# Pathogenicity of oomycete species to different *Prunus* hybrids rootstocks

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**Funding:** This research was funded by the INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria), Spain, through Project RTA2017-00009-C04-04, and matching funds from the ERDF (European Regional Development Fund) and Grant PID2020-114648RR-C33 funded by MCIN/AEI/ 10.13039/501100011033.

**Abstract.** Diseases caused by soil-borne oomycetes are a limiting factor for the cultivation of *Prunus* spp., which makes the choice of a suitable rootstock a key factor. The objective of this study was to evaluate the pathogenicity of 12 oomycete species belonging to the genera *Globisporangium*, *Phytophthora* (*Ph.*) and *Phytopythium* (*Pp.*) to three *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40'. These three rootstocks are widely used to grow stone fruit and almond in the Mediterranean Basin. Pathogenicity tests were conducted using 15 oomycete isolates and 1-year-old rootstock seedlings. Ninety days after inoculation, disease symptoms were evaluated on a severity scale, and the Area Under the Disease Progression Curve and the survival probability of the inoculated seedlings were calculated. Moreover, root dry weight was recorded. All the isolates included in the pathogenicity tests were pathogenic on the rootstock seedlings and were re-isolated from root lesions. Large differences in virulence were detected among the different oomycete species and isolates of *Ph. niederhauserii* for each rootstock. *Phytophthora multivora* and *Pp. helicoides* were generally the most virulent species. The results of the present research offer substantial contribution to increase our knowledge about the pathogenicity of several oomycete species that are frequently isolated in *Prunus* orchards, and the potential risks that they pose for *Prunus* spp. crops.

**Keywords.** Fruit crops, *Globisporangium*, *Phytophthora*, *Phytopythium*, Soil-borne pathogens.

## INTRODUCTION

In the last 5 years, a sustained increase has taken place in the cultivation of *Prunus* spp., including crops like almonds [*P. dulcis* (Miller.) D.A. Webb], apricots (*P. armeniaca* L.), cherries (*P. avium* L.), peaches and nectarines [*P. persica* (L.) Batsch], plums (*P. domestica* L.), sloes (*P. spinosa* L.) and sour cherries (*P. cerasus* L.), with a total world production 50 million tons in 2019 (FAOSTAT, 2021). This was because planted areas have extended, together with new cultivar releases from breeding programs for *Prunus* spp., which have allowed higher yields. These breeding programs have focused mainly on optimizing different tree agronomic aspects, such as floral self-compatibility, blooming and ripening times, productivity, and resistance to pests and diseases (Bielsa and Rubio-Cabetas, 2018).

Significant progress has been made in developing rootstock breeding programs. Rootstocks are essential components in modern fruit production for their ability to adapt a cultivar of commercial interest to several environmental conditions and cultural practices. These capacities are currently being evaluated with the following objectives: shorten or prolong fruit ripening, improve fruit yield and quality, control vigor, compatibility with many cultivars, good tolerance to hypoxia, high water-use efficiency, tolerance to water or soil salinity, and resistance or tolerance to soil-borne pathogens (Gainza et al., 2015).

Most *Prunus* crops grown on their own roots, which were formerly used to cultivate stone fruit and almond, are very susceptible to diseases caused by the genus *Phytophthora* (*Ph.*), such as *Ph. cactorum* [(Lebert and Cohn) Schröter], *Ph. cambivora* [Petri (Buisman)], *Ph. cryptogea* (Pethybr. and Lafferty), *Ph. megasperma* (Drechsler) and *Ph. syringae* [(Kleb.) Kleb.] (Browne and Mircetich, 1995), especially in soils with a fine texture, high bulk density or poor drainage (Reighard and Loreti, 2008). This has been a limiting factor for *Prunus* spp.

64 production, which makes the choice of a suitable rootstock a key factor (Tsipouridis et al.,
65 2005). Therefore, in the 1970s, trees growing on their own roots were progressively replaced
66 by interspecific *Prunus* hybrid rootstocks (Bielsa and Rubio-Cabetas, 2018) to improve
67 qualities of adaptation or tolerance to heavy soils, waterlogging, alkalinity and drought, and
68 to control vigor and tolerance against soil-borne pathogens (Bielsa and Rubio-Cabetas, 2018;
69 Reighard and Loreti, 2008).

70 Currently, the most popular *Prunus* hybrid rootstocks in the Mediterranean Basin are 'GF-
71 677' and 'Garnem' (*P. dulcis* x *P. persica*), being used for almond and peach trees (Reighard
72 and Loreti, 2008; Rubio-Cabetas et al., 2017; Thomidis, 2003b). In the last decade, less
73 vigorous rootstocks have also been developed, and adapted to high-density crops and
74 mechanized harvesting (Yahmed et al., 2016). Examples of such are 'Rootpac-40' (complex
75 almond x peach hybrid), which induces a high yield per *Prunus* tree, similar to that of more
76 vigorous rootstocks like 'Garnem' and 'GF-677' (Jiménez et al., 2011).

77 Despite the positive horticultural qualities conferred by hybrid rootstocks during *Prunus*
78 spp. cultivation, current production intensification is characterized by incorporating
79 irrigation systems together with exploring several soil types. This has increased problems
80 related to waterlogging in association with root asphyxia (Felipe, 2009; Rubio-Cabetas et al.,
81 2017), and the proliferation of diseases caused by oomycetes, to which hybrid rootstocks like
82 'GF- 677' and 'Garnem' are still susceptible (Reighard and Loreti, 2008; Rubio-Cabetas et al.,
83 2017).

84 Several previous studies have investigated the susceptibility of *Prunus* rootstocks to soil-
85 borne pathogens such as *Phytophthora* spp. and *Phytopythium* spp. In Australia, Wicks
86 (1989) evaluated the susceptibility of 'Nemaguard' [*P. persica* x *P. davidiana* (Franch., Pl.

David.)], 'Titan' (*P. dulcis* x *P. persica*), and the hybrid 'Nemaguard' x *P. dulcis* to *Ph cambivora*, which were rated as resistant, moderately resistant and susceptible, respectively. In Greece, Elena and Tsipouridis (2000) reported the susceptibility of rootstocks 'GF-677' and 'J01-ADAFUEL' (both hybrids of *P. dulcis* x *P. persica*) to crown rot caused by *Ph. cactorum, Ph. citrophthora* [(R.E.Sm. and E.H.Sm.) Leonian], and *Ph. megasperma*. In the same country, Thomidis (2000) indicated the susceptibility of peach trees grafted onto rootstocks 'GF-677' and 'KID-I' to *Ph. cactorum*, *Ph. citrophthora*, and *Ph. syringae*. New research into rootstock 'GF-677' showed susceptibility when inoculated with *Ph. cactorum* and *Ph. megasperma* under laboratory conditions (Thomidis, 2003b; Thomidis et al., 2001). Different results were reported by Mehri et al., (2009), who indicated that rootstock 'GF-677' was resistant to two different *Ph. cactorum* strains when inoculated in tests done on excised twigs and plants cultured *in vitro*. In California, Yang et al., (2012) reported a high incidence of *Phytopythium* (*Pp.*) *vexans* [(de Bary) Abad, de Cock, Bala, Robideau, A.M.Lodhi and Lévesque] in a field location with peach trees grafted onto 'Nemaguard' showing replant disease symptoms. *Phytophthora chlamydospora* (Brasier and E.M.Hansen) was reported to attack almond trees grafted onto rootstock 'GF-677' in Turkey (Türkölmez et al., 2016), and almond seedlings grafted onto 'Nemaguard' and peach trees grafted onto 'Lovell' (*P. persica*) in California (Browne et al., 2020). Also in California, Browne (2017) concluded that hybrid rootstocks (almond x peach), such as 'Bright Hybrid-5', 'Bright Hybrid-106', 'Hansen-536' and 'Garnem', were susceptible to *Ph. niederhauserii* (Z.G. Abad et J.A. Abad, sp. nov).

Regarding the genus *Globisporangium*, information about its pathogenicity to *Prunus* rootstocks is scarce. Smither and Jones (1989) reported a low virulence of *G. irregulare* [(Buisman) Uzuhashi, Tojo and Kakish.] in *P. mahaleb* seedlings. However, Schmidt and

Browne (2013) indicated a high virulence of *G. irregulare* in peach seedlings grafted onto 'Nemaguard' roostock.

Recent reports about new oomycete pathogens to *Prunus* crops grafted onto hybrid rootstocks, such as *Ph*. *niederhauserii* on almond trees (Browne et al., 2015; Pérez-Sierra et al., 2010) and *Pp*. *helicoides* (Drechsler) on peach trees (Browne et al., 2019), together with the potential banning of some fungicides currently used for the chemical control of many oomycetes, are of much concern (Thomidis, 2003a). This fact reinforces the need to make new pathogenicity evaluations to obtain updated information about the susceptibility or tolerance of the most widely used rootstocks to well-known and newly described oomycete species.

Thus the objective of this study was to evaluate the pathogenicity of 12 oomycete species belonging to the genera *Globisporangium*, *Phytophthora* and *Phytopythium* to three *Prunus* hybrid rootstocks, 'Garnem', 'GF-677' and 'Rootpac-40', which are widely used to cultivate stone fruit and almond in the Mediterranean Basin.

## MATERIAL AND METHODS

***Oomycete species and isolates*.** Fifteen isolates representing 12 oomycete species were used in the pathogenicity tests (Table 1). They were obtained from extensive surveys of *P. dulcis* plantations and nurseries, which were carried out during the 2018-2021 period by Beluzán et al. (2022) in five Spanish provinces: Córdoba, Huelva, Lérida, Sevilla and Valencia, and identified by Internal Transcribed Spacer (ITS) sequencing.

Additionally, for this study their identity was also confirmed by sequencing cytochrome c oxidase subunit 1 (*cox*1). For this purpose, genomic DNA was extracted from pure cultures grown on PDA at 25 ºC in the dark for 7 days. For DNA extraction, the method of Collado-Romero et al. (2006) was followed, with a slight modification, namely the mycelium was scraped with a sterile pipette tip and placed in a 0.2 mL PCR tube with 20 µL of 25mM NaOH at pH 12. The tubes with the samples were incubated in a PTC 200 thermal cycler (MJ Research Inc., Waltham, MA, USA) following a DNA denaturation program (100 ºC for 15 minutes and 4 ºC for 5 minutes). Subsequently, 40 µL of 40mM Tris-HCl were added at pH 5. The *cox*1 amplification for *Phytopythium* and *Globisporangium* isolates was performed using the forward primer OomCOI-Lev-up (5' TCA WCW MGA TGG CTT TTT TCA AC 3') (Bala et al., 2010) and the reverse primer FM85mod (5' AAC TTG ACT RAT AAT ACC AAA 3') modified from FM85 of Martin and Tooley (2003). For *Phytophthora*, the *cox*1 primers were FM84 (5' TTT AAT TTT TAG TGC TTT TGC 3') and FM50rev (5' CAT CTA AAC CAA CAG TAA AC 3'), the reverse complementary sequence of the FM50 (Martin and Tooley, 2003). Each PCR reaction tube contained water (13.3 µL), Canvax Buffer B (2.5 µL), Canvax $MgCl_2$ (25nM) (2.5 µL), Canvax dNTPs (8nM) (2.5 µL), Canvax Horse Power Taq polymerase (U/µL) (0.2 µL), primers (1 µL of each) and genomic DNA (2 µL), totaling 25 µL (Canvax Biotech SL, Córdoba, Spain). PCR amplification was performed in the same aforementioned thermal cycler by the following program: initial denaturation of 1 cycle at 94 ºC for 3 minutes, 35 cycles of denaturation, annealing and extension at 94 ºC for 30 seconds, 54 ºC for 30 seconds and 72 ºC for 45 seconds, respectively, and a final amplification cycle at 72 ºC for 10 minutes. PCR products were separated by electrophoresis (140 V) on 1.5% agarose gel [1.5% agarose (Conda, Madrid, Spain) dissolved in TAE buffer; Tris-acetate-EDTA, 40mM Tris-acetate, 1mM EDTA]. Nucleic acids staining was done with

155 RedSafe (20000x). To observe band size, a molecular marker (GeneRuler T.M. 100 bp Plus
156 DNA Ladder, Thermo Scientific) was loaded into the first gel well. Presence of bands was
157 observed using a UV light transilluminator. PCR products were sent to the Instituto de
158 Biología Molecular y Celular de Plantas (IBMCP) (Valencia, Spain) for sequencing. The
159 obtained sequences were subjected to a search in NCBI BLAST (http:
160 //https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify isolates at the species level.

161 All isolates were stored in the oomycete collection of the Instituto Agroforestal
162 Mediterráneo (IAM - UPV, Valencia, Spain) in soil solution extract (Mora-Sala et al., 2022)
163 and tubes of oat agar and potato-carrot agar at 10 ºC.

164 ***Rootstocks.*** Pathogenicity tests were conducted using 1-year-old seedlings (≈25 cm
165 height) of three different *Prunus* hybrid rootstocks: 'Garnem', 'GF-677' and 'Rootpac-40'
166 (Table 2). Seedlings were provided by a commercial nursery and, prior to inoculations, they
167 were selected based on morphological homogeneity and healthy appearance.

168 ***Inoculum production and inoculation.*** The inoculum of oomycete isolates was prepared
169 in 500 mL glass flasks with a mixture of 250 mL of vermiculite, 20 mL of oat grains and 175
170 mL of V8 broth medium (200 mL/L V8 juice, 800 mL/L demineralized water and 3 g/L of
171 $CaCO_3$) (Jung et al., 1996). Glass flasks were autoclaved 3 times for 20 min at 120 ºC. Flasks
172 were inoculated separately with each isolate, which was previously grown on V8 juice agar
173 medium (V8A). Then flasks were incubated for 6 weeks in the dark at room temperature
174 (Pérez-Sierra et al., 2013). After this time, the inoculum mixture was rinsed with
175 demineralized water to remove nutrient excess before inoculations.

176 For inoculation, 20 g of inoculum homogenized with 200 g of potting mix that contained
177 peat, vermiculite and sand (1:1:1, v/v/v), and equally autoclaved 3 times prior to use, were
178 added to 350 mL pots. Rootstocks were planted by making a cavity in the pot previously
179 filled with the mixture, then covering the plant with potting mix until the root was completely
180 covered. The control plants were inoculated with a non infected inoculum mixture. Five
181 plants were inoculated for each isolate plus the negative control making a total of 16 inocula
182 x 3 rootstocks x 5 replicates = 240 plants, and the experiment was repeated twice. All the
183 plants were randomly distributed in a growth chamber with a 12-hour day and night
184 photoperiod at 23 ºC. All the seedlings were watered the day before inoculation. Immediately
185 after inoculation, seedlings were flooded for 48 h and flooding repeated every 2 weeks to
186 stimulate zoosporangial formation, as previously described by Pérez-Sierra et al. (2013).

187 ***Pathogenicity evaluation.*** Disease severity evaluations for each plant started 10 days after
188 inoculation, and were then performed every 4 days until the experiment ended 90 days after
189 inoculation. At each evaluation, disease severity was evaluated on this following scale: 0 =
190 asymptomatic, 1 = foliar yellowing, 2 = wilting, dieback and defoliation, 3 = dead plant
191 (Jönsson et al., 2003). With the severity values of each plant over time, the Area Under the
192 Disease Progress Curve (AUDPC) was calculated by the trapezoidal integration method
193 (Campbell and Madden, 1990).

194 At the end of the experiment, seedlings were uprooted, and the root system carefully
195 washed under running water to remove substrate. Re-isolations were done from one
196 symptomatic seedling showing wilting and defoliation (rating scale 2) per oomycete isolate
197 to confirm Koch's postulates. For this purpose, small segments from the primary and
198 secondary roots showing browning and necrosis symptoms were cut. These plant tissue

fragments were disinfested with 70% ethanol for 30 s and then rinsed with sterile distilled water and allowed to dry on sterile absorbent paper. Pieces (1-2 mm long) were cut in a laminar flow chamber and plated onto Petri dishes with CMA-PARPB medium and CMA-PARPBH (CMA-PARPB amended with 0.069 g liter$^{-1}$ Hymexazol) and then incubated at 25 °C for 72 h (Jeffers and Martin, 1986). Pure cultures were obtained when small pieces from the margins of colonies were transferred to potato dextrose agar medium (PDA, Biokar-Diagnostics, Beauvais, France) for their subsequent identification (Erwin and Ribeiro, 1996).

For isolate identification, the ITS region of the ribosomal DNA of the isolates was amplified using universal primers ITS-6 (5' GAA GGT GAA GTC GTA ACA AGG 3') (Cooke et al., 2000) and ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). Genomic DNA extraction, PCR reactions and sequencing was performed as described before for *cox*1.

The root dry weight of the root biomass of each seedling was measured. For this purpose, roots were separated from the main stem and shoots by cutting at the root crown. Then they were placed inside paper bags and dried for 5 days in an oven at 35 ºC. Root dry weight was recorded.

***Statistical analyses.*** A non parametric Kruskal-Wallis analysis was performed for the AUDPC and the root dry weight. When there were statistically significant differences, medians were classified into homogeneous groups by Dunn's test at the 95% confidence level. All the calculations were performed using the InfoStat-2004 statistical software.

The survival probability of the seedlings inoculated with the different oomycete isolates was studied with the Kaplan-Meier estimate (Goel et al., 2010). To test for statistical

differences in the inoculated plants' survival probabilities, the log-rank test was used (Collett, 2003). This non parametric analysis was carried out using STATGRAPHICS Centurion XVI.

## RESULTS

All the rootstock seedlings inoculated with the 12 oomycetes species showed root symptoms, such as dark necrotic lesions and loss of fine roots (Fig. 1). These symptoms were associated with a general aerial decline in seedlings including yellowing, wilting and defoliation, which resulted in variable disease severity values. Re-isolations from symptomatic roots confirmed pathogenicity for all the inoculated species. In contrast, the control seedlings remained healthy and it was not possible to isolate oomycetes from their roots.

The progress of the mean disease severity values obtained throughout a 90-day period after inoculation is shown in Fig. 2. The 'Rootpac-40' seedlings generally obtained higher mean disease severity values than those observed for rootstocks 'Garnem' and 'GF-677'. Severe disease symptoms (between 2 and 3 according to the rating scale) started sooner in 'Rootpac-40' than in the other rootstocks, specifically 1 month after inoculation, and in most inoculated isolates.

The isolate PAL-103 of *Ph. multivora* (P.M.Scott and T.Jung) was particularly virulent. Seedlings began to show symptoms 10 days after inoculation, with mean severity values of 0.6 and 0.7 in 'Garnem' and 'GF-677', respectively, and 1.6 in rootstock 'Rootpac-40'. This resulted in all the seedlings dying 22 days after inoculation in the three evaluated rootstocks. The second species to reach the maximum mean severity was *Pp. helicoides,* in which all the

243 seedlings died 50 days after inoculation, and also for the three rootstocks. For the remaining
244 isolates, the inoculated seedlings generally reached high mean disease severity values (over
245 2). Isolates of *Ph. niederhauserii* showed intraspecific variability. Isolates PAL-21 and PAL-
246 62 reached lower mean disease severity values than isolates PAL-74 and PAL-100 in
247 rootstocks 'Garnem' and 'GF-677', but the highest mean disease severity value in rootstock
248 'Rootpac-40' was obtained for isolate PAL-62.

249 The mean AUDPC values are shown in Fig. 3. There was wide variability among the
250 different isolates, as well as statistically significant differences ($P \leq 0.05$). The mean AUDPC
251 values varied from 28.2 obtained by isolate PAL-21 of *Ph. niederhauserii* in rootstock 'GF-
252 677' to 235.2 obtained by isolate PAL-103 of *Ph. multivora* in rootstock 'Rootpac-40'. In the
253 three evaluated rootstocks, isolates PAL-21 of *Ph. niederhauserii*, PAL-16 of *Ph.*
254 *citrophthora* and PAL-99 of *Pp. chamaehyphon* [(Sideris) Abad, de Cock, Bala, Robideau,
255 A.M.Lodhi and Lévesque] obtained low mean AUDPC values, and showed statistically
256 significant differences ($P \leq 0.05$) with isolate PAL-103 of *Ph. multivora*, which had the
257 highest AUDPC for the three evaluated rootstocks.

258 The mean root dry weight results appear in Fig. 4, which also displays wide variability
259 between oomycete isolates. In the three rootstocks, there were statistically significant
260 differences ($P \leq 0.05$) for some isolates compared to the control. In rootstock 'GF-677', mean
261 root dry weight of the uninoculated control was lower than the values obtained for some of
262 the isolates inoculated, but only statistically significant compared with isolate PAL-16 of *Ph.*
263 *citrophthora*. In rootstocks 'Garnem' and 'GF-677', the lowest mean root dry weight value
264 went to seedlings inoculated with isolate PAL-103 of *Ph. multivora*, with a value of 0.07 g
265 and 0.31 g, respectively, followed by those seedlings inoculated with isolate PAL-96 of *Pp.*

*helicoides* with a value of 0.14 g and 0.36 g, respectively. In these two rootstocks, these differences represented a root dry weight reduction of 92.4% and 57.5% in relation to the uninoculated control for isolate PAL-103 of *Ph. multivora*, and of 84.9% and 50.6% for isolate PAL-96 of *Pp. helicoides*, both respectively. For rootstock 'Rootpac-40', the lowest mean root dry weight value was caused by isolate PAL-71 of *Ph. nicotianae* (Breda de Haan) (0.64 g), which represented a 49.2% reduction in root dry weight compared to the control.

Plots showing the survival probabilities of the inoculated seedlings are in Fig. 5. According to the *P*-value of the log-Rank test ($P \leq 0.05$), there were statistically significant differences among the survival curves of the three inoculated rootstocks. In the evaluated three rootstocks, isolates PAL-103 of *Ph. multivora* and PAL-96 of *Pp. helicoides* displayed a 100% plant mortality at the end of the trial, with the lowest survival probability 50 days after inoculation. On the contrary, on rootstocks 'Garnem' and 'GF-677', isolates PAL-4 of *Ph. cactorum*, PAL-16 of *Ph. citrophthora* and PAL-21 and PAL-62 of *Ph. niederhauserii*, and on rootstock 'Rootpac-40', isolate PAL-101 of *Ph. tropicalis* (Aragaki & J.Y. Uchida) caused no plant mortality at the end of the trial. Hence, the survival probability of these isolates/rootstocks combinations was the highest throughout the trial.

## DISCUSSION

All the isolates included in the pathogenicity tests were pathogenic to the rootstock seedlings and were re-isolated from root lesions. Nevertheless, large differences in virulence were detected between the different oomycete species and *Ph. niederhauserii* isolates for

each rootstock evaluated for disease severity, AUDPC, root dry weight and survival probability.

In this study isolate PAL-103 of *Ph. multivora* was the most virulent. This species is currently considered as an important emerging pathogen because of its worldwide distribution, being associated with a wide range of hosts in nurseries, woody plants in urban environments and natural ecosystems (Migliorini et al., 2019), such as citrus (Meitz-Hopkins et al., 2014), avocado (Rodríguez-Padrón et al., 2018), and macadamia (Jeff-Ego et al., 2021). According to our results, this pathogen significantly reduced the root dry weight of rootstocks in relation to the control. This effect has been previously shown on inoculations of *Ph. multivora* on *Eucalyptus gomphocephala* (DC.) (Scott et al., 2012), *Corymbia calophylla* [(Lindl.) K.D.Hill and L.A.S.Johnson] (Mrázková et al., 2013), and *E. marginata* (Sm.) (Belhaj et al., 2018). In Western Australia, *Ph. multivora* geographical distribution is considerable, and it is active on calcareous soils (Migliorini et al., 2019), which are characteristic of the main Mediterranean stone fruit and almond production areas in Spain. Jung and Burgess (2009) indicated that this pathogen is better adapted to dry climates due to its oospores being thick walled. As far as we know, *Ph. multivora* has not been described to cause infections in stone fruit or almond trees orchards, but our results indicate that this species could represent a serious potential threat to the rootstocks used in these crops.

Isolate PAL-96 of *Pp. helicoides* also performed high virulence. This species has a polyphagous characteristic because it has been reported in fruit crops like kiwifruit and mandarin (Chen et al., 2016; Wang et al., 2015), strawberry (Ishiguro et al., 2014; Marin et al., 2019; Zhan et al., 2020), pistachio (Fichtner et al., 2016), cereals like maize (Xie et al., 2021) and ornamental plants (Chen et al., 2021; Yang et al., 2013), but its worldwide

distribution is still limited or unknown. Although its pathogenicity has been widely documented in China and the United States, information on *Prunus* crops is very scarce. Only the recent studies by Browne et al. (2019) have studied the pathogenicity of *Pp. helicoides* on the 'Nemaguard' rootstock seedlings in California, which caused a fresh root weight reduction and significant root cortex necrosis levels. Subsequently, Beluzán et al. (2022) isolated *Pp. helicoides* from soil (using baiting techniques) in almond orchards in Spain without determining its pathogenicity.

The four evaluated isolates of *Ph. niederhauserii* performed different degrees of virulence. Although intraspecific variability has not been studied in *Ph. niederhauserii*, it has been previously mentioned in the literature for other *Phytophthora* species (Kurbetli et al., 2020; Tian and Babadoost, 2004). Root dry weight reduction and decreased plant survival probability could have been caused by the reduction in lateral roots due to the necrosis produced by this oomycete. This symptom has been previously reported by Rodríguez-Padrón et al. (2018) and Kurbetli et al. (2020) for avocado and pomegranate, respectively. These researches have positioned *Ph. niederhauserii* as a pathogen with more or equal virulence than *Ph. nicotianae*, which coincides with our results in *Prunus* rootstocks.

Another oomycete with high virulence was isolate PAL-58 of *G. ultimum* var. *ultimum* [(Trow) Uzuhashi, Tojo & Kakish]. This pathogen, along with *Pythium* spp. and some fungi, are known to cause damping-off in more than 300 hosts (Toribio et al., 2021). More specifically, *G. ultimum* and *G. irregulare,* together with other *Pythium* species, have been described to cause Prunus Replant Disease (PRD) (Schmidt and Browne, 2013). The authors of this study indicated that the fresh weight of the 'Nemaguard' rootstock is significantly affected when inoculated with *G. ultimum* and *G. irregulare* due to the necrosis produced by

333 both root pathogens. Their results partially agree with ours. Although *G. ultimum* var.
334 *ultimum* was able to significantly reduce root dry weight in rootstocks 'Garnem' and 'Rootpac-
335 40', isolate PAL-90 of *G. irregulare* was unable to significantly reduce it in the three
336 evaluated rootstocks. Research carried out in the 1980s by Smither and Jones (1989) reported
337 low virulence of *G. irregulare* when inoculated into *P. mahaleb* (L.) seedlings. Regarding
338 plant mortality caused by *G. irregulare*, our results partially differ from those obtained by
339 Smither and Jones (1989), because we found that *G. irregulare* caused high mortality in
340 'Rootpac-40'.

341 Regarding isolate PAL-42 of *G. heterothallicum* [(W.A.Campb. and F.F.Hendrix)
342 Uzuhashi, Tojo and Kakish.], in the last decade this species has been associated with
343 grapevine (Spies et al., 2011) and soybean (Rojas et al., 2017), and its pathogenicity has been
344 confirmed in pepper (Derviş et al., 2020) and kiwifruit (Türkkan et al., 2022), but there are
345 no reports about its pathogenicity to stone fruit trees. As to how the disease caused by *G.*
346 *heterothallicum* progresses, Derviş et al. (2020) showed that pepper plants inoculated with
347 this oomycete withered in the first week and died within 3 weeks after inoculation due to
348 severe root and crown rot, as it happened in our pathogenicity test. A very recent study by
349 Türkkan et al. (2022) in kiwifruit indicated *G. heterothallicum* as a pathogen with a
350 significantly lower virulence than that caused by *Pp. vexans*. These results disagree with our
351 results, as both pathogens did not present statistically significant differences in severity
352 according to their AUDPC values. It is important to note that *G. heterothallicum* abundance
353 increases in alkaline soils and at high temperatures (Rojas et al., 2017), such as those used
354 for stone fruit and almond production in the Mediterranean Basin of Spain.

For isolate PAL-98 of *Pp. vexans*, the pathogenicity tests showed a statistically significant reduction in root dry weight in 'Rootpac-40' and high plant mortality. *Phytopythium vexans* has been considered a secondary pathogen in peach trees (Mircetich, 1971). Nevertheless, this oomycete has been confirmed as the main causal agent of root diseases in apple trees (Jabiri et al., 2020), avocado (Rodríguez-Padrón et al., 2018), citrus (Benfradj et al., 2017; Noireung et al., 2020), grapevine (Langenhoven et al., 2018), kiwifruit (Polat et al., 2017; Prencipe et al., 2020; Türkkan et al., 2022) and potato (Santika et al., 2021). Pathogenicity studies of *Pp. vexans* in *Prunus* are scarce, but there has been a recent report about it causing root and crown rot in flowering cherry [*P. serrulata* (Lindl.)] in a nursery in Tennessee, USA (Baysal-Gurel et al., 2021), and also in commercial almond orchards and nurseries (Beluzán et al., 2022).

Isolate PAL-99 of *Pp. chamaehyphon* performed medium virulence compared to the other evaluated oomycetes. This oomycete has been isolated from diverse crops of different botanical families worldwide (Rai et al., 2020), but its pathogenicity has not been widely documented in fruit trees. Recently, a study by Savian et al. (2021) reported the pathogenicity of *Pp. chamaehyphon* to be the cause of Kiwifruit Vine Decline Syndrome (KVDS) in Italy, which causes leaf wilt and root rot in greenhouse trials and commercial orchards.

For isolate PAL-71 of *Ph. nicotianae*, the highest virulence was found in rootstock 'Rootpac-40', showing severe defoliation. Similar results were obtained by Pane et al. (2009), who previously reported that this pathogen affected 1-year-old apricot seedlings in a nursery in Italy by producing leaf yellowing, wilting and defoliation associated with root and crown rot.

377 Infections caused by *Ph. tropicalis* have been reported in several ornamental crops and in
378 some fruit trees (Uchida and Kadooka, 2013). The only report of this pathogen on *Prunus*
379 was on 1-year-old plants in apricot nurseries in southern Italy, where it caused symptoms of
380 leaf yellowing, wilting and defoliation, which were associated with root and crown rot in
381 these plants (Pane et al., 2009). These symptoms are consistent with those obtained in our
382 study for isolate PAL-101, which caused a statistically significant root dry weight reduction
383 in rootstock 'Garnem'.

384 The pathogenicity of *Ph. cactorum* and *Ph. citrophthora* has already been reported on
385 stone fruit trees (Browne, 2017; Thomidis, 2000; Thomidis, 2001; Thomidis, 2003c;
386 Thomidis et al., 2008). In our study, isolate PAL-4 (*Ph. cactorum*) significantly reduced root
387 dry weight only in rootstock 'Rootpac-40' and PAL-16 (*Ph. citrophthora*) only in rootstock
388 'GF-677'. However, for this last isolate, this difference is due to the fact that the uninoculated
389 control presented an unusually low mean root dry weight value. Although the rootstock
390 seedlings were selected with a homogeneous height, perhaps some of them had less
391 developed roots, affecting the mean root dry weight value of the control and, moreover, PAL-
392 16 isolate was not very virulent. In both cases, the severity in 'Garnem' and 'GF-677' was
393 medium and plant mortality was low. Opposite results were obtained by Thomidis (2003c),
394 who pointed out that species *Ph. cactorum* and *Ph. citrophthora* are highly virulent in stone
395 fruit trees. Subsequent research by Thomidis et al. (2008) concluded that *Ph. cactorum* was
396 the most virulent species in 30 different genotypes of cherries.

397 The results of the present research offer substantial contribution to increase our knowledge
398 about the pathogenicity of several oomycete species that are frequently isolated in *Prunus*
399 orchards, and the potential risks that they pose for *Prunus* spp. crops. Our results and the

methodology we used, complemented with additional field trials, could also have immediate implications for the selection of rootstocks for almond and stone fruit cultivation.

## Acknowledgments

Francisco Beluzán was supported by Agencia Nacional de Investigación y Desarrollo/Subdirección de Capital Humano/Doctorado Becas Chile en el Extranjero/72200145.

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671 **Table 1.** Oomycete species and isolates used for the pathogenicity tests.

| Species | Isolate | Source | Location | ITS[1] | | | *cox*1[2] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Identity (%) | Reference Isolate[3] | GenBank Accession[4] | Identity (%) | Reference Isolate[3] | GenBank Accession[4] |
| *Globisporangium heterothallicum* | PAL-42 | Soil | Lérida | 98.0 | HQ643559 | MZ922002 | 100 | HQ708603 | OP067002 |
| *G. irregulare* | PAL-90 | Soil | Huelva | 99.9 | HQ643596 | MZ922042 | 98.1 | HQ708640 | OP067007 |
| *G. ultimum* var. *ultimum* | PAL-58 | 'GF-677' rootstock | Valencia | 99.2 | HQ643869 | MZ922015 | 99.7 | HQ708910 | OP067000 |
| *Phytophthora cactorum* | PAL-4 | Soil | Lérida | 100 | MG783385 | MZ921999 | 99.7 | MH136858 | OP067004 |
| *Ph. citrophthora* | PAL-16 | Soil | Lérida | 99.9 | MG865476 | MZ921978 | 99.0 | MH136872 | OP067006 |
| *Ph. multivora* | PAL-103 | Irrigation water | Valencia | 100 | MG865546 | ON159753 | 99.2 | MH136939 | OP067005 |
| *Ph. nicotianae* | PAL-71 | Soil | Valencia | 99.7 | MG865550 | MZ922025 | 99.9 | MH136943 | OP067003 |
| *Ph. niederhauserii* | PAL-21 | Soil | Lérida | 100 | MG865552 | MZ921966 | 100 | MH136944 | OP066996 |
| *Ph. niederhauserii* | PAL-62 | 'GF-677' rootstock | Valencia | 100 | MG865552 | MZ921954 | 99.9 | MH136944 | OP066997 |
| *Ph. niederhauserii* | PAL-74 | Soil | Valencia | 99.9 | MG865552 | MZ921957 | 99.9 | MH136944 | OP066998 |
| *Ph. niederhauserii* | PAL-100 | 'GF-677' rootstock | Córdoba | 100 | MG865552 | MZ921962 | 99.9 | MH136944 | OP066999 |
| *Ph. tropicalis* | PAL-101 | Soil | Córdoba | 100 | MG865596 | MZ921976 | 98.3 | MH136987 | OP067001 |
| *Phytopythium chamaehyphon* | PAL-99 | Soil | Córdoba | 99.3 | HQ643374 | MZ922051 | 100 | HQ708421 | OP066994 |
| *Pp. helicoides* | PAL-96 | 'GF-677' rootstock | Sevilla | 99.4 | HQ643383 | MZ922048 | 99.6 | HQ708430 | OP066993 |
| *Pp. vexans* | PAL-98 | 'GF-677' rootstock | Córdoba | 99.5 | HQ643400 | MZ922050 | 99.9 | HQ708447 | OP066995 |

672 [1] Isolates identified by ITS (internal transcribed spacer).
673 [2] Isolates identified by *cox*1 (cytochrome c oxidase subunit 1).
674 [3] Reference isolates taken from Abad et al. (2022) and Robideau et al. (2011).
675 [4] Accession number of amplified sequences of ITS and *cox*1 deposited in GenBank.

676 **Table 2.** Rootstocks used in the pathogenicity tests, and the breeding programs from which

677 they were obtained (Bielsa and Rubio-Cabetas, 2018).

| Rootstock | Species | Genetic background | Breeding programs |
|---|---|---|---|
| 'Garnem' | *P. dulcis* x *P. persica* | 'Garfi' x 'Nemared' | CITA[1], Spain |
| 'GF-677' | *P. dulcis* x *P. persica* | Open-pollinated | INRAE[2], France |
| 'Rootpac-40' | (*P. dulcis* x *P. persica*) x (*P. dulcis* x *P. persica*) | ('Marcona' x 'Nemaguard') x 'Felinem' | Agromillora Ibérica, Spain |

678 [1]Centro de Investigación y Tecnología Agroalimentaria de Aragón.
679 [2]Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement.


**Figure 1.** Examples of the root symptoms observed during the pathogenicity tests conducted on three different *Prunus* hybrid rootstocks. (a) Control rootstock 'Garnem'; (b) *Pp. helicoides* (PAL-96) on 'Garnem'; (c) *Pp. helicoides* (PAL-96) on 'Rootpac-40'; (d) *Ph. multivora* (PAL-103) on 'GF-677'; (e) *Ph. multivora* (PAL-103) on 'Garnem'; (f) *Ph. niederhauserii* (PAL-100) on 'Garnem'; (g) *Ph. niederhauserii* (PAL-100) on 'GF-677'; (h) *Ph. nicotianae* (PAL-71) on 'Rootpac-40'; and (i) *G. ultimum* var. *ultimum* (PAL-58) on 'GF-677'.

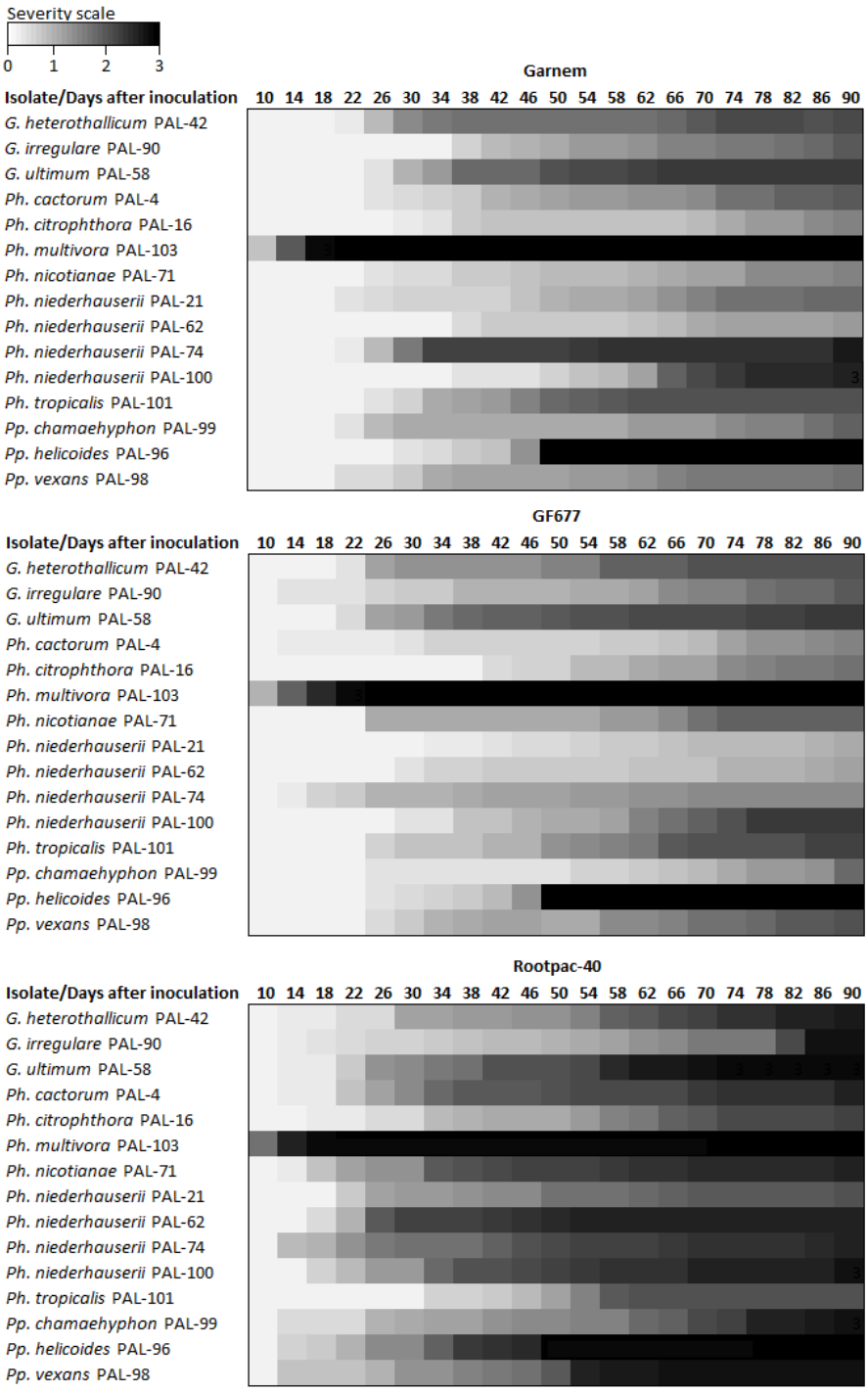
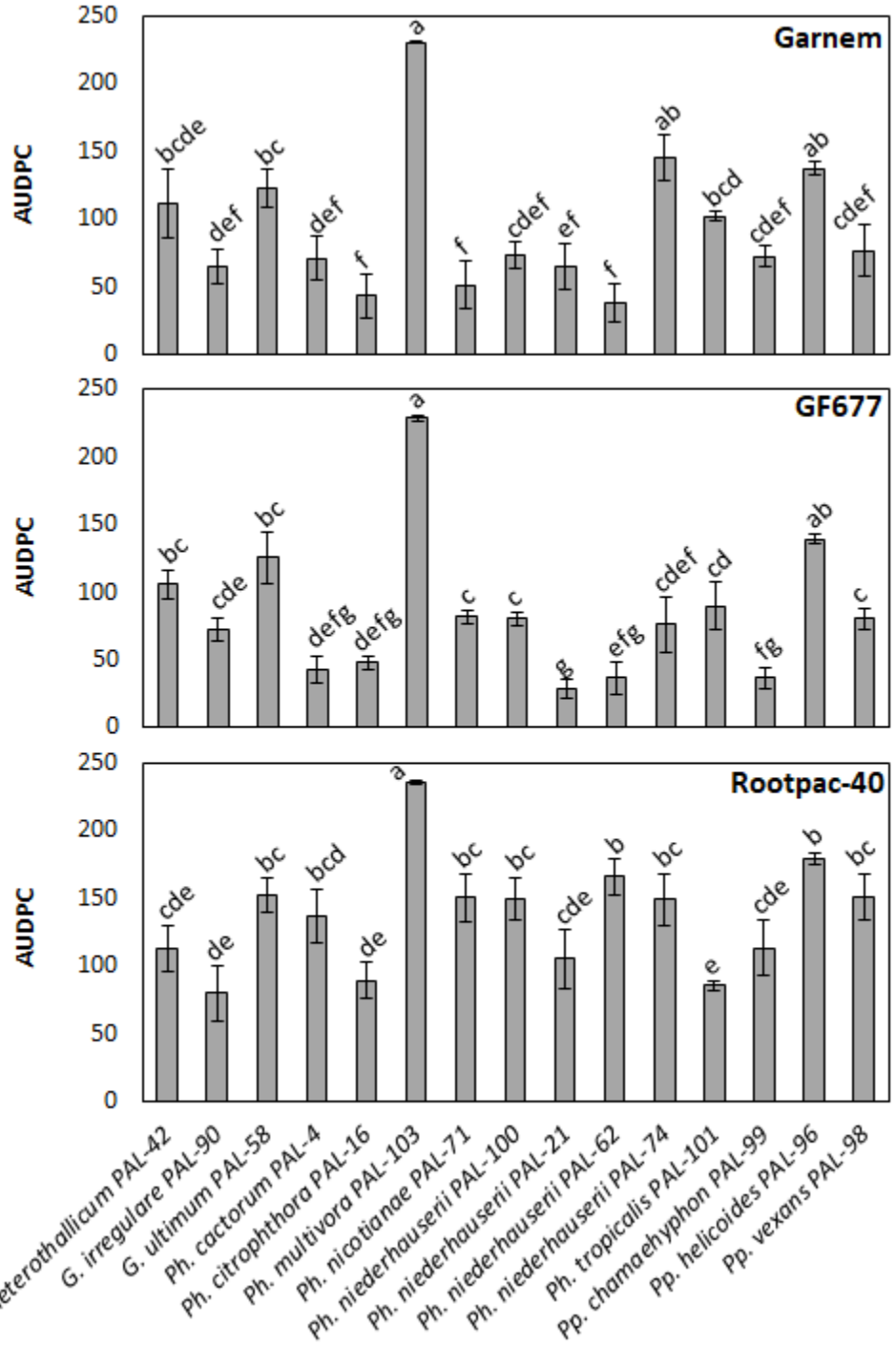



**Figure 2.** Heatmap showing the progress of the mean disease severity values obtained over a 90-day period after the inoculation of three *Prunus* hybrids rootstocks with 15 isolates of 12 oomycete species. Disease severity was evaluated using a 0-3 rating scale (lighter squares represent the lowest disease severity, and darker squares denote the highest disease severity).

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694 **Figure 3.** Area Under the Disease Progress Curve (AUDPC) of rootstocks 'Garnem', 'GF-
695 677' and 'Rootpac-40' inoculated with 15 different oomycete isolates. Bars with different
696 letters indicate statistically significant differences according to Dunn's test ($P \leq 0.05$).

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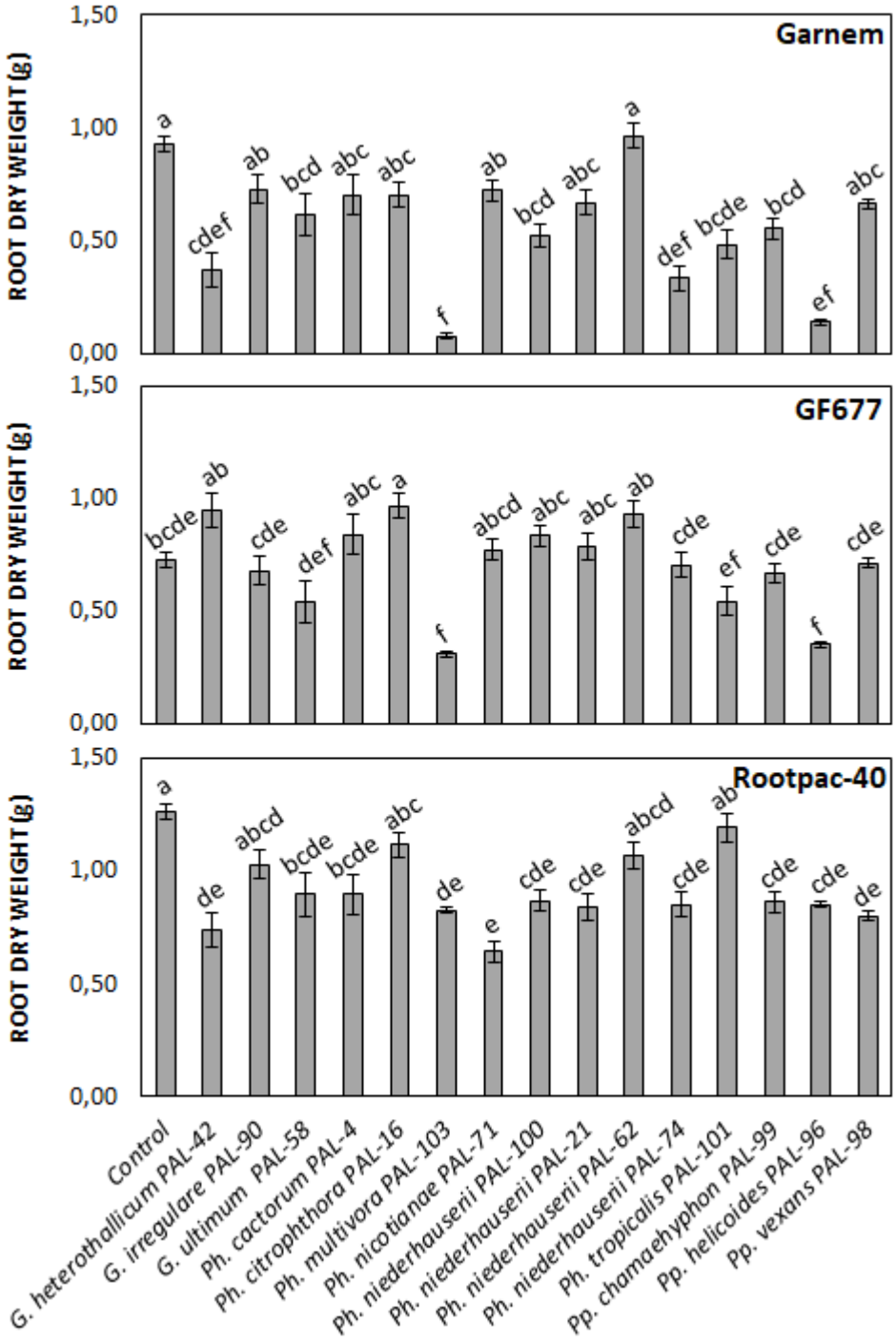


**Figure 4.** Mean root dry weight (± standard error) of hybrid rootstocks 'Garnem', 'GF-677', and 'Rootpac-40' inoculated with 15 different oomycete isolates, and the uninoculated control. Bars with different letters represent statistically significant differences according to Dunn's test ($P \leq 0.05$).

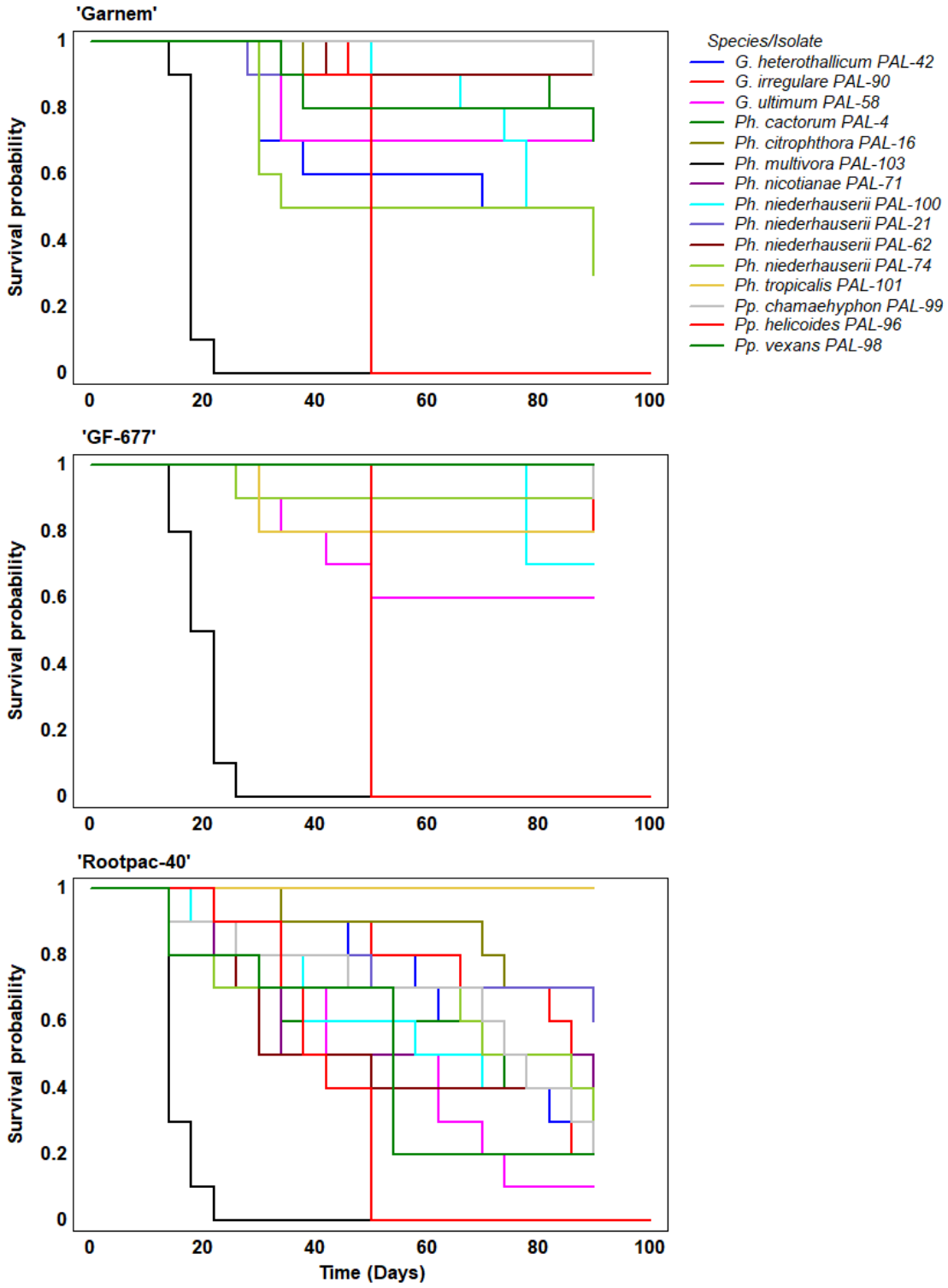


**Figure 5.** Survival probabilities plot obtained using the Kaplan-Meier estimate ($P \leq 0.00$) of the survival function for rootstocks 'Garnem', 'GF-677' and 'Rootpac-40' inoculated with 15 different oomycete isolates.