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Agricultural factors affecting Verticillium wilt in olive orchards in Spain

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Abstract In recent years, the spread of Verticillium wilt in olive orchards, caused by the soil-borne pathogen *Verticillium dahliae,* is often related to intensive modern farming of highly productive cultivars, planted at high densities, usually irrigated, and under a mechanised system. The effects of agricultural factors associated with olive orchards were investigated in an important olive-growing area in southern Spain, as tools in predicting outbreaks of the disease. A stratified double-sampling technique was designed to determine the number of olive orchards needed to survey. A sampling survey was conducted from 2002 to 2005 in 873 olive orchards randomly selected, the owners were interviewed for details of agronomic factors, and orchards were inspected for the presence or absence of the disease. Polymerase chain reaction assays were carried out for identifying *V. dahliae* pathotypes. Pathogen prevalence showed a significant linear correlation with the mean plant density (r^2= 0.93), associated predominantly with a less virulent non-defoliating pathotype (r^2=0.96). Overall, irrigation × high density caused disease incidence to peak in super-high-density olive-tree-planting systems. Olive orchards that had *V. dahliae,* however, did not differ in pathogen prevalence regardless of the olive cultivars. Young olive orchards were significantly more affected by *V. dahliae* than were old ones, particularly orchards with trees 8 to 12 years old. Irrigation increased pathogen prevalence and disease incidence in very young orchards (<7 years old). The prevalence of the non-defoliating pathotype was statistically high in young orchards whereas the prevalence of a highly virulent defoliating pathotype was high in old orchards.

Keywords Disease assessment · Irrigation · *Olea europaea* · Olive landscape

Abbreviations

ND	non-defoliating pathotype
D	defoliating pathotype
VW	Verticillium wilt

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Introduction

Spain, with 2,423,841 ha is the largest producer and exporter of olive and olive oil in the world (COI 2005). This crop is concentrated particularly in the region of Andalusia. In recent years, important new information, methodologies, and technologies have become available for olive-orchard management, bringing about a sustainable increase in olive production (Michelakis 2002).

Starting around the early 1980s, several researchers and farmers, primarily in Spain and Italy, developed irrigation systems for olive orchards and began to increase the plant density in order to reach full production more quickly. Hence, in the Mediterranean area it is possible to distinguish different types of olive production but with diffuse borders (Fontanazza 1986; Araque Jiménez et al. 2002). In general, traditional olive orchards follow old patterns of using the best-adapted cultivars, but under more intensive management, making systematic use of artificial fertilizers and pesticides as well as with more intensive weed control and soil management. Modern olive orchards use young and highly productive cultivars planted at high densities and managed under an intensive and highly mechanized system, usually with irrigation.

Therefore, new problems including Verticillium wilt (VW) have arisen in olive orchards. VW attacks have been increasing in frequency and severity in step with modernization. Currently, VW, caused by a soil-borne pathogen *Verticillium dahliae*, is considered a major cause of olive-crop damage worldwide (Jimenez-Díaz et al. 1998).

The management of VW is difficult and requires an integrated approach, involving the application of control measures before and after planting (Tjamos and Jiménez-Díaz 1998). Some of these include the choice of a planting site and planting material free of *V. dahliae* in addition to disinfestation of contaminated soil with fumigants, soil solarization which causes death of the pathogen by high temperatures, or the incorporation of organic amendments in soil which provide benefits such as improved plant health due to reduction of pathogens.

However, the available control measures have not proved satisfactory because *V. dahliae* can survive in the soil in the form of microsclerotia for long periods of time (Wilhelm 1955) and has a wide host range (Pegg and Brady 2002). Current chemical fungicides cause environmental damage and are not sufficiently effective. Additionally, control of VW is made more difficult because severity depends upon the virulence of the isolates of *V. dahliae* classified into two pathotypes: defoliating (D) and non-defoliating (ND), possessing high and low virulence, respectively (Schnathorst 1981; Rodríguez-Jurado 1993). Of particular concern is the spread of the D pathotype because it represents a major threat to olive trees. Alternative control measures to overcome VW in olive orchards are focused on the use of biological control agents (Mercado-Blanco et al. 2004) and the planting of resistant cultivars (López-Escudero et al. 2004). However, most olive cultivars evaluated are susceptible to *V. dahliae*, although all cultivars assayed were more susceptible to the D pathotype than to ND (López-Escudero et al. 2004).

Microsclerotia germinate in response to root exudates and infect a root by growth and movement of fungal mycelia through soil, and thus epidemics of soil-borne disease, such as VW, depend on the interplay between fungus and agricultural factors (Otten and Gilligan 2006). In this context, further understanding of the relationship between *V. dahliae* and the agricultural methods used in the orchards are needed for control measures and for predicting outbreaks of the disease. Indeed, factors such as soil amendments, crop rotation, plant density, and irrigation are important in the development of VW in many crops (Pegg and Brady 2002). However, there is limited literature on the effects of agricultural factors and VW in different areas of olive cultivation, and there is no information on *V. dahliae* pathotypes.

In the present study, results concerning the relationships between pathogen prevalence, disease incidence of VW, and agricultural factors in olive orchards are presented for different olive-growing areas, as useful tools in predicting outbreaks of the disease.

Materials and methods

Survey sample area

The sampling survey was carried out between 2002 and 2005 in Granada province, in the Andalusia region (southern Spain), a world leader in olive production (Fig. 1). The area is heterogeneous in terms of environmental conditions, crop management, and agricultural factors. The olive landscape and agronomics in Granada province are representative of Spanish olive growing today. Also, many studies regard irrigation as a cause for the spread of VW (Cirulli 1981; Blanco-López et al. 1984; Al-Ahmad and Mosli

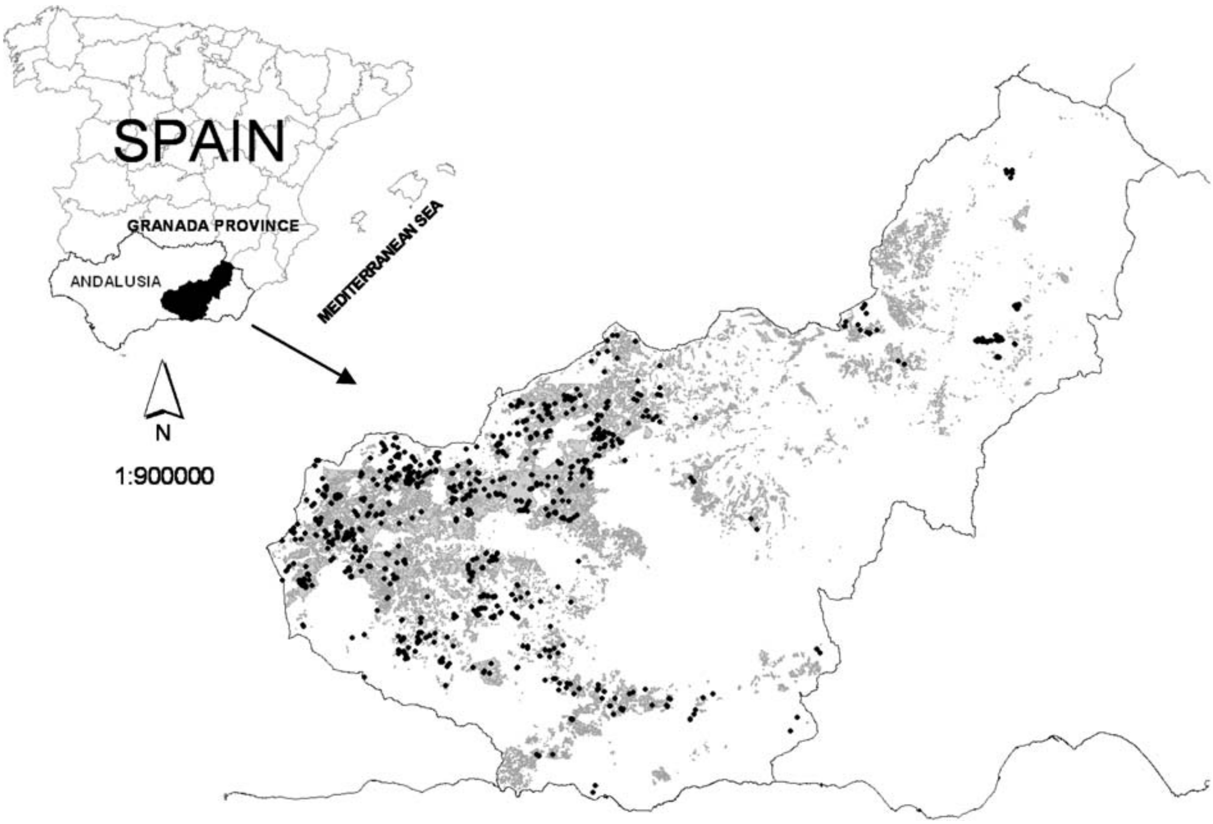


Fig. 1 Distribution of olive orchards in Granada (Andalusia, southern Spain) and regional distribution of randomly assigned olive orchards surveyed for Verticillium wilt. Each *dot* represents the centre point of each olive field

1993; Serrhini and Zeroual 1995; Rodríguez-Navarro 2006; Rodríguez et al. *unpublished data*). Therefore, because of the heterogeneity between areas and irrigation, a stratified double-sampling technique was designed to determine the number of olive orchards needed to make an accurate estimation of the disease. Surveys were conducted in 873 olive orchards, covering an area of 4,087.2 ha and 527,903 olive trees (Fig. 1). The margin of error was of under 3.3% and a confidence level of 95% to estimate proportions ($p=q=0.5$).

Epidemiological surveys

For the area surveyed, the number of olive trees and cultivars of each olive orchard were given by local government (Modelo de Explotaciones Olivareras, Unidad de Prospectiva, Consejería de Agricultura y Pesca, Junta de Andalucía). Thus, olive orchards to be sampled were selected and the owners interviewed. A standardized questionnaire was submitted at random to farmers, who were asked for details on farming factors, management, and symptomatology from each olive orchard. The central point of each olive orchard in this study was also pinpointed using the Ministry of Agriculture and Fisheries system (see http://w3.mapya.es/dinatierra_v3/ website). All the data supplied by the farmers interviewed and olive orchards georeferenced were transferred to a geographic information system (GIS) database. The GIS was suitable for mapping disease and analysing spatial patterns of the pathogen (Rodríguez-Navarro 2006; Rodríguez et al. *unpublished data*), and the GIS database was related and analysed in relation to a detailed land-use/land-cover and soil maps (Rodríguez-Navarro 2006; Rodríguez et al. *unpublished data*).

Isolation of *V. dahliae* from trees

Each spring and autumn, when evaluation of the presence of *V. dahliae* in plants is most appropriate (Levin et al. 2003), samples of shoots, branches, and stems were taken from olives trees showing wilt symptoms. Some six affected olive trees per olive orchard were assessed. Segments 0.5 cm long from different branches were surface-sterilized with NaClO (10%) for 1 min and rinsed with sterile water. Eight pieces were placed in water agar with chlortetracycline (30 mg l^{-1}), incubated at 25°C in the dark. Three Petri dishes per tree were assayed and they were checked every day during a month for the presence of *V. dahliae*.

Pathotype analysis

For DNA extraction the isolates were cultured on potato dextrose broth in 100 ml Erlenmeyer flasks stirred using an orbital shaker (120 rpm) for 7 days at 24°C in darkness. Mycelia were filtered, frozen at −20°C, lyophilised and ground according to Pérez-Artes et al. (2000). DNA was extracted from ground mycelia using the DNeasy Plant Kit (Qiagen, Hilden Germany). DNA concentration were determined using Nanodrop and by agarose-gel electrophoresis according to standard procedures. DNA solutions were stored at −20°C until used. All isolates of *V. dahliae* were characterized for D or ND pathotypes using the polymerase chain reaction technique described by Mercado-Blanco et al. (2003) using a three-primer set in a single reaction.

Statistics

For disease assessment, the terms 'pathogen prevalence' and 'disease incidence' (Nutter et al. 2006) were used. Pathogen prevalence measures the percentage of olive orchards where the pathogen is detected divided by the total number of olive orchards inspected. Disease incidence measures the percentage of olive trees where a disease (expressing symptoms) has been found to occur, divided by the total number of olive trees surveyed. Comparisons between groups were performed using chi-square and Student's *t*-Test. Welch's *t*-test was used to compare means from independent samples with unequal variances.

Results

Plant density

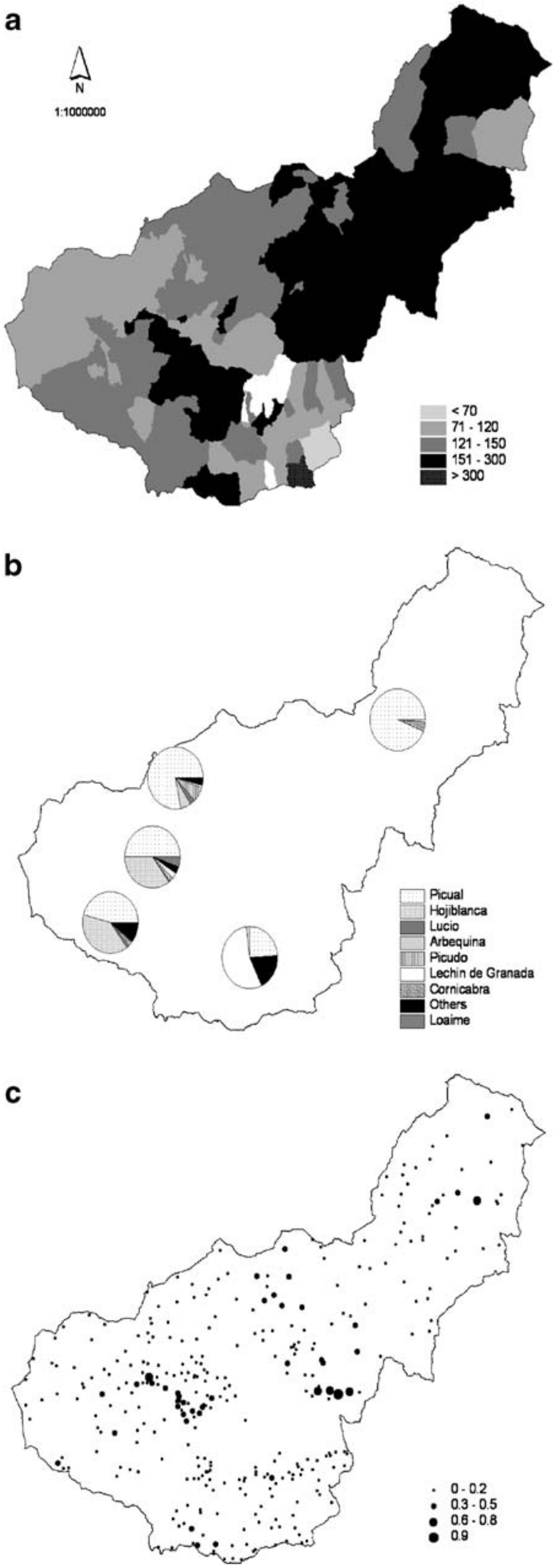
Planting rates varied according to production practices and management technologies (Fig. 2a). Low and moderate densities, (with <71–120 and 121–150 olive trees ha^{-1}, respectively) were typical in traditional olive groves, representing up 42% and 24%, respectively, and were more abundant in the north–west of the area surveyed (Fig. 2a). High densities, with 151–300 olive trees ha^{-1} or more, were distinctive of intensive farming, representing 34% of the total olive orchards surveyed, and were more abundant in east and south of the area (Fig. 2a).

Mean density in olive orchards affected by *V. dahliae* was significantly higher (d=164 olive trees ha^{-1}) than in pathogen-free orchards (d=135 olive trees ha^{-1}; t=4.40; $P<0.05$). Indeed, there was a significant linear correlation between pathogen prevalence and the mean of plant density in all categories ($r^2=0.93$; Fig. 3). Nevertheless, the association was due to ND pathotype data ($r^2=0.96$) since prevalence of D isolates remained unchanged ($r^2=0.28$; $P=0.32$; Fig. 3).

Irrigated olive orchards had higher densities (d=175 olive trees ha^{-1}), than non-irrigated orchards (d=124 olive ha^{-1}; t=2.55 $P<0.05$ Welch *t*-test). Pathogen prevalence appeared to increase (non-significant correlations) with density rates in both systems: irrigated ($r^2=0.82$; $P=0.07$) and non-irrigated olive orchards ($r^2=0.66$; $P=0.11$; Fig. 4a). However, in non-irrigated olive orchards, the incidence of VW decreased with plant density ($r^2=0.96$) but increased exponentially ($r^2=0.98$) in irrigated orchards (Fig. 4b) Hence, irrigation × high density have contributed to the peak of VW incidence registered in super-high-density planting olive systems (which have up to 300 olive trees ha^{-1}) affected by the ND pathotype (Fig. 4b).

Olive cultivars

The main cultivars sampled in Granada were 'Picual' (65%), 'Hojiblanca' (18%), 'Lechín de Granada' (4%), 'Picudo' (4%), 'Lucio' (2%), 'Arbequina' (1%), and other cultivars (6%; Others). Wide variation was found among areas in the composition of cultivars (Fig. 2b), but there were no significant differences in pathogen prevalence among cultivars ($\chi^2=6.51$; $P>0.05$; Fig. 5). Picual, Others, Arbequina, and Picudo registered similar values of pathogen prevalence, ranging from 16% to 11%, whereas Hojiblanca and Lechín de Granada had the lowest levels of pathogen prevalence, with 9% and 8% of the orchards, respectively. No olive orchards of the Lucio



◀ **Fig. 2** Plant-density map (**a**), cultivar map (**b**), and tree-age map (**c**) for olive orchards in Granada (southern Spain), showing the percentage of young olive trees planted before 1 Nov 1995, divided by the total number of olive trees

cultivar were affected by *V. dahliae* (Fig. 5). In all cultivars except Picudo and the mixed group Others, the ND pathotype was more prevalent than the D pathotype (Fig. 5). On the other hand, the composition of olive cultivars depended significantly on the age of the orchards (χ^2=84.731; P<0.05). For instance, Picual, Hojiblanca, and Arbequina were preferentially planted in intensive orchards whereas Picudo, Lechín de Granada, Lucio, and Others typified traditional orchards.

Age of orchard

Olive orchards over 40 years old dominated the area surveyed (35%), followed by different age ranges, including 13–40 years (20%), 8–12 years (14%), 0–7 years (11%). Figure 2c shows the percentage of young olive trees planted before 1 Nov 1995, divided by the total number of olive trees. In some cases, traditional orchards have been interplanted with young olive trees in order to double the tree density. This category, called 'double orchards', represents about 20% of the total orchards surveyed. The results show that young orchards were significantly more affected by VW than were old ones (χ^2=105.42; P<0.05). Pathogen prevalence was ranked as follows: 8–12 years > 0–7 years > 13–40 years > double orchards > (>40 years; Fig. 6a). The pathotypes and plant age (χ^2=14.258; P<0.05) were found to be statistically related, indicating that isolates ND were more prevalent than D in orchards having young olive trees, while isolates D were more prevalent than isolates ND in old orchards (>40 years old; Fig. 6b).

In the sampling survey, 50% of the young olive orchards were irrigated whereas old olive orchards had a lower percentage of irrigation (27%). The prevalence of *V. dahliae* was consistently greater in irrigated olive orchards regardless of the range of orchard age (Fig. 7). Overall, irrigation increased the prevalence of the pathogen and disease incidence in orchards in the first 7 years (Fig. 7).

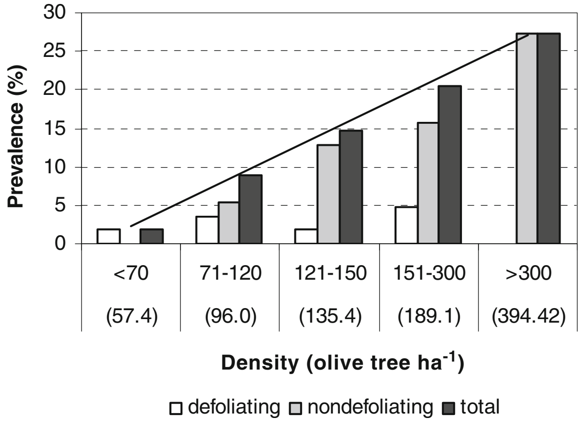


Fig. 3 Prevalence of *V. dahliae* and pathotypes at different planting densities in olive orchards. In parentheses, the mean of plant density in each category

Discussion

Plant density

Pathogen prevalence was significantly affected by the plant density. An increase in olive trees ha^{-1} could encourage several roots belonging to different olive trees to explore simultaneously the same volume of soil infected with microsclerotia, thereby boosting prevalence. In this sense, the transmission of soil-borne pathogens is often related to plant density; specifically, the proportion of plants infected by soil-borne fungi is often greater for high plant densities (Long and Cooke 1969; Burdon and Chilvers 1975; Augspurger and Kelly 1984).

The significant linear correlation between pathogen prevalence and plant density was associated predominantly with the less virulent non-defoliating (ND) pathotype. In previous results (Rodríguez-Navarro 2006; Rodríguez et al. *unpublished data*), an association between isolates D and non-irrigated olive orchards and between isolates ND with irrigated ones was found, implying that wet conditions were more favourable for ND development. For this reason, in non-irrigated olive orchards the incidence of VW by plant density could decrease but rises exponentially in irrigated fields. In the field, root systems develop in response to both endogenous plant design and soil environment. In irrigated olive orchards, wet soil is more extensive and therefore root distribution expands to a larger soil volume (Fernández et al. 2004), enhancing the probability of interactions between olive roots and infective propagules (microsclerotia) in soil.

Irrigation and high plant density also significantly affected incidence VW, causing an outbreak of this disease in very densely planted fields (>300 trees ha^{-1}). For intensive cultivation, trees are planted densely, reducing light penetration while increasing areas with low air circulation and high humidity. High relative humidity in olive orchards tends to favour fungal diseases such as the black scale (*Saissetia oleae*), leaf spot (*Spilocaea oleagina*; Trapero and Blanco 2001), and *Colletotrichum* (Sánchez et al. 2004). For this reason, the density of the olive-tree canopy and microclimatic conditions beneath are related to cultural practices such as tree density and irrigation. Therefore, it could also strongly 'influence the scale of disease development' scale development of the disease.

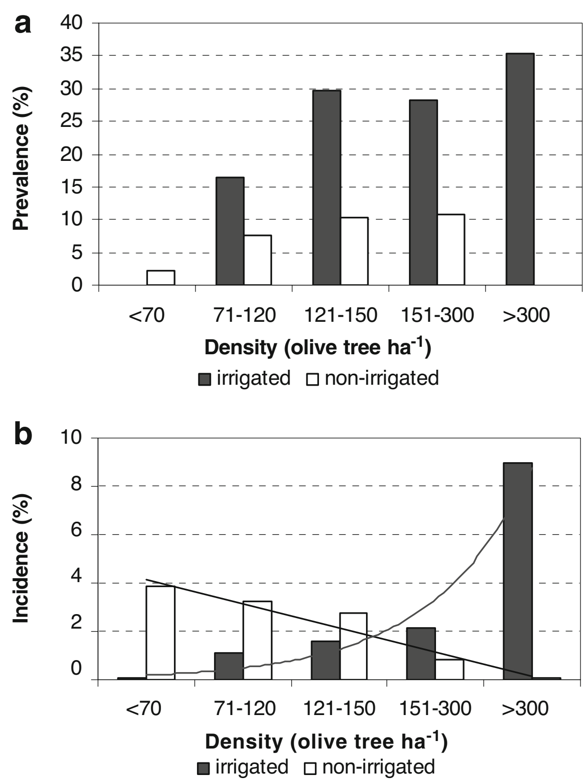


Fig. 4 Pathogen prevalence (**a**) and disease incidence (**b**) of VW dependent on plant density in irrigated and non-irrigated olive orchards

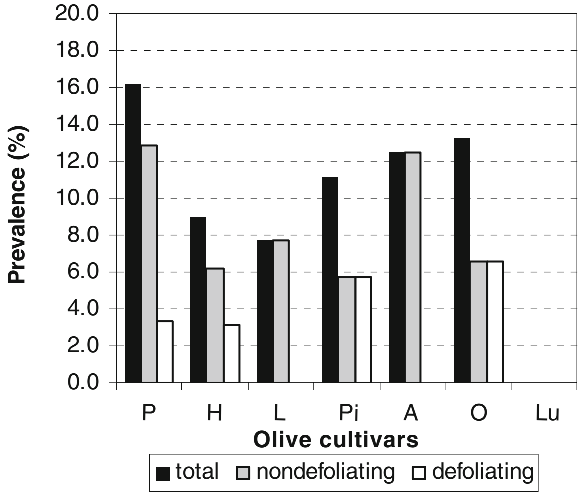


Fig. 5 Pathogen prevalence of olive cultivars (*P*, Picual; *H*, Hojiblanca; *L*, Lechín de Granada; *Pi*, Picudo; *A*, Arbequina; *O*, Others; *Lu*, Lucio) affected by *V. dahliae*

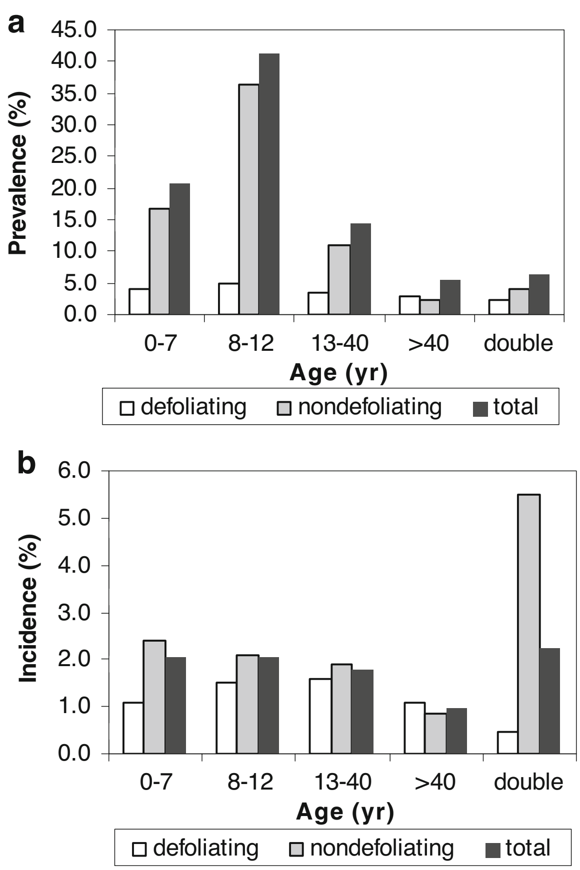


Fig. 6 Pathogen prevalence (**a**) and disease incidence (**b**) of *V. dahliae* and pathotypes affecting different plant ages

Olive cultivars

The evaluation of cultivar resistance to *V. dahliae* is key in disease management. Resistant cultivars could be used for replanting dead olive trees, as rootstocks or as sources for resistance in breeding programmes. In the present work all the cultivars in the olive-growing area, except 'Lucio', were affected by *V. dahliae.* Therefore, these results complement other experiments under controlled conditions with Spanish cultivars in which most of the olive cultivars evaluated were susceptible to both pathotypes of *V. dahliae* (López-Escudero et al. 2004). This epidemiological result of cv. Lucio makes it an appropriate candidate to be used as the rootstock in soils infested with *V. dahliae*. Nevertheless, Lucio variety is typical in traditional olive fields and is not considered among the best cultivars for new high-density olive-production systems, so that more work is required before this rootstock could be recommended for commercial planting in soil infested with *V. dahliae*.

Although studies on the resistance to VW in olive (Ciccarese et al. 2002; López-Escudero et al. 2004) established different levels of susceptibility between olive cultivars, in this study no significant differences in pathogen prevalence among cultivars were found. Therefore these results suggest that several factors are more important than olive cultivar for disease development: irrigation, plant density, plant age, and other factors more difficult to quantify, such as olive

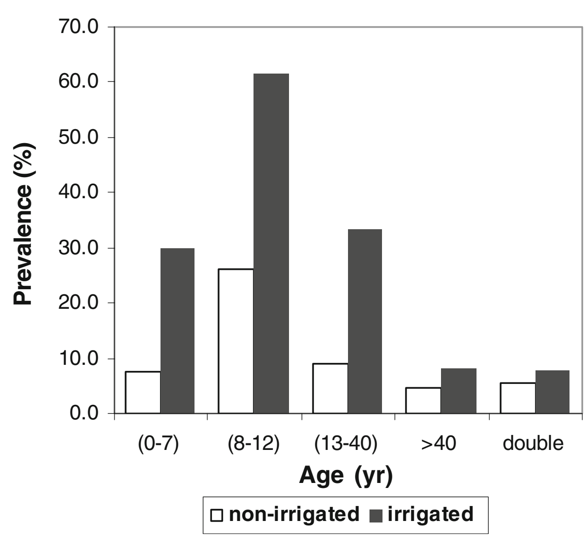


Fig. 7 Pathogen prevalence affecting different ages of olive trees in irrigated and non-irrigated orchards

management and environmental conditions. Indeed, olive cultivars and plant age were found to be significantly associated, so that the parameter olive cultivar probably acted together with other variables such as irrigation and high density, given that some cultivars such as Picual or Arbequina were best for super-high-density systems.

Age of orchard

VW was uncommon in older olive trees and the disease was statistically higher in young orchards, those of 8 to 12 years old being the most susceptible. Also, VW increased in double orchards. Generally, studies indicate that young olive orchards are most at risk (Wilhelm and Taylor 1965; Al-Ahmad and Mosli 1993; Blanco-López et al. 1984; Serrihini and Zeroual 1995). Higher prevalence of VW in young trees is probably linked to the establishment of new plantings in soils having had crops that were highly susceptible to the disease (Cirulli 1981; Tjamos 1993; Blanco-López and Jiménez-Díaz 1995; Serrihini and Zeroual 1995; Nasser and Al-Raddab 1998). The increase in VW recorded in double orchards, where soils are apparently pathogen free indicates the possible use of newly infected planting material and the risk of nurseries in spreading the disease in young orchards.

Irrigation would provide better and quicker conditions for *V. dahliae* development, since throughout the olive growing area surveyed, irrigation increased pathogen prevalence and disease incidence of *V. dahliae* in very young orchards (<7 years old), with the subsequent early expression of symptoms. Nevertheless, although irrigation encourages wilt in olive trees, the incidence of VW was very low in old traditional, irrigated olive orchards of >40 years old. Presumably, traditional old olive agroecosystems have higher numbers and a broader diversity of soil microorganisms, providing a more complex community structure, than intensive young orchards. Indeed, reductions in biodiversity increase ecosystem vulnerability, favouring the spread of plant fungal diseases (Kops et al. 1999).

On the other hand, higher occurrences of the highly virulent pathotype infecting old olive trees in the area surveyed was unexpected. Normally, old traditional orchards are located in dryland-farming areas. Significant differences in the disease levels caused by both pathotypes were found, depending on the type of irrigation used. Previous results (Rodríguez-Navarro 2006; Rodríguez et al. *unpublished data*) show that D isolates were significantly more common in fields using only natural rainfall.

In conclusion, the study demonstrates that *V. dahliae* is critically affected by agricultural factors. Of the three factors—plant density, orchard age, and olive cultivars—only olive cultivars did not affect the occurrence of the disease in the olive-growing area. VW was found in high-density olive and in young orchards. The ND pathotype is particularly affected by high density. The incidence of the disease increase exponentially in irrigated olive orchards. Compared to non-irrigation in olive orchards, irrigation gives rise to more destructive VW during the first 7 years after trees are planted. High density plants and young trees, combined with irrigation, resulted in an outbreak of the disease, implying that irrigation has an important influence on other agricultural factors.

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