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Resistance of pistachio grafted on different *Pistacia* rootstocks against Verticillium wilt under field conditions

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Abstract

Verticillium wilt of pistachio (*Pistacia vera*), caused by *Verticillium dahliae*, is one of the most important diseases in this crop. The most effective and economical control measure is the use of resistant rootstocks. The main objective of this work was to evaluate the resistance of five rootstocks commonly used in pistachio against the disease under field conditions. Pistachio plants (cv. Kerman) grafted on the rootstocks *Pistacia atlantica*, *Pistacia integerrima*, *Pistacia terebinthus* and cv. Platinum and UCBI hybrids, were planted in a field with high inoculum density of *V. dahliae* in the soil. Plants grafted on *P. terebinthus*, followed by *P. integerrima*, showed significantly higher disease severity values than the rest of the rootstocks almost 53 months after planting; also, plants grafted on *P. atlantica* were more resistant than those grafted on *P. integerrima*. These results highlighted the intraspecific variability in resistance to the disease. In addition, the disease was monitored in pistachios grafted onto *P. terebinthus* or UCBI in an established commercial field with three different zones of *V. dahliae* inoculum density in the soil (none, low or high). In the high-density zone, the percentage of diseased plants was significantly lower for plants grafted on UCBI than for those grafted on *P. terebinthus*. The results indicate the importance of rootstock species and specific genotype or seed population used, concentration of *V. dahliae* inoculum in the soil, and previous history of crops on the plot, on the development of the disease under natural conditions.

KEYWORDS
genetic susceptibility, infested soil, *Pistacia vera*, tree nuts, *verticillium dahliae*

1 | INTRODUCTION

Pistachio (*Pistacia vera*) is a nut crop with increasing significance in the cultivated area, production and yield worldwide and throughout the Mediterranean basin. In 2021, the area dedicated to this crop was 817,025 ha with a production of 915,718 t. This means an increase of 58% and 64% in the dedicated surface and production, respectively, in the last 30 years (FAO, 2022). The greatest area of pistachio cultivation is in Iran, followed by Türkiye and the United States, with Spain in fourth place with 70,235 ha; production is also most in the United States, Iran and Türkiye, with Spain in sixth place with 19,889 t in-shell (FAO, 2022; MAPA, 2022). In Spain, Castilla-La

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Mancha emerges as the region with the largest area dedicated to this crop with 53,823 ha, followed by Andalusia with 6654 ha. These regions constitute 77% and 10% of the Spanish pistachio production area, respectively (MAPA, 2022).

Verticillium wilt, caused by the soilborne pathogen *Verticillium dahliae*, is widespread in the Mediterranean basin, affecting diverse herbaceous crops, such as cotton (*Gossypium hirsutum*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*) and eggplant (*Solanum melongena*), as well as woody crops, including pistachios, olive (*Olea europaea*) and almond (*Prunus dulcis*) trees (Ashworth & Zimmerman, 1976; Bhat & Subbarao, 1999; Klosterman et al., 2009; Kowalska, 2021; Pegg & Brady, 2002; Teviotdale et al., 2002). Verticillium wilt was first reported on pistachio trees in California in 1950 (Snyder et al., 1950) and since that time has been identified in several Mediterranean countries, such as Greece (Raabe & Wilhelm, 1978; Thanassoulopoulos & Kitsos, 1972), Cyprus (Stavrides, 1994), Iran (Hadizadeh & Banihashemi, 2007; Mohammadi et al., 2006; Rafiei et al., 2018), Spain (López-Moral et al., 2023; Moral et al., 2010), Türkiye (Guldur & Dikilitas, 2011) and Tunisia (Triki et al., 2014).

Verticillium dahliae survives as long-lasting resistant structures called microsclerotia (MS) in the soil. These MS remain for long periods of time in the soil even without the presence of the host plant and serve as a primary source of infection (Kowalska, 2021). Their germination is stimulated by the exudates released from actively growing roots nearby; they then penetrate these roots and systemically colonize the plant through sap flow (Mol & Van Riessen, 1995). The prolific growth of fungus and occlusion of the xylem vessels results in a restriction in water flow, which causes the wilting and death of the plant (Fradin & Thomma, 2006). New MS are produced in the affected tissues and return to the soil, thus increasing the density of the pathogen in the soil and promoting new infections (Pegg & Brady, 2002).

Pistacia vera is the only species of the genus *Pistacia* that produces nuts with good commercial characteristics, but it is susceptible to soil pathogens such as *V. dahliae* and *Phytophthora* spp. (Kaska, 2002; Vahdati et al., 2021). Control of *V. dahliae* is difficult due to lack of host specificity and high survival of the pathogen in soil. Likewise, the severity of Verticillium wilt in pistachios is increased in plantations where susceptible rootstocks are used, are located in fields where susceptible hosts have previously been grown, or in soils with high densities of the pathogen. Therefore, one of the most economical measures to take against soilborne diseases is the use of resistant or tolerant rootstocks in pistachio orchards (Epstein et al., 2004; Teviotdale et al., 1995; Vahdati et al., 2021). In this framework, rootstock selection is crucial for the long-term health and productivity of pistachio plantations.

Among all the rootstocks used for pistachio, the species *Pistacia terebinthus*, native to the Mediterranean region, is the most widespread in Spain, and is used in other pistachio-producing regions such as Italy. This species has tolerance to drought, salinity and cold and resistance to Armillaria root rot (Teviotdale et al., 1995). However, it is generally regarded as highly susceptible to Verticillium wilt (Ak et al., 2016; Ferguson & Haviland, 2016; Moral et al., 2010), although some studies suggest there might be some intraspecific variability for this trait (Guney et al., 2021; Raabe & Wilhelm, 1978). *Pistacia atlantica* is another rootstock commonly used for pistachio because of its high vigour and its resistance to salinity, drought and low temperatures (Ferguson & Haviland, 2016), but this species also shows some susceptibility to diseases such as Verticillium wilt. Nevertheless, *P. atlantica* can show different levels of susceptibility to disease (Morgan et al., 1992; Triki et al., 2014) and moderately resistant individuals have been successfully used in interspecific crosses with other *Pistacia* species, such as the University of California Berkeley (UCBI) hybrid. *Pistacia integerrima*, although less tolerant than other rootstocks to stresses such as drought, cold and salinity (Ferguson et al., 2002), is valued as a pistachio rootstock due to its resistance to *V. dahliae*. To date, this species is considered the most reliable source of resistance in the genus *Pistacia* (Morgan et al., 1992; Raabe & Wilhelm, 1978). The Pioneer Gold I (PGI) seedling population was the first commercialized rootstock of this species, considered resistant to disease but highly susceptible to frost (Ashworth, 1984; Morgan et al., 1992). Specific individuals of *P. atlantica* and *P. integerrima* have been used in the following interspecific crosses: Pioneer Gold II (PGII) was obtained from the cross *P. integerrima* × *P. atlantica*; and UCBI was developed from *P. atlantica* 'KAC' (Kearney Agricultural Center, Parlier, California) × *P. integerrima* 'KAC'. The PGII hybrid was susceptible to *V. dahliae*, but UCBI showed good resistance to the pathogen, although could present mild symptoms when infested without producing mortality (Epstein et al., 2004; Morgan et al., 1992). Although PGII and UCBI are both hybrids of individual *P. atlantica* and *P. integerrima* genotypes, they have differences in their response to Verticillium wilt, possibly due to the genetic diversity of the parents of each rootstock, and specifically, the *P. atlantica* genotype used (Epstein et al., 2004; Morgan et al., 1992). The UCBI hybrid is acquiring great importance in the Mediterranean basin and worldwide due to its high resistance to *V. dahliae*, tolerance to cold and its high vigour (Epstein et al., 2004). Although the PGII hybrid was not used a great deal due to its susceptibility to the pathogen (Epstein et al., 2004), a selection within this seedling population was identified for its resistance to *V. dahliae*, cloned and released under the name Platinum (Sheikhi et al., 2019).

The presence of intraspecific genetic variability among *Pistacia* rootstocks renders the classification of their resistance to *V. dahliae* notably incomplete. Moreover, *P. terebinthus* has not been tested before against commercial hybrid rootstocks, and the effect of different pistachio rootstocks on resistance to *V. dahliae* has never been evaluated in the conditions of southern Spain, where this crop coexists with other very important hosts of the pathogen, such as olive and cotton. The purpose of this study was to deepen the knowledge of the behaviour of *P. vera* (cv. Kerman) when grafted on *P. atlantica*, *P. integerrima*, *P. terebinthus* and the hybrids Platinum and UCBI in a field with soil naturally infested with *V. dahliae*. In addition, we aimed to monitor the disease on *P. vera* (cv. Kerman) trees grafted on *P. terebinthus* or on the UCBI hybrid, in an established commercial field with three different zones of inoculum density and different history of previous crops grown in the plot.

2 | MATERIALS AND METHODS

2.1 | Plant material

All plant material used for both field trials consisted of 1-year-old seedlings/clones of the rootstock that had been budded in the nursery with buds of the vegetatively propagated *P. vera* 'Kerman'. The five different rootstocks used in the experimental plot to evaluate their effect against *V. dahliae* were *P. atlantica*, *P. integerrima*, *P. terebinthus* and Platinum (*P. integerrima* × *P. atlantica*) and UCBI (*P. atlantica* 'KAC' × *P. integerrima* 'KAC') hybrids. *P. terebinthus* and the UCBI hybrid were the rootstocks used in the commercial pistachio orchard where the disease progress was monitored. These all came from a commercial nursery and were produced by seed propagation, except the clone Platinum, which was obtained by in vitro propagation (Ferguson & Haviland, 2016). *P. atlantica* seeds were harvested from mother trees growing in a commercial nursery in Granada, Spain. The seeds that originated those mother trees were originally collected from Greece, while *P. terebinthus* seeds were directly harvested from wild trees growing in the Sierra de Castril area (Granada province, Spain). Finally, *P. integerrima* and UCBI seeds originated from nursery seed from California.

The identity of the scion and all the rootstocks was confirmed by DNA analysis. Healthy, young leaves of the Kerman scion and from suckers from each rootstock were collected in the field trials and kept in cold conditions until laboratory processing. DNA was extracted from the leaves using a Mag-Bind Plant DNA DS Kit (Omega Biotech). Each PCR included the forward primer, reverse primer and a forward primer labelled with a fluorophore.

For cultivar determination, three primer pairs (CUPVB219, CUPVB306, CUPBV009) developed by Zaloğlu et al. (2015) were used. After PCR amplification, amplicons were analysed with the 3730 Genetic Analyser (ABI). Size calibration was performed with the molecular weight ladder GenScan 500 ROX size standard (ABI). Finally, simple-sequence repeat (SSR) fragment analysis was performed with the Software GeneScan Analysis v. 4.0 (ABI).

Subsequently, for rootstock DNA samples, amplification was performed using the rDNA internal transcribed spacer (ITS) region with primers ITS1/ITS4 (Cheng et al., 2016; White et al., 1990). Amplicons were then purified, and Sanger sequencing carried out using the BigDye 3.0 terminator (ABI) with the provided protocol and run on a 3730 Genetic Analyser (ABI). Sequence results were searched on the BLAST website against the NCBI reference DNA database for candidate species matches (www.ncbi.nlm.nih.gov).

2.2 | Field trials

2.2.1 | Disease resistance of pistachio rootstocks in the field

This trial was established in Pinos Puente (Granada province, Spain; 37.2372° N, 3.7731° W) with a planting area of 0.6 ha. Previous history in that field includes horticultural crops, particularly potato, for more than 10 years. In January 2019, the plants were transplanted into 19 rows with four trees per row. In addition, there were border rows, which were not included in the study. A randomized complete block design was used, with four blocks, and experimental units of four trees (replicates) per rootstock (independent variable) and block (total number of plants in the experiment: $4 \times 4 \times 5 = 80$ trees). The rows were 7 m apart and the distance between planted trees was 6 m. The management of the plantation included spontaneous vegetative cover between rows and herbicide application under the canopy to prevent the appearance of weeds. The annual irrigation was 2500 m^3/ha, applied by surface drippers.

2.2.2 | Disease monitoring in a commercial field

This commercial pistachio field, with a total area of 6.5 ha, was established in Alhama de Granada (Granada province, Spain; 37.0215° N, 4.0317° W). In February 2018, monitoring of the disease in the field was designed to take into account the presence of symptoms in already established pistachios. Thus, the plot was divided into three zones based on three levels of *V. dahliae* inoculum and the different history of previous crops as follows: (1) high-density zone and (2) low-density zone, both previously cultivated with horticultural crops, especially tomato, zucchini (*Cucurbita pepo*), pepper (*Capsicum annuum*) and artichoke (*Cynara scolymus*); and (3) no-inoculum zone, previously cultivated with cereals. The inoculum density was determined as described in section 2.4. The total area of the three monitored areas was 2.2 ha. The high-density zone had 10 rows, of which six were planted with *P. vera* 'Kerman' grafted on the UCBI hybrid and four were grafted on *P. terebinthus*. The no-inoculum zone and low-density zone had four rows each and were both grafted on *P. terebinthus*. The rows were 7 m apart and the distance between trees in the row was 6 m. Planting and irrigation management was performed as described for the Pinos Puente plot.

2.3 | Disease assessment

Disease severity (DS) was evaluated biannually, in July and October, with the first evaluation in July 2020 and the last in July 2023 (53 months after planting), for a total of seven evaluations. A scale comprising 17 values within the range of 0–4 was used to assess the percentage of crown affected by yellowing, interveinal chlorosis, wilting or leaf desiccation, using five principal categories, (0 = no symptoms, 1 = 25%, 2 = 50%, 3 = 75% and 4 = 100%, dead plant). Additionally, three intermediate values (0.25, 0.50 and 0.75) were incorporated between the primary categories (Antón-Domínguez et al., 2023; López-Moral et al., 2021). Upon the conclusion of the trial, the trapezoidal integration method was employed to calculate the relative area under the disease progress curve (RAUDPC) (Campbell & Madden, 1990), as well as the

percentages of affected plants (disease incidence, DI) and dead plants (mortality). In the Alhama de Granada plot, only DI and mortality were evaluated biannually, in July and October, with the first evaluation in October 2020 and the last in July 2023, for a total of six evaluations.

2.4 | Microsclerotia of *V. dahliae* in soil

To determine the inoculum density of the fungus in the soil, soil samples were taken from both fields approximately 1.5 years after planting. For this purpose, a homogeneous sample composed of six soil subsamples (approximately 5 kg each) from the top 30 cm layer under the canopy was randomly collected from each of the four blocks within the Pinos Puente plot in October 2021. Likewise, in the Alhama de Granada plot, a representative sample from each area was collected in July 2019 as described above. Once in the laboratory, the samples were allowed to completely dry at room temperature and, subsequently, organic debris and large particles were removed manually using a 0.8 mm diameter sieve (Trapero et al., 2013). *V. dahliae* inoculum density was ascertained through the wet sieving method (Huisman & Ashworth Jr, 1974), employing 10 replicate Petri dishes containing modified sodium polypectate agar medium (MSPA) (Butterfield & DeVay, 1977) for each soil sample. Inoculum density was quantified and expressed as MS per gram of soil following the protocol described by Varo et al. (2016).

2.5 | Fungal isolation and pathotype characterization

Throughout the trial, plant material from both fields was isolated to confirm *V. dahliae* infections. To achieve this, samples of trunk, stems and bud tissues were collected from symptomatic plants in both trials and were taken to the laboratory for processing. First, the plant material was rinsed under running tap water followed by bark removal. Subsequently, wood chips of affected tissue were cut, and surface disinfected through immersion in a 10% commercial bleach (Cl at 50 g/L) for 1 min. Then, they were dried on sterile filter paper at room temperature and plated on potato dextrose agar (PDA; Difco Laboratories) acidified with lactic acid (APDA; 2.5 mL of 25% vol/vol lactic acid per L of medium) and incubated at 24 ± 2°C in the dark for 7 days. The pathotype of *V. dahliae* isolates recovered from symptomatic pistachio plants was characterized by duplex nested PCR analysis using the protocol described by Mercado-Blanco et al. (2003).

2.6 | Data analysis

In all cases, data were tested for homogeneity of variances and normality. Then, one-way analysis of variance (ANOVA) was conducted with rootstocks as an independent variable, and RAUDPC or DS as dependent variables. All treated means were compared according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985). Absolute data for DI (% plants affected) and mortality (% plants dead) were analysed using multiple comparisons tests for proportions at $\alpha = 0.05$ (Zar, 2010). Data analyses were performed using Statistix 10 software (Analytical Software, 2013).

3 | RESULTS

In general, in both plots, Verticillium wilt symptoms were observed firstly on plants grafted onto the *P. terebinthus* rootstock. These symptoms included interveinal chlorosis and yellowing of the leaves, followed by wilting and desiccation with a scorched appearance. Internally, vascular discolouration of the wood was observed, especially during hot, dry periods, conditions that favour disease progress. As the disease progressed, dieback of branches and, occasionally, death of trees was observed (Figure 1).

3.1 | Disease resistance of pistachio rootstocks in the field

The average concentration of *V. dahliae* inoculum in the soil of this plot was 14.9 ± 1.9 MS per gram of soil. There were significant differences in RAUDPC and final DS between the rootstocks evaluated ($p \leq 0.0001$ and $\alpha = 0.0002$ for RAUDCP and final DS, respectively), with *P. terebinthus* showing significantly higher RAUDPC and DS compared to the remaining rootstocks (47.8 ± 6.97 and 75.8 ± 9.58, for RAUDPC and DS, respectively), followed by *P. integerrima* (22.3 ± 5.74 and 44.5 ± 16.64, respectively). In contrast, *P. atlantica*, and the Platinum and UCBI hybrids showed significantly lower RAUDCP (Figure 2a) and DS (Figure 2b) values. Figure 2b shows the graph of DS progression over time, with a more pronounced increase in DS between October 2021 and July 2022. Trees grafted on *P. terebinthus* rootstock showed the first symptoms of Verticillium wilt in July 2019, 6 months after planting, and reached 100% incidence in October 2021, 32 months after planting; the second rootstock to show symptoms was *P. integerrima*, with first symptoms in the second year, reaching a maximum incidence of 83.3% 32 months after planting (Table 1). It should be noted that pistachios grafted on Platinum or UCBI rootstocks showed the same incidence values throughout the duration of the trial, which were not significantly different from the DI shown by *P. atlantica* (Table 1). Regarding mortality, no pistachios grafted on UCBI died, while those grafted on *P. terebinthus* had significantly higher mortality levels than the other rootstocks, with *P. integerrima*, *P. atlantica* and Platinum showing intermediate reactions (Table 1). Additionally, the final percentage of plants without symptoms, affected (without mortality), and dead for each of the rootstocks are represented in stacked columns in Figure 3. There were significant differences between the rootstocks evaluated for 'symptomless plants', and 'dead plants' ($\alpha = 0.0033$ and $\alpha = 0.0070$



FIGURE 1 Symptoms of Verticillium wilt on *Pistacia vera* 'Kerman' grafted on different rootstocks grown in a soil highly infested by *Verticillium dahliae*, located in Pinos Puente (Granada province, Spain). (a) Comparison of symptoms between *Pistacia terebinthus* (left row) and the hybrid UCBI (right row); (a–d) External symptoms include yellowing, wilting and desiccation of leaves; (e, f) internal symptoms include vascular discolouration of the wood. [Colour figure can be viewed at wileyonlinelibrary.com]

for symptomless and dead plants, respectively), but not for the level of 'affected plants' among the rootstocks ($\alpha = 0.2358$). In summary, all pistachios grafted on *P. terebinthus* showed symptoms of Verticillium wilt at the end of the trial, and no pistachio grafted on the UCBI hybrid died due to the disease.

3.2 | Disease monitoring in a commercial field

This plot was divided into zones according to the mean inoculum concentration of *V. dahliae*. No fungal propagules were detected in the no-inoculum zone (the minimum limit of fungal propagules detected by the technique used was 0.4 MS/g). In contrast, 55.2 MS/g and 6.0 MS/g were detected in the high- and low-density zones, respectively. There were significant differences for the two symptom levels 'symptomless plants' and 'dead plants', between rootstocks and evaluated zones ($\alpha \leq 0.0001$ in both cases), but not for the level of 'affected plants' ($\alpha = 0.1493$). The difference between the three zones for Verticillium wilt symptoms of pistachios grafted on *P. terebinthus* was remarkable. The percentage of plants grafted on *P. terebinthus* that did not show disease symptoms was significantly higher in the no-inoculum zone than in zones with the presence of *V. dahliae* inoculum (high- and low-density zones, Figure 4a,b). In both the high- and low-density zones, the percentage of dead plants on *P. terebinthus* was significantly higher than the percentage of dead plants on *P. terebinthus* in the no-inoculum

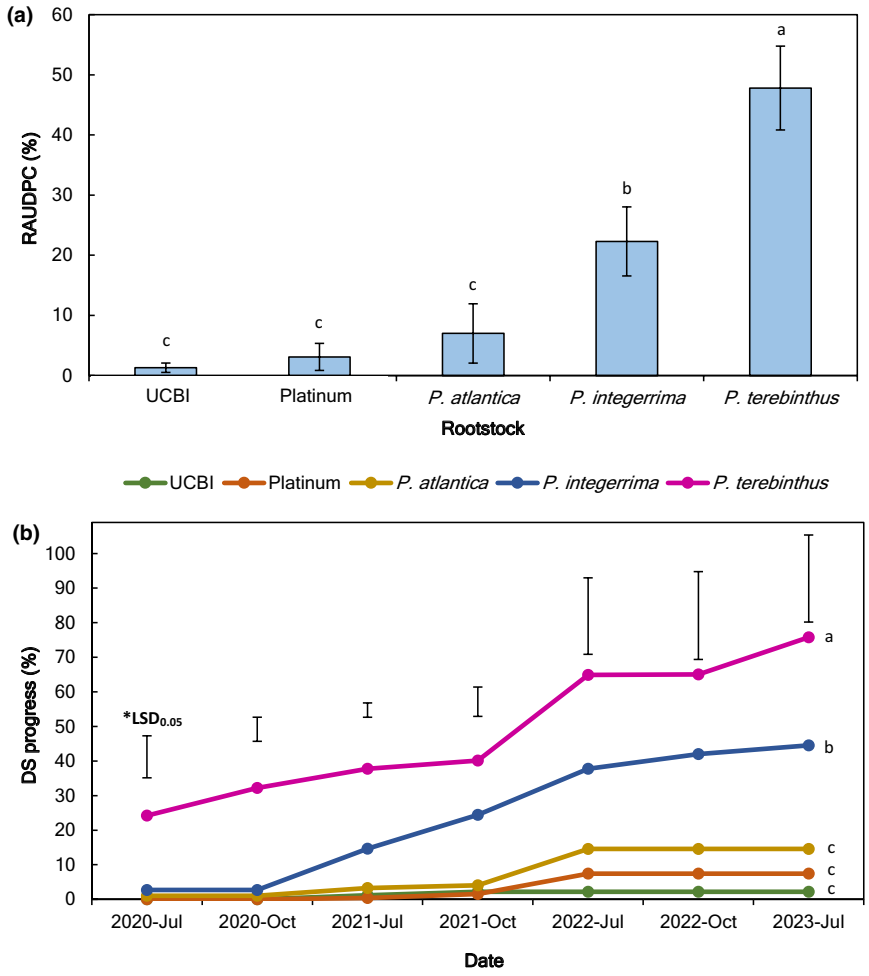


FIGURE 2 Relative area under the disease progress curve (RAUDPC) (a) and disease severity (DS) progress (b) in *Pistacia vera* 'Kerman' grafted on *Pistacia atlantica*, *Pistacia integerrima*, *Pistacia terebinthus*, Platinum and UCBI hybrids rootstocks growing in a soil highly infested by *Verticillium dahliae* located in Pinos Puente (Granada province, Spain). Data are the means of 16 replicated plants per rootstock and vertical bars the standard error of the means. Columns or points with common letters do not differ significantly according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985). LSD bars (*) represent the critical values for comparison at $\alpha = 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Verticillium wilt disease-related parameters for *Pistacia vera* 'Kerman' plants grafted on different rootstocks growing in a soil highly infested by *Verticillium dahliae* located in Pinos Puente (Granada province, Spain).

Rootstock	Incidence (%)[a]	Mortality (%)[a]	Disease severity (%)[b]	RAUDPC (%)[b]
P. terebinthus	100.0 a	62.5 a	75.8 ± 9.58 a	47.8 ± 6.97 a
P. integerrima	83.3 b	18.8 b	44.5 ± 16.64 b	22.3 ± 5.74 b
P. atlantica	29.2 c	12.5 b	14.6 ± 11.97 c	7.0 ± 4.93 c
Platinum	25.0 c	6.3 b	7.4 ± 5.89 c	3.1 ± 2.25 c
UCBI	25.0 c	0.0 c	2.2 ± 1.27 c	1.3 ± 0.78 c

Abbreviations: *P. terebinthus*, *Pistacia terebinthus*; *P. integerrima*, *Pistacia integerrima*; *P. atlantica*, *Pistacia atlantica*; RAUDPC, relative area under the disease progress curve.

[a]In each column, data are the mean of 16 replicated plants ± *SE* and, numbers followed by the same letter do not differ significantly according to multiple comparisons tests for proportions at $\alpha = 0.05$ (Zar, 2010).

[b]In each column, data are the mean of 16 replicated plants ± *SE* and, numbers followed by the same letter do not differ significantly according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985).

zone throughout all the evaluations, except in the first observation in July 2019, where no dead plants were detected (Figure 5). The percentage of dead plants of pistachios grafted on the hybrid UCBI in the high-density zone was not significantly different from that of pistachios grafted on *P. terebinthus* in the no-inoculum zone (Figure 4a,b).

3.3 | Fungal isolation and pathotype characterization

Verticillium dahliae infection was confirmed by isolation of the fungus in both fields. In this study, and according to Mercado-Blanco et al. (2003), only non-defoliating-specific amplicons (824 bp) were

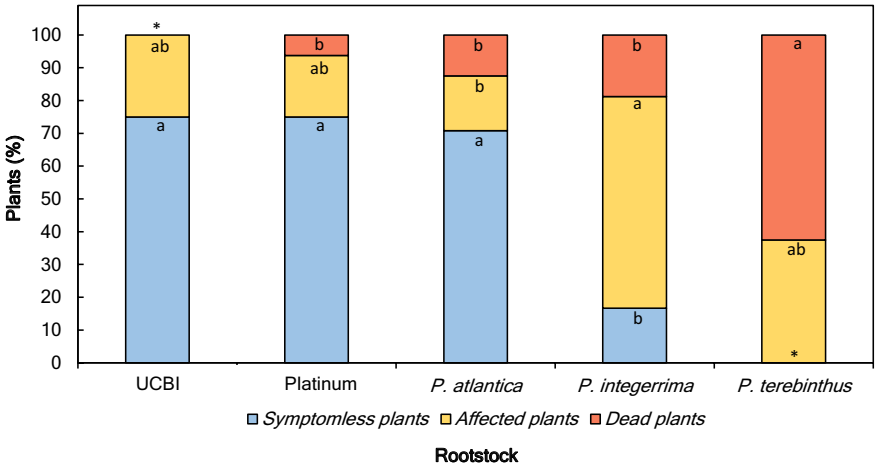


FIGURE 3 Percentage of symptomless, affected and dead plants of *Pistacia vera* 'Kerman' grafted on *Pistacia atlantica*, *Pistacia integerrima*, *Piatacia terebinthus*, Platinum and UCBI hybrids growing in a soil highly infested by *Verticillium dahliae* located in Pinos Puente (Granada province, Spain). For each level of symptom severity, columns with common letters do not differ significantly according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985). No dead plants were observed for UCBI and no symptomless plants for *P. terebinthus* (*). [Colour figure can be viewed at wileyonlinelibrary.com]

obtained; therefore, only the non-defoliating pathotype of the fungus was detected in all symptomatic pistachio trees evaluated.

4 | DISCUSSION

Verticillium wilt is one of the main diseases of pistachio, causing serious damage and production losses. So far, the use of tolerant or resistant rootstocks is the most effective and economical control measure against this disease. To our knowledge, this is the first study that compares *P. atlantica*, *P. integerrima*, *P. terebinthus* and Platinum and UCBI hybrids, in their response to Verticillium wilt, and the first that compares pistachio rootstocks in soils naturally infested with *V. dahliae* under Mediterranean conditions.

In the trial evaluating the effect of rootstocks against the disease progress (Pinos Puente plot), Kerman pistachio trees grafted on *P. terebinthus* showed much higher mortality and disease parameters than the rest of the rootstocks evaluated. This study confirms under field conditions what has been cited by other authors, in which *P. terebinthus* is described as a rootstock susceptible to the disease. (Ak et al., 2016; Ferguson & Haviland, 2016; López-Moral et al., 2023; Moral et al., 2010; Stavrides, 1994; Teviotdale et al., 1995; Vahdati et al., 2021). However, it is important to consider that the seeds used to grow the plants for this species were collected from a group of trees located in a specific region, so it is not representative of the whole species. In this regard, it has previously been reported that important intraspecific genetic variability exists within *P. terebinthus* (Guney et al., 2021; Stavrides, 1994; Ziya-Motalebipour et al., 2016). Although the variation of different populations of *P. terebinthus* to *V. dahliae* has not been extensively investigated, in a preliminary study, Raabe and Wilhelm (1978) found a *P. terebinthus* seed population with a promising level of resistance. However, according to our results and previous studies, it seems *P. terebinthus* is generally susceptible to *V. dahliae*. Nevertheless, exploitation of the intraspecific genetic diversity may lead to the development of *P. terebinthus* populations with increased resistance against the disease, as well as interspecific crosses with other more resistant *Pistacia* species (Werner et al., 2001; Ziya-Motalebipour et al., 2016).

Pistacia atlantica and *P. integerrima* are generally considered susceptible and resistant to *V. dahliae*, respectively (Epstein et al., 2004; Ferguson & Haviland, 2016; Teviotdale et al., 1995). However, in our trial, *P. integerrima* was found to be more susceptible to disease than *P. atlantica*, demonstrating intraspecific variability within these species. Indeed, pistachio trees grafted on *P. atlantica* showed no significant differences in RAUDPC, DS and DI compared to those grafted on UCBI and Platinum hybrids. Even though it usually has a high susceptibility level, variability in *P. atlantica* resistance to *V. dahliae* has been previously reported. For example, Raabe and Wilhelm (1978) and Triki et al. (2014) found variability in Verticillium wilt resistance of *P. atlantica* seedlings from different sources. Morgan et al. (1992) described that the KAC *P. atlantica* clone is more resistant to the disease than the standard *P. atlantica*, based on vascular colonization by *V. dahliae*. This specific KAC *P. atlantica* female tree is the parent of the *V. dahliae* resistant UCBI hybrid, while other *P. atlantica* × *P. integerrima* hybrids obtained from different *P. atlantica* sources, such as the PGII hybrid, have been classified as susceptible to the disease (Epstein et al., 2004; Morgan et al., 1992). This difference in the resistance level must be due to genetic diversity in the parents of the hybrid. However, for the rootstock UCBI, variability in the resistance level to *V. dahliae* has not been reported, although phenotypic resistance for other traits such as vigour exists (Jacygrad et al., 2020). Moreover, all *Pistacia* species are dioecious and can easily cross among them (Sheikhi et al., 2019) and therefore species classification can sometimes be problematic: for example, Morgan et al. (1992) highlighted that the female parent of the UCBI hybrid was first classified as a cross between *P. terebinthus* × *P. lentiscus*, until its classification as *P. atlantica* 'KAC'. The parentage of the entries studied in Morgan et al. (1992) were not authenticated and could be interspecific hybrids. In our work, DNA authentication has secured the identity of both *P. atlantica* and *P. integerrima* seed rootstock used. Previous studies (Epstein et al., 2004; Morgan et al., 1992) found a high level of resistance to Verticillium wilt of two *P. integerrima* seed sources: KAC *P. integerrima* (male parent of UCBI) and the seedling population Pioneer Gold. In contrast, we found a moderate response in *P. integerrima*, intermediate between the highly susceptible

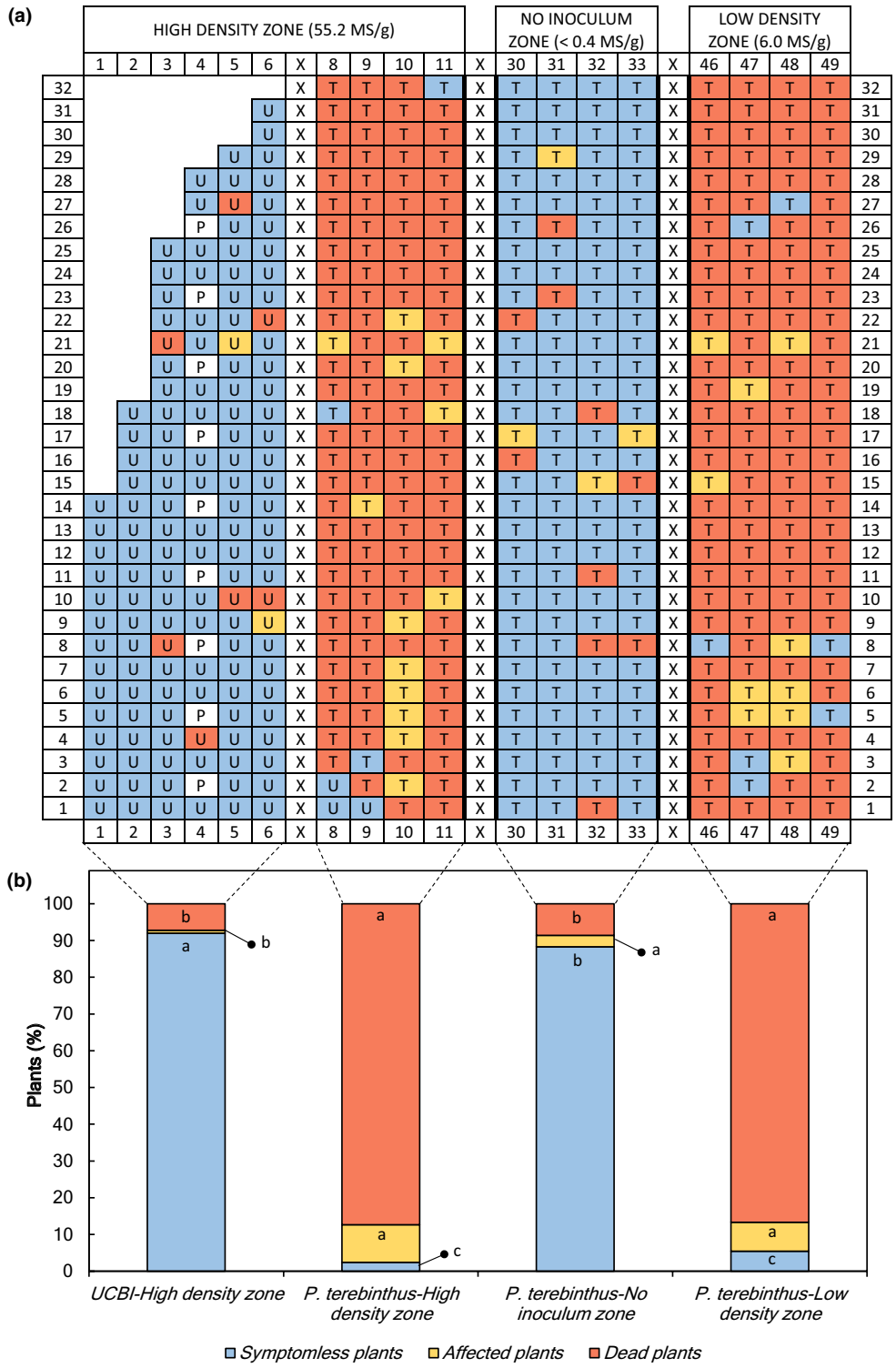


FIGURE 4 (a) Distribution of the high-density inoculum, no-inoculum and low-density zones in Alhama de Granada plot established in a soil infested by *Verticillium dahliae*, showing *Pistacia vera* 'Kerman' plants grafted on UCBI hybrid (U) and *Pistacia terebinthus* (T) rootstocks; P, pollinator. Colours indicate the level of disease incidence as follows: symptomless plants (blue), affected plants (yellow) and dead plants (orange). (b) Percentage of symptomless, affected and dead plants on UCBI hybrid (U) and *P. terebinthus* (T) rootstocks in zones of high-density inoculum (U) or high density, low density and no inoculum (T). Columns with common letters do not differ significantly according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985). [Colour figure can be viewed at wileyonlinelibrary.com]

P. terebinthus and the resistant hybrids UCBI and Platinum. *P. integerrima* has always been considered the main source of resistance to Verticillium wilt in the *Pistacia* genus. However, genetic variability in this species in general, and its resistance to *V. dahliae* in particular, have not been extensively studied. In their study of 1978, Raabe and Wilhelm identified a source of *Pistacia*

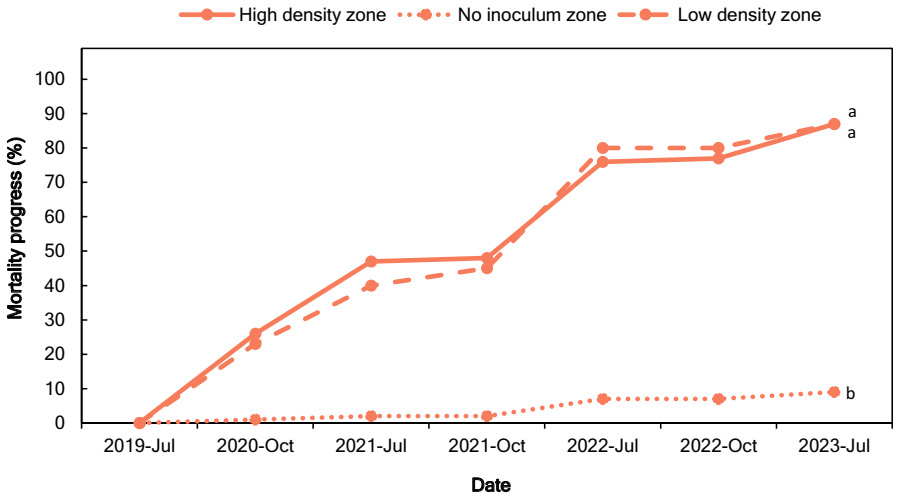


FIGURE 5 Mortality progress in *Pistacia vera* 'Kerman' grafted on *Pistacia terebinthus* in high-density, no-inoculum and low-density zones in Alhama de Granada plot established in a soil infested by *Verticillium dahliae*. Points with common letters do not differ significantly according to Fisher's protected LSD test at $\alpha = 0.05$ (Steel & Torrie, 1985). [Colour figure can be viewed at wileyonlinelibrary.com]

chinensis highly susceptible to the disease. It is noteworthy that what is now recognized as *P. integerrima* was classified at that time as a subspecies of *P. chinensis*. Nevertheless, subsequent genetic analyses, such as those conducted by Yi et al. (2008), have shown a close genetic relationship between both species. It is believed that only five accessions entered the United States and current hybrids were derived from some of them, so only a small portion of the genetic variability has been explored in this species. Intraspecific variability in Verticillium wilt resistance is common in other species such as cacao (*Theobroma cacao*) (Braga et al., 1989) olive (Trapero et al., 2015) and strawberry (*Fragaria* × *ananassa*) (Shaw et al., 2010). According to other studies (Ahmadi-Afzadi et al., 2007; Karci et al., 2022; Khadivi, 2018; Parfitt & Badenes, 1997), there is a high genetic variability within the genus *Pistacia* and, more specifically, of the evaluated species, which agrees with the variability of resistance to Verticillium wilt found in our study. Likewise, the use of wild pistachios in breeding programmes could be beneficial for generating new genotypes with interesting traits (Kafkas et al., 2023; Pourian et al., 2019).

Another interesting result is the lack of significant differences between the UCBI and Platinum hybrids in terms of RAUDPC, DS and DI, although UCBI did not present any dead plants, while Platinum had a mortality of 6.3% (one of the 16 plants). Specifically, the temporal progress of disease incidence since the beginning of the trial was the same for these hybrids. As previously mentioned, Platinum is a clone within the PGII seedling population selected for its greater resistance to the disease, although both UCBI and PGII were obtained from a cross between *P. integerrima* and *P. atlantica* species (Sheikhi et al., 2019). It is notable that no pistachio grafted onto the UCBI hybrid died from the disease at the end of the experiment, an event that highlights its high resistance to *V. dahliae*.

In the Alhama de Granada plot, disease monitoring was carried out in an already established commercial field with trees grafted on *P. terebinthus* and the UCBI hybrid in areas with different inoculum densities of the fungus. There were no significant differences in the percentage of dead trees due to the disease between the plants grafted on the UCBI hybrid established in the high-density zone and *P. terebinthus* in the no-inoculum zone. This highlights the high resistance of the UCBI hybrid to the disease even in the presence of high density of the pathogen in the soil, confirming previous results (Epstein et al., 2004; Morgan et al., 1992). Likewise, for the pistachios grafted on *P. terebinthus*, there were no significant differences in the percentage of symptomless, affected, and dead plants due to the disease between the high-density zone and the low-density zone of fungal inoculum. The high-density zone contained an average of 55.2 MS/g of soil and the low-density zone an average of 6.0 MS/g of soil, which represents an important difference. Even in the no-inoculum zone, we isolated *V. dahliae* from plants and nearly 10% of the plants grafted on *P. terebinthus* died from the disease, possibly due to a very low inoculum level, difficult to detect by the method used but enough to cause symptoms (López-Escudero & Blanco-López, 2007; Varo et al., 2016). From these data, it is concluded that the *P. terebinthus* rootstock used in this study is susceptible to *V. dahliae* when there is inoculum in the soil, regardless of its concentration and even at levels under the detection limits for the wet sieving method. *P. terebinthus* is currently the most widespread pistachio rootstock in Spain and other Mediterranean areas due to its adaptation to the climate and soils in these regions. However, the high susceptibility of the species shown by this and previous observations (Ferguson & Haviland, 2016; Moral et al., 2010; Vahdati et al., 2021) highlights the importance of avoiding planting this rootstock in soils infected by *V. dahliae*, regardless of the inoculum concentrations in the soil and even in soils only suspected to have presence of the fungus.

It should be noted that in this study only the non-defoliating pathotype of *V. dahliae* was detected, in contrast to the defoliating isolates of the fungus, which are more frequent in olive orchards in this region (Mercado-Blanco et al., 2003; Trapero et al., 2013).

In summary, this study presents evidence of the high intraspecific variability within *P. atlantica* and *P. integerrima* in their response to Verticillium wilt. The *P. atlantica* seed source used in this study has proved to be highly resistant, not statistically different from the UCBI and Platinum hybrids. These results highlight the importance of rootstock choice when establishing a pistachio orchard and

its impact on the development of the disease caused by *V. dahliae*. Moreover, they show that average levels of resistance of a given species might be misleading due to intraspecific variability within *Pistacia* species, and specific seedling population or genotype chosen must be considered. Due to this fact, together with the low number of rootstocks highly resistant to *V. dahliae*, the preservation of *Pistacia* genetic resources is critical for the future of pistachio breeding and selection for disease resistance.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Raw data are available on reasonable request to the corresponding author.

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