



Article

Preliminary Findings of New Citrus Rootstocks Potentially Tolerant to Foot Rot Caused by *Phytophthora*

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Abstract: *Phytophthora* spp. are one of the most common soil-borne pathogens in citrus crops, in which *Phytophthora nicotianae* and *P. citrophthora* are the most relevant species, causing disease problems worldwide, such as foot rot and gummosis of the trunk, branch canker, brown rot of fruit, feeder root rot in orchards, and seedling damping-off in nurseries. *Phytophthora*-tolerant citrus rootstocks are essential for its control and for the success of the citrus industry. The aim of this study was to determine the susceptibility of new citrus rootstocks with low HLB incidence to *Phytophthora* diseases. Thus, plants of several commercial and new citrus rootstocks originating in different breeding programs were inoculated with an isolate of *P. nicotianae*. Thirty days post inoculation (DPI), the damage of lesion length in stem was measured for each plant. These results displayed a different susceptibility response to the damage caused by *P. nicotianae* among the citrus rootstocks tested. Thus, eleven new citrus rootstocks (B11R3T25, B11R5T25, B11R5T49, B11R5T60, B11R5T64, N40R1T18, N40R1T19, N40R3T25, WGFT + 50-7, UFR-6, and CL-5146), which have not been previously studied against *Phytophthora* diseases, improved the tolerance effect of Carrizo citrange. Our findings provide useful information for citrus growers on rootstock selection to address incidence problems caused by *Phytophthora* spp.

Keywords: breeding; citriculture; citrus diseases; crop protection; *Phytophthora* diseases; plant tolerance



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1. Introduction

The citrus industry has a great influence on Spanish agriculture. Spain is the top citrus producing country in the European Union and the sixth in the world, with a total production of over six million tons [1].

Phytophthora spp. are the most serious soil-borne pathogens of citrus trees worldwide, and they usually inhabit these crop soils. Ten species of this genus have been described as the causal agents of several citrus diseases, such as foot rot and gummosis of the trunk, branch canker, brown rot of fruit, feeder root rot in orchards, and seedling damping-off in nurseries [2–7]. Nevertheless, *P. nicotianae* (syn. *P. parasitica*) Dastur and *P. citrophthora* (R.E. Sm. & E.H. Sm.) Leonian are the most widespread and relevant species worldwide [5].

Foot rot and gummosis disease infection processes increase under rainy conditions with warm temperatures (20–25 °C). *Phytophthora* propagules can splash from the soil to the trunk through rain drops near the ground level, infecting wounds or growth cracks that produce plant tissue lesions along the trunk [8]. These infections typically initiate at the base of the trunk. Typical symptoms of this disease in trees include growth flush reduction, defoliation, and twig dieback. When the disease is aggressive, infected trees can die, which occurs more frequently in younger than old trees [6].

Phytophthora spp. management has usually been carried out with systemic fungicides from the group of phosphonates (Fosetyl-Al) and phenylamide (metalaxyl), which generate

colonization protection against *P. nicotianae* and *P. citrophthora* for three months [9,10]. However, new environmental trends in European agriculture are conducive to reduce the use of synthetic pesticides and lowering the presence of residues in food, with greater safety from chemical products for growers, and to avoid the emerging risk of pathogen resistance [11,12], such as species of *Phytophthora* [13]. In this sense, there is an additional need to shift agriculture towards sustainable methods that reduce supplies and costs.

In addition, cultural practices can be implemented in citrus orchards to reduce the incidence of diseases caused by *Phytophthora* spp. Thus, citrus growers apply different techniques to reduce excess water in the soil by means of drainage practices or an optimal irrigation dosage, as rainy and waterlogging conditions foster growth of this pathogen and dispersion and infection of its propagules to plant material [14]. These techniques play an important role in reducing propagation and development of the pathogen zoospores (main infective propagules) and mycelia, respectively. While *Phytophthora* spp. can produce other types of propagules, such as chlamydospores and oospores, which are potentially infective for plants, they mainly operate as latent structures of survival with a viability lasting several years. Thus, the use of pathogen-free plant material and disinfection of agricultural equipment and soil are required to avoid the occurrence of these resistant propagules [15]. However, all these cultural techniques are not fully implemented by growers and entail time-consuming and laborious works.

On the other hand, a proper choice of healthy and *Phytophthora* spp.-tolerant citrus rootstock has been reported as an essential factor to avoid these diseases in newly established orchards, as the rootstock confers tolerance to the whole plant against this pathogen [16]. Trifoliolate orange (*Poncirus trifoliata* (L.) Raf.) and its hybrid Swingle citrumelo (*Citrus paradisi* × *P. trifoliata*) have been described as tolerant to *P. nicotianae*; nevertheless, they are not demonstrated to be tolerant against *P. palmivora*. On the other hand, *Citrus volkameriana* is also reported as tolerant to *P. nicotianae* [17], and Carrizo citrange (*Poncirus trifoliata* × *Citrus sinensis*), which is the most commonly cultivated rootstock in Spain (approximately 61% of citrus orchards) [18], is described as having an intermediate level of tolerance to *Phytophthora* spp. [19]. On the contrary, other citrus rootstocks, such as Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) and Rough Lemon (*Citrus jambhiri*), are described as *Phytophthora* spp.-sensitive rootstocks [19,20]. In addition, the occurrence risk of emerging diseases, such as Huanglongbing or citrus greening disease (HLB), have increased in the Iberian Peninsula, due to the dispersal in Spain and Portugal of *Trioza erytreae* [21,22], which is one of the most important insect vectors of the HLB causal agents [23]. Currently, a wide range of commercial citrus rootstocks are available to citrus growers, and in recent years breeding programs are generating new plant material for the citrus industry. To our knowledge, many of these new citrus rootstocks with reported low disease incidence of HBL have not been evaluated against *Phytophthora* diseases yet. The main aim of this work was, therefore, to assess tolerance to foot rot caused by *Phytophthora* in new citrus rootstocks originating in different breeding programs.

2. Materials and Methods

2.1. Plant Material and Experimental Design

Fourteen-month-old citrus plants belonging to twenty-four different rootstocks from in vitro culture were provided by the Agromillora Group nursery (Subirats, Barcelona, Spain) (Table 1). The study was divided into two experiments due to the high plant number and production. Each experiment was carried out in the 2020 and 2021 spring seasons, respectively, as previous described [24], under greenhouse conditions (26 °C average temperature and 96% average relative humidity) located at the Las Torres Center in the Andalusian Institute for Agricultural and Fisheries Research and Training (IFAPA) in Alcalá del Río, Seville, Spain (37°30'43.3" N; 5°57'47.4" W). For each experiment, plants from each rootstock were separated into two treatments [inoculated and control (non-inoculated) plants], with eight replicates for inoculated plants and eight plants for non-inoculated. In all experiments, Carrizo citrange was used as the reference-comparative rootstock. Each

plant was grown in 1.6-L pots with a mix of one part of silica sand and two parts of peat moss substrate (*Sphagnum* moss, wood fiber, and perlite; Gramoflor; Vechta, Germany) kept under an acclimation period of eight months and irrigated with water thrice per week depending on water requirements, non-nutritive solution was applied. Before starting the experiment, plants were distributed under a randomized block design.

Table 1. Citrus rootstocks assayed against foot rot disease.

Rootstocks	Parentals	Ploidy	Origin	Ref.
Carrizo citrange	<i>Poncirus trifoliata</i> × <i>Citrus sinensis</i>	2x		[25]
<i>Citrus macrophylla</i>	<i>Citrus macrophylla</i>	2x		[26]
Forner-Alcaide No. 5	‘Cleopatra’ mandarin × <i>P. trifoliata</i>	2x	IVIA	[27]
UFR-1	Nova + HBP × Cleopatra + Arg trifoliata	4x tetrazyg	CREC	[28]
UFR-4	Nova + HBP × Cleopatra + Arg trifoliata	4x tetrazyg	CREC	[29]
UFR-5	Changsha mandarin + 50-7 trifoliolate orange	4x tetrazyg	CREC	[30]
UFR-6	‘Changsha’ mandarin + Trifoliolate orange 5	4x	CREC	[31]
WGFT+ 50-7	White grapefruit + Trifoliolate orange 50-7	4x	CREC	[32]
B11R3T24	<i>P. trifoliata</i> × ‘Duncan’ grapefruit	2x	CREC	
A + Volk × Orange19-11-8	<i>C. volkameriana</i> × (‘Nova’ + HBP × ‘Cleopatra’ mandarin + Argentine trifoliolate)	4x tetrazyg	CREC	[33]
AMB + CZO	<i>C. amblycarpa</i> + Carrizo citrange	4x	CREC	[34]
B11R5T25	<i>P. trifoliata</i> × ‘Duncan’ grapefruit	2x	CREC	
N40R1T18	<i>P. trifoliata</i> × LB 1-21 (Clementine × ‘Duncan’ grapefruit)	2x	CREC	
2247 × 2075-01-2	‘Nova’ + HBP × ‘Cleopatra’ mandarin + Swingle Citrumelo	4x tetrazyg	CREC	
N40R2T19	<i>P. trifoliata</i> × LB 1-21 (Clementine × ‘Duncan’ grapefruit)	2x	CREC	
N40R3T25	Flying Dragon × LB 1-21 (Clementine × ‘Duncan’ grapefruit)	2x	CREC	
B11R3T53	(‘Cleopatra’ mandarin × <i>C. ichangensis</i>) × USD	2x	CREC	
B11R5T49	Flying Dragon × Ridge Pinneapple sweet orange	2x	CREC	
B11R5T60	Flying Dragon × Ridge Pinneapple sweet orange	2x	CREC	
CL-5146	<i>C. sunki</i> × <i>C. Wingie</i>	2x	CIRAD	
2247 × 6070-02-2	Nova + HBP × Sour orange + <i>P. trifoliata</i>	4x tetrazyg	CREC	
Orange-14	Nova + HBP × Cleopatra + Arg trifoliolate orange	4x tetrazyg	CREC	[35]
B11R3T27	Flying Dragon × duncan grapefruit	2x	CREC	
B11R5T64	Flying Dragon × Ridge Pinneapple sweet orange	2x	CREC	

2x: diploid; 4x: tetraploid somatic hybrid; 4x tetrazyg (origin from crosses of allotetraploid somatic hybrids); CREC: Citrus Research and Education Center (Florida, USA); CIRAD: Centre de Coopération Internationale en Recherche Agronomique pour le Développement (France). Ref.: References.

2.2. Isolate of *Phytophthora*

The isolate of *Phytophthora* (Pn1) was supplied from the fungal collection of Instituto Valenciano de Investigaciones Agrarias (IVIA). DNA identification was carried out to confirm the genus and specie of this oomycete. First, the isolate was grown over cellophane membrane on Petri dishes (60 mm of diameter) with potato-dextrose-agar (15 mL, PDA, Biokar diagnostics, Solabia Group, Cedex, France) and incubated under chamber conditions (25 °C in darkness) for ten days. Next, the isolate was sent to a commercial laboratory (Agricultura y Ensayo S.L.; Alcalá de Guadaira, Seville, Spain) for DNA extraction, PCR amplification, and sequencing. Briefly, DNA extraction process was carried out following the manufacturers’ instructions of HigherPurity™ Plant DNA Purification Kit (Canvax Biotech, S.L., Cordoba, Spain). PCR amplification and further sequencing of the Internal Transcribe Spacer (ITS) region of the nuclear rDNA was carried out with ITS1 and ITS4 primers [36]. The PCR reactions were mixed in a total volume of 25 µL containing DNA product, 0.8 mM dNTPs, 2.5 mM MgCl₂, 1X PCR Buffer, 0.75 µM of each primer, and 0.05 U/µL of Horse-Power-Taq DNA polymerase (Canvax Biotech, S.L., Cordoba, Spain), conducted in a BT1 Thermocycler (Whatman Biometra, Göttingen, Germany) with an initial step of denaturation at 95 °C for 5 min, followed by amplification of 35 cycles of 30 s at 95 °C, annealing 45 s at 56 °C, and extension at 72 °C for 2 min. The amplified fragments were visualized by electrophoresis in 2% agarose gel stained with RedSafe (iNtRON, Biotechnology, Inc., Korea). The PCR products were sequenced by a DNA commercial sequencing service (Secugen, S.L., Madrid, Spain). The raw sequences were edited using the Chromas 2.6.4 program (Technelysium Pty Ltd., South Brisbane, Australia), assembled by the DNAMAN 6.0.3.93 program (Lynnon Corporation, San Ramon, CA, USA) and

compared with sequences from GenBank genetic sequence database using BLAST (version 1.17, Basic Local Alignment Search Tool, National Center for Biotechnology Information).

2.3. Inoculation Process

For the inoculation process in all experiments, Pn1 was grown for refreshing on 15 mL PDA Petri dishes and later incubated under chamber conditions for two weeks. Then, the groups of fourteen-month-old citrus plants were inoculated with Pn1. Disks of bark 5 mm in diameter were incised and cut from the stem of each plant with a sterile cork borer (5-mm diameter) at 30–35 cm above the ground from the rootstock trunks. Mycelial disks (with the same size and tool) were cut from the active PDA culture of Pn1 (Figure 1A) and inserted mycelial face inward into each hole stem section for inoculated plants. PDA disks (with the same size and tool) from non-cultured PDA petri dishes were cut and placed in each sliced stem section for control plants (non-inoculated). Each agar disk was covered with the bark disk (Figure 1B), held, and wrapped in place manually with a strip of cotton moistened with sterile water, and then covered around each stem with aluminum foil (Figure 1C). All inoculated and control plants were incubated under greenhouse conditions, and cotton layers were periodically watered for three days a week to keep the inoculum moist.



Figure 1. *Phytophthora nicotianae* inoculation process in stem of citrus plant: 5-mm mycelial disks of *P. nicotianae* in PDA petri dishes (A); *P. nicotianae* PDA mycelial disks on the citrus plant stem covering with a piece of citrus rootstock bark using laboratory tweezers (B); inoculated citrus plant covered with wet cotton (distilled water) and aluminum paper (C).

2.4. Disease Evaluation and Data Analysis

In all experiments, the aboveground symptoms of foot rot disease caused by Pn1 were evaluated for each citrus plant and treatment 30 DPI from the beginning of the experiment (inoculation day) (Figure 2). Next, all the wrappings were removed, all the rootstocks stems were cut 25–30 cm above and below the inoculation site, and each lesion length caused by *Phytophthora* inoculum was measured using an electronic digital slide gauge (Absolute digimatic caliper, Mitutoyo Corporation, Kawasaki, Japan). The level of rootstock tolerance was evaluated by comparing the lesion length of each. Thus, the values of lesions lengths were statistically analyzed using the free software R version 4.0.2 [37], performing

one-way ANOVA and LSD-Fisher tests ($p < 0.05$) for each experiment [38] with the package “agricolae” [39].

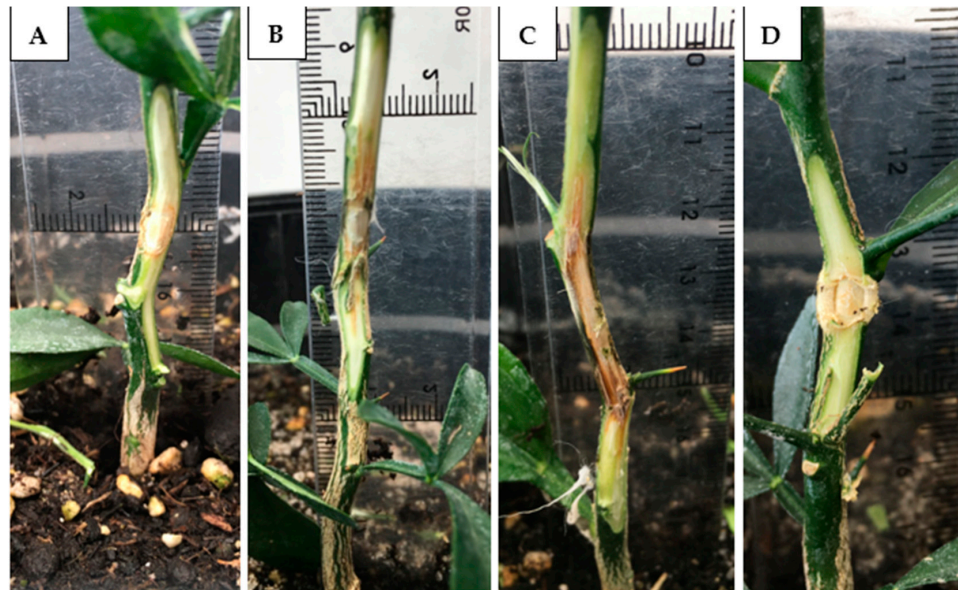


Figure 2. Foot rot lesion length level of *P. nicotianae* 30 days post inoculation in different rootstocks: UFR-6 (7.88 mm) (A); Carrizo citrange (24.03 mm) (B); 2247 × 6070-02-2 (43.57 mm) (C); cork borer lesion (5.00 mm) in non-inoculated UFR-6 (D).

3. Results

3.1. Molecular Identification of Phytophthora Isolate

The oomycete isolate (Pn1) used in this study was corroborated as *P. nicotianae* by sequencing of the ITS region. These sequences showed 96.78% identity with *P. nicotianae* upon BLAST match analysis (Supplementary Material).

3.2. Tolerance Response of Rootstock to Foot Rot Disease

A total of twelve different citrus rootstocks (Carrizo citrange, *Citrus macrophylla*, UFR-4, 2247 × 2075-01-2, A + Volk × orange, AMB + CZO, B11R3T24, B11R5T25, N40R11T18, N40R2T19, N40R3T25, and WGFT + 50-7) were assayed in the first experiment (spring of 2020). Citrus plants inoculated with *P. nicotianae* showed lesion length in the stem with significant response ($F_{11, 83} = 51.60$; $p < 0.001$) among the rootstocks tested. On the contrary, citrus plants inoculated with PDA alone (without inoculum) did not show lesion length in the stem without statistical differences ($F_{11, 84} = 1.12$; $p = 0.35$) among the rootstocks assayed. Hence, 2247 × 2075-01-2 was the citrus rootstock with the highest damage of lesion length in the stem (21.83 mm). This disease incidence was followed by Carrizo citrange (18.11 mm), A + Volk × Orange-E 19-11-8 (17.25 mm), and AMB + CZO (17.13 mm), without significant differences compared with 2247 × 2075-01-2. An intermediate group of lesion length response was comprised of UFR-4 (15.83 mm), N40R1T18 (13.39 mm), *Citrus macrophylla* (12.69 mm), N40R2T19 (11.65 mm), and WGFT + 50-7 (11.29 mm), which showed statistical differences compared with the highest lesion length response. Lastly, the lowest significant incidence of lesion length was accomplished by N40R3T25 (8.16 mm), B11R5T25 (8.37 mm), and B11R3T24 (10.29 mm) (Figure 3).

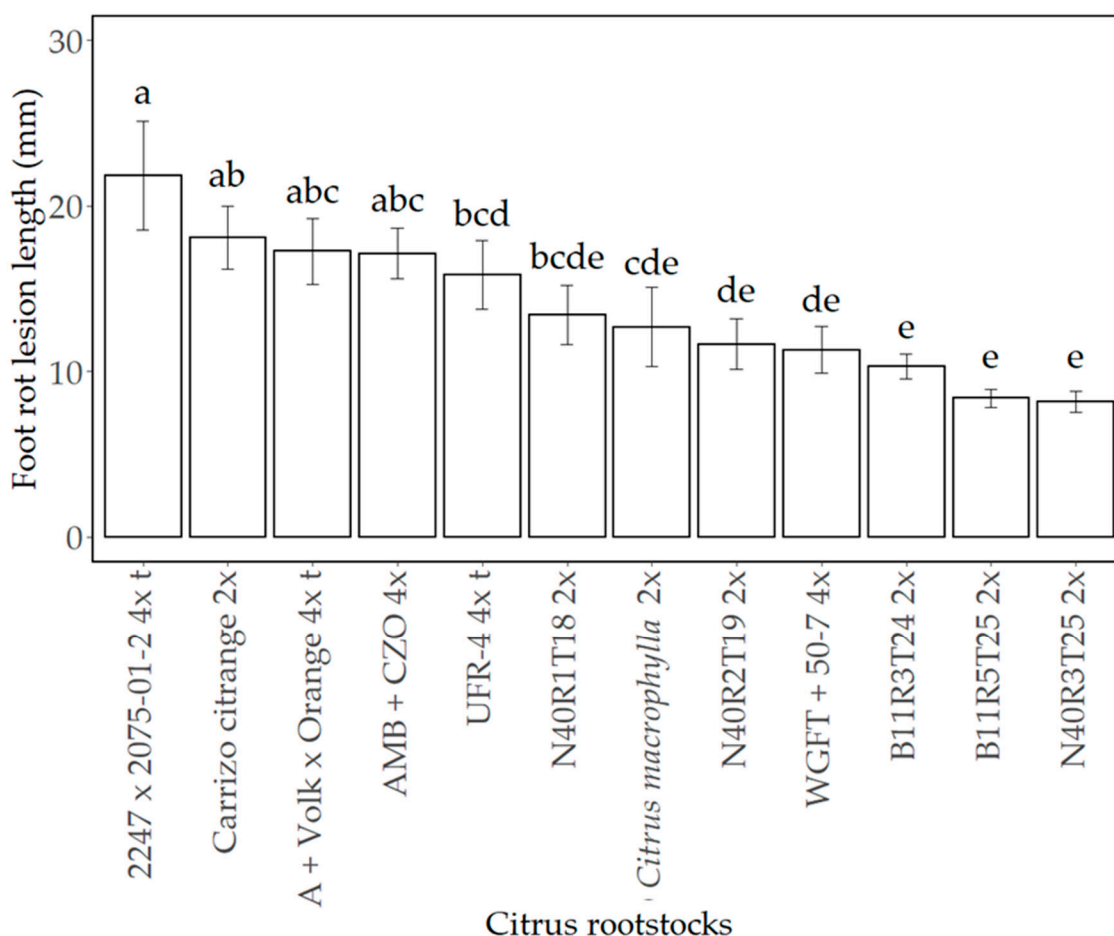


Figure 3. Mean foot rot lesion length response caused by *P. nicotianae* on twelve different citrus rootstocks tested during spring of 2020. Different letters above the bars indicate significant differences among the citrus rootstocks assayed ($p < 0.05$). A + Volk x Orange: A + Volk x Orange19-11-8. Ploidy: 2x; 4x; 4x t: 4x tetrazyg.

In the second experiment (spring of 2021), a total of thirteen different rootstocks (Carrizo citrange, Forner-Alcaide No.5, Orange-14, UFR-1, UFR-5, UFR-6, CL-5146, 2247 x 6070-02-2, B11R3T27, B11R3T53, B11R5T49, B11R5T60, and B11R5T64) were tested for their response to foot rot disease. Citrus plants inoculated with *P. nicotianae* showed lesion length in the stem with significant response ($F_{12, 91} = 93.73$; $p < 0.001$) among the rootstocks tested. On the contrary, citrus plants inoculated with PDA alone (without inoculum) did not show lesion length in the stem without statistical differences ($F_{12, 91} = 1.32$; $p = 0.22$) among the rootstock assayed. Rootstock Orange-14 reported the highest value of lesion length (35.14 mm), which was followed by 2247 x 6070-02-2 (32.21 mm), UFR-5 (30.52 mm), and UFR-1 (29.43 mm), without statistical differences compared with the Orange-14 response. Otherwise, the lowest damage of lesion length was achieved by B11R5T64 (9.16 mm), followed by UFR-6 (9.56 mm), B11R5T60 (10.86 mm), CL-5146 (14.28 mm), and B11R3T53 (14.96 mm), without significant differences compared with the lowest response. An intermediate group of lesion length incidence was obtained by rootstocks B11R5T49 (26.11 mm), B11R3T27 (23.60 mm), Carrizo citrange (22.16 mm), and Forner-Alcaide No. 5 (17.80 mm), showing statistical differences compared with the highest and the lowest response of lesion length (Figure 4).

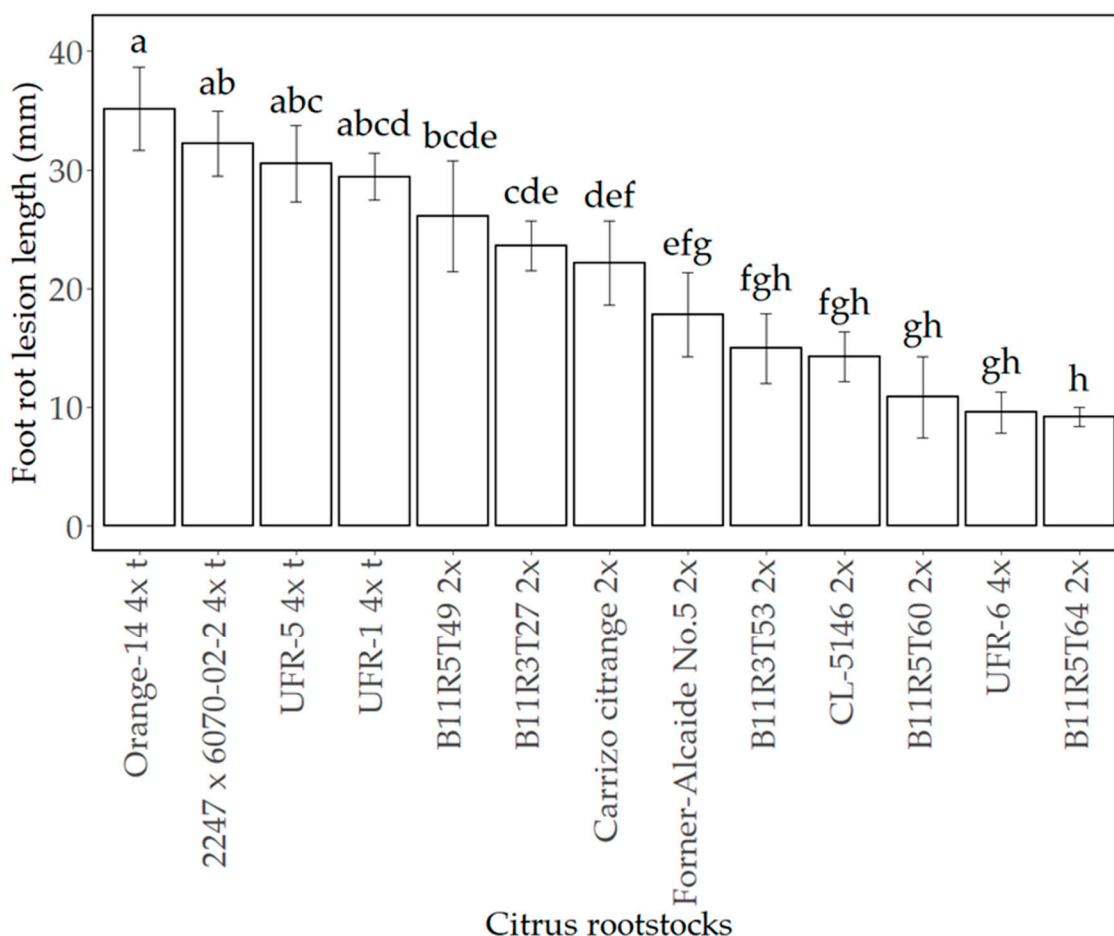


Figure 4. Mean foot rot lesion length response caused by *P. nicotianae* on thirteen different citrus rootstocks tested during spring of 2021. Different letters above the bars indicate significant differences among the citrus rootstocks assayed ($p < 0.05$). Ploidy: 2x; 4x; 4x t: 4x tetrazyg.

4. Discussion

Our findings provide information about the tolerance response to foot rot lesion length in the stem caused by *P. nicotianae* among new different citrus rootstock, which is described as one of the most relevant *Phytophthora* species causing citrus diseases, such as foot rot and gummosis of the trunk [6,40]. To our knowledge, the disease incidence caused by *Phytophthora* on new citrus rootstocks has not been researched recently.

Prior studies have reported Carrizo citrange as having known tolerance and weakness against *P. nicotianae* and *P. palmivora*, respectively [16]. Otherwise, other authors have described an intermediate tolerance to diseases caused by *Phytophthora* spp. However, five recently obtained citrus rootstocks (N40R2T19, WGFT + 50-7, B11R3T24, B11R5T25, and N40R3T25) improved the tolerance behavior of *C. macrophylla* in the 2020 experiment (Figure 3). In the latest experiment, Forner-Alcaide No. 5 rootstock improved the tolerance response of Carrizo citrange, but five others recently obtained citrus rootstocks (B11R3T53, CL-5146, B11R5T60, UFR-6, and B11R5T64) displayed a lower *P. nicotianae* incidence than Forner-Alcaide No. 5 (Figure 4). In this sense, previous authors have reported tolerance to *Phytophthora* spp. on Forner-Alcaide No. 5 rootstock [41].

Furthermore, UFR-1, UFR-4, UFR-5, and UFR-6 have been previously described as tolerant to *Phytophthora* spp. [19]. However, we only identified UFR-6 as being tolerant to *P. nicotianae* in our results. Thus, UFR-4 displayed an intermediate tolerance response to *P. nicotianae*, higher than Carrizo citrange, but more sensitive than *C. macrophylla*. UFR-1 and UFR-5 were reported as having a high level of susceptibility to *P. nicotianae*, with a similar response to 2247 × 6070-02-2 and Orange-14. Lastly, ten recently obtained citrus

rootstocks were found with low level of damage caused by *P. nicotianae*. To the best of our knowledge, the behavior towards *P. nicotianae* by these ten candidates (B11R3T24, B11R5T25, B11R3T53, B11R5T60, B11R5T64, N40R1T18, N40R1T19, N40R3T25, WGFT + 50-7, and CL-5146) has not been previously reported; after these preliminary results, all of these citrus rootstocks will be included for future field research of susceptibility against *Phytophthora* diseases. On the other hand, ten citrus rootstock tested in this work are tetraploid, in which UFR-6, WGFT + 50-7, and AMB + CZO are somatic hybrids, while UFR-1, UFR-4, UFR-5, 2247 × 2075-01-2, 2247 × 6070-02-2, A-Volk × Orange 19-11-8, and Orange 14 are tetrazyg [42]. From these tetraploid rootstocks, only UFR-6 and WGFT + 50-7 reported a high tolerance level to *P. nicotianae*, similar to other diploid citrus rootstocks. Furthermore, most of these new rootstocks have been tested against emerging diseases such as Huanglongbing or citrus greening disease (HLB); hence, some commercial ones are reported with low HLB incidence (UFR-1, UFR-4, and UFR-6) [19] and other new citrus rootstocks have preliminary displayed low HLB incidence (B11R3T24, B11R3T27, B11R5T25, B11R3T53, B11R5T49, B11R5T60, B11R5T64, N40R1T18, N40R2T19, and N40R3T25) [personal communication, F.G. Gmitter Jr.], in which seven rootstocks from this last group are included as the highest tolerance against foot rot disease in the present work.

5. Conclusions

Cultivation of citrus rootstocks tolerant to diseases caused by *Phytophthora* spp. is the most effective methodology to reduce these damages. Our results provide preliminary helpful information for citrus growers to perform an accurate selection of rootstocks in those areas where *Phytophthora* causes serious damage to citrus crops. Thus, this first screening work indicates that eleven new citrus rootstocks, namely, B11R3T24, B11R5T25, B11R3T53, B11R5T60, B11R5T64, N40R1T18, N40R1T19, N40R3T25, WGFT + 50-7, UFR-6, and CL-5146, are potential candidates to be taken into account to solve problems triggered by these diseases. These preliminary results are helpful for the citrus industry to increase the rootstock variability of their orchards, and for the research community and breeding programs aiming for future improvements in this field. Consequently, further studies will involve the field susceptibility of all twenty-four citrus rootstocks against damages caused by *Phytophthora* spp.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/horticulturae7100389/s1>, Sequence 1: Pn1 (950 bp).

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Conflicts of Interest: The authors declare no conflict of interest.

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