

Region-Wide Analysis of Genetic Diversity in *Verticillium dahliae* Populations Infecting Olive in Southern Spain and Agricultural Factors Influencing the Distribution and Prevalence of Vegetative Compatibility Groups and Pathotypes

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ABSTRACT

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Severity of *Verticillium* wilt in olive trees in Andalusia, southern Spain is associated with the spread of a highly virulent, defoliating (D) *Verticillium dahliae* pathotype of vegetative compatibility group 1A (VCG1A) but the extent of this spread and the diversity of the pathogen population have never been documented. VCG typing of 637 *V. dahliae* isolates from 433 trees in 65 orchards from five olive-growing provinces in Andalusia indicated that 78.1% were of VCG1A, 19.8% of VCG2A, 0.6% of VCG2B, 1.4% of VCG4B, and one isolate was heterokaryon self-incompatible. A single VCG prevailed among isolates within most orchards but two and three VCGs were identified in 12 and 3 orchards, respectively, with VCG1A+VCG2A occurring in 10 orchards. VCG1A was the predominant VCG in the three most important olive-growing provinces, and was almost as prevalent as VCG2A in another one. Molecular pathotyping of the 637 isolates using specific polymerase chain

reaction assays indicated that VCG1A isolates were of the D pathotype whereas isolates of VCG2A, -2B, and -4B were of the less virulent nondefoliating (ND) pathotype. The pathotype of isolates correlated with the disease syndrome affecting sampled trees. Only three (*seq1*, *seq2*, and *seq4*) of the seven known sequences of the *V. dahliae*-specific 539- or 523-bp amplicon were identified among the 637 isolates. Distribution and prevalence of VCGs and *seq* sequences among orchards indicated that genetic diversity within olive *V. dahliae* in Andalusia is higher in provinces where VCG1A is not prevalent. Log-linear analysis revealed that irrigation management, source of irrigation water, source of planting stock, and cropping history of soil were significantly associated with the prevalence of VCG1A compared with that of VCG2A. Multivariate analyses using a selected set of agricultural factors as variables allowed development of a discriminant model for predicting the occurrence of D and ND pathotypes in the area of the study. Blind tests using this model correctly identified the *V. dahliae* pathotype occurring in an orchard. The widespread occurrence and high prevalence of VCG1A/D pathotype in Andalusia have strong implications for the management of the disease.

Additional keywords: discriminant analysis, DNA sequencing, molecular markers, *Olea europaea*, soil type.

Verticillium wilt (VW) of olive (*Olea europaea* L.) caused by *Verticillium dahliae* Kleb. is one of the most important diseases of this crop worldwide (27,37,59). In Spain, the disease was first observed in 1979 in experimental fields near Córdoba, Andalusia, southern Spain (9). Subsequently, the pathogen has spread and now causes severe infections in olive throughout that region, as well as other regions in Spain (52,54) (R. M. Jiménez-Díaz, unpublished data), making VW the main current threat for the Andalusia olive industry.

The increase in distribution and importance of VW of olive in Andalusia has occurred concomitant with both the expansion of the olive crop in this region, which has reached ≈65% of the olive acreage in Spain (41), and changes in cropping practices for increasing olive yields. These changes include use of self-rooted

planting stocks to establish high-tree-density, drip-irrigated orchards with reduced tillage and high inputs of fertilizers in newly cultivated soils or fertile soils previously cropped to *V. dahliae* hosts, such as cotton (62). Those changes in the management of olive crops have led to intensively managed orchards and may have played a role in the increased distribution and intensity of VW in olive in Andalusia. However, no information was available on the relationship that might exist between olive husbandry and the extent of the disease.

Severity of *V. dahliae* attacks on olive strongly depends on virulence of the pathogen isolates (here defined as the amount of disease caused on individual hosts or host genotypes). *V. dahliae* isolates infecting olive can be classified into defoliating (D) or nondefoliating (ND) pathotypes, based on their ability to cause defoliation or no defoliation of green leaves from shoots and twigs (46,52). Infections by the D pathotype can be lethal to the plant, whereas ND-infected olive trees can eventually show complete remission from symptoms (27,37,38,63). D and ND pathotypes also occur in *V. dahliae* infecting upland cotton (*Gossypium hirsutum* L.), with D and ND isolates from cotton and olive showing cross-virulence (52,55). In Spain, the D pathotype was

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first reported infecting cotton in Seville province, southwestern Andalusia, in 1981 (3). This pathotype has since been reported in distant (up to 200 km) olive-growing provinces of that region (4,43) (R. M. Jiménez-Díaz, *unpublished data*) but the extent to which the D pathotype has spread in the olive-growing areas of Andalusia has not been documented yet.

Field populations of *V. dahliae* are considered to have a clonal structure, although a level of sexual recombination has recently been suggested to occur (2,14,15). Thus, knowledge of the genetic diversity and structure in the pathogen populations and their relationships with virulence is a key factor for the management of VWs through disease prediction, development and deployment of resistant cultivars, and proper implementation of crop rotations. In spite of the widespread occurrence of VW in olive throughout Andalusia, little was known about the extent of genetic diversity in *V. dahliae* populations infecting olive across the region. Genetic diversity in *V. dahliae* has been studied mainly by analysis of vegetative compatibility groups (VCGs) using nitrate-nonutilizing (*nit*) mutants and analyses of the fungal DNA (14–16,25,31,43,53).

V. dahliae isolates in different VCGs are often thought of as genetically isolated populations which may vary in a number of ecological, physiological, and virulence traits (31,53). Six VCGs (VCG1–VCG6) have been identified using *V. dahliae* isolates from diverse hosts and geographic origins worldwide (5,7,11,28,31,57). VCG1, VCG2, and VCG4 were further divided into two subgroups (designated A and B) based on the frequency and vigor of complementation (5,11,29,56). Isolates and *nit* mutant testers were described that represent the subgroups of those three VCGs (28,29,56,57). The use of this set of isolates and *nit* mutant testers in VCG studies has helped in establishing an internationally uniform system for classification and exchange of information about VCGs in *V. dahliae* populations (31,53). Vegetative compatibility in *V. dahliae* populations has been studied according to two main approaches: (i) using collections of isolates from diverse geographic origin and source plants and (ii) concentrating on populations occurring in particular geographic regions or specific crops. The few studies about VCGs associated with specific crops indicate that some VCGs may prevail on a crop in a given geographic area (7,25,28,29,35,56,57). Moreover, these studies have led to recognition that VCG and virulence of *V. dahliae* isolates from a given crop may be correlated. For example, VCG1A isolates are more virulent to cotton than those in VCG2B and VCG4B (35); VCG2B isolates are more virulent to artichoke and mint than those in VCG1A (25) and VCG4A (30), respectively; and VCG4A isolates are the most virulent to potato (29). All VCG1A isolates from cotton and olive of different geographic origin are of the D pathotype whereas isolates of VCGs 2A, 2B, or 4B are of the ND pathotype (5,11,15,19,20,25,35,36). VCG1B isolates from cotton or woody hosts were proved to be of the ND pathotype when tested on cotton (5,11,15) (R. M. Jiménez-Díaz, *unpublished data*).

Phenetic analysis of amplified fragment length polymorphism (AFLP) fingerprinting indicated that AFLP clustering of *V. dahliae* isolates correlates with VCG subgroups regardless of host source and geographic origin of isolates (15). However, VCGs do not describe fully the overall genetic diversity that may exist within *V. dahliae* populations. High molecular variability occurred within VCGs, as indicated by AFLP and *V. dahliae*-specific DNA sequence analyses as well as by size classes of specific polymerase chain reaction (PCR) amplicons (13–16). Therefore, unravelling the genetic diversity within *V. dahliae* VCGs associated with VW of olive in Andalusia would be of importance to understand mechanisms underlying the pathogen spread through the region and for management of the disease. The main objectives of this study were to (i) determine VCG diversity among *V. dahliae* isolates from olive and their distribution in southern Spain, (ii) characterize those isolates by means of molecular markers previously associated with D and ND pathotypes as well

as by DNA sequence analysis of a polymorphic *V. dahliae*-specific PCR amplicon, and (iii) reveal any association between the distribution pattern of *V. dahliae* VCGs and pathotypes from olive and agricultural factors that characterize olive husbandry and management in Andalusia. A preliminary summary of results from this study has been reported (26).

MATERIALS AND METHODS

VW surveys. In total, 65 VW-affected olive orchards were surveyed in the five most important olive-growing provinces in Andalusia—Córdoba (21 orchards), Granada (10 orchards), Huelva (four orchards), Jaén (16 orchards), and Seville (14 orchards)—in 2005 and 2006 (Fig. 1). Surveys were done in spring each year at the time when conditions are most appropriate for expression of disease symptoms and isolation of *V. dahliae* from affected olive trees (27,37). Orchards were arbitrarily selected with the assistance of growers and extension agents. The incidence of VW in an orchard was estimated in 100 consecutive trees in each of four arbitrarily chosen rows. In all, 4 to 10 trees (depending upon disease incidence) showing symptoms characteristic of the disease (46) were arbitrarily chosen from each orchard for isolation of the pathogen. The disease syndrome affecting each of sampled trees was annotated and the tree location was recorded using geographical positioning equipment (Trimble, Sunnyvale, CA). In total, 433 olive trees were sampled for the study. Eight agricultural factors that are important in the development of VWs in many crops (48) were recorded from each of the sampled orchards: cropping history, crop age, olive cultivar, source of planting stock, tree density, irrigation management, source of irrigation water, and soil texture.

Procurement of *V. dahliae* isolates and culture conditions. A collection of 637 *V. dahliae* monoconidial isolates was studied (Table 1). Isolations were done from four young symptomatic branches per affected tree, as previously described (46). Cultures were incubated at $25 \pm 1^\circ\text{C}$ in the dark for 7 days. Pure cultures of *V. dahliae* were identified based on the morphology of conidiophores and microsclerotia formed on potato dextrose agar (PDA) (Difco Laboratories, Detroit). One or two monoconidial isolates were kept from 229 and 204 olive trees, respectively, for VCG characterization and molecular analyses. Cultures on plum-lactose-yeast extract agar (PLYA) (58) were covered with sterile mineral oil (4) and stored at 4°C in the dark. Isolates are deposited in the culture collection of Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain, and the Department of Plant Pathology, The Pennsylvania State University, University Park, PA. Active cultures of isolates were obtained on water agar and further subculturing on Czapek-Dox agar (CDA; Difco Laboratories) at $25 \pm 1^\circ\text{C}$ in the dark.

Generation and characterization of *nit* mutants and determination of vegetative compatibility. All 637 *V. dahliae* isolates were characterized to VCG by complementation tests of *nit* mutants, which were generated according to Korolev and Katan (33) and previous studies (15,25) and phenotyped according to Correll et al. (17). Complementation tests were performed by pairing phenotypically distinct *nit* mutants of an isolate, as well as by pairing them with complementing *nit* mutants of the international Ohio Agricultural Research and Development Center (OARDC, The Ohio State University, Wooster) reference strains of *V. dahliae* VCGs (VCG1, -3, and -4A) and with Israeli *nit* testers (VCG1A, -2A, -2B, and -4B) (34) in all possible combinations. Testers used and complementation procedures were described in previous studies (15,25). Israeli *nit* testers of VCG2A, VCG2B, and VCG4B were previously demonstrated to correlate with the international OARDC reference strains (31,34,36,53). The *nit* mutants derived from reference strain T9 of D pathotype, kindly provided by T. Katan (The Volcani Center, Bet Dagan, Israel), were used to identify VCG1A. Because T9 belongs to

VCG1A of Bell (5), we assumed that isolates strongly compatible with it should be assigned to VCG1A. The *nit* mutants of isolates 1990.1 and 9.6 (25) from Japanese maple and yellow wood, respectively, were used for the identification VCG1B (5,11). These tester isolates were proved to be of the ND pathotype in virulence assays on cotton (R. M. Jiménez-Díaz, unpublished results). Cultures were incubated at $25 \pm 1^\circ\text{C}$ with a 12-h photoperiod of fluorescent and near-UV light at $36 \mu\text{Em}^{-2} \text{s}^{-1}$ for up to 4 weeks. Pairings were scored for complementation according to Jiménez-Díaz et al. (25). Positive complementation reactions between tested isolates, or between an unknown and a tester strain, allowed the assignment of unknown isolates to the same VCG or to the VCG of the tester strain.

In a previous VCG study, some VCG2B *V. dahliae* isolates from artichoke failed to form heterokaryons with international reference testers of that VCG but did with selected local testers (25). Thus, in the present study, we adopted a two-step strategy for the characterization of *V. dahliae* isolates from olive to VCG. First, 131 isolates were characterized to VCG (VCG1A, 80 isolates; VCG2A, 40 isolates; VCG2B, 4 isolates; and VCG4B, 7 isolates) using OARDC reference strains and Israeli *nit* testers. Then, complementing *nit* mutants of isolates in each of VCGs were paired in all possible combinations to select local tester strains on the basis of strongest complementation. Thereafter, the remaining 506 isolated were assigned to VCG using the OARDC and Israeli tester strains as well as the local tester strains.

Molecular characterization of *V. dahliae* isolates. All 637 *V. dahliae* isolates in this study were characterized by means of PCR assays using primer pairs DB19/DB22 (10), INTD2f/2r and INTND2f/2r (44,45), and DB19/DB22/espdf01 (43). The use of primers DB19/DB22 produces *V. dahliae*-specific polymorphic DNA bands of 539 or 523 bp. The 539-bp marker is present in D isolates of VCG1A and ND isolates of VCG1B, whereas the 523-bp marker is associated with the ND pathotype regardless of VCG (16,43). The joint use of primer pairs INTD2f/2r and INTND2f/2r produce amplicons of either 462 or 824 bp that previously were associated with the D and ND pathotypes of *V. dahliae*, respectively (44,45). The use of primers DB19/DB22/espdf01 in a single reaction yields one of the 539- or 523-bp markers together with an amplicon of 334 bp which is present in D isolates and also in some ND isolates of VCG1B and VCG2B (16,43).

DNA extraction and determination of purity and concentration were as previously reported (15). DNA solutions were stored at -20°C until use. Amplifications using the primer pairs referred to above, and separation and visualization of amplification products, were carried out according to Jiménez-Díaz et al. (25). All PCR assays were performed with a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). Reactions were repeated at least twice and always included negative controls (no DNA) as well as positive controls (template DNA from *V. dahliae* isolates representative of D [V138I isolate] and ND [V176I isolate] pathotypes from previous studies) (43–45). The correlation between molecular typing of

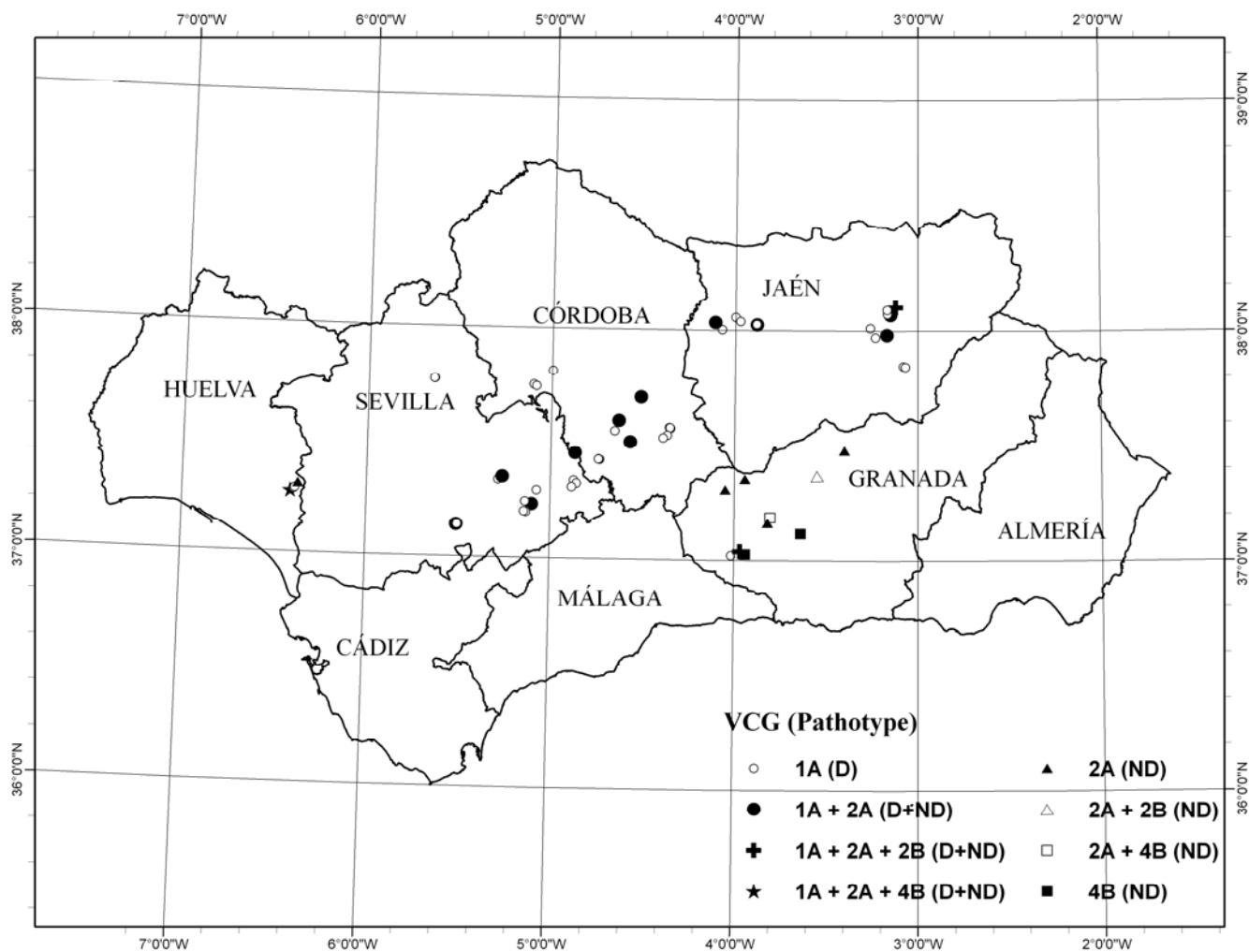


Fig. 1. Regional distribution of *Verticillium dahliae* vegetative compatibility groups (VCGs) 1A, 2A, 2B, and 4B from olive in Andalusia, southern Spain. Isolates of VCG1A isolates are of the defoliating (D) pathotype and isolates of VCG2A, -2B, or -4B are of the nondefoliating (ND) pathotype. Cotton has been grown throughout Cordoba, Jaén, and Seville provinces. Information about latitude and longitude is in degrees, minutes, and seconds, whereas decimal degrees were used as quantitative variable for discriminate analysis in text. This difference does affect positioning of orchards in the figure because scales are shown in degrees.

isolates into D or ND pathotypes and the disease syndrome affecting the tree from which they were obtained was examined. In addition, the pathotype of isolates was confirmed by root-dip inoculation of potted plants of olive cv. Picual using five arbitrarily chosen isolates of each of VCG1A (D isolates) and VCG2A, -2B, and -4B (ND isolates). *V. dahliae* isolates V1381 and V18761 were used as control for pathotypes in those inoculations (24).

Sequencing of the polymorphic *V. dahliae*-specific amplicon. The polymorphic *V. dahliae*-specific DNA band amplified from

the 637 *V. dahliae* isolates in the study using primers DB19 and DB22 was sequenced. PCR products were purified using the AccuPrep PCR Purification kit (Bioneer, Seoul, Korea), quantified spectrophotometrically as described above, and used for direct sequencing with primer DB19. DNA sequencing was done on a multicapillary sequencer (Abiprism 3100 genetic analyzer; Applied Biosystems, Foster City, CA) at the University of Córdoba sequencing facilities. Sequences were aligned using Sequencher 4.1.4 software (Gene Codes Corporation, Ann Arbor,

TABLE 1. Isolates of *Verticillium dahliae* from olive in Andalusia, southern Spain, listed by vegetative compatibility group (VCG), number, geographic origin, molecular marker, DNA sequence type of a polymorphic *V. dahliae*-specific polymerase chain reaction (PCR) amplicon (*seq*), and pathotype

| VCG | No. of isolates ^a | Geographic origin | | Molecular marker (bp) ^b | <i>seq</i> ^c | Pathotype ^d | |
|-----------|------------------------------|-------------------|-------------------|------------------------------------|-------------------------|------------------------|----|
| | | Province | Locality | | | | |
| VCG1A | 10 | Córdoba | Almodóvar del Río | 334 and 462 | 4 | D | |
| | 22 | | Baena | 334 and 462 | 4 | D | |
| | 2 | | Cabra | 334 and 462 | 4 | D | |
| | 5 | | Castro del Río | 334 and 462 | 4 | D | |
| | 16 | | Fuente Palmera | 334 and 462 | 4 | D | |
| | 34 | | Montilla | 334 and 462 | 4 | D | |
| | 12 | | Monturque | 334 and 462 | 4 | D | |
| | 32 | | Puente Genil | 334 and 462 | 4 | D | |
| | 8 | | Santaella | 334 and 462 | 4 | D | |
| | 3 | | Granada | Alhama de Granada | 334 and 462 | 4 | D |
| | 36 | | | Huelva | Hinojos | 334 and 462 | 4 |
| | 37 | | Jaén | Andújar | 334 and 462 | 4 | D |
| | 14 | | | Mengíbar | 334 and 462 | 4 | D |
| | 3 | | | Quesada | 334 and 462 | 4 | D |
| | 58 | | Torreperogil | 334 and 462 | 4 | D | |
| | 45 | | Villacarrillo | 334 and 462 | 4 | D | |
| | 17 | Sevilla | Constantina | 334 and 462 | 4 | D | |
| | 24 | | Estepa | 334 and 462 | 4 | D | |
| | 37 | | Herrera | 334 and 462 | 4 | D | |
| | 36 | | Marchena | 334 and 462 | 4 | D | |
| 6 | Morón | | 334 and 462 | 4 | D | | |
| 40 | Osuna | | 334 and 462 | 4 | D | | |
| Subtotal | 497 | | | ... | ... | ... | |
| VCG2A | 11 | Córdoba | Castro del Río | 824 | 2 | ND | |
| | 1 | | Montilla | 824 | 2 | ND | |
| | 6 | | Monturque | 824 | 2 | ND | |
| | 3 | Granada | Santaella | 824 | 2 | ND | |
| | 4 | | Alhama de Granada | 824 | 2 | ND | |
| | 23 | | Cijuela | 824 | 2 | ND | |
| | 18 | | Iznalloz | 824 | 2 | ND | |
| | 9 | | Montefrío | 824 | 2 | ND | |
| | 1 | | Piñar | 824 | 2 | ND | |
| | 32 | Huelva | Hinojos | 824 | 2 | ND | |
| | 1 | | Jaén | Andújar | 824 | 2 | ND |
| | 3 | | Mengíbar | 824 | 2 | ND | |
| | 2 | | Torreperogil | 824 | 2 | ND | |
| | 6 | | Villacarrillo | 824 | 2 | ND | |
| | 2 | Sevilla | Marchena | 824 | 2 | ND | |
| | 2 | | Morón | 824 | 2 | ND | |
| | 2 | | Osuna | 824 | 2 | ND | |
| Subtotal | 126 | | | ... | ... | ... | |
| VCG2B | 2 | Granada | Iznalloz | 824 | 1 | ND | |
| | 1 | | Alhama de Granada | 824 | 1 | ND | |
| | 1 | Jaén | Villacarrillo | 824 | 1 | ND | |
| Subtotal | 4 | | | ... | ... | ... | |
| VCG4B | 2 | Huelva | Hinojos | 824 | 2 | ND | |
| | 1 | Granada | Alhama de Granada | 824 | 2 | ND | |
| | 5 | | Alhendín | 824 | 2 | ND | |
| | 1 | | Cijuela | 824 | 2 | ... | |
| Subtotal | 9 | | | ... | ... | ... | |
| HSI VCG1A | 1 | Sevilla | Herrera | 334 and 462 | 4 | D | |
| Subtotal | 1 | | | ... | ... | ... | |
| Total | 637 | | | ... | ... | ... | |

^a Vegetative compatibility was determined using nitrate-nonutilizing mutants generated according to Korolev and Katan (33) and Correll et al. (17), and pairing them with complementary mutants of international Ohio Agricultural Research and Development Center reference and Israeli tester strains (34,36,53). HSI = heterokaryon self-incompatible.

^b Amplicons produced in PCR assays using primer sets DB19/DB22/espdef018 (334 bp), INTD2f/INTD2r (462 bp), and INTND2f/INTND2r (824 bp) (43–45).

^c *V. dahliae*-specific sequence amplified in PCR assays using primer pair DB19/DB22 (10). 1 = *seq1* (527 bp); 2 = *seq2* (526 bp); 4 = *seq4* (542 bp) (14).

^d D = olive and cotton defoliating, as indicated by amplification of the 462-bp marker; ND = olive and cotton nondefoliating, as indicated by amplification of 824-bp marker (43–45).

MI) and compared with the seven polymorphic sequences of the 523- or 539-bp amplicons already described (13,14,16): *seq1* (527 bp, GenBank accession no. DQ266246), *seq2* (526 bp, DQ266247), *seq3* (526 bp, DQ266248), *seq4* (542 bp, DQ266249), *seq5* (550 bp, AF363243), *seq6* (543 bp, DQ266244), and *seq7* (541 bp, DQ2666245).

Association among the distribution of *V. dahliae* VCGs and pathotypes from olive and variables that characterize olive orchard husbandry and management in Andalusia. Each of the 65 olive orchards sampled was characterized for the presence and identity of *V. dahliae* VCG and pathotype, as well as the eight agricultural factors mentioned above. The dependent variables (occurrence of *V. dahliae* VCGs and pathotypes) were multinomial. For *V. dahliae* pathotypes, we denoted each component as follows: (i) D pathotype only present, (ii) ND pathotype only present, and (iii) both pathotypes present. Regarding VCGs, only VCG combinations having the highest frequencies (i.e., VCG1A, VCG2A, and their co-occurrence, VCG1A+VCG2A) were used for statistical analyses to avoid use of zero frequency. Consequently, we denoted each component as (i) VCG1A only present, (ii) VCG2A only present, and (iii) both VCG1A and VCG2A present.

Log-linear analyses. Log-linear models were used to analyze contingency tables (1). For the analyses, the agricultural factors listed above were categorized as follows: (i) irrigation management: irrigated and rain fed; (ii) source of irrigation water: well and river; (iii) source of planting stock: woody stems rooted by the farmer at the orchard and nursery-propagated self-rooted leafy stem cuttings; (iv) crop age (years old): <10, 10 to 20, >20 to 30, and >30; (v) cropping history: virgin soil, cereals (e.g., maize, sorghum, and wheat), major *V. dahliae*-hosts in Andalusia (olive and cotton), other *V. dahliae*-host crops (e.g., faba bean, grape vine, potato, sugar beet, sunflower, tomato, and so on), and rotation of *V. dahliae* hosts with cereals; (vi) tree density (number of trees per hectare): low: <125, medium: 125 to 250, high: >250 to 350, and intensive: >350; (vii) soil texture: clay, sandy, clay-loam, and sandy-loam; (viii) VW incidence: <1, 1 to 4, and >4%; (ix) latitude: 37.0 to 37.3°, >37.3 to 37.6°, >37.6 to 37.9°, and >37.9°; and (x) longitude: -6.5 to -5.5°, <-5.5 to -4.5°, <-4.5 to -3.5°, and <-3.5°. The CATMOD procedure in SAS software (Statistical Analysis System, version 9.1; SAS Institute, Cary, NC) with a Poisson error and log link was used.

Multivariate analyses. Canonical discriminant (CD) analysis was used to assess the strength of the association among agricultural factors and *V. dahliae* groups; that is, (i) the three VCGs groups and (ii) the three pathotype groups in separate analyses. Only quantitative factors were included in the analysis (i.e., crop age, tree density, soil texture [expressed as clay, sand, and silt content], and geographic location). First, the STEPDISC procedure of SAS was used to eliminate variables within the model that do not provide additional information or were redundant as determined by the Wilks' lambda method, as well as to add variables outside the model that contribute most to the model (32). The DISCRIM procedure of SAS was then used to generate

a discriminant function capable of determining the classification accuracy of the set of *V. dahliae* groups, based on the pooled covariance matrix and the prior probabilities of the classification groups. A training dataset of 65 olive orchards and a test dataset of 7 olive orchards, each consisting of three *V. dahliae* classes, were created for the STEPDISC selected model. The training set was used to train DISCRIM for classifying *V. dahliae* groups and the test dataset was used to evaluate the model classification accuracy. The data obtained from the stepwise analysis were further subjected to CD analysis using the CANDISC procedure of SAS to separate classification variables (*V. dahliae* groups in this analysis) based on linear combinations of the quantitative variables (agricultural factors). The linear combinations of variables (canonical roots) were then correlated with the original groups. Canonical roots means (centroid values) were calculated for each classification variable and significance between means was determined using Mahalanobis distance (32). Individual values for each canonical root were plotted in a bi-plot for the first and second canonical variable.

RESULTS

Vegetative compatibility of *V. dahliae* isolates. All 637 *V. dahliae* isolates in the study produced *nit* mutants, with a total of 4,545 mutants. Of those *nit* mutants, 86.1% were classified as *nit1*, 11.9% as NitM, and 2% as *nit3*. All *V. dahliae* isolates except one produced *nit1* mutants but only 66% of the isolates produced NitM mutants and 12.2% formed *nit3* mutants. Positive complementation reactions of those mutants with *nit* mutants of the OARDC reference strains and Israeli tester strains allowed the assignment of the 131 initially tested isolates to VCG1A, -2A, -2B, or -4B. Thereafter, use of the local *nit* tester strains and of those specific testers enabled the assignment of the remaining 506 isolates to one of compatibility groups VCG1A, -2A, -2B, or -4B, except for 1 isolate that was heterokaryon self incompatible (HSI). Assignment of isolates to VCG using local tester strains was correlated with assignment made using OARDC and Israeli tester strains, with few exceptions. Thus, two isolates from Huelva province formed positive complementation with the OARDC reference and Israeli tester strains of VCG4B, as well as weak complementation with the local tester strain of VCG2A. Those two latter isolates were assigned to VCG4B. Also, the HSI isolate produced a positive complementation reaction with the local tester strain of VCG1A as well as with the OARDC reference strain of VCG1 and Israeli *nit* tester of VCG1A.

In all, the 637 *V. dahliae* isolates from olive in Andalusia were assigned to VCGs as follows: 497 isolates (78.1%) to VCG1A, 126 (19.8%) to VCG2A, 4 (0.6%) to VCG2B, and 9 (1.4%) to VCG4B and 1 isolate was HSI (Table 2). A single VCG prevailed among isolates within an orchard but two VCGs were identified in 12 (18.5%) orchards (VCG1A+VCG2A, 10 orchards; VCG2A+VCG2B, 1 orchard; VCG2A+VCG4B, 1 orchard) and three VCGs in 3 orchards (VCG1A+VCG2A+VCG2B, 2 orchards; VCG1A+VCG2A+VCG4B, 1 orchard). Also, in four trees from three

TABLE 2. Geographic distribution of vegetative compatibility groups (VCGs) of *Verticillium dahliae* from olive in Andalusia, southern Spain

| Province | Number of isolates | | | | | Total |
|----------|--------------------|-------|-------|-------|-----|-------|
| | VCG1A ^a | VCG2A | VCG2B | VCG4B | HSI | |
| Córdoba | 141 | 21 | 0 | 0 | 0 | 162 |
| Granada | 3 | 55 | 3 | 7 | 0 | 68 |
| Huelva | 36 | 32 | 0 | 2 | 0 | 70 |
| Jaén | 157 | 12 | 1 | 0 | 0 | 170 |
| Sevilla | 160 | 6 | 0 | 0 | 1 | 167 |
| Total | 497 | 126 | 4 | 9 | 1 | 637 |

^a Vegetative compatibility was determined using nitrate-nonutilizing mutants generated according to Korolev and Katan (33) and Correll et al. (17), and pairing them with complementary mutants of international Ohio Agricultural Research and Development Center reference and Israeli tester strains (34,36,53). HSI = heterokaryon self-incompatible.

orchards, the two isolates from a tree were of different VCGs: VCG1A+VCG2A (one tree from one of each orchards sampled at Córdoba, Granada, and Jaén provinces) and VCG2A+VCG2B (one tree from the same orchard at Granada as above). VCG1A and VCG2A occurred in the five provinces surveyed within the region and accounted for 54 (83.1%) and 22 (33.8%) orchards, respectively (Fig. 1; Table 2). Conversely, VCG2B and VCG4B were present in three and four orchards, respectively. Prevalence of the identified VCGs varied among provinces in the surveyed region. Isolates of VCG1A dominated the *V. dahliae* populations in olive orchards at Córdoba, Jaén, and Seville provinces: 87.0% of isolates in 20 of 21 orchards in Córdoba province, 92.3% of isolates in 15 of 16 orchards in Jaén province, and 95.8% of isolates in the 14 orchards surveyed in Seville province. The remaining isolates in those provinces were VCG2A (Córdoba, 13%, one orchard; Jaén, 7.4%, one orchard; Seville, 3.6%, one orchard), except for one of the isolates from Jaén and another from Seville that were identified as VCG2B and HSI, respectively (Table 2). Isolates from Granada, in the southeastern part of the region, showed the highest VCG diversity; 80.9% of isolates belonged to the predominant VCG2A in 7 of 10 orchards, three isolates were of VCG1A in 2 orchards, another three were of VCG2B in 2 orchards, and seven isolates were of VCG4B in 3 orchards (Table 2). Isolates from Huelva, in the southwestern part of the region, were almost equally distributed between VCG1A (51.4%) and VCG2A (45.7%), each in three and two orchards, respectively; and two isolates (2.9%) were of VCG4B in one orchard.

Molecular characterization of isolates by specific PCR and sequencing of the polymorphic *V. dahliae*-specific amplicon. All *V. dahliae* isolates yielded the species-specific 523- or 539-bp polymorphic DNA bands in PCR assays using the DB19/DB22 primer pair. All isolates of VCG1A and the single HSI isolate produced only the 334- and 462-bp amplicons diagnostic of the D pathotype when assayed using primers DB19/DB22/espdef01 or INTD2f/2r and INTND2f/2r, respectively. Consequently, these isolates were classified as D pathotype (Table 1; Fig. 1). Similarly, use of those primers for PCR assays of isolates assigned to VCG2A, -2B, and -4B yielded only the 824-bp amplicon diagnostic of ND *V. dahliae* (Table 1). Isolates of these latter VCGs were then classified as ND pathotype. The pathotype assigned to isolates correlated with the disease syndrome affecting sampled trees. Also, biological pathotyping of isolates on Picual olive plants further confirmed VCG1A/D isolates as defoliating and VCG2A, -2B, and -4B/ND isolates as nondefoliating (data not shown) (24). Sequencing of the 523- or 539-bp amplicons from the 637 *V. dahliae* isolates produced three (*seq1*, *seq2*, and *seq4*) of the seven *seq* sequences previously described (13,14,16) (Table 1; Fig. 1). The sequences and VCGs of isolates were correlated as follows: *seq1* was found only in isolates of VCG2B, *seq2* was amplified from isolates of VCG2A and VCG4B, and

seq4 occurred only in isolates of VCG1A and the single HSI isolate identified in the study (Table 1).

Association between *V. dahliae* VCGs and pathotypes from olive and variables that characterize olive orchard husbandry and management in Andalusia. Most of the agricultural factors

TABLE 4. Maximum likelihood analysis of variance from log-linear analyses for the effects of crop age and previous crop on the frequency of vegetative compatibility groups (VCG) of *Verticillium dahliae* (Vd) infecting olive in Andalusia, southern Spain

| Group | Source | df | χ^2 | $P > \chi^2$ ^a | |
|-------------------------------|--|--------------------|----------|---------------------------|--------|
| Crop age | Global | | | | |
| | VCG (A) | 2 | 29.69 | <0.0001 | |
| | Crop age (B) | 3 | 7.54 | 0.0566 | |
| | A × B | 6 | 16.08 | 0.0133 | |
| By crop age | <10 years old | VCG | 2 | 5.44 | 0.0660 |
| | 10–20 years old | VCG (1A vs. 1A+2A) | 1 | 1.82 | 0.1772 |
| | 20–30 years old | VCG | 1 | 1.03 | 0.5961 |
| | >30 years old | VCG (1A vs. 2A) | 1 | 8.83 | 0.0030 |
| | by VCG | | | | |
| VCG 1A | Crop age | 3 | 10.85 | 0.0125 | |
| | Contrasts | | | | |
| | >30 vs. All others | 1 | 10.28 | 0.0013 | |
| VCG 2A | Crop age | 2 | 1.03 | 0.5961 | |
| | VCG 1A+2A | Crop age | 2 | 0.20 | 0.9055 |
| Cropping history ^b | Global | | | | |
| | VCG (A) | 2 | 29.69 | <0.0001 | |
| | Previous crop (B) | 4 | 10.59 | 0.0315 | |
| | A × B | 8 | 15.92 | 0.0435 | |
| By previous crop | None | VCG (1A vs. 2A) | 1 | 7.69 | 0.0056 |
| | Cereals | VCG (1A vs. 1A+2A) | 1 | 1.51 | 0.2195 |
| | Vd host crops | VCG | 2 | 8.66 | 0.0132 |
| | Contrasts | | | | |
| | 1A vs. 2A | 1 | 4.61 | 0.0317 | |
| | 1A vs. 1A+2A | 1 | 5.50 | 0.0190 | |
| | 2A vs. 1A+2A | 1 | 0.20 | 0.6569 | |
| Olive or cotton | VCG | 1 | 2.28 | 0.3203 | |
| | Rotation/cereal | VCG | 2 | 0.48 | 0.7864 |
| by VCG | VCG 1A | Previous crop | 4 | 12.48 | 0.0141 |
| | Contrasts | | | | |
| | None vs. all others | 1 | 10.51 | 0.0012 | |
| | Cereals vs. (all Vd hosts, rotation) | 1 | 0.23 | 0.6305 | |
| | All Vd hosts vs. rotation | 1 | 4.76 | 0.0291 | |
| | Vd-hosts: Olive, cotton vs. other suscep. vs. rotation | 1 | 0.13 | 0.7178 | |
| VCG 2A | Previous crop | 2 | 1.78 | 0.4097 | |
| VCG 1A+2A | Previous crop | 2 | 1.20 | 0.5479 | |

^a Significant if $P < 0.05$.

^b Cropping history: None = virgin soil; Cereals = maize, sorghum, wheat, and so on; major *V. dahliae* host crops = olive and cotton; other susceptible (suscep.) *V. dahliae* host crops = faba bean, grape vine, potato, sugar beet, sunflower, tomato, and so on; and rotation of *V. dahliae* hosts with cereals.

TABLE 3. Maximum likelihood analysis of variance from log-linear analyses for the effects of irrigation, water source, and tree density on the frequency of vegetative compatibility groups (VCGs) of *Verticillium dahliae* infecting olive in Andalusia, southern Spain

| Source | Irrigation management ^a | | | Source of irrigation water ^b | | | Source of planting stock ^c | | | Tree density ^d | | |
|------------------|------------------------------------|----------|---------------------------|---|----------|---------------------------|---------------------------------------|----------|---------------------------|---------------------------|----------|---------------------------|
| | df | χ^2 | $P > \chi^2$ ^e | df | χ^2 | $P > \chi^2$ ^e | df | χ^2 | $P > \chi^2$ ^e | df | χ^2 | $P > \chi^2$ ^e |
| VCG (A) | 2 | 29.69 | <0.0001 | 2 | 20.06 | <0.0001 | 2 | 29.69 | <0.0001 | 2 | 29.69 | <0.0001 |
| Factor (B) | 1 | 13.93 | 0.0002 | 1 | 18.70 | <0.0001 | 1 | 23.16 | <0.0001 | 3 | 6.03 | 0.1103 |
| A × B | 2 | 6.86 | 0.0324 | 2 | 1.64 | 0.4397 | 2 | 2.56 | 0.2782 | 6 | 8.45 | 0.2069 |
| Contrasts (by A) | | | | | | | | | | | | |
| 1A vs. 2A | 1 | 14.33 | 0.0002 | 1 | 14.33 | 0.0002 | 1 | 18.68 | <0.0001 | 1 | 18.10 | 0.0001 |
| 1A vs. 1A+2A | 1 | 9.05 | 0.0026 | 1 | 9.05 | 0.0026 | 1 | 16.01 | <0.0001 | 1 | 16.01 | <0.0001 |
| 2A vs. 1A+2A | 1 | 2.40 | 0.1214 | 1 | 2.40 | 0.1214 | 1 | 0.52 | 0.4692 | 1 | 0.52 | 0.4692 |

^a Irrigation management: irrigated and rain fed.

^b Source of irrigation water: well and river.

^c Source of planting stock: woody stems rooted by the farmer at the orchard and nursery-propagated self-rooted leafy stem cuttings.

^d Tree density (number of trees per hectare): low: <125, medium: 125 to 250, high: >250 to 350, and intensive: >350.

^e Significant if $P < 0.05$.

in the study were significantly associated with VCG frequency distribution, with the VCG1A/D pathotype being the most prevalent ($P < 0.05$) regardless of the factor analyzed. In addition, there were no significant differences ($P \geq 0.05$) between prevalence of the VCG2A/ND pathotype and that of the co-occurrence of VCG1A+VCG2A (Tables 3 to 5).

The VCG1A/D pathotype was significantly more prevalent in irrigated orchards ($\chi^2 = 8.10$, $P = 0.004$) whereas VCG2A/ND pathotype was similarly prevalent ($\chi^2 = 0.14$, $P = 0.706$) in irrigated and nonirrigated ones (6.9 and 5.2%, respectively). These two VCGs were jointly detected in irrigated orchards but not in nonirrigated ones. In irrigated orchards, both the VCG1A/D and VCG2A/ND pathotype, as well as the joint occurrence of VCG1A+VCG2A, were more prevalent ($P < 0.001$) when the irrigation water was from wells rather than from a river (Table 3; Fig. 2A). Similarly, those three VCG groupings were also more

TABLE 5. Maximum likelihood analysis of variance from log-linear analyses for the effects of soil texture, disease incidence, and geographic location (latitude and longitude) of olive orchards on the frequency of vegetative compatibility groups (VCGs) of *Verticillium dahliae* infecting olive in Andalusia, southern Spain

| Source | df | χ^2 | $P > \chi^2$ ^a |
|--------------------------------|----|----------|---------------------------|
| Soil texture ^b | | | |
| VCG (A) | 2 | 29.69 | <0.0001 |
| Soil texture (B) | 4 | 6.50 | 0.1648 |
| A × B | 8 | 11.28 | 0.1708 |
| Contrasts (A) | | | |
| 1A vs. 2A | 1 | 18.68 | <0.0001 |
| 1A vs. 1A+2A | 1 | 16.01 | <0.0001 |
| 2A vs. 1A+2A | 1 | 0.52 | 0.4692 |
| Disease incidence ^c | | | |
| VCG (A) | 2 | 26.32 | <0.0001 |
| Soil texture (B) | 2 | 7.03 | 0.0298 |
| A × B | 4 | 6.77 | 0.1484 |
| Contrasts (A) | | | |
| 1A vs. 2A | 1 | 16.92 | 0.0001 |
| 1A vs. 1A+2A | 1 | 14.11 | 0.0002 |
| 2A vs. 1A+2A | 1 | 0.52 | 0.4692 |
| Contrasts (B) | | | |
| Di 1 vs. Di 2,3 | 1 | 4.49 | 0.0341 |
| Di 2 vs. 3 | 1 | 4.52 | 0.0334 |
| Latitude ^d | | | |
| VCG (A) | 2 | 26.77 | <0.0001 |
| Latitude (B) | 3 | 9.70 | 0.0213 |
| A × B | 6 | 1.56 | 0.9558 |
| Contrasts (A) | | | |
| 1A vs. 2A | 1 | 18.68 | <0.0001 |
| 1A vs. 1A+2A | 1 | 16.01 | <0.0001 |
| 2A vs. 1A+2A | 1 | 0.52 | 0.4692 |
| Contrast (B) | | | |
| Lt 1,2 vs. Lt 3,4 | 1 | 2.19 | 0.1390 |
| Lt 1 vs. Lt 2 | 1 | 5.15 | 0.0233 |
| Lt 3 vs. Lt 4 | 1 | 0.18 | 0.6702 |
| Longitude ^e | | | |
| VCG (A) | 2 | 26.68 | <0.0001 |
| Longitude (B) | 3 | 12.98 | 0.0099 |
| A × B | 6 | 5.35 | 0.4998 |
| Contrasts (A) | | | |
| 1A vs. 2A | 1 | 18.68 | <0.0001 |
| 1A vs. 1A+2A | 1 | 16.01 | <0.0001 |
| 2A vs. 1A+2A | 1 | 0.52 | 0.4692 |
| Contrast (B) | | | |
| Lg 1,2 vs. Lg 3,4 | 1 | 0.04 | 0.8360 |
| Lg 1 vs. Lg 2 | 1 | 9.86 | 0.0017 |
| Lg 3 vs. Lg 4 | 1 | 1.77 | 0.1830 |

^a Significant if $P < 0.05$.

^b Soil texture levels: St 1 = clay; St 2 = sandy; St 3 = loam; St 4 = clay-loam; and St 5 = sandy-loam.

^c Disease incidence levels: Di 1, <1%; Di 2, 1 to 4%; Di 3, >4%.

^d Latitude levels (south to north, 1° ≈ 112 km): Lt 1 = 37.0 to 37.3°; Lt 2 = 37.3 to 37.6°; Lt 3 = 37.6 to 37.9°; Lt 4 = >37.9°.

^e Longitude levels (west to east, 1° ≈ 90 km): Lg 1 = -6.5 to -5.5°; Lg 2 = -5.5 to -4.5°; Lg 3 = -4.5 to -3.5°; and Lg 4 = <-3.5°.

prevalent ($P < 0.05$) in orchards established using woody stems rooted by the farmer than in those established with nursery-propagated planting stocks (Table 3; Fig. 2B).

Overall, crop age was not significantly associated with VCG frequency ($\chi^2 = 7.54$, $P = 0.057$) but there was a significant ($\chi^2 = 16.08$, $P = 0.013$) crop age–VCG interaction. VCG1A/D was most prevalent ($\chi^2 = 10.28$, $P = 0.001$) in older orchards (>30 years old) and occurred with moderate frequency in olive orchards with trees <20 years old (15.5 to 17.2%), with the lowest frequency (5.2%) occurring in orchards 20 to 30 years old (Table 4; Fig. 3C). That variation in frequency distribution associated with crop age did not occur for VCG2A/ND and the co-occurrence of VCG1A+VCG2A, which were present in ≈5% of orchards in most of the tree-age classes (Fig. 2C).

The cropping history of soil in an orchard was significantly associated with the prevalence of VCGs ($\chi^2 = 10.59$, $P = 0.032$) (Table 4). No significant differences ($\chi^2 < 1.51$, $P > 0.219$) existed among VCG groupings in orchards in which soil had previously been sown to cereals, or to cereals and *V. dahliae* hosts in rotation. Conversely, the VCG1A/D pathotype was significantly more prevalent ($\chi^2 = 11.15$, $P = 0.011$) than VCG2A/ND in orchards in which soil was previously uncropped (virgin soils) or cropped to *V. dahliae*-susceptible hosts (Fig. 2D). VGC frequencies in orchards previously cropped to cotton or olive were similar to those found in orchards previously cropped to other *V. dahliae*-susceptible hosts ($\chi^2 < 0.13$, $P = 0.718$). Fields potentially lacking a previous history of VW (i.e., virgin soils or fields previously cropped to cereals), which represented 48% of the surveyed orchards, harbored VCG1A/D. These fields had been established predominantly using woody stems rooted by the farmer ($\chi^2 = 10.10$, $P = 0.002$) in both irrigated and nonirrigated orchards ($\chi^2 = 3.33$, $P = 0.068$) (data not shown). Tree density in an olive orchard was not associated with the prevalence of VCGs in this study ($\chi^2 = 6.03$, $P = 0.110$). However, prevalence of VCG1A/D decreased with the increase in tree density, ranging from 31.0% at the lowest density (<125 trees ha⁻¹) to 6.9% at the highest (>350 trees ha⁻¹), with similar frequencies (15.5 and 17.2%) occurring in orchards with tree density values in between the two tree density classes. No particular trend of variation with tree density was observed for the other two VCG groupings (Table 3; Fig. 2E).

Soil type in an orchard was not significantly associated with VCG distribution ($\chi^2 = 6.50$, $P = 0.165$). Overall, the three VCG groupings in the study were more prevalent in loam and clay-loam soils compared with clay soil (Fig. 3F). In particular, it was remarkable that the prevalence of VCG1A/D was least in clay soil (7.3%) compared with that in the other four soil types in the study for which prevalence was similar, 21.9 to 24.4% (Table 5; Fig. 2F).

Overall, disease incidence in the surveyed olive orchards averaged 3.45% (0.06 to 42.80%). Disease incidence in orchards harboring the VCG1A/D pathotype averaged 2.66% (0.01 to 33.30%) and that in orchards harboring the VCG2A/ND pathotype averaged 9.03% (0.40 to 42.80%). Overall, disease incidence was significantly associated with the prevalence of VCGs ($\chi^2 = 7.03$, $P = 0.030$) (Table 5). However, whereas prevalence of VCG1A/D tended to decrease with the increase in disease incidence, VCG2A/ND and the joint occurrence of VCG1A+VCG2A were most prevalent in orchards with a disease incidence of 1 to 4% (Fig. 2G).

The geographic location (latitude and longitude) of an orchard also was a significant factor ($\chi^2 > 9.70$, $P = 0.021$) in the distribution of the VCG groupings, but there was a similar effect across them; i.e., no significant interaction between orchard location and VCG frequency distribution ($\chi^2 < 5.35$, $P > 0.500$) (Table 5). VCG1A/D was widely distributed in the full range of latitude and longitude, but particularly at 37.3 to 37.6° latitude that includes most of Jaén, Córdoba, and Seville provinces (Fig.

2H and J). The VCG2A/ND-pathotype showed a narrower distribution at -4.5 to -3.5° longitude, which included mostly olive orchards located in Granada province in the southeastern part of the surveyed area (Fig. 2I and J). The co-occurrence of VCG1A and VCG2A in an orchard was detected at -3.5 to -4.5° longitude range across the full latitude range (Fig. 2H and I).

Discriminant function and canonical analyses. The set of model variables selected in the step forward discriminant analysis is given in Table 6. In this model, agricultural factors that contributed most to discriminating among *V. dahliae* pathotype categories were longitude, plant density, latitude, and crop age, and, to a lesser extent, soil texture (Table 6). Use of the linear discriminant function allowed classifying the sampled orchards in a given *V. dahliae* pathotype category. All orchards harboring the D pathotype were correctly classified in the D category, 72.7% of orchards harboring the ND pathotype were in the ND category, and the co-occurrence of both pathotypes in an orchard was predicted with less accuracy (38.5%). Overall, the achieved classification accuracy of the model was 83.1%. Of the three misclassified ND orchards, two were categorized into the D and one in the D+ND category. Similarly, of the eight orchards harboring D+ND, seven were categorized into the D and one in the ND category. The degree to which these three categories are separated is measured by Mahalanobis distance between centroid values for each pathotype category (Table 7). As expected, all pairwise distances between the three pathotype categories were statistically significant ($P \leq 0.004$), except for the distance between the D and D+ND pathotype categories ($P = 0.052$) (Table 7). The predicted accuracy of the discriminant model was assessed using a cross-

validation sample of 10 unknown olive orchards not included in the original data set. Blind tests using olive tissues sampled from affected trees in those orchards typed the infecting pathotype to D in nine orchards and ND in one orchard. The model correctly

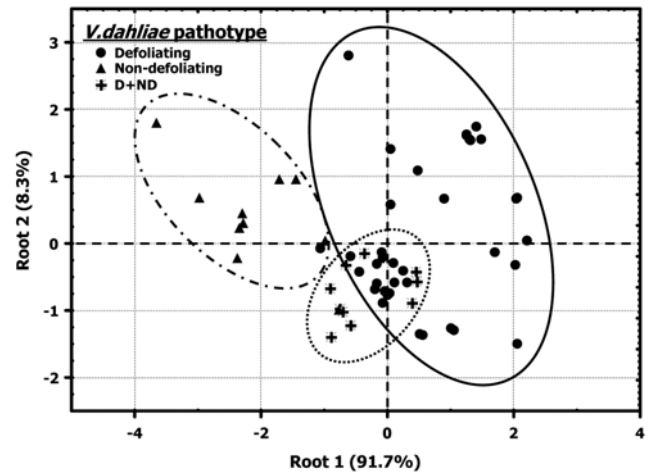


Fig. 3. Classification of 65 olive orchards in Andalusia, southern Spain into three *Verticillium dahliae* pathotype categories based on the first and second canonical functions, from the canonical discriminant analysis. Stepwise multiple discriminant and canonical discriminant analyses identified tree density, crop age, and geographic location of olive orchards (longitude and latitude) as contributing the most to separating the three *V. dahliae* pathotype categories. D = defoliating and ND = nondefoliating pathotypes.

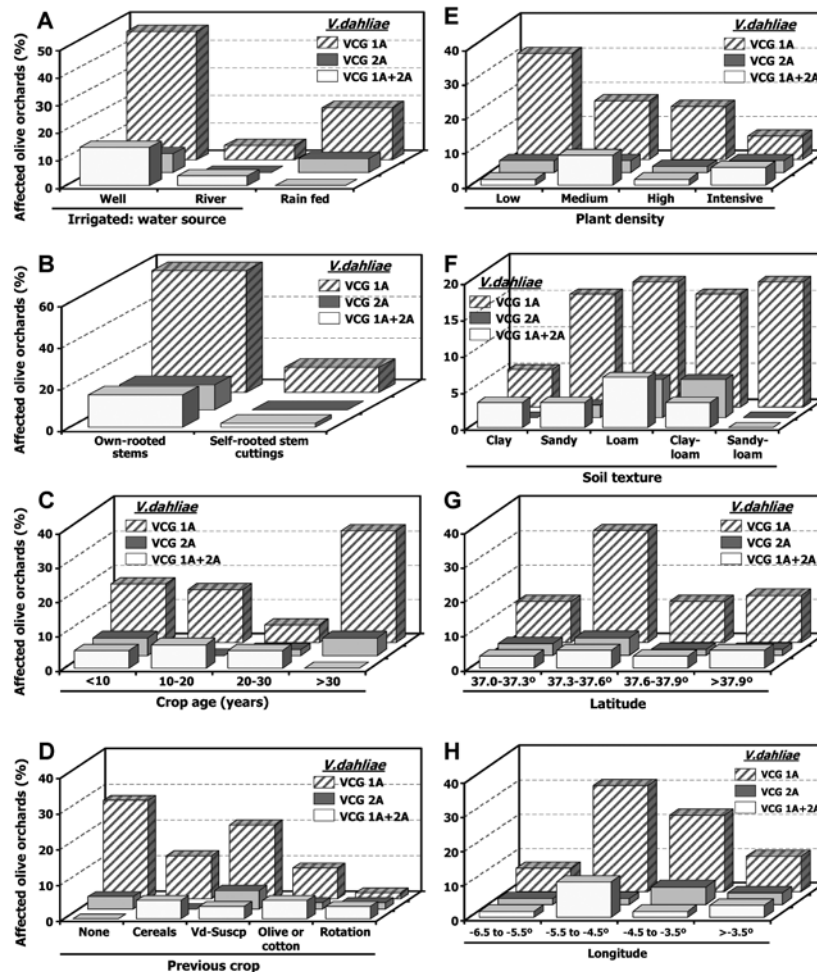


Fig. 2. Prevalence of *Verticillium dahliae* Vegetative Compatibility Groups (VCG) in 65 olive orchards in Andalusia, southern Spain according to **A**, source of irrigation water; **B**, source of planting stock; **C**, crop age; **D**, history of previous crops; **E**, plant density; **F**, soil texture; **G**, latitude; and **H**, longitude.

identified the *V. dahliae* pathotype in all cases, thus demonstrating its usefulness.

Agricultural factors included in the discriminant model were then used in a CD analysis. In this analysis, two canonical functions (roots) were derived. The first canonical root accounted for 91.7% of the variation and was dominated by positive loadings from crop age and latitude and negative loadings from longitude and plant density. The second canonical root, accounting for the remaining 8.3% of the variation, was dominated by positive loadings from crop age, plant density, and longitude and negative loading from latitude (Table 8). According to canonical loadings, the D pathotype occurred in the full range of crop age and latitude but at moderate to low tree density and low longitude (southwestern part of the surveyed area). In contrast, the ND pathotype occurred preferentially in older orchards, high longitude and low latitude (southeastern part of the surveyed area), and high tree density. CD analysis segregated orchards harboring the D pathotype from those harboring the ND one. Orchards harboring D+ND jointly were located close to orchards with the D pathotype (Fig. 3).

DISCUSSION

Genetic and virulence characterization of prevailing *V. dahliae* populations infecting olive in a region are key elements for the efficient management of VW through disease prediction and use of host resistance. In this study, VCG typing, molecular pathotyping, and sequence analysis of a *V. dahliae*-specific PCR marker of a large number of *V. dahliae* isolates indicated that little genetic and virulence diversity exist within *V. dahliae* populations infecting olive in Andalusia, which are dominated by the VCG1A/D pathotype. Also, log-linear analyses identified agricultural factors that were significantly associated with the prevalence of VCGs and pathotypes, some of which (e.g., source of irrigation water and planting stocks) may potentially have contributed to dispersal of VCG1A/D across the region. Moreover, multivariate analyses using a selected set of those factors as variables allowed us to develop a discriminant model for predicting the occurrence of D and ND pathotypes in the area of the study. This is the first time that frequency distribution of genetically and virulence-characterized populations of *V. dahliae* from olive in a region were related to factors involved in crop husbandry and management. A similar statistical approach was used to assess the strength of association of soil variables within *Pythium* communities collected from agronomic production fields in Ohio (8).

Although there were four VCGs identified among the 637 *V. dahliae* isolates in the study, only two of them, VCG1A and VCG2A, accounted for 78.1 and 19.8% of isolates, respectively. These two VCGs occurred singly in an orchard in most cases but, in 13 orchards, in association with either VCG2B or VCG4B. Therefore, the overall prevalence of VCG1A and VCG2A reached 83.1 and 33.8% of the 65 surveyed orchards, respectively. Conversely, VCG2B and VCG4B accounted for the remaining 2% of isolates in three and four orchards, respectively. This limited VCG diversity and predominance of VCG1A among *V. dahliae* infecting olive in southern Spain suggest a clonal structure of the pathogen population within the region. Interestingly, a similar predominance of VCG1A and limited VCG diversity was reported

for olive *V. dahliae* in Turkey, where VCG2A and VCG4B were found to be minor components of the pathogen population (19,20). Studies in other olive-growing countries in the Mediterranean Basin reported VCG2 and VCG4B as predominant groups in Greece (22) and Morocco (12), and VCG2B and VCG4B in Israel (61). Typing of individual *V. dahliae* isolates also indicated the occurrence of VCG2A in olive in Cyprus, Italy, and Syria (15). The limited *V. dahliae* VCG diversity associated with a crop in an area, which has been reported in several crops and regions (7,21,29,34,35,56,57), may reflect the cropping history of *V. dahliae*-susceptible plants in soils and is consistent with the perception of *V. dahliae* isolates being primarily host adapted (6,25,50). Interestingly, the described scenarios concerning VCG1A/D pathotype in Andalusia and Turkey are coincident in that the olive crops in the two countries are expanding to occupy soils previously cropped to cotton, which is as susceptible to VCG1A/D as olive (3,19,20,35,45,52,55).

Assignment of isolates to VCGs with the international reference strains and local tester strains was fully correlated but two VCG4B isolates formed weak complementation with the local tester strain of VCG2A. Such weak heterologous complementation reaction between VCG2A and VCG4B isolates has also been observed to occasionally occur with *V. dahliae* from potato (6,28,57) and it seems to correlate with a degree of molecular similarity and phylogenetic relatedness between those VCGs (6,14,15).

The limited genetic variability in *V. dahliae* populations infecting olive in Andalusia indicated by little VCG diversity is reinforced by the patterns of PCR markers amplified from them in single- and duplex-PCR assays using specific primers, as well as by the type of *seq* sequence of a *V. dahliae*-specific amplicon. All VCG1A isolates produced the 462-bp marker of the D pathotype and *seq4* sequence characteristic of VCG1A, as well as the

TABLE 7. Squared Mahalanobis distances for *Verticillium dahliae* pathotypes categories infecting olive in Andalusia, southern Spain obtained in a stepwise discriminant analysis

| Pathotype category | Squared Mahalanobis distance | | <i>F</i> values (<i>P</i>) | |
|---------------------|------------------------------|--------|------------------------------|--------------------|
| | ND | D+ND | ND | D+ND |
| Defoliating (D) | 5.7570 | 1.0643 | 11.8787 (<0.0001) | 2.4992 (0.0521) |
| Nondefoliating (ND) | ... | 3.0574 | ... | 4.3339 (0.0039) |

TABLE 8. Standardized canonical coefficients (SCCs) and correlation coefficients (CCC) of discriminant canonical functions of agronomic factors that determine the distribution of pathotypes of *Verticillium dahliae* infecting olive in Andalusia, southern Spain, selected by a stepwise discriminant analysis

| Source | SCCs | | CCCs | |
|-----------------------|--------|--------|--------|--------|
| | Root 1 | Root 2 | Root 1 | Root 2 |
| Crop age | 0.515 | 0.926 | 0.337 | 0.839 |
| Longitude | -1.353 | 0.290 | -0.245 | 0.138 |
| Plant density | -0.872 | 0.221 | -0.318 | 0.020 |
| Latitude | 0.839 | -0.657 | 0.259 | -0.272 |
| Eigenvalue | 0.819 | 0.074 | ... | ... |
| Cumulative proportion | 0.917 | 0.083 | ... | ... |

TABLE 6. Summary statistics of discriminant function analysis of agronomic factors that determine the distribution of pathotypes of *Verticillium dahliae* infecting olive in Andalusia, southern Spain

| Source | Wilks' lambda | Partial lambda | <i>F</i> remove | <i>P</i> level | Tolerance | 1 Tolerance |
|---------------|---------------|----------------|-----------------|----------------|-----------|-------------|
| Longitude | 0.7921 | 0.6460 | 16.1635 | <0.0001 | 0.4261 | 0.5739 |
| Plant density | 0.6687 | 0.7653 | 9.0467 | 0.0004 | 0.6786 | 0.3214 |
| Latitude | 0.6486 | 0.7889 | 7.8935 | 0.0009 | 0.6085 | 0.3915 |
| Crop age | 0.6046 | 0.8463 | 5.3557 | 0.0073 | 0.8607 | 0.1393 |

334-bp amplicon that is present in D isolates and some ND isolates of VCG1B and VCG2B (14–16,25,43). Conversely, all isolates in VCG2A, -2B, and -4B produced the 824-bp marker of ND *V. dahliae* and sequences *seq 1* or *seq 2* depending upon VCGs (14). The 462- and 824-bp markers, and the 334-bp one, were developed from a random amplified polymorphic DNA amplicon polymorphic in D and ND isolates and a species-specific amplicon produced by primers derived from a *V. dahliae* genomic library, respectively (10,43–45). These markers are unlikely to be linked to a virulence gene but their association with the D or ND phenotypes has been consistently demonstrated in many studies and by different laboratories (15,16,20,24,25, 36,40,46). The correlation found between molecular and biological pathotyping of D and ND isolates in this present study is consistent with that association.

Previous studies indicated that *V. dahliae* VCG1A/D isolates from cotton and olive are molecularly heterogeneous; isolates from China and Spain harbor the 334- and 462-bp DNA fragments (namely, PCR pattern A) but VCG1A/D isolates from Greece, Israel, and Turkey only carry the 334-bp fragment (namely, PCR pattern B) (15,36). Interestingly, all VCG1A isolates from olive in Turkey failed to amplify the 462-bp marker but did with the 334-bp fragment and caused defoliation in cotton and olive (19,20). Because all olive VCG1A isolates from Andalusia yielded PCR pattern A, it appears that the VCG1A/D pathotype infecting olive comprises at least two distinct populations: one associated with PCR pattern A, located in Spain and perhaps other regions; and another yielding PCR pattern B, existing in the Eastern Mediterranean Basin. The VCG1A/D pathotype is considered indigenous to the southern United States and northern Mexico (5,42). However, VCG1A/D isolates of putative endemic origin were obtained from cotton and cotton soil in Iran and Tadjikistan (18,23,49). Further demonstration of molecular diversity between patterns A and B in VCG1A/D might support earlier suggestions that new strains of VCG1A can develop independently in different geographic areas (15,20).

The VCG1A/D pathotype that now dominates populations of *V. dahliae* infecting olive in Andalusia was first found to be restricted within well-defined zones of an intensively grown cotton marsh area in the Seville province in the southwestern part of that region in 1981 (3). Since then, new infections by this pathotype were reported in cotton and olive crops in a north-eastern geographical pattern (4,43) (R. M. Jiménez-Díaz, unpublished data). The regional distribution and prevalence of the VCG1A/D pathotype infecting olive in Andalusia may reflect factors influencing migration of the D pathotype from the location where it was first found. During the last 15 years, intensive olive cultivation (i.e., new, high-tree-density plantings; drip irrigation; and so on) has significantly expanded in Córdoba and Jaén provinces, which now account for 60% of the 1.5×10^6 ha of the crop in Andalusia. Correspondingly, the VCG1A/D pathotype in Seville, Jaén, and Córdoba provinces accounted for 95.8 to 87% of the total number of isolates in those provinces, respectively. A recent, putative migration and spread of VCG1A/D into those provinces might explain why the increased prevalence of this VCG did not correlate with an increase in incidence (i.e., the time elapsed from first introductions was not enough for subsequent dispersal and completion of disease cycles within orchards) (46).

Irrigation may have contributed to long-distance spread of the D pathotype across the olive-growing area in Andalusia. Log-linear analyses of associations between VCG prevalence and agricultural factors indicated that VCG1A/D was significantly more prevalent in irrigated compared with nonirrigated orchards, and in orchards irrigated with water from wells compared with those irrigated with water from rivers. In a recent study, large numbers of *V. dahliae* propagules were found in water sampled from irrigation drips in VW-affected trees in 18 of 21 orchards in Jaén and Sevilla provinces. Irrigation water was from wells or

from ponds storing river water in 14 and 4 orchards, respectively (51).

The spread of the VCG1A/D pathotype in olive across the region also may have been enhanced by use of infected planting stocks for the establishment of new orchards. For that purpose, farmers use either wooden stems rooted in farm soil or self-rooted planting stocks purchased from nurseries. In 2006 and 2007, in planta molecular detection assays by the Plant Health Service of Andalusia indicated that 5.5% of >600 registered nurseries had *V. dahliae*-infected but symptomless olive planting stocks (P. Holgado, personal communication). Production of planting stocks in those registered olive nurseries satisfies official requirements by the European Union that do not enforce *V. dahliae*-free certification of those stocks. *V. dahliae* may also be spread in the potting soil used for nursery production, in addition to infected plants (47). In Andalusia, on-farm rooting of woody stems by farmers can contribute to spreading *V. dahliae* in infested root balls of planting stocks by inadvertently rooting them in areas with a long history of cotton presumably infested by the pathogen. This is further supported by our finding that orchards potentially lacking a history of VW-susceptible crops, representing 48% of the surveyed orchards, were found harboring the VCG1A/D pathotype, which may have been established using woody stems rooted by farmers.

VW of olive is best managed by combining the use of preventive control measures such as (i) choice of planting date and risk prediction; (ii) use of resistant cultivars, and (iii) use of pathogen-free planting material, and so on (60), the efficiency of which would enhance expression of the infected plant's ability to show remission from symptoms (27,37,63). The widespread occurrence and high prevalence of the VCG1A/D pathotype in Andalusia compromise the efficiency of those control measures and, thus, have strong implications for the management of the disease because (i) VCG1A/D is more virulent than VCG2A, VCG2B, and VCG4B/ND isolates to olive cultivars (20,40,44,45); (ii) the threshold of inoculum density for disease of D isolates is much lower compared with that of ND isolates (39); (iii) olive cultivars of commercial interest and resistant to the ND pathotype are highly to moderately susceptible to the D pathotype (40); and (iv) the ability of olive cultivars to recover from disease caused by the D pathotype is lessened compared with that from disease caused by the ND pathotype (38). Moreover, infected leaves dropping in large amounts from D-infected olive trees are efficient means of dispersal of the pathogen within and possibly among orchards, and a source of inoculum for secondary infections that determine a significant change in the epidemiology of VW in olive (46). In summary, infections by the VCG1A/D pathotype should now be the main target in the management of VW of olive in Andalusia. To that aim, new control measures are needed, such as resistant cultivars and rootstocks, pathogen-free planting stocks, and protection of the root system of healthy or recovered trees from secondary infections by the VCG1A/D pathotype. Use of the developed discriminant model for predicting the occurrence of D and ND pathotypes in an area may help in avoiding high risk soils for new plantings. Avoidance of soils already infested with VCG1A/D would be further facilitated if new, DNA-based methods are developed that overcome limitations of plating soil on semiselective media, for the sensitive and reliable quantification of this strain of the pathogen in soil.

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