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Evaluation of Olive Varieties Resistance for Sustainable Management of Verticillium Wilt

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Abstract: Verticillium wilt resulting from infection by *Verticillium dahliae* is one of the most devastating soilborne fungi of the olive tree (*Olea europaea* L.) worldwide. The pathogen infects a wide variety of plants and can survive in the soil for many years, and chemicals cannot control it. Therefore, sustainable disease management strategies are suggested, with the exploitation of host resistance as the most predominant control measure in practice. In addition, disease risk assessment in commonly used plant genotypes is a prominent issue. In this respect, nine commercially grown Greek olive varieties ('Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', and 'Tragolia') and one variety of international interest ('Picual') were comparatively evaluated for their resistance to *V. dahliae*. The roots of young plants were immersed in a concentrated conidial suspension in order to perform an artificial inoculation. We evaluated disease reactions in a 140-day assessment period based on external symptoms (disease severity, disease incidence, and mortality) and calculated the relative areas under disease progress curves (relative AUDPC). The process of qPCR was used to evaluate *V. dahliae* DNA in vascular tissues and plant growth parameters (height and fresh weight). A cumulative stress response was calculated to consider the overall effect of *V. dahliae* on olive cultivars. The olive varieties resistance to *V. dahliae* varied significantly, with 'Koroneiki', 'Tragolia', and 'Atsiholou' being the most resistant. Interestingly, most tested varieties showed a significantly low resistance level, suggesting increased risk for the Greek olive industry due to *V. dahliae*.

Keywords: disease parameters; evaluation; plant phenotyping; resistance; sustainable management; qPCR quantification; *Verticillium dahliae*

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1. Introduction

Numerous pests and diseases commonly affect the olive tree (*Olea europaea* L.) [1]. *Verticillium dahliae* is a predominant soilborne pathogen that occurs in all olive-growing areas, commonly leading to severe yield losses and tree death [2,3]. Due to its broad host range and capacity to endure for more than a decade in the soil using resistant structures named 'microsclerotia' and benefiting from the lack of effective fungicides, *V. dahliae* is especially difficult to manage [4].

Studies carried out to date report that sustainable management of olive orchards is the most effective approach to control the disease in the field [5]. It has been proven that the most efficient control measures should not be applied individually. Emphasis has been given on sustainable control measures that can be applied before planting [3]. Exploiting plant resistance is the most ecological and effective method for controlling vascular wilt diseases. In particular, using tolerant varieties is the primary cost-efficient and long-lasting means to manage the Verticillium wilt of olive and is considered the basis of integrated

disease control strategy [2]. Furthermore, risk assessment for disease management could be determined by obtaining useful information of resistant genotypes. It is also essential to evaluate the possible climate change effects on the impact of Verticillium wilt on olive groves [6]. Therefore, to date, numerous olive genotypes originating from major olive-producing countries such as Spain, Italy, Turkey, and Greece have been screened for their resistance to *V. dahliae* [7–16]. Additionally, progenies produced from resistant and sensitive varieties have been studied in order to identify the paternal or maternal effect on the heritability of resistant genes related to *V. dahliae* [17]. It was recently reported that exploring genetic resistance through the genes related to *V. dahliae* is one of the most effective [18], environmentally friendly, and economically viable [17] measure to control Verticillium wilt. Moreover, available omics approaches could unravel the basis of Verticillium wilt resistance [5]. Another study investigated Verticillium wilt and olive interaction by a different perspective, focusing on the relationship of olive traits with resistance to *V. dahliae* [19].

Spain, Italy, and Greece are among the leading olive-producing countries. In Greece, olive growing occupies more than 50% of cultivated land, producing 1,525,543 tons of olives in 2019 [20]. Even though olive cultivation has a significant role in the Greek agriculture industry, little is known about the resistance of Greek olive genotypes to this pathogen [7,14,21]. In contrast to other olive-producing countries, only three ('Amfissis', 'Kalamon', and 'Koroneiki') out of dozens of varieties that are widely used in Greece have been experimentally screened for their resistance to *V. dahliae*. Estimating the resistance level of other commercial varieties has been done empirically.

Given all the above, our main goal was the comparative evaluation of the resistance of Greek olive varieties to *V. dahliae* by employing a root-dipping inoculation method and considering several disease and plant growth parameters. *V. dahliae* colonization was also assessed by determining fungal DNA quantity in plant vessels.

2. Materials and Methods

2.1. Plant Material

We used four-month-old rooted cuttings of the varieties 'Amfissis', 'Atsiholou', 'Chalki dikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia'. Plants were derived from semi-hardwood stem cuttings of each variety rooted in a mist unit at Kostelenos Nurseries. The plant nursery has obtained certified initial material from the official plant nursery of our research center (ELGO-DIMITRA). 'Amfissis' and 'Picual' are susceptible, whereas 'Koroneiki' is resistant to *V. dahliae* [12,14,22]; these were included in the experiments as reference varieties with known susceptibility to Verticillium wilt.

2.2. Pathogen Preparation

A *V. dahliae* isolate (code Vd.El.2.6.Kor) originating from a diseased tree of the olive variety 'Koroneiki' in Almyros, Thessaly, Greece, was used in artificial inoculation experiments. The isolate was identified according to its morphological characteristics [23] and an internal transcribed spacer region of ribosomal DNA (rDNA-ITS) gene sequencing. For long-term storage, a suspension of 10^7 conidia mL^{-1} in 25% (v/v) aqueous glycerol solution was preserved at −80 °C. Prior to use, we transferred the fungus to potato dextrose agar (PDA) at 24 °C for two weeks. Conidia were produced by growing the fungus in potato dextrose broth (PDB) at 160 rpm and 24 ± 0.5 °C for six days; we harvested conidia by filtration through three layers of cheesecloth and centrifuged the suspension at 3000× g for 10 min. Spores were resuspended using distilled water to a concentration of 1×10^7 spores mL^{-1}.

2.3. Plant Inoculation

We artificially inoculated twenty-five plants of each of the ten olive varieties following the method of Lopez-Escudero et al. [12]. In brief, we removed plants from the soil mixture and washed the roots with water; then, their roots were immersed in a conidial suspension of *V. dahliae* (1×10^7 spores mL^{-1}) for 30 min. A similar procedure was realized for

five plants of each variety by using sterile distilled water instead of fungal suspension (control). After inoculation, we established the plants in individual 3-L containers filled with sterilized substrate, and grew them in a non-air-conditioned greenhouse at 20 ± 8 °C, applying a 12-h photoperiod for 140 days. We used five trees for each variety and repeated the experiment five times (25 plants per variety and treatment).

2.4. Disease Assessment and Plant Growth

We recorded Verticillium wilt-associated visual evidence on plants at 10-day periods from 10 to 140 days post inoculation (dpi). We estimated the disease severity index at each date, the relative area under the disease progress curve (relative AUDPC), final disease incidence, and mortality, according to [24]. In particular, for the disease severity index we used a visual scale from 0 to 4, by measuring the plant leaves affected (0 = healthy plant or plant with no symptoms, 1 = 1–25% of the plant affected, 2 = 26–50% of the plant affected, 3 = 51–75% of the plant with symptoms, and 4 = deceased or almost deceased plant). We plotted disease rates over time and generated the curves of disease progress. Next, we calculated the area under disease progress curve (AUDPC) by applying the method of trapezoidal integration [25]. We calculated the disease incidence as a percentage of the maximum value reached over the trial period as relative AUDPC [26]. Finally, we estimated the final disease incidence using the percentage of plants that were infected at 140 dpi, whereas mortality was estimated based on the percentage of deceased plants at 140 dpi.

We clipped off all plants at the soil surface level just after the last disease scoring (at 140 dpi) and measured their height and fresh weight for estimating the effect of *V. dahliae* on the growth of plants.

For the verification of *V. dahliae* presence in the plants' vascular system, we performed the following procedure. We surface-sterilized the stems of three plants per variety by spraying 93% ethyl alcohol and then passed them over a flame three times. Subsequently, we removed the phloem, aseptically sampled five xylem chips from each stem, and placed them in Petri dishes containing acidified potato dextrose agar (PDA). We incubated the plates at 24 °C without light for two weeks. We examined the fungal colonies that grew out of tissue excisions using a light microscope and then we identified them as *V. dahliae* based on the colonies' morphology [23].

2.5. DNA Extraction

To quantify the pathogen DNA in vascular tissues of our experimental plants, we randomly selected the stems of three plants per replication and variety (15 stems per variety). In particular, the stems of 15 plants per variety were destructively sampled (5 mixed samples, each of three grouped plants) by cutting into 2–3 mm long pieces after removing the phloem and then stored at −20 °C. Xylem tissues were freeze-dried with liquid nitrogen, and we ground them to a fine powder via autoclaved mortar and pestle. Subsequently, the total DNA was isolated using the cetyltetramethyl ammonium bromide (CTAB) method [27], with slight modifications. We homogenized 100 mg of wood tissue using a mortar and pestle in the presence of liquid nitrogen. We transferred the wood powder to a 1.5 mL Eppendorf tube and added 500 µL of 2X CTAB extraction buffer (100 mMTris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 0.5% *v/v* β-mercaptoethanol) and homogenized it. We incubated our samples at 65 °C for 45 min with periodical vortexing, followed by centrifuging at 10,000 rpm for 10 min. The supernatant (~250 µL) was transferred to clean tubes, and equal amounts (~250 µL) of phenol:chloroform:isoamyl alcohol (25:24:1) were added and mixed by vortexing. We centrifuged the samples at 13,000 rpm for 15 min. We transferred the aqueous phase (~200 µL) into new tubes, added an equal amount of chilled isopropanol, quickly and gently inversed, and then incubated at −20 °C overnight. The DNA pellet was precipitated at 13,000 rpm for 20 min, washed using 500 µL of 70% ethanol, and then precipitated at 13,000 rpm for 5 min. Subsequently, the DNA pellet was suspended in 40 µL of Tris-HCL (10 mM, pH = 8). Next, 2 µL RNAse A (5 mg mL^{-1}) was added, followed by incubation at 50 °C for 15 min. We used a Q5000

UV-Vis Spectrophotometer (Quawell, San Jose, CA, USA) to determine the quantity and purity of DNA. We adjusted the DNA concentration of each isolate to 20 ng mL^{-1} and then stored the samples at −20 °C until further analysis.

2.6. Pathogen qPCR Quantification

We conducted real-time quantitative PCR (qPCR) assays to detect and quantify *V. dahliae* DNA in olive samples. We amplified the *V. dahliae* ITS region via primers ITS1-F (5′-CCGCCGGTCCATCAGTCTCTCTGTTTATAC-3′) and ITS2-R (5′-CGCCTGCGGGACTC CGATGCGAGCTGTAAC-3′) [14,22]. To normalize small differences in total DNA template quantities, we used the olive actin gene as an internal standard. The amplification of the actin gene was performed with the primer pair OeACT-F (5′-ATCCTCACAGAGCGTGG-3′) and OeACT-R (5′-CGATCATTGAAGGCTGG-3′) [14,22]. All qPCR assays were carried out in a QuantStudio 3 Real-Time PCR System (ThermoFisher, Waltham, MA USA) by using the PowerUp™ SYBR® Green Master Mix kit (ThermoFisher, Waltham, MA USA). The qPCR performance included an initial denaturation at 95 °C for 3 min; then 40 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and 30 s of extension at 72 °C; and, finally, an extension step at 60 °C for 1 min. The relative DNA quantity of *V. dahliae* was determined via the $2^{-\Delta\Delta CT}$ method [28]. We performed real-time qPCR reactions twice, and we analyzed melting curves to confirm the absence of nonspecific products and primer dimers.

2.7. Cumulative Stress Response Index (CSRI)

The overall plant response in unfavorable environmental conditions is observed by the cumulative stress response index, which shows how a specific stress affects a genotype: sensitivity or resistance. A CSRI based on Koubouris et al. [29] and Markakis et al. [30], with modifications, was determined to assess the overall response of olive cultivars to Verticillium wilt. The following equation was used:

$$CSRI = [(PHt - PHc)/PHc + (PFWt - PFWc)/PFWc + (PSt - PSc)/PSc] \times 100$$

where

- CSRI = cumulative stress response index,
- PH = plant height,
- PFW = plant fresh weight,
- PS = plant survival,
- t = treatment,
- c = control.

Genotype classification was based on the following: tolerant [>minimum CSRI + 2 standard deviation (stdev)], intermediate (>minimum CSRI + 1 stdev and <minimum CSRI + 2 stdev), and sensitive (<minimum CSRI + 1 stdev).

2.8. Statistics

Analysis of variance (ANOVA) was performed to identify the impact of replication (1, 2, 3, 4, or 5) and variety ('Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia') and the interaction between replication and variety on the relative area under disease progress curve (RAUDPC), final disease severity (FDS), disease incidence (DI), mortality (M), plant fresh weight (W), and plant height (H) (Table 1). Homogeneity of variance across treatments was assessed, and an arcsin transformation was applied to normalize variance, followed by ANOVA. The mean values were compared using Tukey's honestly significant difference test, after a significant F-test was obtained for treatments ($p \leq 0.05$).

Table 1. Analysis of variance for disease incidence (DI), final disease severity (FDS), mortality (M), relative area under disease progress curve (RAUDPC), plant height (H), and plant fresh weight (W) for 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia', artificially inoculated with *Verticillium dahliae*.

Source	*F* Values [a]						
	df [b]	DI	FDS	M	RAUDPC	H	W
Replication	4	0.113	0.687	0.034	0.471	0.956	1.647
Variety	9	33.410 ***	48.778 ***	29.984 ***	28.114 ***	32.850 ***	43.984 ***
Replication × Variety	36	-	1.234	-	1.576	1.178	1.574

[a] Symbol '***' indicates significance at $p \leq 0.001$ level, according to the F-test. [b] Degrees of freedom between groups.

3. Results

3.1. Symptom Development

The onset of symptom development was observed 20 days post artificial inoculation (dpi) in 'Mastoidis' plants showing primarily slight leaf chlorosis and flaccidity. The affected plants in this variety exhibited typical Verticillium wilt symptoms, such as leaf yellowing, wilting, and defoliation, after another ten days (at 30 dpi), and the disease severity index steadily increased with time (Figure 1). Most varieties exhibited rapid progress of symptom severity within a 60-day span (between 50 dpi and 110 dpi), reaching average disease severity indices greater than 3 (on a 0–4 grade scale) at 110 dpi. In the case of 'Megaritiki', the sharp increase in symptom development occurred comparatively later (between 90 dpi and 130 dpi), with the average disease severity index ranging from 0.56 at 90 dpi to 3.43 at 130 dpi. (Figure 1). However, slower disease development reaching final disease severity values 1.62 and 1.64 at 140 dpi, respectively (Figure 1 and Table 2), and less prominent symptoms were observed for 'Atsiholou' and 'Tragolia'. Moreover, disease progress in 'Koroneiki' was practically absent, with disease severity index values ranging from 0.16 to 0.46 over the whole assessment period.

Table 2. Mean values (± standard errors) of disease and growth indicators on 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia' olive varieties, 140 days post artificial inoculation with *Verticillium dahliae*.

Variety	Disease Parameters [a]			Growth Parameters [x]	
	Disease Incidence (%) [b]	Final Disease Severity (Scale 0–4) [c]	Mortality (%) [d]	Plant Height (cm) [y]	Plant Fresh Weight (gr) [z]
Amfissis	**100.00 ± 0.00 a**	**3.66 ± 0.18 a**	**84.00 ± 9.80 a**	**30.28 ± 0.72 c**	**2.43 ± 0.35 b**
Atsiholou	60.00 ± 8.94 b	1.62 ± 0.34 b	32.00 ± 10.20 b	39.28 ± 1.00 a	8.09 ± 0.87 a
Chalkidikis	100.00 ± 0.00 a	3.90 ± 0.10 a	96.00 ± 4.00 a	28.68 ± 0.61 c	2.13 ± 0.19 b
Koroneiki	**24.00 ± 7.48 c**	**0.46 ± 0.18 c**	**0.00 ± 0.00 c**	**40.08 ± 0.92 a**	**9.75 ± 0.51 a**
Kothreiki	100.00 ± 0.00 a	4.00 ± 0.00 a	100.00 ± 0.00 a	29.52 ± 0.49 c	2.46 ± 0.12 b
Koutsourelia	100.00 ± 0.00 a	4.00 ± 0.00 a	100.00 ± 0.00 a	36.68 ± 1.18 a,b	3.09 ± 0.20 b
Mastoidis	96.00 ± 4.00 a	3.67 ± 0.18 a	88.00 ± 8.00 a	34.42 ± 0.65 b	2.78 ± 0.41 b
Megaritiki	100.00 ± 0.00 a	3.65 ± 0.12 a	80.00 ± 10.95 a	29.00 ± 0.62 c	3.62 ± 0.25 b
Picual	**100.00 ± 0.00 a**	**3.78 ± 0.11 a**	**88.00 ± 4.90 a**	**36.80 ± 0.91 a,b**	**2.46 ± 0.27 b**
Tragolia	64.00 ± 7.48 b	1.64 ± 0.32 b	24.00 ± 4.00 b,c	38.96 ± 0.79 a	8.50 ± 0.84 a

[a, x] Disease and growth parameters on plants were evaluated at 140 days after immersing their root system in a 1.0×10^7 conidia mL^{-1} suspension of *Verticillium dahliae*. [b, d] Mean disease incidence and mortality of 25 plants per variety were estimated as the percentage of infected plants and dead plants, respectively. Arcsin transformation was performed prior to statistical analysis. [c] Mean final disease severity of 25 plants per variety was based on a visual scale from 0 (healthy plant) to 4 (dead plant). [y, z] Olive plants were clipped off at the soil surface level, and their height and fresh weight were measured. Varieties with known susceptibility that were used as reference are indicated in bold (Amfissis and Picual are susceptible, whereas Koroneiki is resistant to *V. dahliae*). Within columns, values with different letters differ significantly according to Tukey's honestly significant difference test ($p \leq 0.05$).

At the end of the experiment (140 dpi), final disease severity and disease incidence in 'Amfissis', 'Chalkidikis', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', and 'Picual' were significantly higher compared to 'Atsiholou', 'Koroneiki', and 'Tragolia' (Figure 2, Table 1). Moreover, both final disease severity and disease incidence parameters in Koroneiki were significantly lower than in 'Atsiholou' and 'Tragolia'. Similarly, 'Atsiholou', 'Koroneiki', and 'Tragolia' exhibited significantly lower plant mortality compared to the rest of the varieties tested, with 'Koroneiki' showing significantly lower values than 'Atsiholou' but non-significantly lower values than 'Tragolia'. We observed a non-significant difference between 'Atsiholou' and 'Tragolia', in terms of plant mortality (Table 1). We isolated the pathogen in all olive varieties infested by *V. dahliae*. We did not observe external symptoms nor positive re-isolations in the control throughout the experiment.

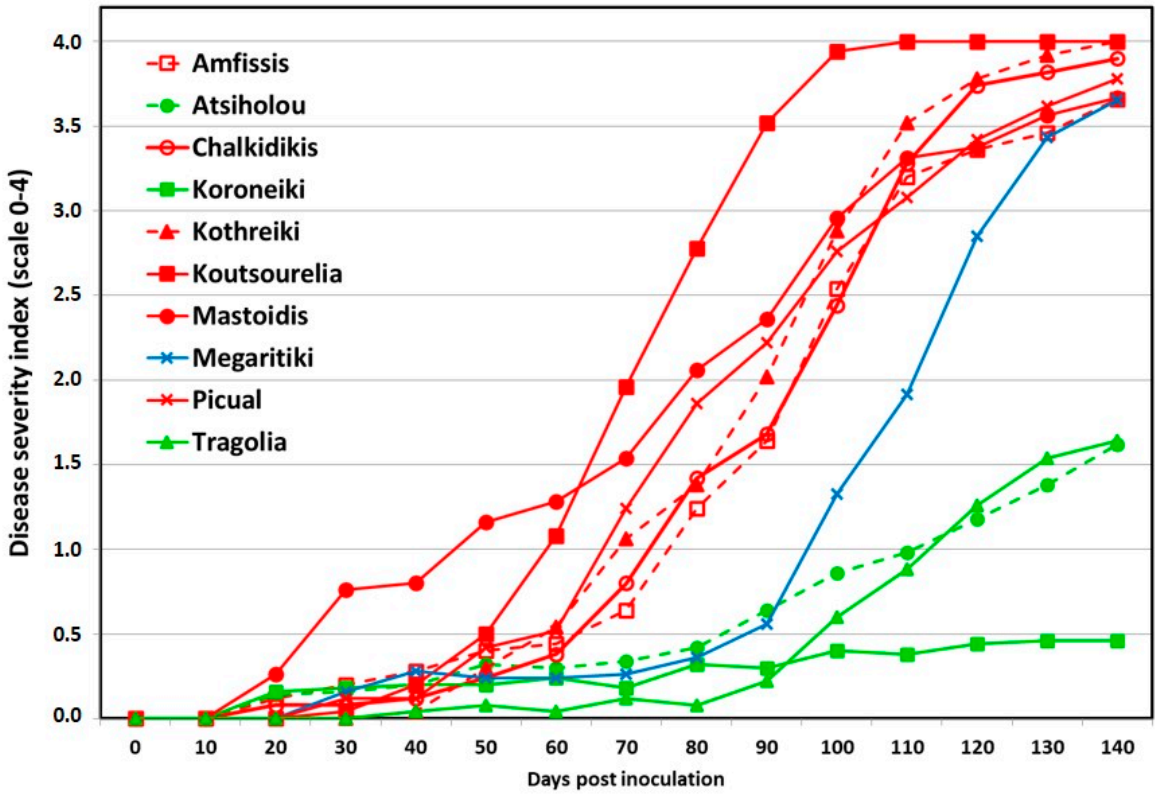

Figure 1. Disease severity index of 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia' olive varieties at 10-day intervals up to 140 days after immersing plant roots into *Verticillium dahliae* conidial suspension. The disease severity was recorded using a 0–4 scale and indicates the percentage of plant leaves with symptoms (0 = healthy plants, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = dead or almost dead plants). Each point represents the mean of 25 plants.

The relative AUDPC analysis showed that symptom development in 'Atsiholou', 'Koroneiki', and 'Tragolia' plants was markedly inferior compared to 'Amfissis', 'Chalkidikis', 'Kothreiki', 'Koutsourelia', 'Mastoidis', and 'Picual', proving their resistance against *V. dahliae* (Figure 3). A moderate resistance was recorded in 'Megaritiki' based on relative AUDPC that was not markedly different from the value of the resistant varieties 'Atsiholou' and 'Tragolia' or the susceptible ones 'Amfissis' and 'Chalkidikis'. We also observed significant differences among the susceptible varieties, with 'Koutsourelia' demonstrating higher relative AUDPC values than 'Amfissis' and 'Chalkidikis' (Figure 3).

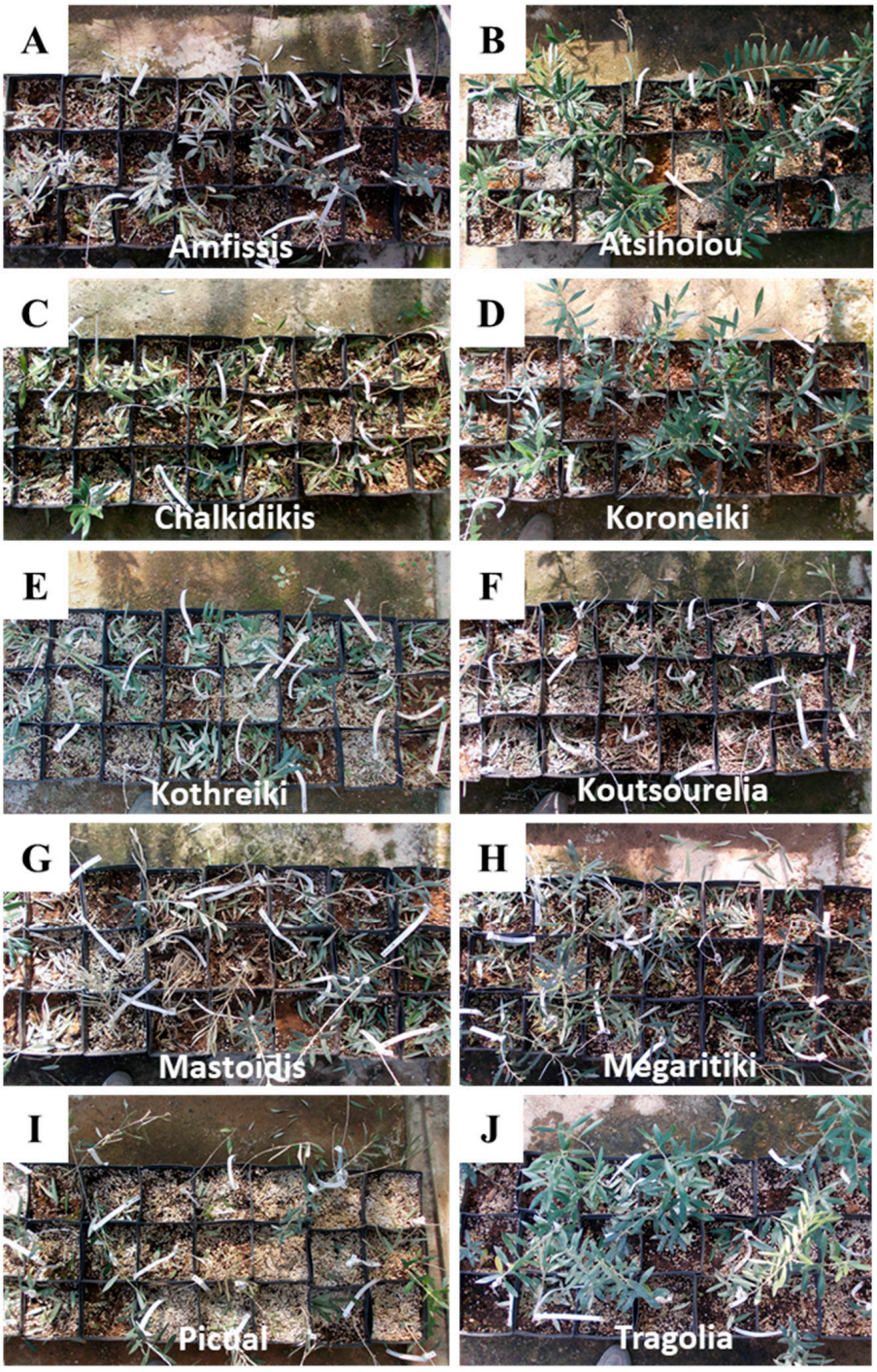

Figure 2. Disease reaction of ten olive varieties treated with Verticillium dahliae, 140 days post artificial inoculation. (**A**) 'Amfissis', (**B**) 'Atsiholou', (**C**) 'Chalkidikis', (**D**) 'Koroneiki', (**E**) 'Kothreiki', (**F**) 'Koutsourelia', (**G**) 'Mastoidis', (**H**) 'Megaritiki', (**I**) 'Picual', and (**J**) 'Tragolia'. Disease reaction of each plant based on a visual scale from 0 to 4, considering the percentage of plant leaves affected (0 = healthy plant or plant with no symptoms, 1 = 1–25% of the plant affected, 2 = 26–50% of the plant affected, 3 = 51–75% of the plant affected, and 4 = dead or almost dead plant).

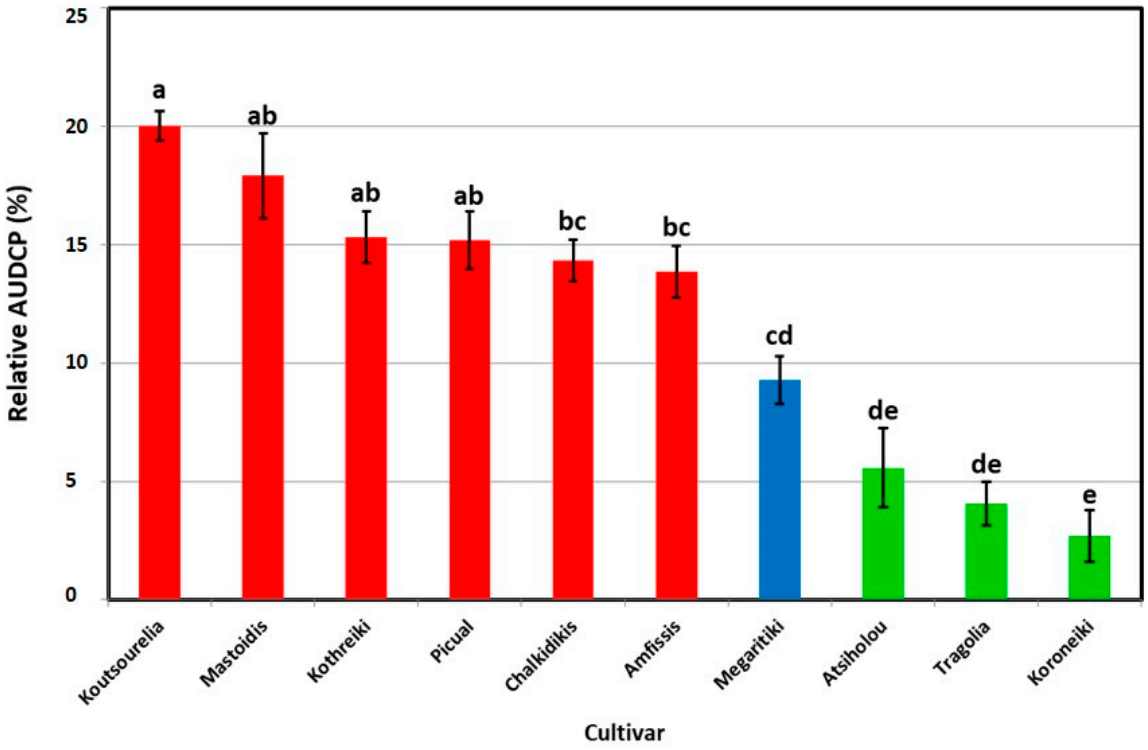

Figure 3. Relative area under the disease progress curve (relative AUDPC) with reference to the maximum value potentially reached over the 140-day assessment period of 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia' olive varieties after immersing their roots into a 1×10^7 mL^{-1} *Verticillium dahliae* conidial suspension (Korolev et al. 2001). When the columns are followed by the same letter (a–e), they are considered statistically similar according to Tukey's honestly significant difference test ($p \leq 0.05$). We present the average of 25 olive trees in each column, with the standard errors depicted as vertical bars.

3.2. Effects of V. dahliae Inoculation on Plant Growth

The observations of plant growth indicators are shown in Table 2. Inoculated plants of 'Atsiholou', 'Koroneiki', and 'Tragolia' developed significantly higher fresh weight than the remaining seven varieties, whereas their plant height was significantly higher than that of 'Amfissis', 'Chalkidikis', 'Kothreiki', 'Mastoidis', and 'Megaritiki' but non-significant compared to 'Koutsourelia' and 'Picual' plants. Furthermore, 'Koutsourelia', 'Mastoidis', and 'Picual' showed significantly higher plant height than 'Amfissis', 'Chalkidikis', 'Kothreiki', and 'Megaritiki' (Table 2).

3.3. Verticillium Dahliae qPCR Quantification

The pathogen DNA was observed in all olive plants artificially infested by *V. dahliae*, but not in the plants used as controls. However, statistically significant differences in *V. dahliae* DNA quantity among varieties were revealed (df = 9, F = 6.753, $p \leq 0.001$). Significantly lower *V. dahliae* DNA quantities in the xylem tissues of Atsiholou', 'Koroneiki', and 'Tragolia' compared with 'Amfissis', 'Chalkidikis', 'Kothreiki', 'Koutsourelia', 'Mastoidis', and 'Picual' was associated with the decreased symptom severity. (Figure 4). Significant differences in *V. dahliae* DNA amounts were not detected between 'Megaritiki' and the susceptible or the resistant varieties, indicating intermediate vascular colonization of 'Megaritiki' tissues by the pathogen.

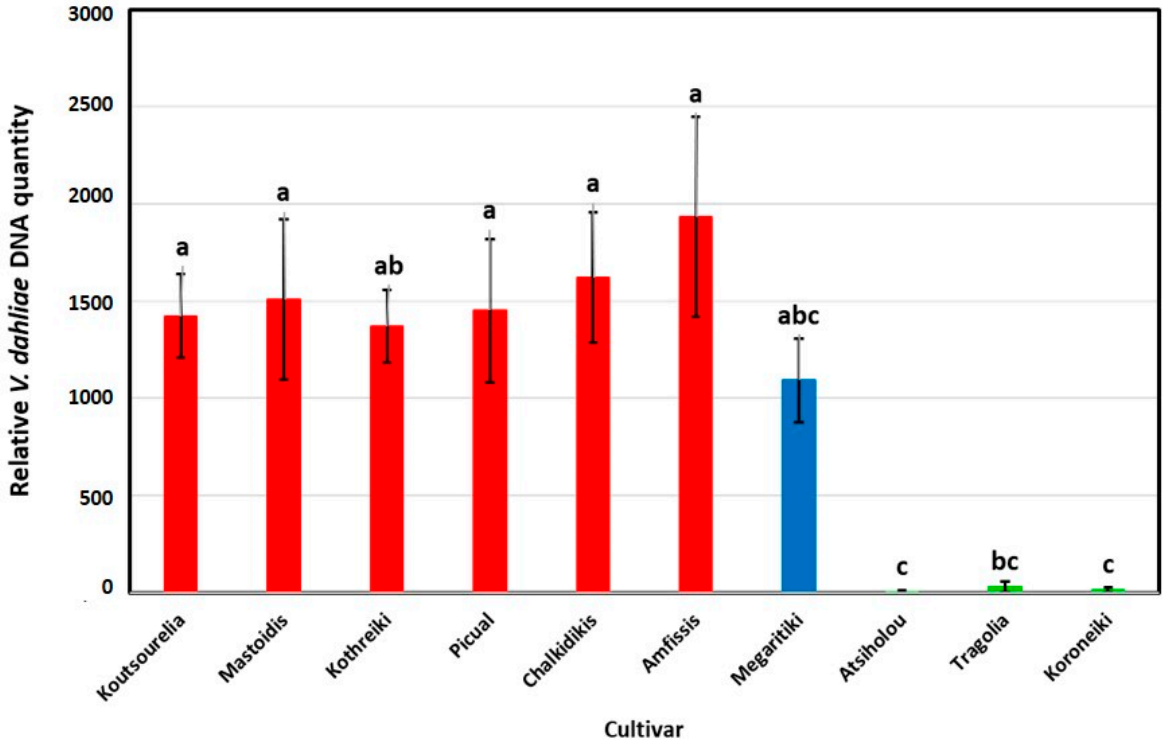

Figure 4. Relative *Verticillium dahliae* DNA quantity in 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia' olive varieties, 140 days after immersing their roots into a 1×10^7 mL^{-1} *Verticillium dahliae* conidial suspension. Columns followed by the same letter (a–c) are not significantly different according to Tukey's honestly significant difference test ($p \leq 0.05$). Each value represents the mean of five composite samples consisting of three pooled plants each, and vertical bars indicate standard errors.

3.4. Cumulative Stress Response Index (CSRI)

All ten olive varieties had negative CSRI for *V. dahliae* infection (Table 3). Based on this index, 'Koroneiki', 'Tragolia', and 'Atsiholou' were classified as tolerant, 'Koutsourelia', Kothreiki', Megaritiki' as intermediate, and 'Amfissis', 'Mastoidis', 'Picual', and 'Chalkidikis' as sensitive.

Table 3. Cumulative stress response index (CSRI) for 'Amfissis', 'Atsiholou', 'Chalkidikis', 'Koroneiki', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', 'Picual', and 'Tragolia' olive varieties artificially infested by *Verticillium dahliae* compared to the control plants, based on plant height and fresh weight.

Genotypes	Cumulative Stress Response Index (CSRI)	Rank [a]	Characterization
Amfissis	−184.52	7	Sensitive
Atsiholou	−79.66	3	Tolerant
Chalkidikis	−219.02	10	Sensitive
Koroneiki	−33.64	1	Tolerant
Kothreiki	−108.95	5	Intermediate
Koutsourelia	−94.41	4	Intermediate
Mastoidis	−188.72	8	Sensitive
Megaritiki	−144.54	6	Intermediate
Picual	−207.51	9	Sensitive
Tragolia	−69.37	2	Tolerant

[a] In this column, the rank of olive cultivars based on their resistance to pathogen infection is presented (1 = most tolerant; 10 = most sensitive).

4. Discussion

Verticillium wilt threatens olive cultivation worldwide, causing severe yield losses and plant death [2,3]. Due to its long-lasting survival and the lack of effective fungicides, *V. dahliae* control focuses on preventive measures and sustainable management. Amongst them, the use of tolerant or resistant varieties and rootstocks is the core of integrated management strategies over time [2,3,31]. Hence, numerous studies have been conducted to screen olive genotypes originating from major olive-producing countries in the effort to identify and employ efficient resistant sources in practice [6–16,20,32,33]. In the present study, we performed a comparative resistance evaluation of nine important olive varieties from Greece and one from Spain. We applied a high inoculum pressure and considered several disease parameters to estimate the olive variety resistance to Verticillium wilt and the disease risk for the Greek olive industry.

Previous studies in Greece have revealed differential susceptibility to *V. dahliae* in a very limited number of Greek olive varieties [7,14]. In Antoniou et al. [7], a trunk drilling inoculation method was employed to differentiate resistance between 'Amfissis' and 'Kalamon', demonstrating the increased susceptibility of the former compared to the latter in terms of symptom development and *V. dahliae* re-isolation ratio. Similarly, Markakis et al. [14] revealed increased resistance of 'Koroneiki' and 'Kalamon' compared to 'Amfissis' against both the non-defoliating and defoliating pathotype of *V. dahliae* by transplanting rooted cuttings in soil artificially inoculated with microsclerotia and considering symptom severity, re-isolation ratio, and qPCR quantification of the pathogen. The variable resistance between 'Koroneiki' and 'Amfissis' has been associated with their differential phenolic response upon *V. dahliae* infection [22]. In addition, authenticated accessions of 'Koroneiki' were characterized as moderately susceptible to the defoliating *V. dahliae* pathotype in artificial inoculation experiments that employed either the root dipping or the stem puncture inoculation methods in Spain [13,15]. In the present study, 'Koroneiki' was used as a reference resistant variety and proved to be the most resistant even when inoculated via the root-dipping technique. The root-dipping inoculation method has been proposed as the most effective, fast, and reliable method to select genotypes at a young stage as potentially resistant to *V. dahliae* [34].

Considering all the disease and plant growth parameters, we conclude that the present study demonstrates a resistance of 'Koroneiki', 'Tragolia', and 'Atsiholou' compared to 'Amfissis', 'Chalkidikis', 'Kothreiki', 'Koutsourelia', 'Mastoidis', 'Megaritiki', and 'Picual'. Serano et al. [35] observed that 'Picual' was susceptible to the disease, which is a result similar to ours. In addition to this study, significant differences were found for crosses of 'Picual' and 'Frantoio' regarding RAUDPC and final severity [17]. According to the same research, 'Frantoio' and crosses derived from 'Koroneiki' and 'Arbosana' and 'Frantoio' and 'Arbosana' are some of the most prominent resistant crosses. Significantly lower pathogen DNA quantity in most cases (apart from 'Megaritiki') was associated with the decreased symptom severity in the resistant varieties, representing less active proliferation of *V. dahliae* into the xylem vessels. 'Megaritiki' demonstrated an intermediate level of vascular colonization by the pathogen, as quantification of *V. dahliae* DNA indicated no significant differences between this variety and the resistant or the susceptible ones. Unfortunately, most of the varieties were proven to be highly susceptible. Even the lesser-known varieties 'Atsiholou' and 'Tragolia' indicated a lower resistance level to Verticillium wilt than 'Koroneiki', suggesting the high risk for olive cultivation in Greece due to *V. dahliae*.

Within susceptible varieties, resistance was most distinguished using the relative AUDPC. Indeed, relative AUDPC was the only disease parameter that discriminated 'Koutsourelia' from 'Chalkidikis' and 'Amfissis', as well as 'Megaritiki' from 'Koutsourelia', 'Mastoidis', 'Kothreiki', and 'Picual'. Moreover, this parameter could separate 'Megaritiki' from 'Koroneiki' but not from 'Atsiholou' and 'Tragolia'. Nevertheless, final disease severity and disease incidence improved resistance evaluation within the resistant varieties, as both parameters differentiated 'Koroneiki' from 'Atsiholou' and 'Tragolia'. Plant mortality measurement was not highly discriminative, as differences were observed between 'Koroneiki'

and 'Atsiholou' but not between 'Koroneiki' and 'Tragolia'. Furthermore, plant growth parameters were less efficient in evaluating resistance. Plant fresh weight distinguished the susceptible from the resistant varieties, but no significant differences were observed within susceptible or resistant variety groups. Moreover, several susceptible varieties did not differ significantly from the resistant ones in plant height. Other research groups have likewise indicated the use of combined instead of individual disease parameters to obtain reliable results, due to the complication in assessing resistance [8,12,14].

Given all the above, apparently a large-scale research is needed to evaluate the resistance of all Greek olive varieties and rootstocks to Verticillium wilt and estimate the disease risk under variable pedoclimatic conditions. In future studies regarding resistance evaluation, a range of disease parameters should be considered in order to ensure reliable results. Furthermore, the mechanisms leading to the observed resistance of 'Koroneiki', 'Tragolia', 'Atsiholou' and other varieties and rootstocks should be elucidated and explored. The use of resistant varieties, certified propagating material, and other cultural practices suggested previously [2] is eventually the only plausible framework for the sustainable management of *V. dahliae*, one of the most severe soilborne pathogens of olive. To completely release and characterize a resistant variety comprehensive evaluation for features such as oil quality and oil content should be determined.

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