Effective inoculation methods to screen for resistance to Verticillium wilt in olive

Carlos Trapero[1*], Concepción M. Díez[1], Luis Rallo[1], Diego Barranco[1] and Francisco J. López-Escudero[1]

[1]Departamento de Agronomía, Universidad de Córdoba-Campus de Excelencia Internacional Agroalimentario ceiA3, Edificio C4, Campus de Rabanales, 14014 Córdoba, Spain

*Corresponding author; Email address, carlostrapero@uco.es, Phone number, +34 957218570; Fax, + 34 957218569

Abstract

Effective inoculation methods to screen for Verticillium wilt resistance are essential for the development of olive cultivars resistant to this devastating disease. Three inoculation methods, ~~soil drenching~~pot immmersion, bare-root dipping and stem injection using a conidial suspension of a highly virulent *Verticillium dahliae* isolate (named V117) were tested in olive seedlings. The root-dipping inoculation performed the best, and its effectiveness was further tested in seedlings aged 40, 80 and 120 days in two different environments (greenhouse and growth chamber). The root-dipping inoculation of the 40-day-old olive seedlings discriminated between resistant and susceptible genotypes. This early screening is less costly and requires less time and space than the

standard inoculation and evaluation methods conducted with older plants. Therefore, we propose the root-dipping inoculation of 40-day-old olive seedlings as a reliable, fast and effective method to select genotypes at a young age that are potentially resistant to *V. dahliae*. The application of this method has allowed for the screening of more than 8,000 genotypes before their evaluation under field conditions.

Keywords: *Verticillium dahliae*, *Olea europaea,* breeding, genetic resistance, seedling.

1. Introduction

Verticillium wilt of olive (*Olea europaea*L.), caused by the fungus *Verticillium dahliae* Kleb., is the most important disease affecting this crop in most olive-growing countries (Hiemstra, 1998;Bubici and Cirulli, 2011;López-Escudero and Mercado-Blanco, 2011; Jímenez-Díaz et al., 2012). Such a importance is due to the wide distribution of the defoliating (highly virulent) pathotypes, the severity of the infections, and the difficulty in controlling the disease, as *V. dahliae* can survive in the soil for long periods of time, has a wide host range and is ineffectively controlled by chemical compounds (Klosterman et al., 2009; Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012).

Control of this disease necessitates an integrated strategy that implements all available control measures because there is no single method sufficiently effective when applied individually. Among these control measures, the use of resistant plant material is widely recognized as the least expensive,

51 easiest, safest and most effective method (Agrios, 2005; Klosterman et al.,
52 2009; Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011;
53 Tsror, 2011; Jiménez-Díaz et al., 2012).

54 Several studies have focused on identifying or screening sources of
55 resistance to Verticillium wilt in olive under controlled or field conditions(Bubici
56 and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011;
57 Jiménez-Díaz et al., 2012; Mercado-Blanco and López-Escudero, 2012).
58 Although several olive genotypes possess some degree of resistance to *V.*
59 *dahliae*, most of them, including cultivars widely grown such as 'Arbequina' and
60 'Picual' (Rallo, 2009; Tous, 2011), are susceptible or extremely susceptible to
61 Verticillium wilt. Among 240 olive cultivars evaluated to date, only three of them
62 ('Changlot Real', 'Empeltre' and 'Frantoio') clearly show a moderate level of
63 resistance, although the level is insufficient when disease pressure is high
64 (López-Escudero et al., 2004; Martos-Moreno et al., 2006; López-Escudero et
65 al., 2007; Markakis et al., 2009; Bubici and Cirulli, 2012; Trapero et al., 2013).
66 Therefore, these cultivars are suitable only to replace dead or severely
67 damaged trees in low or moderately infested soils (Trapero et al., 2013) but not
68 to completely overcome the problem generated by Verticillium wilt. Moreover,
69 these cultivars do not suit the plant architecture and vigor requirements for the
70 new intensive or hedgerow orchards.

71 According to the studies mentioned above, there is no complete
72 resistance in olive to *V. dahliae*. Moreover, all the evaluated olive cultivars are
73 more ~~resistant~~ susceptible to the ~~non-~~defoliating pathotype (highly virulent) than
74 to the non-defoliating one. Besides, every cultivar shows a similar resistance
75 level to different *V. dahliae* defoliating isolates or their mixtures. ~~Subsequently,~~

76 ~~we might hypothesize that the resistance to Verticillium wilt is likely to be~~
77 ~~horizontal. This pattern would simplify the identification of resistant genotypes,~~
78 ~~since it would not be necessary to test the resistance to different isolates of the~~
79 ~~pathogen, pinpointing the key role of new resistant cultivars in the control of the~~
80 ~~disease.~~
81
82 Breeding for disease resistance is a long process, which requires the
83 development of suitable selection and evaluation methods to screen a large
84 number of accessions from different sources of resistance (Johnson and Jellis,
85 1992; Allard, 1999; Eynck et al., 2009). This process is especially slow in fruit
86 crops mainly because of their long juvenility and generation periods (Janick and
87 Moore, 1975), which can last for 12 years in olive plants growing under natural
88 conditions (Bellini, 1992). The availability of accurate screening methods is
89 essential to successfully assess disease resistance (Johnson and Jellis, 1992;
90 Blanco-López et al., 1998; Infantino et al., 2006). Screening methods are often
91 applied under controlled conditions that allow the evaluation of breeding
92 genotypes using well-characterized isolates and optimum conditions for disease
93 development. However, the limited availability of labor and space in
94 greenhouses or growth chambers is a major constraint to screening a large
95 number of genotypes under controlled conditions. The screening methods must
96 perform three main functions: i) easily differentiate between susceptible and
97 resistant genotypes, ii) minimize the number of plants that escape infection and
98 iii) produce results that correlate highly with the performance of plants in the
99 field (Grau et al., 1991; Johnson and Jellis, 1992; Debode et al., 2005; Gordon
100 et al., 2005)

101 Resistance to *V. dahliae* is often evaluated in olive using artificial
102 inoculations. Root dipping, soil drenching and trunk drilling to infect the plants
103 with spore suspensions are the most used methods. In general these methods
104 are costly and labor-intensive and may also be highly time-consuming if many
105 genotypes must be inoculated and evaluated. For instance, the inoculated
106 plants are often nearly one year old, and the time required for their evaluation
107 ranges from 3 to 15 months (Mercado-Blanco et al., 2003; López-Escudero et
108 al., 2004; López-Escudero et al., 2007;Antoniou et al., 2008; Cirulli et al., 2008).
109 The evaluation period may last for 6-24 months if the resistance assessment is
110 conducted with soil inoculum (microsclerotia) (López-Escudero and Blanco-
111 López, 2007; Antoniou et al., 2008).
112 Resistance to fungal vascular wilts may change during plant growth and
113 development. In addition, information about the effect of host age on the
114 infection of *V. dahliae* is quite limited and inconclusive, especially in woody
115 hosts where it is possible to find a wide range of sizes and developmental
116 stages (Develey-Riviere and Galiana, 2007; Häffner et al., 2010). Certain
117 authors found that disease severity decreases with host age (Parker, 1959;
118 Evans et al., 1966), such as in olive (López-Escudero et al., 2010), but others
119 reported the reverse situation (Presley and Taylor, 1969; Martin et al., 1993;
120 Resende et al., 1995). Nevertheless, the assessment of the resistance of young
121 seedlings to *V. dahliae* has been frequently used to develop faster and less
122 expensive inoculation techniques (Raabe and Wilhelm, 1978; Chambers and
123 Harris, 1997; Steventon et al., 2002; Klosterman and Hayes, 2009; Bae et al.,
124 2011).

125 The main goal of this study was to develop effective methods to screen
126 olive seedlings for resistance to *V. dahliae* with the aims of: i) clearly
127 distinguishing resistant from susceptible genotypes, ii) shortening the incubation
128 period of infections, and iii) reducing the age of the screened plants and the
129 space and time necessary for their evaluation.
130
131 **2. Materials and methods**
132
133 In a first step, three different methods to inoculate *V. dahliae* in olive were
134 tested using seedlings. Subsequently, the method that performed the best was
135 optimized by assessing the possible effects of the environmental growing
136 conditions and the age of the seedlings at inoculation on their subsequent level
137 of resistance.
138
139
140 **2.1. Evaluation of three methods to inoculate olive seedlings with**
141 ***Verticillium dahliae***
142
143
144 **2.1.1. Plant material**
145
146 Approximately 180 seedlings (90 inoculated and 90 control) from the
147 cross between the cultivars 'Arbequina' (♀, moderately susceptible to
148 Verticillium wilt) x 'Picual' (♂, susceptible to Verticillium wilt) were used.
149 Hereafter, this olive progeny will be named A x P. The cross was performed in

150 the spring of 2010 by applying male pollen to reproductive structures on bagged
151 branches. The fruits were harvested in October, and the seeds were germinated
152 and grown under controlled conditions for 40 days after germination in 0.2 L
153 pots according to Santos-Antunes et al. (2005). Microsatellite-based paternity
154 tests were conducted to assess the genitors of the crosses following the
155 protocol described by de la Rosa et al. (2004). 24 plants of the 'Picual' cultivar
156 (12 inoculated and 12 control) were also included as a reference of well-known
157 susceptible reaction to the disease (López-Escudero et al., 2004; Martos-
158 Moreno et al., 2006; López-Escudero et al., 2007). These plants were self-
159 rooted by stem cutting and root-dip inoculated at the age of 6 months. Both
160 germinated olive seedlings and self-rooted olive plants were grown in the
161 greenhouse until their inoculation.
162
163 **2.1.2. Fungal material and inoculum production**
164 Plants were inoculated in all the experiments with the V117 defoliating *V.*
165 *dahliae* isolate from the collection of the Agronomy Department, University of
166 Córdoba (Blanco-López et al., 1984). This isolate was collected from cotton in
167 southern Andalucía (Spain). The high virulence in olive of this isolate has been
168 previously reported in several artificial inoculations (López-Escudero et al.,
169 2004; Martos-Moreno et al., 2006; López-Escudero et al., 2007). The inoculum
170 was prepared from single-spore stock cultures maintained on potato dextrose
171 agar (PDA) slants at 4 °C. Mycelium was spread on the PDA plates and grown
172 for 8 days at 23 °C in the dark. The plates were flooded with tap water and
173 rubbed gently with a rubber-tipped glass rod. The resulting suspension was
174 filtered through double cheesecloth, counted with a hemocytometer and diluted

to 10^7conidia/ml. This final conidial suspension was used to test the three inoculation methods.

2.1.3. Inoculation methods

Three different inoculation methods, root-dipping, stem injection and ~~soil~~ pot immersion~~drenching~~, were ~~each~~ tested using 60 A x P seedlings (30 inoculated and 30 controls). Additionally, 12 ‘Picual’ self-rooted plants were inoculated using the root-dip method, and 6 ‘Picual’ plants were used as control. In order to ensure inoculum absorption by the plants, all self-rooted and seedling plants were not watered 2 days prior to the inoculation. Plants were arranged in a completely randomized design. The inoculation applying the different methods was performed as follows:

i) Root dipping: the seedlings and self-rooted plants were inoculated by dipping their bare root systems in the *V. dahliae* conidial suspension for 30 min. Then, the plants were transplanted to pots (whose size were 0.19 l for seedlings and 1.5 l for self-rooted plants) with sterile soil (1:1:1, peat:sand:lime) and maintained in a growth chamber during a 12-week evaluation period. Control plants were handled identically except that tap water was substituted for the conidial suspension.

ii) Stem injection: the seedlings were inoculated with one stem puncture between the cotyledons and the first pair of true leaves. The conidial suspension was delivered using a syringe fitted with a 21-gauge needle. The needle was inserted into the stem until the needle point was visible on the opposite side of the stem. One drop of inoculum was dispensed, and the drop disappeared rapidly inside the stem. Approximately 5 μl of inoculum suspension

was absorbed with each puncture. The control seedlings were similarly punctured, but the syringe dispensed a drop of tap water.

iii) Pot immersion~~Soil drenching~~: whole pots containing olive seedlings were immersed simultaneously in a 10-liter *V. dahliae* conidial suspension for 30 minutes, so that the suspension was absorbed from the basement and completely drenched the soil contained in the pots. The pots with control plants were treated the same, except that they were immersed in tap water. The plants were not watered for the first 3 days after the immersion.

2.2. Effect of growing environment and seedling age in the root-dip inoculation method

2.2.1. Plant material, inoculation protocol and experimental design.

In a second step, we optimized the best performing inoculation method by testing the effect of two growing environments and the age of the plant at inoculation on the expression of resistance to Verticillium wilt. To do so, we evaluated seedlings from the crosses A x P and 'Frantoio' (♀, moderately resistant to Verticillium wilt) x 'Picual' (♂), hereafter F x P, and self-rooted plants of the 'Picual' and 'Frantoio' cultivars, which served as examples of well-known resistance in both environments (López-Escudero and Mercado-Blanco, 2011). All the plants for both experiments were inoculated by root dipping as described in 2.1.3., and the same number of plants was treated with water for use as controls. The plants were arranged in a completely randomized design.

2.2.2. Effect of growing environment and seedling age

Seedlings from both crosses and the two cultivars were inoculated, incubated and evaluated in two different environments: the greenhouse and a

225 growth chamber. Approximately 70 seedlings per cross and 12 self-rooted
226 plants per cultivar ('Picual' and 'Frantoio') were evaluated in each environment.
227 The temperature was 22±2°C for the plants incubated in the growth chamber
228 and 20±5°C for those incubated in the greenhouse. Both the greenhouse and
229 the growth chamber were set to 16 h day/8 h night cycles and 85±10% relative
230 humidity.

231 We also tested the expression of resistance in seedlings inoculated at
232 three ages: 40, 80 and 120 days after the beginning of the radicle growth.
233 Approximately 40 seedlings per cross and age were inoculated. Plants were
234 incubated in a growth chamber at 22±1°C under a 16 h day/8 h night
235 photoperiod. The relative humidity was maintained at 85±10%.

236

237 **2.3. Evaluation of the experiments and statistical analysis**

238 **2.3.1. Assessment of disease severity**

239 From the third week after inoculation, olive plants were scored weekly for
240 disease symptoms using a 0 to 4 scale based on the percentage of plant tissue
241 displaying the symptoms of *V. dahliae* infection. Self-rooted olive plants were
242 scored according to the scale used in previous works (López-Escudero et al.,
243 2004). The 0 to 4 rating scale was adapted for small olive seedlings. Because
244 young olive seedlings have very few leaves (usually 2 to 6 pairs, depending on
245 seedling age), the disease severity was based primarily on the number of
246 defoliated or wilted leaves: (0=no symptoms, 1=1 to 33% shed or wilted, 2=34
247 to 66%, 3=67 to 99% and 4=dead plant)

248

249 The relative area under the disease progress curve (RAUDPC) was
250 considered the main parameter to assess the disease intensity. It was
251 calculated from the disease severity values according to the following formula
252 (Campbell and Madden, 1990):

253 $$RAUDPC = \frac{100}{(s_{\max} \times t_e)} \times \sum_{i=1}^{n} \frac{(s_i + s_{i+1})}{2} \times (t_{i+1} - t_i)$$

254 Where s_i = disease severity value for observation number i, s_{max} = maximum
255 value of severity (4), t_i= number of days between planting and observation i, t_e =
256 total evaluation period and n = number of observations.

257

258 The incidence or percentage of symptomatic plants, percentage of dead
259 plants, incubation period and recovery from the disease were also calculated to
260 assess the intensity of the reactions (Wilhelm and Taylor, 1965; López-
261 Escudero et al., 2004; López-Escudero and Blanco-López, 2005).

262

263 **2.3.2. Pathogen isolation**

264 The pathogen was isolated from symptomatic plants to confirm the infection.
265 The seedling stems were washed in running tap water and surface disinfected
266 in 0.5% sodium hypochlorite for 1 min. The stem pieces were placed on PDA
267 plates and incubated at 24°C in the dark for 6 days.

268

269 **2.3.3. Assessment of the efficiency of the inoculation methods**

270 To accurately assess the labor needed for each inoculation method, all
271 the experiments were conducted by the same team. The hours of labor and
272 number of inoculated plants were counted for each experiment and type of plant
273 material. The space needed to maintain the plant material in individual pots was

274 also calculated. The costs for materials, labor, and greenhouse and growth
275 chamber space were recorded to compare the total costs for each inoculation
276 method.
277

278 **2.3.4. Statistical analysis of data**

279 An analysis of variance (ANOVA) of the RAUDPC was performed for
280 each experiment. To analyze the effects of the growing environment and the
281 seedling age, a factorial analysis was performed for each variable. Data were
282 transformed in order to fulfill the ANOVA requirements (Levene's homogeneity
283 of variances test *P* values were 0.21 and 0.06 respectively for the log-
284 transformed RAUDPC and the inverse-transformed incubation period in the
285 growing environment experiment; and 0.24 and 0.09 for the log-transformed
286 RAUDPC and the inverse-transformed incubation period in the seedling age
287 experiment). Mean values were compared using Fisher's protected least
288 significant difference test at $P = 0.05$.

289 Both incidence and mortality were analyzed by Pearson's Chi-squared
290 nonparametric test, considering the observed and expected frequencies of
291 symptomatic or dead plants, respectively. Incubation period was analyzed by
292 the nonparametric Kaplan-Meier survival analysis (Kaplan and Meier, 1958), in
293 which survival times were calculated as the day in which a plant showed
294 disease symptoms for the first time. Pair-wise comparisons were tested for
295 significance using the log-rank test.

296 In the experiment comparing the two environmental treatments, the
297 distribution of data within each plant material was analyzed and compared by
298 calculating summary statistics and by drawing box and whisker plots. Statistical

299 analyses were performed using the programs SPSS 21.0 (SPSS Inc., Chicago,
300 USA) for analyzing the incidence, mortality and incubation period; and Statistix
301 9.0 (Analytical Software, Tallahassee, USA) for the rest of the analyses.
302
303 **3. Results**
304 **3.1. Methods of inoculation**
305 Both root dipping and stem injection inoculation using *V. dahliae* conidial
306 suspensions were able to induce Verticillium wilt symptoms in every inoculated
307 olive seedling. However, no symptoms were observed in the plants inoculated
308 using the pot immersion soil-drenching method. No visually observable
309 symptoms were detected in any of the control plants.
310 Disease symptoms in the young seedlings were the same with both the
311 root-dipping and the stem injection inoculation methods and similar to those
312 observed in 6-month-old self-rooted plants inoculated by root dipping. In
313 addition to the defoliation and sudden wilt observed in both types of infected
314 plant material, a purple coloration on the leaf underside was observed in
315 inoculated seedlings just 1 or 2 weeks before the beginning of symptoms.
316 Disease progressed faster and was more severe in the olive seedlings
317 than in the self-rooted plants of the 'Picual' cultivar after both were inoculated by
318 root dipping. In both cases, the first symptoms generally appeared 4-5 weeks
319 after inoculation, regardless of the method of inoculation. The increase of the
320 disease lasted for 8 weeks in the self-rooted plants and 6 weeks in the young
321 seedlings (Fig. 1).
322 RAUDPC (66.9), final severity (3.8) and percentage of dead plants
323 (91.7%) in the seedlings inoculated by root dipping were greater than in those

inoculated by stem injection (56.7, 3.2 and 58.3%, respectively). However, none of the parameter values differed significantly between the inoculation methods at $P = 0.05$ according to the Fisher's protected LSD test ($P > 0.05$ for RAUDPC and the final severity).

The stem injection inoculation of the young olive seedlings produced two main issues. First, we observed the development of new sprouts just below the injection site some weeks after the inoculation. Second, the time needed to obtain the maximum disease severity in the seedlings inoculated by this method was longer than the time needed in those inoculated by root dipping (Fig. 1).

3.2. Effect of growing environment and seedling age in the root dip inoculation method

Significant differences in the RAUDPC were found between the evaluated plant material (two crosses and two self-rooted cultivars) and between the two environmental conditions, but there was no significant interaction between ~~both~~ them (Table 1). The A x P seedlings showed higher values of the disease parameters than those shown by the F x P seedlings (Table 1). After inoculation, the seedlings and self-rooted plants kept in the growth chamber showed more severe symptoms than the plants in the greenhouse.

The incubation period was therefore longer in the plants maintained in the greenhouse (Table 1, Fig. 2 A and B). The mean temperature during the experiment was 20.7 or 22.3 °C for the greenhouse or growth chamber, respectively, whereas the minimum and maximum temperatures were 17.0 and 24.1°C in the greenhouse and 21.2 and 23.5 °C in the growth chamber.

349 Following inoculation by root dipping, the seedlings from the two crosses
350 showed consistent symptoms of *V. dahliae* infection independently of their age
351 of inoculation. The differences in the RAUDPC values between the crosses and
352 among the seedling inoculation ages were both significant, but the interaction
353 between the cross and age factors was not (Table 2). Like the effect of the
354 growing environment, the values of the disease parameters were higher for the
355 seedlings from the A x P cross than for those from the F x P cross (Table 2, Fig.
356 3). The A x P seedlings inoculated at the age of 40 days were the most
357 susceptible to the infection according to the disease parameter values, while F x
358 P seedlings inoculated 120 days after inoculation were, by far, the most
359 resistant (Table2, Fig. 3).
360

361 **3.3. Pathogen isolation and plant recovery from the infection**

362 The pathogen was isolated from nearly all the affected plants that were
363 tested and from many of the inoculated asymptomatic plants (data not shown).
364 Note that no plant inoculated with the fungus was able to grow during the first 8
365 weeks after inoculation except for the sprouts growing below the injection site in
366 the stem-inoculated seedlings. After 8-12 weeks, the plants that had been free
367 of symptoms and some of the plants that had shown slight symptoms were able
368 to resume growth.
369

370 **3.4. Assessment of the efficiency of the inoculation methods**

371 The stem injection of olive seedlings was the quickest inoculation
372 method, whereas the root dipping of self-rooted 6-month-old (50 cm high) plants
373 was the slowest (Table 3). Reducing the plant age at inoculation to 40 days and

374 the height to approximately 7 cm hastened5 times the root dipping method, so
375 that 75 seedlings could be inoculated per person and hour (Table 3).

376 These age and height reductions also quite effectively reduced the space
377 required in the greenhouse or growth chamber. Consequently, the use of young
378 seedlings reduced the cost per plant by 82% considering the materials, labor
379 and greenhouse or growth chamber expenses (Table 3).

380

381 **3.5. Resistance of genotypes to *Verticillium dahliae***

382 According to the RAUDPC and other disease parameters (incidence,
383 mortality and incubation period), the seedlings from the A x P cross were
384 significantly more susceptible than those from the F x P cross for all the
385 seedling inoculation ages and growing environments (Tables 1 and 2).

386 From 200 olive genotypes evaluated in the growth chamber, 17 (14.4%)
387 and 28 (34.2%) genotypes from the A x P and F x P crosses, respectively,
388 remained free of symptoms during the disease evaluation period and were
389 selected for resistance to Verticillium wilt. In the greenhouse, 18 A x P (24.0%)
390 and 33 F x P (48.3%) genotypes were selected for their resistance out of 146
391 seedlings evaluated. This difference in the disease reaction observed between
392 the two growing environments is shown in the box and whisker plots (Fig. 4 A
393 and B). The plants incubated in the growth chamber reacted more severely than
394 the plants in the greenhouse, but the responses were highly variable in both
395 growing environments and plant groups, especially in the progeny seedlings
396 from the crosses between cultivars, which are comprised of different genotypes.

397

398 **4. Discussion**

The infection and inoculation of olive seedlings with *V. dahliae* have been poorly studied and have always involved the evaluation of large plants, usually more than one year old (Wilhelm and Taylor, 1965; Colella et al., 2008). This is the first report of consistent infection of young olive seedlings with *V. dahliae*. The inoculation of young seedlings may have enormous potential for application in programs to breed for Verticillium wilt resistance in olives, as has occurred in other woody crops affected by this pathogen, such as *Acer platanoides* (Chambers and Harris, 1997; Hiemstra and Van Holsteijn, 2000), avocado (Pinkas and Kariv, 1981), cocoa (Resende et al., 1995), apricot (Taylor and Flentje, 1968) and pistachio (Raabe and Wilhelm, 1978; Ashworth, 1984; Morgan et al., 1992). The application of this methodology in olive breeding programs may be quite important considering the lack of complete resistance in traditional olive cultivars and the spread of the disease worldwide (Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012).

In the present study, we demonstrate that both root dipping and stem injection are effective inoculation methods to evaluate young olive seedlings for resistance to Verticillium wilt under controlled conditions. The ~~soil-drenching~~pot-immersion inoculation did not induce symptoms in the olive seedlings. This result differs from those reported by Cirulli et al. (2008) although the inoculation methods used in both studies were not exactly the same. We immersed the pots with plants in the conidial suspension, while Cirulli et al. (2008) inoculated self-rooted olive plants by the immersion of their root balls previously injured. Besides, we did not sterilize the soil as it was done in the cited study and the substrate might have retained or inactivated the conidia, preventing the infection

424 of the roots (Bubici and Cirulli, 2011; López-Escudero and Mercado-Blanco,
425 2011; Jiménez-Díaz et al., 2012). The stem injection of seedlings was the
426 quickest inoculation method, but it had several drawbacks. For instance, the
427 disease severity was less than that obtained with the root-dipping method, as
428 reported for self-rooted olive plants (López-Escudero et al., 2007; Cirulli et al.,
429 2008). Moreover, the lower portion of the injected seedlings did not seem to be
430 affected by the pathogen, so plants were able to recover from the disease. This
431 pattern could be due to the upward movement of the *V. dahliae* conidia in the
432 xylem vessels, as reported by Presley et al. (1966).

433 The fact that no seedling inoculated by root dipping grew for several
434 weeks after the inoculation supports the efficacy of this method. It is likely that
435 no inoculated plant escaped systemic infection, which is consistent with
436 previous studies in self-rooted olive plants (López-Escudero et al., 2004; Cirulli
437 et al., 2008; Markakis et al., 2009). This fact also emphasizes the need of
438 further research before using these putative resistant seedlings as rootstocks
439 since nothing is known about the possible transmission of the fungi to the
440 grafted cultivar. The dipping of roots was a rapid inoculation procedure when
441 using small olive seedlings and additionally allowed us to shorten the incubation
442 period. Moreover, the reduced requirements for greenhouse or growth chamber
443 space, labor and time devoted to plant evaluation make this method
444 exceptionally convenient for screening a large number of olive seedlings for *V.*
445 *dahliae* resistance.

446 The environmental conditions were critical for evaluating the disease
447 resistance of the olive seedlings. The higher disease severity observed in the
448 growth chamber compared with the greenhouse was likely attributable to the

449 higher and more stable temperature recorded in the chamber. Although
450 temperature has not been studied as a factor in *V. dahliae* symptom
451 development in olives, approximately 22-25 °C has been reported to be optimal
452 for the *in vitro* growth of the defoliating pathotype of *V. dahliae* (Soesanto and
453 Termorshuizen, 2001; Xu et al., 2012) and for infecting olives (López-Escudero
454 et al., 2004; López-Escudero and Blanco-López, 2007). Our results do not
455 directly address the effect of temperature on the infection caused by *V. dahliae*,
456 but they demonstrate that the growth chamber conditions are most likely the
457 better choice for screening olive genotypes for high *V. dahliae* resistance,
458 easing the selection of the highly resistant genotypes.
459 The results of inoculating seedlings at different ages suggest that age is
460 an important factor in evaluating olive genotypes for resistance to *V. dahliae*.
461 Apparently, younger seedlings are more susceptible to the infection than older
462 ones and develop the disease much faster. There were also some differences
463 between seedlings and self-rooted older plants regarding disease reaction
464 (López-Escudero et al., 2004; Cirulli et al., 2008).These differences may be due
465 to several sources of variation such as, genetic, root morphology but especially
466 to the size of the plant, because it takes several weeks for the pathogen to
467 reach the upper portion of the plant and induce foliar symptoms in 6-month-old
468 plants (Mercado-Blanco et al., 2003; Prieto et al., 2009). This process likely
469 occurs faster in extremely small plants, which is consistent with our results and
470 those reported in other species (Evans et al., 1966; Hiemstra and Van Holsteijn,
471 2000; Bae et al., 2007). It is also noticeable that the infection and the symptom
472 development occurred without wounding the roots, probably because the

473 fungus is able to penetrate by microscopic wounds in the roots (Prieto et al.,
474 2009).

475 Our study analyzed four disease parameters (incidence, mortality,
476 incubation period and RAUDPC) to assess the most suitable inoculation
477 method. These parameters were used also to select the most resistant
478 genotypes of the evaluated progeny. In many studies, the final severity score or
479 the proportion of plants with no symptoms appears to be the most suitable
480 parameter (Johnson and Jellis, 1992). In the present study, both the plants
481 having no symptoms and the plants alive at the end of the experiment were the
482 selection parameters considered, provided in both cases that the plants showed
483 consistent growth as a result of recovery from the disease. However, the most
484 convenient parameter of the two would depend on the evaluation environment
485 and the mean resistance of the progeny evaluated.

486 The results also provided initial information about valuable genitors to
487 breed for Verticillium wilt resistance in olive. Progeny from the cross between
488 the susceptible cultivars (‘Arbequina’ and ‘Picual’) were more susceptible than
489 those derived from the cross between the moderately resistant (‘Frantoio’) and
490 the susceptible (‘Picual’) cultivars. Thus, it seems likely that progeny resistance
491 is correlated with the resistance of the genitors, even at the seedling stage. The
492 resistance of the ‘Frantoio’ cultivar has been previously confirmed using artificial
493 inoculations (Blanco-López et al., 1998; López-Escudero et al., 2004; Martos-
494 Moreno et al., 2006; Bubici and Cirulli, 2012) as well as under field conditions
495 (Trapero et al., 2013). Therefore, our results are consistent with those of a
496 preliminary study conducted under controlled conditions (Trapero et al., 2011)
497 and those observed under field conditions by Wilhelm and Taylor (1965), which

498 indicate that 'Frantoio' may be a suitable genitor to breed for Verticillium wilt
499 resistance in olive.
500

501 **5. Conclusions**

502

503 The success of a breeding program for disease resistance depends upon the
504 methods employed for the inoculation, evaluation and selection of plants within
505 the target host population (Johnson and Jellis, 1992; Resende et al., 1995). Our
506 results showed that the inoculation of young seedlings by root dipping is a fast,
507 effective and reliable method to screen a large number of olive genotypes for *V.*
508 *dahliae* resistance. Stem injection inoculation may also be suitable in
509 experiments requiring speed or low cost. The root-dip inoculation of young
510 seedlings (40 days old) subsequently evaluated in growth chambers was shown
511 to be the most effective inoculation method, the better environment and the best
512 age to begin the screening process for the large number of genotypes
513 generated in an olive breeding program. These results provide information
514 useful to optimize the evaluation and selection of olive genotypes resistant to
515 Verticillium wilt, saving labor, space and economic resources. Actually, more
516 than 8,000 olive seedlings from different sources of resistance have been
517 screened for Verticillium wilt resistance by applying the procedures described in
518 this study.
519

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521

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525

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712

713 **Figure captions**

714

715 Fig. 1. Disease severity progress curves for olive seedlings from the 'Arbequina'
716 x 'Picual' cross and for self-rooted olive plants of the 'Picual' cultivar inoculated
717 by root dipping or stem injection with a conidial suspension of a highly virulent

isolate (V117) of *Verticillium dahliae* and maintained in a growth chamber. ~~Soil drenching~~Pot immersion method is not shown in the Figure as no symptoms were observed in seedlings inoculated by this method. The disease severity was rated weekly using a 0 to 4 scale, indicating the percentage of plant tissue affected by defoliation and sudden wilt (0 = healthy plant or plant with no symptoms, 1 = 1 to 33%, 2 = 34 to 66%, 3 = 67 to 99% and 4 = dead plant).

Fig. 2. Disease severity progress curves in olive seedlings from crosses between 'Arbequina' x 'Picual' (A x P) and 'Frantoio' x 'Picual' (F x P) cultivars and in self-rooted olive plants of 'Picual' and 'Frantoio' cultivars inoculated by root dipping with a conidial suspension of a highly virulent isolate (V117) of *Verticillium dahliae* and maintained in a growth chamber (A) or greenhouse (B). The disease severity was rated weekly using a 0 to 4 scale, indicating the percentage of plant tissue affected by defoliation and sudden wilt (0 = healthy plant or plant with no symptoms, 1 = 1 to 33%, 2 = 34 to 66%, 3 = 67 to 99% and 4 = dead plant).

Fig. 3. Disease severity progress curves in olive seedlings derived from crosses between 'Arbequina' x 'Picual' (A x P) and 'Frantoio' x 'Picual' (F x P) cultivars inoculated by root dipping at 40, 80 and 120 days after germination with a conidial suspension of a highly virulent isolate (V117) of *Verticillium dahliae* and

743 maintained in a growth chamber. The disease severity was rated weekly using a
744 0 to 4 scale, indicating the percentage of plant tissue affected by defoliation and
745 sudden wilt (0 = healthy plant or plant with no symptoms, 1 = 1 to 33%, 2 = 34
746 to 66%, 3 = 67 to 99% and 4 = dead plant).

747

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749

750 Fig. 4. Box and whisker plots showing the distribution of the relative area under
751 the disease progress curve (RAUDPC) of olive seedlings from crosses between
752 'Arbequina' x 'Picual' (A x P) and 'Frantoio' x 'Picual' (F x P) cultivars and self-
753 rooted olive plants of 'Picual' and 'Frantoio' cultivars. All plants were inoculated
754 by root dipping with a conidial suspension of a highly virulent isolate (V117) of
755 *Verticillium dahliae* and maintained in a growth chamber (A) or greenhouse (B).
756 Disease severity was rated weekly using a 0 to 4 scale. The rectangles show
757 the values below which 25% (lower side of box), 50% (the center line or
758 median), and 75% (upper side of box) of the observations fall. The whiskers
759 extend to the highest and lowest observation unless they are more than 1.5
760 box-lengths long. Observations outside this range are plotted as black circles
761 (outlying data). The disease severity progress of these plants is shown in Figure
762 2 A and B.

763

Table 1. Disease parameters for olive seedlings from the ‘Arbequina’ x ‘Picual’ and ‘Frantoio’ x ‘Picual’ crosses between cultivars and for self-rooted plants of the ‘Picual’ and ‘Frantoio’ cultivars all inoculated by root dipping in a conidial suspension of a highly virulent isolate (V117) of *Verticillium dahliae* and evaluated in the growth chamber and greenhouse environments.[a]

Plant material[b]	RAUDPC[c]			Incidence[d] (%)		Mortality[e] (%)		Incubation period[f] (days)	
	Chamber	Greenhouse	Mean	Chamber	Greenhouse	Chamber	Greenhouse	Chamber	Greenhouse
A x P seedlings	61.4	15.9	40.9 a	93.5 a	76.0 b	71.7 a	9.3 b	35.6 a	60.3 b
F x P seedlings	34.6	9.3	23.3 b	76.5 a	51.8 b	52.9 a	3.5 b	50.3 a	61.2 b
‘Picual’ plants	51.2	17.4	37.7 a	100.0 a	100.0 a	90.0 a	50.0 a	37.1 a	61.8 b
‘Frantoio’ plants	12.5	0.7	6.8 c	80.0 a	25.0 b	0.0 a	0.0 a	56.9 a	74.7 b
Mean	39.9 a	14.3 b		87.5 a	63.6 b	51.2 a	15.9 b	41.7 a	61.1 b

[a]Values are the means by environment and cross or cultivar estimated 12 weeks after inoculation of plants inoculated at all ages.

[b]Genitors are ‘Arbequina’ (A), ‘Frantoio’ (F), and ‘Picual’ (P).

[c]Mean value for the relative area under the disease progress curve potentially reached over the assessment period. Data were analyzed after ~~its~~ their log transformation in order to fulfill the ANOVA (factorial design) requirements. Interaction between the main factors was not significant (P = 0.698). Mean values in the same column (for plant materials) and mean values in the same row (for growing environment) followed by the same letter are not statistically significant according to Fisher’s protected least significant difference test at P = 0.05.

11 [d]Percentage of plants showing symptoms 12 weeks after inoculation. Values in rows followed by the same letter are not statistically

12 significant according to Pearson's Chi-squared test at *P* = 0.05.

13 [e]Percentage of plants killed by *V. dahliae* 12 weeks after inoculation. Values in rows followed by the same letter are not statistically

14 significant according to Pearson's Chi-squared test at *P* = 0.05.

15 [f]Mean number of days from inoculation to the appearance of symptoms. Data were analyzed by Kaplan-Meier's survival analysis.

16 Values in rows followed by the same letter are not statistically significant according to log-rank test at *P* = 0.05

17

18

19

20

21 Table 2. Disease parameters for olive seedlings from the ‘Arbequina’ x ‘Picual’ and ‘Frantoio’ x ‘Picual’ crosses between cultivars

22 and inoculated by root dipping in a conidial suspension of a highly virulent isolate (V117) of *Verticillium dahliae* at 40, 80 and 120

23 days after germination.[a]

Age at inoculation (pairs of leaves)	RAUDPC[b]			Incidence[c] (%)		Mortality[d] (%)		Incubation period[e] (days)	
	A x P	F x P	Mean	A x P	F x P	A x P	F x P	A x P	F x P
40 (2.7)	61.4	34.6	50.1 a	93.5 a	76.5 b	71.7 a	52.9 a	35.6 a	50.3 b
80 (4.3)	52.0	30.4	42.7 b	87.5 a	60.0 b	68.8 a	40.0 b	40.8 a	46.3 b
120 (7.2)	30.8	10.6	22.1 c	66.7 a	55.6 a	41.7 a	5.6 b	37.7 a	49.7 a
Mean	51.4 a	27.8 b		82.6 a	64.0 b	60.7 a	32.8 b	38.0 a	48.8 b

24 [a]Values are the means by cross and inoculation age estimated 12 weeks after inoculation of the inoculated plants maintained in

25 both environments. Genitors are ‘Arbequina’ (A), ‘Frantoio’ (F), and ‘Picual’ (P).

26 [b]Mean value for the relative area under the disease progress curve potentially reached over the assessment period. Data were

27 analyzed after ~~its~~ their log transformation in order to fulfill the ANOVA (factorial design) requirements. Interaction was not significant

28 (P = 0.909). Mean values in the same column (for inoculation ages) and mean values in the same row (for crossess) followed by the

29 same letter are not statistically significant according to Fisher’s protected least significant difference test at P = 0.05.

30

31 [c]Percentage of plants showing symptoms 12 weeks after inoculation. Values in rows followed by the same letter are not statistically

32 significant according to Pearson's Chi-squared test at $P = 0.05$.

33 [d]Percentage of plants killed by *V. dahliae* 12 weeks after inoculation. Values in rows followed by the same letter are not statistically

34 significant according to Pearson's Chi-squared test at $P = 0.05$.

35 [e]Mean number of days from inoculation to the appearance of symptoms. Data were analyzed by Kaplan-Meier's survival analysis.

36 Values in rows followed by the same letter are not statistically significant according to log-rank test at $P = 0.05$

37

38

39

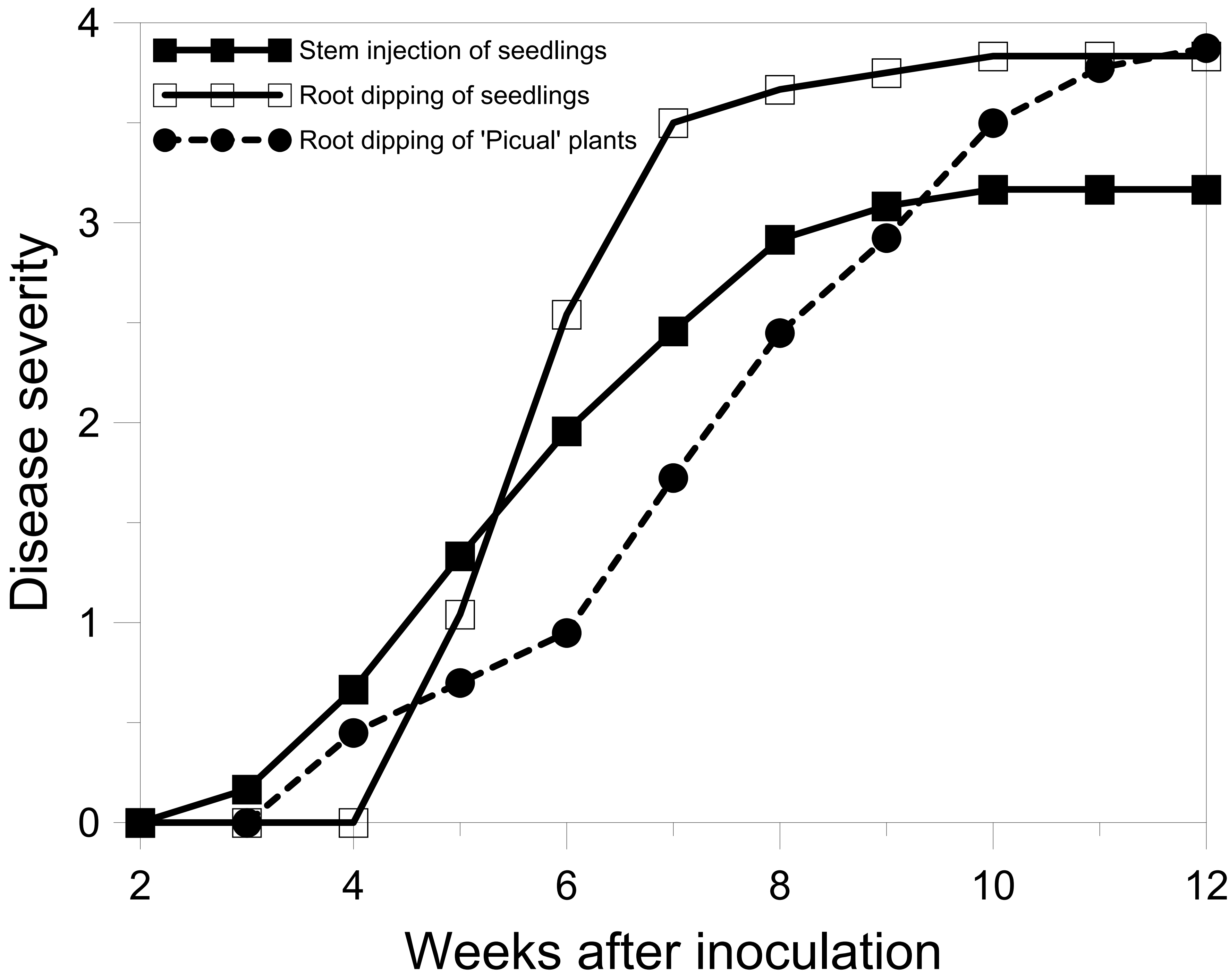
Table 3. Efficiency parameters of several *Verticillium dahliae* inoculation methods performed in seedlings and self-rooted olive plants.

Inoculation method	Plants/h[a]	Plants/m^2 [b]	Cost[c]
Root dipping of self-rooted	15	19	4
Root dipping of seedlings	75	222	1
Stem injection of seedlings	120	222	1
Pot immersion~~Soil~~	50	222	2

[a]Plants inoculated by one person in one hour. This calculation includes the whole process from inoculum preparation until the plants ~~a~~were in the greenhouse/growth chamber ready to be evaluated.

[b]Number of plants that was possible to evaluate in 1 square meter.

[c]Estimation of the total economic costs of each inoculation method. Total costs were ranged in four groups: (1: very low; 2: low; 3: medium; and 4: high). This cost includes production of the plants and inoculum, materials, labor and energy costs.



Stem injection of seedlings
Root dipping of seedlings
Root dipping of 'Picual' plants
Disease severity
0
1
2
3
4
2
4
6
8
10
12
Weeks after inoculation

A

Growth chamber

'Picual'
'Frantoio'
A x P seedlings
F x P seedlings

Disease severity

0 1 2 3 4

2 4 6 8 10 12 14

Weeks after inoculation

B

Greenhouse

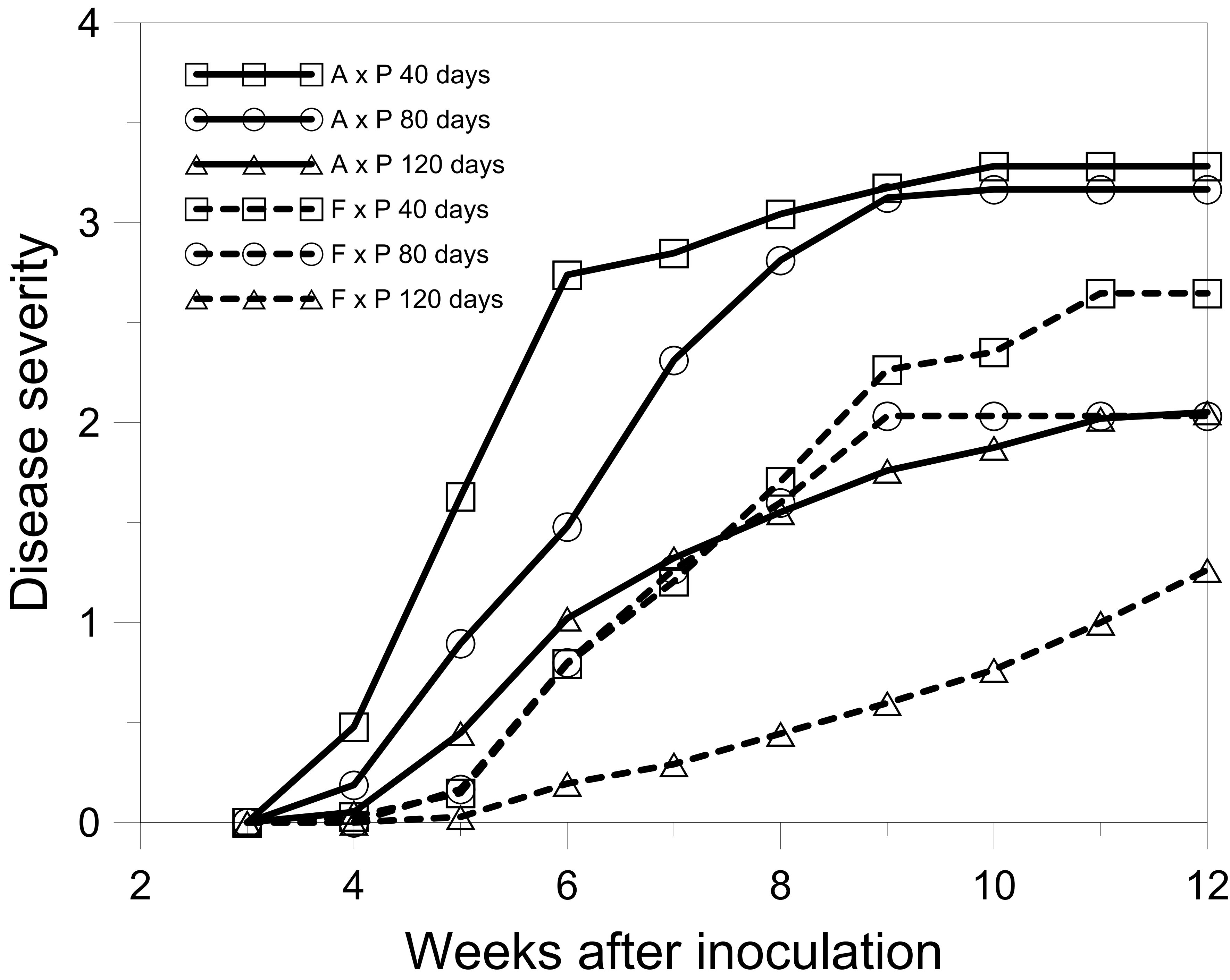
'Picual'
'Frantoio'
A x P seedlings
F x P seedlings

Disease severity

0 1 2 3 4

2 4 6 8 10 12 14

Weeks after inoculation


A x P 40 days
A x P 80 days
A x P 120 days
F x P 40 days
F x P 80 days
F x P 120 days
Disease severity
0
1
2
3
4
2
4
6
8
10
12
Weeks after inoculation

A

Growth chamber

RAUDPC

100
80
60
40
20
0

A x P
F x P
'Picual'
'Frantoio'

B

Greenhouse

RAUDPC

100
80
60
40
20
0

A x P
F x P
'Picual'
'Frantoio'