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ORCID:
GC: 0000-0002-0519-1200
CC: 0000-0002-1748-3294
BEB: 0000-0002-5562-6751
SS: 0009-0000-2889-9140
MDG: 0000-0003-3339-2007
GD: 0000-0002-6080-6466
SLM: 0000-0001-5869-0348
AG: 0009-0005-1312-4808
GP: 0000-0001-8630-2760

Research Papers

Susceptibility of *Citrus* germplasm to canker caused by *Neofusicoccum parvum*

Giorgio GUSELLA, Chiara CATALANO*, Bassma EL BAKKOURI, Sebastiano SEMINARA, Mario DI GUARDO, Gaetano DISTEFANO, Stefano LA MALFA, Alessandra GENTILE, Giancarlo POLIZZI

Department of Agriculture, Food and Environment (Di3A), University of Catania, via S. Sofia 100, 95123 Catania, Italy.
*Corresponding author. E-mail: chiara.catalano@unict.it

Summary. *Citrus* trees are important for fruit production, and canker pathogens can be constraints to tree productivity. In Italy, field surveys showed presence of different pathogens causing trunk and branch cankers, gummoses and shoot blight. Among these pathogens, *Neofusicoccum parvum* is aggressive and widely distributed. Susceptibility of 47 *Citrus* genotypes was assessed using detached twig assays and an *in planta* assessment. The detached twig assay identified mandarin as the most susceptible variety (mean lesion length = 3.80 cm), followed by citrumelo 'Swingle' (2.83 cm), and lemons (2.47 cm), whereas grapefruits (1.34 cm) and citrons (1.76 cm) had greatest levels of tolerance. These results were consistent with those obtained in the *in planta* assay, in which grapefruits showed greatest tolerance (mean lesion length = 1.24 cm), while the lemon cultivars 'Monachello' (7.32 cm) and 'Femminello Siracusano 2Kr' (6.48 cm) were confirmed as the most susceptible genotypes. This study provides the first overview assessment of susceptibility of different *Citrus* species to infection by *N. parvum*.

Keywords. *Botryosphaeriaceae*, citrus gummosis, phenotyping, resistance, detached twig assay, in planta assay.

INTRODUCTION

Citrus species are important tree crops in terms of total production and harvested areas (FAOSTAT, 2025). Among the top producing countries, China, Brazil, and India account for the greatest production of tangerines, oranges and lemons (Pereira Gonzatto and Scherer Santos, 2023). In Europe, Spain and Italy are key citrus fruit producers (Beshara *et al*., 2024). In Italy, the southern regions of Sicily, Calabria and Campania are the most suitable areas for citrus cultivation, with Sicily having the largest plantations (Ciriminna *et al*., 2024). These regions produce important local citrus products that are protected by the EU geographical indications system, including protected designation of origin (PDO) and protected geographical indication (PGI). Historically, Italian citrus orchards have been affected by severe diseases, including the "mal secco" caused by *Plenodomus tracheiphi-*

lus (homotypic synonym, *Phoma tracheiphila*), and the virus disease "Tristeza" caused by *Closterovirus tristezae* (formerly citrus tristeza virus; CTV). These pathogens shortened orchard lifetimes and severely reduced economic returns for growers (Catalano *et al*., 2021; Leonardi *et al*., 2023; Sun *et al*., 2024).

Citrus and other perennial crops, represent long-term grower investments, as productivity increases and stabilizes several years after orchard establishment, and then lasts for many years. Canker pathogens are severe threats to fruit orchard lifespans. Canker-causing pathogens usually do not completely compromise annual production, but overall yields are reduced over time (Moral *et al*., 2019).

Although canker pathogens can infect many *Citrus* species, these fungi in Italy were restricted to lemon, with only a few reports on other citrus (Scaramuzzi, 1965). However, canker caused by fungi has long been recognized in other countries (Adesemoye *et al*., 2014; Mayorquin *et al*., 2016; Vakaloumakis *et al*., 2019; Bezerra *et al*., 2021; Kayim *et al*., 2024; Gusella *et al*., 2025), but studies focused on the citrus canker etiology. *Botryosphaeriaceae*, *Diaporthaceae*, and *Diatrypaceae* have been identified as the main taxonomic groups involved on the hosts *Citrus limon*, *C. paradisi*, *C. sinensis*, *C. latifolia*, *C. unshiu*, *C. aurantifolia*, *C. medica*, *C. aurantium*, *C. reticulata*, *Microcitrus australasica*, citrumelo Swingle, and citrange Carrizo (Adesemoye *et al*., 2014; Mayorquin *et al*., 2016; Vakaloumakis *et al*., 2019; Bezerra *et al*., 2021).

In Italy, recent investigations conducted in different citrus production areas, on crops of *C. limon*, *C. bergamia*, *C. sinensis* × *C. clementina*, *C. aurantium*, or *C. paradisi*, showed a wide fungus pathogen diversity associated with branch and trunk cankers, referred to as "Bot gummosis disease". *Neofusicoccum parvum* (*Botryosphaeriaceae*) was in the most common pathogen in all the surveyed areas and was the most aggressive in the pathogenicity tests (Aloi *et al*., 2021; Gusella *et al*., 2025). Other species, in addition to the well-recognized *Botryosphaeriaceae* spp., have been characterized and were particularly aggressive, particularly *Boeremia exigua* and *Neocosmospora metavorans* (Gusella *et al*., 2025). *N. parvum* was isolated from symptomatic hybrids of *C. sinensis* × *C. reticulata* in Greece (Vakalounakis *et al*., 2019), mandarin in Turkey (Kurt *et al*., 2025), sweet orange in California (Adesemoye *et al*., 2014), and Satsuma mandarin in China (Xiao *et al*., 2021).

Phenotypic screening of plant species and/or cultivars, based on artificial inoculations, has been an important starting point for research in plant breeding and plant pathology. For *Botryosphaeriaceae* pathogens, and *N. parvum* in particular, information on host susceptibility is currently available only for a limited number of *Citrus* species. Specifically, the rootstock citrumelo Swingle (*Poncirus trifoliata x Citrus paradisi*) is considered to be tolerant to *N. parvum*, while 'Piretto' and 'Lia Kritis' citrons have intermediate tolerance, and 'Femminello Siracusano 2Kr' and 'Monachello' lemons are considered to be susceptible to Bot gummosis disease (Vakaloumakis *et al*., 2019; Aloi *et al*., 2021).

The objective of the present study was to assess the phenotypic responses of 47 *Citrus* genotypes to the infection by *N. parvum,* using detached twig assays and *in planta* inoculations. This allowed us to verify consistency between the two inoculation approaches and evaluate their respective strengths and limitations in plant pathology research, and for phenotypic screening for marker-trait association studies.

MATERIAL AND METHODS

Plant material

Responses to infection by *N. parvum* were investigated in 47 *Citrus* genotypes. Thirty-eight were used in detached twig assays and 24 were assessed in an *in planta* assay, with 15 of the genotypes used in both experiments (Table 1). Plant samples for the detached twig assays were collected from the *Citrus* germplasm collection located at the Experimental farm of the University of Catania (UniCt) (Sicily, Italy; latitude, 37°24′33″N; longitude, 15°03′20″E; altitude, 10 m asl). The UniCt germplasm collection is mainly composed of local varieties complemented with some of the most widely cultivated at national and international levels. Plants were all of similar age (~ 15-years) and were maintained in four replicates under standard agronomic practices. The plants used for the *in planta* assay were supplied by a local citrus-specialized nursery, were maintained in open-air conditions, and were of 2-years-old after grafting. They were grafted onto sour orange rootstock and grown in 5 L capacity pots containing a mixture of natural soil and peat.

Detached twig assays

Detached twig inoculations were conducted as follows: a total of eight growing twigs (each approx. 20 cm long), lignified but still green, were sampled from three plants (biological replicates) of each selected host genotype. The sterilization and inoculation procedures were conducted according to Catalano *et al.* (2025), as outlined in Figure 1.

Table 1. List of the plant genotypes used in detached twig and/or *in planta* assays.

Species	Genotype	Detached twig assays	*In planta* assay
Sweet orange (*C. sinensis*)	Valencia		x
	Ovale		x
	Navelina	x	x
	Lane Late	x	
	Tarocco Sant'Alfio	x	
	Tarocco Meli	x	
	Washington navel	x	
	Tarocco Sciré	x	x
Sour orange (*C. aurantium*)	Sour orange	x	
Mandarin and mandarin-like hybrids (*C. reticulata*)	Tardivo di Ciaculli (6022A2 clone)	x	x
	Nova	x	x
	Tacle	x	x
	Clementine	x	x
	Tardivo di Ciaculli o.l. (old line)	x	
	Clementine tardivo	x	
	Mandalate	x	
	Comune		x
Lemon (*C. limon*)	Sfusato Amalfitano		x
	Sfusato di Procida		x
	Interdonato	x	x
	Femminello Zagara Bianca	x	x
	Femminello Siracusano 2Kr	x	x
	Monachello		x
	Femminello Siracusano m296	x	
	Quattrocchi	x	
	Fino	x	
	Femminello Fior d'Arancio	x	
	Monachello Continella o.l.	x	
	Femminello Adamo	x	
Citron (*C. medica*)	Diamante		x
Citron-like (*C. limoni-medica*)	Vozza_vozza (lemon *x* pummelo hybrid)	x	
	Spatafora	x	
	Piretto	x	x
	Limone Rosso		x
Grapefruit (*C. paradisi*)	Ruby	x	x
	Star Ruby	x	
Bergamot (*C. bergamia*)	Femminello	x	x
	Castagnaro	x	
	Fantastico	x	x
Lime, limetta and their hybrids	Lime (*C. aurantifolia*)	x	x
	Eustis (limequat, *C. aurantifolia x F. japonica*)	x	
	Limetta Romana (*C. limetta*)	x	
	Palestina sweet lime (*C. limettoides*)	x	
Fortunella spp.	Kumquat (*F. japonica*)	x	x
	F. obovata	x	
Other	Citrumelo Swingle (*Poncirus trifoliata x C. paradisi*)	x	
	Meyer (*C. meyerii*)		x

Inoculum consisted of *N. parvum* mycelium/agar plugs (5 mm diam., obtained using cork borer), derived from active 7-d-old colonies *N. parvum* isolate CT84, grown on potato dextrose agar (PDA; Lickson). Isolate CT84 was previously molecularly characterized by Gusella *et al.* (2025). Eight twigs were used per host genotype. Inoculation points were not sealed to allow further manipulation of detached shoots, since the humidity inside the plastic boxes containing the twigs was sufficiently high for infection establishment. The plastic boxes (20 × 15 × 8 cm) were each filled with approx. 20 g of sterile perlite, to which 200 mL of water was added to maintain optimal humidity, and were used as humid chambers for the inoculated hos twigs. The plastic boxes were then moved into a growth chamber, set at 25 °C (± 1 °C), and a 12 h light 12 h dark cycle. r photoperiod at 25 ± 1 °C for five days. Wounded twigs inoculated with non-colonized sterile PDA plugs served as negative inoculation controls. Five days after inoculation, photographs of the inoculated stems were taken, and lesion lengths were assessed using the software ImageJ (Schneider *et al.*, 2012). Representative shoots were randomly collected from ten genotypes (three twigs per genotype) after disease assessments, and fungus re-isolations were conducted to determine if Koch's postulates were fulfilled. Re-isolations was conducted as follows: discoloured parts of twig tissue (0.5 × 0.5 cm) were excised, then surface sterilized for 1 min. in 1.5% sodium hypochlorite (NaOCl) solution, rinsed in sterile distilled water, dried on sterile absorbent paper, and then placed in Petri plates containing PDA amended with 100 mg L^{-1} of streptomycin sulfate to prevent bacterial growth. The Petri plates were then incubated in the dark at 25 (± 1 °C) for 3-5 d. until fungus colonies emerged and were ready for morphological examinations.

In planta *inoculations and disease assessments*

An *in planta* assay was carried out using a total of 24 plant genotypes (Table 1). Three plants (biological replicates) were used for each genotype, and three twigs

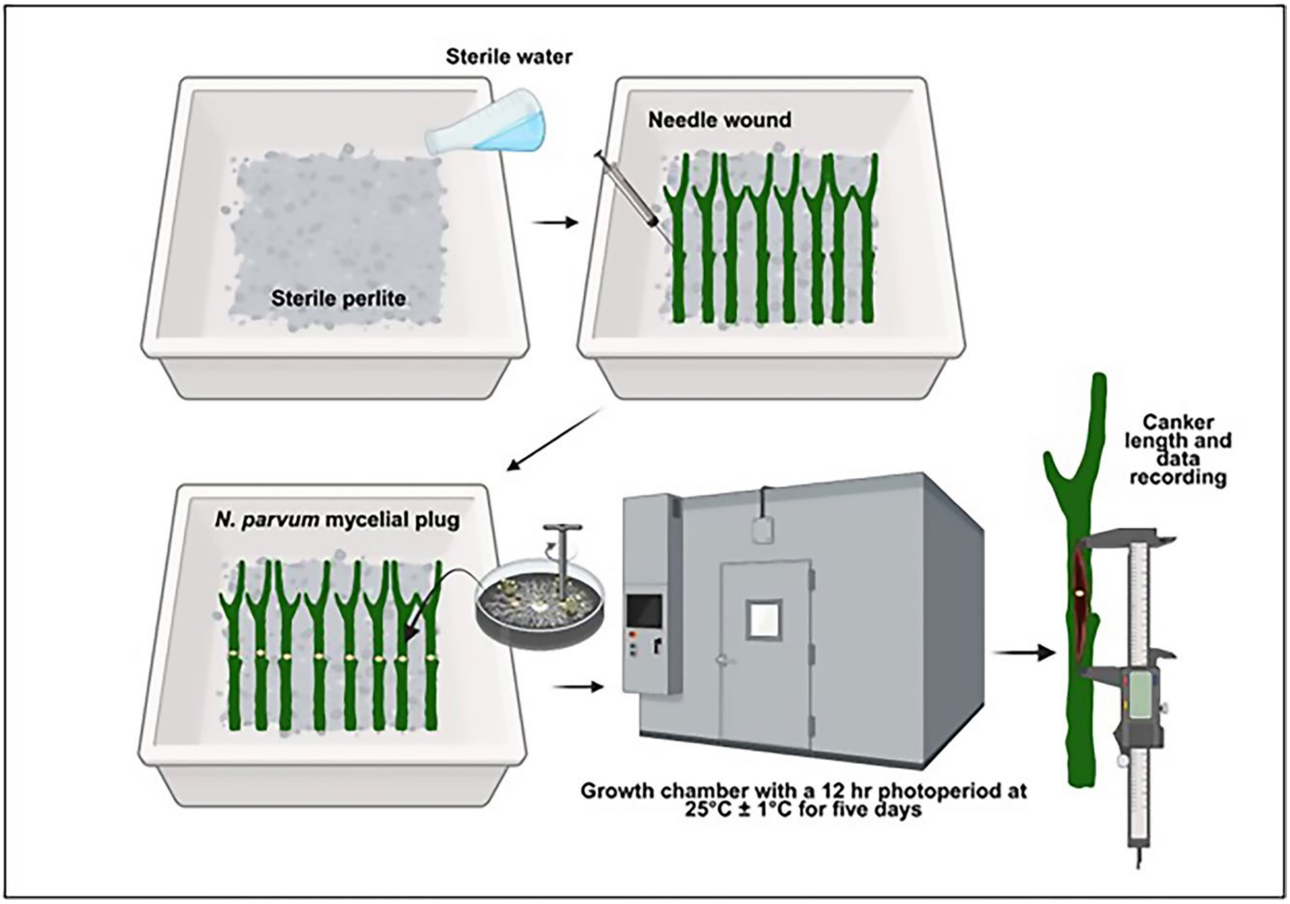

Figure 1. Scheme for detached twig assay procedure. Graphic created using BioRender.com.

were inoculated for each plant. Inoculations were conducted using the mycelium plug technique (described above) and *N. parvum* isolate CT84. The plants were maintained outside, to expose them to high humidity conditions required for infection, and the inoculation site on each plant was sealed with plastic film. Disease assessments were carried out 15 d after the inoculations. Inoculation controls consisted of plants (one for each genotype) that were wounded and inoculated with sterile PDA plugs. Plants were visually inspected, lesion lengths were measured using a caliper, and presence of gummosis, wilting, blight, and pycnidia were recorded for each plant genotype. The plants were kept outside, were regularly watered, and temperature and humidity were monitored using a datalogger. Re-isolations were carried out (as described above) for a randomly selected group of inoculated plants.

Statistical analyses of data

Descriptive statistical analyses (means, standard deviations, standard errors) were obtained using the 'stat' package, and plots using the 'ggplot2' package, both within R software (version 4.1.0, R Core Team, 2021, Wickham *et al.*, 2016). Levene's test (median-centered) indicated heteroscedasticity among genotypes ($P = 0.007$). Therefore, the data were analyzed using linear mixed-effects models to account for variance heterogeneity and biological replication, using the 'lme4' package in R. Data analyses were carried out separately for each host species and genotype, to account for potential species-specific responses to *N. parvum* inoculations. A subset of 15 host genotypes, which were tested with using the detached stem and *in planta* methods, was further analyzed to evaluate agreement between the two inoculation methods, for classifying citrus genotypes as low susceptible (LS), mild susceptible (MS), high susceptible (HS), and very high susceptible (VHS). To classify genotypes into these susceptibility classes, an unsupervised k-means clustering approach was applied to the mean disease severity values obtained from the detached twig and *in planta* inoculation assays. For each dataset, k-means clustering was carried out with $k = 4$, and the resulting cluster centroids were ordered from least to greatest susceptibility. Class boundaries were then defined as the midpoints between adjacent centroids, yielding the four ordinal categories (Supplementary Table 1). Each genotype was assigned independently to one of these categories for the two inoculation methods. Agreement between the detached and the *in planta* classifications was subsequently evaluated using Cohen's Kappa coefficient, which quantified the level of concordance between the four category ratings while adjusting for chance agreement. These analyses were carried out using the *irr* and *classInt* packages in R.

RESULTS

Detached twig assays

Necroses due to *N. parvum* infections were clearly visible 5 d after inoculations, when samples were assessed (Figure 2). The calculated mean lesion lengths were varied between the host genotypes, ranging from 1.24 cm (± 0.41 cm) for the grapefruit 'Ruby' to 5.66 cm (± 0.49 cm) for the mandarin 'Clementine' (Figure 3 A). Eighteen genotypes out of the 37 assessed had mean necrosis lengths less than 2 cm, while the lemon 'Femminello Zagara Bianca' (mean = 3.07 ± 0.49 cm) and the mandarins 'Tacle' (4.1 ± 0.40 cm), 'Mandalate' (5.10 ± 0.49 cm), 'Tardivo di Ciaculli' (5.16 ± 0.42 cm), and 'Clementine' (5.66 ± 0.49 cm), developed lesions of lengths greater than 3 cm (Figure 3 A, Supplementary Table 2).

Mandarin was the most susceptible to *N. parvum* infections (mean lesion length = 3.80 ± 0.20 cm), followed by citrumelo Swingle (2.83 ± 0.46 cm), and lemons (2.47 ± 0.19 cm), while the most tolerant host were grapefruits (1.34 ± 0.34 cm) and citrons (1.76 ± 0.27 cm). Limes and citrons had medium to low susceptibility, with respective mean lesion lengths of 1.82 cm (± 0.29 cm) and 1.75 cm (± 0.27 cm) (Figure 3 B, Supplementary Table 3).

In all samples tested, re-isolations confirmed presence of the inoculated fungus *N. parvum*. Wounded control twigs inoculated with non-colonized sterile PDA plugs did not develop necroses.

In planta *assay*

In planta inoculations were carried out in open air, and a datalogger was used to monitor temperature and humidity during the assay (Supplementary Table 4). Average temperature and relative humidity when symptoms became visible were, respectively, of 19.8 °C and 62.1%. These conditions contributed to rapid onset of symptoms (Figure 2 B).

The lemons 'Monachello' (mean lesion length = 7.32 ± 1.04 cm) and 'Femminello Siracusano 2Kr' (6.48 ± 1.04 cm) developed the largest necrotic lesions (Figure 4 A). Sweet orange 'Valencia' had the smallest lesions (0.72 ± 1.04 cm) (Figure 4A; Supplementary Table 5). Most of the genotypes (18 of the 24 tested) had mean necrosis lengths greater than 2 cm.

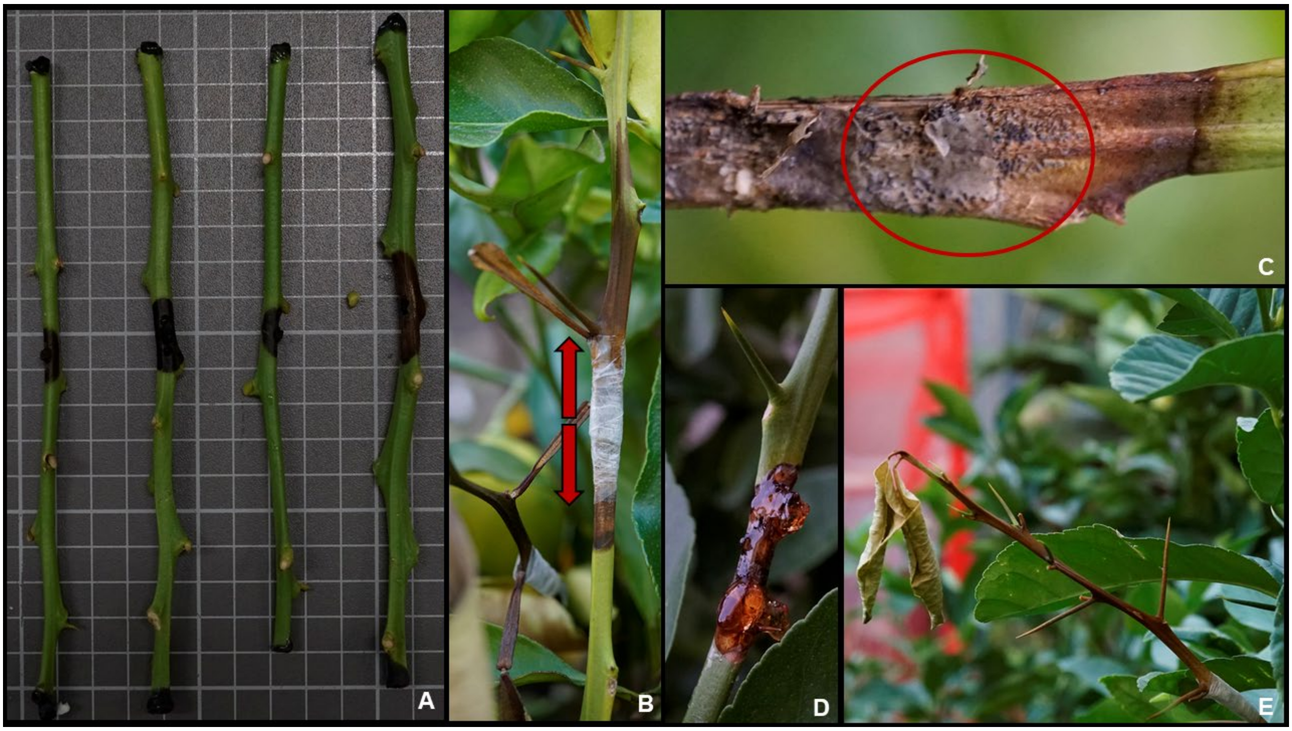

Figure 2. A) necroses developed from inoculation points on detached *Citrus* twigs (each background square is 1 cm^2). B) *In planta* assay showing necrosis upward and downward from an inoculation point sealed with Parafilm. C) Black pycnidia (red circle) developed on a necrotized area. D) Gum exudate from a canker lesion. E) Shoot blight on a plant shoot.

The *in planta* assay evaluated a total of eight host species. Grapefruits were confirmed to be the most tolerant to *N. parvum*, with a mean lesion length = 1.24 cm (± 1.30 cm), while the most susceptible were the *Fortunella* spp. Mean length = 5.14 cm; ± 1.30 cm) (Figure 4 B, Supplementary Table 3).

Re-isolations confirmed presence of *N. parvum* in all the processed samples, whereas the wounded non-inoculated control plants showed no necroses and only slight suberization at the wound sites.

Most genotypes (19 out of 24) exhibited gum production at inoculation sites in at least one replicate, whereas 'Comune' mandarin was the only genotype that produced pycnidia on the lesions without other symptoms (Supplementary Table 6, Figure 2, C to E). The mandarins 'Clementine', 'Tacle', and 'Tardivo di Ciaculli', and the sweet orange 'Tarocco Sciré', only developed blighting, with no pycnidia or gummoses. In contrast, the sweet oranges 'Navelina' and 'Ovale', and the lemons 'Femminello Siracusano 2Kr', 'Femminello Zagara Bianca', 'Interdonato', and 'Monachello', developed gummoses, pycnidia, and shoot blight.

Comparison of results from the two pathogenicity test methods gave a low positive correlation (Cohen's Kappa coefficient of 0.26). According to the Landis and Koch (1977) scale, this value corresponds to "fair agreement". The confusion matrix (Table 2) shows that seven genotypes were assigned to the same categories for the two inoculation methods, namely 'Ruby' grapefruit, 'Femminello' bergamot, lime, 'Nova' mandarin, 'Navelina' sweet orange, 'Femminello Zagara Bianca' lemon, and Clementine mandarin (Supplementary Table 7). Three genotypes were assigned into different susceptibility categories, with a difference of one category (the citron-like 'Piretto', 'Interdonato' lemon and 'Tacle' mandarin), four differed by two categories (kumquat, 'Fantastico' bergamot, 'Tarocco Sciré' sweet orange, 'Femminello Siracusano 2Kr' lemon), and one ('Tardivo di Ciaculli' mandarin) differed by three categories. Where discrepancies in susceptibility classification were observed between the two inoculation methods, susceptibility was generally greater in the *in planta* assays than in the detached twig assay. The only exception was 'Tardivo di Ciaculli' mandarin, which was classified as very high susceptibility in the detached twig assays but as low susceptible in the *in planta* assay.

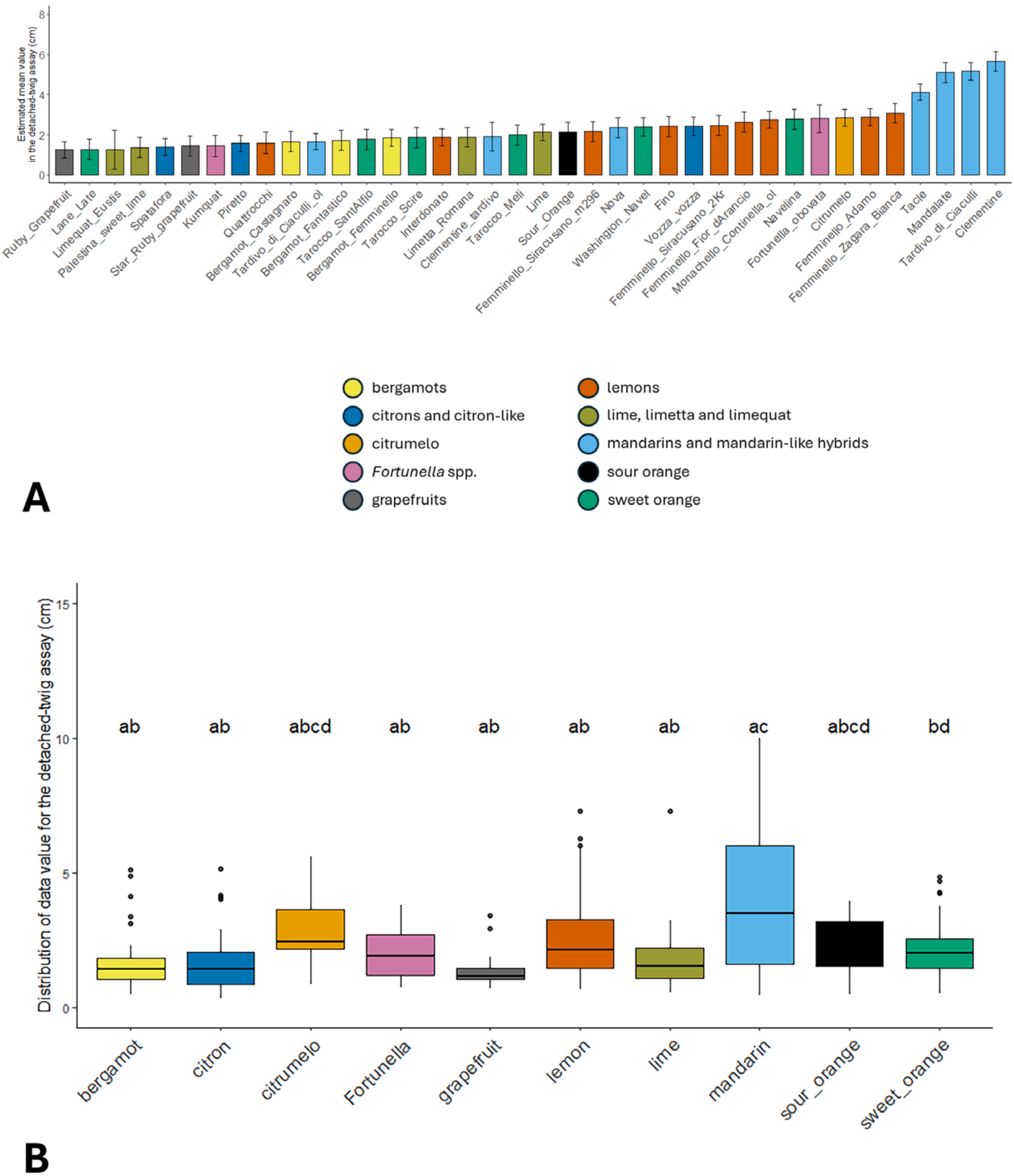

Figure 3. Barplots (A) and boxplots (B) showing results obtained in the citrus genotypes analyzed through the detached twig assay for susceptibility to *Neofusicoccum parvum*. In the boxplots (B), different letters accompanying the means indicate statistically significant differences ($P < 0.001$), according to *post hoc* Tukey HSD tests.

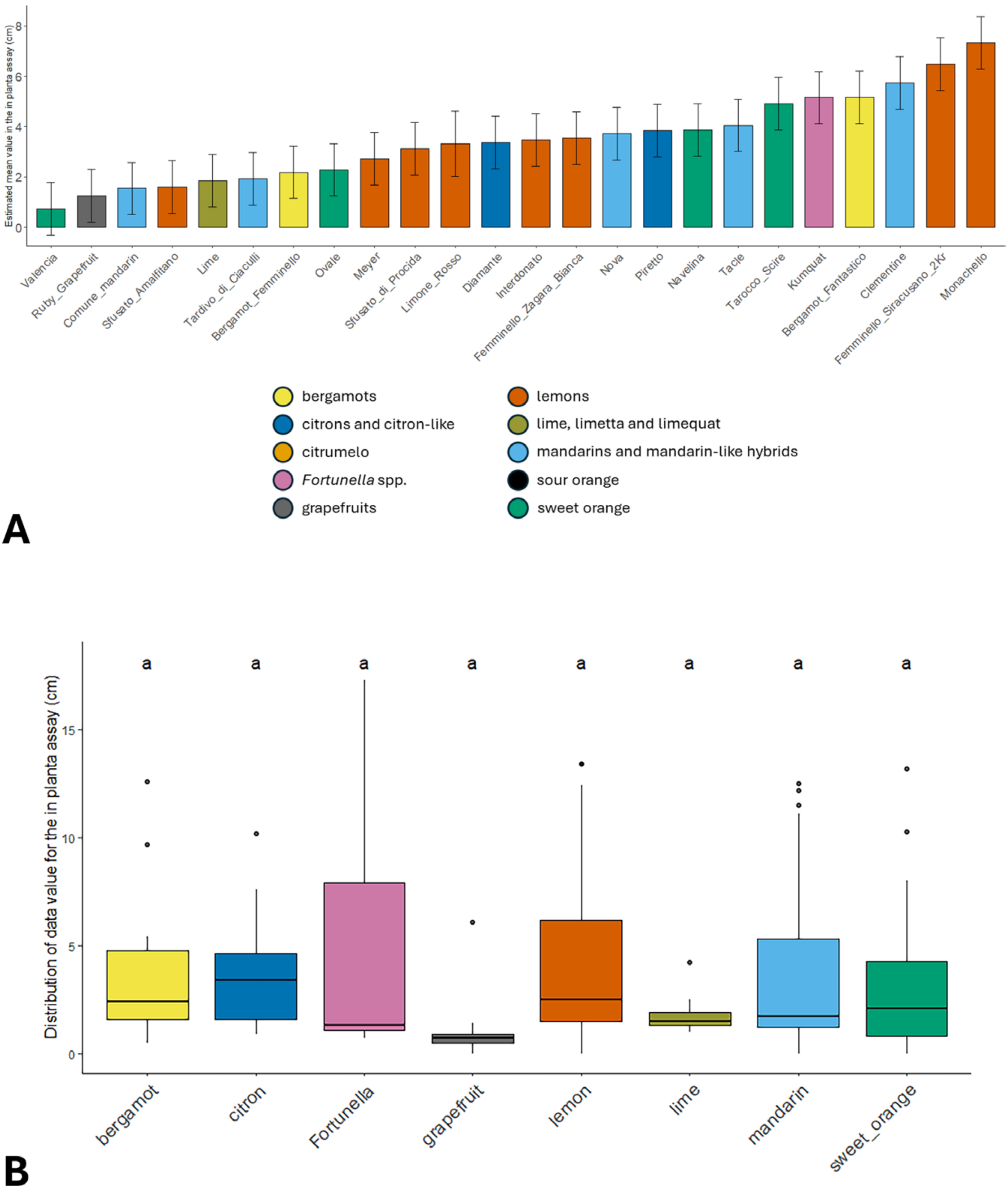

Figure 4. Bar plots (A) and boxplots (B) showing results obtained for *Citrus* genotypes analyzed using the *in planta* assays for susceptibility towards *Neofusicoccum parvum*. In the boxplots (B), different letters accompanying the means indicate statistically significant differences ($P < 0.001$), according to *post hoc* Tukey's HSD tests.

Table 2. Confusion matrix showing agreement between susceptibility class assignments obtained from detached twig assays and the *in planta* assay. Susceptibility classes are: low susceptible (LS), mild susceptible (MS), high susceptible (HS), and very high susceptible (VHS).

		Categorization in the *in planta* assay			
		LS	MS	HS	VHS
Categorization in the detached twig assays	LS	3	2	3	0
	MS	0	3	0	1
	HS	0	1	0	0
	VHS	1	0	0	1
Colour legend		same categorization	1 class difference in categorization	2 classes difference in categorization	3 classes difference in categorization

DISCUSSION

Previous field and laboratory studies conducted in Italy have elucidated the etiology of traditionally named "Dothiorella gummosis" of citrus plants, showing that more than one fungus is causes canker and canker-related symptoms in different *Citrus* species in Italy (Gusella *et al*., 2025). Symptoms of canker and gummoses along trunks and at branch insertion sites were attributed to *Diplodia natalensis* and *Dothiorella gregaria* (Eckert and Brown 2000; Klotz 1978; Savastano 1932). Among the fungi involved in these diseases, *N. parvum* has been the most common pathogen in all previously surveyed areas, showing also the greatest aggressiveness in pathogenicity tests (Gusella *et al*., 2025).

The present study is the first overview, using the experimental conditions described, of susceptibility of different citrus species to the *N. parvum* infections. Only sporadic and outdated observations have been reported in Italy (Aloi *et al*., 2021; Savastano 1932; Grasso and Polizzi, 1988). The present study is the first to assess *N. parvum* susceptibility of a large germplasm collection that includes many economically important species of economic interest, for their importance for fruit production and/or as ornamental plants or rootstocks. The citrus market is characterized by diverse products, geographical dynamism, and is important to the economies of many countries (Zhong and Nicolosi, 2020).

Investigations conducted on trunk and shoot canker and gummoses have mainly focused on disease etiology, but many fungi (*Botryosphaeriaceae*, *Diaporthaceae* and *Diatrypaceae*) have been isolated from symptomatic *Citrus* species and assessed for the pathogenicity (Adesemoye *et al*., 2014; Mayorquin *et al*., 2016; Vakaloumakis *et al*., 2019; Bezerra *et al*., 2021; Kayim *et al*., 2024; Gusella *et al*., 2025). Only no or limited data were available on susceptibility of different *Citrus* species. Susceptibility comparisons among different hosts have not been the immediate goal of etiology studies, which have concentrated on ascertaining the roles of the potential etiological agents isolated from samples. In the present study, the predominant role of *N. parvum* was determined in the etiology and distribution of disease (Gusella *et al*., 2025). The responses of broad and varying germplasm collection to this pathogen were evaluated.

Results from these experiments confirmed host/pathogen compatibility, and identified mandarins and mandarin-like species, and lemons, as the most susceptible to *N. parvum* under the experimental conditions used. Detached twig inoculation is a widely used technique, especially for preliminary pathogenicity tests or other similar experiments (León *et al*., 2020; Beluzán *et al*., 2022; Luo *et al*., 2022). This technique is simpler to set up than *in planta* inoculations, so was adopted for preliminary screening of large numbers of host genotypes. Only some representative host genotypes were selected for the *in planta* assay. Although the detached twig assay gave positive assay results, as also previously confirmed for almond inoculated with *Diaporthe amygdali* (Catalano *et al*., 2025), results from the *in planta* assay suggest that the detached twig inoculations need to be confirmed by *in planta* inoculations. The detached twig assays were reliable and rapid, as a starting point for large host phenotype screening, but should not be considered the only method for host screening.

Experiments conducted on walnut blight showed requirement for field testing in conjunction with detached leaf assays (Jiang *et al*., 2019). In contrast, for almond, tolerance levels assessed by detached twig and *in planta* assays were consistent (Catalano *et al*., 2025). In the present study with *Citrus* hosts, discrepancies between the two inoculation methods were detected. Seven genotypes were classified in the same susceptibility categories by the two inoculation methods, including 'Ruby' grapefruit, 'Femminello' bergamot, lime, 'Nova' mandarin, 'Navelina' sweet orange, 'Femminello Zagara Bianca' lemon and Clementine mandarin, while three genotypes were classified with one class difference (citron-like 'Piretto', 'Interdonato' lemon and 'Tacle' mandarin), four differed by two classes (kumquat, 'Fantastico' bergamot, 'Tarocco Sciré' sweet orange, 'Femminello Siracusano 2Kr' lemon), and one ('Tardivo di Ciaculli' mandarin) differed by three classes. Where these discrepancies occurred, susceptibility was generally greater in the *in planta* assay than in the detached twig assays, with the only exception being 'Tardivo di Ciaculli' mandarin, which was classified as very-high susceptible in the detached twig assay but as low susceptible in the *in planta* assay.

These discrepancies in assignment to different susceptibility classes emerged from the Cohen's Kappa coefficient calculations and the confusion matrix between the two inoculation methods, were probably a result of the small sample size that amplified differences between the class categorizations (Sim and Wright, 2005). The susceptibility classes were calculated independently for each assay, which may have further contributed to these differences. Plant susceptibility tests are generally considered more reliable when performed *in planta*, as detached assays, or assays conducted on excised plant tissues, may lead to overestimation of host plant susceptibility (Orlowska *et al*., 2013). This was not the case in the present study, where susceptibility was consistently greatest for particular host genotypes when inoculated *in planta*, highlighting high aggressiveness of *N. parvum*.

The aim of the present study was to evaluate responses of the different *Citrus* germplasms to *N. parvum*, and both the methodologies used showed that all the tested plant lines were susceptible to infection by this fungus, but with differences in susceptibility. While *in planta* inoculations provide accurate assessment of susceptibility, the detached twig method enabled evaluation of many genotypes, so was suitable for preliminary large-scale phenotyping that could be used for crop breeding. The fair agreement between the two inoculation methods (Cohen's Kappa coefficient = 0.26) further supports the suitability of the detached twig assay for this application. The importance of *in planta* inoculation is also highlighted by the appearance of symptoms that were not observed in the detached twig assays, due to specific technical time within the protocol. Typical shoot blight, gum exudation and pathogen pycnidium formation were observed from the *in planta* inoculations.

Symptomatology of some non-lemon citrus species observed after artificial inoculations, compared to field surveys, could be due to several factors. Disease development in the field is unpredictable due to variable inoculum pressure in different groves, and the different environmental/agronomic conditions within each grove (Ben-Hamo *et al*., 2020; Veresoglou *et al*., 2013). Different host susceptibility responses have been observed elsewhere between controlled and field conditions (Jiang *et al*., 2019). Grapefruit was the least susceptible to *N. parvum* in both of the present study assays. Field observations (Gusella *et al*., 2025) showed that grapefruit is highly susceptible to another *Botryosphaeriaceae* species, *Neoscytalidium dimidiatum*, as also observed in California (Dr. Themis Michailides, personal communication). These observations highlight the difficulties for disease management, especially when etiologies are complex. Results from the present study confirm that all citrus are potentially susceptible to the canker-causing pathogen *N. parvum*, but, until now, the disease seems to be particularly evident and aggressive on lemon species in the region of this research. However, the polyphagous behaviour of this pathogen, its wide distribution, the susceptibility of different *Citrus* species, and the intercropping of citrus in the local territory, as well as in many Mediterranean environments, emphasize the risks from potential inter-species infections.

CONCLUSIONS

This study has provided the first assessment, using the described experimental conditions, of susceptibility of a broad group of *Citrus* genotypes to the important pathogen *N. parvum*. Although only one isolate of this fungus was used, the results provide insights into the responses of host germplasm to *N. parvum* infection. Nevertheless, a broader picture of susceptibility could be obtained by testing multiple fungal isolates, since populations of a fungal species consist of numerous isolates that may differ in their levels of virulence. Our research also establishes practical criteria for reliable phenotyping, forming a solid basis for future screening efforts aimed at citrus genetic improvement. While the detached twig assay represents a valuable tool preliminary for large-scale phenotyping, *in planta* assay confirmed to provide a more accurate assessment of susceptibility. Our results showed that all tested genotypes are susceptible under artificial inoculation, revealing mandarins and mandarin-like species, as well as lemons, as the most susceptible. The variability in susceptibility observed suggests the possible presence of tolerance sources, which merits further investigation to elucidate the genetic determinants underlying citrus tolerance towards *N. parvum*.

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