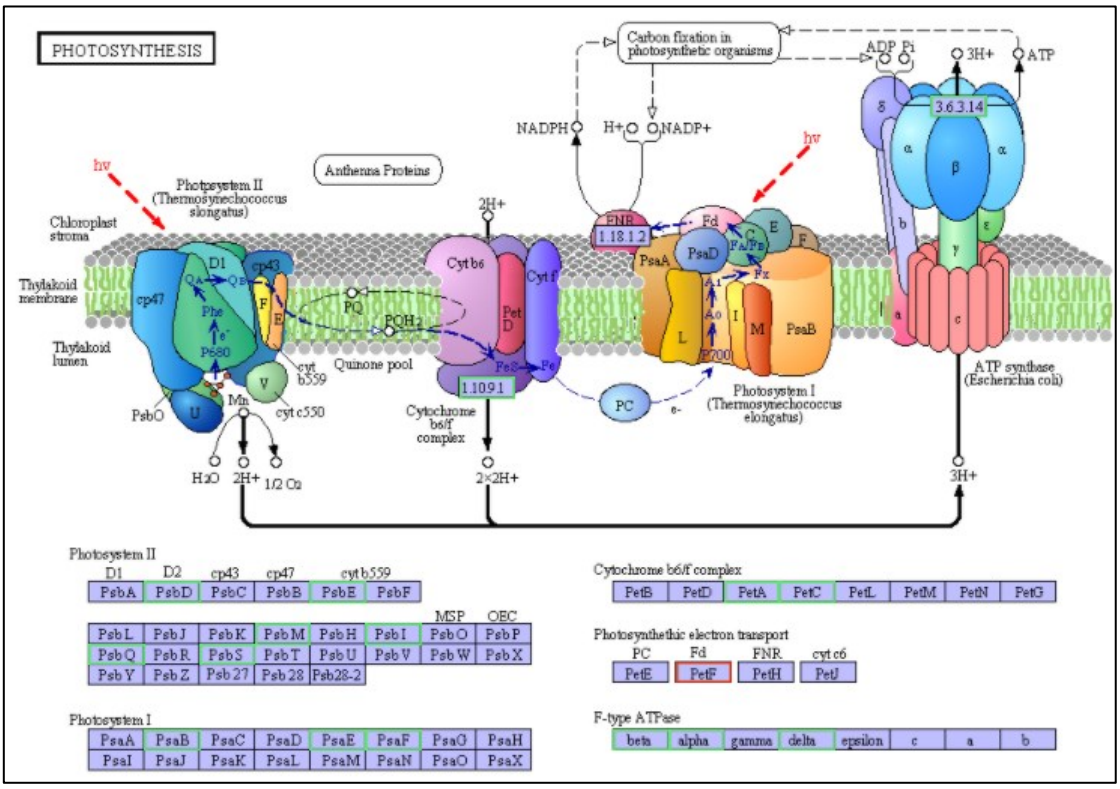


Figure 41. Scheme and components of the photosynthetic electron flow including CF0F1-ATP synthase (clusters in boxes surrounded by a green line are down regulated).

#### 7.3.4.4.3 Iron homeostasis

As shown in Table 15, genes involved in iron uptake and reduction were differently regulated in the *Pt* vs. CK comparison. In particular, ferric reduction oxidase 6 (FRO6), FRO7 and FRO8 were repressed by the infection. These genes are proposed to be involved in iron transport across the membrane in green part of the plant, FRO6 being localized in the plasma membrane, FRO7 in the chloroplasts and FRO8 in mitochondria (Jeong et al., 2008). These results clearly indicate that the iron homeostasis is sharply impaired in the organelles of inoculated plants and in chloroplasts where it plays a crucial role in the heme biosynthesis and photosynthesis. Ferric reduction oxidase 2 (FRO 2) and 4 (FRO4) which normally are expressed in plant roots were upregulated by fungal infection, as well as the gene encoding 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase which are involved in sideretin biosynthesis, a metabolite exuded by roots in response to iron deficiency to facilitate iron uptake. The stress induced expression of genes, both FROs and 2OG, normally involved in iron uptake in roots might be explained as an ultimate attempt to cope with the shoot iron deficiency caused by the down regulation of leaf-specific FRO genes. Regarding other nutrients such as nitrogen and phosphate, the results show that gene involved in nitrate uptake was down-regulated, whereas glutamine synthase and aspartate aminotransferase involved in nitrogen fixation and in amino acid and Krebs cycle metabolisms were up-regulated. The high-affinity transporter for external inorganic phosphate functioning as $H^+$: Phosphate symporter was also up-regulated (Table 15).

#### 7.3.4.4.4 Carbohydrate metabolism

The analysis of carbohydrate metabolism highlighted that several genes involved in sugar metabolism were clearly induced in response to fungal infection (Table 15). Specifically, sucrose-phosphate synthase 4, which plays a role in photosynthetic sucrose synthesis by catalyzing the rate-limiting step of sucrose biosynthesis from UDP-glucose and fructose- 6-phosphate, was down-regulated. On the contrary, transcripts encoding sucrose synthase, a cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways, were among the up-regulated genes. Table 15 also reports that transcripts encoding the acid beta-fructofuranosidase and alkaline/neutral invertase, respectively involved in the continued mobilization of sucrose to sink organs and in the cleavage of sucrose into glucose and fructose, were up-regulated. Overall, these data suggest that both sucrose synthesis and therefore the export of photo assimilates out of the leaf were impaired, whereas cleavage seems to be the favorite route undertaken by this metabolite. However, the fructose-bisphosphate aldolase 1 and hexokinase 1 were down-regulated in diseased plants indicating that glycolysis might be repressed in the inoculated plants (Table 15).

#### 7.3.4.4.5 Cell wall modification and degradation

During pathogen infections, the cell wall undergoes dramatic structural and chemical changes of cell wall constituents. Necrotrophic pathogens are sensed by a plasma membrane receptor, leading to activation of defense signaling cascades and eventual mounting of inducible defense responses (Nafisi et al., 2015). In our study, several DEGs encoding pectin lyase-like superfamily protein and pectin acetylesterases were identified (Table 15). However, these transcripts were both up- and down-regulated, making it difficult to extrapolate unequivocal conclusions. Certainly, as expected, cell walls of inoculated plants underwent remodeling processes likely involved in the response to pathogen.

## 8. Discussion

Environmental stresses severely affect plant and crop growth and reproduction. Therefore, determining the critical molecular mechanisms and cellular processes in response to stresses will provide knowledge for identifying genes that might be target of modification, by *knocking out* or by *knocking down* procedures, especially in susceptible host–pathogen interactions. RNA sequencing (RNA-Seq) using next-generation sequencing (NGS) provides opportunity to isolate genes of interest, develop of functional markers, quantify of gene expression and carry out comparative genomic studies. It has been successfully applied to unravel the transcriptome profile of several *Citrus* varieties in response to *Phytophtora parasitica* infection (Naveed et al., 2019) and to *Candidatus* Liberibacter asiaticus (Hu et al., 2017; Yu et al., 2017) providing new insight into host responses to both pathogens. In this work, we described the results of RNA sequencing and de novo transcript assembly in rough lemon (*C. jambhiri*) leaves subjected to artificial inoculation by *P. tracheiphilus*, the causal agent of "mal secco" disease used as model of a compatible host–pathogen interaction. At harvest time (15 days after inoculation infected plants showed the typical disease symptoms, and the pathogen was detected by molecular analysis. Globally, a deep reprogramming of the leaf transcriptome emerged as 4986 (2865 up-regulated and 2121 down regulated) DEGs have been identified confirming that the attempt of an active defense against microbial pathogens involved the induction of elaborate defense signaling pathways. In plants, some of these defense strategies can provide protection at the site of infection, whereas others provide systemic resistance throughout the plant including in non-infected

tissue. Local resistance includes basal immunity, or PAMP/MAMP (pathogen/microbe associated molecular patterns)—triggered immunity (PTI) which is induced when pattern recognition receptors (PRRs) from the plant recognize pathogen-derived elicitors. To establish a successful infection, plant pathogens can suppress PTI by injecting effectors into the host cells (Shine et al., 2019). To counter this virulence strategy, plants have evolved the so-called resistance (R) proteins, which can either directly detect the effectors or indirectly detect their activity. In plants where the activity of effectors is detected by the R proteins, effector-triggered immunity (ETI) is activated rendering the pathogen avirulent (Noman et al., 2020). ETI in plants is often associated with rapid, localized programmed cell death (PCD) at the infection site, a visible phenotype known as the hypersensitive response HR, to prevent the spread of the pathogen. HR is generally associated with race-specific resistance to biotrophic pathogens and it is less effective against necrotrophics which require dead host tissue to complete their life cycle (Noman et al., 2020). Necrotrophic pathogens such as *P. tracheiphilus* are well able to block HR by initiating systemic signals for defense activation in distal parts of plant that ultimately results in the activation of systemic acquired resistance (SAR) (Noman et al., 2020). Induction of SAR involves the generation of mobile signals at the site of primary infection, which translocate to distal tissue and prepare the plant against future infections. Several chemical inducers of SAR have been identified and some of these have been shown to translocate systemically. The SAR associated chemicals include salicylic acid (SA), free radicals, and reactive oxygen species (ROS), among others (Fu and Dong, 2013). Upon SA accumulation, NPR1 monomers are transported into the nucleus. Here, NPR1 interacts with TGA proteins, which belong to the basic leucine zipper (bZIP) protein family of transcription factors and binds TGACG motifs to activate defense-related transcription (Fu and Dong, 2013). The analysis of the transcriptomic data reported in this work unequivocally indicated that the entire gene set encoding the components of SAR from salicylic acid biosynthesis on was strongly up-regulated. In addition, *P. tracheiphilus* is able to overcome the basal immunity of rough lemon plants (PTI) as the essential regulator of plant defense (RPM1 interacting protein 4) was down-regulated, and the expression of BIR2, which is negative regulator of basal level of immunity was up-regulated in the diseased plants. In the inoculated plants, the observed repression of auxin signaling by the SA pathway might also contribute to increase rough lemon susceptibility to *P. tracheiphilus* as reported in *Arabidopsis* infected by the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* (Llorente et al., 2008).

Chitin, found in the cell walls of true fungi, is a well-established elicitor of plant defense responses and it appears to play a significant role in plant defense to fungal pathogens (Gentile et al., 2007). The fact that chitin elicits de novo gene expression suggests the involvement of transcription factors (TFs) with WRKY TF family strongly represented (Eulgem et al., 2000; Ülker and Somssich, 2004; Zhang and Wang, 2005). To regulate gene expression, WRKY proteins bind specifically to a DNA sequence motif (T)(T)TGAC(C/T) known as the W box (Du and Chen, 2000; Eulgem et al., 1999; Turck et al., 2004; Yu et al., 2001), which occurs in the promoters of genes under the control of WRKY proteins. A number of defense-related genes, including PR genes, contain a W box in their promoter regions (Eulgem et al., 1999; 2000; Du and Chen, 2000). The promoters of pathogen and/or salicylic acid (SA) regulated *Arabidopsis* WRKY genes (Dong et al., 2003) are substantially enriched in W boxes, suggesting that defense-regulated expression of WRKY genes involves transcriptional activation and repression through self-regulatory mechanisms mediated by transcription factors of the WRKY gene superfamily (Ryu et al., 2006). For example, expression of the *Arabidopsis NPR1* is known to be controlled by WRKY factors (Yu et al., 2001). In this study, transcription factors

interacting specifically with the W box motif such as *WRKY14*, *WRKY23*, *WRKY49*, *WRKY72*, *WRKY75*, and *WRKY71* were up-regulated in infected plants, indicating a strong activation of the defensive mechanism. The up-regulation of both *WRKY4*, that is reported to have a positive role in plant resistance to necrotrophic pathogens (Lai et al., 2008) and *WRKY51*, acting as positive regulator of salicylic acid (SA)-mediated signaling (Gao et al., 2011) confirms that the rough lemon plants tried to resist the *P. tracheiphilus* infection by the activation of salicylic acid-mediated signaling pathway, in accordance with the whole results of this study. Furthermore, the strong induction of *WRKY40*, *WRKY18,* and *WRKY70* transcription factors accounts for a defense response specifically addressed towards fungi as they specifically respond to chitin (Libault et al., 2007). *P. tracheiphilus* infection induced the expression of oxidative burst peroxidases (RBOHs) in rough lemon (Table 14). The apoplastic oxidative burst could directly kill pathogens by generating ROS with antimicrobial activity; otherwise, a second, stronger phase can occur, which is associated with the hypersensitive response (Daudi et al., 2012). However, the role of RBOHs is controversial as a relatively limited role for NADPH oxidases in the HR has been observed in tobacco (*Nicotiana tabacum*), where RBOHD-mediated hydrogen peroxide production does not seem to be essential for the development of the HR or systemic acquired resistance (SAR) (Rouet et al., 2006; Lherminier et al., 2009). More recently, these genes have been studied in detail in *A. thaliana* and are reported as the major component of PTI (Daudi et al., 2012)). Considering that rough lemon plant is susceptible to *P. tracheipilus*, their effectiveness in overcoming the pathogen is not sufficient to block it, and probably they have a major role in transducing the signal of the "presence" of the pathogen by increasing ROS concentration.

Although important in biotic stress signal transduction, reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide ($O^{2-}$), and singlet oxygen ($^1O_2$) are highly reactive and could cause oxidative damage to DNA, proteins and other molecules of the cell. There are different cellular mechanisms in place to deactivate the excess of these damaging ROS molecules. These include enzymatic reactions through catalase, superoxide dismutase, glutathione peroxidase and ascorbate peroxidase but also small antioxidants such as ascorbic acid and glutathione (Chen and Yang, 2020). This study revealed that a subset of these ROS-scavenging genes was induced in the infected plants (Table 15). Interestingly, a wide number of glutathione transferases belonging to phi and tau classes were also up-regulated by the infection in accordance with early studies on the role of GSTs in plant biotic stress (Gullner et al., 2018). Notably, the expression of multiple GSTs was massively activated by salicylic acid and some GST enzymes were demonstrated to be receptor proteins of salicylic acid (Gullner et al., 2018). Functional studies revealed that overexpression or silencing of specific GSTs can markedly modify disease symptoms and pathogen multiplication rates (Liao et al., 2014). As reported in the case of other necrotrophic fungi such as *B. cinerea* (Berger et al., 2004), the main metabolic effect upon inoculated plants was the down-regulation of either light harvesting components or photosynthetic electron flows or CF1F0-ATPase. This might have led to an apparent, persistent "dark" or "shade" condition: Plants are in regular light/dark alternation but they cannot use light to provide energy. The sucrose mobilization suggested by the regulation of the two main genes involved in sucrose biosynthesis and cleavage is in accordance with this energy requirement. In the dark, plant mitochondria generate the required ATP molecules for basic cellular function (Braun, 2020). However, two main genes involved in glucose catabolism were down-regulated (Table 15) indicating that sugars seem not be routed towards glycolysis and Krebs cycle. On the contrary, as fungal genes involved either in sugar fermentation or in mitochondrial synthesis of ATP were strongly

expressed in rough lemon leaves, the plant sugar resources might be hijacked towards the fungus to feed it. This mode of nutrition is the rule for biotrophic pathogens, but also necrotrophics might exhibit a similar behavior (Talbot, 2010). In this study, we also show that FRO7 (chloroplast Fe(III) chelate reductase), involved in chloroplast iron homeostasis and required for survival under iron-limiting conditions, was down regulated. It has been shown that chloroplasts isolated from *fro7* loss-of-function mutant plants have significantly reduced Fe(III) chelate reductase activity, reduced iron content, and altered photosynthetic complexes, providing genetic proof that chloroplasts do rely in part on a reductive strategy for iron acquisition (Jeong et al., 2008). Consequently, the lack of a regular input of reducing power from water photolysis induced by light might be in turn responsible for the iron deficiency observed in the apical part of the leaves of diseased rough lemon.

## 9. Conclusions

The global transcriptome analysis of *Pt* vs. control plants led to the identification of genes and metabolic pathways involved in rough lemon response to *P. tracheiphilus*. As far as we know, this is the first manuscript that describes at molecular level the "mal secco" disease induced by *P. tracheiphilus* in citrus and makes *C. jambhiri* genetic resources available for the scientific community interested in citrus breeding. The results highlight most of the events occurring during this compatible host–pathogen interaction, which now it is known relies on the activated SA signal cascade that, in turn, induces systemic acquired resistance (SAR). As the main scope of the work was the identification of putative target genes for genome editing experiments, a wide range of genes belonging to structural and transcription factors have been identified and they could be taken in consideration for targeted mutagenesis, RPM1 and BIR2 being only two of them. This strategy fits the increased demand for economical and environmentally friendly approaches to cope with plant diseases, while avoiding the use of agrochemicals.

## 10. General Conclusion

This project was useful to categorize many citrus accessions belonging to true and derived species based on their field tolerance to MSD. Many accessions were found immune or resistant to the disease under natural pathogen pressure, but also a broad range of tolerance was observed. Several degrees of field tolerance cannot be explained by a single gene involved in the resistance (Russo, 1977). The presence of many genes involved in host-pathogen interaction was also supported by Reforgiato Recupero et al. (Recupero et al., 1997). We found that DNA isolation from twigs coupled with real-time PCR detection of the pathogen is a reliable method for field phenotyping. This method could be routinely used to validate phenotyping of mapping populations or germplasm collections to select tolerant of resistant accessions. A putative source of resistance was found in Doppio Lentini (autotetraploid lemon) and 46321 (probably a tetraploid Monachello) since no symptoms were found during the three years of visual monitoring and no pathogen was detected by real-time PCR analysis. This resistance seems not to be exclusively related to tetraploidy (Grosser et al., 2015), since other autotetraploids included in the phenotyping, namely 46515 and 46245, showed clear symptoms confirmed by real-time PCR detection. This project was also helpful to identify sources of resistance among other citrus species that could be used to introgress resistance genes into the lemon genome. Therefore, based on the phenotyping results, two monoembryonic mal secco-resistant species, namely Khasi papeda and Clementine, were chosen as female parents and crossed with Femminello Siracusano 2KR, a very susceptible lemon clone. The first population, Khasi papeda × Femminello Siracusano 2Kr, composed of 150 seedlings grafted on Carrizo Citrange, while the second population, Clementine × Femminello Siracusano 2Kr, was composed by 140 seedlings grafted on Carrizo Citrange. These two populations might be useful in the future for studying the segregation of MSD susceptibility and for identifying candidate genes and QTLs associated with the disease. A genome-wide mapping of QTLs controlling the resistance to MSD will be a desirable tool to use in the next years, with the final purpose to identify the most valuable molecular markers for MAS. The identification of a molecular marker linked to the resistance or susceptibility to the disease can allow an early selection of the seedlings (screened at a 3-4 leaves stage), resulting in cost reduction and in a much more efficient space management, since only the individuals with the favourable genotype for the trait of interest will be considered. We also used a transcriptomic approach (RNAseq) to investigate the host responses that occurred in a susceptible genotype upon *P. tracheiphilus* infection. The results indicated that several PR genes, kinase genes, and PTI signalling pathway genes were significantly induced, suggesting a specific role of these genes in the *P. tracheiphilus*- rough lemon interaction. In addition, the RNAseq data suggests that many different pathways were negatively affected by the pathogen, such as the photosynthetic system that showed an important downregulation of the genes involved in the production of ATP. RNAseq gave a complete picture of most of the events occurring during this compatible host-pathogen interaction, which was shown to be an activated SA signal cascade that, in turn, induces systemic acquired resistance (SAR). As the main scope of the work was the identification of putative target genes for genome editing experiments, a wide range of structural genes , for example RPM1 and BIR2, that could be taken in consideration for targeted mutagenesis. These genes could be modified using the New Plant Breeding Techniques (NPBTs), providing in the next years alternative methods to conventional methods for lemon genetic improvement. NPBTs represent innovative alternative methods to conventional breeding, with a shortened process, and their precise mechanisms of action that produce minimal modifications to

selected genotypes, without altering the genetic background, are particularly important for elite cultivars.

In conclusion, this project developed useful breeding and pre-breeding material that could be used in the future in the framework of the CREA and University of Catania lemon breeding programs. The KxS and CxS populations represents the first examples of reference segregating populations specifically developed for understanding the genetic basis of MSD.

Finally, as far as we know this is the first project that describes at molecular level the MSD induced by *Plenodomus tracheiphilus* and makes *C. jambhiri* transcriptome resources available for the scientific community interested in *Citrus* breeding. The development of new techniques in plant breeding has not led to the replacement of the older methods. The use of all available technologies is essential for plant breeding. Conventional breeding techniques, transgenesis and new plant breeding techniques are essential components of what could be called the plant breeders' toolbox (Lusser et al., 2011).

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- 62°Congresso annuale della Società Italiana di Genetica Agraria (SIGA), Verona, 25-28/09/2018
- Metodologie statistiche per le scienze agrarie, modelli lineari e generalizzati, Roma, CREA Agricoltura e Ambiente, 21-25/01/2019
- XV Eucarpia Fruit Breeding and Genetics Symposium, Praga, 3-7/06/2019
- Summer School in "Plant Phenotyping", Metaponto, 3-5/07/2019
- 63°Congresso annuale della Società Italiana di Genetica Agraria (SIGA) – Napoli 10-13/09/2019
- XXV National Congress Italian Phytopathological Society (SIPaV) Milano 16-18/09/2019

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# Identification of Field Tolerance and Resistance to Mal Secco Disease in a Citrus Germplasm Collection in Sicily

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**Abstract:** Mal secco is a tracheomycotic disease caused by the fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp, and Verkley that has caused severe damage and loss of yield in the citrus industry in the Mediterranean area, for 100 years. While the disease can affect different cultivated citrus species, lemon (*C.* × *limon* var. *limon* (L.) Burm. f.) and citron are the most susceptible. The identification of resistant or field-tolerant clones and hybrids is a major goal for lemon growers and breeders. To identify sources of resistance or tolerance to the disease, we performed a phenotypic survey on a lemon and lemon-like open-field germplasm planted at CREA (Research Centre for Olive, Fruit and Citrus Crops), Italy, in an area with high pathogen pressure. Phenotyping was performed visually, four times, for three consecutive years, on a total of 50 accessions, with two or three replicate trees per accession. Moreover, molecular screening based on real-time PCR was performed, for two consecutive years, on twigs, young leaves, and mature leaves of all plants, to detect the pathogen in the absence of clear symptoms. The accessions were categorized into seven groups based on the presence of visual symptoms, real-time PCR pathogen detection, and canopy volume. The results revealed sources of tolerance in lemon and citron hybrids. The molecular screening identified *P. tracheiphilus* in all lemon clones, with mean Ct values ranging from 17 to 39. The screening also identified *P. tracheiphilus* in clones without clear symptoms, indicating their ability to tolerate the disease. Moreover, a strong negative correlation was found between the Ct values in twigs and symptom severity ($r = -0.72$). This indicates that the DNA from twigs is the most appropriate for use in performing reliable phenotyping of mal secco susceptibility in adult plants. An autotetraploid lemon (Doppio Lentini) seems to be immune to the disease, under natural pressure, since *P. tracheiphilus* was not detected by real-time PCR and visual screening. Overall, the data obtained are a valuable resource for identifying both the most tolerant lemon varieties suitable for areas with high pathogen pressure and the best breeding parents for the introgression of resistance genes into lemon genotypes.

**Keywords:** lemon; phenotyping; *Plenodomus tracheiphilus*; polyploids; real-time PCR

## 1. Introduction

Mal secco is a vascular disease caused by the quarantine fungus *Plenodomus tracheiphilus* Petri Gruyter, Aveskamp, and Verkley [1]. It was previously classified as *Phoma tracheiphila* and is now included in the A2 list of quarantine pests of the European and Mediterranean Plant Protection Organization [2,3].

This fungus was discovered in 1984 on two Aegean Greek islands, Chios and Poros, and later it spread to other Mediterranean and Black Sea countries. Recently, it was also found in Spain, although it is not present in Portugal, Morocco, Malta, and Croatia (https://gd.eppo.int/taxon/DEUTTR/distribution). Lemon is one of the most susceptible sensitive species to this pathogen. The fungus penetrates through wounds [2,4–6] caused by heavy rains, hail, and wind; these atmospheric conditions favor the spread of the disease.

The symptoms usually begin with leaf vein chlorosis and leaf drop. Afterward, the pathogen reaches twigs and branches, and it is possible to observe red discolored strands in the xylem of stems. This is followed by the dieback of twigs and branches and the eventual death of the tree.

The disease severity shows a seasonal fluctuation and varies in different growing areas, depending on the climatic conditions. Ruggieri [7] reported that, in the years from 1918 to 1953, mal secco disease (MSD) destroyed no less than 12,000 ha of lemon groves in Sicily, Italy. According to Salerno and Cutuli [8], the mean yield of the production of lemon orchards in Sicily was approximately 20 tons/ha in the presence of MSD, whereas, in lemon orchards not affected by MSD, the yield could reach 60–80 tons/ha.

The pathogenicity of the different isolates collected in different Mediterranean countries was characterized in many studies [4,9–19], and efficient protocols were optimized to detect fungal infection in different plant tissues [16,20].

Chemical treatments in commercial orchards can only be used to prevent infections [21]. Therefore, the selection of field-tolerant lemon varieties is the most effective strategy to control the disease [2,22].

Lemons have a narrow genetic base, since most of them are bud sports of a single ancestor, which is a hybrid between sour orange and citron [23,24]. Such a genetic background exposes the species to the threat of the pathogen and hampers the identification of resistant varieties. Although most lemons are susceptible to the disease, some sources of tolerance were observed in field conditions, specifically in Monachello [25–27], Interdonato [25–27], Santa Teresa [28,29], Quattrocchi [30], Zagara Bianca, and Continella [31–33]. Unfortunately, none of them combine high fruit quality and productivity with tolerance to the disease.

Many citrus and citrus relatives were classified as susceptible or resistant to MSD [2], but the classification was based on a comparison among few citrus species and lemon varieties by visual screening or artificial inoculum [34–39]. Most of the bibliographic information is based on observations of single or few cultivars grown in the same field, while phenotypic studies comparing several accessions in the same field block are lacking.

Obtaining a lemon cultivar with good qualitative and pomological traits, as well as resistance to MSD, is a major challenge for the Mediterranean citrus industry [22]. The use of genetic transformation might be useful to improve the resistance to MSD or other diseases [40,41], but the use of genetically modified organisms (GMOs) raises public concerns regarding their safety. Consequently, traditional breeding approaches are, so far, the only means of releasing improved cultivars. To achieve this aim, identifying and characterizing sources of tolerance in lemon germplasm is needed to provide growers with improved varieties that could be grown under high pathogen pressure, reducing the yield losses caused by MSD and achieving acceptable productivity and fruit quality [5]. Moreover, identifying sources of resistance within the lemon-like germplasm, and, more generally, in other citrus species, is essential for the introgression of resistance genes into lemon commercial cultivars as a part of a long-term strategy.

In this study, we analyzed the behavior of a germplasm field collection, which mostly comprises lemons, in response to *P. tracheiphilus* natural infections by visual observation of symptoms and detection by real-time PCR. The objectives of the present study were (i) the identification of sources of MSD tolerance or resistance in the lemon and lemon-like germplasm by comparison of several clones and hybrids grown in the same field block under the same high pathogen pressure; (ii) the successful application of a fast and reliable method to detect *P. tracheiphilus* in natural infection conditions;

and (iii) the identification of sources of resistance in other citrus species that could be used to introgress resistance genes into lemon interspecific hybrids.

## 2. Materials and Methods

### *2.1. Plant Material and Phenotyping*

Phenotyping started in 2018, at the CREA (Research Centre for Olive, Fruit and Citrus Crops) germplasm collection of Acireale (Catania, Italy; 37°37′23″ N, 15°09′50″ E). The original collection was planted in 2002. Plants were grafted onto the sour orange (*C.* × *aurantium* L. var. *aurantium*), in which lemon clones were replicated three times, and the other genotypes were replicated two times. The plants were grown with standard cultural practices, allowing comparative evaluation of the MSD symptoms under similar natural pathogen pressure.

The studied germplasms included 1 citron clone, 27 lemon clones, 15 lemon and citron hybrids (most of them of unknown parentage), and 7 varieties belonging to other citrus species. The list of analyzed accessions and their reported parentage is included in Table 1. Information regarding yield and fruit quality of 18 of the 27 lemon clones was previously reported by Di Vaio et al. [42].

Phenotyping was carried out through a visual screening, in four different periods, for three consecutive years, when the symptoms were more pronounced. Field evaluation was always performed by the same personnel. The wood of desiccated or defoliated twigs was examined for pink salmon discoloration, which is typical of MSD infection (Figure 1A), by removing the bark.

Phenotyping also included measurement of the canopy volume of each tree, because pruning was routinely performed to remove infected branches since the establishment of the collection field, influencing the canopy development of the most susceptible trees. Canopy volume was measured at the end of the last vegetative flush each year and was approximated as one-half prolate spheroid with the following formula [43]:

$$V = 4/6\pi h(d/2)^2 \quad (1)$$

where h is the tree height, and d is the tree diameter.

For each survey, symptom severity was scored according to an empirical scale based on the following assigned values:

0 = no symptoms—the plant did not show any twigs or branches with symptoms (Figure 1B);
1 = few symptoms—fewer than 5 twigs had visible symptoms (Figure 1C);
2 = medium symptoms—more than 5 twigs had visible symptoms (Figure 1D);
3 = strong symptoms—all branches had visible symptoms (Figure 1E);
4 = dead plant (Figure 1F).

**Table 1.** List of accessions phenotyped for mal secco disease (MSD) susceptibility at the CREA experimental farm of Acireale, Italy. Botanical names refer to the latest proposal of taxonomical classification by Ollitrault et al. [44]. Asterisks in the description of the citrus species refer to the species parentage, as revealed by Curk et al. [23] (*) and Wu et al. [45] (**).

| Common/Cultivar Name | Description | Origin | Botanical Name | Reference |
|---|---|---|---|---|
| 'Adamo VCR' lemon | Shoot tip grafted clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [46] |
| 'Akragas' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [47] |
| 'CNR L58' lemon | Shoot tip grafted clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [48] |
| 'Cerza' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [46] |
| 'Continella M84' lemon | Shoot tip grafted clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [31] |
| 'Dosaco M503' lemon | Shoot tip grafted clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [31] |

**Table 1.** *Cont.*

| Common/Cultivar Name | Description | Origin | Botanical Name | Reference |
|---|---|---|---|---|
| 'Fino VCR' lemon | Shoot tip grafted clonal selection | Spain | *C.* × *limon* var. *limon* (L.) Burm. f. | [46] |
| 'Erice' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [47] |
| 'Interdonato' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [24] |
| 'Kamarina' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [47] |
| 'Lo Porto' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [49] |
| 'Mascali seedless' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | unknown |
| 'Ovale di Sorrento' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | unknown |
| 'Pink Fleshed' lemon | clonal selection | USA | *C.* × *limon* var. *limon* (L.) Burm. f. | [49] |
| 'Quattrocchi' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [30] |
| 'Femminello-S' lemon | Nucellar callus of ‚Femminello' lemon, selected in vitro for tolerance to *P. tracheiphilus* toxin | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [50] |
| 'Scandurra' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | unknown |
| 'Segesta' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [47] |
| 'Selinunte' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [47] |
| 'Sfusato Amalfitano' lemon | clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [49] |
| 'Siracusano 2Kr' lemon | Lemon obtained from nucellus from irradiated fruits by CO60 | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [51] |
| 'Zagara Bianca M79' lemon | Shoot tip grafted clonal selection | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [31] |
| '46515' tetraploid lemon | Autotetraploid Femminello lemon | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [52] |
| '46245' tetraploid lemon | Autotetraploid Femminello lemon | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [52] |
| '46321' tetraploid lemon | Autotetraploid Monachello lemon | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | [52] |
| 'Doppio' tetraploid lemon | Autotetraploid lemon | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | unknown |
| 'Doppio Lentini' tetraploid lemon | Autotetraploid lemon | Italy | *C.* × *limon* var. *limon* (L.) Burm. f. | unknown |
| 'Vozza Vozza' | Lemon × pummelo hybrid | Italy | *C.* × *lumia* | [53] |
| 'India CRC 2476' rangpur lime | Mandarin × citron hybrid *, ** | India | *C.* × *limonia* Osbeck var. *Limonia* | [49] |
| 'India CRC 2322' lemon hybrid | lemon hybrid of unknown parentage | India | *C.* × *limonia* Osbeck var. *Limonia* | [49] |
| Volkamer lemon | Mandarin × citron hybrid * | Italy | *C.* × *limonia* var. *volkameriana* Pasquale | [49] |
| 'Fantastico' bergamot | Sour orange × lemon hybrid * | Italy | *C.* × *limon* var. *bergamia* ined. | [49] |
| 'Femminello' bergamot | Sour orange × lemon hybrid * | Italy | *C.* × *limon* var. *bergamia* ined. | [49] |
| 'Cardinale' | Lemon × pummelo hybrid | Italy | *C.* × *lumia* | [53] |
| 'Spatafora' | Lemon × citron hybrid | Italy | *C.* × *limon* | [53] |
| 'Incomparabile' | Sour orange × citron hybrid | Italy | *C.* × *lumia* | [53] |
| 'Mangiagli lemon | lemon hybrid of unknown parentage | Italy | *C.* × *lumia* | unkown |
| 'Palestinian' sweet lime | (Pummelo × mandarin) × citron * | India | *C.* × *limon* var. *limettioides* ined. | [49] |
| 'Limetta romana' sweet lime | Sour orange × citron hybrid * | India | *C.* × *limon* var. *limetta* ined. | unkown |
| 'Corrugated red lime' rangpur lime | Mandarin × citron hybrid *, ** | India | *C.* × *limonia* Osbeck var. *limonia* | [49] |
| 'Ponderosa lemon' | Pummelo × citron hybrid | Italy | *C.* × *lumia* var. *pyriformis* ined. | [53] |
| Sour orange | pummelo × mandarin F1 hybrid *, ** | Italy | *C.* × *aurantium* L. var. *aurantium* | [49] |
| 'ISA' Clementine | Clonal selection of clementine (Mandarin x sweet orange **) | Italy | *C.* × *aurantium* var. *clementina* ined. | [54] |
| 'Khasi' papeda | Wild nonedible citrus species | India | *C. latipes* | [55,56] |
| 'Tachibana' | Wild nonedible mandarin ** | Taiwan | *C. reticulata* var. *tachibana* ined. | [45] |
| 'Changshou' kumquat | Considered to be a chance hybrid between two *Fortunella* species | Japan | *Fortunella spp.* | [57] |
| 'Doppio Sanguigno' orange | clonal selection of Sweet orange (pummelo × mandarin complex hybrid *) | Italy | *C.* × *aurantium* var. *sinensis* L. | [58] |
| 'Chandler CRC 3224' pink pummelo | Hybrid of Siamese Pink pummelo and Siamese Sweet pummelo | USA | *C. maxima* (Burm.) Merr. | [49] |
| 'Siamelo CRC 2586' tangelo | King tangor × grapefruit | USA | *C.* × *aurantium* var. *Tangelo* | [49] |
| 'Diamante' citron | Citron cultivar | Italy | *C. medica* | [23,49] |

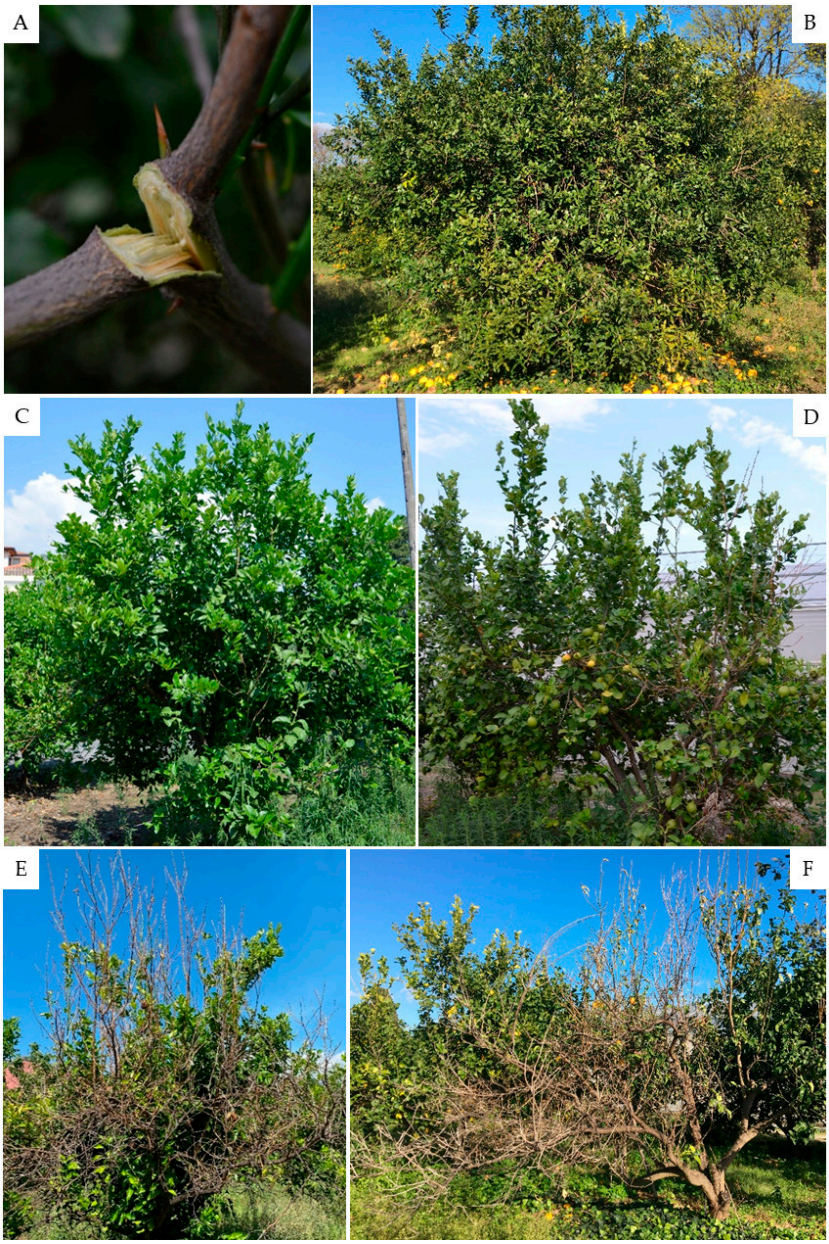


**Figure 1.** Citrus plants of the CREA germplasm showing different MSD symptoms: (**A**) infected shoot shows a yellow or pink-salmon to reddish discoloration of the wood; (**B**) a plant of Khasi papeda that shows no symptoms of MSD, scored with 0; (**C**) a plant of Mascali seedless lemon that shows few symptoms of MSD, scored as 1; (**D**) a plant of Zagara Bianca M79 lemon that shows medium symptoms of MSD, scored as 2; (**E**) a plant Femminello Dosaco M503 lemon that shows strong symptoms of MSD, scored as 3; (**F**) a plant of Akragas lemon that died of MSD, scored as 4.

### *2.2. DNA Extraction*

Samples of plant tissues for real-time PCR analysis were collected in the four cardinal directions, for each plant, in July 2018 and July 2019. For each tree, three types of samples were collected: one consisted of bulks of 10 young leaves (less than 6 months), one consisted of bulks of 10 mature leaves (6–12 months), and one consisted of 5 twigs, for a total of 9 samples per accession (3 biological replicates per tissue type). When only two plants per accession were present, the third biological replicate consisted of bulks of tissues from the two plants. For accessions with one or two replicates that were missing due to plant death, the samples were taken from the survivor plant to obtain nine samples from each accession. A total of 828 samples were collected from the 84 surviving plants.

All samples were first surface-sterilized in a solution of 2.5% sodium hypochlorite and then washed twice with sterile distilled water. Ten grams of each sample was homogenized, using liquid nitrogen, and less than 0.1 g was collected for DNA extraction [18]. The *P. tracheiphilus* Pt10 strain (kindly provided by Professor Vittoria Catara, Di3A, University of Catania) was cultured for DNA isolation, as a reference for the real-time PCR experiments. The fungus was cultured for 10 days, at 21 °C ± 2 °C, in Petri dishes containing potato dextrose agar medium. One hundred micrograms of mycelium were harvested with a sterile loop from the surface of the colony, placed into an Eppendorf tube, frozen at −80 °C, and homogenized with a grinder (TissueLyser—Qiagen, Hilden, Germany). DNA extraction of both plant and fungal tissues was performed by the CTAB method, as described in Caruso et al. [59], with slight modifications. Briefly, tubes containing 0.1 g of powdered plant tissues were mixed with 400 µL of extraction buffer (2% CTAB, 20 mM EDTA, 1.44 mM NaCl, 100 mM Tris HCl, pH 8) and 0.1% β-mercaptoethanol. Samples were vortexed and incubated at 65 °C, for 60 min, agitating for the first 5 min. After adding 300 µL of chloroform-isoamyl alcohol (24:1), the vials were vortexed for 15 s and finally centrifuged at 20,800× *g* for 10 min. The supernatant was recovered, 500 µL of 100% ethanol was added and incubated at −20 °C, for at least 30 min, or at 4 °C, overnight, followed by centrifugation at 20,800 *g* for 10 min. The pellet was rinsed with 1000 µL of 70% ethanol, resuspended in 50 µL of sterile distilled water, and stored at 4 °C until analysis. The quality and concentration of the isolated DNA were measured by using a Nanodrop 2000 spectrophotometer (Thermo Scientific™, Waltham, MA, USA). The 260/280 and 260/230 ratios were approximately 1.80 and 2.20, respectively, and the concentrations ranged from 50 to 300 ng $\mu L^{-1}$. All the samples were diluted at 10 ng $\mu L^{-1}$.

### *2.3. Real-Time PCR Analysis*

Real-time PCR amplifications were performed according to the protocol described by Licciardello et al. [16], using GR70 forward primer (5′-GATCCGTACGCCTTGGGGAC-3′), GL1 reverse primer (5′-AGAAGCGTTTGGAGGAGAGAATG-3′), and the probe PP1 (5′-FAM-CACGCAATCTTGGCGACTGTCGTT-TAMRA-3′). Each sample was amplified, using the following mix: 2X real-time PCR master mix (TaqMan™ Fast Advanced Master Mix Applied Biosystems™), 200 nM forward primer, 200 nM reverse primer, 100 nM fluorogenic probe, and 40 ng/µL genomic DNA. Negative controls, using water in place of DNA, were routinely included. Amplifications were carried out in an ABI 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA), using the following program: 50 °C for 2 min, 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s and 62 °C for 30 s.

Calibration of the standard curve for fungal DNA quantification by real-time PCR was assessed by using *P. tracheiphilus* DNA (100 µg $mL^{-1}$) extracted from the Pt10 strain and serially diluted in sterile distilled water, as described in Licciardello et al. [16].

### *2.4. Statistical Analysis*

To perform the comparison of means, a value of 40 was assigned to all runs where *P. tracheiphilus* was non-detectable. The correlation between the five variables measured (severity symptom scores, Ct value from young leaves, Ct values from old leaves, Ct values from twigs, and canopy volume) was performed, using Spearman's method, at the 95% confidence level. Statistical analysis and the analysis of correlation among the variables were performed, using R software, version 3.6.3 [60], using the packages "corrplot" [61] and "corrgram" [62].

## 3. Results and Discussion

### *3.1. Field Phenotyping*

In the present study, we evaluated the disease responses of 50 accessions belonging to the *Citrus* and *Fortunella* genera to natural MSD infections. The main purpose of this work was the identification of sources of tolerance and resistance in lemons and in other citrus species and hybrids. Information

is essential to planning lemon-breeding programs based on hybridization and somaclonal variation, to generate new varieties with improved tolerance to the disease. The field trial was conducted in an area where the environmental conditions are particularly favorable to the disease. The field trial originally planted in 2002 included 50 accessions replicated two or three times, for a total of 123 plants. The accessions initially included in the field are listed in Table 1. Some plants died from MSD within the first years after planting [46], and were replanted in 2009 (Table 2). At the end of the survey (May 2020), just 84 plants belonging to 46 accessions survived. Specifically, 79 plants were the original plants (16 years old), and five were the replanted replicates (nine years old). All the trees of the following selections died before the beginning of the survey: Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon, and Diamante citron. Attempts to replant them, to re-constitute the original collection, were made, but plants died again due to MSD, confirming their high susceptibility. The rest of the missing plants were replicates of other lemon clonal selections or susceptible citrus accessions, as indicated in Table 2.

Visual screening was performed four times, starting from May 2018, for three consecutive years, to check the behavior of each plant in response to 18 years of natural infection and to follow the possible progression of the disease during the three years of observations.

The scores used for the estimation of symptom severity ranged between 0 (absence of symptoms) and 4 (plant death). Score 4 was also assigned to the replicates that died of MSD before May 2018 or during the visual screening. The mean scores of symptom severity recorded in the three years are shown in Table 2. Several accessions showed no symptoms and had a score of 0 (Table 3). This group includes only a true lemon, Quattrocchi, a clone very similar to Monachello, already known for its high tolerance to the disease. Other accessions with citron ancestry, such as Palestinian sweet lime, Ponderosa lemon, and Incomparabile, showed no symptoms, indicating that it is theoretically possible to generate mal secco–resistant lemon-like phenotypes through hybridization. In this group, the only accession not included in the genus *Citrus* was Changshou kumquat, which differs from the most common kumquat (*Fortunella margarita*) in its rounded shape. Interestingly, sour orange also had a score of 0. This species is reported as susceptible [2,5] and is often used to evaluate the pathogenicity of the *P. tracheiphilus* strains at the seedling stage [18,63–67]; however, we observed no symptoms during the three years of evaluation.

The genotypes with very few symptoms that had a score less than 1 were Spatafora (*C* × *limon*), Cardinale (*C.* × *lumia*), Vozza Vozza (*C.* × *lumia*), and Fantastico bergamot (*C.* × *limon* var. *bergamia* ined.). These genotypes are all lemon or citron hybrids and show a high tolerance to the disease. The accessions India CRC 2476' rangpur lime (*C.* × *limonia* Osbeck var. *limonia)*, 'India CRC 2322' lemon (*C.* × *limonia* Osbeck var. *limonia)*, Volkamer lemon (*C.* × *limonia* var. *volkameriana* Pasquale), corrugated red lime rangpur lime (*C.* × *limonia* Osbeck var. *limonia),* Femminello bergamot *(C.* × *limon* var. *bergamia* ined.), and limetta romana sweet lime (*C.* × *limon* var. *limetta* ined.) showed a range of symptoms, with a score from 2 to 3. These genotypes are all citron hybrids and showed susceptibility to MSD. Some lemon clones revealed field tolerance, such as Continella M84, Segesta, Interdonato, Zagara Bianca M79, Lo Porto, and Mascali seedless, with scores ranging between 0.6 and 2. Other lemon clones with scores between 2 and 3 were Femminello S, Kamarina, Dosaco M503, Selinunte, Scandurra, Sfusato Amalfitano, Ovale di Sorrento, Pink Fleshed, CNR L58, and Akragas. Higher scores among the two lemon clones were assigned to Erice and Cerza, with 3.3 and 3.4, respectively. Finally, the highest score was assigned to three lemons, Siracusano 2Kr, Adamo VCR, and Fino VCR, and citron Diamante, with a 0% survival rate before the beginning of the survey, which can be considered the most susceptible to the disease.

**Table 2.** Results of MSD germplasm phenotyping based on real-time PCR, visual observation of symptoms, and canopy volumes. Values of the real-time PCR refer to the mean values of the three replicates sampled in 2018 and the three replicates of 2019 approximated to the nearest integer. Values of symptoms represent the average of four scores recorded between May 2018 and May 2020. The table shows the number of original plants, the number of replicates replanted in 2009, and the number of surviving plants at the end of the survey. The list also includes the accessions that died of MSD before the beginning of the survey.

| Cultivar | Ct Value Young Leaves | | Ct Value Mature Leaves | | Ct Value Twigs | | Symptom Severity Scores | Average Canopy Volume ($M^3$) | Number of Original Plants (2002) | Replanted Replicates (2009) | Number of Dead Plants (May 2020) | Number of Surviving Plants (May 2020) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2018 | 2019 | 2018 | 2019 | 2018 | 2019 | | | | | | |
| 'Adamo VCR' lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| 'Akragas' lemon | 23 | 37 | 34 | 36 | 40 | 28 | 2.66 | 16.64 | 3 | 0 | 2 | 1 |
| 'CNR L58' lemon | 36 | 38 | 38 | 38 | 28 | 30 | 3.00 | 11.80 | 3 | 0 | 1 | 2 |
| 'Cerza' lemon | 35 | 39 | 36 | 38 | 25 | 27 | 3.16 | 8.28 | 3 | 3 | 5 | 1 |
| 'Continella M84' lemon | 40 | 40 | 31 | 29 | 34 | 34 | 1.83 | 41.67 | 3 | 0 | 1 | 2 |
| 'Dosaco M503' lemon | 35 | 37 | 38 | 36 | 28 | 34 | 2.25 | 14.71 | 3 | 1 | 2 | 2 |
| 'Fino VCR' lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| 'Erice' lemon | 36 | 38 | 37 | 33 | 26 | 40 | 2.33 | 6.69 | 3 | 3 | 5 | 1 |
| 'Interdonato' lemon | 30 | 18 | 34 | 32 | 28 | 30 | 1.12 | 14.03 | 2 | 0 | 1 | 1 |
| 'Kamarina' lemon | 34 | 40 | 36 | 38 | 25 | 31 | 2.16 | 22.00 | 3 | 0 | 1 | 2 |
| 'Lo Porto' lemon | 40 | 36 | 30 | 32 | 28 | 38 | 0.50 | 8.51 | 2 | 0 | 0 | 2 |
| 'Mascali seedless' lemon | 21 | 23 | 23 | 25 | 20 | 30 | 1.50 | 14.90 | 2 | 1 | 1 | 2 |
| 'Ovale di Sorrento' lemon | 34 | 32 | 29 | 31 | 22 | 30 | 3.16 | 7.28 | 3 | 0 | 1 | 1 |
| 'Pink Fleshed' lemon | 38 | 40 | 37 | 37 | 32 | 34 | 3.00 | 21.75 | 2 | 0 | 1 | 1 |
| 'Quattrocchi' lemon | 34 | 40 | 34 | 40 | 32 | 40 | 0 | 11.00 | 2 | 0 | 0 | 2 |
| 'Femminello-S' lemon | 40 | 36 | 38 | 34 | 25 | 31 | 3.16 | 2.98 | 3 | 0 | 2 | 1 |
| 'Scandurra' lemon | 40 | 34 | 38 | 36 | 23 | 35 | 3.16 | 25.88 | 3 | 0 | 2 | 1 |
| 'Segesta' lemon | 34 | 40 | 28 | 36 | 30 | 34 | 0.66 | 20.74 | 3 | 0 | 0 | 3 |
| 'Selinunte' lemon | 28 | 40 | 40 | 36 | 30 | 34 | 2.83 | 75.63 | 3 | 0 | 2 | 1 |
| 'Sfusato Amalfitano' lemon | 38 | 40 | 39 | 37 | 30 | 32 | 3.16 | 6.35 | 3 | 0 | 2 | 1 |
| 'Siracusano 2Kr' lemon | - | | - | | - | | 4.00 | 0.00 | 3 | 3 | 6 | 0 |
| 'Zagara Bianca M79' lemon | 31 | 39 | 40 | 38 | 23 | 31 | 2.75 | 16.08 | 3 | 0 | 1 | 2 |
| '46515' tetraploid lemon | 33 | 39 | 34 | 38 | 29 | 27 | 1.00 | 19.31 | 2 | 1 | 1 | 2 |
| '46245' tetraploid lemon | 33 | 35 | 28 | 30 | 26 | 30 | 1.87 | 11.48 | 2 | 1 | 2 | 1 |
| '46321' tetraploid lemon | 38 | 40 | 38 | 40 | 38 | 38 | 0 | 5.65 | 2 | 0 | 0 | 2 |
| 'Doppio' tetraploid lemon | 37 | 39 | 36 | 38 | 38 | 38 | 0 | 7.27 | 2 | 0 | 0 | 2 |
| 'Doppio Lentini' tetraploid lemon | 37 | 39 | 38 | 40 | 37 | 39 | 0 | 36.82 | 2 | 0 | 0 | 2 |
| 'Vozza Vozza' | 32 | 36 | 40 | 40 | 35 | 35 | 0.11 | 5.69 | 3 | 0 | 0 | 3 |
| 'India CRC 2476' rangpur lime | 29 | 33 | 36 | 36 | 33 | 35 | 2.87 | 5.03 | 2 | 1 | 2 | 1 |
| 'India CRC 2322' lemon | 31 | 33 | 40 | 40 | 33 | 35 | 2.87 | 5.30 | 2 | 0 | 1 | 1 |

**Table 2.** *Cont.*

| Cultivar | Ct Value Young Leaves | | Ct Value Mature Leaves | | Ct Value Twigs | | Symptom Severity Scores | Average Canopy Volume ($M^3$) | Number of Original Plants (2002) | Replanted Replicates (2009) | Number of Dead Plants (May 2020) | Number of Surviving Plants (May 2020) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2018 | 2019 | 2018 | 2019 | 2018 | 2019 | | | | | | |
| Volkamer lemon | 36 | 38 | 34 | 38 | 31 | 33 | 2.00 | 9.32 | 3 | 0 | 0 | 3 |
| 'Fantastico' bergamot | 34 | 36 | 34 | 36 | 39 | 39 | 0.62 | 33.91 | 3 | 0 | 0 | 3 |
| 'Femminello' bergamot | 30 | 34 | 32 | 34 | 35 | 37 | 2.12 | 12.00 | 2 | 0 | 1 | 1 |
| 'Cardinale' | 36 | 38 | 35 | 35 | 37 | 37 | 0.12 | 7.19 | 2 | 0 | 0 | 2 |
| 'Spatafora' | 36 | 36 | 37 | 37 | 36 | 38 | 0.25 | 16.39 | 2 | 0 | 0 | 2 |
| 'Incomparabile' | 34 | 36 | 32 | 36 | 37 | 39 | 0 | 1.61 | 2 | 0 | 0 | 2 |
| 'Mangiagli lemon | 40 | 40 | 40 | 40 | 38 | 40 | 0 | 19.16 | 2 | 0 | 0 | 2 |
| 'Palestinian' sweet lime | 36 | 38 | 37 | 37 | 38 | 38 | 0 | 22.20 | 2 | 0 | 0 | 2 |
| 'Limetta romana' sweet lime | 30 | 36 | 33 | 35 | 30 | 34 | 3.00 | 1.34 | 2 | 0 | 1 | 1 |
| 'Corrugated red lime' rangpur lime | 40 | 40 | 40 | 40 | 35 | 37 | 2.00 | 17.38 | 2 | 0 | 0 | 2 |
| 'Ponderosa lemon' | 37 | 37 | 40 | 40 | 38 | 40 | 0 | 18.85 | 3 | 0 | 0 | 3 |
| Sour orange | 40 | 40 | 40 | 40 | 37 | 39 | 0 | 7.39 | 3 | 0 | 0 | 3 |
| 'ISA' Clementine | 40 | 40 | 40 | 40 | 38 | 40 | 0 | 9.29 | 2 | 0 | 0 | 2 |
| 'Khasi' papeda | 38 | 38 | 38 | 38 | 38 | 38 | 0 | 33.21 | 2 | 0 | 0 | 2 |
| 'Tachibana | 40 | 40 | 40 | 40 | 37 | 37 | 0 | 1.64 | 3 | 0 | 0 | 3 |
| 'Changshou' kumquat | 40 | 40 | 40 | 40 | 34 | 38 | 0 | 2.32 | 2 | 0 | 0 | 2 |
| 'Doppio Sanguigno' orange | 39 | 39 | 40 | 40 | 38 | 38 | 0 | 13.55 | 2 | 0 | 0 | 2 |
| 'Chandler CRC 3224' pink pummelo | 33 | 37 | 34 | 38 | 35 | 37 | 0 | 1.75 | 2 | 0 | 0 | 2 |
| 'Siamelo CRC 2586' tangelo | 40 | 40 | 40 | 40 | 40 | 40 | 0 | 28.93 | 2 | 0 | 0 | 2 |
| 'Diamante' citron | - | | - | | - | | 4.00 | 0.00 | 2 | 2 | 4 | 0 |

Among the autotetraploid lemon clones (Doppio Lentini, Doppio, 46321, 46245, and 46515), we generally noticed a high tolerance or the absence of symptoms, with some differences. Specifically, no symptoms were found in Doppio Lentini, Doppio, and 46321 (probably Monachello 4×) during the three years of visual monitoring, while few infected branches were observed in 46245 and 46515. In addition to symptom observation, we measured the canopy volume of all surviving plants, not as an indication of plant vigor, but as an additional parameter to describe the sensitivity of each accession to MSD. The canopy volume can be drastically reduced by pathogen attack and by pruning infected branches. Indeed, pruning is one of the few effective measures to contain the spread of the disease in lemon orchards. In our survey, we analyzed many different citrus species, and a lower canopy volume of some accessions was due to the different growth habits and not necessarily to MSD infections (Table 2). Specifically, some citrus species, such as Chandler pink pummelo or tachibana, showed a very low canopy volume in the absence of MSD symptoms, probably because they are poorly adapted to the growing environment. Consequently, we found no general correlation (r = 0.01) between canopy volume and symptoms when analyzing the whole dataset (Figure 2). However, a higher correlation between canopy volume and symptoms (r = −0.40) was found when the comparison was limited to the lemon clonal selections (Figure 3). The accessions that had lower canopy volumes, such as CNR L58 lemon, Erice lemon, and Cerza lemon, were generally the ones that underwent severe pruning due to the presence of more symptoms. All the most susceptible clones had canopy volumes below 10 $m^3$, whereas many tolerant clones showed values ranging from 11.00 and 75.63 $m^3$, with some exceptions, such as Doppio, 46321, and Lo Porto.

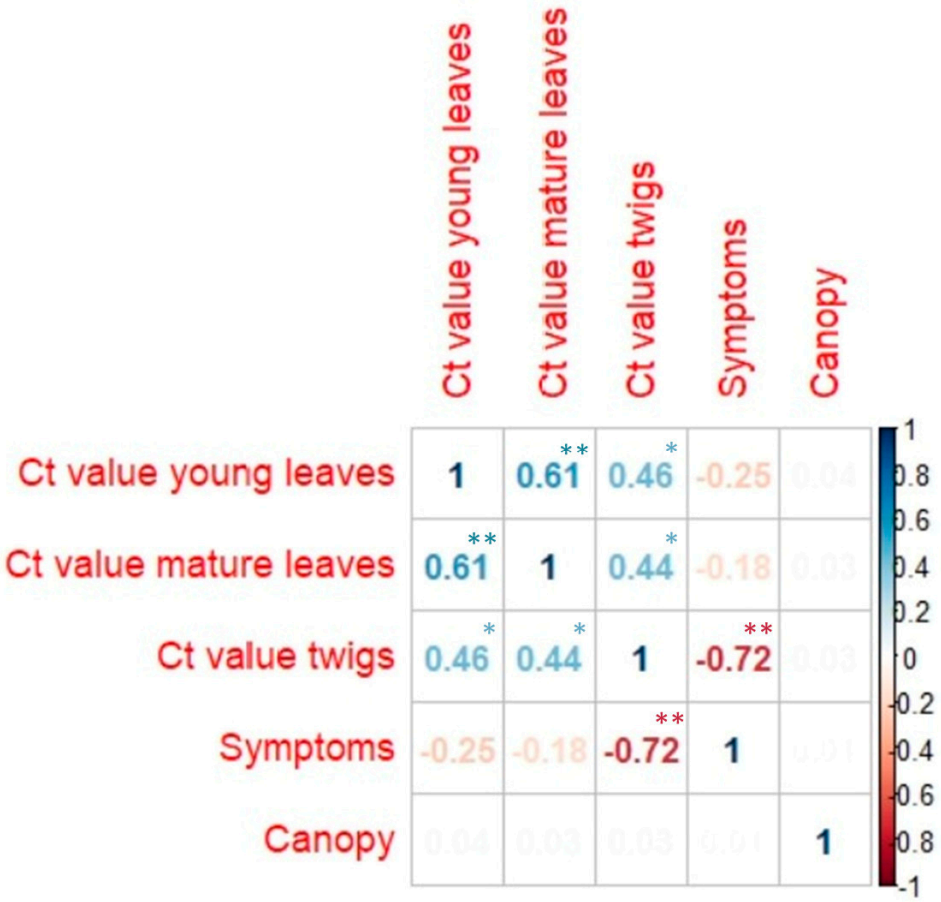


**Figure 2.** Pairwise correlation matrix of five traits measured in the CREA germplasm. Blue numbers represent positive correlations, and red ones represent negative correlations. Faded numbers correspond to very low correlation values (<0.1). Correlation values were statistically significant with the following *p*-values: * <0.05 and ** <0.001. Values without asterisks were not statistically significant.

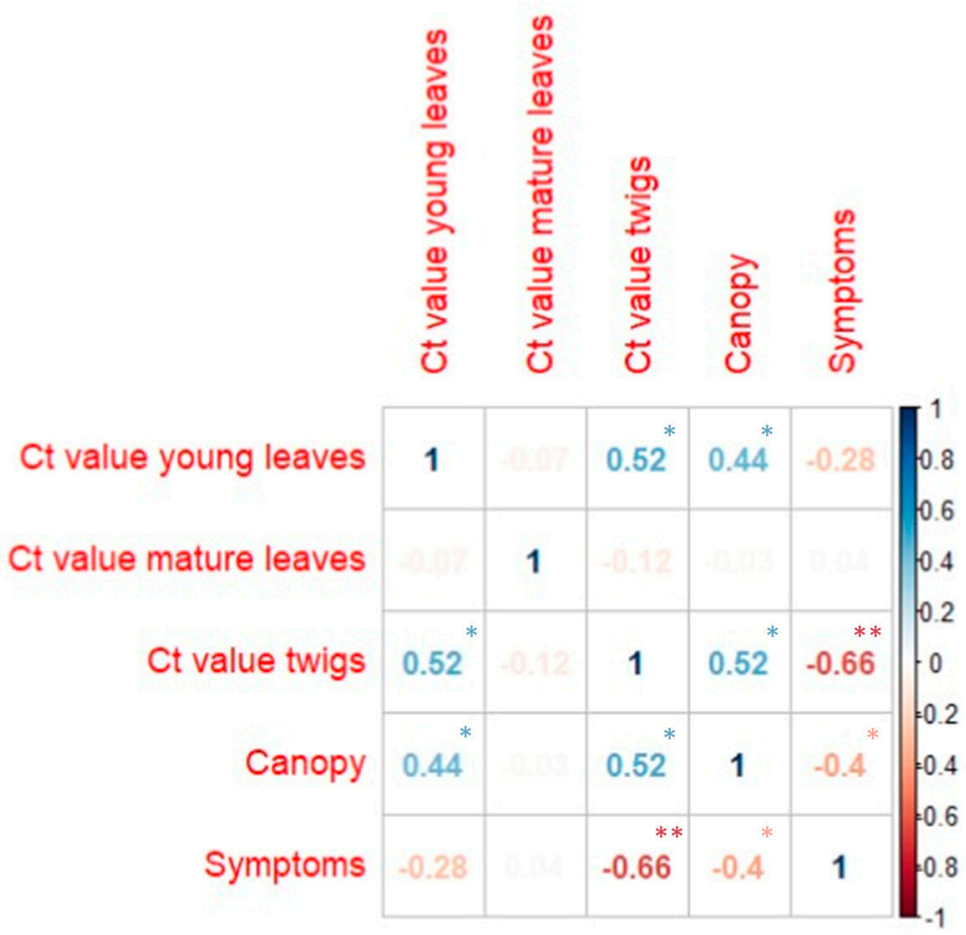


**Figure 3.** Correlation index between the five variables of the lemon clones in the CREA germplasm. Blue numbers represent positive correlations, and red ones represent negative correlations. Faded numbers correspond to very low correlation values (<0.1). Correlation values were statistically significant with the following *p*-values: * <0.05 and ** <0.001. Values without asterisks were not statistically significant.

### 3.2. Real-Time PCR Detection of *P. tracheiphilus*

In addition to visual phenotyping, we performed molecular screening, to obtain a more exhaustive and reliable assessment of the *P. tracheiphilus* infection in all replicate trees of the germplasm collection, especially in the absence of clear symptoms. Sample collections for DNA isolation were conducted in the first week of July, because symptoms usually appear during spring and early summer [2]. Molecular detection was performed by using different types of tissues (twigs, young leaves, and mature leaves), amplifying a genomic region of the fungus by real-time PCR, as reported by Licciardello et al. [16]. In our experiment, Ct values ranged from 22 to 39 in young leaves, from 24 to 39 in mature leaves, and from 17 to 39 in DNA from twigs. Licciardello et al. [16] reported that the minimum amount of pathogen DNA that could be quantified accurately by using real-time PCR was 1 pg, corresponding to a Ct value of 37.93. Therefore, Ct values above 38 cannot reflect the occurrence of *P. tracheiphilus* infection (Table 2).

Leaf samples were included because the pathogen is able to penetrate through leaf wounds, so this survey could be potentially useful to identify early infections. However, the correlation between leaf Ct values and symptom scores was generally weak (Figure 2), and, in some cases, it was not useful to detect infections that were clearly visible in parts of the canopy, such as in the lemon clones CNR L58, Kamarina, Sfusato Amalfitano, Pink fleshed, and Cerza.

DNA samples from twigs were the most effective for *P. tracheiphilus* detection. The real-time PCR analysis of twig samples confirmed the presence of *P. tracheiphilus* in all genotypes where the symptoms

were present. Furthermore, the molecular analysis detected *P. tracheiphilus* in xylem tissues of many accessions where no symptoms were present from any of the phenotyping data. This phenomenon occurred in Quattrocchi lemon and Chandler pink pummelo. These cases may include plants that were infected recently, so that symptoms were not yet visible or plants that showed some tolerance and that were able to block the movement of the pathogen and recover from the disease. In many replicates, the pathogen was detected only in the twigs and not in leaves, such as in Vozza Vozza, India CRC 2322, Corrugated red lime, Sour orange, ISA clementine, Tachibana, and Changshou Kumquat. Moreover, Ct values from twigs showed a high correlation ($r = -0.72$; $p$-values $< 0.001$) with the symptom scores (Figure 2). In particular, they ranged between 17 and 30 in the susceptible clones showing field symptoms, while they were higher (Ct value > 30) in tolerant clones and hybrids. Figure 4 shows the relationship between symptoms and Ct values in twigs and provides a view of the different degrees of susceptibility to MSD observed in the germplasm. The most susceptible accessions are in the lower right part of the plot, while the field tolerant or resistant accessions are grouped in the upper left part. Specifically, the right side of the plot includes all the lemon clones with the exceptions of Quattrocchi and Segesta, which are in the upper left side, grouped with some tetraploid lemons (Doppio Lentini and Doppio, 46321), different citron and lemon hybrids (Vozza Vozza, Cardinale, Incomparabile, Spatafora, and Palestinian sweet lime), and other citrus species that are resistant to MSD (Khasi papeda, Doppio Sanguigno, Mangiagli lemon, Chandler pink pummelo, and ISA clementine).

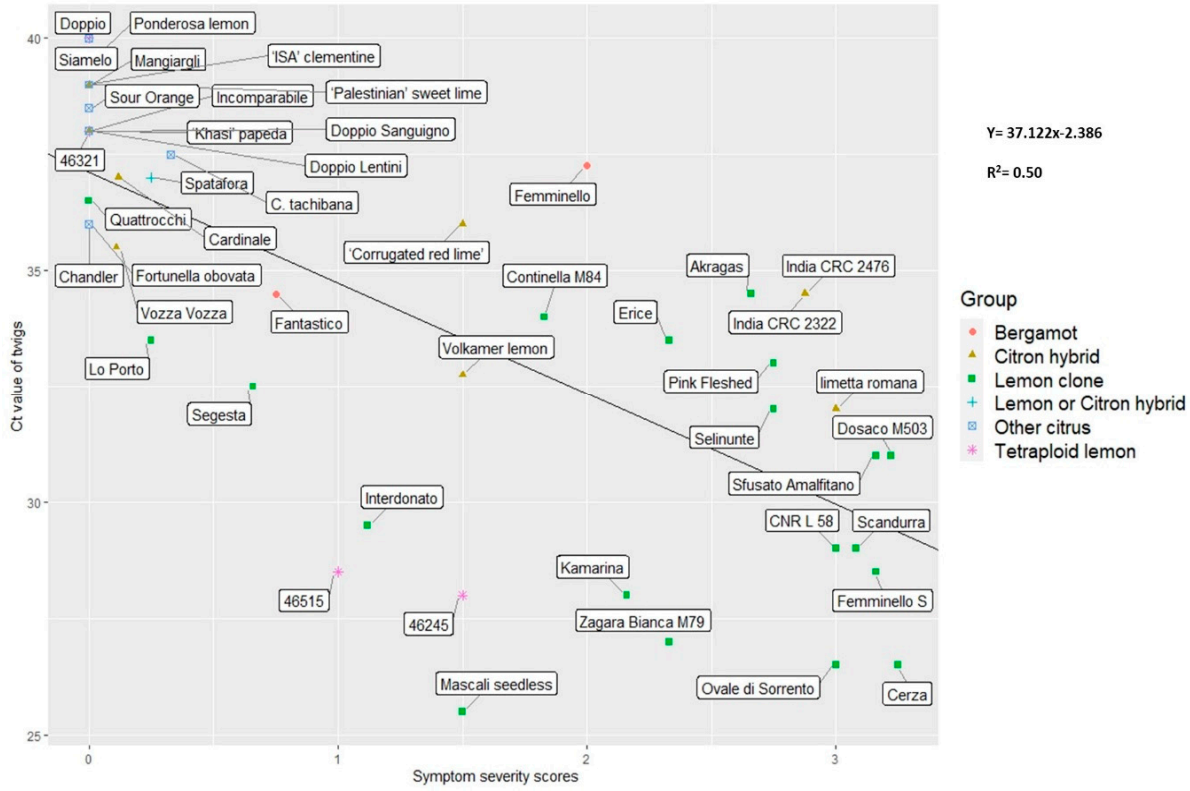


**Figure 4.** Scatterplot with regression line showing the relationships between Ct value of twigs and symptoms of the germplasm collection of CREA considered in this study.

Significant correlations were also found analyzing the subset of the lemon clonal selections between Ct values of twigs and symptoms ($r = -0.66$; $p$-values $< 0.001$; Figure 3), and between Ct values of twigs and canopy volumes ($r = 0.52$; $p$-values $< 0.05$; Figure 3). A scatterplot revealing the relationship Ct values of twigs and canopies is shown in Figure 5.

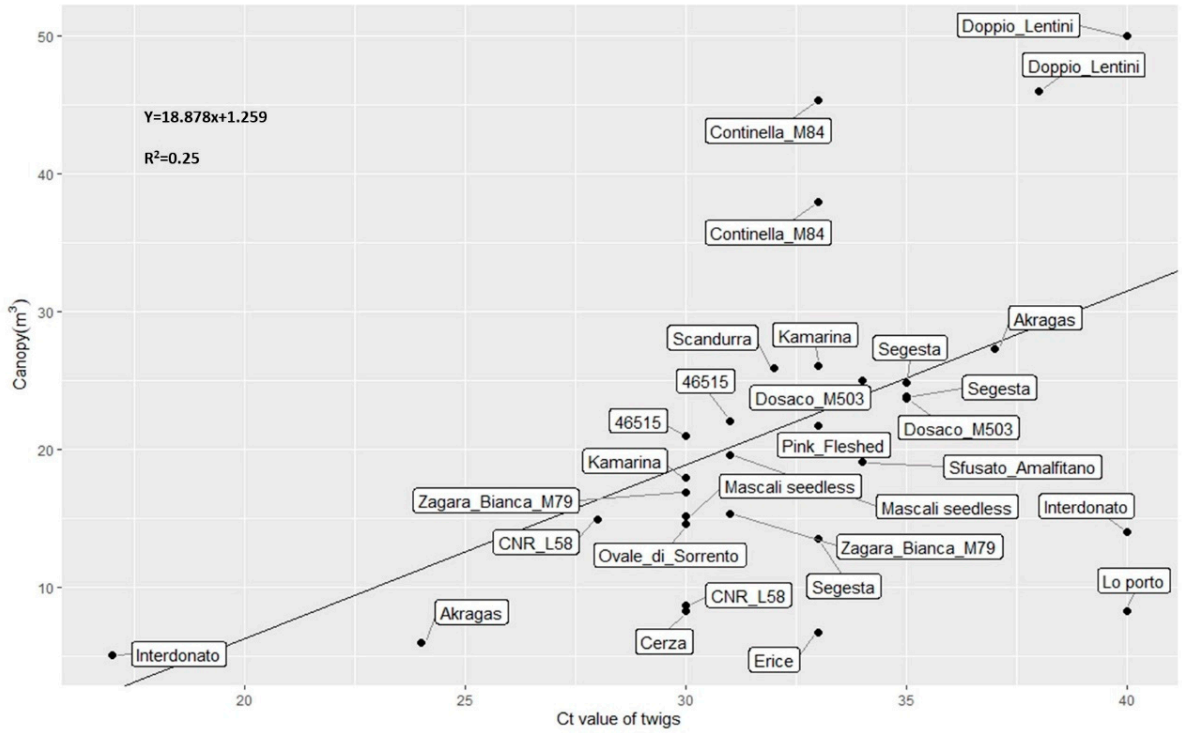


**Figure 5.** Scatterplot with regression line showing the relationships between canopy of each surviving replicate of the lemon clonal selections and Ct values of twigs.

### *3.3. Assignment of the Accessions to Disease-Severity Groups*

Based on the symptom-severity scores, canopy volumes, and real-time PCR results, we assigned the analyzed accessions to seven different disease-severity groups.

For determining disease groups, we considered the complete absence of the pathogen (immunity), the cases of very limited pathogen movement in the xylem with no visible symptoms (field resistance), the presence of very few symptoms with the ability of the plant to recover from infections (field tolerance), and successful colonization of the pathogen leading to clear disease symptom expression and, in some cases, to plant death (susceptibility). The groups and the list of accessions assigned to each group are listed in Table 3. Pictures of plants representative of each severity group are included in Supplementary Figures S1 to S6.

**Table 3.** List of disease-severity groups based on visual observations, real-time PCR results of twig samples and canopy volumes, and accessions assigned to each group.

| Disease Severity Group. | Accessions |
| --- | --- |
| Group 1: the most susceptible accessions, all plants died. | Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon and Diamante citron. |
| Group 2: susceptible to MSD. Very severe symptoms, some replicates died. | Erice and Cerza. |
| Group 3: medium to severe symptoms, two of the three original replicates died of MSD. In some cases, a slight recovery of the plants during the three years of observation was recorded. | Akragas lemon, Femminello S, Selinunte, Dosaco M503, Sfusato Amalfitano, Scandurra, Ovale di Sorrento, Mascali seedless, 46245, Pink Fleshed, Limetta romana India CRC 2476, India CRC 2322 and Femminello bergamot. |
| Group 4: tolerant to the MSD, different range of symptoms from mild to severe, but real-time PCR showed Ct values between 30 and 31. | Continella M84 Lemon, Interdonato lemon, CNR L58 lemon, Zagara Bianca M79 lemon, Kamarina lemon, Lo Porto lemon, 46515 tetraploid lemon, Corrugated red lime and Volkamer lemon. |
| Group 5: high tolerance to MSD, and very few symptoms were detected during the visual screening. Mean real-time PCR Ct value of 32. All replicates planted in 2002 are still alive. | Segesta. |
| Group 6: very few symptoms during the field phenotyping. Real-time PCR mean Ct values between 35 and 37. | Chandler pink pummelo, Fantastico bergamot, Vozza Vozza and Cardinale. |
| Group 7: no symptoms in the field, mean Ct value > 37. | Doppio Lentini tetraploid lemon, 46321 tetraploid lemon, Doppio tetraploid lemon, Palestinian sweet lime, sour orange, Khasi papeda, ISA Clementine, Doppio Sanguigno orange, Ponderosa lemon, Tachibana, Changshou kumquat, Quattrocchi lemon, Siamelo CRC 2586 tangelo, Spatafora, Incomparabile and Mangiagli lemon. |

Group 1: In this group, we included the most susceptible accessions. Specifically, the lemon cultivars Siracusano 2Kr lemon, Adamo VCR lemon, Fino VCR lemon, and Diamante citron died before the beginning of the survey. Reforgiato Recupero and colleagues [46] reported that MSD was the cause of death of all replicates of the original field, and later attempts to replace the dead plants were not successful, since the new plants died again, due to MSD.

Group 2: Accessions in this category are susceptible to MSD and were also planted twice. Very severe symptoms were found on all plants, and some replicates died. This group includes the lemon clones Erice and Cerza.

Group 3: The accessions in this group showed a different range of symptoms, from medium to severe, and two of the three original replicates died of MSD. In some cases, a slight recovery of the plants during the three years of observation was recorded. This group includes Akragas lemon, Femminello S, Selinunte, Dosaco M503, Sfusato Amalfitano, Scandurra, Ovale di Sorrento, Mascali seedless, 46245, Pink Fleshed, Limetta romana India CRC 2476, India CRC 2322, and Femminello bergamot.

Group 4: This group includes accessions that can be considered tolerant to the disease, namely Continella M84 Lemon, Interdonato lemon, CNR L58 lemon, Zagara Bianca M79 lemon, Kamarina lemon, Lo Porto lemon, 46515 tetraploid lemon, and Corrugated red lime. The plants showed a different range of symptoms, from mild to severe, but real-time PCR showed medium levels of the pathogen, with Ct values between 30 and 31. Moreover, their canopy volume is generally higher than the accessions included in the previous groups, confirming their ability to tolerate the disease and guarantee canopy growth. This group also includes Volkamer lemon. This species was reported to exhibit a medium level of susceptibility by Russo [38], while other reports described it as highly susceptible [35,68]. We also observed different responses among the three replicates, with one healthy plant with very limited symptoms and the other two with severe dieback and reduced canopy volume.

Group 5: In this group, we can find just a lemon clone, Segesta. It showed high tolerance to the disease, and very few symptoms were detected during the visual screening. The plants had a high

canopy volume, and real-time PCR confirmed the low level of infections in twigs, with a mean Ct value of 32. All replicates planted in 2002 are still alive.

Group 6: In this group, we were able to detect very few symptoms during the field phenotyping, but a low level of the fungus was detected periodically by real-time PCR (mean Ct values were between 35 and 37). Under field conditions, the pathogen could not be established in these hosts. The accessions are Chandler pink pummelo, Fantastico bergamot, Vozza Vozza, and Cardinale.

Group 7: This group includes all the accessions where the pathogen was detected in very low quantities, with a Ct value > 37, and the plants did not show any symptoms in the field. Therefore, these accessions showed resistance in the field conditions of natural pathogen pressure. The accessions in this group are Doppio Lentini tetraploid lemon, 46321 tetraploid lemon, Doppio tetraploid lemon, Palestinian sweet lime, sour orange, Khasi papeda, ISA Clementine, Doppio Sanguigno orange, Ponderosa lemon, Tachibana, Changshou kumquat, Quattrocchi lemon, Siamelo CRC 2586 tangelo, Spatafora, Incomparabile, and Mangiagli lemon. The possible resistance of these accessions needs confirmation on a larger number of replicates, since some of these genotypes, such as a mandarin hybrid or sweet orange, showed sporadically mild infections; however, the pathogen caused the typical symptoms of "mal nero", a form of the disease where the fungus enters the plant through the roots [69–71]. Sour orange is reported to be very sensitive to the disease [2,5,30,34,35,72,73], but in our study, no symptoms or pathogens were detected by phenotyping or real-time PCR, respectively. This might be due to different degrees of susceptibility to different clonal selections. Some sour orange clones are reported as being resistant to MSD, as already confirmed by Reforgiato Recupero [74] and Nigro [75]. It is also well-known that plant age is a determinant of susceptibility, since adult plants are more tolerant than young seedlings [5].

## 4. Conclusions

This survey was useful to discriminate many citrus accessions belonging to true and derived species based on their field tolerance to MSD. Many accessions were found to be immune or resistant to the disease under natural pathogen pressure, but a broad degree of tolerance was also observed. Several degrees of field tolerance cannot be explained by a single gene involved in the resistance [38]. The presence of many genes involved in host–pathogen interaction was also supported by Reforgiato Recupero et al. [76].

We found that DNA isolation from twigs, coupled with real-time PCR detection, is a reliable method for field phenotyping. This method could be routinely used to validate phenotyping of mapping populations or germplasm collections, to better understand the genetic basis of MSD resistance.

A putative source of resistance was found in Doppio Lentini (autotetraploid lemon) and 46321 (probably a tetraploid Monachello), since no symptoms were found during the three years of visual monitoring, and no pathogen was detected by real-time PCR analysis. This resistance seems not to be exclusively related to tetraploidy [77], since other autotetraploids included in the phenotyping, namely 46515 and 46245, showed clear symptoms confirmed by real-time PCR detection.

This survey was also useful to identify sources of resistance among other citrus species that could be used to introgress resistance genes into the lemon genome. Therefore, based on the phenotyping results, two monoembryonic mal secco-resistant species, namely Khasi papeda and Clementine, were chosen as female parents and crossed with Femminello Siracusano 2Kr, a very susceptible lemon clone, to create two populations that might be helpful in the future for studying the segregation of MSD susceptibility and for identifying candidate genes and QTLs associated with the disease.

**Supplementary Materials:** The following materials are available online, at http://www.mdpi.com/2073-4395/10/11/1806/s1. Pictures of plants representative of each disease severity group (Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, and Figure S6).

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# De Novo Transcriptome Sequencing of Rough Lemon Leaves (*Citrus jambhiri* Lush.) in Response to *Plenodomus tracheiphilus* Infection

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**Abstract:** Mal secco is one of the most severe diseases of citrus, caused by the necrotrophic fungus *Plenodomus tracheiphilus*. With the main aim of identifying candidate genes involved in the response of citrus plants to "Mal secco", we performed a de novo transcriptome analysis of rough lemon seedlings subjected to inoculation of *P. tracheiphilus*. The analysis of differential expressed genes (DEGs) highlighted a sharp response triggered by the pathogen as a total of 4986 significant DEGs (2865 genes up-regulated and 2121 down-regulated) have been revealed. The analysis of the most significantly enriched KEGG pathways indicated that a crucial role is played by genes involved in "Plant hormone signal transduction", "Phenylpropanoid biosynthesis", and "Carbon metabolism". The main findings of this work are that under fungus challenge, the rough lemon genes involved both in the light harvesting and the photosynthetic electron flow were significantly down-regulated, thus probably inducing a shortage of energy for cellular functions. Moreover, the systemic acquired resistance (SAR) was activated through the induced salicylic acid cascade. Interestingly, RPM1 interacting protein 4, an essential positive regulator of plant defense, and BIR2, which is a negative regulator of basal level of immunity, have been identified thus representing useful targets for molecular breeding.

**Keywords:** *Plenodomus tracheiphilus*; *Citrus jambhiri*; rough lemon; mal secco; RNAseq; de novo assembly; SAR



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## 1. Introduction

Citrus, one of the most important fruit crops in the world, is sensitive to many environmental stresses of both abiotic and biotic nature, often leading to poor tree growth and reductions in fruit yield and quality [1]. "Mal secco" disease (MSD) is a severe vascular disease of citrus caused by the mitosporic fungus *Plenodomus tracheiphilus* (Petri) Gruyter, Aveskamp and Verkley (syn. *Phoma tracheiphila* (Petri) Kantschaveli and Gikashvili). It appeared in the second half of 19th century (1894) in Chios and Poros, two Greek Aegean islands, from which it derived its first name ("Poros's disease"). In Italy, MSD was first reported in 1918 in the district of Messina (eastern Sicily), probably following the introduction of infected plants from Greece [2]. The current geographical distribution of MSD comprises the east coast of the Black Sea (Georgia) and mainly all citrus-growing countries of the Mediterranean Basin, except for Morocco and Portugal [3].

The MSD pathogen infects mainly lemon (*Citrus limon* (L.) Burm. F.) [4]. Citron (*C. medica* L.) and other citrus species and hybrids having citron or lemon as parent, such as

lime (*C. aurantifolia* Christ.), bergamot (*C. bergamia* Risso), Volkamer lemon (*C. volkameriana* Ten. et Pasq.), Alemow (*C. macrophylla* Wester), and rough lemon (*C. jambhiri* Lush) are also particularly susceptible to the disease [4,5]. Rough lemon is counted among the most "mal secco" susceptible species [6]. *C. jambhiri* Lush is native to northeastern India and is a mandarin × citron F1 natural hybrid [7]. Due to fruit typology, as the name implies, characterized by a very coarse exterior, it is unsuitable as a scion cultivar but it has been widely used in many countries as a rootstock [8].

The distinct symptomatology of the disease, characterized by desiccation of twigs, branches, or the whole plant, suggested its extant name "mal secco" meaning "dry disease" [9,10], a denomination ever since adopted internationally [5]. The first symptoms of the disease usually appear in spring on the leaves of the uppermost shoots, which display a slight discoloration of the primary and the secondary veins [11,12]. The leaves then turn yellow or sometimes brown and fall. Newly infected shoots show a yellow or pink-salmon to reddish discoloration of the wood, which occurs also in the wood of the main and secondary branches, as well as in the trunk, where the pathogen is advancing. A progressive basipetal desiccation of shoots, branches, and trunk follows and, finally, the whole plant may die [13]. Glycoproteins of 93 KDa and 60 KDa (called Pt60) belonging to the malseccin complex have been isolated from culture filtrates and host plants infected by *P. tracheiphilus* [14–16]. Both were able to reproduce all the symptoms of the disease when injected into different plants [16]. The toxic effects of the malseccin complex on citrus leaves are clearly visible only under illuminated conditions, suggesting that light plays a role in the toxin activity. In light conditions, the induction and formation of reactive oxygen species (ROS) can damage cellular structures as ROS induce lipid membrane peroxidation leading to the loss of membrane integrity, electrolyte leakage, and cell death. Oxidative stress in plant pathology has been a general subject of investigation and its ability to drive the metabolism of both host and pathogen during their interaction has been demonstrated [17]. It has been shown that the synchronous presence of hydrolytic enzymes, toxic compounds, oxidative stress inducers, and membrane transporters in the fungus, and the differential ability to modulate the lipoperoxidative pathway in the host can play a central function in *P. tracheiphilus* infection of *C. limon* [18].

The knowledge at the molecular level of the mechanisms that occur in plant–pathogen interaction, not only in tolerant but also in susceptible interactions, is the basis for the development of innovative tools for phytosanitary control and that may lead to eco-sustainable interventions to minimize or replace the massive use of agro-pharmaceuticals. Gene expression profiling by RNA-Seq provides an unprecedented high-resolution view of the global transcriptional landscape. A primary objective of many gene expression experiments is to detect transcripts showing differential expression across various conditions. In this context, next-generation high-throughput sequencing techniques have become an increasingly useful tool for exploring whole plant genomes, providing a means for analyzing plant molecular regulatory mechanisms in specific abiotic and biotic stress conditions. The identification of candidate genes is a prerequisite for the application of new genome editing techniques by which targeted genetic modifications can lead to the introduction of precise changes directly into the genome of commercial varieties, offering an alternative to traditional methods of genetic improvement [19–21]. Different authors in the last years conducted transcriptomic analysis to better understand *Citrus* plants response to biotic stress caused by pathogens [22–27]. Specifically, a study evaluated the transcriptional reprogramming of both rough lemon and sweet orange leaf tissue during the asymptomatic stage of infection caused by *Candidatus* Liberibacter asiaticus. Functional analysis of the differentially expressed genes (DEGs) indicated that genes involved in the mitogen activated protein kinase (MAPK) signaling pathway involving WRKY transcription factors were highly upregulated in rough lemon. Among the most biologically relevant transcripts in the gene set enrichment analysis were those related to several functional categories suggesting that DEGs with different functions were subjected to reprogramming. Therefore, using global transcriptome analysis approach, both a wide range of candidate genes and

information that could be useful for genetic engineering to control Huanglongbing disease have been identified [25]. Considering the impact of mal secco in the Mediterranean citrus industry, the aim of this work was to unravel the transcriptomic reprogramming of a highly susceptible citrus species subjected to *P. tracheiphilus* infection by applying a de novo sequencing and assembly RNAseq approach. This is the first report concerning the transcriptome analysis of a susceptible Citrus species challenged by the causal agent of "mal secco" disease.

## 2. Results

### 2.1. Effect of Plenodomus tracheiphilus Infection on Citrus jambhiri Phenotype and Fungus Detection

The effectiveness of fungal inoculation was evaluated by both visual inspection of inoculated leaves and by detection of fungus genome by Taqman real time PCR. As shown in Figure S1A, the typical symptoms consisting of the midrib and main vein chlorosis were detected 15 days after inoculation. All the inoculated plants were chlorotic on the adaxial leaf surface (Figure S1B); that chlorosis symptom is different from the aforementioned vein chlorosis and more specifically indicates that a pathogen-induced micronutrient deficiency has occurred. As expected, the untreated plants appeared healthy (Figure S1B). As described in [28], quantitative detection of *P. tracheiphilus* was performed by real-time PCR assay. The fungus was detected in inoculated rough lemon plants, whereas no fluorescence emission was detected in the case of DNA extracted from healthy samples as well as from negative control (NTC, inoculated with water) (Table S1). The standard curve for fungal DNA quantification gave a coefficient of determination $R^2 = 0.98$ (data not shown).

### 2.2. Transcript Assembly and Annotation

In this work, a comprehensive identification of the transcriptional response of rough lemon (*Citrus jambhiri* Lush) to *P. tracheiphilus* infection was carried out by RNAseq approach (see the experimental design in the "Material and Methods" section). The quality of RNA samples has been assessed before libraries preparation by RIN measurement. The mean RIN value was 8.2 (Table 1) indicating that very low level of RNA degradation occurred and that it was suitable for further downstream analysis.

**Table 1.** Summary statistics of the RNA quality and sequencing results.

| Parameter | Value |
|---|---|
| Average RIN | 8.2 |
| Clean reads | 228 million |
| N° of transcripts | 115,100 |
| N° of Unigenes | 77,631 |
| Average of read mapped rate | 83.40% |
| Transcripts N50 (bp) | 2372 |
| Unigenes N50 (bp) | 2060 |
| Q30 (%) | 92.82 |
| GC content (%) | 44.22 |

After library construction and sequencing, reads containing adapters or reads of low quality were removed by filtering the raw reads, so that the downstream analyses are based on a total of 228 million clean reads with an average of ~38 million reads (~11.4 G) per sample, the average percentage of Q30 and GC being 92.8% and 44.2%, respectively. De novo assembly of clean reads resulted in 115,100 transcripts and 77,631 unigenes with N50 length of 2372 and 2060, respectively (Table 1), indicating that a good coverage of the transcriptome had been achieved. The assembly consistency was evaluated by mapping back the filtered unique reads to the final assembled leaf transcriptome and the average read mapping rate using the alignment software Bowtie2 was 83.40%. Both transcript and unigene length distributions are reported in Figure S2. These data showed that

the throughput and sequencing quality were high enough to warrant further analysis. To achieve comprehensive gene functional annotation, all assembled unigenes were blasted against public databases, including National Center for Biotechnology Information (NCBI), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), SwissProt, Ortholog database (KO), and Gene Ontology (GO) (Figure 1). The 80.89% of the obtained total unigenes were annotated in at least one searched database. Among them, 72.93% and 78.25% assembled unigenes showed identity with sequences in the Nr and Nt databases, respectively. The percentage of assembled unigenes homologous to sequences in KO, KEGG, Swiss-Prot, Pfam, GO, and KOG databases were 27.13%, 15.36%, 53.35%, 24.52%, 15.53%, and 23.59%, respectively (Figure 1).

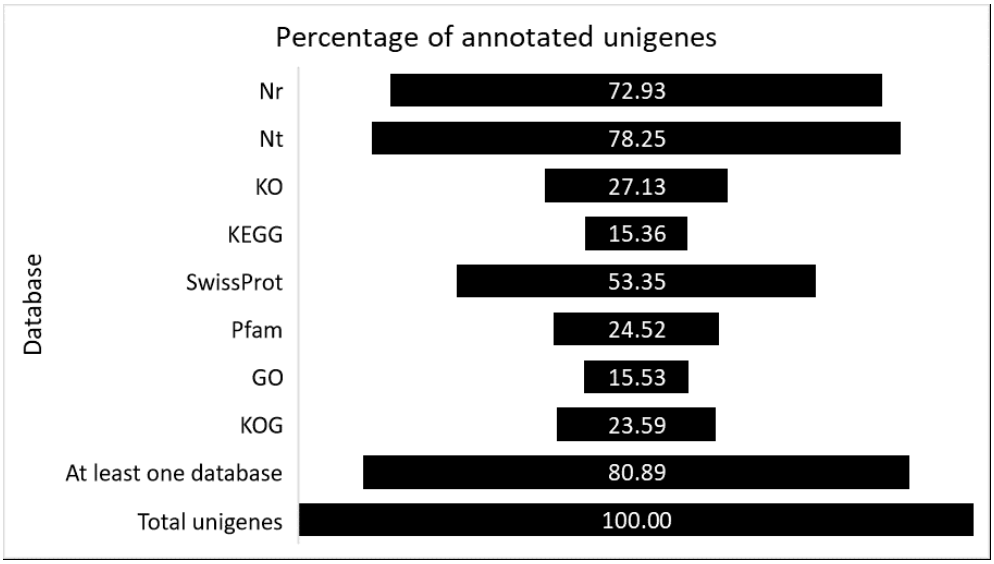


**Figure 1.** The percentage of successful annotated genes in several databases.

### *2.3. Identification of Differentially Expressed Genes (DEGs)*

The unigenes whose expression level changed upon pathogen infection were identified as differentially expressed genes (DEGs) and they were used to characterize the transcriptomic response of *C. jambhiri* to fungal attack. A total of 4986 differentially expressed genes were identified from the comparison *Pt* vs. CK (*P. tracheiphilus* sample set versus control sample set), of which 2865 were up-regulated and 2121 were down-regulated (Figure 2). Validation of expression levels for ten selected DEG candidates was carried out by quantitative real-time PCR (qRT-PCR). The results show high congruence between RNA-Seq results and qRT-PCR (coefficient of determination $R^2 = 0.92$) indicating the reliability of RNA-Seq quantification of gene expression (Figure S3). Therefore, the selected genes could also constitute useful markers of pathogen infection in rough lemon.

### *2.4. Functional Classification of DEGs*

Gene Ontology (GO) terms, Clusters of Orthologous Groups of proteins (KOG) classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway functional enrichments were performed to identify possible biological processes or pathways involved in the response of plant to pathogen. Considering the GO enrichment, "oxidoreductase activity" (GO:0016491) (104 up-regulated and 65 down-regulated), "transmembrane transporter activity" (GO:0022857) (75 up-regulated and 27 down-regulated) and "DNA-binding transcription factor activity" (GO:0003700) (37 up-regulated and 14 down-regulated) are the three most enriched terms in Molecular Function (MF) category, while "transport" (GO:0006810) (86 up-regulated and 36 down-regulated) and "transmembrane transport" (GO:0055085) (70 up-regulated and 27 down-regulated) are the two most enriched terms in Biological Process (BP) category (Figure 3).

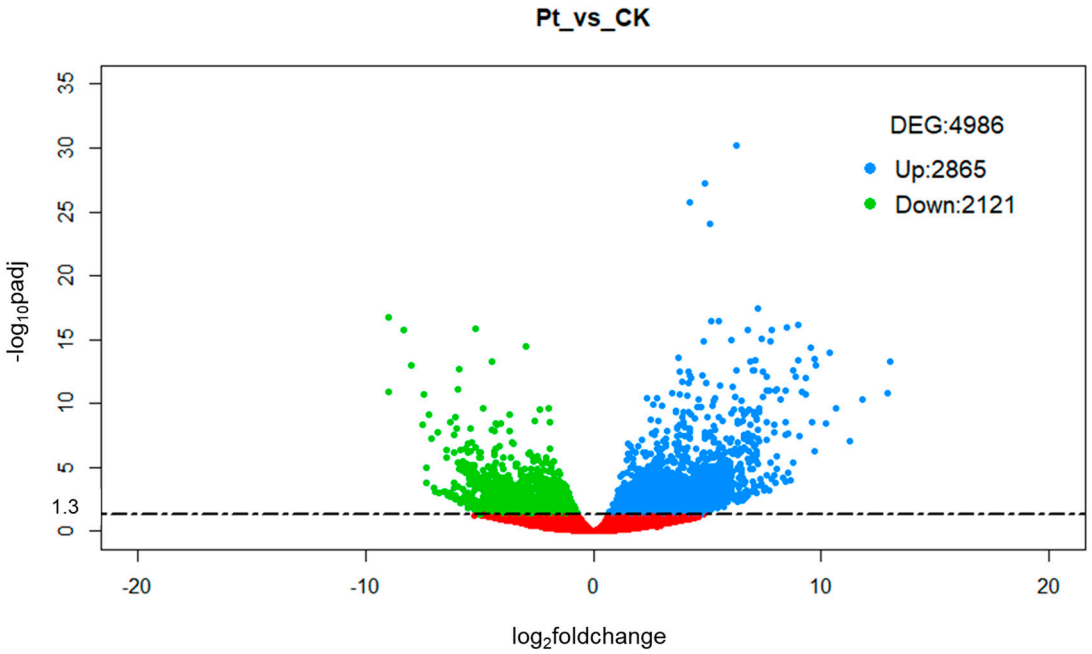


**Figure 2.** Volcano plot showing the DEGs of *Pt* vs. CK comparison. The up-regulated genes with statistically significance are represented by blue dots, the green dots represent the down-regulated genes and the red dots are DEGs with $-\log_{10}$padj < 1.3, adopting $\log_2$FoldChange threshold of 0.58 (1.5 fold change). The X-axis is the gene expression change, and the Y-axis is the *p* value adjusted after normalization.

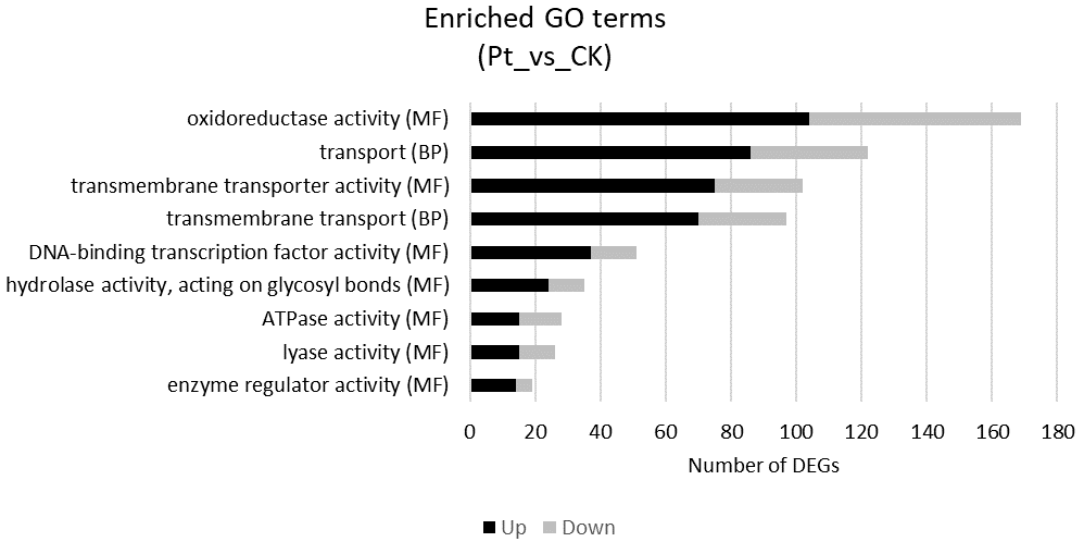


**Figure 3.** Gene Ontology (GO) enrichment analysis for the DEGs in *Citrus jambhiri* (*Pt* vs. CK comparison). The X-axis indicates the numbers related to the total number of GO terms, and the Y-axis indicates the subcategories. BP, biological processes; MF, molecular functions.

To predict and classify possible functions, all the 77,631 unigenes were aligned to the KOG database and were assigned to the KOG categories (Figure S4). Among the KOG categories, the cluster for "General function prediction only" (15.8%) represented the largest group, followed by "Posttranslational modification, protein turnover, chaperones" (12.9%) and "Signal transduction mechanisms" (9.1%). "Translation, ribosomal structure and biogenesis" (7.3%) and "RNA processing and modification" (6.8%) were the largest next categories (Figure S4).

The main KEGG pathway terms were in the "Carbon metabolism" and "Phenylpropanoid biosynthesis" categories, followed by and "Biosynthesis of amino acids" indicating that a deep cellular rearrangement occurred in presence of the fungus (Figure 4). The reprogramming activity of the metabolic pathways is supported by the involvement of other important pathways such as "Plant hormone signal transduction" and "Starch and sucrose metabolism". The strong involvement of "Plant hormone" category in the response to pathogen is also indicated by the presence of different pathways involved in amino acid biosynthesis and metabolism such as "Tyrosine metabolism", "Phenylalanine metabolism", "Phenylalanine, tyrosine and tryptophan biosynthesis", and "Arginine biosynthesis", known to be precursors of plant hormones (Figure 4).

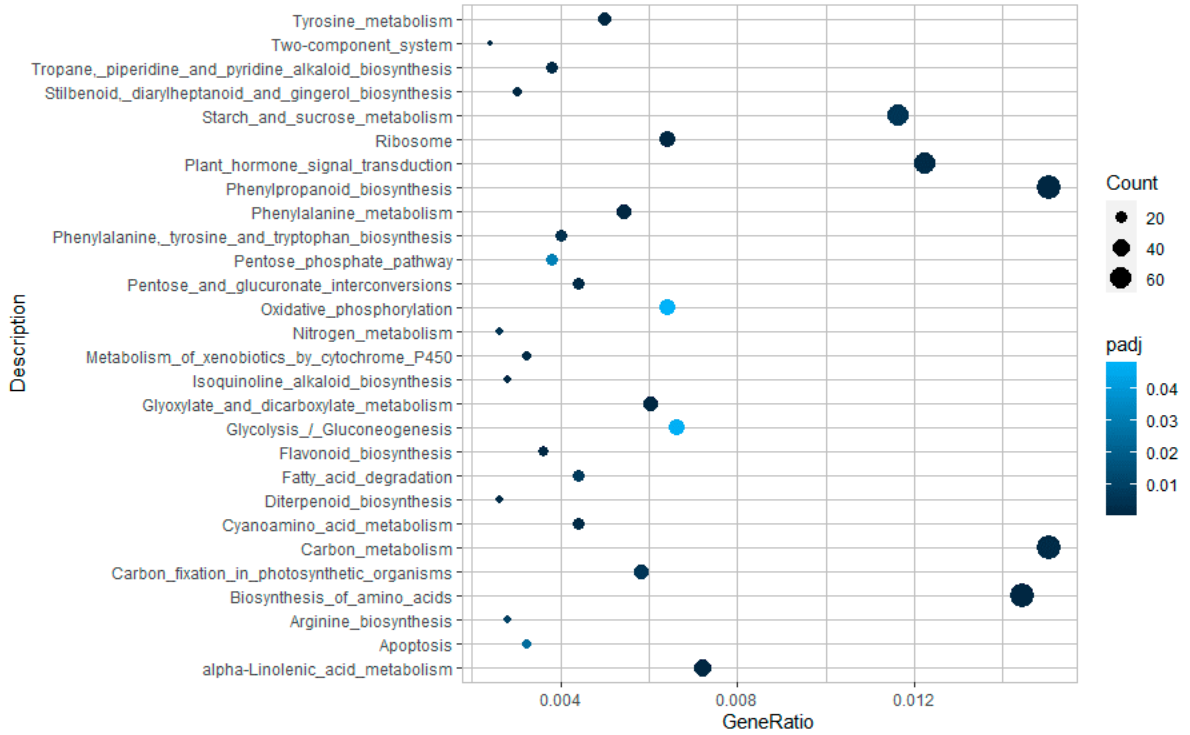


**Figure 4.** Distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for differential expressed genes (DEGs) in the *Pt* vs. CK sample set.

Because of their fundamental involvement of "Plant hormone" (Table 2, Figure S5), "Transcription factors" (Figure 5) and "Defense and pathogenesis" related genes (Table 3) in host–pathogen interaction, we have analyzed them further. The following description of DEGs belonging to the above-mentioned pathways was carried out considering a $\log_2$foldchange threshold of $\pm 2.32$ (corresponding to a fold change = $\pm 5$). In the following tables, the coding sequence of each clusters were identified as orthologs of *A. thaliana* genes (http://plantgdb.org/prj/GenomeBrowser, accessed on 23 November 2020). Congruously, tables report clusters whose % of identity was higher than 50 and the e value < 0.05.

**Table 2.** List of "Plant hormone" related DEGs identified in *Pt* vs. CK comparison.

| Cluster | Symbol | Annotation | TAIR Code | $Log_2$Fold Change | Identity Score | e-Value |
|---|---|---|---|---|---|---|
| | | *Auxin* | | | | |
| 5112,0 | YUC6 | Flavin-binding monooxygenase family protein | AT5G25620 | −4.22 | 69% | $4 \times 10^{-69}$ |
| 15782,1 | AUX1 | Transmembrane amino acid transporter family protein | AT2G38120 | −5.18 | 74% | 0.0 |
| 14701,68946 | TIR1 | F-box/RNI-like superfamily protein | AT3G62980 | −2.62 | 66% | $2 \times 10^{-26}$ |
| 10078,0 | IAA18 | Indole-3-acetic acid inducible 18 | AT1G51950 | −3.18 | 79% | $7 \times 10^{-9}$ |
| 16281,1 | IAA4 | AUX/IAA transcriptional regulator family protein | AT5G43700 | −2.91 | 75% | $3 \times 10^{-63}$ |
| 16862,0 | IAA32 | Indole-3-acetic acid inducible 32 | AT2G01200 | −3.39 | 70% | $3 \times 10^{-20}$ |
| 14701,19495 | IAA7 | Indole-3-acetic acid 7 | AT3G23050 | −5.42 | 83% | $5 \times 10^{-71}$ |
| 16281,0 | IAA3 | AUX/IAA transcriptional regulator family protein | AT1G04240 | −3.61 | 81% | 0.001 |
| 14701,30415 | ARF3 | Auxin-responsive factor AUX/IAA-related | AT2G33860 | +4.30 | 73% | $2 \times 10^{-168}$ |
| 14701,26809 | GH3.3 | Auxin-responsive GH3 family protein | AT2G23170 | +3.42 | 72% | $5 \times 10^{-123}$ |
| 17976,0 | | SAUR-like auxin-responsive protein family | AT2G36210 | +3.41 | 82% | $3 \times 10^{-4}$ |
| | | *Ethylene* | | | | |
| 20624,0 | ACS2 | 1-amino-cyclopropane-1-carboxylate synthase 2 | AT1G01480 | +7.94 | 68% | $2 \times 10^{-131}$ |
| 14701,32226 | ACO1 | ACC oxidase 1 | AT2G19590 | +6.30 | 72% | $9 \times 10^{-133}$ |
| 17499,2 | ACS6 | 1-aminocyclopropane-1-carboxylic acid (acc) synthase 6 | AT4G11280 | +5.09 | 69% | $4 \times 10^{-139}$ |
| 14701,57239 | ETR2 | Signal transduction histidine kinase, hybrid-type, ethylene sensor | AT3G23150 | +3.46 | 66% | $2 \times 10^{-170}$ |
| 14701,58523 | EBF1 | EIN3-binding F box protein 1 | AT2G25490 | +8.86 | 66% | $2 \times 10^{-87}$ |
| 14701,46599 | EIN3 | Ethylene insensitive 3 family protein | AT3G20770 | +7.72 | 76% | 0.0 |
| 6645,0 | ERF1 | Ethylene response factor 1 | AT3G23240 | +3.32 | 72% | $6 \times 10^{-58}$ |
| 14701,24495 | ERF2 | Ethylene responsive element binding factor 2 | AT5G47220 | +2.49 | 76% | $3 \times 10^{-46}$ |
| 14701,21798 | ERF13 | Ethylene-responsive element binding factor 13 | AT2G44840 | +2.53 | 78% | $1 \times 10^{-37}$ |
| 14701,35256 | ERF4 | Ethylene responsive element binding factor 4 | AT3G15210 | +2.42 | 78% | $2 \times 10^{-36}$ |
| 14701,7830 | ERF110 | Ethylene response factor 110 | AT5G50080 | +4.23 | 77% | $6 \times 10^{-28}$ |
| | | *Salicylic acid* | | | | |
| 14701,45136 | PAL1 | Phenylalanine ammonia lyase 1 | AT2G37040 | +3.82 | 74% | 0.0 |
| 14701,67897 | 4CL | 4-coumarate–CoA ligase-like 5 | AT1G51680 | +3.89 | 72% | $1 \times 10^{-30}$ |

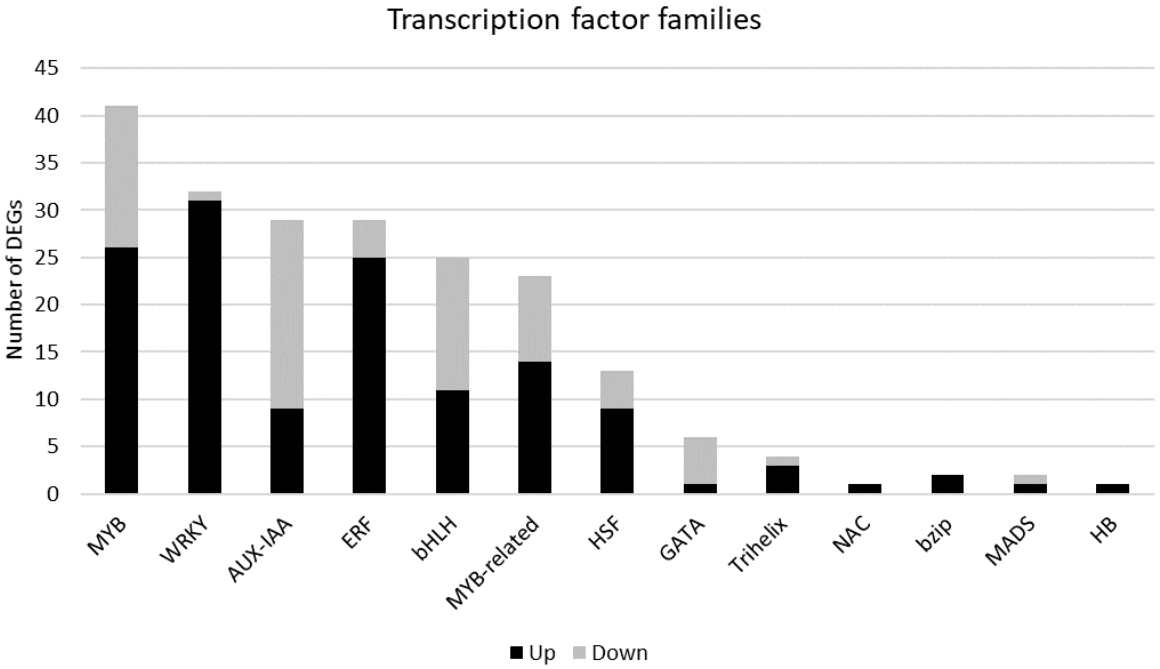


**Figure 5.** Distribution of rough lemon transcription factors responsive to *Plenodomus tracheiphilus* infection. Each bar represents the number of DEGs belonging to a transcription factor family.

**Table 3.** List of defense and pathogenesis related DEGs identified in *Pt* vs. CK comparison.

| Cluster | Symbol | Annotation | TAIR Code | $Log_2$Fold Change | Identity Score | e-Value |
|---|---|---|---|---|---|---|
| | | *Response to pathogen* | | | | |
| 14701,26919 | CPK33 | calcium-dependent protein kinase 33 | AT1G50700 | +3.13 | 67% | $4 \times 10^{-22}$ |
| 14701,66288 | CRCK3 | calmodulin-binding receptor-like cytoplasmic kinase 3 | AT2G11520 | +2.33 | 71% | $5 \times 10^{-59}$ |
| 17682,2 | CML11 | calmodulin-like 11 | AT3G22930 | +3.33 | 76% | $2 \times 10^{-77}$ |
| 14701,33952 | MPK3 | mitogen-activated protein kinase 3 | AT3G45640 | +5.42 | 76% | $2 \times 10^{-28}$ |
| 14701,16139 | MAPKKK15 | mitogen-activated protein kinase kinase kinase 15 | AT5G55090 | +2.49 | 67% | $1 \times 10^{-58}$ |
| 20990,0 | MAPKKK17 | mitogen-activated protein kinase kinase kinase 17 | AT2G32510 | +3.46 | 64% | $1 \times 10^{-33}$ |
| 14701,65619 | CERK1 | chitin elicitor receptor kinase 1 | AT3G21630 | +5.56 | 75% | $3 \times 10^{-18}$ |
| 8490,0 | PR1 | pathogenesis-related gene 1 | AT2G14610 | +4.09 | 68% | $8 \times 10^{-31}$ |
| 14701,26429 | PRB1 | basic pathogenesis-related protein 1 | AT2G14580 | +8.96 | 68% | $2 \times 10^{-32}$ |
| 16905,0 | NPR1 | regulation of innate immune response | | +5.40 | | |
| 13144,0 | NPR1 | *Citrus sinensis* protein NIM1-INTERACTING 3 (LOC107177379) | | +4.21 | 100% | 0.0 |
| 18290,0 | CF-9 | *Citrus clementina* receptor-like protein 9DC3 (LOC18042467) | | +5.75 | 97% | $3 \times 10^{-121}$ |
| 6996,2 | BIR2 | Inactive LRR receptor-like serine/threonine-protein kinase | AT3G47570 | +5.22 | 98% | 0.0 |
| 14701,81960 | CES101 | lectin protein kinase family protein | AT3G16030 | +5.49 | 77% | $7 \times 10^{-11}$ |
| 14701,15619 | IOS1 | Leucine-rich repeat transmembrane protein kinase protein | AT2G19230 | +5.15 | 68% | $2 \times 10^{-16}$ |
| 14701,38537 | EIX2 | *Citrus sinensis* receptor-like protein EIX2 (LOC102609951) | | +4.79 | 80% | 0.0 |
| 14701,79574 | LECRK3 | *Citrus clementina* G-type lectin S-receptor-like serine/threonine-protein kinase (LOC18049964) | | +4.20 | 99% | 0.0 |
| 14701,84653 | RGS1 | G-protein coupled receptors; GTPase activators | AT3G26090 | +4.76 | 70% | $4 \times 10^{-81}$ |
| 14701,13865 | TGA2 | transcription factor TGA2.3 isoform X1 | AT5G06950 | +3.44 | 70% | $5 \times 10^{-102}$ |
| 14701,40930 | BAD1 | Ankyrin repeat family protein BAD1 | AT1G14500 | +3.18 | 75% | $2 \times 10^{-7}$ |
| 19125,1 | RIN4 | RPM1 interacting protein 4 | AT3G25070 | −4.22 | 69% | $2 \times 10^{-14}$ |
| 14701,27598 | RBOHF | respiratory burst oxidase protein F | AT1G64060 | +2.41 | 75% | 0.0 |
| 14701,78394 | RBOHB | respiratory burst oxidase homolog B | AT1G09090 | +6.89 | 72% | $4 \times 10^{-154}$ |
| 14701,77930 | RBOHC | NADPH/respiratory burst oxidase protein D | AT5G51060 | +3.51 | 72% | $1 \times 10^{-8}$ |
| 14701,55000 | RBOHD | respiratory burst oxidase homologue D | AT5G47910 | +2.74 | 72% | 0.0 |
| | | *WRKY transcription factors* | | | | |
| 16089,0 | WRKY35 | WRKY DNA-binding protein 35 | AT2G34830 | +2.39 | 83% | $1 \times 10^{-92}$ |
| 15844,0 | WRKY49 | WRKY DNA-binding protein 49 | AT5G43290 | +2.89 | 77% | $1 \times 10^{-8}$ |
| 14701,6540 | WRKY23 | WRKY DNA-binding protein 23 | AT2G47260 | 2.78 | 76% | $5 \times 10^{-80}$ |
| 14701,12356 | WRKY4 | WRKY DNA-binding protein 4 | AT1G13960 | +2.35 | 77% | $5 \times 10^{-24}$ |
| 21223,0 | WRKY72 | WRKY DNA-binding protein 72 | AT5G15130 | +5.75 | 82% | $7 \times 10^{-87}$ |
| 14701,60912 | WRKY50 | WRKY DNA-binding protein 50 | AT5G26170 | +5.31 | 78% | $3 \times 10^{-36}$ |
| 14701,18458 | WRKY40 | WRKY DNA-binding protein 40 | AT1G80840 | +5.28 | 74% | $5 \times 10^{-27}$ |
| 16962,0 | WRKY75 | WRKY DNA-binding protein 75 | AT5G13080 | +5.07 | 76% | $4 \times 10^{-53}$ |
| 14701,3630 | WRKY71 | WRKY DNA-binding protein 71 | AT1G29860 | +4.71 | 84% | $2 \times 10^{-24}$ |
| 14701,66972 | WRKY18 | WRKY DNA-binding protein 18 | AT4G31800 | +4.34 | 77% | $2 \times 10^{-17}$ |
| 14701,51257 | WRKY70 | WRKY DNA-binding protein 70 | AT3G56400 | +4.03 | 72% | $1 \times 10^{-18}$ |
| 14701,2889 | WRKY44 | WRKY family transcription factor family protein | AT2G37260 | −2.43 | 80% | $2 \times 10^{-43}$ |

#### 2.4.1. Plant Hormone Related Genes

A significant deviation in the expression of genes involved in "Plant hormone" category was observed between the infected and control samples (Table 2, Figure S5). Considering auxin, known to be required for plant growth, the gene encoding one of the main biosynthetic enzymes, such as flavin-binding monooxygenase family protein YUC6 [29] was downregulated as well as the transmembrane amino acid transporter protein (AUX1) and three auxin-responsive IAA proteins (IAA32, IAA7, and IAA3) indicating that auxin biosynthesis and signaling are impaired in the inoculated plants. However, auxin-responsive transcription factors have been found up regulated suggesting that several pathways might be differently regulated. In this study, transcripts encoding several isoforms of the 1-amino-cyclopropane-1-carboxylate synthase, involved in the ethylene biosynthesis, were up-regulated. Moreover, many genes belonging to the ethylene signal transduction pathway and acting downstream of ethylene (signal transduction histidine kinase, hybrid-type, ethylene sensor (ETR2), mitogen-activated protein kinase 1 (MPK1), EIN3-binding F box protein 1 (EBF1/2), ethylene insensitive 3 family protein (EIN3), and ethylene response factor 1 (ERF1/2) were up-regulated (Table 2, Figure S5), clearly indicating an activation

of ethylene signaling which might lead to the inhibition of plant growth and changes in a plant's life cycle. Salicylic acid (SA) is synthesized via the shikimic acid pathway, with chorismic acid serving as an important precursor that can be converted to SA via two distinct branches. In one branch, chorismic acid is converted to SA via phenylalanine and cinnamic acid intermediates by the key enzyme phenylalanine ammonia lyase (PAL). In the other branch, chorismic acid is converted to SA via isochorismic acid by the enzyme isochorismate synthase (ICS1/SID2) [30]. Among the up-regulated transcripts, phenylalanine ammonia-lyase and 4-coumarate-CoA both implicated in one branch of salicylic acid biosynthesis have been found induced in the *Pt* vs. CK comparison. Moreover, genes encoding ICS1 were not among the DEGs suggesting that the main route for salicylic acid biosynthesis under biotic stress in rough lemon is that starting by phenylalanine and catalyzed by PAL.

#### 2.4.2. Transcription Factors

Reprogramming of gene expression upon *P. tracheiphilus* infection is regulated by many transcription factors. In Figure 5 the most represented transcription factor (TF) families in terms of number of DEGs are reported. The results showed that 41 DEGs belong to MYB family (26 up-regulated and 15 down-regulated), 29 to both auxin responsive protein (AUX/IAA) and ethylene-responsive transcription factor (ERF) families, these latter already cited above (*"Plant hormone related genes"* section) indicating that they play a key role in regulating the transcriptional response induced by the pathogenic fungal infection (Figure 5). In addition, 32 genes encoding WRKY transcription factors were among the DEGs, most of which were over-expressed (31 up-regulated and 1 down-regulated). Due to their involvement in plant response to pathogenic fungi infection [31–35] the analysis of their role are included in the following paragraph (Table 3).

#### 2.4.3. Defense and Pathogenesis Related Genes

In Table 3 differentially expressed genes involved in defense mechanisms and pathogenesis are summarized to provide a complete picture of the rough lemon response to pathogen attack. A plethora of genes encoding calmodulin-like protein, calcium-dependent protein kinase, mitogen-activated protein kinase 3, mitogen-activated protein kinase kinase kinase 15, mitogen-activated protein kinase kinase kinase 17, and GTPase activators were up-regulated in the *Pt* vs. CK sample set. These results clearly indicate that fungal infection triggers a wide reprogramming of the cellular signal transduction. Among the DEGs, several leucine rich repeat (LRR) domains, which might have a role as plant resistance (R) genes (IOS1, EIX2, and LECRK3) were up-regulated in the inoculated plants. However, the up-regulation of BIR2, which is negative regulator of basal level of immunity (namely PTI, pathogen-associated molecular patterns triggered immunity) strongly suggests that plant defense is already impaired at this first level [36]. Nevertheless, some of R genes are also known to activate prolonged resistance by inducing phytohormones and pathogenicity related genes (PR genes) that collectively give rise to broad spectrum systemic acquired resistance (SAR) against future infections [37]. Indeed, the members of the pathogenesis-related protein 1 (PR-1) family, which are among the most abundantly produced proteins in plants on pathogen attack, were up-regulated in rough lemon infected plants (Table 3). Concomitantly, genes encoding the positive regulator protein NPR1, which is involved in the induction of defense gene and PR-1 gene expression, and the TGA transcription factor that NPR1 interacts with in the nucleus, were up-regulated in the inoculated plants. These findings suggest that systemic acquired resistance (SAR) mechanism occurred in the rough lemon interaction with the pathogen, probably giving rise to broad-spectrum systemic protection against future infections. According to these results, another signal component of the SAR pathway BAD1, functioning upstream of NPR1 to regulate defense responses, was found to be induced by the pathogen in the *Pt* vs. CK comparison (Table 3). Finally, transcript encoding CERK1 Lysin motif (LysM) receptor kinase that functions as a cell surface receptor in chitin elicitor signaling involved in the resistance to pathogenic

fungi [38] was up-regulated in the infected plants (Table 3). It probably acts by sensing microbe-associated molecular patterns (MAMP) and pathogen-associated molecular patterns (PAMP) as a component of the PTI. Finally, RPM1 interacting protein 4 is an essential regulator of plant defense, which plays a central role in resistance in case of infection; it acts in association with avirulence proteins with which it triggers a defense system including the hypersensitive response (HR) limiting the spread of disease. Interestingly, this transcript was found down-regulated in the inoculated plant (Table 3) suggesting that it might have a role in susceptibility of rough lemon which is not able to avoid the pathogen circulation inside the plant. Transcriptional regulation of defense related genes is crucial for defeating pathogens. The involvement of chitin elicitation that is suggested by the up-regulation of CERK1 appears to play a significant role in plant defense to fungal pathogens through the activity of transcription factors belonging to WRKY family [31]. Different genes encoding for WRKY DNA-binding protein were overexpressed in *C. jambhiri* infected plants. In detail, we observed the up-regulation of *WRKY14*, *WRKY23*, *WRKY49*, *WRKY72*, *WRKY75*, and *WRKY71*. Moreover, *WRKY4*, that is reported to have a positive role in resistance to necrotrophic pathogens [34], *WRKY51*, acts as positive regulator of salicylic acid (SA)-mediated signaling [33], *WRKY40*, *WRKY18,* and *WRKY70* specifically that responds to chitin [31] were also induced by *P. tracheiphilus* attack (Table 3). Finally, in response to pathogen infection, the induction of the calcium-dependent respiratory burst oxidase homologues (RBOHB, RBOHC, RBOHD, and RBOHF), which represent the major sources of ROS production in plants induced by pathogen infection, was observed in inoculated rough lemon plants [39].

#### 2.4.4. Main Processes or Pathways Affected by *P. tracheiphilus* Infection

In order to have a comprehensive view of the metabolic changes occurring in rough lemon infected by *P. tracheiphilus*, all the 4986 significant DEGs were mapped to the MapMan 3.6.0RC1 pathways, and the metabolism overview is shown in the Figure 6. Overall, the analysis indicates that the pathways which are more specifically involved in the response to *P. tracheiphilus* infection are "Reactive oxygen" (both up- and down- regulated genes), "Light reaction" (mostly down-regulated genes), "Nutrient homeostasis" (both up- and down- regulated genes), "Carbohydrate metabolism" (up-regulated genes), all of these will be singularly analyzed (Table 4).

##### Reactive Oxygen

Table 4 reports the DEGs related to "reactive oxygen" category. Two main gene sets were found to be strongly up-regulated in the *Pt* vs. CK comparison: Genes involved in the oxidoreductase activity and glutathione transferases. In particular, genes encoding copper/zinc superoxide dismutase, ascorbate peroxidase were induced by pathogen to overcome the damage induced by ROSs that play a central role during plant–necrotrophic fungus interactions through the stimulation of the plant's defense responses [40]. The gene encoding allene oxide synthase, involved in the pathway of oxylipin biosynthesis starting from unsaturated fatty acids was found strongly up-regulated. Their chemical nature renders unsaturated fatty acids intrinsic antioxidants; that is, they can directly react with ROS and thus consume them. Their oxidation gives rise to various oxylipins that, in turn, modulates ROS levels and signaling [41]. Transcript of aldehyde dehydrogenase 3H1 involved in oxidative stress tolerance by detoxifying reactive aldehydes derived from lipid peroxidation was also found up-regulated in diseased rough lemon plants (Table 4). Interestingly, numerous genes encoding glutathione transferases (GSTs) belonging to different GST classes have been induced by the fungal infection. This gene family can positively contribute to antimicrobial resistance in host plants by mostly unknown mechanisms, although a recognized GST function is their participation in the elimination of ROSs and lipid hydroperoxides that accumulate in infected tissues [42,43].

Light Reactions

As shown in Table 4 and Figure S6, the light reactions of the photosynthetic pathway were strongly affected by *P. tracheiphilus* inoculation as most of the components of both light harvesting and photosynthetic electron flows (cyclic and non-cyclic) as well as subunits of the CF0F1-ATP synthase were down regulated in inoculated plants (Figure S6). In detail, the PSAE-2 photosystem I subunit E-2 and the PSBE photosystem II reaction center protein as well as thylakoid-associated phosphatase 38 (Table 4) were down regulated in seedlings the diseased plant. This last gene is involved in light-harvesting complex of photosystem II (LHCII) dephosphorylation, facilitating its relocation to photosystem I. The expression of NDH-dependent cyclic electron flow 1 complex, that is involved in the cyclic electron transport by accepting electrons from ferredoxin (Fd), was sharply repressed. Moreover, the expression of the CF1-ATP synthase subunit was downregulated suggesting that the photophosphorylation of ADP leading to the ATP synthesis is strongly impaired because of fungal infection. Considering that photosynthesis is the main metabolic pathway devoted to energy supply in the green part of the plants, these findings clearly indicate that inoculated plants were suffering of energy shortage.

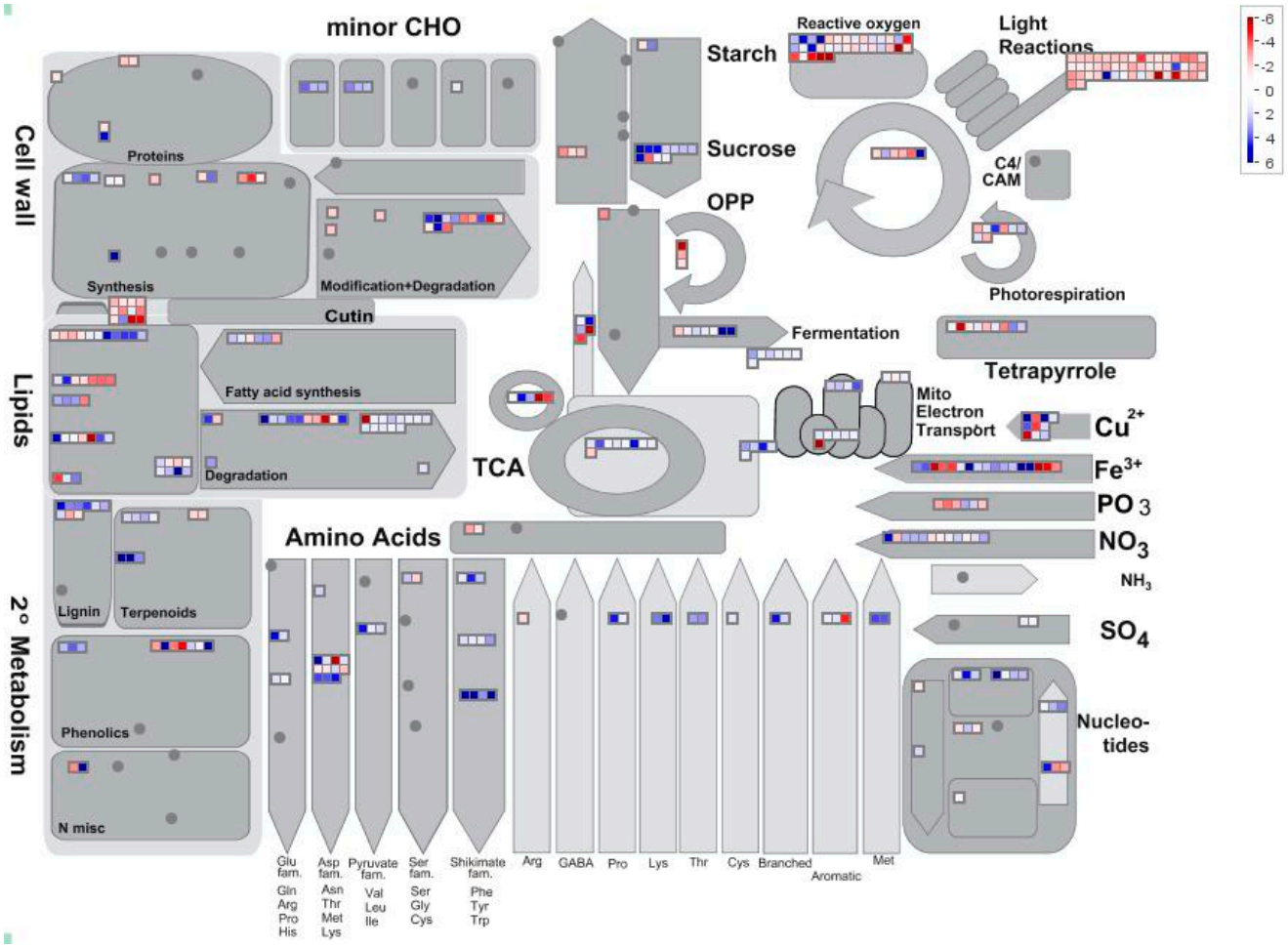


**Figure 6.** MapMan analysis of differentially expressed genes in *C. jambhiri* affected by *P. tracheiphilus.* Red dots represent up-regulated genes, blue dots represent down-regulated genes in the *Pt* vs. CK comparison.

**Table 4.** List of DEGs identified in *Pt* vs. CK comparison.

| Cluster | Symbol | Annotation | TAIR Code | $Log_2$Fold Change | Identity Score | e-Value |
|---|---|---|---|---|---|---|
| | | *Reactive oxygen—Oxidoreductase activity* | | | | |
| 14701,18284 | CSD1 | Copper/zinc superoxide dismutase 1 | AT1G08830 | +9.31 | 69% | $2 \times 10^{-4}$ |
| 14701,15083 | APX2 | Ascorbate peroxidase 2 | AT3G09640 | +5.11 | 78% | $2 \times 10^{-166}$ |
| 14701,14158 | PMP22 | Peroxisomal membrane 22 kDa | AT4G04470 | −4.28 | 80% | $2 \times 10^{-30}$ |
| 14701,8276 | AOS | Allene oxide synthase | AT5G42650 | +9.59 | 67% | $6 \times 10^{-26}$ |
| 14701,29676 | ALDH3H1 | Aldehyde dehydrogenase 3H1 | AT1G44170 | +3.70 | 68% | $3 \times 10^{-6}$ |
| | | *Reactive oxygen—Glutathione metabolism* | | | | |
| 14701,7488 | GSTU19 | Glutathione S-transferase TAU 19 | AT1G78380 | +7.58 | 70% | $1 \times 10^{-56}$ |
| 14701,35413 | GSTU10 | Glutathione S-transferase TAU 10 | AT1G74590 | +5.23 | 73% | $1 \times 10^{-17}$ |
| 14701,48103 | GSTF9 | Glutathione S-transferase PHI 9 | AT2G30860 | +5.22 | 72% | $1 \times 10^{-53}$ |
| 14701,17358 | GSTU7 | Glutathione S-transferase TAU 7 | AT2G29420 | +4.54 | 71% | $1 \times 10^{-33}$ |
| 14701,48102 | GSTF9 | Glutathione S-transferase PHI 9 | AT2G30860 | +4.32 | 69% | $3 \times 10^{-5}$ |
| | | *Light reaction* | | | | |
| 14701,61813 | PSAE-2 | Photosystem I subunit E-2 | AT2G20260 | −2.58 | 77% | $1 \times 10^{-6}$ |
| 14701,4480 | PSBE | Photosystem II reaction center protein E | ATCG00580 | −2.31 | 95% | $9 \times 10^{-110}$ |
| 14701,34255 | ATPD | ATP synthase delta-subunit gene | AT4G09650 | −3.10 | 72% | $2 \times 10^{-59}$ |
| 14701,26690 | PSBS | Chlorophyll A-B binding family protein | AT1G44575 | −3.50 | 76% | $2 \times 10^{-24}$ |
| 14701,72032 | TAP38 | Thylakoid-associated phosphatase 38 | AT4G27800 | −3.42 | 68% | $5 \times 10^{-42}$ |
| 14701,83115 | FKBP16 | FK506-binding protein 16-2 | AT4G39710 | −5.55 | 75% | $2 \times 10^{-90}$ |
| 14701,66882 | NDF4 | NDH-dependent cyclic electron flow 1 complex | AT3G16250 | −7.12 | 74% | $1 \times 10^{-61}$ |
| 14701,65295 | NDHB.2 | NADH-Ubiquinone/plastoquinone (complex I) protein (chloroplastic) | ATCG01250 | −2.44 | 98% | 0.0 |
| 14701,64497 | PPDK | Pyruvate orthophosphate dikinase (chloroplastic) | AT4G15530 | −2.79 | 77% | 0.0 |
| | | *Nutrient homeostasis* | | | | |
| 14701,19268 | | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | AT1G55290 | +7.77 | 68% | $2 \times 10^{-86}$ |
| 14701,28407 | FRO4 | Ferric reduction oxidase 4 | AT5G23980 | +7.14 | 66% | $2 \times 10^{-120}$ |
| 19914,4 | FRO2 | Ferric reduction oxidase 2 | AT1G01580 | +3.94 | 69% | $1 \times 10^{-36}$ |
| 14701,21908 | FRO7 | Ferric reduction oxidase 7 (chloroplastic) | AT5G49740 | −3.93 | 73% | $1 \times 10^{-81}$ |
| 14701,21905 | FRO6 | Ferric reduction oxidase 6 | AT5G49730 | −4.33 | 75% | $3 \times 10^{-105}$ |
| 14701,82040 | FRO8 | Ferric reduction oxidase 8 (mithocondrial) | AT5G50160 | −5.65 | 69% | $9 \times 10^{-106}$ |
| 4412,0 | IREG2 | Iron regulated 2 | AT5G03570 | −5.78 | 79% | $6 \times 10^{-40}$ |
| 14701,68697 | ASP3 | Aspartate aminotransferase 3 (chloroplastic) | AT5G11520 | +2.74 | 80% | 0.0 |
| 14701,45698 | GLN1;1 | Glutamine synthase clone R1 (cytosolic isozyme 1) | AT5G37600 | +2.66 | 77% | 0.0 |
| 20088,0 | NRT2:1 | Nitrate transporter 2:1 | AT1G08090 | −2.41 | 73% | $2 \times 10^{-111}$ |
| 14701,24935 | PHT1;4 | Phosphate transporter 1;4 | AT2G38940 | +2.78 | 73% | 0.0 |
| | | *Carbohydrate metabolism* | | | | |
| 14701,30461 | SUS2 | Sucrose synthase 2 | AT5G49190 | +5.52 | 79% | $7 \times 10^{-63}$ |
| 14701,11795 | SUS6 | Sucrose synthase 6 | AT1G73370 | +2.33 | 71% | 0.0 |
| 14701,60145 | SPS4F | Sucrose-phosphate synthase 4 | AT4G10120 | −2.33 | 82% | $4 \times 10^{-29}$ |
| 14701,28539 | BETAFRUCT4 | Acid beta-fructofuranosidase | AT1G12240 | +8.66 | 70% | 0.0 |
| 14701,71035 | INV-E | Alkaline/neutral invertase (chloroplastic) | AT5G22510 | +2.36 | 72% | $6 \times 10^{-162}$ |
| 14701,25319 | FBA1 | Fructose-bisphosphate aldolase 1 | AT2G21330 | −3.27 | 74% | $8 \times 10^{-10}$ |
| 14701,77303 | HXK1 | Hexokinase 1 | AT4G29130 | −3.81 | 73% | $2 \times 10^{-97}$ |
| | | *Cell wall modification and degradation* | | | | |
| 11195,0 | QRT3 | Pectin lyase-like superfamily protein | AT4G20050 | +6.07 | 67% | $2 \times 10^{-38}$ |
| 14701,45234 | | Pectinacetylesterase family protein | AT4G19420 | +5.45 | 69% | $1 \times 10^{-75}$ |
| 8874,0 | | Pectin lyase-like superfamily protein | AT1G11920 | +3.10 | 72% | $3 \times 10^{-64}$ |
| 13011,0 | | Pectate lyase family protein | AT1G67750 | −3.55 | 77% | 0.0 |
| 14701,76034 | | Pectinacetylesterase family protein | AT3G05910 | −3.75 | 73% | $1 \times 10^{-101}$ |
| 14701,45231 | | Pectinacetylesterase family protein | AT4G19420 | −4.87 | 69% | $1 \times 10^{-75}$ |

Iron Homeostasis

As shown in Table 4, genes involved in iron uptake and reduction were differently regulated in the *Pt* vs. CK comparison. In particular, ferric reduction oxidase 6 (FRO6), FRO7 and FRO8 were repressed by the infection. These genes are proposed to be involved in iron transport across the membrane in green part of the plant, FRO6 being localized in the plasma membrane, FRO7 in the chloroplasts and FRO8 in mitochondria [44]. These

results clearly indicate that the iron homeostasis is sharply impaired in the organelles of inoculated plants and in chloroplasts where it plays a crucial role in the heme biosynthesis and photosynthesis. Ferric reduction oxidase 2 (FRO 2) and 4 (FRO4) which normally are expressed in plant roots were upregulated by fungal infection, as well as the gene encoding 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase which are involved in sideretin biosynthesis, a metabolite exuded by roots in response to iron deficiency to facilitate iron uptake. The stress induced expression of genes, both FROs and 2OG, normally involved in iron uptake in roots might be explained as an ultimate attempt to cope with the shoot iron deficiency caused by the down regulation of leaf-specific FRO genes. Regarding other nutrients such as nitrogen and phosphate, the results show that gene involved in nitrate uptake was down-regulated, whereas glutamine synthase and aspartate aminotransferase involved in nitrogen fixation and in amino acid and Krebs cycle metabolisms were up-regulated. The high-affinity transporter for external inorganic phosphate functioning as $H^+$: Phosphate symporter was also up-regulated (Table 4).

Carbohydrate Metabolism

The analysis of carbohydrate metabolism highlighted that several genes involved in sugar metabolism were clearly induced in response to fungal infection (Table 4). Specifically, sucrose-phosphate synthase 4, which plays a role in photosynthetic sucrose synthesis by catalyzing the rate-limiting step of sucrose biosynthesis from UDP-glucose and fructose-6-phosphate, was down-regulated. On the contrary, transcripts encoding sucrose synthase, a cleaving enzyme that provides UDP-glucose and fructose for various metabolic pathways, were among the up-regulated genes. Table 4 also reports that transcripts encoding the acid beta-fructofuranosidase and alkaline/neutral invertase, respectively involved in the continued mobilization of sucrose to sink organs and in the cleavage of sucrose into glucose and fructose, were up-regulated. Overall, these data suggest that both sucrose synthesis and therefore the export of photo assimilates out of the leaf were impaired, whereas cleavage seems to be the favorite route undertaken by this metabolite. However, the fructose-bisphosphate aldolase 1 and hexokinase 1 were down-regulated in diseased plants indicating that glycolysis might be repressed in the inoculated plants (Table 4).

Cell Wall Modification and Degradation

During pathogen infections, the cell wall undergoes dramatic structural and chemical changes of cell wall constituents. Necrotrophic pathogens are sensed by a plasma membrane receptor, leading to activation of defense signaling cascades and eventual mounting of inducible defense responses [45]. In our study, several DEGs encoding pectin lyase-like superfamily protein and pectin acetylesterases were identified (Table 4). However, these transcripts were both up- and down-regulated, making it difficult to extrapolate unequivocal conclusions. Certainly, as expected, cell walls of inoculated plants underwent remodeling processes likely involved in the response to pathogen.

## 3. Discussion

Environmental stresses severely affect plant and crop growth and reproduction. Therefore, determining the critical molecular mechanisms and cellular processes in response to stresses will provide knowledge for identifying genes that might be target of modification, by *knocking out* or by *knocking down* procedures, especially in susceptible host–pathogen interactions. RNA sequencing (RNA-Seq) using next-generation sequencing (NGS) provides opportunity to isolate genes of interest, develop of functional markers, quantify of gene expression and carry out comparative genomic studies. It has been successfully applied to unravel the transcriptome profile of several *Citrus* varieties in response to *Phytophtora parasitica* infection [26] and to *Candidatus* Liberibacter [24,25] providing new insight into host responses to both pathogens. In this work, we described the results of RNA sequencing and de novo transcript assembly in rough lemon (*C. jambhiri*) leaves subjected to artificial inoculation by *P. tracheiphilus*, the causal agent of "mal secco" disease

used as model of a compatible host–pathogen interaction. At harvest time (15 days after inoculation), infected plants showed the typical disease symptoms, and the pathogen was detected by molecular analysis. Globally, a deep reprogramming of the leaf transcriptome emerged as 4986 (2865 up-regulated and 2121 down regulated) DEGs have been identified confirming that the attempt of an active defense against microbial pathogens involved the induction of elaborate defense signaling pathways. In plants, some of these defense strategies can provide protection at the site of infection, whereas others provide systemic resistance throughout the plant including in non-infected tissue. Local resistance includes basal immunity, or PAMP/MAMP (pathogen/microbe associated molecular patterns)—triggered immunity (PTI) which is induced when pattern recognition receptors (PRRs) from the plant recognize pathogen-derived elicitors. To establish a successful infection, plant pathogens can suppress PTI by injecting effectors into the host cells [46]. To counter this virulence strategy, plants have evolved the so-called resistance (R) proteins, which can either directly detect the effectors or indirectly detect their activity. In plants where the activity of effectors is detected by the R proteins, effector-triggered immunity (ETI) is activated rendering the pathogen avirulent [47] ETI in plants is often associated with rapid, localized programmed cell death (PCD) at the infection site, a visible phenotype known as the hypersensitive response HR, to prevent the spread of the pathogen. HR is generally associated with race-specific resistance to biotrophic pathogens and it is less effective against necrotrophics which require dead host tissue to complete their life cycle [47]. Necrotrophic pathogens such as *P. tracheiphilus* are well able to block HR by initiating systemic signals for defense activation in distal parts of plant that ultimately results in the activation of systemic acquired resistance (SAR) [47]. Induction of SAR involves the generation of mobile signals at the site of primary infection, which translocate to distal tissue and prepare the plant against future infections. Several chemical inducers of SAR have been identified and some of these have been shown to translocate systemically. The SAR associated chemicals include salicylic acid (SA), free radicals, and reactive oxygen species (ROS), among others [48]. Upon SA accumulation, NPR1 monomers are transported into the nucleus. Here, NPR1 interacts with TGA proteins, which belong to the basic leucine zipper (bZIP) protein family of transcription factors and binds TGACG motifs to activate defense-related transcription [48]. The analysis of the transcriptomic data reported in this work unequivocally indicated that the entire gene set encoding the components of SAR from salicylic acid biosynthesis on was strongly up-regulated. In addition, *P. tracheiphilus* is able to overcome the basal immunity of rough lemon plants (PTI) as the essential regulator of plant defense (RPM1 interacting protein 4) was down-regulated, and the expression of BIR2, which is negative regulator of basal level of immunity was up-regulated in the diseased plants. In the inoculated plants, the observed repression of auxin signaling by the SA pathway might also contribute to increase rough lemon susceptibility to *P. tracheiphilus* as reported in *Arabidopsis* infected by the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* [49].

Chitin, found in the cell walls of true fungi, is a well-established elicitor of plant defense responses and it appears to play a significant role in plant defense to fungal pathogens [50]. The fact that chitin elicits de novo gene expression suggests the involvement of transcription factors (TFs) with WRKY TF family strongly represented [51–53]. To regulate gene expression, WRKY proteins bind specifically to a DNA sequence motif (T)(T)TGAC(C/T) known as the W box [54–57] which occurs in the promoters of genes under the control of WRKY proteins. A number of defense-related genes, including PR genes, contain a W box in their promoter regions [51,54,55]. The promoters of pathogen and/or salicylic acid (SA) regulated *Arabidopsis* WRKY genes [58] are substantially enriched in W boxes, suggesting that defense-regulated expression of WRKY genes involves transcriptional activation and repression through self-regulatory mechanisms mediated by transcription factors of the WRKY gene superfamily [32]. For example, expression of the *Arabidopsis NPR1* is known to be controlled by WRKY factors [57]. In this study, transcription factors interacting specifically with the W box motif such as *WRKY14*, *WRKY23*,

*WRKY49*, *WRKY72*, *WRKY75*, and *WRKY71* were up-regulated in infected plants, indicating a strong activation of the defensive mechanism. The up-regulation of both *WRKY4*, that is reported to have a positive role in plant resistance to necrotrophic pathogens [34] and *WRKY51*, acting as positive regulator of salicylic acid (SA)-mediated signaling [33] confirms that the rough lemon plants tried to resist the *P. tracheiphilus* infection by the activation of salicylic acid-mediated signaling pathway, in accordance with the whole results of this study. Furthermore, the strong induction of *WRKY40*, *WRKY18,* and *WRKY70* transcription factors accounts for a defense response specifically addressed towards fungi as they specifically respond to chitin [31]. *P. tracheiphilus* infection induced the expression of oxidative burst peroxidases (RBOHs) in rough lemon (Table 3). The apoplastic oxidative burst could directly kill pathogens by generating ROS with antimicrobial activity; otherwise, a second, stronger phase can occur, which is associated with the hypersensitive response [39]. However, the role of RBOHs is controversial as a relatively limited role for NADPH oxidases in the HR has been observed in tobacco (*Nicotiana tabacum*), where RBOHD-mediated hydrogen peroxide production does not seem to be essential for the development of the HR or systemic acquired resistance (SAR) [59,60]. More recently, these genes have been studied in detail in *A. thaliana* and are reported as the major component of PTI [39]. Considering that rough lemon plant is susceptible to *P. tracheipilus*, their effectiveness in overcoming the pathogen is not sufficient to block it, and probably they have a major role in transducing the signal of the "presence" of the pathogen by increasing ROS concentration.

Although important in biotic stress signal transduction, reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide ($O^{2-}$), and singlet oxygen ($^1O_2$) are highly reactive and could cause oxidative damage to DNA, proteins and other molecules of the cell. There are different cellular mechanisms in place to deactivate the excess of these damaging ROS molecules. These include enzymatic reactions through catalase, superoxide dismutase, glutathione peroxidase and ascorbate peroxidase but also small antioxidants such as ascorbic acid and glutathione [61]. This study revealed that a subset of these ROS-scavenging genes was induced in the infected plants (Table 4). Interestingly, a wide number of glutathione transferases belonging to phi and tau classes were also up-regulated by the infection in accordance with early studies on the role of GSTs in plant biotic stress [43]. Notably, the expression of multiple GSTs was massively activated by salicylic acid and some GST enzymes were demonstrated to be receptor proteins of salicylic acid [43]. Functional studies revealed that overexpression or silencing of specific GSTs can markedly modify disease symptoms and pathogen multiplication rates [62].

As reported in the case of other necrotrophic fungi such as *B. cinerea* [63], the main metabolic effect upon inoculated plants was the down-regulation of either light harvesting components or photosynthetic electron flows or CF1F0-ATPase. This might have led to an apparent, persistent "dark" or "shade" condition: Plants are in regular light/dark alternation but they cannot use light to provide energy. The sucrose mobilization suggested by the regulation of the two main genes involved in sucrose biosynthesis and cleavage is in accordance with this energy requirement. In the dark, plant mitochondria generate the required ATP molecules for basic cellular function [64]. However, two main genes involved in glucose catabolism were down-regulated (Table 4) indicating that sugars seem not be routed towards glycolysis and Krebs cycle. On the contrary, as fungal genes involved either in sugar fermentation or in mitochondrial synthesis of ATP were strongly expressed in rough lemon leaves, the plant sugar resources might be hijacked towards the fungus to feed it. This mode of nutrition is the rule for biotrophic pathogens, but also necrotrophics might exhibit a similar behavior [65]. In this study, we also show that FRO7 (chloroplast Fe(III) chelate reductase), involved in chloroplast iron homeostasis and required for survival under iron-limiting conditions, was down regulated. It has been shown that chloroplasts isolated from *fro7* loss-of-function mutant plants have significantly reduced Fe(III) chelate reductase activity, reduced iron content, and altered photosynthetic complexes, providing genetic proof that chloroplasts do rely in part on a reductive strategy for iron acquisition [44].

Consequently, the lack of a regular input of reducing power from water photolysis induced by light might be in turn responsible for the iron deficiency observed in the apical part of the leaves of diseased rough lemon.

## 4. Materials and Methods

### 4.1. Plant Material and Inoculum

Seeds of rough lemon (*C. jambhiri*) were sowed on sterile peat in May 2019. After 6 months of growing in a chamber at 25 °C and 90% humidity, the plants were inoculated with the pathogen *Plenodomus tracheiphilus* PT10 strain (kindly provided by Professor Vittoria Catara, University of Catania). Rough lemon was chosen as plant material for the following reasons: (I) It was previously reported as very susceptible to the disease [6]; (II) it has a high degree of polyembryony, higher than true lemons or other citron hybrids [66], allowing the production of true-to-type seedlings; and (III) it is very vigorous, with seedlings growing faster than those of other citrus species. Moreover, our preliminary inoculation tests indicated that symptoms after artificial inoculations were easier to detect in rough lemon than in *C. limon* seedlings.

The inoculum was prepared according to a slight modification of the method described in [67]. Briefly, three pieces of young fungus grown at 21 °C ± 2 in Petri dishes containing potato dextrose agar medium (PDA) were placed in 7 different flasks containing 100 mL of carrot broth and incubated for 5 days in a heidolph unimax 2010 shaker at 22 °C. Successively, the growth medium was filtered and centrifugated at 8000 rpm × 20 min. The pellet was recovered and the phialoconidia were counted with a counting chamber to adjust the inoculum concentration at $10^6$ $mL^{-1}$. The inoculation was performed by depositing 10 µL on wounds obtained by cutting the midvein of three leaves for each plant with a sharp sterile blade. Overall, five plants were inoculated with the pathogen and five plants were inoculated with water as control. Both inoculated and control samples were collected 15 days after inoculation, considering that inoculated plants showed evident symptoms of the disease. Leaves were immediately frozen with liquid nitrogen and stored at −80 °C until both DNA and RNA extractions were performed.

### 4.2. DNA and RNA Extraction

DNA extraction was performed according to [68]. Briefly, samples were powdered using liquid nitrogen in mortar and pestle. Two hundred milligrams of grinded leaves were mixed approximately with 500 µL of CTAB buffer (2% CTAB, 20 mM EDTA, 1.44 mM NaCl, 100 mM Tris HCl, pH 8.0) and 0.2% β-mercaptoethanol. Samples were vortexed and incubated at 65 °C for 30 min, then the CTAB-plant extract mixture was transferred into a microfuge tube. After adding 300 µL of chloroform-isoamyl alcohol (24/1), the tubes were mixed by inversion and centrifuged at 14,000 rpm for 10 min. The supernatant was recovered into a clean microfuge tube and 7.5 M ammonium acetate (50 µL) followed by 1000 µL of ice cold 100% of ethanol were added to each tube. The tubes were mixed by inversion and then centrifuged at 10,000 rpm for 10 min. The pellet was rinsed twice with 1000 µL of ice cold 70% ethanol, resuspended in 50 µL of sterile distilled water and stored at 4 °C until analysis. The DNA concentration and purity were checked by a Nanodrop 2000 spectrophotometer (Thermo Scientific™, Waltham, MA, USA).

The RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were checked using the NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Before sequencing, sample RNA integrity (RIN) was assessed using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

### 4.3. Real-Time Confirmation of Infected Plants

Taqman Real-time PCR was performed to reveal the presence of the pathogen within the inoculated plants using an ABI 7500 Real-Time PCR System (Applied Biosystems™,

Foster City, CA, USA). The analysis was performed according to the method described in [28], using DNA extracted from both inoculated and control leaves as template. Forward primer GR70 (5′-GATCCGTACGCCTTGGGGAC-3′) and reverse primer, GL1 (5′-AGAAGC GTTTGGAGGAGAGAATG-3′), dual-labeled fluorogenic probe, PP1, (5′-FAM-CACGCAATCTTGGCGACTGTCGTT-TAMRA-3′) were used with the aim to amplify a 84-bp segment of the pathogen DNA. Each reaction contained 200 nM forward primer, 200 nM reverse primer, 100 nM fluorogenic probe, and 4 µL of genomic DNA in a final volume of 15-µL. Negative control contained the same mixture, with sterile water replacing the DNA template. The assay was performed on three biological replicates, each one repeated twice. The thermal cycling conditions for *P. tracheiphilus* DNA template amplification were 50 °C for 2 min (1 cycle), 95 °C for 30 s (1cycle), 95 °C for 10 s, 62 °C for 30 s (40 cycles). Standard curve for fungal DNA quantification was constructed using *P. tracheiphilus* DNA (100 µg $mL^{-1}$) extracted from the Pt10 strain and serially diluted in sterile distilled water as described in [28].

### 4.4. Library Preparation and Sequencing

After the QC procedures, sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA) following manufacturer's recommendations and as reported in [69]. Briefly, mRNA was enriched using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X), followed by cDNA synthesis using random hexamers and M-MuLV Reverse Transcriptase (RNase H-). After first-strand synthesis, a custom second-strand synthesis buffer (Illumina) was added containing dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand by nick-translation. After adenylation of 3′ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. To select cDNA fragments preferentially 150~200 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Then, 3 µL USER Enzyme by NEB was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min followed by 5 min at 95 °C before PCR. Successively, PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. Library concentration was first quantified using a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and then diluted to 1 ng/µL before checking insert size on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Cluster generation and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). After cluster generation, the libraries were sequenced on Illumina HiSeq2000 platform to generate pair-end reads. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data were obtained by removing reads containing adapters, reads containing poly-N and low-quality reads. Sequences putatively belonging to pathogen in inoculated rough lemon samples were removed by filtering out the reads mapped to the fungus genome (https://mycocosm.jgi.doe.gov/Photr1/Photr1.info.html, accessed on 18 November 2020). Then Q20, Q30, GC-content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

### 4.5. De novo Transcriptome Assembling and Gene Functional Annotation

De novo transcriptome assembly was accomplished using Trinity (r20140413p1 version) with min_kmer_cov:2 parameters (k = 25). Then Hierarchical Clustering was performed by Corset (v1.05 version, https://github.com/Oshlack/Corset/wiki) to remove redundancy (parameter -m 10) and the longest transcripts of each cluster were selected as Unigenes. The flow chart of the rough lemon de novo transcriptome assembly is stackable to that reported in [69]. The *Citrus jambhiri* transcriptome was uploaded to NCBI (https://www.ncbi.nlm.nih.gov/geo/, accessed on 29 December 2020) accession number GSE164096.

Gene function was annotated based on the following databases: National Center for Biotechnology Information (NCBI) non-redundant protein sequences (Nr), NCBI non-redundant nucleotide sequences (Nt), Protein family (Pfam), Clusters of Orthologous Groups of proteins (KOG/COG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Ortholog database (KO) and Gene Ontology (GO). A pathway analysis was conducted using MapMan3.6.0RC1 (https://mapman.gabipd.org/, accessed on 19 October 2020). All the unigenes were annotated and mapped using Mercator4 V2.0, an on-line tool of MapMan (https://www.plabipd.de/portal/mercator4, accessed on 5 November 2020) which accurately assigns hierarchal ontology providing visual representation of genes in different plant processes. The significant DEGs (padj < 0.05), with the corresponding $\log_2$FoldChange values, were used as dataset to align with the Mercator map.

### *4.6. Quantification of Gene Expression and Differential Expression Analysis*

Gene expression levels were estimated by RSEM (v1.2.26 version, http://deweylab.github.io/RSEM/) with bowtie2 mismatch 0 parameters to map the Corset filtered transcriptome. For each sample, clean data were mapped back onto the assembled transcriptome and readcount for each gene was then obtained. Differential expression analysis between control (CK) and infected (*Pt*) samples was performed using the DESeq R package (1.12.0 version, padj < 0.05, https://bioconductor.org/packages/release/bioc/html/DESeq.html). The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate [70]. Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed. A $\log_2$FoldChange threshold of 0.58 (1.5 fold change) was adopted. The GO enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages (1.10.0, 2.10.0 version, corrected *p* value < 0.05 based) Wallenius non-central hyper-geometric distribution. Furthermore, all of the unigenes were submitted to the KEGG pathway database for the systematic analysis of gene functions. KOBAS software (v2.0.12 version, corrected *p*-Value < 0.05, kobas.cbi.pku.edu.cn) was used to test the statistical enrichment of differential expression genes in KEGG pathways.

### *4.7. Real-Time Validation of Selected DEG Candidates Using qRT-PCR*

Total RNA (2.5 µg) extracted from sample leaves as described above, was reversed transcribed using the SuperScript™ Vilo™ cDNA synthesis kit by ThermoFisher Scientific (Warrington WA1 4SR, UK), according to the manufacturer's instructions. Real-time qRT-PCR was performed for a total of 10 DEGs with PowerUp SYBR Green Master mix by ThermoFisher Scientific and carried out in the Bio-Rad iQ5 Thermal Cycler detection system. All the genes were normalized with *Citrus clementina* actin (LOC18039075). All reactions were performed in triplicate and fold change measurements calculated with the $2^{-\Delta\Delta CT}$ method. The selected DEGs and their corresponding primer sequences are provided in Table S2.

## 5. Conclusions

The global transcriptome analysis of *Pt* vs. control plants led to the identification of genes and metabolic pathways involved in rough lemon response to *P. tracheiphilus*. As far as we know, this is the first manuscript that describes at molecular level the "mal secco" disease induced by *P. tracheiphilus* in citrus and makes *C. jambhiri* genetic resources available for the scientific community interested in citrus breeding.

The results highlight most of the events occurring during this compatible host–pathogen interaction, which now it is known relies on the activated SA signal cascade that, in turn, induces systemic acquired resistance (SAR). As the main scope of the work was the identification of putative target genes for genome editing experiments, a wide range of genes belonging to structural and transcription factors have been identified and they could be taken in consideration for targeted mutagenesis, RPM1 and BIR2 being only two of

them. This strategy fits the increased demand for economical and environmentally friendly approaches to cope with plant diseases, while avoiding the use of agrochemicals.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/1422-0067/22/2/882/s1, Figure S1: Effect of *P tracheiphilus* on *C. jambhiri* phenotype, Figure S2: Overview of the number of transcripts and unigenes in different length intervals, Figure S3: Validation of DEGs in *Pt* vs. CK comparison by Real Time qRT-PCR, Figure S4: KOG function classification, Figure S5: Scheme of the metabolic pathways involved in the "Plant hormone" category, Figure S6: Scheme and components of the photosynthetic electron flow including CF0F1-ATP synthase, Table S1: Real-time detection of *P. tracheiphilus* in inoculated plants, Table S2: Primers used to validate the RNAseq experiment by real time PCR.

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