HortScience 59(5):658–665. 2024. https://doi.org/10.21273/HORTSCI17673-23

Phenotypic Evaluation of a Lemon Hybrid Population to Identify Sources of Resistance to *Plenodomus tracheiphilus*

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Keywords. citrus, disease resistance, lemon breeding, mal secco, phenotyping

Abstract. **Mal secco, caused by the fungus *Plenodomus tracheiphilus*, is a xylem disease that is a limiting factor for lemon production in the Mediterranean. Resistance or field tolerance are major goals for lemon breeders; however, there is scant information regarding the heritability of mal secco resistance in breeding populations. As with other vascular diseases, phenotyping is the bottleneck for ascertaining resistance and susceptibility, and a validated protocol for greenhouse phenotyping would be valuable to accelerate the selection of tolerant trees before field evaluation. We report phenotyping of 148 hybrids of Khasi papeda (*Citrus latipes*; tolerant to mal secco) × lemon (susceptible to the disease) in field and greenhouse conditions. Field evaluation was performed on all hybrids for 2 to 3 consecutive years on trees subjected to high natural-pathogen pressure. Detection of the fungal infection was performed by visual observation and real-time polymerase chain reaction (PCR). The first infections occurred ≈6 months after planting, but 2 years of observations were needed for a reliable estimation of susceptibility. The spread of the disease did not occur uniformly throughout the plot, with patterns of spread within rows, probably resulting from infections from plant to plant. The possible errors in the estimation of susceptibility as a result of the uneven distribution of infections in the plot were reduced by using more than one replicate tree per hybrid. The correlation between phenotyping scores and cycle threshold values was weak ($r = -0.48$, $P < 0.001$). Three years after planting, hybrids clustered into three groups—susceptible, tolerant, and intermediate—based on symptom progression. A subset of 65 self-rooted hybrids was also subjected to stem inoculation in an unheated greenhouse, with two to seven biological replicates per hybrid. Three months after inoculation, the samples were monitored for symptoms appearance and subjected to real-time PCR pathogen quantification. We observed a weak ($r = 0.41$) but significant ($P < 0.001$) correlation between phenotypes in the field and the greenhouse, indicating that, in our conditions, field evaluation remains the best method for phenotyping. However, artificial inoculations might help to discard the highly susceptible hybrids before field evaluation.**

Mal secco disease is caused by the fungus *Plenodomus tracheiphilus* Petri Gruyter, Aveskamp, and Verkley (De Gruyter et al. 2013). It is a xylem disease that affects different citrus species, mainly lemon (*Citrus limon*). Citron (*Citrus medica*), bergamot (*Citrus bergamia*), Tahiti (*Citrus latifolia*) and Mexican (*Citrus aurantiifolia*) limes, chinotto (*Citrus myrtifolia*), *Citrus macrophylla*, Volkamer lemon (*Citrus volkameriana*), rough lemon (*Citrus jambhiri*), and sour orange (*Citrus aurantium*) are also very sensitive to the disease (Migheli et al. 2009; Nigro et al. 2011). Mal secco is established primarily in the Mediterranean area and is the major limiting factor for lemon production in several countries, including Italy, Turkey, Greece, Israel, Tunisia, and Algeria. The pathogen was also occasionally found in Spain (https://gd.eppo.int/taxon/DEUTTR/distribution/ES), and a recent study (Krasnov et al. 2023) indicated that this country, as well as Morocco and Portugal, which are still free of the disease, have a suitable environment for its potential spread. The inoculum is dispersed by wind and rain, and the penetration of the fungus occurs through wounds on leaves and other plant organs. The first manifestations are usually leaf vein chlorosis, wilting, and leaf drop, which might be followed by twig and branch desiccation, and the death of the tree. Root infections are also possible, leading to a more rapid decline of the plant. In the infected branch tissues, it is possible to observe a yellow or salmon-pink color on the xylem, typical of the presence of the pathogen (Batuman et al. 2020; Nigro et al. 2011). Curative treatments are not effective in controlling the disease (Catara and Catara 2019), and treatments based on copper are used to prevent it. The only effective strategies to control the disease are the use of certified plant material, proper field management (pruning of infected twigs and branches, burning of infected wood, preventive fungicide treatments, reduction of nitrogen fertilization to reduce vegetative growth), and the use of tolerant lemon varieties. All lemon commercial varieties come from bud sports of a single ancestor (Barry et al. 2020): a hybrid between sour orange and citron (Di Guardo et al. 2021). This leads to a very low level of genetic variability, which could be used to identify sources of resistance to the disease. Despite the narrow genetic diversity, some lemon clonal selections have shown field tolerance (Ben-Hamo et al. 2020; Continella 1992; Russo et al. 2020). No citrus species is considered immune to P. *tracheiphilus* (Catara and Cutuli 1972), but it is known that some lemon-like hybrid cultivars and species, such as Palestinian lime (*Citrus limettioides*), 'limone cedrato Spatafora' or 'Meyer', are field tolerant (Russo 1977; Russo et al. 2020). This indicates that it could be possible to generate mal secco–tolerant lemon-like phenotypes through conventional breeding approaches.

In the absence of clear information regarding the genetic basis of mal secco resistance, the generation of large populations using different tolerant or resistant parents should be a prerequisite to obtaining tolerant lemon hybrids with acceptable quality traits. Breeding efforts to generate resistant lemon hybrids were made

in past decades (Carrante and Bottari 1952; Russo 1977; Tuzcu et al. 1992), but it is not known how many resistant hybrids have been obtained from lemons crossed with resistant citrus accessions. Carrante and Bottari (1952) and Russo (1977) described the Italian breeding program based on hybridization, which was performed between 1945 and the late 1970s, and indicated the obtained hybrids were, in some cases, resistant to mal secco, but were characterized by poor fruit quality or low productivity. The described hybridization strategies used predominantly lemon, citron, or their hybrids as parents to produce new hybrids resembling the true lemons; however, citron and lemon hybrids are, on average, more susceptible to the disease compared with other citrus species (Russo 1977; Russo et al. 2020). Moreover, Russo (1977) indicated that a low number of hybrids was generated in the crosses between true lemons, because of the polyembryony of most varieties used as parents, which decreased the efficiency of the program. Russo (1977) also reported that most of the hybrids died at early developmental stages. Russo and Starrantino (1996) reported the generation of very few triploid lemon hybrids backcrossing lemons and lemon hybrids with a tetraploid lemon. Some of them had acceptable fruit quality. One of these hybrids was released as 'Lemox' (Reforgiato Recupero et al. 2005), a hybrid resembling a true lemon in terms of fruit characteristics, but was characterized by susceptibility to mal secco. Strategies based on the screening of large lemon progenies using distantly related, resistant parents have not been performed extensively.

Although the generation of the hybrids is relatively easy, the bottleneck is represented by a reliable phenotyping protocol to estimate susceptibility. The identification of sources of resistance in accessions or breeding populations is based on visual screening of plants subjected to artificial inoculation or natural infection. Selection is hampered by the long period between infection and the appearance of typical symptoms, and fluctuations in the severity of symptoms from year to year and in different environments. The time needed to phenotype lemon hybrids properly for mal secco resistance and fruit quality traits has discouraged the use of hybridization in past decades.

In addition to the field evaluation, several artificial inoculation protocols on leaves or stems have been described that might help in the assessment of tolerance and susceptibility, accelerating the evaluation of resistance and susceptibility compared with field evaluation (Dimaria et al. 2023; Russo et al. 2021; Salerno and Catara 1967; Solel and Spiegel-Roy 1978; Solel et al. 1995). Russo et al. (2021) carried out artificial inoculation in rough lemon leaves, detecting symptoms after 15 d, whereas stem inoculation has been used to test the susceptibility of different lemon varieties (Cacciola et al. 2010; Gentile et al. 2000; Solel and Spiegel-Roy 1978). Some inoculation protocols took advantage of real-time polymerase chain reaction (PCR) analysis to detect symptomatic and symptomless infections (Demontis et al. 2008; Licciardello et al. 2006; Russo et al. 2020). Gentile et al. (2000) found correspondence between symptom severity after artificial inoculation and known behavior in the field of some lemon cultivars and somaclones. However, Solel and Spiegel-Roy (1978) revealed discrepant behaviors between specific lemon cultivars subjected to natural infections in field conditions and artificial inoculations in controlled conditions. Moreover, these protocols have been applied mostly to test the susceptibility of lemon cultivars or sour orange seedlings, or to characterize *P. tracheiphilus* strains (Ziadi et al. 2014). Tuzcu et al. (1992) reported preliminary results of screening young lemon nucellar seedlings and intraspecific hybrids by artificial inoculation. Since their work, there have been no reports of artificial inoculation protocols applied in the framework of a breeding program based on hybridization.

Considering the described background, we phenotyped a lemon hybrid population using two approaches with the following aims: 1) to understand the evolution of natural infections for reliable phenotyping of a hybrid population generated from a very susceptible, high-quality lemon and a resistant citrus accession; 2) to estimate the ratio of resistant or tolerant hybrids that could be used as breeding material; and 3) to understand whether prescreening methods based on *P. tracheiphilus* artificial inoculation in the greenhouse could be used to accelerate selection in a systematic breeding program for mal secco disease resistance. This information is crucial to estimate the feasibility of a lemon breeding program for mal secco resistance.

Materials and Methods

Plant material. The hybrid population of Khasi papeda (*C. latipes*; resistant to mal secco) × 'Femminello Siracusano 2KR' lemon (*C. limon;* susceptible to mal secco) was generated in 2018 from crosses made in Spring 2017 performed at the San Salvatore experimental farm of the Council for Agricultural Research and Economics, Research Center for Olive, Fruit, and Citrus Crops, Acireale, Catania, Italy (lat. 37°37023″N, long. 15°09050″E). To confirm the hybrid origin of the population and eliminate F1 individuals resulting from self-pollination and any other pollen sources, each plant was genotyped using six simple sequence repeat markers: AG14, CAC39, CT19, GT03, TAA15, and TAA41 (Barkley et al. 2006; Kijas et al. 1997). The amplified fragments were separated using capillary electrophoresis, performed using the ABI3130 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) (data not shown).

One hundred forty-eight hybrids, each replicated twice, were grafted in Jun 2019 onto Carrizo citrange. The mother trees of each hybrid were maintained in a screen house to keep them free of mal secco. Approximately 16 months after grafting, 197 propagated plants of 128 hybrids plus the two parents, which were considered suitable for field growth, were planted at the San Salvatore experimental farm. Seventy-three additional plants belonging to 44 hybrids (20 hybrids that were not represented in the first planting and 24 hybrids that were represented by a single replicate in the first planting) were grafted in Summer 2020 and planted in Sep 2021. By that date, the total number of studied plants included in the experimental field was 270, with 148 hybrids replicated one to three times in a completely randomized design. The different number of replicates of each hybrid depended on the variable number of propagated plants considered suitable for field growth. Hybrids plus parents were planted in twin rows at a distance of 1 m between plants in each row, with 0.5 m between adjacent rows, and 1.50 m between each set of twin rows. The plot was surrounded by infected lemon plants and is in an area of high pressure of mal secco, as described previously by Russo et al. (2020). Plants were grown using conventional cultural practices. Fertilization was performed four times per year, with a 25N–10P inorganic fertilizer at a dose of 0.4 kg/plant per year. Irrigation was performed by drip, with emitters spaced 0.20 m apart. No fungicides or pesticides were applied during the 3 years of evaluation.

Field phenotyping, sample collection, and PCR detection of P. tracheiphilus. Plants subjected to the natural pressure of the pathogen were phenotyped for 2 to 3 years, depending on the planting date. Phenotyping was performed 13 times from Apr 2021 until Aug 2023. In 2021 and 2022, phenotyping started in winter or spring after the appearance of new symptoms and continued monthly until July or August. In 2023, phenotyping was performed twice (in late May and early August). Considering that symptoms appear mostly during late winter and spring, and that xylem colonization is inhibited by winter and summer temperatures (Migheli et al. 2009), phenotyping was not performed in late summer, autumn, and early winter. Phenotyping was carried out through a visual screening based on the observation of typical leaf vein chlorosis, the desiccation of the twigs and branches, and the observation of pink-salmon coloration of the xylem (Fig. 1). For each survey, symptom severity was scored according to an empirical scale, and the following values were attributed: 0 = no symptoms, 1 = very mild symptoms on healthy plants (one or two desiccated twigs for a length of ≤ 5 cm), 2 = a few desiccated twigs

Received for publication 19 Dec 2023. Accepted for publication 1 Feb 2024.
Published online 10 Apr 2024.
This work was supported by FREECLIMB, Fruit Crops Resilience to Climate Change in the Mediterranean Basin, a project funded in the framework of the Partnership for Research and Innovation in the Mediterranean Area (PRIMA) and INNO.VI.A (innovative products and processes in the horticultural nursery and agrifood sectors), a project funded in a Sicilian operative program (European Social Fund 2014–2020).
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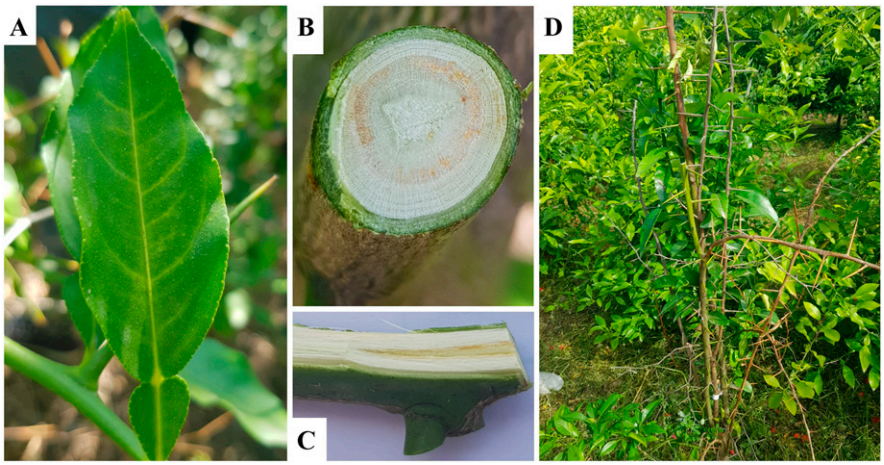

Fig. 1. Typical mal secco symptoms observed on leaves (**A**), and cross (**B**) and longitudinal (**C**) sections of branches, and desiccation of twigs (**D**) of the lemon hybrids.

(length > 10 cm) on a healthy canopy, 3 = symptoms extended to at least half of the canopy, and 4 = dead plants (Supplemental Fig. 1).

Real-time PCR was performed on three twigs of all replicates in Jun 2022 to confirm that the observed symptoms were caused by *P. tracheiphilus* and to verify the possible presence of the pathogen in the xylem of nonsymptomatic plants. In the case of symptomatic plants, the three twigs per plant were sampled as follows to have a homogeneous sampling: one portion of ~10 cm was sampled from desiccated twigs, below the desiccation; one portion of ~10 cm was sampled from a healthy twig with symptoms in leaves; and one portion of ~10 cm was sampled from a healthy twig without desiccation or symptomatic leaves. In the case of plants with no symptoms, samples of the same size were collected randomly about 15 to 20 cm from the apex from at least three twigs. Samples were surface-sterilized in a solution of 2.5% sodium hypochlorite for 2 min and then washed twice with sterile distilled water as described by Russo et al. (2020). The sterile twigs were cut into rounds using sterile scissors to facilitate homogenization and were stored at –80 °C until DNA extraction. The rounds of frozen whole stems were homogenized with grinding jars (TissueLyser; Qiagen, Hilden, Germany) frozen in dry ice, and 10 mg of samples were collected for DNA extraction.

DNA extraction from stems was performed using the method described by Russo et al. (2020). DNA concentration was estimated by measuring ultraviolet absorption at 260 and 280 nm to assess DNA purity using a Nanodrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All samples were diluted at 10 ng·μL^{-1} for the real-time PCR analysis. Real-time PCR amplifications were performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) according to the protocol described by Licciardello et al. (2006). 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 (kindly provided by Professor Vittoria Catara, University of Catania, Italy) and diluted serially in sterile distilled water. The cycle threshold (Ct) values obtained from the individuals were used for statistical analysis.

Artificial inoculation and phenotyping in the greenhouse. Five to 10 cuttings of all hybrids plus the parents were collected from the mother plants grown in a screen house from May 2021 until May 2022, self-rooted using a commercial powdered rooting hormone (Rigenal P PFnPE; VAR5, Cifo, Italy), and cultivated in a growth chamber under controlled conditions (25 °C and 16/8h of light/dark) for ≈2 months to facilitate rooting. Only cuttings of 65 hybrids developed roots on two or more biological replicates and showed vigorous growth. The replicates of these 65 hybrids were transplanted in 2-L pots using a commercial substrate (Pindstrup citrus mix; Pindstrup Mosebrug A/S, Denmark) and were grown in an unheated greenhouse with temperatures ranging from 4 °C at night to 27 °C during the day and relative humidity between 35% and 80%. Rooted cuttings were also obtained from the susceptible parent ('Femminello Siracusano 2KR'). We were not able to generate rooted cuttings of the resistant parent (*C. latipes*), so the three replicates used for artificial inoculation were obtained through grafting onto 1-year-old seedlings of Carrizo citrange.

Plantlets of the 65 hybrids plus the parents were stem-inoculated with the *P. tracheiphilus* Pt10 strain. Two to seven biological replicates per genotype were used and arranged randomly in the greenhouse. Inoculations were performed between Nov 2021 and Nov 2022 on the self-rooted plants with a stem length > 20 cm. The inoculum was prepared according to the method described by Russo et al. (2020). The phialoconidia were quantified in a Bürker counting chamber and the concentration was adjusted to 10^6·mL^{-1}. The inoculation was carried out by depositing 10 μL conidia suspension on wounds obtained by cutting the stem longitudinally with a sharp, sterile blade. One or two stems per plant were inoculated depending on the plant architecture, and wounds were protected with parafilm. The evaluation of symptoms started about 2 months after inoculation and was monitored every 2 weeks, for a total of three times. The following score was assigned to every survey: 0 = no symptoms, 1 = chlorosis of leaf veins, 2 = desiccation of stems or leaves. Different phenotype classes were attributed to the artificially inoculated plantlets compared with the plants in the field, because in the field we performed phenotyping for a longer period and on more developed plants, and we observed a more heterogeneous behavior in response to the infections.

Real-time PCR was performed on a subset of inoculated replicates (symptomatic and nonsymptomatic) belonging to 53 hybrids that, during the first year, reached a stem length > 20 cm, to correlate Ct values with the presence of symptoms and verify that the symptoms of chlorosis and wilting were a result of the progression of the pathogen in the xylem and not a result of other biotic or abiotic stresses. At the end of the phenotypic survey, the inoculated stem tissues were sampled 5 to 10 cm above the wound site, depending on the length of the stem. DNA isolation and real-time PCR analysis protocols were the same as described for field samples.

Data analysis. The spatial distribution analysis was conducted to estimate the field distribution of infection over the years of the evaluation. This was done in R software ver. 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) using the kde2d function for kernel density estimation in the MASS package (Venables and Ripley 2002) and using the ggplot2 package (Wickham 2016). The kernel density distribution analysis was overlaid with the plant positions in the field and plotted in a north-up orientation. Each plant was represented by a point on the plot, with the point color indicating the severity of the symptom.

For the analysis of symptom progression in the field trial, only the 128 hybrids planted in 2020 and phenotyped for three consecutive seasons were considered, whereas for the comparisons between field and greenhouse phenotyping, we considered the performance of the hybrids planted in 2020 and 2021. The analysis of symptom progression and the principal component analysis in the field trial were performed in R (R Foundation for Statistical Computing), using ggfortify (Tang et al. 2016), ggrepel (Slowikowski 2023), stats (R Foundation for Statistical Computing), and ggplot2 (Wickham 2016). Data analysis was also performed to compare field and greenhouse phenotyping and to correlate field Ct values with symptom scores in all field-planted genotypes. To perform the comparison of means, a value of 40 was assigned to all runs for which the fungus was undetectable. The analysis of the distribution of the phenotyping data was computed using the function modetest, and the corresponding density plot was performed using the multimode package in R ambient (Ameijeiras-Alonso et al. 2018). The mean values by genotype for all the collected data were determined using the aggregate function in stats. Considering that our dataset did not show

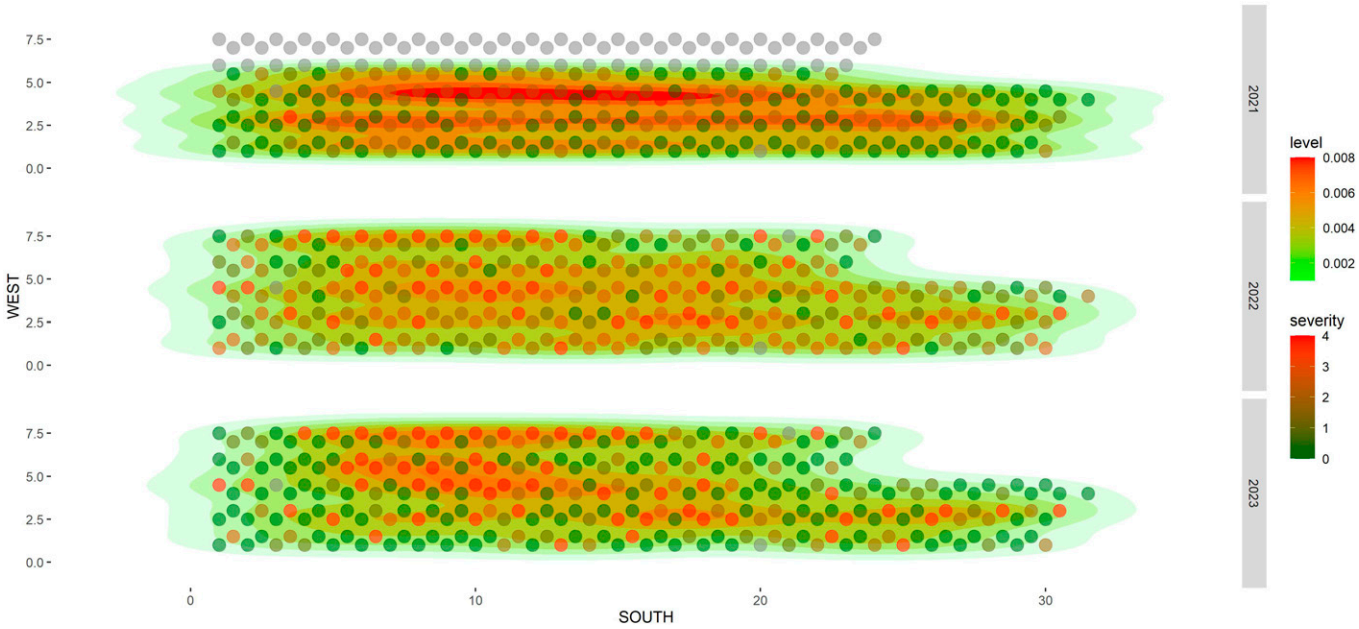

Fig. 2. Spatial distribution of symptomatic plants during the 3 years of field phenotyping. The different colors of the points from green to red (representing the plants) indicate different symptom severity. Levels were generated using a spatial kernel density estimation algorithm, and indicate the spatial distribution of infections. Red layers indicate greater concentrations of infected plants in specific areas of the plot. Gray points indicate missing plants.

a normal distribution, for the comparison between field and greenhouse phenotyping we converted the continuous values of the means of field phenotyping scores into two categories—tolerant and susceptible—and defined tolerant as all accessions showing a phenotyping score ≤ 1. These two categories were plotted against the means of phenotyping scores of the artificially inoculated plants. An analysis of variance was conducted using the anova_test function in the rstatix package (Kassambara 2023b) and then plotted into a boxplot using the ggboxplot and stat_*P* value_manual functions in ggpubr (Kassambara 2023a). The scatterplots were made using the ggscatter function of in ggpubr.

Results

Field phenotyping. The most severe infections were concentrated in specific areas in the middle of the plot, and the disease progression occurred mostly within twin rows (Fig. 2). However, plants with at least mild symptoms were evenly distributed throughout the plot, especially in 2022, confirming that the source of inoculum was distributed in the whole block. The inoculum spread from surrounding adult lemon trees severely affected by mal secco. Figure 2 also shows that 2022 was the year in which the symptoms were generally more severe. The severe symptoms observed in 2022 might have been favored by a hurricane-like storm in Oct 2021, followed by a hailstorm in Nov 2021, probably causing multiple wounds on leaves and branches, facilitating pathogen penetration.

Based on these findings, we decided to consider the replicate with the highest phenotyping score for each hybrid and each phenotyping time point to analyze symptom progression for 3 years. The analysis was performed on the 128 hybrids plus parents planted in 2020 and allowed us to distinguish them into three clusters (Fig. 3A). The first cluster included 37 hybrids in which at least one replicate tree was found with very severe symptoms or died of mal secco, and also included the susceptible male parent 'Femminello Siracusano 2KR'. All replicates of the male parent died within 2 years of observations. The second cluster included 42 hybrids with intermediate behavior. The third cluster contained the 49 hybrids that showed mild or no symptoms, and included Khasi papeda. Only one of the three replicates of Khasi papeda (the resistant parent) showed mild symptoms in 2021 and 2022 (maximum score = 2), and no symptoms in 2023, whereas the others showed no symptoms during the 3 years.

The progression of symptoms during the 3 years is shown in Fig. 3B. The first symptoms of mal secco appeared in early Spring 2021, ≈6 months after planting. During the first year, symptoms were observed in 109 of the 197 plants. During the second year, a dramatic increase in the number of symptomatic plants was observed. At the end of year 1, we were able to distinguish symptomatic vs. nonsymptomatic plants, whereas from 2022, three clusters were differentiated based on symptom severity. During the third year, we observed, on average, less severe symptoms than in 2022, likely resulting from less favorable climatic conditions for new infections or fungal movement in the xylem. Although the symptoms were less evident in the third year, the three major clusters were confirmed. A high correlation ($r = 0.8$; $P < 0.001$) was observed between symptoms observed in Jul 2022 and Aug 2023.

Real-time PCR was performed on all replicates of the 148 hybrids, and *P. tracheiphilus* DNA was detected in most of them, with average Ct values ranging from 19.5 (high infection) to 40 (no detection). Kashi papeda showed an average Ct value of 34 (low infection), whereas 'Femminello Siracusano 2KR' had a Ct value of 21.6 (high infection). When considering all hybrids, a significant but weak correlation of –0.48 ($P < 0.001$) was observed between symptoms and Ct values (Fig. 4). In addition, only 16 plants (including Khasi papeda) had Ct values ≥ 34, suggesting low colonization of the xylem. However, 6 of the 16 plants showed severe symptoms on the same date when sampling for DNA isolation was performed, confirming that Ct values did not always correlate with symptom severity. This was possibly a result of limits in the detection technique, compartmentalization of the fungus in specific plant sectors, or twig desiccation resulting from other biotic or abiotic stresses.

Greenhouse phenotyping and comparison with field phenotyping. Sixty-five of the 148 hybrids generated enough vigorous, self-rooted replicates to perform stem inoculations. The rest of the hybrids rooted poorly (n = 44) or did not root (n = 39) in the three attempts made with the same commercial rooting agent. Two hundred twenty-seven plants (217 replicates of the hybrids, 3 of *C. latipes*, and 7 of 'Femminello Siracusano 2KR') were stem-inoculated to compare the performance of each hybrid in the field under natural infection and were subjected to artificial inoculations in greenhouse conditions. Self-rooted replicates were obtained from different rooting cycles, and the results of the inoculations of Nov 2021 and Nov 2022 were merged to obtain the minimum quantity of biological replicates for each of the 65 hybrids. Symptoms were visible ≈2 months after inoculation, and started with leaf vein chlorosis and wilting, and progressed in some cases to twig desiccation (Fig. 5).

Phenotyping ended in Mar 2022 and 2023, when greenhouse temperatures were > 30 °C,

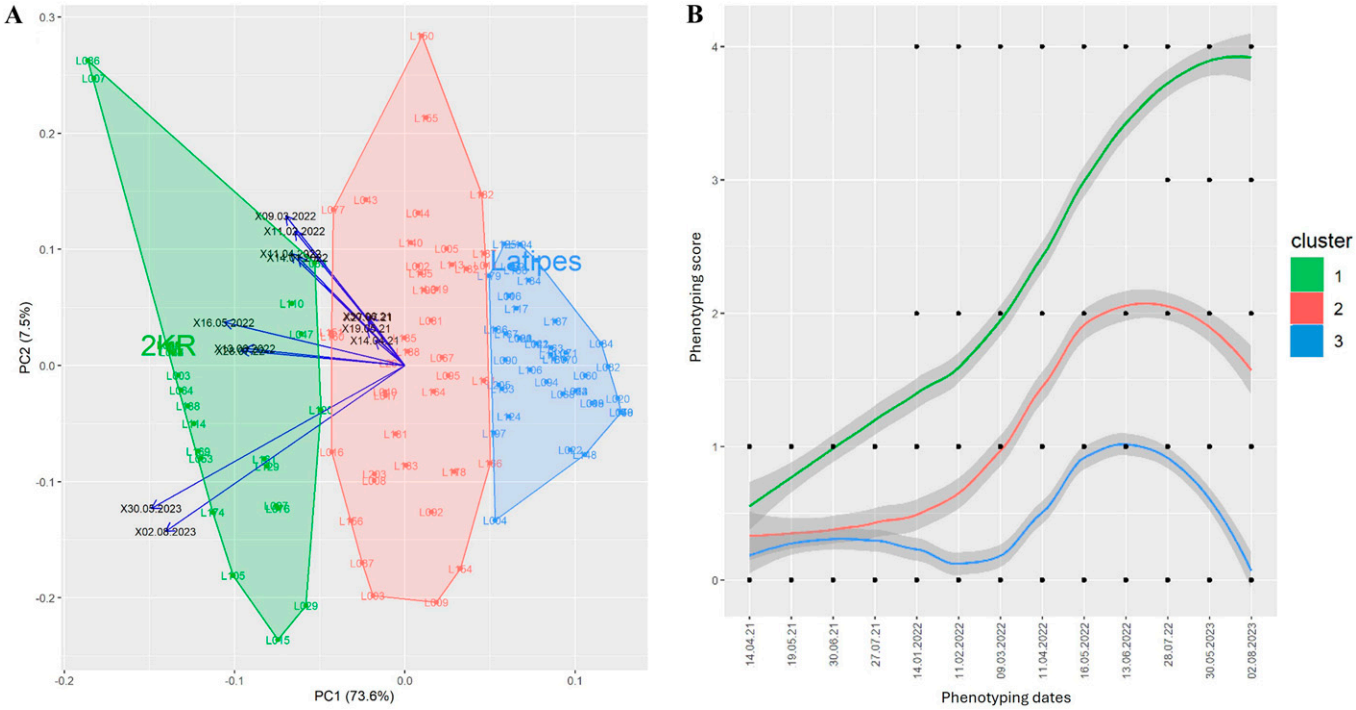

Fig. 3. Principal component (PC) analysis based on 3 years of phenotyping (**A**) and plot showing the progression of symptoms during the 3 years (**B**) in 128 Khasi papeda × lemon hybrids subjected to natural infections of *Plenodomus tracheiphilus*. Hybrids are divided into three clusters with different behavior in response to *P. tracheiphilus*: 1) susceptible, 2) intermediate, and 3) tolerant.

which were too high to allow further fungal movement in the xylem tissue. At the end of the phenotyping cycle, the typical pink discoloration of the wood was checked in the wound site and other portions of the stems (Supplemental Fig. 2).

Real-time PCR detection of *P. tracheiphilus* was performed 3 months after inoculation on a subset of 112 plants belonging to 53 hybrids inoculated in Nov 2021. The presence of the symptoms (leaf chlorosis or desiccation of twigs) showed a high negative correlation with Ct values ($r = -0.71$, $P < 0.0001$) (Arlotta et al. 2022). Consequently, visual symptoms were considered reliable enough to assess the resistance or susceptibility of the rest of the inoculated plants.

Plants were categorized as resistant (with no symptoms), moderately susceptible (showing the typical leaf vein chlorosis), or very susceptible (showing stem desiccation). Mean values of the scores of each hybrid subjected to artificial inoculation were compared with the ones of field phenotyping, where at least two replicate trees for each of the 65 hybrids were evaluated for at least two seasons. The boxplot in Fig. 6A shows the susceptibility to artificial inoculations of the hybrids categorized as tolerant in the field (score ≤ 1) vs. the hybrids categorized as susceptible (score > 1). Figure 6B shows the scatterplot comparing the same plants subjected to field and greenhouse phenotyping. The correlation was low but significant ($r = 0.41$, $P < 0.001$).

Discussion

We generated and evaluated a population of Khasi papeda (*C. latipes*; resistant to the disease (Russo 1977; Russo et al. 2020) × 'Femminello Siracusano 2KR' (*C. limon*; a very susceptible lemon cultivar (Starrantino et al. 1988) in response to natural and artificial infections of *P. tracheiphilus*. Our study provided important guidelines for reliable phenotyping aimed at the selection of resistant breeding material to be used in future backcrosses.

The two parents chosen for the generation of the population confirmed a contrasting behavior for mal secco disease resistance, as reported previously (Russo 1977; Russo et al. 2020). Two years after planting, all lemon replicates died, whereas the Kashi papeda ones were healthy and, in one case only, showed very mild symptoms that did not affect canopy development. Field phenotyping of 128 hybrids for 3 consecutive years clustered them into three groups (Fig. 3) based on symptom progression and revealed that 38.3% of them were classified as field tolerant.

There are few reports regarding the generation of lemon hybrids using distantly related citrus accessions or species (Carrante and Bottari 1952; Russo 1977; Starrantino et al. 1997) to introgress genes of mal secco resistance into commercial lemon varieties, and there is no clear information regarding the ratio between resistant and susceptible hybrids. Few studies hypothesized the segregation of resistance to mal secco in citrus populations with different genetic backgrounds. Reforgiato Recupero et al. (1997) analyzed the segregation of the incidence of natural infections in three populations resulting from the crosses of susceptible and resistant parents and hypothesized a multigenic control of the resistance. However, none of these populations were designed specifically to generate lemon varieties or involved true lemons. Tuzcu et al. (1992) reported the development of resistant lemon hybrids that were subjected to artificial inoculation at the seedling stage and suggested that the resistance was controlled by several recessive genes. However, it is not clear how the genetic basis of susceptibility was hypothesized.

The ratio between resistant and susceptible hybrids observed in the field evaluation seems to confirm the multigenic control of mal secco resistance, but our observed ratios differed substantially from those reported by Reforgiato Recupero et al. (1997). Specifically, the resistant individuals reported by them were the majority, even in the populations derived from susceptible accessions (sour orange and Volkamer lemon), whereas in our case, field resistance (with phenotyping scores ≤ 1) was observed in 27 of 148 individuals. This could be a result of 1) differences in the estimation of the symptoms, 2) different environmental conditions of the phenotyping sites, 3) different degrees of susceptibility of the parents and the progenies, 4) different ages of the plants, and 5) different disease pressure and erratic spread of the disease.

The severity of the symptoms was influenced by the spatial distribution of the plants. Specifically, we observed some areas of the field that could be considered infection sources for the nearby plants (Fig. 2). The infections spread mostly within rows, probably as a result of the closer distance of the plants within the twin rows compared with the one between twin rows. We also observed an underrepresentation of highly susceptible individuals

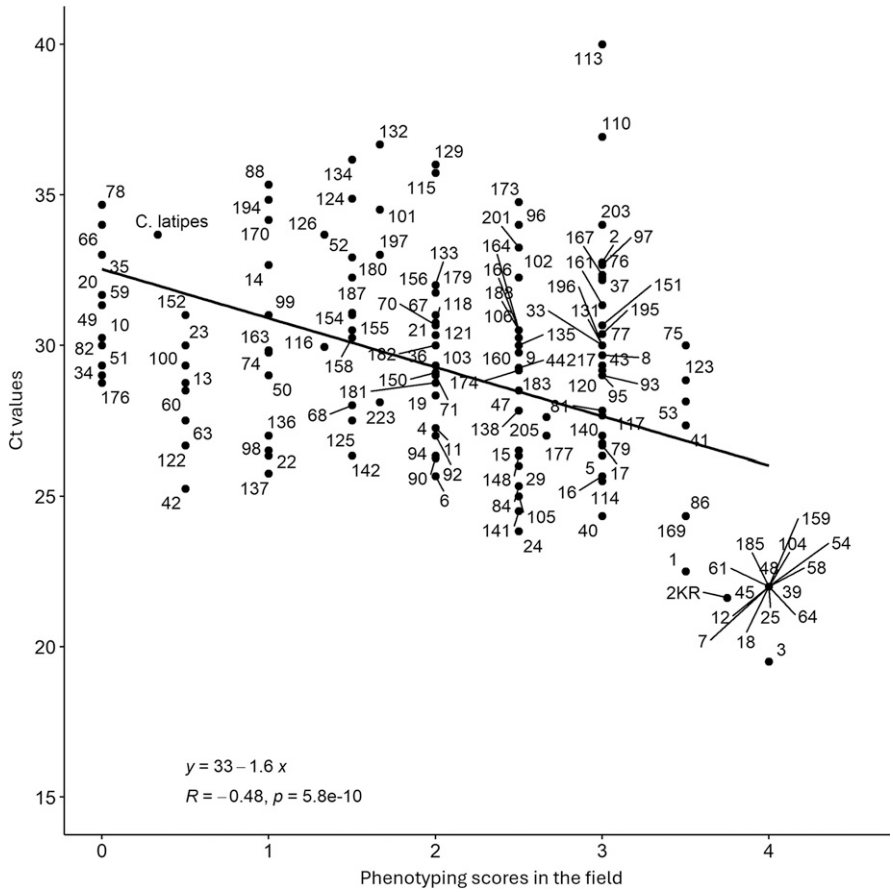

Fig. 4. Scatterplot with regression line showing the relationship between the scores of field phenotyping and real-time polymerase chain reaction cycle threshold (Ct) values for *Plenodomus tracheiphilus* detection in 148 Khasi papeda (*Citrus latipes*) × 'Femminello Siracusano 2KR' hybrids and their parental genotypes.

and the absence of clear infection sources in some specific rows, especially the first and the second (Fig. 2, bottom part of the plot). This is likely a result of the presence of walls (the borders of the experimental farm) near rows 1 and 2, which might have protected these plants from wounds caused by wind, reducing severe infections. The reduction in the percentage of highly susceptible plants in specific areas of the plot could also be a result of the absence of preexisting infected plants nearby. In agreement with our observations, Ben-Hamo et al. (2020) recently indicated that *P. tracheiphilus* could be spread, at least in some cases, preferentially within rows from plant to plant. The use of more than one replicate per hybrid, placed in different areas of the plot, allowed us to reduce errors in the estimation of susceptibility that could be caused by different spatial distribution of the pathogen. Given the differences between rows, and considering that one of our aims was to select promising resistant plants as breeding material for future backcrosses, we decided to assign to each hybrid the highest phenotyping score for the analysis of symptom progression (Fig. 3).

The severity of the symptoms also changed from year to year, indicating the need for multiyear phenotyping for this vascular disease. In 2022 we observed the most severe infections.

Fig. 5. Representative self-rooted hybrids showing no symptoms (**A**), leaf vein chlorosis (**B**), and twig desiccation (**C**) 3 months after artificial inoculations. Arrows indicate the inoculation sites.

The third season confirmed the results of the previous one (a clear differentiation of three clusters), although we observed milder infections, especially in the hybrids categorized as tolerant or intermediate. At the same time, the most susceptible plants died or confirmed their high susceptibility (Fig. 3B). Our results indicate that at least two seasons of field phenotyping are needed in our environmental conditions to estimate accurately the phenotypes of young lemon hybrids.

Ct values correlated negatively but weakly with symptom severity. Consequently, real-time PCR data are useful to confirm that wilting, desiccation, or other nonspecific symptoms are caused by *P. tracheiphilus*, but it was not helpful to provide additional information for a better phenotyping protocol. Ct values of the best-performing hybrids (with phenotyping scores between 0 and 1) ranged from 25 to 35 (Fig. 4). This result could suggest that the tolerant plants might use different mechanisms to cope with *P. tracheiphilus* infection. This aspect requires further investigation.

Considering the length of field phenotyping, and the space needed to test a high number of hybrids, another aim of our work was to test an artificial inoculation protocol on the same population tested in the field, as an alternative to field phenotyping. In the absence of molecular markers associated with tolerance to the disease, a rapid phenotyping protocol based on artificial inoculation could speed up the selection of tolerant individuals and reduce the number of plants subjected to field evaluation.

Results comparing a subset of hybrids revealed many discrepancies between field and greenhouse phenotyping. In our conditions, artificial inoculations confirmed the susceptibility of several hybrids (Fig. 6B, top-right corner of the plot) and the tolerance of a small group of plants (Fig. 6B, bottom-left corner); however, the correlation between the two methods was low. An example of the discrepancies between field and greenhouse phenotyping is represented by the behavior of the susceptible parent. The 'Femminello Siracusano 2KR' replicates in the field died within 2 years, whereas the self-rooted replicates subjected to artificial inoculations showed milder symptoms on average, which could result from the failure of inoculation (Gentile et al. 2000) or, more likely, those plants might have needed more time to develop symptoms after artificial inoculation (Arlotta et al. 2022). Our results suggest that phenotyping based on our artificial inoculation method could be useful to test a large number of plants before field evaluation, but it cannot replace field phenotyping. The low correlation between field and greenhouse results could be, primarily, a result of 1) different environmental and growing conditions, 2) differences in plant age and canopy development, and 3) differences in the duration of phenotyping. Moreover, plants in the field might have been subjected to multiple natural infections of *P. tracheiphilus* during the 3 years, whereas a single infection occurred in the greenhouse.

It could be possible that the use of more efficient phenotyping facilities and the growth in

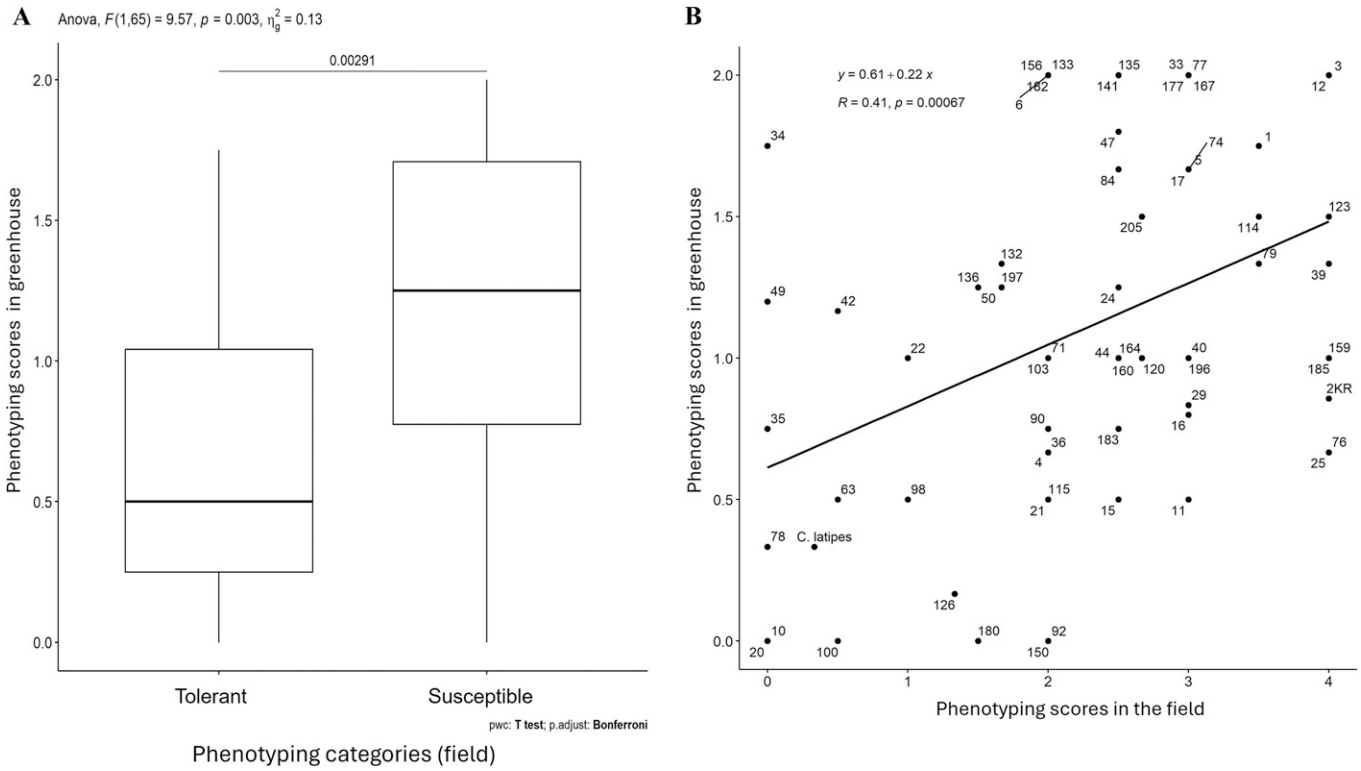

Fig. 6. Boxplot (**A**) and scatterplot with regression line (**B**) showing the relationship between symptoms observed in the field caused by natural infections and symptoms caused by artificial inoculations on self-rooted replicates in 65 Khasi papeda × lemon hybrids and their parental genotypes. Anova = analysis of variance; 2KR = Femminello Siracusano 2KR lemon; C. latipes = *Citrus latipes*; p.adjust = adjusted *P* value; pwc = pairwise comparison.

more controlled conditions (controlled temperatures, artificial light, and so on) could promote stronger and quicker symptom development, improving the efficiency of the screening.

Conclusion

In our conditions, field phenotyping remains the best method to estimate the degrees of susceptibility of a lemon population and to identify new, useful sources of resistance. Field evaluation could be influenced by the position of the hybrids in the plot, but this influence can be reduced using two or more replicates per hybrid. The combination of field and greenhouse phenotyping allowed the selection of promising hybrids that could be used for lemon breeding programs for mal secco resistance. Although the evaluation of two replicates in the field is not enough to confirm full resistance of the hybrids, our strategy allowed us to identify the most susceptible individuals. The resistance of the promising hybrids is still under evaluation on three additional replicates that were planted in the same field in Sep 2022.

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