Short title: Resistance to Verticillium wilt of olive

Variability and selection of Verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus

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Abbreviations: OP = Open pollinated

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Abstract

Developing Verticillium wilt resistant genotypes is currently a major objective in olive breeding. In this study, 6017 genotypes derived from 48 crosses obtained by open pollination and crosses between olive cultivars, wild olive genotypes and other *Olea* species and *Olea europaea* subspecies were individually evaluated for Verticillium wilt resistance. More than 800 genotypes were identified as resistant to the disease based on the absence of symptoms. High genetic variability and wide segregation in resistance were observed. The inheritance of resistance was studied, and the best parents and crosses to breed resistant genotypes were identified. According to the

results, Verticillium wilt resistance in olive appears to be a quantitative trait. The results obtained by comparing the level of resistance between different crosses as well as by estimating heritability suggest that is possible to breed for Verticillium wilt resistance in olive.

INTRODUCTION

Olive (*Olea europaea* L.) is the most important oil tree crop in temperate areas worldwide (FAO, 2012). Verticillium wilt, caused by the fungus *Verticillium dahliae* Kleb., is currently the most important disease affecting this tree in the majority of olive growing countries (López-Escudero & Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012). Verticillium wilt causes significant economic losses due to the severity of the disease and the difficulty of preventing its spread. A mean incidence of 0.4% has been reported in Spain, but in some areas, the incidence is as high as 9%, with more than 50% of orchards affected (Ruiz Torres, 2010). Indeed, Verticillium wilt is among the most important olive farmers' concerns in Spain (Areal & Riesgo, 2014). The most severe attacks are caused by the defoliating (highly virulent) isolates and usually result in death of the tree (Bubici & Cirulli, 2011; López-Escudero & Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012).

The control of Verticillium wilt is challenging for three main reasons: first, *V. dahliae* is able to survive for up to 14 years in soil (Wilhelm, 1955); second, it is able to infect a wide host range, from annual plants to woody crops (McCain *et al.*, 1981; Bhat & Subbarao, 1999; Klosterman *et al.*, 2009); and third, no effective therapeutic treatments for infected trees have yet been developed (Klosterman *et al.*, 2009; López-Escudero & Mercado-Blanco, 2011; Tsror, 2011; Jiménez-Díaz *et al.*, 2012). Where available, host resistance is the most effective, environmentally friendly and least

51 expensive method to control this disease (Agrios, 2005; Klosterman *et al.*, 2009; Tsror,
52 2011).

53 Unfortunately, most olive cultivars evaluated to date are susceptible to *V.*
54 *dahliae* to varying degrees (López-Escudero *et al.*, 2004, 2007; Martos-Moreno *et al.*,
55 2006; Trapero *et al.*, 2013b; Garcia-Ruiz *et al.*, 2014). However, an extensive and as yet
56 unevaluated genetic diversity exists within cultivated olive (Rallo *et al.*, 2005; Díez *et*
57 *al.*, 2012) and the wild olive species, called oleaster (*Olea europaea* subsp. *europaea*
58 var. *sylvestris*) (Belaj *et al.*, 2010; Lavee, 2013). This variability could be invaluable for
59 breeding for resistance to Verticillium wilt and other diseases and for tolerance to
60 abiotic stresses (Klepo *et al.*, 2013). Moreover, there are several wild species and
61 subspecies within the *Olea* genus that have never been explored for these traits (Green,
62 2002; de la Rosa *et al.*, 2003; Besnard *et al.*, 2009; Lavee, 2013).

63 New genotypes coming from open pollination or crosses between selected
64 parents in breeding programs constitute another potential source of resistance. These
65 genotypes exhibit new allelic combinations that might confer higher resistance to
66 Verticillium wilt. Breeding programs in other species, such as in *Acer* spp. (Hiemstra &
67 Van Holsteijn, 2000), avocado (Pinkas & Kariv, 1981), pistachio (Ashworth, 1984;
68 Epstein *et al.*, 2004) and strawberry (Shaw *et al.*, 2010), have been successful in
69 obtaining genotypes resistant to Verticillium wilt. In many of these species, the
70 resistance was found within the cultivated germplasm, but in others, such as pistachio,
71 the resistance was provided by wild relatives (Schnathorst, 1988; Epstein *et al.*, 2004).

72 The generation and evaluation of these new genotypes (normally large progenies
73 resulting from crosses between known parents) also constitute a useful approach to
74 understanding the mechanisms underlying resistance to Verticillium wilt. To date, only
75 progenies coming from the open pollination of wild and cultivated olive genotypes have

been evaluated (Wilhelm & Taylor, 1965; Colella *et al.*, 2008; Trapero *et al.*, 2011). This approach is very valuable considering the difficulties of generating pure crosses in olive (Diaz *et al.*, 2007). However, the offspring from controlled crosses between known genotypes would enhance the evaluation of the influence of both parents as well as of the mechanisms determining resistance to Verticillium wilt.

Thus far, Verticillium wilt resistance has been described as either qualitative or quantitative (Pegg & Brady, 2002). Major genes conferring resistance have been identified in host species such as cotton (Mert *et al.*, 2005), lettuce (Hayes *et al.*, 2011), mint (Vining & Davis, 2009), potato (Jansky *et al.*, 2004) and tomato (Schaible *et al.*, 1951; Bender & Shoemaker, 1984; Fradin *et al.*, 2009), while a polygenic control of resistance has been reported in alfalfa (Vandemark *et al.*, 2006), cotton (Li *et al.*, 2013), hop (Jakse *et al.*, 2013), rapeseed (Happstadius *et al.*, 2003; Rygulla *et al.*, 2007) and spinach (Villarroel-Zeballos *et al.*, 2012). Knowledge of Verticillium wilt resistance is more limited in fruit trees but presumably also relies on complex mechanisms with a species-specific component. For instance, a qualitative type of resistance is likely to operate in cocoa according to Braga and Silva (1989), while quantitative genes most likely drive the resistance in *Pistacia* spp. (Raabe & Wilhelm, 1978; Morgan *et al.*, 1992; Teviotdale *et al.*, 1995).

Bearing these ideas in mind, the main goals of this study were as follows: a) selection of Verticillium wilt-resistant genotypes among the progenies of cultivars, wild olives and other wild *Olea* species (*Olea europaea* subsp. *cuspidata* and *Olea exasperata*); b) characterization of the relationship between parents and the distribution of resistant and susceptible genotypes in their offspring; c) identification of the most suitable parents to improve breeding for resistance to Verticillium wilt. To achieve these goals, we applied a recently developed method to screen a great number of olive

genotypes for Verticillium wilt resistance, identifying new sources of Verticillium wilt resistance in olive (Trapero *et al.*, 2013a).

MATERIALS AND METHODS

Plant Material

During three different trials conducted in 2009, 2010 and 2011, we evaluated 6017 olive genotypes derived from 48 different crosses for Verticillium wilt resistance. During the first trial (2009), we evaluated 5 different potential sources of resistance: i) 16 open pollinated olive cultivars located in the World Olive Germplasm Bank at IFAPA (Andalusian Institute for Research and Training in Agriculture) in Cordoba, Spain (Table 1) (Caballero *et al.*, 2006). These cultivars were selected because of their resistance to Verticillium wilt or for other valuable agronomic traits (Table 1). ii) 'Frantoio' x 'Picual' and the reciprocal cross. All the controlled crosses between cultivars were performed by applying male pollen to female-bagged branches according to (Diaz *et al.*, 2007). iii) 11 wild olive genotypes in open pollination scattered through the Andalusia region following an altitudinal gradient (Table 2). Elevation has a large impact on species distribution and is considered a proxy for a suite of environmental variables (Gaston, 2000; Lookingbill & Urban, 2003; Díez *et al.*, 2013). Therefore, following an altitudinal gradient, we aimed to sample the range of environmental diversity of the wild olive in the Andalusia region. iv) Five genotypes of *O. europaea* subsp. *cuspidata* (originally from Croatia, Pakistan and South Africa) in open pollination and provided by the germplasm collection of the USDA (United States Department of Agriculture) in Davis, USA (Koehmstedt *et al.*, 2010). v) Seedlings from a mixture of several mother parents of the species *O. europaea* subsp. *cuspidata* and *O.*

125 *exasperata* in open pollination. These seeds were collected in Southern South Africa
126 and provided by Silverhill Seeds (Table 3).
127 During the second and third trials (2010 and 2011, respectively), we evaluated
128 the progenies resulting from controlled crosses between six different cultivars and one
129 wild olive (HU-AC-102) (Table 4). Moreover, we analyzed the open pollinated (OP)
130 progenies of these seven genotypes used as parents for the controlled crosses (Table 1
131 and Table 2).
132 The fruits for all the trials were collected at the beginning of their maturation in
133 order to achieve satisfactory seed germination (Morales-Sillero *et al.*, 2012). The seeds
134 were stratified and germinated according to the method optimized by Santos-Antunes *et*
135 *al.* (2005). In order to assure the parents used in the crosses, we performed
136 microsatellite-based paternity tests following the protocol described by de la Rosa *et al*.
137 (2004). Seedlings were inoculated 40 days after germination when they were
138 approximately 7 cm high and had 2-3 pairs of leaves, which has been reported as the
139 best time to achieve high disease severity (Trapero *et al.*, 2013a).
140 In addition, 6-month-old plants of 'Frantoio' and 'Picual' obtained by self-rooting stem
141 cuttings (Caballero & Del Río, 2010) were included in the experiments as controls
142 because of their known response to *V. dahliae* infection (resistant and susceptible,
143 respectively) (López-Escudero & Mercado-Blanco, 2011). The role of these plants
144 (hereafter "controls") in the experiments was to confirm the effectiveness of the
145 inoculation and not to compare their resistance with that of the seedlings, as they were
146 different types of plant material.
147
148 **Fungal inoculation, experimental design and evaluation of the seedlings**

All plants were inoculated with the defoliating *V. dahliae* cotton isolate V117 (Blanco-López *et al.*, 1989), which has been described as highly virulent in olive (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013a). Preparation of the conidial suspension and the inoculation of plants by root dipping were performed as described for young olive seedlings by Trapero *et al.* (2013a). Briefly, seedlings were inoculated by dipping their bare root systems in a conidial suspension (10^7 conidia/ml) of the *V. dahliae* isolate V117 for 30 min. Inoculated seedlings were transplanted to pots with sterile soil (1:1:1, peat:sand:lime) and incubated in a greenhouse for 15 weeks, with 16 h of light/day and temperatures of 23±2°C (day) and 18±2°C (night). Non-inoculated plants were treated in the same manner but were immersed in tap water instead of a spore suspension. Plants were arranged according to a completely randomized design with different numbers of plants for each set of progeny (Tables 1-4).

We scored plants for disease severity every week from the 4th to the 12th week after inoculation on a scale ranging from 0 (healthy plant) to 4 (dead plant) depending on the number of defoliated or wilted leaves in the seedlings (Trapero *et al.*, 2013a). Plants were also visually evaluated every week for the presence of new vegetative growth after the inoculation. Four percent of the seedlings, randomly selected, were assayed to determine if they were infected by *V. dahliae*. For this analysis, seedling stems were washed in running tap water and surface disinfested in 0.5% sodium hypochlorite for 1 min. Stem pieces were placed on PDA plates and incubated at 24ºC in the dark for 6 days. The identity of *V. dahliae* was confirmed by microscopic observations of verticillate conidiophores and by the formation of microsclerotia.

Data analysis

The main parameter to assess the resistance of each progeny was the RAUDPC (Relative Area Under the Disease Progress Curve). This parameter was calculated from the disease severity values according to the following formula (Campbell & Madden, 1990):

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

where s_i = the disease severity value for observation number i; s_{max} = the maximum value of severity (4); t_i = the number of days between planting and observation i; t_e = the total evaluation period; and n = the number of observations.

The percentage of dead plants, calculated from the disease severity values, was used as an additional parameter to assess the resistance level of the genotypes (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013a). Lastly, as some plants did not show symptoms but were not able to restart growing after the inoculation, we considered as resistant genotypes only those plants showing no symptoms during the evaluation period and that were able to consistently grow after the inoculation.

To estimate the differential response to Verticillium wilt, first we calculated the differences between trials, considering only the crossings that were common to the three experiments (2009, 2010 and 2011) and applied an analysis of variance (ANOVA) to the RAUDPC. After that, the RAUDPC and the percentage of resistant plants in each set of progeny were analyzed for each trial separately by applying parametric and non-parametric tests when appropriate.

For the 2009 trial, we applied the Kruskal-Wallis non-parametric test to the RAUDPC ($P = 0.05$) because the data did not satisfy the assumptions of ANOVA.

An ANOVA of the RAUDPC was applied to the 2010 and 2011 trials because they fulfilled the ANOVA requirements. For these experiments, mean values were compared by the least significant difference test at $P = 0.05$. While in the 2011 trial the date

198 satisfied the homogeneity of variances, in the 2010 one data were log-transformed to
199 satisfy this requirement before applying the ANOVA.
200 For all trials (2009, 2010 and 2011), the mean RAUDPC values for each set of
201 progeny were compared with the values of the 'Picual' OP progeny (hereafter,
202 "susceptible check") (Figure 1) due to its reported susceptibility (Trapero *et al.*, 2011).
203 The percentage of resistant genotypes was analyzed by a Chi-square test at $P = 0.05$.
204 We compared the percentage of resistant progeny from each cross with the percentage
205 of resistant genotypes in the 'Picual' OP progeny. These statistical analyses were
206 performed using the program Statistix 9.0 (Analytical Software, Tallahasee, FL, USA).
207 Lastly, we calculated the frequency distribution histograms for the RAUDPC
208 values to illustrate the response of several representative progenies (Figure 2). A normal
209 distribution curve was fitted using the program Grapher 9.6 (Golden Software, Golden,
210 CO, USA) according to the least squares method (Press *et al.*, 1992). The Shapiro-Wilk
211 W test was applied to determine the departure of the frequency distributions from
212 normality using the program Statistix 9.0.
213
214 **Estimation of heritability of resistance in open pollinated progenies**
215 To estimate the additive component of the genetic variation in the performed
216 experiments, the narrow-sense heritability (h^2) was calculated separately for the OP
217 progenies from the 2010 and 2011 trials. Heritability was not estimated in the 2009 trial
218 because the assumptions for ANOVA were not satisfied. The value of h^2 was estimated
219 by a one-way ANOVA according to the half-sib analysis for progenies with unequal
220 numbers of individuals (Lynch & Walsh, 1998), which is based on the linear model:
221 $$z_{ij} = \mu + m_i + e_{ij}$$

Where z_{ij} is the phenotype of the *j*th offspring of the *i*th mother, m_i is the effect of the *i*th mother, and e_{ij} is the residual error resulting from segregation, dominance genetic variance among mothers, and environmental variance. A basic assumption of linear ANOVA models is that the random factors are uncorrelated to each other. Thus, for the half-sib model, the residual deviations are uncorrelated with the mother effects:

$$\sigma(m_{i,}e_{ij}) = 0$$

and the total phenotypic variance equals the variances due to mothers plus the residual variance:

$$\sigma_z^2 = \sigma_s^2 + \sigma_e^2$$

Assuming that the measurement error is independent of the progeny mean and by making some substitutions, we obtain the following unbiased estimators of σ_s^2, σ_e^2 and σ_z^2:

$$\text{Var (s)} = \frac{\text{MS}_\text{s}\text{-MS}_\text{e}}{\text{n}_0}$$

$$Var\ (e) = MS_e$$

$$Var\ (z) = Var(s) + Var(e)$$

The quantity

$$\text{t}_{\text{PHS}} = \frac{\text{Var (s)}}{\text{Var(z)}}$$

is the intraclass correlation and provides an estimate of the fraction of the phenotypic variance attributable to differences among mothers. Considering that $\sigma_s^2 \approx \frac{\sigma_A^2}{4}$, the maternal half-sib ANOVA estimator of the heritability was calculated as follows:

$$h^2 = 4 \times t_{PHS}$$

The standard error of h^2 was calculated according to Swiger et al. (Swiger *et al.*, 1964).

RESULTS

Disease symptoms

Inoculation of seedlings with *V. dahliae* by root dipping was successful in the three trials. The main symptoms observed were partial or complete defoliation and wilting occurring indistinctly in each plant, and typically followed by necrosis. These symptoms were common to all the inoculated susceptible seedlings, regardless of the cross, with the fungus consistently isolated from randomly selected seedlings. Non-inoculated plants remained free of symptoms and started growing between the 2nd and 4th week after the treatment with water. Conversely, no inoculated plant was able to grow during the first six weeks after the inoculation. All these observations were confirmed in the three trials.

Overall resistance of the progenies and variability between and within trials

We found statistically significant differences ($P < 0.05$) between trials according to the analysis of the RAUDPC of the progenies that were common to the 3 experiments ('Changlot Real', 'Empeltre', 'Frantoio', 'Picual' and 'Sikitita' OP; and 'Frantoio' x 'Picual' and 'Picual' x 'Frantoio'). Interaction between trial and cross was also significant ($P < 0.05$). Given these results, the same crossing showed differences in RAUDPC values and in the percentage of resistant plants depending on the year of evaluation (Figure 1).

The average RAUDPC in the three evaluated trials and in all progenies evaluated was 28.0, with values ranging from 3.4 to 72.6. (Tables 1-5). The disease reactions were significantly lower ($P < 0.05$) in the progenies in comparison with the susceptible check.

The percentage of resistant genotypes selected from each set of progenies ranged from 0.0 to 48.4. A total of 877 (14.6%) of the 6017 genotypes evaluated were selected for their Verticillium wilt resistance. The number of selected resistant genotypes from each origin was: 380 (12.6%) from OP cultivars, 331 from crosses between cultivars (15.9%), 121 from wild olives (20.1%), 19 from *O. europaea* subsp. *cuspidata* (15.03%) and 26 from *O. exasperata* (43.3%).

Variability and resistance of the progenies within trials

Analyzing the variability within trials we found that:

a) In the 2009 trial, progenies from the crosses 'Frantoio' x 'Picual' and 'Picual' x 'Frantoio', as well as OP progenies from the cultivars 'Frantoio' and 'Koroneiki', two wild olives and *O. exasperata* had RAUDPC values significantly lower than the susceptible check according to the Kruskal-Wallis test (Tables 1-3). The percentage of resistant plants was significantly higher in seven of the progenies ('Frantoio' x 'Picual', 'Frantoio' OP, three wild olives, *Olea europaea* subsp. *cuspidata SA-01* and *O. exasperata*) than in the susceptible check.

b) In the 2010 trial, none of the evaluated progenies outperformed the susceptible check except the crossing between 'Frantoio' and the wild olive 'HU-AC-102' (Table 4).

c) In the 2011 trial, the offspring of 'Frantoio' in OP, 'Frantoio' x 'HU-AC-102' and 'Frantoio' x 'Picual' showed significantly lower RAUDPC values, and in the last case, a higher percentage of resistant plants, compared with the susceptible check (Figure 1). Similar to 2010, the cross between 'Frantoio' and the wild olive 'HU-AC-102' led again to progeny with a high percentage of resistant individuals, although its

294 resistance level was not higher that that of the OP progenies of the two parents (Table
295 4).

296 In the 2011 trial, we compared the performance of the offspring of six cultivars
297 involved in controlled crosses and in OP (Figure 2, Table 5). As a general pattern, the
298 cultivars 'Arbosana' and 'Sikitita' gave rise to susceptible offspring, while 'Koroneiki'
299 produced intermediately susceptible progeny and 'Frantoio' produced resistant
300 offspring. The resistance of the 'Arbosana' offspring was consistently higher when
301 'Koroneiki' and especially 'Frantoio' participated in the crossing. In fact, progenies
302 having 'Arbosana' as the female parent and 'Sikitita', 'Koroneiki' or 'Frantoio' as the
303 male parent showed significantly different RAUDPC values ($P < 0.05$) (Table 5). The
304 lowest RAUDPC corresponded to the 'Arbosana' x 'Frantoio' progeny, while the
305 'Arbosana' x 'Sikitita' progeny showed the highest value (Table 5). The percentages of
306 resistant plants were not over 10%, except in the progeny of 'Frantoio' in open
307 pollination, which were the only progeny showing significant differences in this respect.
308

309 **Heritability and RAUDPC frequency distributions**

310 The heritability (h^2) was estimated for the OP progenies evaluated in the 2010
311 and 2011 trials as they were the only ones complying with the ANOVA assumptions.
312 Values of h^2 and its standard error (SE) were 0.26 and 0.20, respectively, for the 2010
313 trial and 0.31 and 0.18, respectively, for the 2011 trial.

314 The variability in resistance within progenies was normally greater in the most
315 susceptible progenies than in the resistant ones, as can be observed in the frequency
316 distribution histograms for the RAUDPC (Figure 2). These frequency plots show a
317 continuous but not normal distribution (Shapiro-Wilk W test: $P < 0.05$). We found a
318 certain percentage of resistant plants in all the progenies, even in the highly susceptible

ones, such as that resulting from the ‘Arbosana’ x ‘Sikitita’ cross. This percentage was remarkably high in the crosses that involved ‘Frantoio’ as a parent, with the RAUDPC frequency distribution skewed towards the left (0-30) (Figure 2). In the rest of the progenies, the RAUDPC values were concentrated between the values 20-80, indicating a general susceptibility to Verticillum wilt.

DISCUSSION

To date, no source of complete resistance to Verticillium wilt in olive has been found. Still, a wealth of cultivated and wild genetic resources within the *Olea* genus remains unexplored for this trait. For this reason, we carried out three experiments to individually evaluate 6017 genotypes belonging to 48 progenies across the *Olea* genus for their resistance to Verticillium wilt. Seedlings were screened under controlled conditions by applying a previously optimized root dip inoculation method with a highly virulent *V. dahliae* isolate (Trapero et al. 2013a). It appeared that none of the inoculated genotypes escaped infection because they did not begin growth as quickly as the non-inoculated plants. Similar observations have been reported for studies using self-rooted olive plants (López-Escudero & Blanco-López, 2005; Cirulli *et al.*, 2008; Markakis *et al.*, 2009).

There was notable variation in the response to *V. dahliae* between and within the evaluated offspring. In addition, there was variability in the response of progeny obtained from the same parents in different experiments, likely caused by environmental and genetic factors. The OP progenies showed high variability in this respect, most likely due to variation in the fluxes of pollen and therefore in the identity and proportions of male parents each year (Barranco *et al.*, 1994; Mookerjee *et al.*, 2005; Diaz *et al.*, 2006).

Despite this variation, we also found consistent patterns. Cultivar ‘Frantoio’, one of the most resistant olive cultivars to Verticillium wilt (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Trapero *et al.*, 2013b), clearly transmitted a high level of resistance to its offspring as either a female or a male parent (Table 1 and Table 4). This result was in agreement with previous evaluations of OP ‘Frantoio’ progenies under controlled (Trapero *et al.*, 2011) and field conditions (Wilhelm & Taylor, 1965). ‘Koroneiki, which is considered a moderately susceptible cultivar (López-Escudero *et al.*, 2007; Markakis *et al.*, 2009; Trapero *et al.*, 2013b), also conferred relatively good performance to its offspring, though not as high as that of ‘Frantoio’ (Table 1 and Table 5). Of 22 cultivars evaluated, only ‘Frantoio’ and ‘Koroneiki’ yielded progenies significantly more resistant than the susceptible check in at least one experiment. Moreover, 491 of the 877 genotypes selected as resistant had ‘Frantoio’ as one of the parents. The superiority of ‘Frantoio’ over ‘Koroneiki’ in transmitting resistance to offspring was also observed when both cultivars were crossed as male parents with the moderately susceptible cultivar ‘Arbosana’; the offspring of ‘Koroneiki’ were more resistant than the offspring of ‘Sikitita’ but more susceptible than the ‘Frantoio’ offspring (Table 5). ‘Frantoio’ gave rise to a high number of resistant seedlings, even when crossed with a very susceptible cultivar, such as ‘Picual’ (Table 4). Curiously, the offspring obtained from crossing ‘Frantoio’ and ‘Picual’ were as resistant as the offspring between ‘Frantoio’ and other resistant genotype, the wild olive ‘HU-AC-102’ (Table 4).

However, not all the resistant cultivars, such as ‘Frantoio,’ conferred resistance to their offspring. The cultivars ‘Changlot Real’ and ‘Empeltre’, the most resistant olive cultivars along with ‘Frantoio' (López-Escudero *et al.*, 2004; Martos-Moreno *et al.*, 2006; Trapero *et al.*, 2013b), produced mostly susceptible offspring (Table 1). The

crosses between these three resistant cultivars, which might have increased the probability of obtaining resistant genotypes, were repeatedly unsuccessful, presumably due to incompatibility phenomena (Rodríguez-Castillo *et al.*, 2009).

Another consistent pattern in our results was the general susceptibility to disease of progenies obtained from susceptible or moderately susceptible cultivars, such as 'Dolce Agogia', 'Sevillenca' and 'Bodoquera' (Table 1).

Exceptionally, the offspring of susceptible cultivars, such as 'Morisca' OP, showed high but not significant resistance values (low RAUDPC and a high proportion of resistant seedlings). Massive natural pollination with a resistant cultivar such as 'Frantoio' could explain this pattern. However, further experiments including replicates and a larger number of seeds are needed to evaluate this effect.

We observed high variability in the response to *V. dahliae* particularly within susceptible progenies, such as 'Picual' OP, with some genotypes being either more resistant or more susceptible than their parents (Figure 2, 'Picual' OP). This phenomenon, called transgressive segregation (Robinson, 1996), has also been reported for the inheritance of Verticillium wilt resistance in cotton (Bolek *et al.*, 2005), *Medicago truncatula* (Negahi *et al.*, 2013) and pepper (Palloix *et al.*, 1990). Due to this phenomenon, it would be possible to select resistant genotypes in progenies that, although showing a high susceptibility mean value, also have other interesting agronomical traits, such as the low vigor found in the offspring of 'Arbosana' x 'Sikitita' (Hammami *et al.*, 2012). However, considering the distribution shown in Figure 2, a large number of genotypes of susceptible progenies would have to be screened to select resistant genotypes.

We evaluated the offspring of 11 wild olives collected in different edaphoclimatic conditions, reproducing the finding of resistant genotypes reported by Colella *et al.* (2008), who sampled wild olives in the Mediterranean Basin.

In agreement with our observations in the cultivated material, the level of resistance between and within the wild olive progenies was quite variable, ranging from resistant to very susceptible. However, four progenies showed outstanding resistance, with percentages of resistant genotypes that in some cases were close to 50% (Table 2). These high proportions of resistant genotypes, which exceeded those observed by Colella *et al.* (2008), are promising for the development of resistant rootstocks. However, broader sampling is necessary to corroborate this observation and to reinforce the value of the wild olives as a genetic resource for the breeding of resistant rootstocks (Bubici & Cirulli, 2012) or even cultivars (Klepo *et al.*, 2013). Curiously, three of the most resistant wild progenies were collected at low altitude. Again, the low number of wild progenies evaluated in this study is insufficient to determine a consistent correlation; however, our observation raises questions about the possible relationship between elevation and resistance to Verticillium wilt in wild olive.

The value of other wild *Olea* species as donors of valuable agronomical traits or as possible rootstocks in olive breeding remains unexplored (Besnard *et al.*, 2012; Lavee, 2013). We have characterized, for the first time, the differential performance of *O. europea* subsp. *cuspidata* and *O. exasperata* to Verticillium wilt. In general, *O. europea* subsp. *cuspidata* seedlings were highly susceptible, while those of *O. exasperata* were highly resistant. In fact, the level of resistance of *O. exasperata* seedlings was comparable to that of the wild olives considered highly resistant, such as HU-AC-102. A broader evaluation of genotypes from these species and from other

417 unexplored *Olea* species, may identify additional sources of resistance that could be
418 used as rootstocks to control the disease.

419 Olive breeding, as in the breeding of other fruit species, is primarily based on the
420 evaluation of large progenies coming from parents with desirable traits, such as
421 resistance to Verticillium wilt. A high heritability value for a certain trait indicates that
422 most of the observed variability for this trait is due to genotypic instead of
423 environmental variation (Falconer, 1989). Therefore, the higher the heritability, the
424 more feasible is breeding for that trait. In recent studies, heritability values have been
425 estimated for some olive traits, such as fruit weight, flowering behavior and fatty acid
426 composition, with the values ranging from low-medium (0.3) to high (0.97) (Leon *et al.*,
427 2004; Zeinanloo *et al.*, 2009; Ruiz-Domínguez *et al.*, 2013; Ben Sadok *et al.*, 2013). In
428 our case, we obtained a general medium-low heritability value for Verticillium wilt
429 resistance, in agreement with the high variability observed among and within progenies
430 and also between years for a given set of progeny. However, a higher heritability value
431 could be obtained using controlled crosses instead of OP progenies, whose variability
432 highly depends on the fluxes of pollen and environmental conditions.

433 Complex genetic interactions might be involved in resistance to Verticillium wilt
434 in olive. We found possible patterns of transgressive segregation in the offspring of
435 susceptible and moderately susceptible cultivars, which might be indicative of
436 polygenic control (Robinson, 1996). However, under this hypothesis, it is difficult to
437 explain why only one resistant cultivar, 'Frantoio', differentially transmitted this
438 condition to its offspring, or why the proportion of resistant seedlings was not enhanced
439 when crossing two resistant genotypes (Frantoio and AC-HU-102) from that observed
440 in both parents in OP. These puzzling results led us to presume that: a) there are
441 possibly different allelic or even gene configurations that might lead to resistant

442 genotypes; b) some genes might play a major role in resistance; c) these two non-
443 exclusive options might lead to genotype-specific resistant configurations. Interactions
444 between major and minor genes in resistance to this pathogen have recently been
445 reported in hop (Jakse *et al.*, 2013).

446 Despite difficulties of breeding for Verticillium wilt resistance in olive, we
447 identified 14.6% of all the evaluated genotypes as highly resistant. The most suitable
448 parents to increase this percentage in further crosses were also identified. A second
449 cycle of selection, this time under field conditions, will be needed to corroborate the
450 resistance of selected genotypes. The presence of other valuable agronomical traits,
451 such as vigor, production or oil quality, will determine the genotypes to be discarded or
452 considered as rootstock or olive cultivars.

453 Considering our results, obtaining fully resistant progenies may be a slow
454 process, especially taking into account the presumably polygenic nature of this trait.
455 Massive screening of seedlings is necessary to maximize the possibilities of finding a
456 resistant genotype. 'Frantoio' is currently the best parent to breed olive genotypes with
457 increased resistance to Verticillium wilt. Some wild olives and even some *O.*
458 *exasperata* genotypes might also be useful sources of resistance, mainly for rootstock
459 breeding. Verticillium wilt seems to be quantitatively inherited in olive. Due to the
460 narrow range of compatible crosses between resistant cultivars, developing new
461 cultivars with improved resistance and other useful traits will depend on the
462 identification of new sources of resistance (cultivars or wild genotypes) and on the
463 recovery of transgressive segregants from non-resistant parents.

464

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703

704 **FIGURE LEGENDS**

705

706 Figure 1. Relative Area Under the Disease Progress curve (RAUDPC) of several olive
707 and *Olea* progenies, obtained by open pollination or cross-breeding and inoculated with
708 isolate V117 of *Verticillium dahliae* in years 2009, 2010 and 2011. Columns and bars
709 represent mean values and standard errors, respectively. Number of plants was variable
710 for each progeny and year. Progenies with an asterisk above their bars had a

711 significantly lower RAUDPC value than the 'Picual' OP progeny (striped bar),
712 according to Krukal-Wallis test at P = 0.05 (2009 trial) or according to Least Significant
713 Difference (LSD) test at P = 0.05 (2010 and 2011 trials).
714
715 Figure 2. Histograms indicating the frequency distributions of the Relative Area Under
716 the Disease Progress Curve (RAUDPC, x axis) of several olive progenies inoculated
717 with isolate V117 of *Verticillium dahliae* in the 2011 trial (see also table 5). The fitted
718 normal distribution curve is also shown, according to the least squares method.
719

2009 Trial

RAUDPC

0
20
40
60
80

* * * * * *

Frantoio x Picual
HU-AC-102 OP
O. exasperata OP
CA-AC-101 OP
HU-AC -04 OP
Picual x Frantoio
Koroneiki OP
JA-AC-21 OP
SE-AC-154 OP
Frantoio OP
Morisca OP
JA-AC-03 OP
10-54 OP
Lechin de Sevilla OP
JA-AC-27 OP
Arbequina OP
Manzanilla de Sevilla OP
DOLE 132 OP
Sikitita OP
Hojiblanca OP
SE-AC-101 OP
CA-AC-33 OP
10-30 OP
DOLE176-6 OP
Changlot Real OP
Picual OP
O. e. subsp. Cusp. OP
HU-AC -03 OP
Peq. de Casas Ibañez OP
Menya OP
DOLE90 OP
Cornicabra OP
Arbosana OP
Empeltre OP
CA-AC-001 OP
Bodoquera OP
DOLE177-9 OP
DOLE116 OP

2010 Trial

RAUDPC

0
20
40
60
80

*

Frantoio x HU-AC-102
HU-AC-102 OP
Frantoio OP
Frantoio x Picual
Picual OP
Picual x Frantoio
Arbosana x Koroneiki
P x Arbequina
Sikitita OP
Koroneiki x Arbosana
Changlot Real OP
Arbosana x Sikitita
Empeltre OP
Arbequina OP

2011 Trial

RAUDPC

0
20
40
60
80

* * *

Frantoio x HU-AC-102
Frantoio x Picual
Frantoio OP
Arbosana x Frantoio
HU-AC-102 OP
Cipresino OP
Picual OP
Picual x Frantoio
Koroneiki OP
Koroneiki x Arbosana
Changlot Real OP
Empeltre OP
Calcaudete OP
Arbosana x Koroneiki
Leccino OP
Chemlali OP
Sevillenca OP
Arbosana x Sikitita
Dolce Agogia OP
Arbosana OP
Sikitita OP