The increased presence of Verticillium wilt (Verticillium dahliae Kleb.) in olive groves is often related to the use of infected propagation material and to the planting of new olive trees in contaminated soils. This study assessed the implications of plant propagation, land-use history and soil properties on disease prevalence in southern Spain, the most important olive-growing area worldwide. To this purpose, a large-scale sampling survey was carried out in this area, V. dahliae pathotypes were identified by PCR, and GIS was used to analyze soil properties and cropland-use history. Finally, multiple correspondence analysis was performed to show the statistical association between the variables taken into account. Results strongly indicated the potential risk of planting olive in valleys with irrigated cropland history, especially those that had hosted herbaceous crops, highlighted the importance of using pathogen-free certified planting material as a key component for a successful disease management, and confirmed the role played by saline, alkaline, and steep-slope soils in enhancing V. dahliae prevalence.

Key words: defoliating Verticillium pathotypes, land-use, nondefoliating pathotypes, Olea europaea, soil, disease management.

INTRODUCTION

Verticillium wilt (VW), caused by the soil-borne fungus Verticillium dahliae Kleb., is a disease affecting a wide variety of host plants (Harris, 1998), and is considered the most important olive disease worldwide (Jiménez-Díaz et al., 1998).

Soils pathogen-contaminated by previous crops or inter-cropped with susceptible hosts are a major factor for V. dahliae occurrence (Cirulli, 1981; Tjamos, 1993; Blanco-López and Jiménez-Díaz, 1995; Serrhini and Zeroual, 1995; Naser and Al-Raddab, 1998). In these soils the pathogen can survive for long time (in excess of 10 years) thanks to the microsclerotia produced in dying tissues of the host plant, which represent the main inoculum source for VW development (Wilhelm, 1955; Schnathorst, 1981). Therefore, the knowledge of land-use history can help estimating the risk of disease outbreaks in any given plot to be planted with olive. Since the use of infested plant propagation material determines the appearance of VW in pathogen-free soils, nurseries may have been instrumental in spreading accidentally the highly virulent V. dahliae pathotype (Jiménez-Díaz et al., 1998; Nigro et al., 2003).

According to their virulence to cotton and olive, V. dahliae isolates can be classified as highly virulent defoliating (D) or non-defoliating (ND) pathotypes, depending on their ability or not to induce leaf shedding (Schnathorst and Sibbet, 1971; Schnathorst, 1973). The D pathotype was first reported in south-western Spain in an area of intensive cotton cropping (Bejarano-Alcázar et al., 2001), and in the last 5-10 years has spread to distant pathogen-free olive-growing areas in southeastern Spain. Currently, spreading of the D pathotype is a very serious threat to olive crops since no resistant cultivars are available, and control measures are not efficient (Bejarano-Alcázar et al., 1996; López-Escudero and Blanco-López, 2001; Navas-Cortés et al., 2001; Rodríguez et al., 2009). Knowing the origin of plant propagation material would allow a better understanding of the spread and spatial patterns of the highly virulent pathotype for improved management and protection programs of olive groves.

Soil properties have a pronounced effect on disease spread and development. Tenuta and Lazarovits (2004) found that effectiveness of nitrogenous amendments (meat and bone meal) is related to soil properties such as organic carbon content and pH. Addition of meat and bone meal to a sandy soil killed microsclerotia but had no effect in loamy soil. Overall, VW is more of a problem in neutral or alkaline soils, rather than acid soils (Pegg and Brady, 2002), but there is no information on a soil-specific effect on VW in olive groves. Such knowledge would be vital to identify the factors
involved in wilt development and to predict which soils facilitate VW occurrence.

In this study, a large-scale sampling survey was carried out to assess the implications of the origin of plant propagation materials, land-use history and soil properties for VW prevalence in olive groves. GIS was employed to identify agricultural land-use history and soil properties impacting on disease risk. PCR assays were used to identify \textit{V. dahliae} pathotypes and multiple correspondence analysis was done to analyze the patterns of relationships of categorical variables with pathogen prevalence.

**MATERIAL AND METHODS**

**Study area.** The study was carried out in Granada province (Andalusia, south-east Spain) where olive is the most extensive crop, covering a surface of 175,000 ha (Fig. 1). Olive is characterized by a high degree of heterogeneity, production regions differing greatly from one another for physical characteristics of the groves, management practices, socioeconomic situations and environmental factors (Rodríguez et al., 2008, 2009).

Many studies blame irrigation as a cause of VW spread (Cirulli, 1981; Blanco-López et al., 1984; Al-Ahmad and Mosli, 1993; Serrhini and Zeroual, 1995; Rodríguez et al., 2009). Due to the heterogeneity of different olive regions and use of irrigation, we designed a stratified double-sampling technique to determine the number of olive groves needed to make an accurate estimation of the disease (Rodríguez et al., 2009). Surveys were conducted in 873 olive groves, covering an area of 4,087.2 ha and comprising 527,903 olive trees with a standard error ≤ 3.3% (95% confidence interval).

From 2002 to 2005 a questionnaire was distributed to randomly chosen farmers with queries on the agronomic features (Rodríguez et al., 2008), origin of plant propagation materials, previous cropping history and symptoms present in their plantations. To reduce bias from farmer’s subjectivity, all groves with VW symptoms were visited directly to validate survey results.

**Isolation of \textit{V. dahliae} from olive trees.** Samples were collected from March to June and from September to November, the most appropriate time for detecting \textit{V. dahliae} presence within plants (Levin et al., 2003). Olive trees with wilt symptoms were inspected, counted and sampled. Branches and stems from at least 10 affected trees were collected, and analysed in the laboratory. Twenty-four small internal fragments (~1 cm$^2$) of vascular tissue from different affected branches of each tree were surface-sterilized with NaClO (10%) for 1 min, rinsed with sterile water for 1 min, and placed in Petri plates in water agar with chlorotetracycline (30 mg/l). Fungal colonies were periodically examined for \textit{V. dahliae} identification (Rodríguez et al., 2009).

**Characterization of \textit{V. dahliae} pathotypes.** Fungal isolates were PCR-assayed using specific primers for \textit{V. dahliae} pathotype identification. For DNA extraction, isolates were cultured on Potato Dextrose Broth (PDB) in 100 ml Erlenmeyer flasks, agitated on an orbital shaker (120 rpm) for 7 days at 24°C in the dark. Mycelia mats were harvested by filtration, frozen at -20°C, lyophilized and ground as in Pérez Artes et al. (2000). DNA was extracted from ground mycelia using the DNeasy Plant Kit (Qiagen, Germany) and its concentration was determined using Nanodrop and by agarose gel electrophoresis, according to standard procedures. DNA preparations were stored at -20°C for future use. Primers DB19 and DB22 amplify \textit{V. dahliae}-specific products 523 or 539 bp in size (Carder et al., 1994). \textit{V. dahliae} population was screened for D or ND pathotypes using the primers DB19/DB22/espedf01, which, in a single-reaction, yield one of the 523 or 539 bp amplicons together with an additional amplicon of 334 bp in the case of D isolates (Mercado Blanco et al., 2003).

**GIS and data analysis.** The very center of each olive orchard was identified using the “Sistema Integrado de Gestión” (SIG) (integrated olive-management system) of the Spanish Ministry of Agriculture and Fisheries (http://w3.mapya.es/dinatierra_v3/). All information from questionnaires and georeferenced olive groves were transferred to a GIS database. ArcGIS (version 9.1) was used to display data and analyze the correlation between prevalence of \textit{V. dahliae} and agricultural properties (i.e. soils and land-use history). The layers used were the 1:400 000 soil map of Andalusia (IARA-CSIC, 1998) to analyze soil properties and 1995-96 land-crop/land-use map (Consejería de Agricultura y Pesca, Junta de Andalucía, Province of Granada) to analyze land-use history. The 1:400 000 soil map of Andalusia consists of 64 cartographic units (c.u). Four basic variables are included on the soil map. The soils themselves are divided into major groups and further subdivided to give individual soil units. The map also specifies categories of texture and slope. Finally, information is provided on different soil phases, such as stoniness and salinity, i.e. land attributes that are particularly important to land management. The map is composed of soil associations, each of which may include up to eight soil units. In each association, the dominant soil is indicated by a capital letter and a lower case letter; associated soils occupy at least 20% and inclusions at least 10% percent of the area (FAO, 1974; European soil map, Commission of the European Communities, 1985).

**Statistics.** “Pathogen prevalence”, the term used for disease assessment, describes the proportion (or percentage) of olive groves where the pathogen was detect-
Main characteristic of the cartographic units (FAO 1974) taxonomy:

42: Bk (Re Jc Lk). Medium and fine-textured material. Terraces, undulating or hilly terrain. Calcaric cambisols with moderate to deep soil profiles are very productive soils and intensively used. On steep slope are covered with almond and olive.

44: Bk Rc I (E). A weakly developed soil found on unconsolidated materials. They are typical of the mountainous regions. High percentage of limestone. Land use vary widely. Suitable for olive and dry land farming.

13: Rc Bk (I Jc E). Hilly landscape with white and loamy soils called “albarizas” that provide an excellent soils for vineyards, olive, sunflower and cereal. High percentage of limestone depleted of N and organic matter.

14: Rc Bk (Le Jc). Hilly terrain with unconsolidated material. Young and poorly developed soils with continuous threat of erosion. Vegetation includes Retama sp., shrubs, olive and cereal.

19: I Lc E (Bk). Steep slope (more than 30%) with shallow and stony soils. Limited potential for tree crop production. Only suitable for forest and livestock.

58: Lk Bk Le (Re). Luvisols calcic are a clayey fertile soils suitable for a wide range of agricultural uses. These soils are commonly used for cereals and for sugar beet while the upper slopes are best suited for fruit trees, vineyards, olives and grazing.

2: Jc. Clay, deep and rich soils, with great agricultural capacity located in valleys (<2% slope). Soils particularly suitable for intensive irrigated crops.

41: Bk (Re). Strongly sloping and loamy soils with high salt concentrations in solum. Xerophytic and halophytic vegetation.

15: 1 Re Lc (Be). Various kinds of rock and unconsolidated materials over shallow soils. Stony and steep slope (>25% slope) only suitable for livestock and forest.

32: Be Re Lc (I). Mountainous terrain with loamy-sandy soils. Soils with steep slope and with low water-holding capacity. Xerophytic vegetation, forest, vineyards, olive and fig.

47: Bk Lk Lc (I Jc). Hilly soils in which amount of calcium carbonate are found. Soils with a strong summer drought. The land use of these soils is highly variable including orange tree, olive, horticultural crops and cereals.

48: Bv Re Vc (Bk). Deep, rich and dark clay soils called “bueo” that are excellent for dry land farming.

40: Bk (Rc). Loamy soils on nearly level to undulating terrain with gypsum concentrations. Land-use include Quercus coccifera, Rhamnus lycioides, Ephedra fragillis and dry land farming.

† Types of soils were characterized by cartographic units (c.u.). Source: 1:400 000 soil map of Andalusia (IARA-CSIC, 1998).

Fig. 1. Percentage of olive groves surveyed and *V. dahliae* prevalence related to types of soils. Chi-square test was performed $\chi^2=30.883$ at the 0.05 probability level.
ed, divided by the total number of olive groves that were inspected (Nutter et al., 2006). Chi-square tests were used to assess the statistical significance of associations between categories. Statistical analysis excluded soil type with less than five observations. Multiple correspondence analyses (MCA) was used to analyze associations among qualitative variables and their influence on V. dahliae prevalence in olive groves (Savary et al., 1995). MCA is an extension of correspondence analysis which allows one to analyze on the same graph the pattern of statistical relationships of several categorical dependent variables (Abdi and Valentine, 2007). In this study, the nominal variables and levels considered were: (i) origin of propagation material; (ii) land-use history and (iii) cartographic units. Prevalence of the pathogen was described as one of the two unique categorical variables: high risk or low risk.

The interpretation in MCA is often based upon proximity between points in a low-dimensional map (i.e., two or three dimensions). Proximity was meaningful only between points from the same set (i.e., rows with rows, columns with columns). When two row points were close to each other they tended to select the same level of nominal variables. For the proximity between variables, two cases were needed for discrimination. First, the proximity between levels of different nominal variables meant that these levels tended to appear together in the observations. Second, because the levels of the same nominal variable could not occur together, a different type of interpretation was needed in this case. Here the proximity between levels meant that the groups of observations associated with these two levels were themselves similar (Savary et al., 1995; Abdi and Valentine, 2007).

**RESULTS**

Implications of plant propagation material. The most traditional technique of olive tree reproduction in Spain is by cuttings from the annual pruning which are planted directly in the field. The majority of the olive groves surveyed (52.7%) were established in this way. Development of modern olive growing has increased the establishment of intensive olive groves with plants from nurseries. In fact, 30% of the groves surveyed originated from nursery productions. Conversion of traditional olive groves into more productive plantations requires inter-planting with young trees from nurseries so as to double tree density. This type of orchards represented about 5.3% of the total. Finally, 12% of the farmers interviewed did not know the origin of their trees.

Olive groves planted with nursery material registered the highest percentage of V. dahliae prevalence (24.5%), followed by groves with double density (17.4%) and those with unknown tree origin (13.2%). Conversely, groves planted with cuttings from old trees had the lowest V. dahliae prevalence since infection rate was only 8.5% (|2= 105.42; p< 0.05) (Table 1). However, no significant differences were detected between the prevalence of V. dahliae pathotypes in relation to the differ-

<table>
<thead>
<tr>
<th>Land use history</th>
<th>Prevalence V. dahliae</th>
<th>Surface surveyed (ha)</th>
<th>Prevalence of pathotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agricultural land</td>
<td></td>
<td></td>
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<tr>
<td>Irrigated cropland</td>
<td></td>
<td></td>
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<tr>
<td>Orchards and mixed crops</td>
<td>27.1</td>
<td>60</td>
<td>91</td>
</tr>
<tr>
<td>Herbaceous crops</td>
<td>56.4</td>
<td>212</td>
<td>86</td>
</tr>
<tr>
<td>Non irrigated cropland</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Orchards and crops</td>
<td>10.6</td>
<td>887</td>
<td>73</td>
</tr>
<tr>
<td>Olive groves</td>
<td>7.5</td>
<td>1915</td>
<td>54</td>
</tr>
<tr>
<td>Herbaceous crops</td>
<td>15.6</td>
<td>611</td>
<td>70</td>
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<tr>
<td>Heterogeneous agricultural land</td>
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<tr>
<td>Irrigated and non-irrigated mixed cropland</td>
<td>33.3</td>
<td>161</td>
<td>100</td>
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<tr>
<td>Mixed crops with natural vegetation</td>
<td>4.2</td>
<td>51</td>
<td>100</td>
</tr>
<tr>
<td>Irrigated surfaces</td>
<td>8.3</td>
<td>101</td>
<td>100</td>
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<tr>
<td>Forest land</td>
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<tr>
<td>Shrub land</td>
<td>0.0</td>
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<td>-</td>
</tr>
<tr>
<td>Pasture and shrubland with or without oak woodland</td>
<td>30.0</td>
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<td>100</td>
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<tr>
<td>Natural areas with scarce vegetation</td>
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<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Mixed forest land</td>
<td>0.0</td>
<td>14</td>
<td>-</td>
</tr>
</tbody>
</table>

† Source: 1995-96 land-use digital maps. Province of Granada. Consejería de Agricultura y Pesca, Junta de Andalucía
ent origin of plant propagation material (t2= 6.51 p> 0.05). In all cases, prevalence was around 76% for the ND pathotype and around 24% for the D pathotype.

Implications of land-use. *V. dahliae* prevalence in olive groves varied according to previous land-use. Prevalence was higher in olive groves established in lands classified as irrigated herbaceous crops (56.4%), followed by irrigated and non-irrigated mixed cropland (33.3%) (Table 1). Questionnaires indicated that the top 10 crops grown in the irrigated herbaceous category were potato, beet, bean, pepper, tomato, garlic, tobacco, alfalfa, maize and asparagus. Likewise, sunflower, vegetables, cereals, and legumes (chick-pea, lentil and bean) accounted for the largest crops in the irrigated and non-irrigated mixed cropland category. Typically, olive groves established in certain forest land had the lowest *V. dahliae* incidence (Table 1).

Prevalence of the ND pathotype appeared to increase with irrigated land-use, whereas the prevalence of the D pathotype was higher in olive groves established in non irrigated than irrigated cropland (Table 1). Although areas classified as olive groves made up most of the surface surveyed, the prevalence of the disease was low (7.5%). An unexpectedly high prevalence of pathotype D was registered in olive grove lands (Table 1).

Implications of soil properties. Olive groves surveyed were mainly located in c.u. 42, 44 and 13, followed by 14 and 19 (Fig. 1). The five c.u. (42, 44, 13, 14, 19) have similar soils and climatic patterns. The most important soils in c.u. 42 were calcic cambisols. C.u. 42 is usually located on hilly to mountainous terrain with high potential for cereals, almond and olive, but with strong limitations due to summer drought. Calcic cambisols and calcic regosols, the typical soils in c.u. 44, are suitable for non irrigated crops, olive and ilex. Their main limitations are summer drought and the high percentage of active limestone. Calcic regosols, the most abundant soils in c.u. 13, are fairly poor in organic matter (less than 2%) and have a calcium carbonate content ranging from 30% to 70%. Regosol/cambisol is the association mainly found in unit 14. These soils are typical of mountainous regions. Regosols are shallow soils found on loosely arranged materials but when deep soil occurs, cambisols support scrubs, *Retama* spp. and can be used for dry land farming. The continuous threat of erosion is the main limitation of this c.u. The most important soils in c.u.19 are leptosols, i.e. shallow soils lying directly over a calcareous bank, or soils having less than 20% (by volume) fine earth material, or extremely stony deeper soils. Slope are often more than 30%.

VW was significantly more prevalent in olive groves located in c.u. 2, 41, and 15, with pathogen prevalence of 39% for c.u. 2, 29% for c.u. 41 and 25% for c.u. (t2=30.883; p< 0.05) (Fig. 1). C.u. 2 included eutric fluvisols, i.e. deep soils derived from recent alluvial sediments, slightly basic at the surface (pH nearly 9) and having a sandy-clay to clayey-loam constitution. C.u. 2 was located in fertile valleys of Andalusia on flat terrain.
around 400 m a.s.l. and with a slope of less than 2%. The cover and land-use is calcicole plants, mainly grass. Natural vegetation was scarce due to intensive cropping. C.u 15 included thin rocky soils located on mountain sides with steep slopes (more than 25%). Leptosols predominated in craggy and highly eroded areas whereas eutric regosols with chronic luvosols prevailed in levelled areas. Those soils were distributed from 200 to 1600 m a.s.l. The principal land-use was forest (Quercus rotundifolia, Retama sphaerocarpa) as well as extensive livestock rearing. Calcic cambisols, the dominant soils in c.u. 41, are located from 750 to 900 m a.s.l. and are characterized by a loamy texture and high salt concentrations. Their natural vegetation consists of saline plants and xerophytes typical of a semi-arid climate. The principal land-use is low-productive dry land farming as well as extensive livestock rearing.

**Multiple correspondence analysis (MCA).** Relationship among the origin of plant propagation material, land-use history, c.u. and prevalence of VW risk in olive groves is shown in Fig. 3. There were two clusters of risk prevalence, i.e. high risk in the left half, and low risk in the right half. High risk prevalence was graphically associated with propagation material from nurseries (intensive olive groves), irrigated herbaceous crops as previous land-use and soil types corresponding to c.u. 2, 14, 44. Low risk was associated with propagation material from cuttings, and with olive groves with double density (called in the graph “double”). Furthermore, low risk were associated with forest, non-irrigated cropland as previous land-use and with soil types corresponding to c.u. 42, 13 and the rest of the soil units grouped and called “rest”. This indicates that c.u. 2, nurseries and irrigated cropland are strongly correlated with *V. dahliae* prevalence in olive groves (r = + 0.734), and negatively correlated with the rest of the variables (r = - 0.734).

**DISCUSSION**

**Implications of plant propagation.** There was a statistical relationship between VW and intensive olive cropping, consequent to the origin of plants from nurseries, whereas olive groves established with the traditional propagataion system had the lowest level of infection. In previous reports, *V. dahliae* spreading via greenhouse and nursery stock had been reported from Greece (Thanassoulopoulos, 1993) and Italy (Nigro et al., 2003). In our case, traditional olive groves that doubled the density by inter-planting old with young trees from nurseries, had an intermediate level of disease. Therefore, these “mixed olive groves” disclosed the role played by nurseries in *V. dahliae* dissemination, with serious implications for the spread of the highly virulent pathotype.

In Spain, the D pathotype was restricted to cotton crops in the south-west in the mid-1980s (Bejarano-Alcázar et al., 2001) but now it is well established in the olive area surveyed in the south-east of the country (Rodríguez et al., 2009). As fungal isolates from cotton and olive show cross-virulence in olive (Schnathorst and Sibbett, 1971; Schnathorst, 1973), the spread of pathotype D in olive groves has been related with previous cotton growing in areas where new olive groves have been established (Collins et al., 2005). However, cotton was not grown in the area we have surveyed or in surrounding fields, suggesting that the introduction of D pathotype could have occurred through infected planting stocks. Thus, nurseries had a central role in D pathotype spreading in pathogen-free areas of eastern Spain.

**Implications of agricultural land-use history.** The spread of pathotype D in olive groves has been related with previous cotton growing in areas where new olive groves have been established (Collins et al., 2005). However, cotton was not grown in the area we have surveyed or in surrounding fields, suggesting that the introduction of D pathotype could have occurred through infected planting stocks. Thus, nurseries had a central role in D pathotype spreading in pathogen-free areas of eastern Spain.

**Implications of soil properties.** VW prevailed more in olive groves established in saline, alkaline, and poor soils (steep slope soils) with low levels of organic matter. In cotton, the disease was favoured by neutral to alkaline soils (pH 6-9) and natural soil salinity or salinity induced by recycling of salt-rich irrigation water (Pegg and Brady, 2002). Saline irrigation can exacerbate the disease in olive groves established (Collins et al., 2005). However, cotton was not grown in the area we have surveyed or in surrounding fields, suggesting that the introduction of D pathotype could have occurred through infected planting stocks. Thus, nurseries had a central role in D pathotype spreading in pathogen-free areas of eastern Spain.
pected in olive groves established in these kind of soils naturally located in fertile valleys and characterized by a long land-use with crops susceptible to V dahliae (Zachos, 1963; Wilhelm and Taylor, 1965; Cirulli and Montemurro, 1976; Thanassoulopoulos et al., 1979; Blanco-López et al., 1984; Al-Ahmad and Mosli, 1993; Serrhini and Zeroual, 1995).

In conclusion, the application of multiple correspondence analyses of data relative to soil properties, land-use history and origin of propagation material of the groves surveyed, made it possible to acquire a wider knowledge of certain epidemiological aspects of VW in olive groves. The role of saline, alkaline and steep slope soils favouring a higher V dahliae incidence was confirmed. Furthermore, the noteworthy potential pathway for introduction of the pathogen into olive orchards from infected nursery plants was shown. It is clear from these analyses that it is best not to plant olive in soils previously cropped with irrigated crops, especially herbaceous crops, without making use of preventive measures such as soil disinfestation. On the other hand, establishing olives in soils with non-irrigated, olive or forest land-use history seems to allow prolonged olive culture without an excessive risk of V dahliae prevalence.

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