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Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*

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Abstract An innovative inoculation process, involving the drilling of a trunk hole in 3 year-old olive trees and injecting a dense conidial suspension of *Verticillium dahliae*, was developed to study differentiation in foliar symptom expression between olive cultivars tolerant or susceptible to the pathogen. It was demonstrated that *V. dahliae* conidia could be translocated and colonize the xylem at the same distance above and below the point of trunk injection in both cultivars. However, the pathogen could be subsequently isolated at statistically significant percentages in susceptible cv. Amphissis compared to the tolerant cv. Kalamon, indicating operation of resistance mechanisms in the vascular phase of the disease. Consequently symptom development in the susceptible cultivar was at least sixfold more intensive compared to the tolerant cultivar, 6–11 months after trunk inoculation. Perennial olive orchard experiments, aimed at selecting Verticillium-resistant root-stocks, were conducted by applying the novel method in 2–3 year-old root-stock suckers of Amphissis olive trees and in the tolerant cvs Lianolia of Corfu and Koroneiki. It was indicated that potentially resistant root-stocks could be obtained following the trunk drilling technique. Resistance differentiation between cvs Amphissis and Kalamon was further verified through root inoculation by various *V. dahliae* microsclerotial concentrations and demonstrated that the trunk drilling inoculation procedure is equally efficient in resistance evaluation of olives to Verticillium wilt. The trunk inoculation procedure could be useful in selecting and screening root-stocks for resistance to *V. dahliae* and other vascular pathogens and could elucidate resistance mechanisms in woody plants against vascular wilt diseases.

Keywords *Olea europea* · Trunk drilling inoculation · Verticillum wilt

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Introduction

Olive (*Olea europea*) is considered as the most vulnerable tree host of *Verticillium dahliae.* Verticillium wilt occurs throughout the range of olive cultivation, substantially reducing the production of olive orchards and potentially causing tree death (Tjamos 1993; Hiemstra and Harris 1998; Jimenez-Diaz et al. 1998). *Verticillium dahliae* survives in the soil by means of microsclerotia that are formed in the dead and dying tissues of colonised annual susceptible host plants or weeds (Thanassoulopoulos et al. 1981). It is also capable, through the xylem vessels, of colonizing leaves of the infected olive trees, thus contributing to the increase of the

inoculum level and to the dissemination of the microsclerotia in the olive orchards (Tjamos and Tsougriani 1990). In the soil, microsclerotia are stimulated to germinate by root exudates (Schreiber and Green 1963) or by the addition or release of nutrients in the soil (Green and Papavizas 1968; Farley et al. 1971). The hyphae of the germinated microsclerotia penetrate the root cortical tissue and spread systemically in the plant via the xylem (Huisman 1982; Huisman and Gerik 1989; Schreiber and Green 1963). Microsclerotial populations in fields show a wide variation (Johansson et al. 2006) directly affecting disease severity. Therefore, the use of various microsclerotial concentrations could be a strategy in screening experiments for host resistance. Since this is a time-consuming procedure, the development of preliminary rapid and easy to apply screening methods could boost the research for Verticillium-resistant olive cultivars or root stocks.

It is known that the spread of *V. dahliae* in the vascular tissues may be limited by several resistance mechanisms of the host (Dimond 1970). There are physical and biochemical components involved in defensive reactions of infected plants (Harrison and Beckman 1982). Production of phytoalexins such as terpenoid aldehydes in cotton (Harrison and Beckman 1982) and rishitin in tomato (Tjamos and Smith 1974) occur in pathogen-challenged xylem, often in conjunction with host-produced vascular occlusions (Dimond 1970). In addition, elemental sulphur appears to be part of this multiple defence response, which presumably serves to provide a physical and chemical barrier to vertical and lateral colonisation by *V. dahliae* (Cooper et al. 1996; Novo et al. 2007). According to Beckman (1989) and Mace (1989), the resistance responses in susceptible hosts are essentially the same as in the resistant ones, but the rates, intensities or spatial arrangement of these responses are inadequate to contain the pathogen.

Verticillium wilts are generally controlled by a combination of measures and strategies, including: use of resistant or tolerant cultivars or root-stocks, reducing soil inoculum, limiting disease spread and manipulating factors which influence disease severity (Tjamos 1993). The use of host resistance is considered as the most effective and ecologically sound method for managing Verticillium wilt in olive trees (Blanco-Lopez et al. 1998; Lopez-Escudero et al. 2004).

Resistance evaluation of olive root-stocks to Verticillium wilt constitutes one of the most promising approaches in solving the problem for newly-established olive orchards. However, selection and screening for resistant root-stocks is a particularly time-consuming procedure and lacks rapidity and reliability. We therefore attempted the development and evaluation of a rapid method, based on the differentiation between two olive cultivars of known susceptibilities to the disease.

The main objectives of this work were to: (1) develop a fast inoculation method by drilling a trunk hole and injecting a conidial suspension of *V. dahliae* to induce vascular wilt symptoms in 3 year-old Verticillium-tolerant and susceptible table Greek olive cvs Kalamon and Amphissis, respectively, under glasshouse conditions; (2) study colonization of *V. dahliae* in the vascular tissues of cvs Amphissis and Kalamon injected trees; (3) examine the ability of the fast procedure to differentiate resistance in root-stock suckers of old olive trees of susceptible or tolerant cultivars in Fthiotis, Preveza and Messinia prefectures of Greece, in order to select potentially resistant olive root-stocks; and (4) evaluate the resistance of the olive cvs Amphissis and Kalamon to different concentrations of *V. dahliae* microsclerotia.

Materials and methods

Fungal culture

A severe non-defoliating *V. dahliae* isolate from a diseased Amphissis olive tree was used throughout these experiments. For short-term storage the fungus was maintained on potato dextrose agar (PDA, Merck) at 4°C; while for long-term, it was maintained at −80°C. For the experiments, conidia of *V. dahliae* were produced in sucrose sodium nitrate (SSN) liquid growth medium. *Verticillium dahliae* microsclerotia were also prepared in SSN in Erlenmeyer flasks of 250 ml capacity, containing 100 ml of the medium. Pieces of 7 day-old *V. dahliae* cultures in PDA were transferred into the liquid medium. The liquid cultures were shaken in the orbital incubator at 22°C for 3 weeks. Abundant microsclerotia were formed and centrifuged to remove growth medium, dried and re-suspended in sterile distilled water (SDW). Microsclerotia were further re-suspended in SDW and

filtered through 70 μm mesh to select those microsclerotia to be used for root inoculation (Tjamos and Fravel 1995). It is well known that large microsclerotia germinate easily and show high levels of pathogenicity (Hawke and Lazarovits 1994). A water suspension of 3, 10 and 20 microsclerotia g^{-1} soil was prepared in 500 ml water and was poured and gradually mixed with the soil to obtain the most uniform distribution of microsclerotia.

Plant material for developing the method under glasshouse conditions

Differentiation in the resistance to Verticillium wilt was studied in two olive cultivars of known susceptibility to the disease. The cv. Amphissis, the most susceptible, and the tolerant cv. Kalamon (Tjamos 1993), were evaluated for resistance to *V. dahliae* under glasshouse conditions. The plant material consisted of 100 uniform 3 year-old trees per cultivar (regularly 1.50–1.80 m high), grafted on wild olive root-stocks. Differentiation of the cultivars was based on the genetic behaviour of each variety per se since the root-stock was not preventing or restricting symptom development through the applied inoculation procedure described below. The experiment was performed in a randomized block design. Olive trees were maintained at 24±5°C with a 12-h light and dark cycle.

Trunk drilling inoculation method

The inoculation method for resistance evaluation to *V. dahliae* was adapted from Resende et al. (1995), except that an oblique half-way hole was made in the trunk, and a measured amount of conidial suspension was introduced into the hole. The experimentation took place under controlled glasshouse conditions (randomized block design, 24±5°C, with a 12/12 h day/night regime). Inoculation was made in the trunk under the sites of the first ramification of branches or to the base of the trunk by making holes 3 mm in diam and 5 mm long using a Bosch PSB 1000 RCA drilling apparatus at low speed to avoid development of high temperatures, and introducing 100 μl of conidial suspension (10^8 conidia ml^{-1}) of a selected non-defoliating virulent olive isolate of *V. dahliae* immediately after making the hole, which was then sealed with Vaseline and covered with adhesive paper tape (Antoniou et al. 2000, 2002).

Plants were assessed 3 months after inoculation. The disease index was based on an arbitrary scale from 0–5 where 0 = no symptoms, healthy plants; 1 = initial to mild symptoms with dull green leaves; 2 = intermediate symptoms with internally rolled leaves; 3 = severe symptoms with scattered necrotic leaves; 4 = extremely severe symptoms with several defoliated or dead twigs and 5 = trees dead. The presence of the pathogen in the xylem tissue of symptomatic plants was checked by attempting to isolate the pathogen from the tissue at three points: (1) 5 cm above the inoculation point, (2) 5 cm below the inoculation point and (3) 50 cm below the inoculation point. In brief, bark was removed and chips of wood were placed onto acidified PDA. Plates were incubated at 22°C in the dark for 10 days.

Evaluation of the trunk drilling inoculation method by inoculating 3 year-old olive trees of cvs Amphissis and Kalamon

Further resistance evaluation to *V. dahliae* under glasshouse conditions was also attempted on plant material consisting of 3 year-old trees of cvs Amphissis and Kalamon. The plant material consisted of 130 uniform trees per cultivar (regularly 1.50–1.80 m high), grafted onto unknown wild olive root-stocks. The experimentation took place under controlled glasshouse conditions (randomized block design, 24±5°C, with a 12/12 h day/night regime) using the trunk drilling inoculation procedure and introducing 100 μl of *V. dahliae* conidial suspension (10^8 conidia ml^{-1}) to each hole. Ten trees per cultivar were sampled every 5 days for 50 consecutive days after inoculation for pathogen presence, while 30 trees per cultivar after inoculation were retained for recording symptom development, and 10 trees per cultivar were inoculated with 100 μl of SDW and kept as negative controls.

Spreading of *V. dahliae* conidia, vascular system colonization and assessment of symptom severity

To assess disease spread, pathogen isolation as described above was carried out by taking pieces of plant tissue from 15 sites at a distance of 3–45 cm above and below the injection site. Foliar symptoms were recorded for up to 190 days following inoculation in the canopy of 30 inoculated but not sampled

trees per cultivar. Symptoms were also recorded in the new sprouts of 90 sampled trees per cultivar up to 1 year after inoculation. The scale, described below, refers to data recorded for each twig of all branches or sprouts of a tree: 0 = no symptoms, twigs or sprouts healthy; 1 = dull green leaves; 2 = internally rolled leaves; 3 = necrotic leaves; and 4 = defoliated or twigs or sprouts dead. The disease index was calculated by the formula:

$$\text{Disease index}(\%) = \left[\Sigma\left(\begin{array}{c}\text{rating no.} \times \text{no. of twigs}\\ \text{or sprouts in the rating}\end{array}\right) \Big/ \begin{array}{c}\text{Total no. of twigs or}\\ \text{sprouts} \times \text{highest rating}\end{array}\right] \times 100\%$$

The experiment was performed in a randomized block design. The trees were held at 24±5°C, with a 12/12 h day/night regime. No fungicides were applied. The experiment was repeated three times with ten replicates per treatment.

Plant material of root-stock suckers from selected olive trees in established olive orchards of the susceptible cv. Amphissis and the tolerant cv. Lianolia of Corfu and Koroneiki for resistance evaluation under field conditions

Olive orchard experiments were carried in a 70 year-old irrigated olive orchard of nearly 800 trees of the susceptible cv. Amphissis in Fthiotis prefecture. Long-standing observations of the olive orchard owner during the last 30 years identified scattered trees of the susceptible cultivar without apparent Verticillium wilt symptoms. Almost 20% of trees remained symptomless among highly infected ones for several consecutive years indicating a degree of resistance of the randomly used wild root-stocks. Based on these observations, two 3-year old root-stock suckers (1.5 to 2.0 m tall and 2–3 cm thick in diam) originating from the base of the root-stocks of 65 selected symptomless olive trees with four to five root-stock suckers per tree were inoculated following the above procedure by introducing 100 μl of a suspension of 2×10^7 conidia ml^{-1} per hole instead of the 10^8 conidia ml^{-1} used in the glasshouse experiments, to avoid any possible negative effects on the trees of the olive orchard. Root-stock sucker inoculation was applied in April (spring inoculation), while a second one was carried out in November (autumn inoculation).

A second field resistance evaluation was carried out in two olive orchards (site Logidia and Kalamitsi) of cv. Lianolia of Corfu in Preveza prefecture, where root-stock suckers of 15 olive trees per orchard were inoculated. Verticillium wilt incidence of cv. Lianolia, considered as tolerant to Verticillium wilt, is extremely rare. Both orchards, of 250 year-old trees, were located in a highly Verticillium-infested region due mainly to adjacent cultivation of potatoes and eggplants severely infected by the pathogen (nearly 30 microsclerotia g^{-1} soil had been detected, prior to inoculation). The inoculation procedure was applied on wild root-stock suckers used locally for cv. Lianolia with five root-stock suckers per tree inoculated by introducing 100 μl suspension of 2×10^7 conidia ml^{-1} per hole. Root-stock sucker inoculation was applied in September and November respectively (autumn inoculation). Infected root-stocks were examined for symptom development both on cv. Lianolia and on the wild root-stocks at monthly intervals.

The degree of tolerance of cv. Lianolia of Corfu to Verticillium wilt was also tested by applying the trunk drilling technique in a fourth field in the Eternity region of Preveza prefecture, by injecting 3–4 year old branches (2–3 cm in diam and 1–2 m long) with a 100 μl suspension of 2×10^7 conidia ml^{-1} per hole of 25, 30 year-old trees. Tree branch drilling inoculation was applied in October (autumn inoculation).

A fifth experiment was established in the Messinia prefecture in Kyparissia region in 20, 50 year-old trees of the cv. Koroneiki (also considered as a tolerant Verticillium cultivar). Two year-old root-stock suckers (1.5 to 2 m long and 2–3 cm thick in diam) originating from the base of the trees were inoculated in October (autumn inoculation).

Resistance evaluation of olive cvs Kalamon and Amphissis to root inoculation with various concentrations of *Verticillium dahliae* microsclerotia

This work refers to the inoculation of olive trees originating from self-rooting procedures. Three year-old rooted cuttings of the susceptible cv. Amphissis and the tolerant cv. Kalamon were transferred from 5 kg capacity plastic pots to nearly 20 kg of soil. During transfer, root inoculation with 3, 10 and 20

microsclerotia g^{-1} soil of a *V. dahliae* non-defoliating strain was applied by uniformly drenching the root system with 500 ml suspension of microsclerotia. The experiment was repeated three times with ten replicates per treatment using a randomized block design. Olive trees were maintained at 24±5°C with a 12-h light and dark cycle. Percentage disease index was recorded 244 days after *V. dahliae* microsclerotia application using the scale and formula described previously.

Statistics

In order to evaluate the data from the experiments, statistical analysis of variance (ANOVA) for each treatment was performed. The results of the multivariate analysis of the data, when a significant ($P \leq 0.05$) *F*-test was obtained for treatments, were subjected to means separation by Duncan's multiple range test.

Results

Effectiveness of the trunk drilling inoculation method for resistance evaluation of the olive cvs Kalamon and Amphissis to *Verticillium dahliae*

Symptom development was evident 1 month after inoculation but the evaluation of this method was terminated 3 months after the inoculation when most of tested olive trees of the cv. Amphissis showed intense Verticillium wilt symptoms. On the contrary, disease symptoms in cv. Kalamon were mild with most of the trees not developing apparent symptoms of the disease. Data of Fig. 1, presenting percentages of trees in every disease index category ranging from 0–5, demonstrate that the severity of developed symptoms of the disease in cv. Kalamon was significantly lower in comparison with the susceptible cv. Amphissis.

The variation in symptom severity as calculated from the average of the specific disease indices used in this experiment showed that almost 63% of the cv. Kalamon trees did not develop or developed very mild symptoms, 25% showed intermediate while only 13% developed severe symptoms, with necrotic leaves and dead branches. On the contrary, the susceptible cv. Amphissis developed very severe or necrotic symptoms in >64% of the trees, mild to intermediate in 29% while very mild or healthy in 7% only.

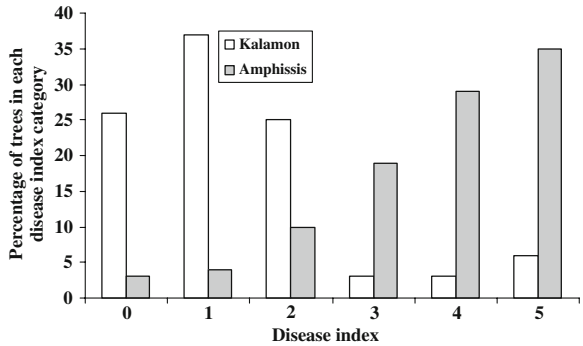


Fig. 1 Differential behaviour of Kalamon (100 trees) and Amphissis (100 trees) cultivars in symptom development following trunk drilling inoculation of 3 year-old trees with conidia of *V. dahliae*. Disease index categories: *0* no symptoms, healthy plants; *1* initial to mild symptoms with dull green leaves; *2* intermediate symptoms with internally rolled leaves; *3* severe symptoms with scattered necrotic leaves; *4* extremely severe symptoms with several defoliated or dead twigs and *5* trees dead

As for the progression of the pathogen into the infected tissues of symptomatic trees, it was shown that the pathogen was isolated at 5 cm above and below the inoculation point from cv. Amphissis. However it was not isolated from 50 cm above and below the inoculation point. In cv. Kalamon, the pathogen was isolated 5 cm above the inoculation point in symptomatic branches only. Symptomless Kalamon trees did not harbour the pathogen.

Differentiation between tolerant and susceptible olive cultivars to *Verticillium dahliae* spread and colonization of the vascular system, using the trunk drilling inoculation technique

Data of Fig. 2a demonstrate that *V. dahliae* spread at the same distance above and below the point of inoculation in both cultivars. However, the pathogen could be isolated at a higher percentage in cv. Amphissis than cv. Kalamon trees, reaching a statistically significant difference ($P \leq 0.05$) above and below the inoculation point 50 days after inoculation (Fig. 2b). The first Verticillium wilt symptoms were observed 70 days after inoculation in both cultivars, while from 80 days on a statistically significant difference in the percentage disease index was observed between the two cultivars. The disease index was 60% in Amphissis and 10% in cv.

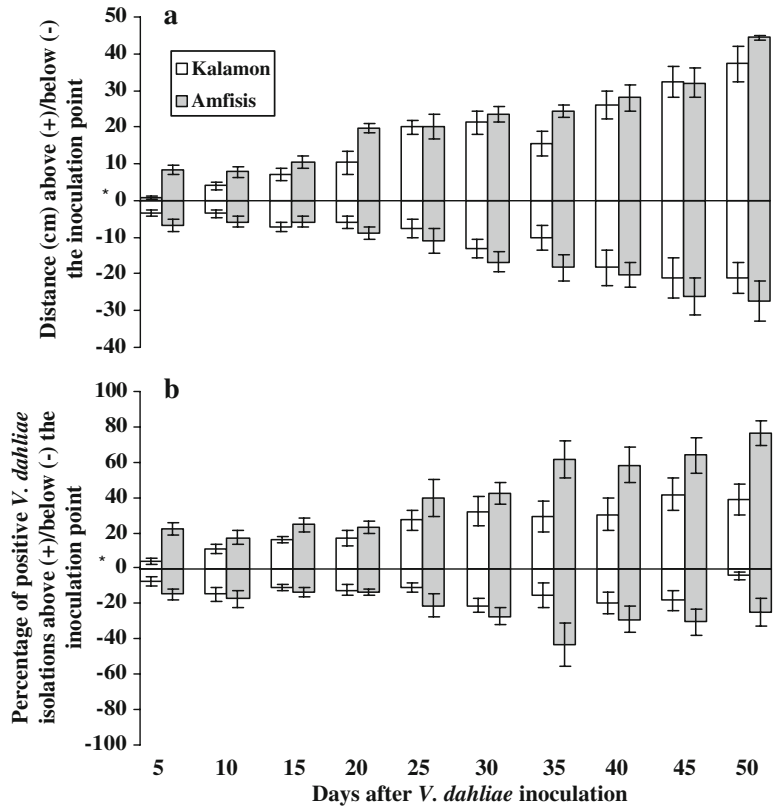


Fig. 2 Spread (**a**) and percentage of positive *V. dahliae* isolations (**b**) above and below the inoculation point (*0) in the trunk of Amphissis and Kalamon cultivars. To assess spread and percentage of positive *V. dahliae* isolations, bark was removed and chips of wood from 15 sites at a distance of 3–45 cm above and below the injection site were placed onto acidified PDA. Plates were incubated at 22°C in the dark for 10 days. Means of ten trees per cultivar with three replications. *Vertical bars* indicate standard errors

Kalamon, 190 days after *V. dahliae* inoculation (Fig. 3). These results coincide with the results presented in Fig. 4, where the young sprouts that emerged from the inoculated plants of both cultivars were infected but at a different percentage. In cv. Amphissis sprouts, the disease index was 57.8%, while in cv. Kalamon it was 10% 330 days after inoculation.

Resistance evaluation of root-stock suckers from selected olive trees in existing olive orchards of the susceptible cv. Amphissis in Fthiotis prefecture to *Verticillium dahliae*

Data of Fig. 5a show that application of the trunk drilling technique (April, spring inoculation) in root-stock suckers of 65 selected symptomless olive trees belonging to an irrigated olive orchard of 800 of Verticillium-susceptible cv. Amphissis trees in Fthiotis prefecture, resulted in differential susceptibility of the suckers to *V. dahliae* inoculation. Two months after inoculation most of the infected root-stock suckers showed intense Verticillium wilt symptoms with dull green leaves, leaf necrosis and defoliation in almost all inoculated branches of 52 inoculated root-stock suckers. However, 13 root-stocks remained initially symptomless and 3 months after inoculation the number of healthy symptomless trees decreased to seven, and after 5 months was only six.

Verticillium dahliae isolations attempted 1–3 months after symptom development at several sites along the axis of the inoculated suckers, were positive. On the contrary, fungal isolations, attempted in asymptomatic suckers belonging to the group of six healthy root-stocks were negative, even 7 months after inoculation. New root-stock suckers from six trees with symptomless root-stock were inoculated again with *V. dahliae* spore suspension 7 months after

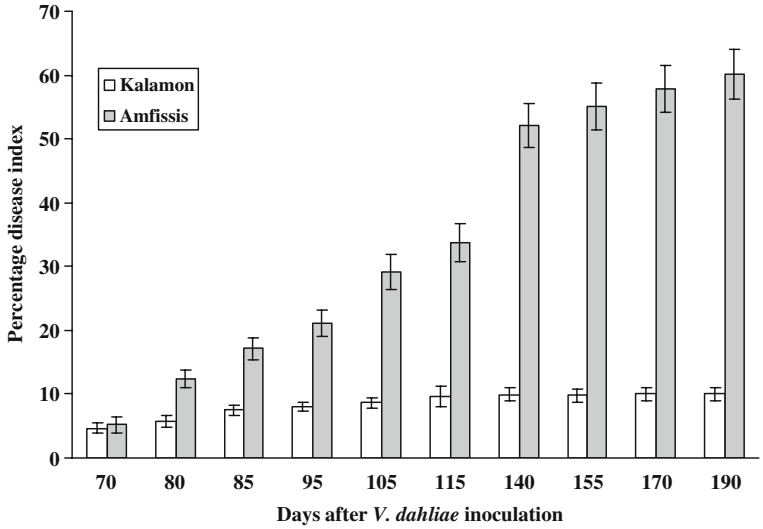


Fig. 3 Effect of the trunk drilling inoculation procedure on Verticillium wilt disease severity in tree canopy of Amphissis and Kalamon cultivars. Means of ten trees per cultivar with three replications. *Vertical bars* indicate standard errors. Disease index categories: *0* no symptoms twigs healthy; *1* dull green leaves; *2* internally rolled leaves; *3* necrotic leaves; and *4* defoliated or dead twigs. The percentage disease index was calculated from the disease rating by the formula: Disease index(%)=[Σ(rating no. × no. of twigs or sprouts in the rating)/Total no. of twigs or sprouts × highest rating] × 100%

the initial inoculation (November). These new root-stock suckers did not develop symptoms and did not harbour the pathogen.

One year after the initial sucker inoculation, downward movement of the pathogen was indicated by symptom development in the corresponding trees and was demonstrated by positive isolation of the fungus in diseased branches of 14 trees with symptomatic root-stocks.

Seventeen months after inoculation five out of 65 infected root-stock suckers remained eventually symptomless. It was unexpectedly shown that although five root-stocks remained symptomless, in four of them the corresponding trees developed intense Verticillium wilt symptoms. It was concluded that the 18 Amphissis trees (14 with symptomatic and 4 with symptomless root-stock suckers) were apparently infected by the propagules of the pathogen drawn into the trees through negative water movement from the root-stock base. Twenty-four months after inoculation, five root-stock suckers still remained symptomless to *V. dahliae* infection. It was, however, demonstrated that the suckers of one root-stock designated as Ionia and its corresponding Amphissis tree remained symptomless over 2 years after the second inoculation.

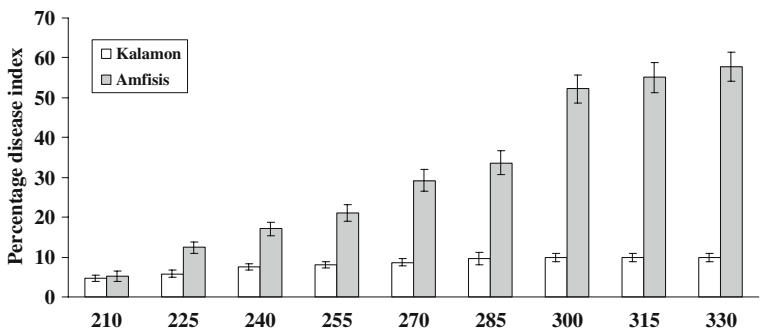
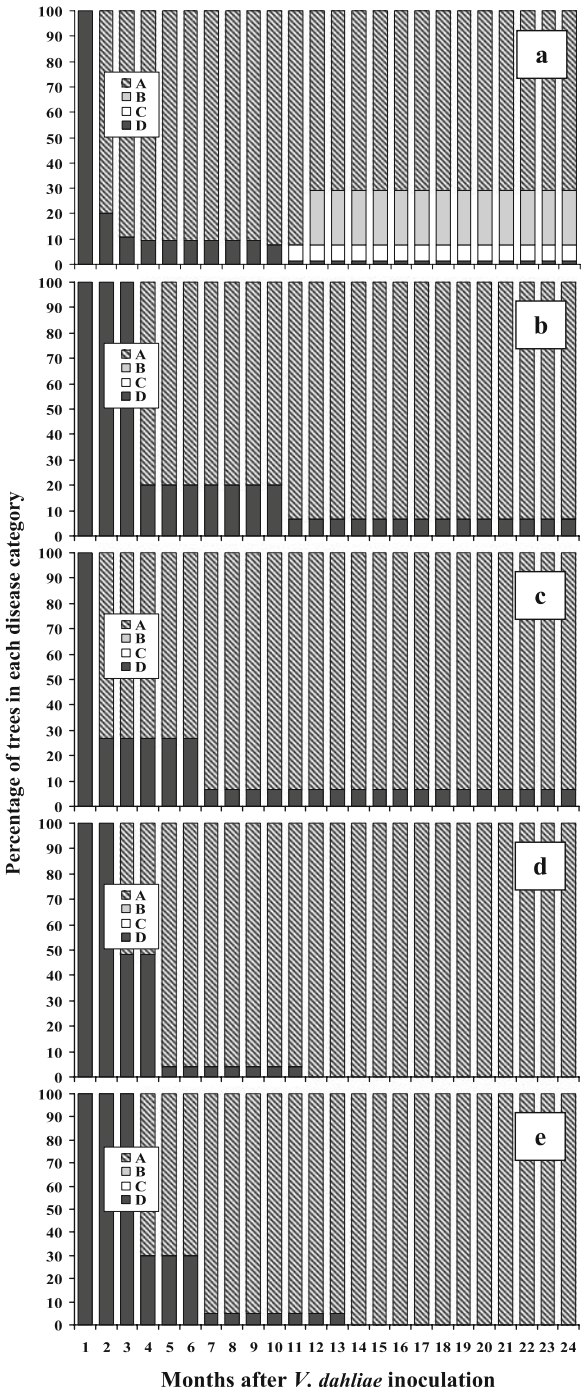


Fig. 4 Effect of the trunk drilling inoculation procedure on Verticillium wilt disease severity in sprouts from Amphissis and Kalamon trees. Means of 30 sprouts per cultivar with three replications. *Vertical bars* indicate standard errors. Disease index categories: *0* no symptoms, sprouts healthy; *1* dull green leaves; *2* internally rolled leaves; *3* necrotic leaves; and *4* defoliated or dead sprouts



◀ **Fig. 5** Effect of trunk drilling inoculation on Verticillium symptom development in inoculated root-stock suckers and/or on the corresponding trees of the susceptible cv. (**a**) Amphissis (65 trees) and the tolerant cvs (**b**) Lianolia of Corfu (Site Logidia, 15 trees) (**c**) Lianolia of Corfu (Site Kalamitsi, 15 trees) (**d**) Lianolia of Corfu (Site Eternity, 25 trees) (**e**) Koroneiki (20 trees) surveyed at monthly intervals. Disease categories: (**a**) diseased suckers on healthy trees, (**b**) diseased suckers on diseased trees, (**c**) healthy suckers on diseased trees, (**d**) healthy suckers on healthy trees

It was shown that there were four types of responses of the *V. dahliae* infected root-stock sprouts: (1) those that developed symptoms of the disease on the inoculated root-stocks within 2–6 months after trunk drilling inoculation. Symptoms of the disease were not observed on the corresponding Amphissis trees 19 months later (46 out of 65, 71%); (2) those that developed symptoms of the disease on the inoculated root-stock and on the on the corresponding Amphissis trees 3–17 months after trunk drilling inoculation (14 out of 65, 21.5%); (3) those that did not develop any foliar symptoms of the disease 12–24 months after trunk drilling inoculation but the corresponding Amphissis trees, developed intense symptoms of the disease (4 out of 65, 6%) and (4) those that remained symptomless for over 24 months after inoculation without any symptoms on the corresponding Amphissis tree (1 out of 65, almost 1.5%).

Resistance evaluation of root-stock suckers from selected olive trees in existing olive orchards of the tolerant cv. Lianolia of Corfu in Preveza prefecture to *Verticillium dahliae*

Data of Fig. 5b and c (site 1, 2, Loggidia and Kalamitsi) demonstrate that application of the trunk drilling inoculation technique in root-stock suckers of 15 selected symptomless olive trees belonging to the Verticillium tolerant cv. Lianolia of Corfu, resulted in differential susceptibility of the suckers to *V. dahliae* inoculation. Although root-stock suckers remained symptomless 2 months after inoculation, intense Verticillium wilt symptoms developed in three out of 15 trees 4 months after inoculation. However, 11 months after inoculation the number of healthy symptomless root-stock suckers declined to one in the Loggidia and Kalamitsi sites while in the Eternity site, all root stock suckers were symptomatic 1 year after inoculation.

The two root stock suckers (one in Loggidia and one in the Kalamitsi sites) that remained symptomless over 24 months after inoculation were named Ambrakia and Nicopolis, respectively. Although *V. dahliae* was isolated from the diseased root-stock suckers, it was not isolated from the potentially resistant root-stocks Ambrakia and Nicopolis. In addition, symptom

development was not demonstrated in adjacent branches of the experimental trees in the three sites, indicating lack of downward movement of the fungus in cv. Lianolia of Corfu even 24 months after inoculation

Resistance evaluation of root-stock suckers from selected olive trees in existing olive orchards of the tolerant cv. Koroneiki in the Kyparissia region of Messinia prefecture to *Verticillium dahliae*

Data of Fig. 5e demonstrate that the severity of symptoms from the trunk injection technique on 2 year-old suckers of self-rooted plants of the tolerant cv. Koroneiki was extremely pronounced. The 20 trees of cv. Koroneiki that were infected (October, autumn inoculation) remained asymptomatic 3 months after inoculation. Four months after inoculation only six out of 20 root-stock suckers remained symptomless, while 12 months after inoculation only one sucker remained symptomless. It was however, demonstrated that 14 months after inoculation all root-stock suckers developed symptoms of the disease.

Evaluation of the olive cvs Kalamon and Amphissis for resistance to root inoculation with different concentrations of *Verticillium dahliae* microsclerotia

Tested cultivars showed different levels of susceptibility to *V. dahliae*. Cultivar Kalamon proved less susceptible than Amphissis and 244 days after *V. dahliae* application, the disease index in cv. Kalamon ranged from 0.3 to 8.8% at 3 and 20 microsclerotia g^{-1} soil, respectively. On the contrary, at the same sampling time, in cv. Amphissis the disease index (12.8%) at 3 microsclerotia g^{-1} soil was higher than in cv Kalamon (8.8%) at 20 microsclerotia g^{-1} soil. In addition, 10 and 20 microsclerotia g^{-1} soil resulted in a steady increase in the disease index in cv. Amphissis, significantly higher than cv. Kalamon (Fig. 6). In both cultivars, the use of 10 and 20 microsclerotia g^{-1} soil did not result in a significant difference between the trees of the same cultivar, with cv. Amphissis showing a higher disease index initially at 10 than at 20 microsclerotia.

Discussion

Verticillium wilt has long been a serious problem in the cultivation of a wide range of economically important herbaceous and woody plants in many parts of the world. Although preventive or therapeutic measures have been suggested, new methods are badly needed. The single most important and valuable control method remains the use of resistant or tolerant cultivars or root-stocks (Tjamos et al. 1991; Tjamos 1993; Hiemstra and Harris 1998). Resistance of olive root-stocks to Verticillium wilt is one of the most promising approaches in solving the problem world-

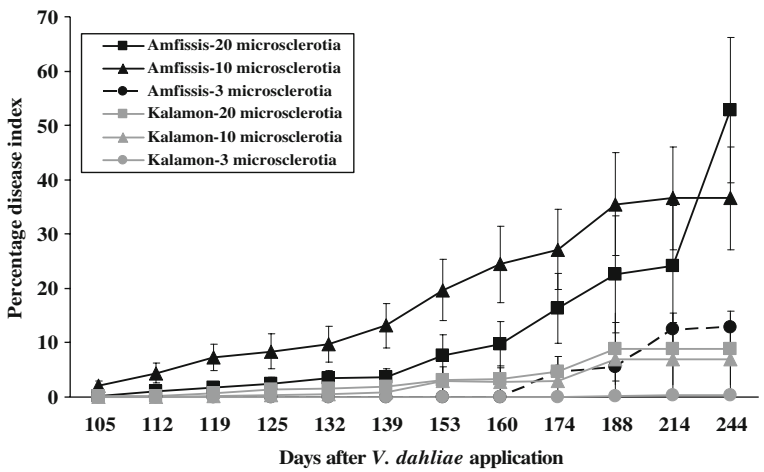


Fig. 6 Verticillium wilt disease index on Amphissis and Kalamon cultivars potted in soil infested with 3, 10, and 20 *V. dahliae* microsclerotia g^{-1} soil. Means of ten trees per treatment with three replications. *Vertical bars* indicate standard errors. Disease index categories: *0* no symptoms, twigs healthy; *1* dull green leaves; *2* internally rolled leaves; *3* necrotic leaves; and *4* defoliated or dead twigs

wide. However, resistance evaluation is a particularly time-consuming procedure and lacks rapidity and reliability.

In the present work, a rapid resistance evaluation method was developed to differentiate resistance among olive cultivars to *V. dahliae*. The method focused on examining differential behaviour between 3 year-old trees of the most susceptible Greek cv. Amphissis and the tolerant cv. Kalamon in symptom development, by trunk drilling and injecting a dense conidial pathogen suspension directly into the vascular tissue of the trees (100 μl of 10^8 conidia ml^{-1}). Regardless of resistance mechanisms operating in the pre-vascular phase of the disease, application of a high concentration conidial suspension clearly differentiated symptom severity 2–3 months after inoculation of 3 year-old susceptible or tolerant olive trees.

Our data also constitute the first evidence that *V. dahliae* spreads the same distance above and below the point of inoculation in both cultivars, reaching 40 cm above or below the site of injection 50 days after inoculation. Data also suggest a lack of mechanisms involved in the dispersal of the pathogen in the tested cultivars. However, data referring to vascular tissue colonization demonstrated that *V. dahliae* could colonize more profusely the xylem tissues in cv. Amphissis than cv. Kalamon reaching a statistically significant difference above and below the inoculation point 50 days after inoculation. The evidence is further supported 80 days after inoculation ($P \leq 0.05$).

It is known that upward spread of the pathogen in the vascular tissues is primarily by means of conidia transported in the transpiration stream (Talboys 1962; Garber and Houston 1966; Presley et al. 1966). However, downward spread has never been observed or suggested in the vascular tissues. This can be considered as an important factor in the epidemiology of the disease.

The trunk drilling inoculation technique applied on potentially resistant root-stocks suckers of 65 Verticillium-susceptible trees (100 μl of 2×10^7 conidia ml^{-1}) in an olive grove of cv. Amphissis, resulted in the development of intensive Verticillium wilt symptoms in susceptible suckers almost within 2 months after inoculation, while 5 months after inoculation 59 out of 65 root-stock suckers were symptomatic. This demonstrates that the drilling inoculation can be a rapid evaluation and selection procedure.

Our field data also demonstrate that 18 Amphissis trees were apparently infected by propagules of the pathogen drawn into the trees through negative water movement from the root-stock base. Twenty-four months after inoculation, five root-stock suckers still remained symptomless to *V. dahliae* infection. It was further shown that although four out of five root-stocks remained symptomless, their corresponding trees developed intense Verticillium wilt symptoms. However, it was very promising to demonstrate that the suckers of one root-stock designated as Ionia and its corresponding Amphissis tree remained symptomless over 2 years after the second inoculation

Unpredictably, a downwards spread of *V. dahliae* in the vascular tissues was also evidenced in aged trees of the cv. Amphissis olive orchard. Occurrence of Verticillium-infected trees through the artificially inoculated root-stocks is possibly due to the downward movement of water to the base of the suckers and from the base up the susceptible tree (Drossopoulos et al. 1996).

It seems that the susceptibility of cv. Amphissis facilitated infection of the corresponding trees through negative sap movement via susceptible or resistant root-stock suckers. However, this phenomenon was not observed with the tolerant cv. Lianolia of Corfu regardless of the presence of susceptible or potentially resistant root-stocks. Lack of symptom development on the corresponding trees could mean operation of resistance mechanisms in the vascular phase of the disease in the tolerant cv. Lianolia of Corfu.

Resistant-type interactions in vascular wilts involve gel plugs that completely block off the lumen of the infected vessel above the trapping sites, resulting in immobilization of the pathogen (Beckman 1987, 1989); moreover, these sites become infused with several secondary metabolites like phytoalexins that are exuded from paratracheal parenchyma cells (Tjamos and Smith 1974, 1975; Mace 1989). Studies on resistance mechanisms of trees against vascular pathogens are generally restricted and not extensively clarified so far. The phenomenon of recovery related to the operation of resistance mechanisms was studied initially by Wilhelm and Taylor (1965) and reported later by several other workers (Tjamos et al. 1991; Hiemstra 1995). As for biochemical factors, Resende et al. (1996) and Cooper et al. (1996) working with Verticillium wilt of cocoa have reported that elemental sulphur acts as a phytoalexin. Data focusing on

olives and referring to the role of phenolics or glycoalcaloids are very inconclusive. Our future experiments will try to elucidate resistance mechanisms involved in the observed resistance of potentially resistant root-stocks.

Our work also demonstrated differential behaviour of cv. Amphissis and cv. Kalamon to various concentrations of *V. dahliae* microsclerotia. Verticillium wilt symptoms appeared 3–4 months after microsclerotial application. Kalamon proved to be less susceptible than Amphissis at different concentrations of microsclerotia. Moreover, the disease index in cv. Kalamon was nearly zero (0.33%) at 3 microsclerotia g^{-1} soil, a concentration close to the naturally occurring inocula (Tjamos et al. 1991), while cv. Amphissis trees at that concentration were infected as much as cv. Kalamon at 10 and 20 microsclerotia g^{-1} soil. In both cultivars the use of 10 and 20 microsclerotia g^{-1} soil did not result in significant differences between the trees of the same cultivar, therefore 10 microsclerotia g^{-1} soil might be a threshold in screening experiments. Our data suggest that both cultivars are susceptible to *V. dahliae*; however, cv. Kalamon showed restricted pathogen ramification in the vascular tissue compared to cv. Amphissis.

These differences are in agreement with the results obtained by the trunk drilling inoculation technique, carried out throughout the 4-year experimentation period, under glasshouse or field conditions, and during the fourth year of our experimentation, in particular. Our data also demonstrated that the applied root inoculation technique is very reliable in evaluating Verticillium resistance of potentially resistant olive root-stocks. The susceptible cv. Amphissis was extremely susceptible to the 20 microsclerotia g^{-1} soil inoculum level, while the tolerant cvs Kalamon and Lianolia of Corfu hardly developed symptoms and did not harbour the pathogen in the vascular tissue.

Preliminary data concerning resistance evaluation of three potentially resistant rooted root-stocks obtained from olive root-stock suckers originating from three experimental regions of Greece suggested a valuable genetic pool able to provide resistant olive root-stocks (unpublished data). Therefore, our current investigation also involves glasshouse resistance evaluation of the three selected rooted root-stocks designated as Ionia, Ambrakia and Nicopolis using root inoculation with 20 microsclerotia g^{-1} soil to verify resistance levels after inoculation. Our studies will also be concentrated on RT-PCR evaluation to evaluate the degree of colonization and pathogen ramification along the trunk to confirm the degree of resistance in the pre-vascular and vascular phases of the disease and the potential for release and use as commercial olive root-stocks.

The early development and differentiation in Verticillium wilt symptoms between the tested cultivars, along with the feasibility of inoculation by trunk drilling make the method appealing for resistance-screening experiments in olive, because a large number of trees can be quickly inoculated.

The phenomenon of the recovery already described by several authors (Wilhelm and Taylor 1965; Hiemstra 1995) along with the reported or implicated mechanisms (Resende et al. 1996) could be further elucidated by our method. Indeed because of the very considerable vascular tissue inoculation obtained by the described method, it could be considered as a valuable procedure in studying resistance mechanisms involved in the differentiation among tolerant versus susceptible olive cultivars, or potentially resistant root-stocks. It could also be useful in rapid screening programme for resistance to Verticillium wilt of other woody host plants, such as ash and maple, and also against *Ophiostoma ulmi* of elms and *Ophiostoma platani* of plane trees.

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