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An efficient method for *in vitro* screening of susceptibility of wild cherry (*Prunus avium* L.) to *Phytophthora* species

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Summary. A method was developed for *in vitro* screening clones of wild cherry (*Prunus avium* L.) for susceptibility to four *Phytophthora* species (*P. uniformis*, *P. megasperma P. citrophthora*, and *P. cinnamomi*), by using inoculation tests on micropropagated plants. Susceptibility was evaluated with a disease severity scale based on visual assessments of external symptoms. All tested wild cherry clones were very susceptible to *P. citrophthora,* while susceptibility to *P. cinnamomi* was variable among the clones, and resistance to *P. uniformis* and *P. megasperma* was generally high. These results were consistent with those obtained previously using inoculated wild cherry seedlings. The method offers advantages compared to seedling or sapling assays, including reduced time requirement, evaluation of cloned material under controlled conditions, and easy replication at any time of year. Compared to field tests, the method does not depend on the availability of land and does not involve risks of soil or water contamination, which is always a concern in Phytophthoras. The method is suitable for mass screening of plant material in programmes for selecting genetically improved wild cherry clones for disease resistance.

Keywords. Micropropagation, early screening, host resistance.

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INTRODUCTION

Prunus avium L. (wild cherry), is a valuable diversity component in temperate forest ecosystems in Europe (Bornand, 1973; Bernetti and Padula, 1984). Wild cherry is also an important species for wood production in

Europe, and has been increasingly planted for this purpose in artificial stands and in semi-natural forests. After the European Common Agricultural Policy (CAP) encouraged finding alternative uses for farmland, wild cherry has become a common species planted in pure or mixed deciduous stands (Welk *et al*., 2016). Interest in *P. avium* for its valuable timber is also increasing outside its natural distribution area, for example in New Zealand (Ledgard and Giller, 1998) and southwestern Australia (CAB International, 2003). Wild cherry ornamental varieties are also used in landscape architecture (Ducci *et al*., 2013).

In the Mediterranean region, wild cherry is a component of deciduous oak forests and beech forests. Small groups of trees and isolated specimens can be found scattered along moist river valleys, in the edges of woods, and in hedgerows (Ducci *et al*., 1988; Savill, 1991; Ducci and Proietti, 1997; Joyce *et al*., 1998). *Prunus avium* trees may be found on a wide range of soil types (Welk *et al*., 2016), and Teissier du Cros (1980) described wild cherry as one of the most productive species adapted to hydromorphic soils in France and Germany. Wild cherry clones may be useful for nature restoration interventions due to ability to develop adventitious roots for deep soil reinforcement, for soil stability, and for mitigation of erosion (Welk *et al*., 2016).

The new broadleaf stands for timber production were generally established on sites where agricultural production had been poor, mainly because the soil was compact and heavy. These soils expose trees to a variety of fungal diseases and, particularly, to root rot pathogens. Several *Phytophthora* species have been repeatedly found to be associated with root rots of wild and cultivated clones of cherry trees (Mircetich and Matheron, 1976; Thomidis, 2001; Thomidis and Sotiropoulos, 2003; Vettraino *et al*., 2007; Thomidis *et al*., 2008; Türkölmez *et al*. 2015; Sarandi-Kovacs *et al*., 2016), and these diseases are significant obstacles to *P. avium* cultivation.

Breeding is a key strategy for development of disease resistance in forest trees, which involves recursive processes of hybridisation, artificial inoculation, selection of resistant material, and clonal multiplication (Showalter *et al*., 2018). The main problem with artificial inoculation of *Phytophthora* is the risk of soil and water contamination, which can lead to biosecurity issues. To address this risk, alternatives to inoculating roots or collars of plants in the field have been adopted, such as inoculating excised twigs or shoots or inoculating stems (Thomidis *et al*., 2008). *In vitro* assays using host callus tissue cultures prevent environmental contamination, and can speed up selection of resistant genotypes (Miller *et al*., 1984; McComb *et al*., 1987; Jang and Tainter, 1990a; 1990b; 1991).

In the present study, micropropagated plants were used to develop a rapid and effective *in vitro* method for testing resistance of wild cherry clones to *Phytophthora cinnamomi*, *P. citrophthora* and *P. megasperma*, which are commonly associated with cherry root rot, and to *P. uniformis,* which is associated with *Alnus cordata* Loisl., a nitrogen-fixing species commonly planted with wild cherry in mixed forestry plantations (Leslie, 2024).

MATERIALS AND METHODS

Plant material and culture method

Ten wild cherry clones (Table 1) from a clonal collection of the Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria - Foreste e Legno (CREA-FL) were selected for this study. Plant propagation protocols were adapted for the different clones tested, since *in vitro* culture of wild cherry is strongly genotype-dependent (Grant *et al*., 1998). In February, buds were collected from each clone. The buds were sterilised with 99% ethanol for 25 sec, and aseptically added to MS medium (Murashige and Skoog, 1962) supplemented with 0.054 µM naphtalenacetic acid (NAA) or 0.49 µM indol-butyric acid (IBA) and 6-benzylaminopurine (BAP) with varying concentrations from 8.8 to 4.4 µM. Plant cultures were grown in 50 mL glass vials each containing 10 mL of MS medium. After 20 d, plant shoots were transferred onto MS medium supplemented with 0.054 µM NAA or 0.49 µM IBA plus 3.8 µM BAP. After 28 d, resulting multiplied shoots were transferred to 500 mL capacity glass jars each containing 120 mL of MS medium supplemented with 0.49 µM IBA and 2.2 µM BAP. These cultures were then sub-cultured at 28 d intervals by separating individual shoots from each pro-

Table 1. Wild cherry clone id codes, provenance and geographical coordinates.

Clone ID	Site of origin, Region (Italy)	Coordinates
ACW10	Alpe Catenaia, Toscana	43°44N 12°06'E
AP06	Alpago, Veneto	46°10'N 12°21'E
FL07	Foresta Lama-Campiglia, Emilia Romagna	43°47'N 11°50'E
ML10	Monti Lessini, Veneto	45°40'N 11°12'E
PUZZOLO	Val Tiberina, Toscana	43°40'N 12°02'E
ROTI	Val Tiberina, Toscana	43°34'N 12°08'E
TO08	Val Sangone, Piemonte	45°02'N 07°21'E
VC04	Valle del Senio, Emilia Romagna	44°07'N 11°22'E
VM02	Valle del Metauro, Marche	43°38'N 12°20'E
VTS02	Val Cerfone, Umbria	43°25'N 11° 58'E

liferating shoot culture before transfer to fresh medium. Shoots were rooted in MS medium supplemented with IBA at concentrations varying between 12.25 and 17.15 µM. All media were supplemented with 0.6% (w/v) agar (Sigma A4550), 3% (w/v) sucrose, and were adjusted to pH 5.65 before autoclaving for 20 min at 121 °C. Cultures were maintained at 24.0 ± 1 °C with a 16 h daily photoperiod at 70 µmol m^{-2} s^{-1}, from Philips 58 W / 840 fluorescent tubes positioned 25 cm above the cultures.

As soon as the plantlet root systems were well established, each plant was transplanted into a sterile 1000 mL capacity glass jar containing 1:1 peat + vermiculite that had been sterilised twice at an interval of 24 h. The substrate was watered with 50 mL deionised sterile water.

Phytophthora *cultures and inoculum preparation*

Four *Phytophthora* isolates belonging to species known to be pathogenic to wild cherry and used to inoculate wild cherry seedlings in a previous susceptibility trial (Santini *et al*., 2006), were used for inoculation assays. These were: *P. cinnamomi* (Pc), previously isolated from *Quercus rubra*, and supplied by C.M. Brasier, Forestry Commission, United Kingdom; *P. citrophthora* (Pct) isolate, previously isolated from *Convolvolus* sp., and provided by S.O. Cacciola, Università di Catania, Italy); *P. megasperma* var. *megasperma* (Pm) isolate CBS 118733, from wild cherry; and *P. uniformis* (Pa) isolate CBS 109280, from *Alnus cordata* (Santini *et al*., 2001).

Phytophthora cinnamomi has been commonly recovered from nurseries and can be an important pathogen in the Mediterranean region due to its adaptation to warm temperatures. *Phytophthora citrophthora* was selected for the present study because it is particularly aggressive to cherry (Thomidis, 2001; Thomidis and Sotiropoulos, 2003; Santini *et al*., 2006), although it also has wide host range. *Phytophthora megasperma* was detected from an orchard where the clonal collection used in the present study (see below) was growing. *Phytophthora uniformis* was chosen because *Alnus cordata* Loisl., commonly used as an auxiliary species in wild cherry plantations, is a preferred host for this pathogen (Santini *et al*., 2003).

Phytophthora colonies were sub-cultured for 2 weeks at 20 °C in the dark in 90 -mm diam. Petri dishes, each containing 20 mL of sterilised V8 agar (100 mL L^{-1} V8 juice [Campbell Grocery Products], 3 g L^{-1} $CaCO_3$, 20 g L^{-1} Technical Agar [DIFCO]).

Inoculations

The inoculation experiment involved a total of 291 micropropagated wild cherry plants of ten clones. Plants were inoculated in sterile conditions 15 d after transplantation, and were then maintained at 21 °C under 12h daily photoperiods. Light was provided by fluorescent tubes (OSRAM L58W/77 Fluora). Photosynthetically active radiation was approx. 60 µmol m^{-2} s^{-1}. The glass jars with inoculated plants were sterilised at the end of the experiment to prevent contamination.

Each plant received one inoculation. Each *Phytophthora* isolate was inoculated onto a variable number (four to 12) of ramets from each clone, depending on the number of micropropagated plants available. Due to difficulties in multiplication of some wild cherry genotypes, not all the combinations of cherry clone × *Phytophthora* isolate were tested. The clones TO08 and VC04 were not inoculated with Pa. Five additional ramets for each clone were inoculated with two sterile V8 agar plugs as inoculation controls. Inoculations were carried out in a laminar flow cabinet, by placing two 0.6 mm diam V8 plugs colonized by the respective pathogen onto the principal roots of the plantlets near the plantlet collars. To ensure uniform and comparable inoculation method, the same technique was also used for inoculating Pa, although the species is not a typical root pathogen. This choice was supported by the results from a previous test in greenhouse conditions, which showed that inoculating the roots or the stems of wild cherry seedlings with Pa produced the same results (Santini *et al*., 2003). The inoculated plants were then maintained for 2 months under diffuse light at 21 °C.

Disease assessments

Assessments of symptoms (numbers of yellowing or wilting leaves, presence of necroses on leaf margins, presence of necroses on stems) were carried out at 1, 2, and 3 weeks post inoculation. Each plant was scored using a disease index as follows: 0 = healthy plant; 1 = plant with 10 to 25 % yellowing/wilting; 2 = plant with 26 to 50 % yellowing/wilting; 3 = plant with 51 to 75 % yellowing/wilting; 4 = plant with 76 to 100 % yellowing/wilting; or 5 = dead plant. Any necrosis at the leaf margin, base or apex of a stem was scored adding 0.5 to the plant score. The Area Under the Injury Progress Curves (AUIPCs) were calculated using the midpoint rule method (Simko and Piepho, 2012).

Data analyses

Two-way factorial analysis of variance (ANOVA) with Type-III sum of squares for unbalanced design (Maxwell *et al*., 2017) was applied to AUIPC (depend-

ent variable data) and to damage index data (damagew4), measured 4 weeks post inoculation, to assess the effects of three sources of variation.

The following linear model was used to analyse the data:

$$yijk = \mu + \alpha i + \beta j + \alpha\beta ij + \varepsilon ijk,$$

where yijk = individual disease severity score or AUIPC value belonging to the kth ramet (k = 1, 2, ..., 10), of the jth wild cherry clone;
(j = 1, 2) inoculated with the ith *Phytophthora* isolate (i = 1, 2, 3, or 4),
μ = overall mean;
αi = effect of the ith "*Phytophthora* isolate";
βj = effect of the jth "wild cherry clone";
αβij = interaction effect between *Phytophthora* isolate and wild cherry clone; and
εijk = experimental error.

Homogeneous groups of means for the statistically significant interaction effects were identified by the Tukey's Honestly Significant Difference (HSD) test. The χ^2 test was used for determining differences in mortality among wild cherry clones and among the *Phytophthora* isolates. The normality assumption of ANOVA was verified *a posteriori* by analysing the distribution of residuals. All statistical analyses were carried out in R version 4.1.3 (2022-03-10). HSD tests were carried out using R package 'agricolae', Statistical Procedures for Agricultural Research, version 1.3-7 (2023-10-22), author Felipe de Mendiburu.

RESULTS

Incipient symptoms were detectable on wild cherry clones at 1 week after inoculation in a few cases, but generally occurred in the second week post-inoculation (data not shown). Death of the first plants occurred at 2 to 3 weeks when Pc or Pct were inoculated, while Pa and Pm only rarely caused death of plants, and then at more than three weeks post-inoculation (Figure 1).

ANOVAs revealed statistically significant effects of all sources of variation for both dependent variables analysed, i.e. the disease severity scores (diseasedamagew4) and the AUIPC (auipcw4) (Tables 2 and 3). Since the interaction (clone × *Phytophthora* isolate) was statistically significant for both variables, subsequent *post hoc* tests were only carried out for this effect.

Pct had the greatest virulence to all the tested wild cherry clones, and HSD *post hoc* tests did not detect any significant differences among the clones inoculated with this isolate. Pc aggressiveness was variable depending on the challenged host clone, while Pa and Pm were always much less aggressive than the other two isolates, although they killed a few plants in some of the host clones (Table 3, Figures 1 and 2).

Some of the wild cherry clones showed partial resistance to some *Phytophthora* isolates: in particular clones FL07, ML10 and VC04 generally developed low disease severity scores, even when inoculated with Pct, although these differences were not statistically significant. In contrast, the clones ROTI, PUZZOLO, and TO08 always developed the greatest disease severity scores (Table 4, Figure 2).

Plant mortality rates (Figure 1) differed significantly between the clones after inoculations with Pc ($\chi^2 = 56.53$, $P \leq 0.01$) or with Pct; ($\chi^2 = 26.33$, $P \leq 0.05$). There were no statistically significant differences in survival after inoculations with Pa or with Pm. There were also significant differences among the *Phytophthora* isolates in their capacities to cause plant death ($\chi^2 = 33.91$, $P \leq 0.01$).

DISCUSSION AND CONCLUSIONS

The propagation technique applied in this study made it possible to obtain *in vitro* micropropagated wild cherry plants that were uniform and of high quality. This plant material proved to be suitable for *in vitro* screening of susceptibility to *Phytophthora* pathogens.

The inoculation system used in this study was functional, reliable and repeatable, and the results were consistent with those previously obtained by inoculating wild cherry seedlings *in vivo* with the same *Phytophthora* isolates (Santini *et al*., 2006). The results were also consistent with the few other data in the literature on this subject (Thomidis, 2001; Thomidis and Sotiropoulos, 2003; Thomidis *et al*., 2008). There are advantages to using the method presented here: clones can be tested early in the selection process; the risk of environmental contamination by *Phytophthora* spores is negligible; the space required to set up a trial is small; plants can be available all year round; the method is more rapid than using seedlings, saplings or adult plants. For rapidity of responses, Santini *et al*. (2003; 2006) required almost 3 months after inoculation before susceptibility to *Phytophthora* could be assessed when screening visible symptoms in seedlings of wild cherry and other broadleaf tree species.

The present study used a six-step disease scoring scale, which allowed detailed estimation of host plant resistance to the pathogens, giving results similar to those of Santini *et al.* (2003; 2006) on lignified plants.

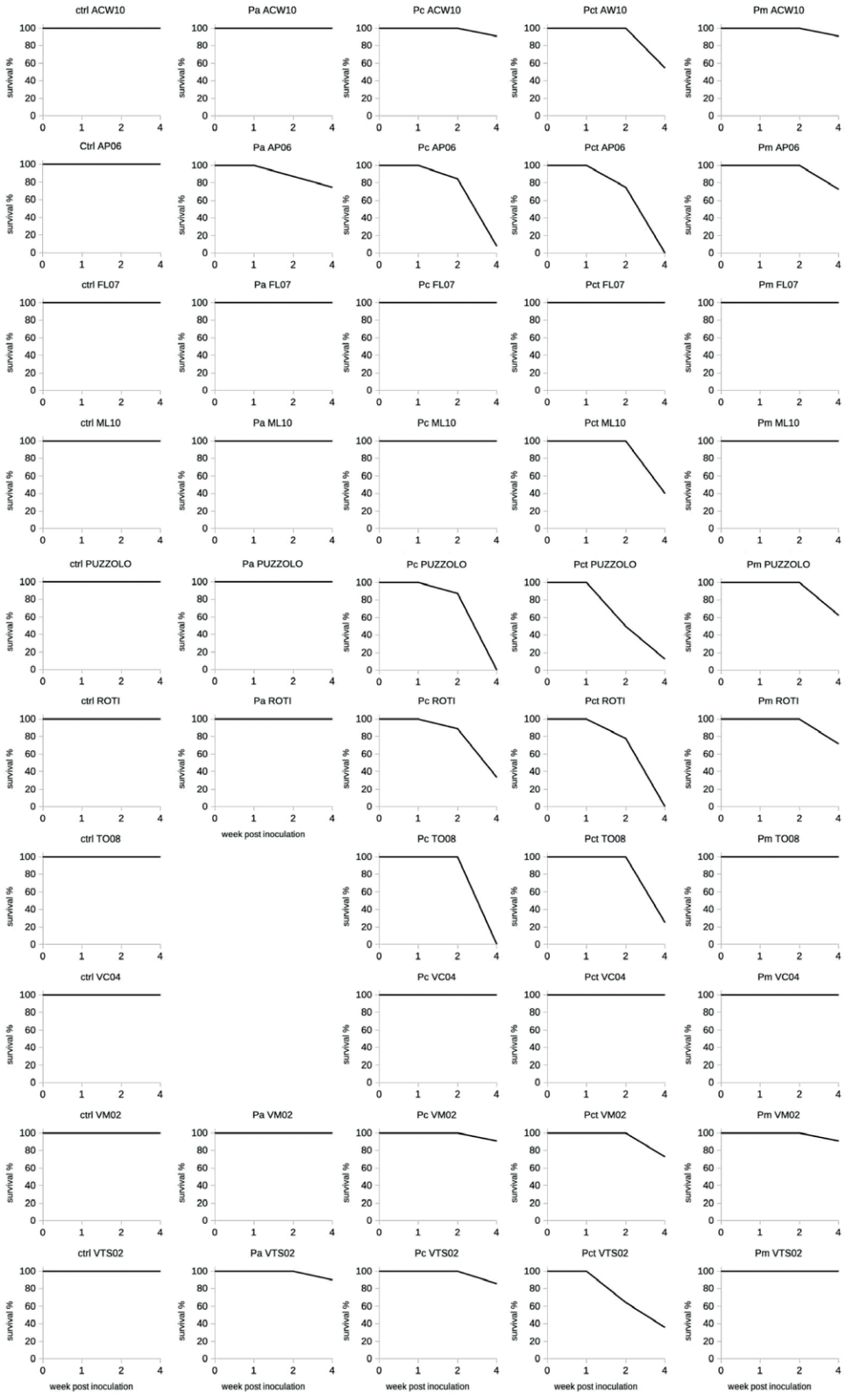

Figure 1. Survival rate (%) of the micropropagated plants inoculated *in vitro* for each combination of clone (rows) and *Phytophthora* isolate (columns) one, two and 4 weeks after inoculation. Ctrl = mock inoculation; *Phytophthora* isolates: Pa (*P. uniformis*); Pc (*P. cinnamomi*); Pct (*P. citrophthora*); Pm (*P. megasperma* var. *megasperma*)

Table 2. Two-way analysis of variance (ANOVA) table (Type-III sum of squares for unbalanced design) considering the effects of *Phytophthora* isolate, wild cherry clone, and their interactions on the Area Under Injury Progress Curve (auipcw4), as dependent variable.

Dependent variable auipcw4	df	SS	RSS	F value	Pr(>F)
Phytophthora isolate (A)	3	144925	284746	104.6874	$<2.2e^{-16}$
Cherry clone (B)	7	28170	167991	8.7209	$9.164e^{-10}$
A × B	34	25059	164880	1.5972	0.02215

auicpw4 = Area Under Injury Progress Curve during 4 weeks after inoculation; df = degrees of freedom; SS = Sums of Squares; RSS = Residual Sums of Squares.

Table 3. Two-way analyses of variance (ANOVA) table (Type-III sum of squares for unbalanced design) considering the effects of *Phytophthora* isolate, wild cherry clone, and their interaction on the disease severity score (damagew4), as dependent variable.

Dependent variable damagew4	df	SS	RSS	F value	Pr(>F)
Phytophthora isolate (A)	3	587.48	1066.67	123.8242	$< 2.2e^{-16}$
Cherry clone (B)	7	136.17	615.36	12.3005	$7.467e^{-14}$
A × B	34	106.76	585.95	1.9855	0.001363

damagew4 = Disease severity score 4 weeks post inoculation; df = degree of freedom; SS = Sums of Squares; RSS = Residual Sums of Squares.

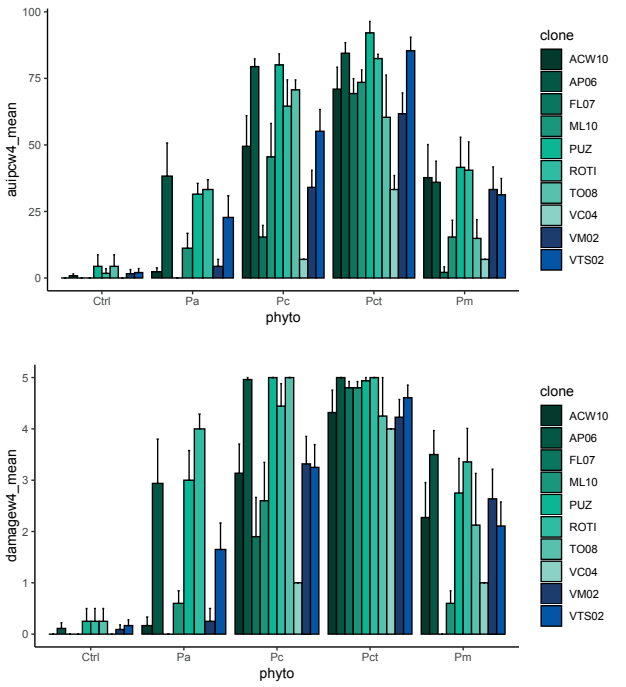

Figure 2. Mean value (+ SE) of the Area Under Injury Progress Curve (auipcw4_mean, top) and of the Disease severity score (damagew4_mean, bottom) 4 weeks post inoculation for all combination of cherry clone and treatment (Ctrl = mock inoculation; *Phytophthora* isolates (phyto): Pa (*P. uniformis*); Pc (*P. cinnamomi*); Pct (*P. citrophthora*); Pm (*P. megasperma* var. *megasperma*).

In vitro plants typically have small numbers of leaves, making percentage-based visual estimations potentially observer-dependent, even if experienced personnel can distinguish subtle differences among plants.

It is important to highlight that working with micropropagated plants is not equivalent to testing plants with well-developed root systems. Differences in physiology, root architecture, and plant maturity can influence susceptibility and disease development. Micropropagated wild cherry plants were also successfully used also for screening resistance to bacterial canker: the method highlighted differences in responses to different bacterial isolates, and results were consistent with those obtained by inoculating seedlings in field trials (Vicente and Roberts, 2003).

The disadvantages of the method presented here lie mainly in complications during regeneration and multiplication of clones, which is genotype-dependent and may require the development of clone-specific protocols (Grant *et al*., 1998; 1999).

Among the pathogen isolates tested, *P. citrophthora* (Pct) was the most virulent, causing the most severe symptoms and the greatest mortality of most of the clones assessed. After 4 weeks, Pct killed 67% of the inoculated plants, and the remaining plants were dead by the end of the experiment. These results confirm the high virulence of Pct to wild cherry. This pathogen could therefore be a risk for *P. avium* cultivated varieties and wild host populations. This threat is particularly important in warm climates, such as those typical of the Mediterranean region, because *P. citrophthora* is considered a thermophilic species.

Susceptibility of wild cherry clones to *P. cinnamomi* (Pc) was variable. Pc caused death of more than 40% of inoculated plants within 4 weeks post-inoculation. These results confirm the previous report of significant variability in susceptibility to the same Pc isolate among wild cherry genotypes (Santini *et al*. 2006).

Inoculations with *P. megasperma* (Pm) only sporadically caused severe symptoms on host plants, and killed less than 15% of the Pm-inoculated plants. This confirms the low susceptibility of wild cherry trees to this *Phytophthora* isolate, as previously observed after inoculations of seedlings (Santini *et al*., 2006).

Phytophthora uniformis (Pa) rarely caused symptoms, and less than 5% of the plants inoculated with this

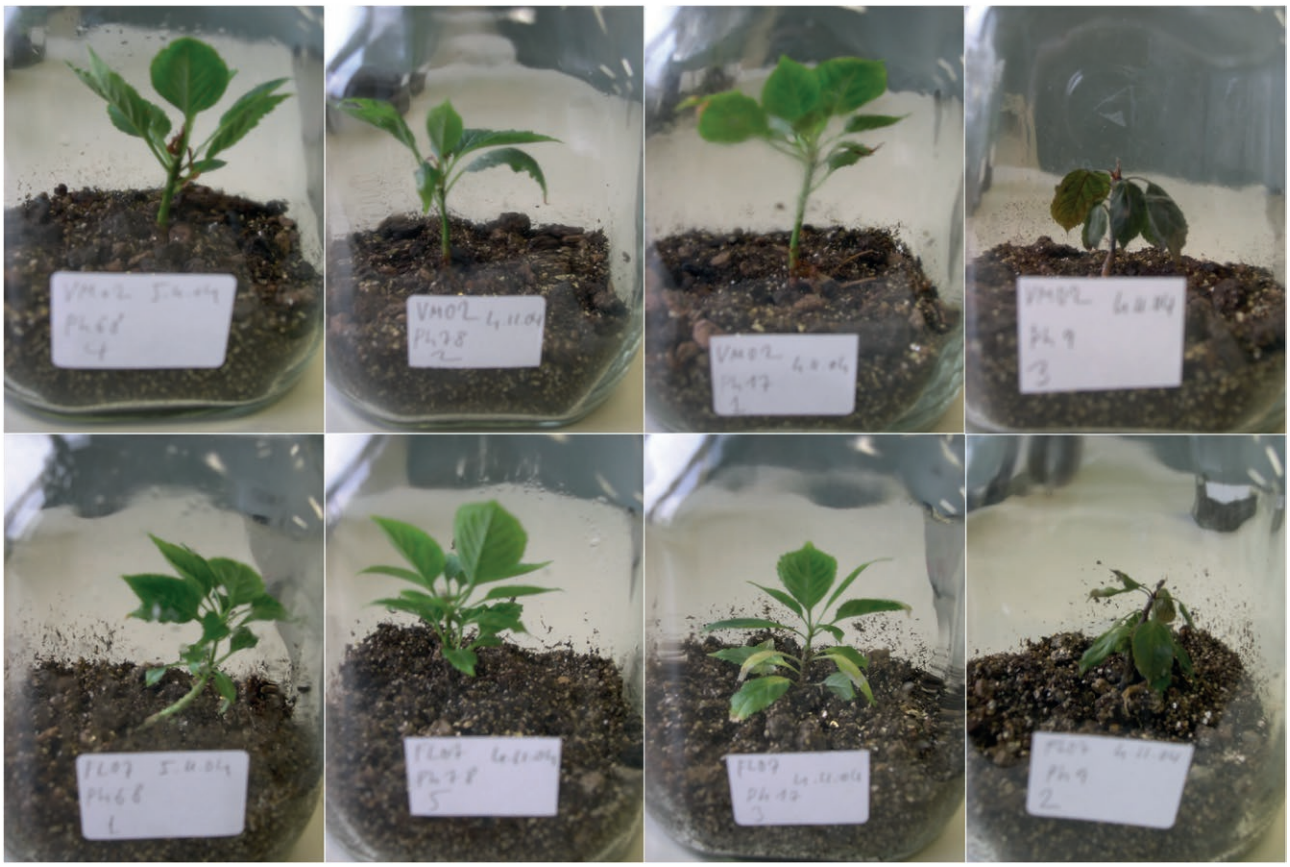

Figure 3. Micropropagated plants of two wild cherry clones inoculated with different *Phytophthora* isolates.

Table 4. Number of ramets, absolute mortality and mean disease severity score (damagew4) for each wild cherry clone inoculated with each *Phytophthora* isolate. Mean values not sharing the same letter are significantly different for HSD multiple test. Ctrl = control (mock inoculation); Pct = *Phytophthora citrophthora*; Pc = *P. cinnamomi*; Pa = *P. uniformis*; Pm= *P. megasperma* var. *megasperma*.

Cherry clone	Ctrl 0.11			Pct 4.68-			Pc 3.73-			Pa 1.56-			Pm 2.37-		
	n[a]	nk[a]	damagew4[a]	n	nk	damagew4	n	nk	damagew4	n	nk	damagew4	n	nk	damagew4
ACW10	9	0	0.00f	11	5	4.32abc	11	1	3.14abcde	6	0	0.17ef	11	1	2.27cdef
AP06	9	0	0.11ef	12	12	5.00ab	13	12	4.96ab	8	2	2.94abcdef	11	3	3.50abcde
FL07	4	0	0.00f	5	3	4.80abc	5	0	1.90def	5	0	0.00f	5	0	0.00f
ML10	5	0	0.00f	5	3	4.8abc	5	0	2.60bcdef	5	0	0.60ef	5	0	0.60ef
PUZZOLO	4	0	0.25ef	8	7	4.94ab	8	8	5.00a	5	0	3.00abcdef	8	3	2.75abcdef
ROTI	4	0	0.25ef	9	9	5.00ab	9	6	4.44abc	6	0	4.00abcd	7	2	3.36abcde
TO08	4	0	0.25ef	4	3	4.25abcd	5	5	5.00ab	-	-	--	4	0	2.12cdef
VC04	2	0	0.00f	2	0	4.00abcde	2	0	1.00def	-	-	--	2	0	1.00def
VM02	11	0	0.00f	11	3	4.23abcd	11	1	3.32abcde	5	0	0.25ef	11	1	2.64bcdef
VTS02	12	0	0.17ef	14	9	4.61abc	13	2	3.25abcde	10	1	1.65def	14	0	2.11def

[a] Numbers of micropropagated plants; nk = numbers of dead plants 4 weeks post inoculation; damagew4 = mean disease severity scores 4 weeks post inoculation. Means accompanied by different letters are different ($P < 0.05$), according to HSD tests.

organism were dead at 4 weeks post-inoculation. This mortality rate remained unchanged at the end of the trial (data not shown). These results are in agreement with those previously obtained after inoculation of Pa in wild cherry seedlings (Santini *et al*., 2006).

Susceptibility to *Phytophthora* isolates among wild cherry clones was variable, highlighting a potential source of resistance to the pathogen even within a small sample of wild cherry genotypes, except for Pct.

The method developed in the present study was effective, rapid and reliable as the results were consistent with those obtained through *Phytophthora* inoculation in seedlings (Santini *et al*., 2006). In addition, the assays did not require land space for field tests, and did not risk pathogen contamination in soil or water, The procedure allowed evaluation of host clones under controlled conditions, reduced evaluation time compared to testing seedling or saplings, and was replicable yearround. This method is for mass screening of susceptibility to *Phytophthora* sp. in wild cherry, and could be adapted for testing other tree species susceptible to *Phytophthora* and able to be propagated *in vitro*. The method could be used to detect natural resistance to *Phytophthora* species in *ex situ* host collections and native populations, contributing to ongoing processes for conservation of native genetic resources (Namkoong, 1989; Lefèvre *et al*., 2020).

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