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Inexpensive and Reliable On-Site Solution for Olive Producers to Contain Verticillium Wilt

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1. Introduction

The European Union leads the global olive oil sector, producing 80% and consuming 70% of the average total world output of over 2.5 million tonnes. Olive production is distributed into a large number of small and medium enterprises with low profit margins, thus particularly vulnerable to drops in productivity.

The competitiveness of the European olive production sector, already faced with growing international competition and other adverse factors such as a steady reduction in olive oil prices, is additionally threatened by the soil-borne fungus *Verticillium dahliae*.

In recent years, Verticillium wilt is occurring with increasing frequency and severity in most olive growing areas of the Mediterranean basin due to intensive farming of highly productive cultivars, planting at high densities and flood irrigation techniques, all of which favour the spread of the pathogen.

Driving consortium SME-AGs partners INOLEO, EDOEE, AAR and their members are certain that containment and even eradication of this disease is feasible, but only if timely and accurate detection and quantification of the *Verticillium* fungus in soil and trees is achieved at a comparative low price and appropriate effective and sound economic measures are applied within an Integrated Pest Management strategy based on both preventive and eradication measures.

The VERTIGEEN project proposes a new, cost-effective, reliable system for early on-site detection and quantification of *Verticillium dahliae* which will allow the olive producers to significantly reduce the losses caused by this pathogen and consequently increase their profit margins, preserve employments and maintain competitive edge in the global market.

2. Objectives

The main objective of this deliverable is to provide the Olive sector with a guide containing protocols to control the disease in established orchards and prevent contamination of new olive groves or healthy parts of existing groves. This guide is the result of an extensive literature review and contains a decision scheme based on disease risk categories established on the results of previous tasks of WP6.

The contents of the guide have been extensively discussed among the consortium and especially concerns of the SME-AGs and other end-users were taken into account.

The text of Chapter 3 (the actual BP Guide) has been written and formatted in such a way that it can be distributed to growers on its own, without further editing, as a practical guide to control Verticillium wilt of olive.

3. Guide for Best Practices

Control of Verticillium Wilt in Olive

Guide of Best Practices

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Symptoms of Verticillium wilt

Infected trees may develop different symptoms depending on the amount and virulence of the Verticillium inoculum in the soil, the susceptibility of the cultivar affected and the environmental conditions. Traditionally two main disease syndromes have been distinguished: an acute form called Apoplexy and a chronic form called Slow Decline (Jiménez-Díaz *et al.* 2012; López-Escudero & Mercado-Blanco 2011). Apart from these two disease syndromes, symptomless infection is a common phenomenon (Barranco *et al.*, 2010).

Apoplexy, the acute form, is characterized by the rapid death of one or more limbs of a tree. Leaves on affected branches lose their intense green colour, turn light brown, roll back and dry up, necrotic leaves remaining attached to the shoots (Fig. 1). Under Mediterranean conditions this type of symptoms usually develops in late winter till early spring. When it occurs on young plants, death of the affected plants is common.

Slow decline is the chronic form of the non-defoliating syndrome. It appears mainly in spring and may slowly progress to early summer and is characterized by heavy defoliation of green or dull green leaves (Fig. 2), foliar chlorosis and desiccation and mummification of inflorescences that remain attached to the twigs (Fig. 3). Often some still green leaves remain attached at the end of affected shoots (Fig. 4). The bark of affected branches often shows a purple-reddish discoloration.

As can be seen from the above, symptoms are rather variable and little specific. Moreover different symptoms may occur on the same tree simultaneously or in different seasons. Therefore diagnosis of the disease always needs to be confirmed by identification of the pathogen, either by isolating it and growing it on an agar plate, or by examining a sample of the plant for the presence of DNA of the pathogen (molecular detection).

Fig. 1 Apoplexy: rapid death of branches with leaves still attached

Fig. 2 Slow decline: sudden loss of green leaves

Fig. 3 Slow decline: mummification of inflorescences and fruits

Fig. 4 Slow decline: chlorosis, necrosis and loss of leaves with some green leaves remaining on affected shoots

Disease cycle of Verticillium in trees

Introduction

Verticillium wilt is caused by the soil-borne fungus *Verticillium dahliae.* This fungus has hundreds of host plants including important agricultural crops such as cotton, potato and sunflower; horticultural crops such as eggplant, tomato and pepper; and many shade and fruit tree species including olive, stone fruits and pistachio (Pegg & Brady 2002; Smith *et al.* 1988). Among the hosts are also many herbaceous weeds (Thanassoulopoulos *et al.* 1981). The disease cycle of Verticillium wilt in trees (see Fig. 5) has several phases that are shortly described below. More detailed information can be found in reviews by Hiemstra & Harris (1998) for trees in general and by Jimenez-Diaz *et al.* (2012), Lopez-Escudero & Mercado-Blanco (2011) and Tsror (2011) for olive.

Survival of the pathogen

The fungus survives in soil by means of so-called microsclerotia (ms), clumps of very thick-walled dormant cells, that are formed in the dying tissues of its host. After decomposition of the plant tissue these microsclerotia become incorporated in the soil where they can survive for 10 or more years.

Infection of the root

Microsclerotia are not mobile and stay dormant till they are stimulated to germinate by root exudates. Then hyphae penetrate the cortex of young roots and start colonizing the root tissues. Because trees continuously form new roots and because of the large size of a tree root system, even in soils with very low inoculum levels, the tree may encounter many microsclerotia resulting in many local root infections.

Colonization of the host plant

In susceptible plants the fungus grows into the xylem vessels in the root. There its behaviour changes and besides hyphae many conidia (spores) are formed. These conidia are carried upward with the sap stream. Xylem vessels in trees form a complex branching network from root till top of a tree. As a result

even large trees can become completely colonized within weeks after the first xylem infection. Because the transport is mainly upward, single root infections may result in one-sided symptom development.

Symptom development

Trees are not completely helpless against xylem invading pathogens. Their defence reaction usually includes mechanisms to seal off infected vessels by means of tyloses or gel plugs. When this is fast and effective the pathogen may be localised and not be able to colonise the whole tree. However, in susceptible hosts these mechanisms apparently are inadequate to contain the pathogen resulting in widespread local occlusions because the plant starts defence again every time the pathogen escapes to a new part of the vascular system. In addition degradation products from vessel cell walls resulting from activities of the fungus and fungal structures may contribute to blockage of the vessels. Finally the water transport in the xylem is completely disrupted resulting in the typical wilt symptoms.

Formation of new inoculum and dissemination of the pathogen

When the host plant starts to die the fungus again changes its behaviour. Up to then its growth was limited almost completely to the xylem vessels, but now it starts colonizing the dying tissues of its host and especially the leaf tissues. Here large numbers of new microsclerotia are formed. Therefore these leaves are a very important source of infection, not only for the soil below infected trees, but also for other trees nearby or in neighbouring fields. Usually the disease is monocyclic, meaning that the new inoculum does not cause new disease in the same year, but because of the perennial character of trees secondary inoculum, that remains active in soil over many years, can substantially contribute to disease during the production time of an olive grove.

Natural recovery

Trees infected by *Verticillium* do not always die. The fungus enters the tree through one or more roots and initially often only part of a tree is colonized by the pathogen. Because of the way trees are built and the annual formation of new xylem tissue, trees are highly compartmented organisms. As a result a tree may survive and outgrow attack by *Verticillium* when (1) the tree can limit the distribution of the pathogen effectively (compartmentalize it); (2) its cambium survives, enabling the tree to form new healthy tissues to replace infected parts; and (3) new infections do not occur or are limited. Natural recovery has been described for several tree species, but trees vary in their capacity to recover (Hiemstra & Harris, 1998). Also in olive this phenomenon has been reported repeatedly (e.g. Levin, Lavee & Tsror, 2003b; Lopez-Escudero & Blanco-Lopez, 2001 and 2005a; Tjamos, Biris & Paplomatas, 1991). However, recovery often is only temporary (Bubici & Cirulli, 2014), probably because on infected soils new infections may take place continuously.

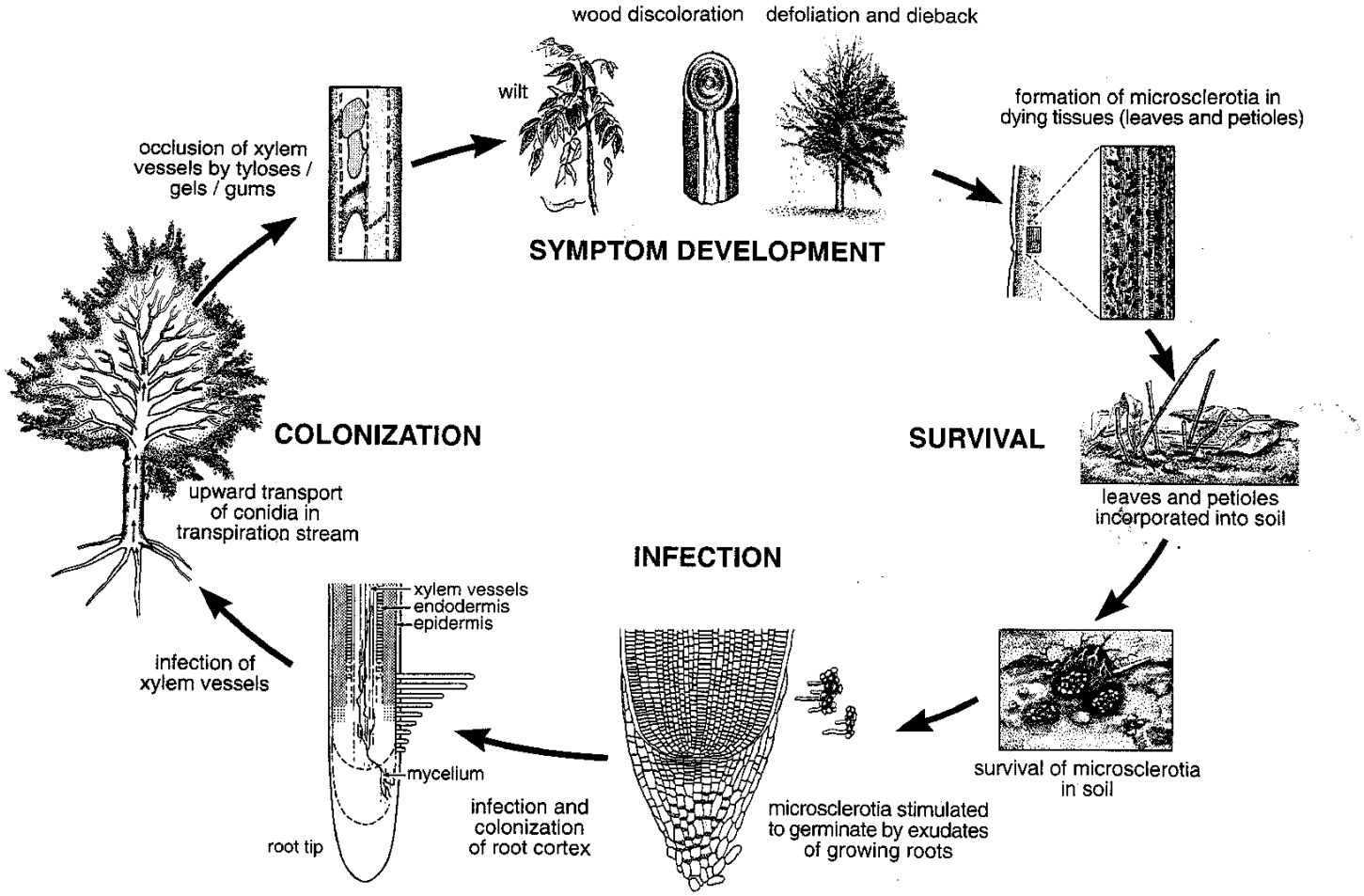


Figure 5: Schematic representation of disease cycle of Verticillium wilt in trees (from: Hiemstra & Harris, 1998)

Main factors affecting disease

Pathogen factors

- **Virulence of specific isolates: D/N-D type**

Generally two main types are distinguished in *Verticillium* isolates infecting olive; defoliating (D-type) and non-defoliating (ND-type), the names of which refer to their capability to cause complete fall of green leaves in cotton (Schnatthorst & Sibbett, 1971). D-type isolates are more virulent also in olive resulting in faster disease development and more severe symptoms (Lopez-Escudero & Mercado-Blanco, 2011). The effect of the two types is influenced by soil temperature with D-type isolates having higher optimum temperatures for infection (16-24 ºC) than ND-type isolates (16-20 ºC) (Calderón *et al.*, 2014). ND-type isolates are commonly present in all areas with *Verticillium*; D-type isolates apparently have developed more recently and are still spreading. In Europe they are known to be widely spread in southern Spain (Jimenez-Diaz *etal.* 2009; López-Escudero *et al.* 2010b) and Turkey (Dervis *et al.*, 2010). So far the D-type has not been reported on olive in Italy and Greece, although this type has been detected on cotton in these countries (Elena & Paplomatas, 2001; Nigro *et al.*, 2005).

- **Inoculum density**

Even very low amounts of *Verticillium* in soil can result in serious disease, especially when D-type strains are present. In an experiment with artificially infested soils inoculum levels of 3 microsclerotia of the D-type per gram soil and higher resulted in over 50% disease in a susceptible cultivar (Picual) and even 1 ms/g resulted in serious disease. As a result it was concluded that on soils infested by the D-type susceptible olive cultivars should not be planted at all (Lopez-Escudero & Blanco-Lopez, 2007). Further information on the relation between inoculum density (ID) in soil and resulting disease incidences is very limited, probably because this relation is influenced by many factors. From the information available, however, it is clear that low ID levels of the ND type also may cause substantial disease in olive, especially in highly susceptible cultivars. Roca *et al* .(2015) for example reported > 10% diseased Picual trees on a field with an inoculum density of only 1 ms/g soil.

Also for highly susceptible trees like maple (Goud, 2003) and pistachio (Ashworth & Zimmermann, 1976) it was reported that ID levels of 1-2 ms/g soil may result in DI of 5% and 10-14%, respectively. Moreover, in soil testing for the planting of susceptible tree nursery crops in The Netherlands and Germany 1-2 ms/g soil is the threshold level for the category “low level of infestation” (Hiemstra, 2014).

Host factors

- **Cultivar**

Olive cultivars vary in susceptibility to *Verticillium* with most of the commercially grown cultivars being susceptible to very susceptible. Some of the common cultivars in the countries taking part in the Vertigeen project as well as some promising resistant cultivars identified so far are listed below. (Data from Lopez-Escudero & Mercado-Blanco (2011) unless indicated differently; ES

extremely susceptible, S susceptible, MS moderately susceptible, R resistant, T tolerant: can get infected without sustaining severe losses in yield or quality).

Greece:	Amfissis ES, Konservolia ES, Kalamon MS, Koroneiki T, Oblonga R
Italy:	Coratina S, Frantoio R, Leccino ES (Bubici & Cirulli, 2012)
Portugal:	Cobrancosa R (ND-type) / ES (D-type) (Lopez-Escudero *et al.*, 2004), Galega S (Trapero *et al.*, 2011)
Spain:	Arbequina MR, Cornicabra S/ES, Hojiblanca S, Manzanilla S, Picual S/ES

Cultivars with high resistance: Changlot Real, Empeltre, Frantoio, Oblonga
Cultivars with moderate resistance: Cipresino, Koroneiki, Sevillenca

- **Age of the trees**

Apparently young trees are more susceptible than older trees. Several authors have reported higher disease incidences in young orchards than in older ones (over 25 years) or decreasing disease incidence with time (Al-Ahmad & Mosli, 1993; Blanco-Lopez, Jimenez-Diaz & Caballero, 1984; Lopez-Escudero *et al.*, 2010; Rodriguez *et al.*, 2008).

Agronomic factors

- **History of field/ Intercropping/Neighbouring fields**

Cropping history of a field affects the risk of attack by Verticillium wilt. When susceptible crops are grown on a field microsclerotia produced on these crops will lead to increased soil inoculum levels. This new inoculum may also be distributed to nearby fields by windblown leaves etc. Indeed in several surveys higher prevalence of Verticillium wilt was reported in fields in or near areas where previously other *Verticillium* susceptible crops such as alfalfa, cotton, safflower, sunflower or sugar beet had been grown (Lopez-Escudero *et al.*, 2010; Rodriguez *et al.*, 2009). Similarly intercropping with horticultural crops such as tomato, potato, eggplant or pepper or alternative cropping with cotton may increase *Verticillium* inoculum in soil and therefore also cause higher disease incidence of Verticillium wilt.

- **Soil disinfestation/solarization**

Infested soils can be restored for planting susceptible crops if the inoculum level is significantly reduced. Chemical treatments (chloropicrin, formalin, metham-Na, methylbromide and others) have been used in the past successfully especially in high-value crops, but the large scale use of this chemicals because of environmental and consumer aspects is undesirable and in many countries not anymore allowed. An alternative approach also suitable for tree plantings is solarization of soil (Hiemstra & Harris, 1998). Solarization of soil (covering the soil surface with transparent plastic sheeting, usually after moistening) increases the temperature in the upper soil layers and can be an effective method to lower inoculum density of soil pathogens such as V. dahliae (Katan, 1980; Stapleton & DeVay, 1986). However, the effect is not 100%, there is always a residual population left in the soil, especially in the deeper soil layers. Also the decrease in soil inoculum not necessarily corresponds to a similar reduction in disease severity. After field experiments in southern Spain it was reported that solarization significantly reduced V. dahliae

populations in the top 20 cm of soil for at least 3 years in relation to control plots but disease incidence was reduced only in orchards with medium or high initial inoculum densities (Lopez-Escudero & Blanco-Lopez, 2001; Lopez Escudero & Blanco Lopez, 2001). Several other authors, however, report beneficial effects of soil solarization either on disease incidence or disease severity (Otero *et al.*, 2014; Tjamos *et al.*, 1991; Yildiz & Benlioglu, 2010). Apparently in these cases the reduction in soil inoculum supports natural recovery of infected trees by reducing the number of reinfections from soil, and also stimulating the presence of antagonistic microorganisms (Tjamos & Paplomatas, 1987). The positive effects of soil solarization are temporary (Bubici & Cirulli, 2014) but may be lasting for up to at least three years (Tjamos *et al.*, 1991).

- **Plant density**

It is often suggested that Verticillium wilt incidence is higher in high density orchards but the few reports are contradictory and recently Roca *et al*. (2015) reported that in commercial orchards with Arbequina and Picual varieties in southern Spain planting density had no effect on incidence of Verticillium wilt. Probably other factors such as cultivar susceptibility, pathogen virulence and irrigation are more important (Lopez-Escudero & Mercado-Blanco, 2011). High density orchards are usually irrigated by drip irrigation which may increase *Verticillium* problems (see below). In a survey in southern Spain indeed it was reported that the combination of irrigation and high density caused disease incidence to peak in super-high-density olive-tree-planting systems (Rodriguez *et al.*, 2008, 2009).

- **Irrigation**

Irrigation clearly can increase the incidence of *Verticillium* wilt. Surveys in Spain (Blanco-Lopez *et al.*, 1984; Lopez-Escudero *et al.*, 2010; Rodriguez *et al.*, 2009) and Syria (Al-Ahmad & Mosli, 1993) reported higher disease incidences in irrigated orchards than in non-irrigated ones with average disease incidence in irrigated orchards being 3 (Rodriguez *et al.*, 2009) to 5 (Al-Ahmad, Moselli & Doksi, 1992) times higher. The higher disease incidences in irrigated orchards probably result from an increase of inoculum in soil as Lopez-Escudero & Blanco-Lopez (2005) reported that the inoculum density in the wet areas around the drippers in *Verticillium* infested fields were higher than in the dry areas. Also the lower temperature in daily irrigated soils may contribute to a soil environment more conductive to establishment of infection (Perez Rodriguez *et al.*; 2015). The same authors conclude that daily irrigation especially in a susceptible cultivar (Picual) stimulates the onset and development of Verticillium wilt. In a more resistant cultivar (Frantoio) the higher level of resistance apparently has more impact. In addition it has been demonstrated that irrigation water may contain *Verticillium* propagules and directly contribute to distribution of the pathogen (García-Cabello *et al.*, 2012; Rodriguez-Jurado & Bejarano-Alcazar, 2007). Here it also should be mentioned that salinity as in natural saline soils or because of the use of salt-rich irrigation water increases the incidence and severity of Verticillium wilt (Levin, Lavee & Tsror, 2003a; Levin *et al.*, 2003b; Pegg & Brady, 2002)

- **Weed control**

Verticillium not only attacks olive, but has many host plants including many non-crop plants. As a result the fungus can survive and even increase on weeds present in or near fields with *Verticillium* susceptible crops (Thanassoulopoulos, Biris & Tjamos, 1981). The higher disease incidences in non-tilled orchards as compared to regularly tilled orchards as reported by (Lopez-Escudero *et al.*, 2010) may be related to more weeds being present in the non-tilled orchards. Moreover such orchards also may have been tilled in the past.

- **Tilling/ploughing**

Generally it is accepted that root wounding may stimulate the occurrence of Verticillium wilt by providing root entries to the pathogen (Tjamos, 1993). In addition tilling may contribute to spreading the pathogen throughout the orchard. In a survey in Syria (M. Al-Ahmad *et al.*, 1992; M. A. Al-Ahmad & Mosli, 1993) disease incidence indeed was reported to be correlated with the number of ploughings in olive fields. On the other hand regularly applied superficial disking for weed control may be beneficial because of limiting the build-up of soil inoculum (see section on weeds).

- **Pruning/pollarding**

Pruning away diseased branches is a common practice in management of olive trees. Theoretically this practice may help to support natural recovery. Therefore it has been tried to use severe pruning or even pollarding of trees in managing schemes aimed at stimulation of natural recovery (Bubici & Cirulli, 2014). The effect on recovery however appeared to be only temporarily with the regrowth developing new symptoms 3-21 months after the start of the experiment. Also pruning debris should be removed from the field or burned because it is an important source of new inoculum (Lopez-Escudero & Mercado-Blanco, 2011).

- **Fertilization**

In agricultural crops much research has been done on the effect of nutrients on Verticillium wilt. In general it is assumed that high levels of N fertilization and deficiency in K can increase incidence and severity of Verticillium wilts (Pegg & Brady, 2002). Data on olive are very limited but the results of a small scale study on young diseased olives cv Nabali in Jordan are in line with this general assumption as they report that fertilization with 150 gr/tree NPK 15:15:30 decreased disease severity and percentage infection (Abu-Qamar & Al-Raddad, 2001).

Best Practices for Control

General aspects

The only fully effective method to avoid losses by Verticillium wilt is **prevention.** For the establishment of new fields this means planting healthy planting material in clean soils. To be effective the planting material should be guaranteed free from *V. dahliae* and the soil of new planting sites should be tested thoroughly for infection by the pathogen before planting.

The long life of olive trees is a complicating factor in management of Verticillium wilt. Inoculum densities before planting only give an idea of the disease risk in the first years after planting. During the life-span of the trees, however, newly arriving inoculum as well as inoculum build-up in infected fields may add substantially to the disease risk. Therefore prevention of introduction of *V. dahliae* into and spread within fields should be important aspects of the management of olive fields throughout their life-span.

In existing olive fields there is no single effective method to control the disease. Also there is no cure for infected trees. Therefore the only way to effectively control the disease in existing fields is through an **integrated approach** using all possible measures, even if individually their efficacy is limited. Such an approach combines measures focusing on three main themes: exclusion, eradication and escape (Barranco *et al.*, 2010; Lopez-Escudero & Mercado-Blanco, 2011). As long as a field is free from *Verticillium* preventive measures are sufficient. However, once *Verticillium* has infested a field or part of it also measures from the other categories should be included. The optimal strategy depends on the local conditions. The main options are listed below and a schematic decision scheme is given in the figure on the next page.

Measures for an integrated approach

- **Exclusion measures** (preventing introduction and spread of inoculum)
    - Only use healthy (*Verticillium* free) propagation and planting material
    - Avoid planting olive near fields where Verticilium susceptible crops like cotton are grown and avoid planting on soils with a history of such crops
    - Test the soil before planting. New trees should only be planted in *Verticillium* free soil; if the soil is infested try to eradicate the pathogen as much as possible before (re)planting
    - Avoid horticultural crops (vegetables) to be grown in or near olive plantations
    - Prevent remains from susceptible crops on neighboring fields being blown into olive fields
    - Clean machinery before moving to another field to prevent transport of contaminated soil
    - Avoid soil being spread by water (run-off) or wind by using reduced tillage systems or cover crops (never use species susceptible to *Verticillium* for this purpose)
    - Avoid leaves from diseased trees being spread

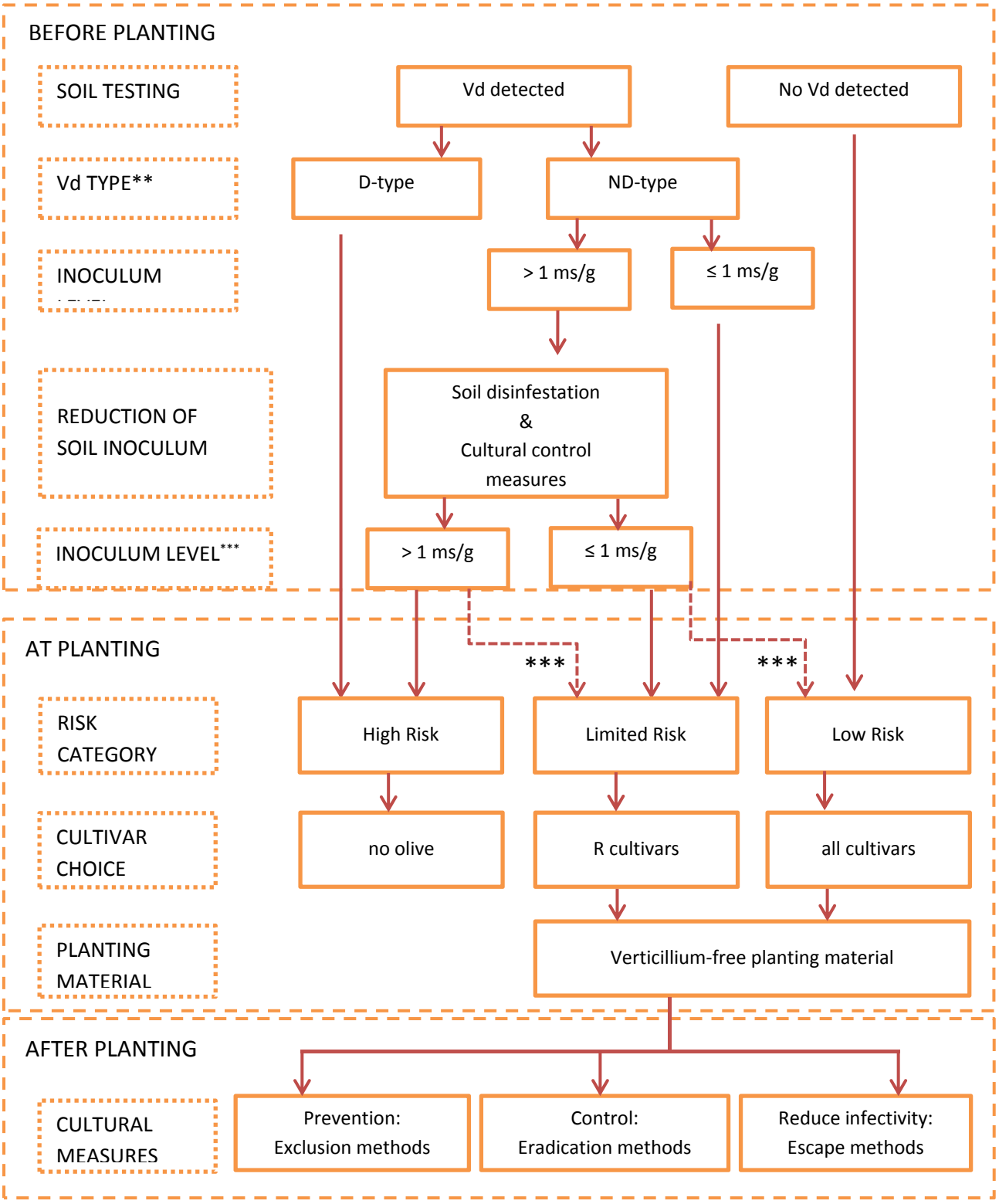
- Disinfect pruning tools when moving to the next tree
- Avoid using natural cover crops that include pathogen hosts
- Avoid using pruning material from infected trees for mulching
- Check plant material that is used as organic amendment (e.g. leaves from the cleaning in olive oil cooperatives) for infection by *Verticillium*

- **Eradication measures** (reducing inoculum levels in soil and prevent build-up as much as possible)
  - Remove and destroy (burn) single diseased trees in young plantings completely (including leaves) as soon as possible to prevent build-up of new inoculum
  - Prune and burn diseased parts from affected trees, as well as fallen leafs from these trees
  - Keep soils free of weeds by using herbicides and/or superficial disking; cover crops could also be used (between rows or full-field) to suppress weeds. However, species susceptible to *Verticillium* should never be used as cover crops.
  - Reduce soil inoculum by soil solarization or chemical methods (when allowed); this can be applied before planting of new fields as well as in existing fields after removing individual diseased trees. Complete eradication of the pathogen, however, is virtually impossible and non-eliminated *Verticillium* propagules may lead to re-infestation

- **Escape measures** (reducing the efficiency of the soil inoculum; i.e. measures aimed at escaping from disease in situations with soil inoculum present)
  - Do not plant olives when D-type *Verticillium* is present or on soils with moderate to high levels of ND-type *Verticillium* (see decision scheme)
  - When planting on infested soils use resistant or tolerant cultivars, or susceptible cultivars on a resistant rootstock but only when inoculum levels are low
  - Avoid extensive root damage
  - Minimize dose and limit frequency of irrigation; especially avoiding daily irrigation frequency
  - Decrease the dose of nitrogen fertilizer and fertilize in a balanced manner

In future biological control by using antagonistic organisms may be an useful addition to eradication and escape strategies, but these methods currently are still being developed.

Decision scheme*



* Figure redrawn and adapted after (Barranco *et al.*, 2010 and Lopez-Escudero, 2011 #70).

** Vd – Verticillium dahliae, D-type – defoliating strains, ND-type – non-defoliating strains

*** The threshold level in the scheme is based on the very scarce information available on threshold levels for damage in olive and nursery trees (see text), and should be seen as an indication only. Depending on local conditions it might be possible to grow resistant cultivars when soil inoculum levels are somewhat higher. For that reason there is also a (dotted) line from the situation with > 1 ms/g soil of the ND type to the use of resistant cultivars. However, it should be stressed that from the available information (see text) it follows clearly that very low levels of soil inoculum already can result in substantial damage.

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Appendix 1 Protocol for soil sampling and DNA extraction

1. Sampling:
    a. Collect 25 different subsoil samples for each hectare.
    b. Collect the subsamples within 30 cm of olive trees using a hand trowel to sample 5-15 cm deep at the base.
    c. Combine the different soil samples.

2. DNA extraction:
    a. Add 2 heaped spoonfuls of soil in the bottle with the ball bearings. Add content of bottle “A” and shake vigorously for 2 minutes.
    b. Pour soil sludge into extraction tube and centrifuge for 5 minutes.
    c. Transfer 1 mL of liquid phase into tube “B”, knock and shake to get an opaque liquid.
    d. Add content of tube “C” into tube “B” and mix by inverting the tube several times. Centrifuge for 10 minutes.
    e. Transfer 750 µL of liquid phase of tube “B” to tube “D” and mix by inverting the tube. Allow to stand for 1 minute.
    f. Place tube on magnetic stand, leave for a few seconds and then remove liquid phase using a pipette.
    g. Remove tube “D” from magnetic stand and add 1 mL of liquid from tube “E”. Mix by inverting the tube several times and leave for 1 minute. Replace tube on the magnetic stand, leave for a few seconds and then remove the liquid phase.
    h. Repeat step g and leave the tube with lid open on the stand for 10 minutes.
    i. Remove tube from magnetic stand and add 100 µL of liquid from tube “F”. Mix by tapping the bottom of the tube, leave to stand for 2 minutes.
    j. Replace tube “D” on magnetic stand, wait until the magnetic bead suspension stands in the side, and transfer the liquid phase into the collection tube.

Appendix 2 Protocol for plant sampling, DNA extraction and analysis by Q-PCR

Protocol for sampling of olive trees for detection of *V. dahliae*

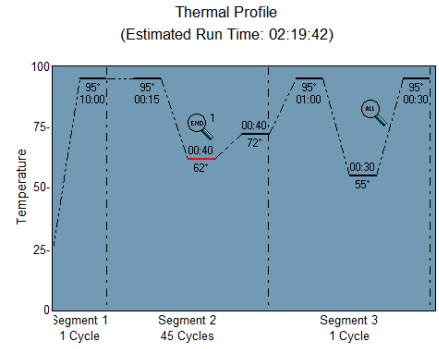
1. Per tree 5 branch samples are taken from the middle and lower part of the crown at different sides of the tree. Each sample consists of the top end of a shoot containing this year's growth and last year's growth and is about 20-30 cm of length.
2. Samples are stored in a plastic bag with 5 samples per tree and marked with a code identifying the tree.
3. When processing the sample for analysis 3 subsamples from top and middle part of each branch are combined in one sample per tree screening for detection of *V. dahliae.*
4. From here the protocol for detection of *V. dahliae* by LAMP-PCR or Q-PCR is followed..

Protocol for DNA extraction (laboratory version: DNeasy kit)

For woody samples (branch) 200-400 mg was ground in 0,5 - 1 ml lysis buffer AP1 (DNeasy Plant Mini Kit) in 2 ml reaction tube (Eppendorff) using a Retsch mixer mill.
From this sample, 400 µl of crude extract was used for DNA isolation according to the manufacturer's instruction using the Qiagen column based extraction (DNeasy Plant Mini Kit):
1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor(R), the TissueLyser II or a mortar and pestle.
2. Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation
3. Add 130 µl Buffer P3. Mix and incubate for 5 min on ice.
4. Centrifuge the lysate for 5 min at 20,000 x g (14,000 rpm).
5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x g.
6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting
7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x g (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.
8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x g. Discard the flowthrough.
9. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x g.
10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
11. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g
12. Repeat step 11.
13. DNA is now ready to use.

Protocol for detection of *V. dahlia* by real Time (SYBRGreen) PCR

1. SYBRGreen Real Time PCR was carried out using a Stratagene Mx3000P Real Time cycler
2. Reactions were prepared in total reaction volumes of 25 µl, using Brilliant II Master Mix (Stratagene)
3. The reaction mix contained ca. 100 ng DNA (in 1µl sterile distilled water), 12.5 µl of 2X mastermix (Promega) and 1 µl of the forward and reverse primers (approx. 10 µM each).
4. The thermal cycling profile consisted of an initial cycle of 95 ^{o}C for 10 min and 45 cycles at 95 ^{o}C for 30 s, 62 ^{o}C for 40 s and 72 ^{o}C for 40 s.
5. The Ct value and the specificity of the reaction were viewed using the Stratagene Program software.



Thermal profile Q-PCR assay

Protocol for on farm DNA extraction

1. Crude extracts are prepared by rubbing with a battery powered electric drill.
2. 300 mg of woody material (saw dust) is added to 5 ml LFD Buffer C (Forsite Diagnostics) in a bottle containing 5 stainless steel balls
3. The bottle with olive sample is thoroughly shaken by hand during 2 min. to further macerate the sample.
4. Subsequently, an DNA LFD (Forsite) is put into the crude extract during 2 minutes
5. DNA is extracted after two times washing with buffer C (200 µl)
6. DNA is eluted from the LFD by using sterile (MQ) water (200 µl)

	
Drill	LFD Buffer C (Forsite Diagnostics), shaking by hand
	
2-minute DNA LFD (Forsite) , drying	Use eluted DNA for LAMP PCR (or Q-PCR)