Received: 16 December 2020 | Accepted: 18 July 2021

DOI: 10.1111/ppa.13442

Plant Pathology An International Journal edited by the British Society for Plant Pathology WILEY

ORIGINAL ARTICLE

Unravelling the relationships among Verticillium wilt, irrigation, and susceptible and tolerant olive cultivars

Antonio Santos-Rufo[1,2] | **Dolores Rodríguez-Jurado**[2]

[1]Department of Agronomy (Excellence Unit 'María de Maeztu' 2020-23), University of Cordoba, Córdoba, Spain

[2]Laboratory of Plant Pathology, Area of Sustainable Crop Protection, Centro 'Alameda del Obispo', Andalusian Institute of Agricultural Research and Training (IFAPA), Córdoba, Spain

Correspondence
Antonio Santos-Rufo, Department of Agronomy (Excellence Unit 'María de Maeztu' 2020-23), University of Cordoba, Campus de Rabanales, Córdoba 14071, Spain.
Email: g02sarua@uco.es

Funding information
Andalusian Institute of Agricultural Research and Training, Grant/Award Number: PEI.PEI2011.1; European Regional Development Fund; National Institute for Agricultural and Food Research and Technology, Grant/Award Number: RTA2006-0012-00-00

Abstract

Survival, germination, olive colonization, and water-use efficiency (WUE) impairments by *Verticillium dahliae* could be influenced by cultivar susceptibility or irrigation, and this could modify the irrigation–pathogen–disease relationship. In this study, the combined effects of irrigation and cultivar susceptibility on Verticillium wilt (VW) development were modelled by the temporary assessment of *V. dahliae* propagules (total inoculum density, density of micropropagules, and sclerotia in wet and air-dried soil; ID, MpD, S_wD, and S_dD, respectively), root (RCI) and shoot (SCI) colonization indexes, and WUE. The relationship of disease severity to the measured parameters was then explored. Under controlled conditions, plants of cultivars 'Picual' and 'Frantoio' were irrigated to a high and low rate by varying drip-irrigation frequencies: daily, twice weekly, and a combination of daily for 11 days and then twice weekly. Disease severity and colonization parameters were higher in 'Picual', while WUE was higher in 'Frantoio'. However, high rate and twice weekly and combination treatments significantly increased disease incidence and reduced time-to-symptoms-onset only in 'Picual', while high rate reduced WUE and increased relative ID, MpD, and S_wD in both cultivars. Irrigation did not affect SCI, but a higher RCI was found at high rate during the development of symptoms in 'Picual'. By using classification trees to examine parameters—disease severity relationships, it was possible to determine the degree to which VW was affected by irrigation and/or cultivar susceptibility. MpD was the best indicator for VW detection at any time, WUE was best before symptoms developed, and RCI, total ID, and S_dD after symptoms developed.

KEYWORDS
classification trees, irrigation management, micropropagules, *Olea europaea*, *Verticillium dahlia*, water-use efficiency

1 | INTRODUCTION

Verticillium wilt (VW) of olive, caused by *Verticillium dahliae*, is one of the most destructive fungal diseases affecting olive worldwide and the primary phytopathological problem for olive cultivation in Andalusia, mainly due to the prevalence of the defoliating pathotype of the fungus and of the extremely susceptible olive cultivar, 'Picual' (Jiménez-Díaz et al., 2012; López-Escudero & Mercado-Blanco, 2011; Montes-Osuna & Mercado-Blanco, 2020). VW requires integrated disease management mainly based on the use of more resistant cultivars coupled with other measures before and after planting (López-Escudero & Mercado-Blanco, 2011). These measures include risk assessment and choice of planting site, and farming practices that mitigate the disease and reduce the levels of pathogen in the soil, such as the use of biological antagonists, soil solarization, and efficient irrigation (Jiménez-Díaz et al., 2012; Montes-Osuna & Mercado-Blanco, 2020). In this regard, there are irrigation schedules that can increase the population of *V. dahliae* in the soil, and create favourable conditions for the infection of the plant and the onset of disease (Pérez-Rodríguez, Alcántara, et al., 2015; Pérez-Rodríguez, Orgaz, et al., 2015; Santos-Rufo et al., 2017, 2018). However, there are very different outcomes to irrigation experiments depending on the type of inoculum (natural or artificial), the methodology followed to define irrigation treatments (gravimetric or volumetric), and the environmental conditions where plants are grown (natural environmental, microplot, or field conditions). Thus, the mechanisms by which irrigation affects VW severity are still unknown, even though almost 40% of the olive groves in Andalusia are currently irrigated (MAPA, 2019).

Concerning the effect of irrigation intervals and/or rates on VW incidence and severity, Santos-Rufo et al. (2017) found—by using artificial inoculum, a gravimetric method (by pot weight) to determine irrigation treatments, and maintaining plants under natural environmental conditions—that daily irrigation events (2.05 ± 0.05 days per irrigation event) of cv. 'Picual' led to greater reductions in the disease incidence (DI) and area under disease progress curve than the weekly and daily during some periods and otherwise weekly frequencies (7.40 ± 0.20 and 5.95 ± 0.05 days per irrigation event, respectively), regardless of the irrigation rate (defined here as range of soil water content). Other disease parameters, such as mortality and incidence of plants recovered from the disease, did not differ between treatments under mentioned conditions. However, these results contrast with those of Pérez-Rodríguez, Alcántara, et al. (2015) obtained in microplots with naturally infested soil when four irrigation frequencies (determined by a volumetric method) were studies for three olive cultivars with differing levels of disease resistance. These authors indicated that DI, mortality, and area under the disease progress curve increased more in 'Picual' plants subjected to a daily irrigation schedule (unknown days per irrigation event, with the possibility of not being purely daily) than to either weekly or biweekly schedules, or in plants subjected to a water deficit. Also, time-to-symptom expression, expressed as weeks from planting until 50% of the plants were affected, was drastically reduced (almost 40 weeks) in plants subjected to a daily irrigation schedule with respect to other treatments. Therefore, it seems that frequency of irrigation exerts more influence on VW than irrigation rate, which is supported by the absence of irrigation rate effect in the whole olive orchards surveyed at Guadalquivir Valley (Spain) reported by Pérez-Rodríguez, Orgaz, et al. (2015). Nevertheless, both the DI at first year and the incidence of infected plants at the end of the trial by Santos-Rufo et al. (2017) were affected by the irrigation rate, with higher values in plants irrigated to high (field capacity) than to low (65% field capacity) irrigation rate. Other factors related to the effect of water on plant or plant–pathogen interaction are likely to play a role in disease development. This was confirmed by Pérez-Rodríguez et al. (2016) under field conditions, with natural inoculum and irrigation treatments determined by a volumetric method. In this study, area under the disease progress curve was greater on most of the recording dates in the daily compared with biweekly and dryland-rainfed treatment, but mortality varied with the experimental field, and DI and time to symptom expression was scarcely different between treatments. On the other hand, irrigation treatments that led to greater *V. dahliae* infections and visible symptoms reported by Santos-Rufo et al. (2017), also reduced water-use efficiency (WUE) of biomass production by the same proportion under the same conditions (Santos-Rufo et al., 2018). Although this could explain how these treatments increase the incidence of the disease, only 'Picual', which is the most susceptible olive cultivar to VW (López-Escudero et al., 2004; Rodríguez Jurado, 1993) was evaluated in these conditions. Thus, there could be cultivar-specific mechanisms underlying the irrigation treatments that increase the disease rates. Pérez-Rodríguez, Alcántara, et al. (2015) evaluated the influence of irrigation on Arbequina (moderately susceptible) and 'Frantoio' (tolerant) cultivars, and irrigation frequency did not seem to influence the disease progress, with low disease rates reported for both cultivars. These authors did not evaluate WUE, and it was only evaluated at the end of the trial in 'Picual' (Santos-Rufo et al., 2018). Thus, a hypothesis worth exploring could be related to how efficiently olive plants use water throughout the VW development depending on the susceptibility of cultivars, as has been studied in other pathosystems (Ploetz et al., 2015). Also, it is hypothesized that irrigation (through its influence on the pathogen in the soil and on the plant) could have a different effect on the colonization process of olive tissues by *V. dahliae* before symptoms develop compared to when trees are colonized and show wilt. To the best of the authors' knowledge, the influence of irrigation at this point remains unknown.

It is known that the increase in microsclerotia (size ≥ 35 and <150 μm), estimated after drying soil at room temperature for 4 weeks, is greater in the wet zones around the drippers than in the dry zones out of them (López-Escudero & Blanco-López, 2005). However, the number of mentioned propagules does not differ between irrigation treatments under the above-mentioned conditions follows by Pérez-Rodríguez, Alcántara, et al. (2015) and Pérez-Rodríguez et al. (2016). According to Isaac et al. (1971), *V. dahliae* may exist in soil as conidia, mycelium, or their resting structures (microsclerotia), so an isolation technique should distinguish between these propagules and estimate their relative proportions within the soil. By estimating these proportions in artificially infested soils, Santos-Rufo et al. (2017) found that the area under the progress curve of the fraction of *V. dahliae* propagules >20 μm was higher (for two and a half years) when soil was at 65% of field capacity than at field capacity. However, irrigation events to maintain soil at field capacity (specifically at weekly or daily during some periods and otherwise weekly), increase the quantity of propagules in wet soil, other than the above-mentioned fraction >20 μm, named as "micropropagules", presumably by increasing sclerotium germination

(Santos-Rufo et al., 2017). Germination rate and number of germinating hyphae are positively correlated with the size of the microsclerotia in soil that have been air dried and remoistened (Ben-Yephet & Pinkas, 1977; Farley et al., 1971). Thus, irrigation events other than daily maintaining soil at field capacity, which increase VW incidence and severity and reduce the fraction of *V. dahliae* propagules >20 μm (Santos-Rufo et al., 2017), favour the presence in soil of microsclerotia greater in size, but in a lower amount. Microsclerotia (35–150 μm) density of 3.33 and 10.0 propagules per gram of air-dried soil, resulted in not significantly different final VW incidence of 47.2% and 63.8%, respectively, for 'Picual' olive trees after 2.5 years (López-Escudero & Blanco-López, 2005). Smaller and less persistent propagules than microsclerotia, although not quantified in air-dried soil, could play a part as an infective structure affecting olive trees (Santos-Rufo et al., 2017). Thus, the analysis of wet rather than air-dried soil could enable the estimation of less persistent propagules in addition to microsclerotia. These micropropagules, not previously quantified in relation to VW epidemics, are propagules of the fungus with size ≥ 1 and <20 μm collected from wet soil, among the fraction >20 μm, by filtering over a series of tandem filters using a Millipore analytical filter holder connected to a high suction pump and sonication (Santos-Rufo et al., 2017). This methodology enables estimation of the relative proportions of less (≥1 and <20 μm; micropropagules mainly composed of conidia and mycelium) and more (≥20 μm; mainly composed of microsclerotia) persistent propagules in the sample and was based on the modified filtration technique of Rodríguez-Jurado and Bejarano-Alcázar (2007). A similar approach was follows by Isaac et al. (1971) in artificially infested soils, successfully separating conidia and microsclerotia by using filter papers of differing pore sizes (1 and ≥5 μm pore size for retaining conidia and microsclerotia, respectively).

However, even following a similar trend, neither micropropagules, nor the fraction >20 μm, correlate with the incidence of *V. dahliae*-infected olives, as well as the incidence and intensity of visible symptoms (Santos-Rufo et al., 2017). Similarly, it was impossible to establish a relationship between the amount of *V. dahliae* in soil and VW incidence over time (by years) in 'Picual' olive plants transplanted into microplot soil artificially infested with given densities of microsclerotia of defoliating *V. dahliae* (López-Escudero & Blanco-López, 2007). It seems that, as has been studied in various herbaceous hosts of this pathogen, the inoculum density is not a unique parameter indicator of the potential development of VW, with this relationship variably influenced by diverse environmental factors such as soil texture (Harris & Yang, 1996). Irrigation frequency and rates influence the development of VW in olive, and so these factors could also affect the relationship between VW severity and *V. dahliae* in soil. Considering olive cultivar, *V. dahliae* pathotype, and soil temperature as factors, Calderón et al. (2014) studied the relationship between several stress-related parameters and VW severity as a basis for disease risk prediction in olive. Machine-learning algorithms such as tree decision were required for the study of this relationship. The complexity of studying the relationship between *V. dahliae* in soil and VW severity over time considering olive cultivar, irrigation frequency, and rates as factors, could make it even more necessary.

The objectives of this study were to model (a) the combined effects of irrigation regimes (rate and frequency) and olive cultivar susceptibility on the onset and development of VW, with an assessment of sclerotium survival and germination in soil, and colonization and water-use impairments by *V. dahliae* in the host before, during, and after symptoms develop; and (b) how VW severity is related to levels of more and less persistent *V. dahliae* propagules in the soil, root, and shoot colonization by the pathogen, and olive WUE. These research objectives were based on the hypothesis that a holistic approach, accounting for the temporary effects of water and cultivar susceptibility on the disease cycle of *V. dahliae*, would help to explain why some irrigation schedules exalt the virulence of VW more than others.

2 | MATERIAL AND METHODS

2.1 | Irrigation experiment with potted plants growing in soil infested by *V. dahliae*

A pot experiment was set up with susceptible and tolerant olive cultivars and *V. dahliae* defoliating isolate combinations, under optimal conditions for disease development (growth chamber set at 24 ± 1°C, 65%–70% relative humidity (RH), and a photoperiod of 14 h per day of fluorescent light at 290 μmol/m^2/s) for a total of 120 days.

The plants used were 3-month-old rooted olive plants of cv. 'Picual', which is the most widely cultivated in Spain and highly susceptible to the defoliating pathotype of *V. dahliae* (López-Escudero et al., 2004; Rodríguez Jurado, 1993), and 'Frantoio', which is wilt tolerant (López-Escudero et al., 2004; Martos-Moreno et al., 2006). Plants were provided by La Conchuela S.L. (Villarrubia, Cordoba, Spain).

The highly virulent *V. dahliae* isolate VO161, classified as a defoliating pathotype (Moraño Moreno et al., 2011), was used in this study. This single-spore isolate belongs to the culture collection of the Area of Crop Protection, IFAPA "Alameda del Obispo" Centre (Cordoba, Spain). The culture was refreshed on water agar amended with chlorotetracycline (CWA), subcultured on potato dextrose agar (PDA), and multiplied in a cornmeal-sand substrate (CSS). The inoculum was prepared by placing colonized PDA plugs in flasks containing the autoclaved CSS and incubated. Inoculum density in CSS was determined by dilution plating on CWA as described by Santos-Rufo et al. (2017).

Olive plants were transplanted from their original substrate in plastic pots (1 L) containing autoclaved potting soil (sieved loam and peat and supplemented with basal nutrients) with 20% (wt/wt) CSS colonized by *V. dahliae* (Santos-Rufo et al., 2017). Then, a volume of sterile distilled water equivalent to that required to achieve either field capacity or 65% of field capacity was added; referred to here as high and low rate, respectively. As a control, plants treated in the same way were transplanted into pots with a similar amount of soil mixed with pathogen-free CSS and watered to field capacity or 65% of field capacity. Total weight per pot was recorded immediately after transplanting (weights at the beginning of trial). 'Picual' and 'Frantoio' olive plants in infested and noninfested soil (72 of each cultivar in infested soil and 48 of each as controls, for both high and low rates) were subjected to three drip-irrigation frequencies: daily, twice weekly, and a combination of daily and twice weekly (combination treatment). Irrigation was performed by applying sufficient amounts of water to compensate for the amounts lost through transpiration and evaporation, thereby maintaining the soil water content of the pots at either high or low rate. The mean amount of water required was determined by recording daily (for daily treatments), or every 3–4 days (for twice weekly treatments), the total weight of two pots per treatment. In the combination treatment, plants were irrigated twice weekly except at the beginning of experiment, when they were watered daily for 11 days.

The irrigation system was composed of six secondary polypropylene tubes controlled by an automatic head unit programmer connected to six valves (one valve provided the same irrigation treatment to infested and noninfested soils for 'Picual' and 'Frantoio' plants) bearing one 1.3 L/h compensating dripper per pot. Plants were fertilized weekly (equally for all treatments) with 50–60 ml per pot of Hoagland's nutrient solution.

The experimental design was a 2 × 2 × 3 × 2 factorial combination of treatments arranged in a split-split-split-plot completely randomized design with four replications. Each replication consisted of four (*V. dahliae*-free soil) or six (*V. dahliae*-infested soil) pots (one plant per pot). Four out of the six inoculated plants were used purely for disease assessment, while the remaining two, plus one from the disease assessment group upon termination of the experiment, were used for measurements taken before, during, and after symptom onset, as explained below. The main plot was fungal soil infestation (infested and noninfested soil), the subplot was the rate or range of soil water content (low and high), the sub-subplot was the irrigation frequency (daily, twice weekly, and combination frequency) and the sub-sub-subplot was the cultivar ('Picual' and 'Frantoio'). The full experiment was repeated twice.

2.2 | Assessment of symptom development

Incidence and severity of symptoms were assessed at 3- to 4-day intervals throughout the duration of the experiment. Symptom severity was assessed according to the percentage of the aerial part affected by chlorosis, green defoliation, wilt, necrosis, and/or death of each individual plant, using a rating scale from 0 to 4 (Rodríguez Jurado, 1993). At the first appearance of symptoms, three fallen leaves per plant were tested for the presence of *V. dahliae*, following the procedure described by Santos-Rufo et al. (2017). Leaves were thoroughly washed under running tap water before sectioning petioles and five representative pieces of the total length. Pieces were then surface-disinfected, rinsed with sterile distilled water, plated onto CWA, and incubated. *V. dahliae* was identified by microscopic observations.

Disease progress curves were obtained from accumulated disease intensity index (0%–100%; Santos-Rufo et al., 2017) scores over time in days from the date of inoculation. The nonlinear form of the Gompertz model was evaluated for goodness of fit to disease intensity index progress data using nonlinear regression analyses. The Gompertz equation was as follows: disease intensity index (t) = K exp[−B exp(−rt)], where K = asymptote parameter, B = constant of integration, r = relative rate of disease increase, and t = time of disease assessment in days after inoculation. The coefficient of determination (R^2), mean square error, standard errors associated with the parameter estimates, confident intervals of predicted values, and pattern of standardized residuals plotted against either predicted values or the response variable were used to evaluate how well the models describe the data.

Survival curves were obtained from survival times, or the day on which a plant showed symptoms minus the day on which plants were inoculated. The disease score was transformed into binary data, with a disease severity below 0.25 on the 0–4 scale corresponding to 0, and a disease severity equal to or higher than 0.25 corresponding to 1. This transformation allowed us to apply the survival analysis statistical protocols. Kaplan–Meier estimates were analysed using parametric regression models, and four different distributions (logistic, log normal, Gaussian, and Weibull) were used. As it is a parametric fit, two parameters of the distribution were estimated for the model. One is the scale of the model, and the other is referred to as location or shape, which is the centre of the distribution (Schandry, 2017).

To further assess disease development, five additional variables associated with disease progression and survival were explored. These variables were (a) the final DI (percentage of affected plants); (b) the disease intensity index assessed at the end of the experiment; (c) the area under the disease progress curve of disease intensity index plotted against time (3–4 days) relative to the maximum possible area (0%–100%; Campbell & Madden, 1990); (d) the rate of disease increase (*rho* [ρ]] parameter) of the Gompertz function fitted to disease intensity index progress data; and (e) the time-point when 50% of the population are estimated by a survival fit (location parameter) to have experienced the event (onset of symptoms). The overall effects of experimental *V. dahliae*-infested treatment combinations on these parameters were explored to determine the amount of variability explained by the main factors in the study (i.e., irrigation rate, frequency, and cultivar) using mixed effects models. Final DI and intensity index were arcsine-transformed before analyses. Experiments and blocks were considered as random effects. Irrigation rate, frequency, cultivar, and their interactions were considered fixed effects. Upon finding significant interactions of irrigation frequencies and cultivar, the whole data set was divided into two subsets according to the cultivar. The effects of irrigation factors were then tested by performing an analysis of variance on each of the subsets. Tukey's honestly significant difference test was used for all pairwise comparisons among treatments.

2.3 | Assessment of sclerotium survival and germination in soil, and colonization and water-use impairments by *V. dahlia* in the host

The densities of the pathogen in the soil, extent of stems and roots colonized by *V. dahliae*, and WUE were assessed before, during, and after symptom expression in another group of plants (two out of the six inoculated plants per replication, plus one from the disease assessment group upon termination of the experiment). The dates selected for the assessments before, during, and after symptom expression were 12, 40, and 120 days post-soil infestation (DPI), respectively.

Propagule levels of *V. dahliae* in the soil were estimated in air-dried soil (dried at room temperature for 4 weeks at 22–27°C and 30%–35% RH), and wet soil (stored at 4°C in closed polythene bags for a maximum of 48 h). Two or three (upon termination of experiment) 20 g soil samples were collected (one per pot), thoroughly mixed, and assayed for total inoculum density (ID), wet soil sclerotia (not mentioned as microsclerotia, normally used in the *V. dahliae* literature, due to fact that the size of propagules was considered including micropropagules as a type of propagules) density (S_wD), micropropagule density (MpD), and air-dried soil sclerotia density (S_dD). The methods used to estimate total ID and S_dD were direct plating of diluted wet soil and impaction plating of pulverized dry soil, respectively (Butterfield & DeVay, 1977; Santos-Rufo et al., 2017). To estimate S_wD and MpD, samples of 2 g per pot were subjected to the filtration technique described by Rodríguez-Jurado and Bejarano-Alcázar (2007), slightly modified (Santos-Rufo et al., 2017). Different sized propagules of *V. dahliae* were collected using a 47 mm-diameter Millipore analytical filter holder connected to a high suction pump. Briefly, each sample inmediately after collection (stored at 4°C for a maximum of 48 h) was suspended in 100 ml sterile distilled water; particle fractions were then collected by filtering over a series of tandem Sefar-Nitex nylon filters (Sefar Headquarter) of 20 and 1 μm pore size and a diameter of 47 mm. The residue retained in each filter was immediately resuspended in 10 ml sterile distilled water by sonication for 6 min, and 1 ml of the suspension was distributed on each of five plates of modified sodium polypectate agar (MSPA) before the suspension was either diluted (1:10) one to three times, or not diluted, and plated on MSPA. Colonies of *V. dahliae* developing on the medium, presumably originating from microsclerotia (20 μm pore size filter; more persistent propagules; ≥20 μm), and conidia and mycelium (1 μm pore size filter; less persistent propagules; <20 and ≥1 μm), were then quantified to determine sclerotia density (S_wD) and micropropagule density (MpD), respectively. Colony-forming units (cfu) on MSPA were counted under the microscope and expressed per g of dry soil (cfu/g dry). For each block, inoculum densities estimated at each sampling time (12, 40, and 120 DPI) were related to the initial values (0 DPI) and expressed as a percentage. These relative inoculum densities were the soil inoculum parameters assessed in the study.

In planta quantification of *V. dahliae* was assessed by isolations of the fungus in CWA. For each plant, randomly chosen tissues were treated as mentioned, and three 5 mm-long pieces of each of eight randomly selected roots, and twenty 5 mm-long pieces of debarked woody aerial tissues were incubated. At each time-point during the experiment, 176 pieces were plated per treatment, and a total of 6336 pieces were plated over the entire experiment. Data from pathogen isolation from the stem and roots were used to calculate the stem and root colonization indexes (SCI and RCI, respectively) as follows: $SCI/RCI = \sum N_i/N_j \times 100$, where N_i is the number of plated stem or root pieces yielding *V. dahliae* colonies and N_j is the total number of stem or root pieces plated.

To investigate differences in plant water uptake efficiency, the total biomass was determined at the beginning for all plants, and then at the same times and in the same pots where colonization indexes and relative densities of the pathogen were estimated. Plants were removed from the soil, the roots were washed free of soil, and the total biomass was recorded. Absolute biomass, which was calculated by subtracting the biomass at the beginning of the experiments from that at each date assessed, was divided by the cumulative amount of water used throughout the experiment for each treatment, and WUE was determined for each plant.

These parameters were analysed to test the (fixed) effects of factors, and also to assess the consistency of these effects over the three DPIs assessed (12, 40, and 120 DPI). SCI and RCI data were square root-transformed before analyses. Experiments and replicates were considered as random effects. Fungal soil infestation (only considered for the analysis of WUE data), irrigation rate, frequency, cultivar, DPI, and their interactions were considered fixed effects.

2.4 | Assessment of the relationship between measures of VW severity and *V. dahlia* soil inoculum, colonization, and olive WUE

The overall response of experimental treatment combinations to disease, soil inoculum, colonization, and WUE parameters was first explored by cluster analyses. The purpose of this analysis was to establish functional groups of correlated experimental treatments at each sampling time. Then, the relationship between the classes of disease intensity index and relative total ID, S_wD, S_dD, and MpD, *V. dahliae* RCI and SCI, and olive WUE was estimated using six different linear and nonlinear machine learning algorithms: logistic regression, linear discriminant analysis, k-nearest neighbours, classification and regression trees, Gaussian naive Bayes, and support vector machines. These algorithms were fitted to the above-mentioned parameters as explanatory variables, and disease intensity index class was the response variable, taking the lowest disease intensity index class as the reference category. The data set was partitioned 4:1 into training and test sets, and 10-fold cross validation was used to estimate the accuracy of the models, with precision as a parameter for scoring. The best learned classifier was then evaluated on the test set and used to identify the groups that were homogeneous in terms of disease intensity index. The results were summarized as a final accuracy score, a confusion matrix, and a classification report, which are common performance measurements for machine learning classification of severity classes in several plant diseases (Fenu & Malloci, 2021).

2.5 | Software

The nonlinear, survival, mixed effects models and cluster analyses were conducted in R (http://www.R-project.org) with the packages nlme (Pinheiro et al., 2021), survival (Therneau, 2020), lme4 (Bates et al., 2015), and cluster (Maechler et al., 2019), respectively. Machine-learning algorithms were implemented using the v. 0.19.1 of the Python (https://www.python.org/psf/) library Scikit-learn (Pedregosa et al., 2011).

3 | RESULTS

3.1 | VW onset and development

The most common qualitative symptoms in inoculated plants consisted of chlorosis partially or completely affecting the leaf, colour change of the leaf from intense green to matt green, defoliation of green, matte, or chlorotic green leaves, and/or shortening of internodes at the distal end of the stem of the plant.

Time-to-symptom onset, assessed by survival statistical protocols, showed that the best survival regression fit (the model with the lowest AIC score) was produced when the lognormal distribution was used in both cultivars (Figure 1c,d). On the other hand, the disease intensity index progress over time for cv. 'Picual' was adequately described by the Gompertz model ($R^2 > 0.95$; root mean square error < 0.3972; Figure 1a). However, the experiment did not provide sufficient precision to fit curves of disease progression for 'Frantoio' to any model (Figure 1b). For cv. 'Picual', the increase in disease intensity index became asymptotic (disease intensity index > 28.0%) at all irrigation treatments except for low rate-daily frequency (disease intensity index = 16.1%) and high rate-daily frequency-irrigated (disease intensity index = 18.0%) plants. Analyses performed on the intrinsic rate of disease progression (*rho* parameter of the Gompertz model fitted to temporal disease intensity index progress) showed that this parameter was significantly higher on average for plants irrigated with the combination frequency ($\rho = 0.89$) than the mean value for the other irrigation frequencies (0.34 and 0.45 for the daily and twice weekly irrigation frequencies, respectively; Table 1).

Type III tests of fixed effects from the mixed-model analysis showed significant effects of olive cultivar, irrigation frequency, and the interaction of irrigation frequency and cultivar on area under disease progress curve of VW (data not shown). The effects

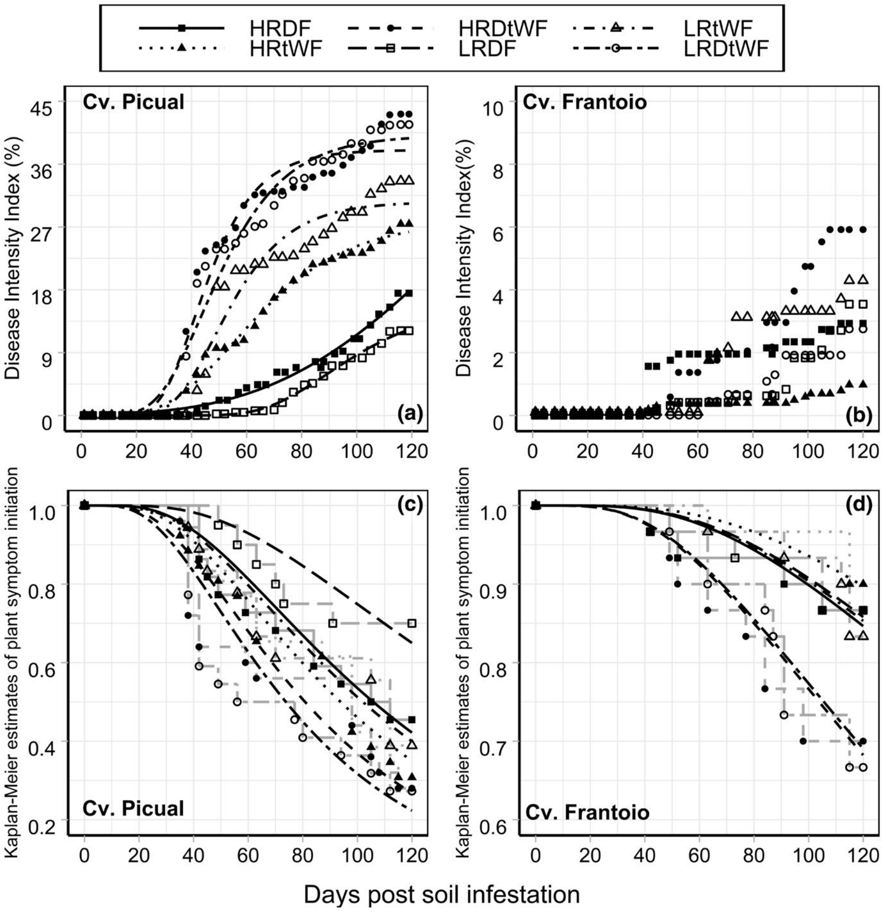

FIGURE 1 Progress of Verticillium wilt intensity index (a, b) and Kaplan–Meier estimates of survival (c, d) fitted with Gompertz model and survival regression analysis to log normal distribution, respectively, in 'Picual' (a, c) and 'Frantoio' (b, d; no fits with Gompertz model were found for progress of Verticillium wilt intensity index) olive cultivars. The plants were grown in soil artificially infested with *Verticillium dahliae* and irrigated to two rates of soil water content (high and low; HR and LR, respectively) at three irrigation frequencies (daily, twice weekly, and daily/twice weekly; DF, tWF, and DtWF, respectively) over 120 days under growth chamber conditions. Each point represents the mean of data from two repeated experiments, each comprising 16 pots with one plant per pot. Grey lines represent the Kaplan–Meier estimates and black lines the resulting fitted curves for the survival analyses

TABLE 1 Disease parameters in a trial where olive cultivars 'Picual' and 'Frantoio' were grown in soil artificially infested with *Verticillium dahliae* and irrigated to two ranges of soil water content at three irrigation frequencies over a period of 120 days under growth chamber conditions

		'Picual'					'Frantoio'			
Range of soil water content	Irrigation frequency	AUDPC[a]	DI[a]	DII[a]	ρ[b]	Exp (location)[c]	AUDPC	DI	DII	Exp (location)
High	Daily	6.3[d]	36.5	19.8	0.54	107.3 Aa	1.6	12.5	2.9	293.7
	tWeekly	12.3	67.7	34.1	0.47	96.2 Aa	2.0	9.4	1.0	353.0
	Daily-tWeekly	21.9	65.6	41.6	0.98	84.1 Aa	1.4	30.2	6.0	187.4
	mean	13.5	56.6 A	31.8	0.66	95.9	1.7	17.4	3.3	244.4
Low	Daily	2.9	19.8	14.7	0.15	151.2 Ab	0.7	12.5	3.3	220.6
	tWeekly	8.3	42.7	26.6	0.42	103.9 Aab	0.3	15.6	4.3	218.3
	Daily-tWeekly	18.6	54.2	39.1	0.81	77.0 Aa	3.7	30.2	2.9	152.2
	mean	9.9	38.9 B	26.8	0.46	110.7	1.6	19.4	3.5	261.6
Mean	Daily	4.6 b	28.1 b	17.2 b	0.34 b	129.2	1.1	12.5 b	3.1 a	257.2
	tWeekly	10.3 ab	55.2 a	30.4 ab	0.45 b	100.1	1.2	12.5 b	2.6 a	290.5
	Daily-tWeekly	20.3 a	59.9 a	40.3 a	0.89 a	80.6	2.6	30.2 a	4.5 a	165.8

Abbreviations: AUDPC, area under disease intensity index progress curve; DI, disease incidence; DII, disease intensity index.

[a] Severity of wilt symptoms of each individual plant was determined twice every week for 120 days and used to calculate relative AUDPC. DI and DII are, respectively, the disease incidence and the disease intensity index.

[b] Rate of disease increase parameter of the Gompertz function fitted to disease progress data.

[c] Time-point where 50% of the population are estimated by a survival log normal fit to have experienced the event (onset of symptoms).

[d] Values are the mean of four replications (six plants per replication). Different upper case or lower case letters in the same column indicate significant differences between values for ranges of soil water content or irrigation frequencies, respectively, according to Tukey test ($p \le 0.05$).

of irrigation factors were then analysed separately for each cultivar. Disease severity, area under disease progress curve, and disease intensity index for the susceptible cultivar 'Picual' was higher for the combination treatment (daily/twice weekly) compared with the daily irrigation frequency (Table 1; Figure 1a). The twice weekly irrigation frequency was generally intermediate between the daily and combination frequency treatments. This result was also supported by the probability of when 50% of the plants expressed symptoms (Figure 1c; Table 1 [exp(Location) parameter]). Fifty percent of plants were estimated to show VW symptoms at 77 days in the combination frequency compared with 151 days in the daily frequency at low irrigation rate, and 84 days from the combination frequency compared with 107 days for the daily frequency at the high irrigation rate. However, irrigation frequency was not different for these same parameters for the resistant 'Frantoio'. DI was similar in the twice weekly (55%) and the combination (60%) frequencies and higher than daily (28%) irrigation frequency in 'Picual', while in 'Frantoio', DI was higher in the combination (30%) frequency than either the daily (13%) or twice weekly (13%) irrigation frequencies (Table 1).

3.2 | Soil inoculum, colonization, and WUE parameters

Time or DPI effects were significant ($p < 0.05$) in all *V. dahliae* soil inoculum parameters (Table 2; Figure 2). A generalized decline was found for initial high-level densities of *V. dahliae* estimated in wet soil, as has been cited in other similar *V. dahliae*–olive experiments (Jiménez Díaz et al., 2009). Initial total ID, MpD, and S_wD values ranged, according to treatments, from 0.7 to 9.2×10^7, from 0.2 to 3.2×10^6, and from 0.8 to 6.1×10^5 cfu/g dry, respectively, reaching final mean relative values (in both soils where 'Picual' and 'Frantoio' were grown) from 45.8%, 2.1%, and 31.5% to 67.9%, 73.9%, and 82.9%, respectively, according to treatments (Figure 2a–f). However, the relative levels of persistent propagules in air-dried soil (relative S_dD; initial values ranged, according to treatments, from 3.9 to 6.2×10^4 cfu/g dry, as has been cited by Calderón et al. (2014), in a similar experiment) followed a different pattern over time in both 'Picual' and 'Frantoio' soils, with a marked increase during symptom development (40 DPI), before slightly decreasing to final mean relative values of 43.8%–92.4% at 120 DPI, according to treatments (Figure 2g,h). As shown by type III tests, the interaction irrigation rate × frequency × cultivar × time was significant ($p < 0.05$), or almost significant ($p < 0.08$), for these parameters (Table 2). However, soil inoculum parameters were not significantly different for the cultivar factor ($p > 0.05$), but the interaction irrigation frequency × cultivar did turn out to be significant ($p < 0.05$), or almost significant ($p = 0.069$–0.085). Mean relative inoculum parameters were similar in soils daily irrigated regardless of the cultivar, although these parameters were higher (cv. 'Picual') or lower (cv. 'Frantoio'), when this soil was twice weekly- or daily/twice weekly-irrigated, respectively. Irrespective of irrigation frequency and olive cultivar factors, relative inoculum parameters estimated in wet soil (total ID, S_wD, and MpD) were significantly higher at high rate (70.1%, 76.7%, and

TABLE 2 Type III tests of fixed effects from the mixed-model analysis of the soil inoculum parameters of *Verticillium dahliae* estimated at 12, 40 and 120 days post-soil infestation in a trial where olive cultivars 'Picual' and 'Frantoio' were grown in soil artificially infested by the pathogen and irrigated to two ranges of soil water content at three irrigation frequencies over a period of 120 days under growth chamber conditions

	Total ID				MpD		S_wD		S_dD	
Effect	df_{num}	df_{den}	F	$p > F$	F	$p > F$	F	$p > F$	F	$p > F$
Range of soil water content (*R*)	1	251	9.80	0.0020	19.31	<0.0001	6.38	0.0122	1.35	0.2457
Irrigation frequency (*F*)	2	251	2.65	0.0729	1.77	0.1717	3.51	0.0314	13.39	<0.0001
R × F	2	251	3.45	0.0334	0.54	0.5845	1.16	0.3151	0.83	0.4392
Cultivar (*C*)	1	251	0.11	0.7362	0.19	0.6651	0.22	0.6407	2.61	0.1073
R × *C*	1	251	1.83	0.1778	1.23	0.2693	1.98	0.1606	0.00	0.9955
F × C	2	251	2.71	0.0686	2.49	0.0849	6.66	0.0015	12.55	<0.0001
R × *F* × C	2	251	2.32	0.1001	4.70	0.0099	4.30	0.0145	0.48	0.6221
Days post-soil infestation (DPI)	2	251	88.34	<0.0001	171.98	<0.0001	68.26	0.0000	6.24	0.0023
R × DPI	2	251	9.32	0.0001	5.15	0.0065	3.27	0.0395	4.26	0.0152
F × DPI	4	251	7.22	<0.0001	3.96	0.0039	4.41	0.0018	2.78	0.0273
R × F × DPI	4	251	2.86	0.0239	2.21	0.0685	3.63	0.0068	2.66	0.0331
C × DPI	2	251	7.95	0.0004	5.41	0.0050	4.04	0.0188	3.03	0.0503
R × *C* × DPI	2	251	5.37	0.0052	3.24	0.0406	4.76	0.0094	6.67	0.0015
F × *C* × DPI	4	251	6.40	0.0001	0.90	0.4670	0.74	0.5625	3.49	0.0086
R × F × *C* × DPI	4	251	5.48	0.0003	2.07	0.0855	2.53	0.0409	2.18	0.0717

Note: Verticillium dahliae inoculum in soil was estimated in wet soil by direct plating soil dilution method (total ID; total inoculum density), by plating wet soil fractionated as indicated by Santos-Rufo et al. (2017) to separately quantify more persistent sclerotia (size ≥ 20 μm; S_wD, sclerotia density in wet soil) and less persistent micropropagules (size < 20 and ≥1 μm; MpD, micropropagules density), and by impaction plating of pulverized dry soil (S_dD; sclerotia density in dried soil). For each block, inoculum densities estimated at each sampling time (12, 40, and 120 DPI) were related to the initial values (0 DPI) and expressed as a percentage.

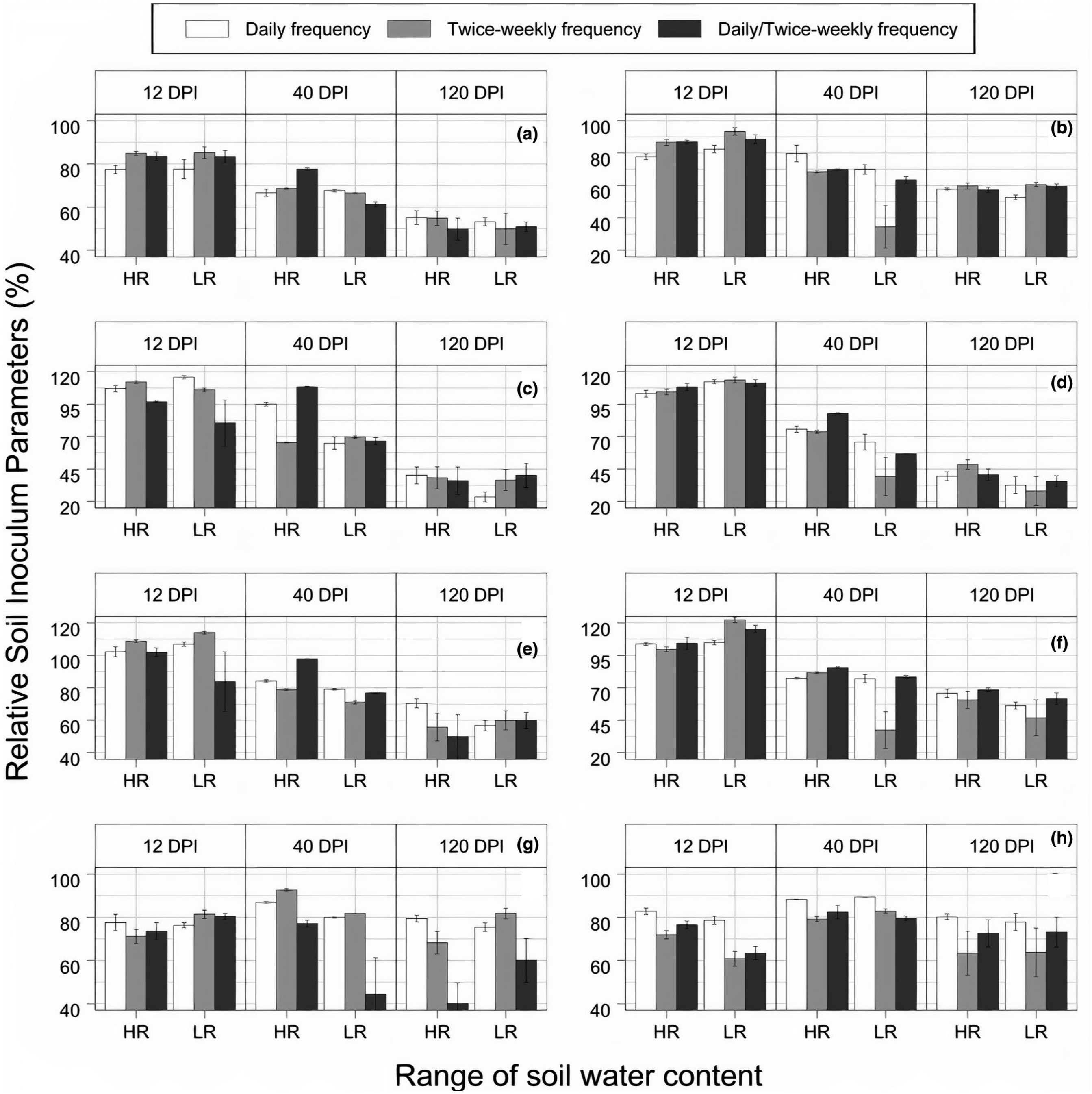

FIGURE 2 Relative density of total inoculum (a, b) and less (c, d) and more (e–h) persistent propagules of *Verticillium dahliae* in infested soil irrigated to two rates of soil water content (high and low; HR and LR, respectively) at three irrigation frequencies (daily, twice weekly, and daily/twice weekly) over 120 days under growth chamber conditions. Densities were calculated at 3 days post-soil infestation (DPI; 12, 40, and 120 DPI) as colony-forming units (cfu) per g dry soil and related to their initial values by dividing the number of propagules at each sampling time by the number of propagules at the beginning (0 DPI), expressed as a percentage (a, b) total inoculum density (ID) in wet soil; (c, d) density of less persistent propagules (micropropagules; MpD) of size < 20 μm and ≥1 μm, in wet soil; (e, f) density of more persistent propagules (sclerotia; S_wD) of size ≥ 20 μm in wet soil; (g, h) sclerotia density (S_dD) in air-dried soil. Left panels (a, c, e, g) correspond to soil where cultivar 'Picual' was grown and right panels (b, d, f, h) to soil where cultivar 'Frantoio' was grown. Each point represents the mean of data from two repeated experiments, each comprising four pots with one plant per pot

83.1%, respectively) than at low rate (66.7%, 66.6%, and 78.2%, respectively; Table 2; Figure 2).

Verticillium dahliae colonization exhibited different trends to those of soil inoculum (Figure 3). Analysis showed that RCI and SCI of VW were significantly higher in 'Picual' (RCI values ranged from 0.0% to 0.5%, 0.5% to 5.2%, and 0.0% to 7.2%, and SCI values ranged from 0.0% to 0.0%, 5.0% to 15.0%, and 6.9% to 10.3% at 12, 40, and 120 DPI, respectively; Figure 3a,c) than in 'Frantoio' (RCI values ranged from 0.0% to 0.5%, 0.0% to 2.6%, and 0.4% to 1.3%, and SCI values ranged from 0.0% to 0.0%, 0.0% to 5.0%, and 0.4%

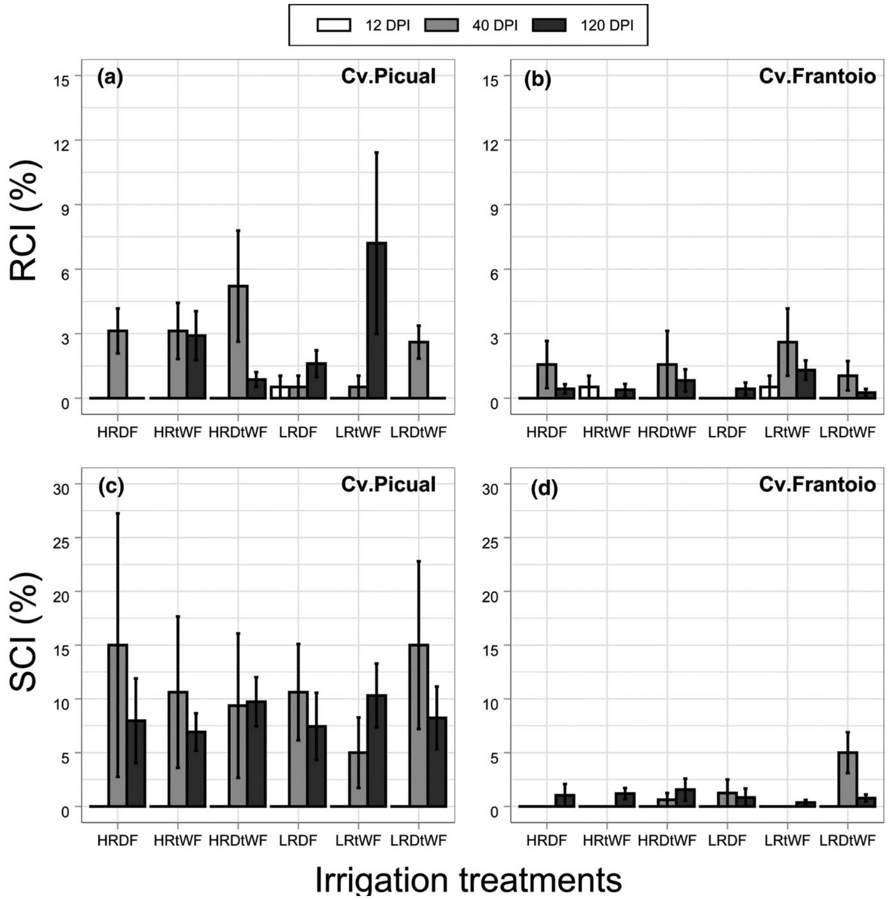

FIGURE 3 Root (a, b) and shoot (c, d) colonization indexes (RCI and SCI, respectively) in 'Picual' (a, c) and 'Frantoio' (b, d) cultivar olive plants grown in soil artificially infested with *Verticillium dahliae* and irrigated to two rates of soil water content (high and low; HR and LR, respectively) at three irrigation frequencies (daily, twice weekly, and daily/twice weekly; DF, tWF, and DtWF, respectively) over 120 days under growth chamber conditions. Colonization indexes were assessed at 3 days post-soil infestation (DPI; 12, 40, and 120 DPI). Each bar represents the mean of data from two repeated experiments, each comprising four pots with one plant per pot

to 1.6% at 12, 40, and 120 DPI, respectively; Figure 3b,d; Table 3). However, the effects of irrigation rate and frequency on these parameters were identical in 'Picual' and 'Frantoio' (Table 3). Time effects and time × cultivar interaction were significant ($p < 0.05$) for both RCI and SCI parameters (Table 3). No other interactions were significant for SCI ($p > 0.05$), suggesting that this parameter varied similarly with time regardless of irrigation factors. On the other hand, time × irrigation frequency interaction was significant for RCI ($p < 0.05$), as well as the triple interactions time × irrigation frequency × cultivar and time × irrigation rate × cultivar (Table 3) in that the difference among irrigation factors and cultivar varied with time: differences in the mean were generally greater at 40 (at time of symptom onset) than at 12 and 120 (before and after symptom onset) DPI, with higher RCI values at high than at low rate found at this date (Figure 3a,b).

Water-use efficiency was not significantly different ($p > 0.05$) between *V. dahliae*-inoculated and control plants in this study (data not shown), but the effects of time and the interaction time × irrigation rate were significant (data not shown; $p < 0.05$). At high rate, no differences were found between WUE estimates at different sample times, whereas a strong decline was found after 12 DPI at low rate (Figure 4). All other interactions with time were not significant (data not shown; $p > 0.05$) except for time × irrigation frequency (data not shown; $p < 0.05$). On the other hand, the simple effect of cultivar and most of the double and triple interactions significantly affected WUE (data not shown). This parameter declined after 12 DPI at low rate in both cultivars regardless of whether *V. dahliae* was present in the soil or not; however, this was most pronounced in 'Picual', in which WUE was 40% lower at low than high rate by 40 DPI (Figure 4).

3.3 | Relationships among disease, soil inoculum, colonization, and WUE parameters

The hierarchical cluster analysis yielded four functional groups (A to D) among the 36 experimental treatment combinations (two rates of soil water content × three irrigation frequencies × two cultivars × three DPIs; Figure 5). Group A comprised four experimental treatments with a high level of disease intensity index, including 'Picual' plants that were high rate-twice-weekly-, high rate-daily/twice-weekly-, low rate-twice-weekly-, and low rate-daily/

TABLE 3 Type III tests of fixed effects from the mixed-model analysis of the root and shoot colonization indexes by *Verticillium dahliae* estimated at 12, 40, and 120 days post-soil infestation in a trial where olive cultivars 'Picual' and 'Frantoio' were grown in soil artificially infested by the pathogen and irrigated to two ranges of soil water content at three irrigation frequencies over a period of 120 days under growth chamber conditions

	RCI				SCI	
Effect	df_{num}	df_{den}	*F*	$p > F$	*F*	$p > F$
Range of soil water content (*R*)	1	248	0.17	0.6838	0.24	0.6281
Irrigation frequency (*F*)	2	248	2.52	0.0825	1.08	0.3408
$R \times F$	2	248	1.39	0.2503	0.31	0.7310
Cultivar (*C*)	1	248	9.31	0.0025	47.86	<0.0001
$R \times C$	1	248	1.02	0.3124	0.04	0.8447
$F \times C$	2	248	0.14	0.8683	0.05	0.9554
$R \times F \times C$	2	248	1.29	0.2766	0.08	0.9202
Days post-soil infestation (DPI)	2	248	14.89	<0.0001	24.81	<0.0001
$R \times$ DPI	2	248	2.75	0.0659	0.62	0.5401
$F \times$ DPI	4	248	3.74	0.0057	0.98	0.4211
$R \times F \times$ DPI	4	248	1.86	0.1177	0.98	0.4195
$C \times$ DPI	2	248	3.17	0.0436	12.15	<0.0001
$R \times C \times$ DPI	2	248	3.55	0.0301	0.27	0.7652
$F \times C \times$ DPI	4	248	2.93	0.0217	0.40	0.8089
$R \times F \times C \times$ DPI	4	248	0.83	0.5091	0.23	0.9209

Note: Root and stem colonization indexes (RCI and SCI, respectively) were calculated as follows: SCI/RCI $= \sum N_i/N_j \times 100$, where N_i is the number of plated stem or root pieces yielding *V. dahliae* colonies and N_j is the total number of stem or root pieces plated.

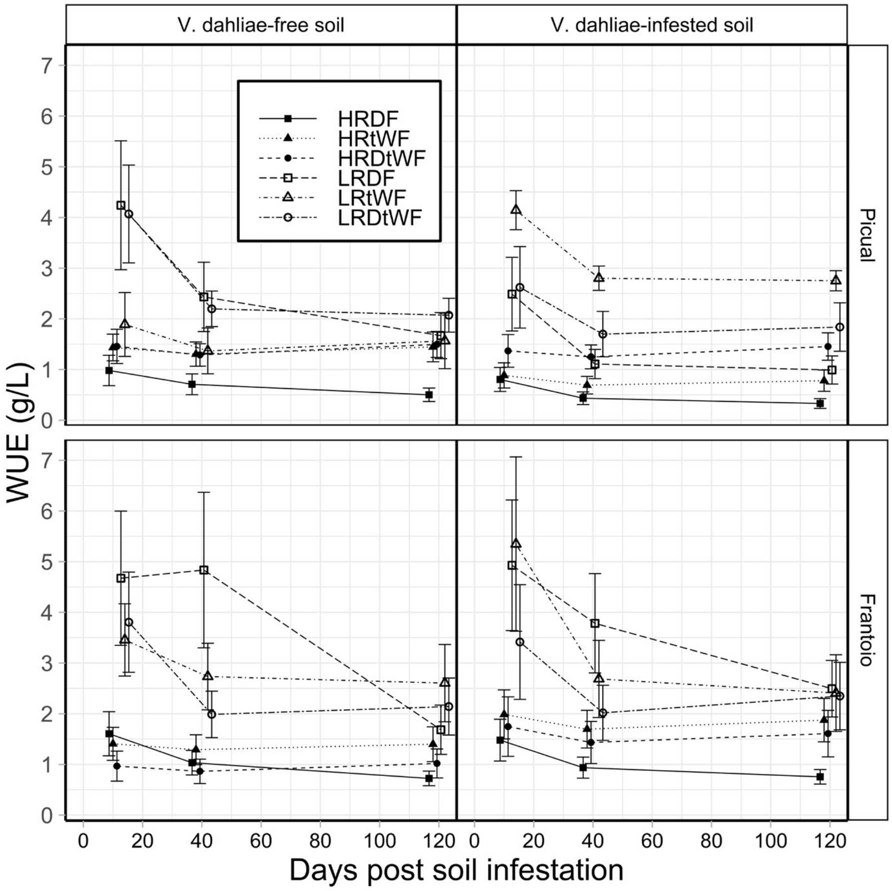

FIGURE 4 Water-use efficiency (WUE) of biomass production in 'Picual' and 'Frantoio' cultivar olive plants grown in *Verticillium dahliae*-infested and *V. dahliae*-free soils, irrigated to two rates of soil water content (high and low; HR and LR, respectively) at three irrigation frequencies (daily, twice weekly, and daily/twice weekly; DF, tWF, and DtWF, respectively) over 120 days under growth chamber conditions. Each point represents the mean of data from two repeated experiments, each comprising four pots with one plant per pot

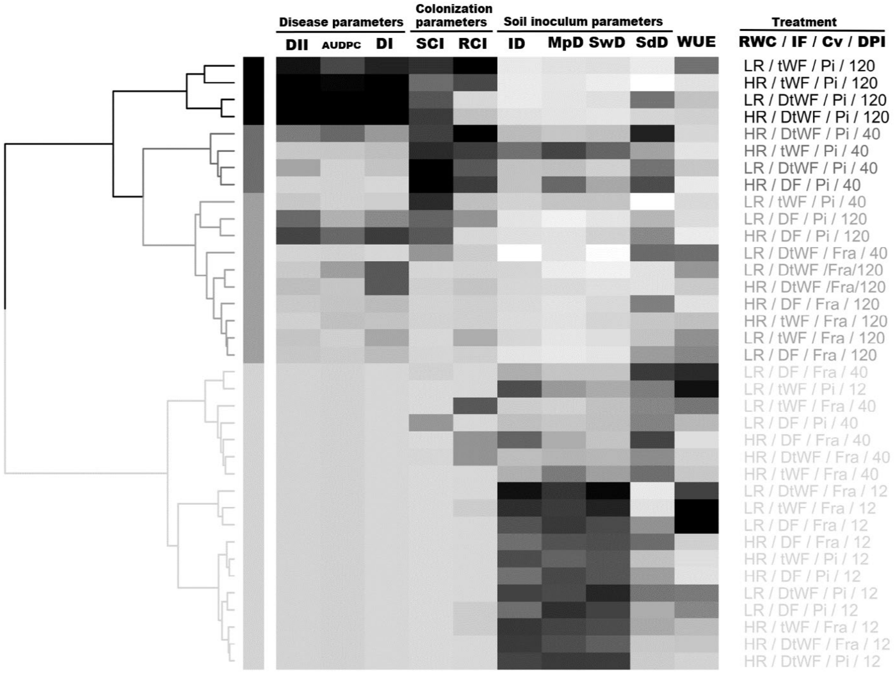

FIGURE 5 Dendrogram derived from cluster analysis of 36 experimental treatment combinations in a trial where the effect of irrigation was assessed on Verticillium wilt of olive, and heat map representation of the disease, *Verticillium dahliae* colonization, soil inoculum, and water-use efficiency (WUE) parameters for the different sampling dates. The 10 parameters selected for the heat map representation were related to visual Verticillium wilt reaction (three parameters): final disease incidence (DI), final disease intensity index (DII), and area under disease intensity index progress curve (AUDPC); to *V. dahliae* colonization (two parameters): shoot (SCI) and root (RCI) colonization index; to *V. dahliae* soil inoculum (four parameters): relative total inoculum density (ID) in wet soil, density of less persistent propagules (micropropagules; MpD) of size < 20 μm and ≥1 μm in wet soil, density of more persistent propagules (sclerotia; S_wD) of size ≥ 20 μm in wet soil, and sclerotia density (S_dD) in air-dried soil; and related to plant water use (one parameter): WUE of biomass production. Agglomerative cluster analyses were performed based on the Spearman correlation matrix calculated from values of the different parameters using the Ward method. Clusters of experimental treatment combinations, depicted in different shades of grey, were estimated on the basis of the average silhouette width according to the Mantel statistic. In the heat map, in each column, cells represent the relative value of each parameter for each experimental treatment combination of two rates of soil water content (RWC; high and low; HR and LR, respectively), three irrigation frequencies (IF; daily, twice weekly, and daily/twice weekly; DF, tWF, and DtWF, respectively), two olive cultivars (cv; 'Picual' and 'Frantoio'; Pi and Fra, respectively) and 3 days post-soil infestation (DPI; 12, 40, and 120 DPI) from two repeated experiments

twice-weekly-treated at 120 DPI. In general, treatments with a severe disease reaction were associated with intermediate to high colonization index values, low relative levels of total ID, MpD, and S_wD, intermediate relative levels of S_dD, and low levels of WUE. Group B included five experimental treatments with a moderate level of disease intensity index but very high colonization index values. It included all cv. 'Picual'-irrigation treatments at 40 DPI except for low rate-daily frequency. This group of treatment combinations exhibited intermediate values for relative total ID, MpD, and S_wD, high relative levels of S_dD and low levels of WUE. Group C comprised 10 experimental treatments associated with a low disease reaction and an intermediate value of SCI and low levels of RCI indexes. The treatments included low rate-daily frequency in cv. 'Frantoio' at 120 DPI, high rate-daily frequency in cv. 'Picual' at 120 DPI, and 'Picual'-low rate-daily frequency and 'Frantoio'-low rate-daily/twice-weekly combinations at 40 DPI. The low levels of disease intensity index and the intermediate or low colonization index values observed in treatment combinations within this group was associated with low relative levels of total ID, MpD, and S_wD and moderate values of relative S_dD and WUE. Group D was made up of the remaining 17 treatments with no visual symptoms, very low values of SCI and low levels of RCI, including all cultivar and irrigation treatment combinations at 12 DPI and combinations of all irrigation treatments in cv. 'Frantoio' at 40 DPI with the exception of low rate-daily/twice-weekly-treated plants. High values of soil inoculum parameters, except for relative S_dD with intermediate values, and WUE were exhibited for this group of plants (Figure 5).

Classification and regression trees yielded the highest accuracy (>83%) in classifying very low, low, moderate, and severe disease intensity index classes with the *V. dahliae* colonization, soil inoculum, and WUE parameters in this study. Mean accuracies of k-nearest neighbours, Gaussian naive Bayes, linear discriminant analysis, support vector machines, and logistic regression were, respectively, 76.1%, 75.2%, 71.3%, 69.0%, and 64.0% (data not shown). Classification and regression trees algorithm was then used to determine the thresholds of *V. dahliae* colonization, WUE, and soil inoculum parameters that discriminated between disease intensity index classes (Figure 6). This was performed using the

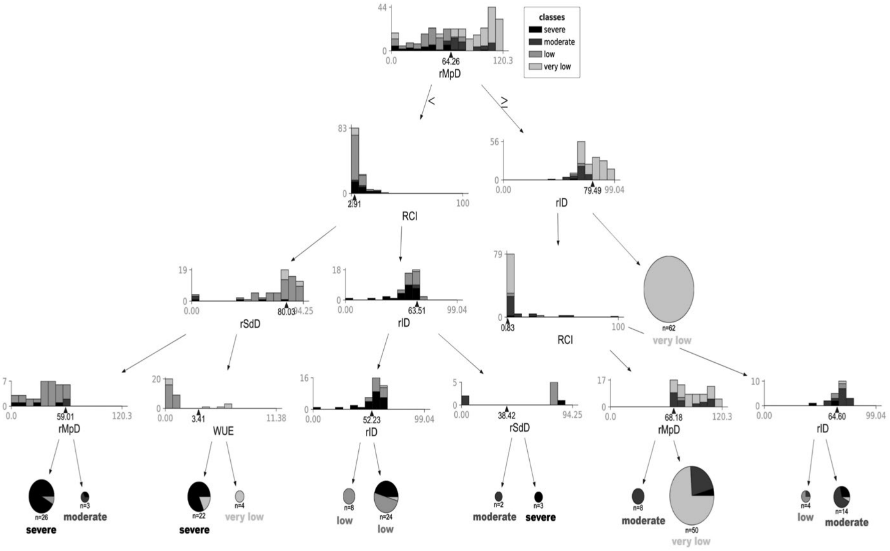

FIGURE 6 Pruned classification tree for predicting Verticillium wilt (VW) intensity index classes (very low, low, moderate, and severe) in olive based on *Verticillium dahliae* soil inoculum (relative density of total inoculum and of micropropagules; ID and MpD, respectively), colonization (shoot and root colonization index; SCI and RCI, respectively) and water-use efficiency (WUE) parameters. The histogram for each node represents the percentage of plants in each VW intensity index class. For each terminal (decision) node, the most prevalent VW severity class is indicated showing feature distributions as overlapping stacked-histograms, one histogram per target class. Leaf size is proportional to the number of samples in that leaf. Data include a training set (80%) from a data set (288 plants) comprising all experimental combinations of rate of soil water content, irrigation frequency, olive cultivar, and days postinoculation from two repeated experiments

TABLE 4 Classification report of a decision tree classifier directly run on the test set to estimate the relationship between the classes of Verticillium wilt intensity index in olive and the *Verticillium dahliae* soil inoculum, colonization, and water use efficiency of biomass production parameters

	Precision	Recall	F1 score	Support
Class[a]				
Very low	1.00	0.96	0.98	27
Low	0.76	0.94	0.84	17
Moderate	0.71	0.71	0.71	7
Severe	0.50	0.29	0.36	7
Accuracy	—	—	0.84	58
Macro average	0.74	0.73	0.73	58
Weighted average	0.84	0.84	0.83	58

Note: The data set (288 plants) was partitioned 4:1 into training and test sets, including relative values of each parameter for each experimental treatment combination of range of soil water content, irrigation frequency, olive cultivar, and days post inoculation from two repeated experiments.

[a] Verticillium wilt intensity index classes in olive.

XGBoost library for Python (Chen & Guestrin, 2016). The pruned tree generated by classification and regression trees contained five parameters (relative MpD, total ID and S_dD, RCI, and WUE) with 13 terminal nodes (Figure 6). The relative soil inoculum parameter MpD was the main factor (i.e., first splitting parameter) that differentiated between plants in the severe disease intensity index class and those in moderate, low, and very low affected classes, with a relative MpD threshold of 64.3%.

The selected tree was validated with the test set for an independent final check on the accuracy of the model. With the test sets, the confusion matrix indicated that few errors were made for the very low, low, and moderate classes, but some more errors were made for the severe class (data not shown). This was confirmed by the classification report, which showed a precision of 100.0%, 76.0%, 71.0%, and 50.0% for the classes very low, low, moderate, and severe, respectively, with a mean F1-score of 84.0% (Table 4).

4 | DISCUSSION

We demonstrated the potential capabilities of machine learning techniques for studying the influence of irrigation on VW in most of the points of the disease cycle of *V. dahliae* in olive. Although such influence has been well reported (Pérez-Rodríguez, Alcántara, et al., 2015; Pérez-Rodríguez et al., 2016; Pérez-Rodríguez, Orgaz, et al., 2015; Santos-Rufo et al., 2017), the resulting evidence has been contradictory (Montes-Osuna & Mercado-Blanco, 2020). In this research, the effect of differential irrigation treatments—based on the resistance level of olive cultivars to VW—was assessed by their influence on the pathogen and on the plant simultaneously. Other factors affecting the disease (e.g., soil texture, fertilization schedule, and age of plantation) were not considered in this study, and thus, all the findings shown here must be taken with caution in the choice of an irrigation schedule for VW-diseased olive orchards.

Symptoms in affected plants were identical to those previously described in similar inoculations with the D *V. dahliae* pathotype (Calderón et al., 2014; Jiménez-Fernández et al., 2016). Severe foliar symptoms were developed in 'Picual' and 'Frantoio' olive cultivars, respectively, but their reactions varied when subjected to differing irrigation treatments, as has been reported under microplot and natural environmental conditions in 'Picual' (Pérez-Rodríguez, Alcántara, et al. 2015; Santos-Rufo et al., 2017). However, time-to-symptom onset (survival time) was similarly reduced in 'Picual'- and 'Frantoio'-low rate-daily/twice-weekly treatments (almost 75 and 69 days shorter, respectively) compared with 'Picual'- and 'Frantoio'-low rate-daily frequency treatments (one of the less symptomatic ones), showing a great impact of irrigation in the early stages of the infection process in comparison with cultivar susceptibility. Although it would have been interesting to assess how cultivar susceptibility affects disease development, the experiment did not provide sufficient precision to fit curves of disease progression for 'Frantoio' to any model, either in this study or in the study by Pérez-Rodríguez, Alcántara, et al. (2015). However, while no significant differences in any disease parameters were detected in cv. 'Frantoio' subjected to different irrigation treatments in the study by Pérez-Rodríguez, Alcántara, et al. (2015), irrigation frequency significantly affected D in both cultivars in this study, also suggesting a significant influence of irrigation, compared with cultivar susceptibility, after symptom onset.

Average initial total ID, MpD, and S_wD levels, quantified in wet soil, tended to decrease continuously over time. In the case of total ID, this finding coincided with results previously reported under similar conditions (Gómez-Gálvez & Rodríguez-Jurado, 2018). By contrast, the average inoculum level of more persistent pathogen propagules quantified in dried soil (S_dD) followed a different pattern over time, partially coinciding with results previously reported (Santos-Rufo et al., 2017), with a marked increase as symptoms develop (40 DPI). In addition, although RCI values of inoculated 'Picual' plants did not display a clear maximum during the course of sampling, SCI tended to be higher at 40 DPI, as found in a DNA quantification assay for root-dip inoculated 'Picual' plants (Mercado-Blanco et al., 2003). However, RCI values of inoculated 'Frantoio' plants were (with some exceptions) higher at 40 DPI, but no clear maximum was found for the SCI parameter during the course of sampling; conversely, very high values were found when assessing this parameter after 3 months of growing in infested soil (Jiménez-Fernández et al., 2016). Consistent with our findings, it is interesting to note that irrespective of the cultivar susceptibility, higher RCI values were found at high than at low rate as symptoms developed (40 DPI). Thus, irrigation rate had a similar effect on the olive root colonization by *V. dahliae* as symptoms develop compared to when trees are colonized and show wilt, as the DI parameter was also higher at high than low rate at the end of the experiment. By contrast, there was a strong influence of cultivar susceptibility on WUE, compared with irrigation, as symptoms develop. Regardless of *V. dahliae* infection, this parameter declined after 12 DPI at low rate in both cultivars, but this was most pronounced in 'Picual', in which WUE was 40% lower at low than high rate by 40 DPI, as reported by Ploetz et al. (2015) for avocado cultivars that differed in susceptibility to laurel wilt. In that study, WUE declined in all cultivars after inoculation when plants were treated with standard irrigation practices (presumably at low rate); however, it was most pronounced in the most susceptible cultivar (Ploetz et al., 2015).

A multivariate hierarchical cluster analysis confirmed the results of the mixed-model analysis, highlighting a clear effect of time, followed by cultivar, among several interactions with irrigation treatments and time. The analysis led to the creation of four functional groups which were associated with very low, low, moderate, and severe disease classes, as reported in a study evaluating the effects of soil temperature on olive cultivars with different susceptibility to VW (Calderón et al., 2014). However, while logistic regression yielded the lowest accuracy in classifying these disease intensity index classes with the *V. dahliae* soil inoculum, colonization, and WUE parameters under the above-mentioned conditions of this study (below 64.0%; data not shown), it was chosen as the best predictor to discriminate among disease severity classes by stress-related parameters (accuracy of 76.6%) in the study by Calderón et al. (2014). Between six different linear and nonlinear algorithms assessed in this study, classification and regression trees yielded the highest classification accuracy with the training set (data not shown) and was then used to determine the thresholds of *V. dahliae* colonization, WUE, and soil inoculum parameters that discriminated between disease intensity index classes. This algorithm was also used to determine the thresholds of stress parameters that discriminated between disease severity classes in the study by Calderón et al. (2014), reaching a similar mean F1-score with the test set (85.1%) to the value obtained in this study (84.0%). According to classification and regression trees, the soil inoculum parameter MpD could be a good indicator for disease detection at both early and advanced stages of *V. dahliae* infection. These results obtained under growth chamber conditions are in line with those obtained under natural environmental conditions, where it was reported that the density of micropropagules or propagules of low persistence in the soil played a part in disease development, though no correlation was found with final disease parameters (Santos-Rufo et al., 2017). At the second level, plants with a mean disease intensity index of 35.4% (severely affected) were separated from plants with a mean disease intensity index of 0.3% (very low disease intensity index) according to an RCI threshold of 2.9%. Plants below this threshold with a relative S_dD in soil $>80.03\%$ and WUE $< 3.41\%$ were severely affected, while plants with a higher WUE threshold had very low disease intensity index. In general, WUE values higher than 3.41% were registered before symptom onset (12 DPI), so this parameter could be considered a good indicator in the early stages of *V. dahliae* infection. In addition, plants with RCI above 2.9%, relative total ID value $> 63.5\%$, and relative $S_dD > 38.42\%$ were severely affected and those with a lower relative S_dD value exhibited moderate symptoms (mean disease intensity index = 6.9%). Pearson correlation revealed a significant relationship between WUE and AUDPC under natural environmental conditions ($r = 0.83$; $p < 0.05$; Santos-Rufo et al., 2018), and between the progress of relative S_dD and RCI over time in this study ($r > 0.63$; $p < 0.05$; data not shown), which could partially explain their high explanatory power. However, although RCI was one of the most important parameters in the tree classifier (i.e., second splitting parameter), neither this parameter nor relative S_dD could clearly distinguish between low (mean disease intensity index = 4.6%)/very low classes and severe classes, which means that RCI and relative S_dD are only suitable as indicators for disease detection in advanced stages. On the other hand, very low, low, and moderate VW classes separated by a relative MpD in soil ≥8.3% were divided into two groups by relative total ID. Plants grown in soil with relative total ID > 79.5% (threshold mainly reached before symptom onset, at 12 DPI) were directly classified as very low, which rules out relative total ID as a good indicator for early stages of *V. dahliae* infection. Plants grown in soil with a relative total ID threshold in the range 64.6%–79.5% (threshold mainly reached during symptom onset, at 40 DPI) and RCI > 0.8% had a moderate disease intensity index, while plants grown in soil with a lower relative total ID value were scarcely affected (low class).

There are irrigation schedules that can increase the population of *V. dahliae* in the soil and create favourable conditions for the infection of olive plant and the development of VW (Santos-Rufo et al., 2017, 2018), so it is important to uncover the mechanisms in the disease cycle of the pathogen through which irrigation generates such increases. In this study, 'Picual' plants were more susceptible to these irrigation schedules than 'Frantoio' plants, even though irrigation with low rate and daily frequency treatments benefited both cultivars. Soil inoculum, colonization, and WUE parameters were able to detect the degree to which VW was exalted by irrigation and/or cultivar susceptibility. Density of micropropagules, which is related to sclerotium germination, was the best indicator for VW detection before, during, and after symptoms develop. Other parameters can only be used before symptoms develop (WUE) or after symptoms develop (root colonization indexes, relative total inoculum density, and density of sclerotia in air-dried soil). By providing a better understanding of the relationships between irrigation, VW, and olive cultivar, these results will be useful for those seeking to optimise the use of irrigation in an integrated approach to VW management in olive.

ACKNOWLEDGEMENTS

This research was supported by the PEI.PEI2011.1 project of the Andalusian Institute of Agricultural Research and Training (IFAPA) and by the RTA2006-0012-00-00 project of the National Institute for Agricultural and Food Research and Technology (INIA), both partially funded by the European Regional Development Fund (ERDF). Funding for open access charge: Universidad de Córdoba / CBUA.

CONFLICT OF INTEREST

The authors of this work declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Antonio Santos-Rufo https://orcid.org/0000-0002-2740-4473

REFERENCES

Bates, D., Mächler, M., Bolker, B.M. & Walker, S.C. (2015) Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48.

Ben-Yephet, Y. & Pinkas, Y. (1977) Germination of individual microsclerotia of *Verticillium dahliae*. *Phytoparasitica*, *5*, 159–166. https://doi.org/10.1007/BF02980349

Butterfield, E.J. & DeVay, J.E. (1977) Reassessment of soil assays for *Verticillium dahliae*. *Phytopathology*, *67*, 1073–1078. https://doi.org/10.1094/Phyto-67-1073

Calderón, R., Lucena, C., Trapero-Casas, J.L., Zarco-Tejada, P.J. & Navas-Cortés, J.A. (2014) Soil temperature determines the reaction of olive cultivars to *Verticillium dahliae* pathotypes. *PLoS One*, *9*, e110664. https://doi.org/10.1371/journal.pone.0110664

Campbell, C.L. & Madden, L.V. (1990) *Introduction to plant disease epidemiology*. New York: John Wiley & Sons.

Chen, T. & Guestrin, C. (2016) XGBoost: a scalable tree boosting system. In: Krishnapuram, B., Shah, M., Smola, A. J., Aggarwal, C., Shen, D. … Rastogi, R. (Eds.) *Proceedings of the ACM SIGKDD international conference on knowledge discovery and data mining, 13–17 August*. pp. 785–794. New York, NY: Association for Computing Machinery. https://doi.org/10.1145/2939672.2939785

Farley, J.D., Wilhelm, S. & Snyder, W.C. (1971) Repeated germination and sporulation of microsclerotia of *Verticillium albo-atrum* in soil. *Phytopathology*, *61*, 260–264. https://doi.org/10.1094/Phyto-61-260

Fenu, G. & Malloci, F.M. (2021) Forecasting plant and crop disease: an explorative study on current algorithms. *Big Data and Cognitive Computing*, *5*, 2. https://doi.org/10.3390/bdcc5010002

Gómez-Gálvez, F. & Rodríguez-Jurado, D. (2018) Potential efficacy of soil-applied disinfectant treatments against Verticillium wilt of olive. *Crop Protection*, *106*, 190–200. https://doi.org/10.1016/j.cropro.2018.01.002

Harris, D.C. & Yang, J.R. (1996) The relationship between the amount of *Verticillium dahliae* in soil and the incidence of strawberry wilt as a basis for disease risk prediction. *Plant Pathology*, *45*, 106–114. https://doi.org/10.1046/j.1365-3059.1996.d01-96.x

Isaac, I., Fletcher, P. & Harrison, J.A.C. (1971) Quantitative isolation of *Verticillium* spp. from soil and moribund potato haulm. *Annals of Applied Biology*, *67*, 177–183. https://doi.org/10.1111/j.1744-7348.1971.tb02918.x

Jiménez Díaz, R.M., Trapero Casas, J.L., Boned, J., Landa, B. & Navas Cortés, J.A. (2009) Uso de Bioten para la protección biológica de plantones de olivo contra la Verticilosis causada por el patotipo defoliante de *Verticillium dahliae*. *Boletin Sanidad Vegetal Plagas*, *35*, 595–615.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., Jiménez-Gasco, M.M., Antoniou, P.P. & Tjamos, E.C. (2012) Verticillium wilt, a major threat to olive production: current status and future prospects for its management. *Plant Disease*, *96*, 304–329. https://doi.org/10.1094/PDIS-06-11-0496

Jiménez-Fernández, D., Trapero-Casas, J.L., Landa, B.B., Navas-Cortés, J.A., Bubici, G., Cirulli, M. et al (2016) Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. *Plant Pathology*, *65*, 1279–1291.

López-Escudero, F.J. & Blanco-López, M.A. (2005) Effects of drip irrigation on population of *Verticillium dahliae* in olive orchards. *Journal of Phytopathology*, *153*, 238–239. https://doi.org/10.1111/j.1439-0434.2005.00961.x

López-Escudero, F.J., del Río, C., Caballero, J.M. & Blanco-López, M.A. (2004) Evaluation of olive cultivars for resistance to *Verticillium* *dahliae*. *European Journal of Plant Pathology*, *110*, 79–85. https://doi.org/10.1023/B:EJPP.0000010150.08098.2d

López-Escudero, F.J. & Mercado-Blanco, J. (2011) Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant and Soil*, *344*, 1–50. https://doi.org/10.1007/s11104-010-0629-2

Maechler, M., Rousseeuw, P., Struyf, A., Hubert, M. & Hornik, K. (2019) *cluster: cluster analysis basics and extensions*. *R package version 2.1.0*. Available at: https://CRAN.R-project.org/package=cluster [Accessed 28th July 2021].

MAPA (2019) *Encuesta sobre superficies y rendimientos de cultivos*. Madrid: Ministerio de Agricultura, Pesca y Alimentación: Distribución General de Cultivos específicos. Available at: https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/esyrce/ [Accessed 18th November 2020].

Martos-Moreno, C., López-Escudero, F. & Blanco-López, M.A. (2006) Resistance of olive cultivars to defoliating pathotype of *Verticillium dahliae*. *HortScience*, *41*, 1313–1316.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D. & Jiménez-Díaz, R.M. (2003) Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiological and Molecular Plant Pathology*, *63*, 91–105. https://doi.org/10.1016/j.pmpp.2003.10.001

Montes-Osuna, N. & Mercado-Blanco, J. (2020) Verticillium wilt of olive and its control: what did we learn during the last decade? *Plants*, *9*, 1–31. https://doi.org/10.3390/plants9060735

Moraño-Moreno, R., Bejarano-Alcázar, J. & Rodríguez-Jurado, D. (2011) Grupos de virulencia de *Verticillium dahliae* sobre olivo presentes en las aguas subterráneas y superficiales utilizadas para el riego de olivares en Andalucía. *Communications of the 15th scientific-technical symposium expoliva, olive grove and the Environment Forum, OLI-08. 11–13 May 2011* (pp. 0–6). [CD-ROM, ISBN 978-84-938900-0-1]. Jaen: Fundación del Olivar.

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O. et al (2011) Scikit-learn: machine learning in python. *Journal of Machine Learning Research*, *12*, 2825–2830.

Pérez-Rodríguez, M., Alcántara, E., Amaro, M., Serrano, N., Lorite, I.J., Arquero, O. et al (2015) The influence of irrigation frequency on the onset and development of Verticillium wilt of olive. *Plant Disease*, *99*, 488–495. https://doi.org/10.1094/PDIS-06-14-0599-RE

Pérez-Rodríguez, M., Orgaz, F., Lorite, I.J. & López-Escudero, F.J. (2015) Effect of the irrigation dose on Verticillium wilt of olive. *Scientia Horticulturae*, *197*, 564–567. https://doi.org/10.1016/j.scienta.2015.10.016

Pérez-Rodríguez, M., Serrano, N., Arquero, O., Orgaz, F., Moral, J. & López-Escudero, F.J. (2016) The effect of short irrigation frequencies on the development of Verticillium wilt in the susceptible olive cultivar 'Picual' under field conditions. *Plant Disease*, *100*, 1880–1888. https://doi.org/10.1094/PDIS-09-15-1018-RE

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2021) *nlme: linear and nonlinear mixed effects models*. Available at: https://CRAN.R-project.org/package=nlme [Accessed 29th July 2021].

Ploetz, R.C., Schaffer, B., Vargas, A.I., Konkol, J., Salvatierra, J. & Wideman, R. (2015) Impact of Laurel wilt, caused by *Raffaelea lauricola*, on leaf gas exchange and xylem sap flow in avocado, *Persea americana*. *Phytopathology*, *105*, 433–440.

Rodríguez Jurado, D. (1993) *Interacciones huésped–parásito en la marchitez del olivo (*Olea europaea *L.) inducida por* Verticillium dahliae *Kleb.* Cordoba: University of Cordoba. PhD thesis.

Rodríguez-Jurado, D. & Bejarano-Alcázar, J. (2007) Dispersión de *Verticillium dahliae* en el agua utilizada para el riego de olivares en Andalucía. *Boletin Sanidad Vegetal*, *33*, 547–562.

Santos-Rufo, A., Hidalgo, J.J., Hidalgo, J.C., Vega, V. & Rodríguez-Jurado, D. (2018) Morphophysiological response of young olive trees to verticillium wilt under different surface drip irrigation regimes. *Plant Pathology*, *67*, 848–859. https://doi.org/10.1111/ppa.12788

Santos-Rufo, A., Vega, V., Hidalgo, J.C., Hidalgo, J.J. & Rodríguez-Jurado, D. (2017) Assessment of the effect of surface drip irrigation on *Verticillium dahliae* propagules differing in persistence in soil and on Verticillium wilt of olive. *Plant Pathology*, *66*, 1117–1127.

Schandry, N. (2017) A practical guide to visualization and statistical analysis of *R. solanacearum* infection data using R. *Frontiers in Plant Science*, *8*, 623. https://doi.org/10.3389/fpls.2017.00623

Therneau, T. 2020. *A package for survival analysis in R*. *R package version 3.2-3*. Available at: https://CRAN.R-project.org/package=survival [Accessed 28th July 2021].

How to cite this article: Santos-Rufo, A. & Rodríguez-Jurado, D. (2021) Unravelling the relationships among Verticillium wilt, irrigation, and susceptible and tolerant olive cultivars. *Plant Pathology*, 00, 1–16. https://doi.org/10.1111/ppa.13442