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Mini review

Quality of methods to quantify microsclerotia of *Verticillium dahliae* in soil

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Abstract

Existing methods used to quantify microsclerotia of *Verticillium dahliae* in soil are reviewed. Most quantification methods are soil-type dependent, but are useful for disease prediction within certain soils. The major factor determining the accuracy of dry plating methods is the amount of soil plated per Petri dish. Wet plating methods are less sensitive to higher amounts of soil, especially when the fraction smaller than 20 μm is removed by wet sieving. Despite general assumptions, wet plating methods do not have lower detection limits than dry plating methods. Dry plating methods are less variable at higher inoculum levels, but more variable at low inoculum levels. Bioassays are helpful tools in answering specific research questions, but are not convenient for large scale use. Molecular quantification techniques are promising, because they are not hampered by antagonistic effects, but data on their disease predictive abilities are still largely lacking. Suggestions are given for a better comparison of techniques, and some original results are presented to illustrate certain arguments.

Abbreviations: CFU – colony forming units.

Introduction

The soil-borne fungus *Verticillium dahliae* causes severe wilt in many herbaceous and woody plant species. Among them are agricultural crops (cotton, potato), vegetables (artichoke, eggplant, pepper), fruits (apricots, avocado, cocoa, grapevine, olive, strawberry), flowers (chrysanthemum), fibre and oilseed crops (flax, sunflower) and woody ornamentals (ash, catalpa, maple, lilac, rose). *V. dahliae* is widely distributed throughout the subtropical and temperate zones, but is generally absent from tropical lowlands (Pegg and Brady, 2002). Crop losses of 13–25% have been reported in potato (Bollen et al., 1989; Haverkort et al., 1989; Locke, 2001; Nagtzaam, 1998), or even higher when nematodes were also present (Martin et al., 1982), and up to 90% in maple and olive (Goud et al., 2000; Harris, 1998; Jiménez-Díaz et al., 1998).

Microsclerotia, the surviving structures of *V. dahliae*, which are formed in senescing plant tissues, may persist in soil for many years in the absence of hosts (Pegg and Brady, 2002). Quantifying the density of microsclerotia in soil is important for disease prediction and for assessing the effect of control measures. Many methods have been described for quantifying *V. dahliae* in soil, but despite huge efforts, no reliable method exists: the best method detects only 50% of the microsclerotia. Moreover, methods are highly soil-type dependent and large differences are observed between quantification performed by different laboratories (Termorshuizen et al., 1998), even when the same method is used (Termorshuizen et al., unpublished data). In this review, we will try to uncover what are the reasons for these differences and give suggestions for a better comparison of methods. Existing methods will be grouped according to working mechanisms

and discussed with respect to their quality of performance, convenience and disease predictive abilities. Quality of performance includes: (1) the detection percentage (i.e. the nearness to the true value, also called accuracy or recovery); (2) the detection limit (i.e. the lowest density in the soil that can be detected, also called sensitivity or threshold); and (3) the variability (i.e. the variation between measurements; the reverse of precision). Convenience includes the expertise, facilities, time and money needed to perform a given method. The relation between measured inoculum densities and observed disease or yield loss of the crop of interest shows the usefulness of a method in practice. This relation is affected by the microbial, chemical and physical factors in the soil, so a quantification method that is affected by the same soil factors could be better with respect to disease prediction than a method that detects 100% of the microsclerotia.

Some of the references in this review apparently refer to *V. albo-atrum*. This is because many researchers have regarded *V. dahliae* as a subgroup within *V. albo-atrum*. Strains were referred to as 'microsclerotial' and 'dark mycelial' strains. After 1976, there was a general international agreement that earlier references to 'the microsclerotial form of *V. albo-atrum*' actually referred to *V. dahliae* (Pegg and Brady, 2002).

Throughout the text, results will be presented of own experiments on optimisation of existing quantification methods or refining steps. Until now, one inter-laboratory comparison has been published (Termorshuizen et al., 1998), with erratum (Termorshuizen et al., 1999), which will be discussed thoroughly and partly re-analysed. The aims of the present study were: (1) to review the published methods used to quantify microsclerotia of *V. dahliae* in soil; (2) to discuss their quality of performance, convenience and biological relevance; (3) to give suggestions for a better comparison; and (4) to optimise existing quantitative detection methods.

Methods to quantify *V. dahliae* in soil

Methods to quantify *V. dahliae* in soils can be divided into plating methods (dry and wet plating), bioassays, molecular methods and immunoassays. Plating methods have often been used in risk analysis studies and therefore they will be treated here extensively, together with published refinement steps and media.

Plating methods

Dry and wet plating involve spreading a known amount of soil onto a Petri dish containing agar media semi-selective for *V. dahliae*, followed by incubation, which enables the microsclerotia to germinate and form colonies in the medium. Prior to plating, the soil sample has to be air dried for 2–4 weeks to kill conidia and mycelial fragments of *V. dahliae* (Butterfield and DeVay, 1977). After incubation, the soil is washed off and the developed colonies containing newly-formed microsclerotia can be counted under a dissecting microscope. The observed inoculum level is expressed as the number of colony forming units (CFU) per unit of weight (or volume) of dry soil.

Media

To obtain detectable colonies of *V. dahliae* on agar plates, microsclerotia plated onto the agar need to germinate, show hyphal growth and form new microsclerotia. Germination of microsclerotia in soil is inhibited through the process of microbiostasis, which is overcome by excess availability of carbon and nitrogen sources occurring in root exudates (Emmatty and Green, 1969; Olsson and Nordbring-Hertz, 1985; Powelson, 1966; Schreiber and Green, 1963). However, rich media are not useful for soil plating, because other fungi will overgrow *V. dahliae*. Use of poor media amended with undefined medium ingredients such as soil extracts (Harris et al., 1993; Menzies and Griebel, 1967) and cellophane extract (Taylor, 1969) have been advocated. Care should be taken in using undefined compounds in media, as these diminish reproducibility. Our results of effects of adding root exudates of eggplant (*Solanum melongena*) or smoketree (*Robinia pseudo-acacia*), both hosts of *V. dahliae*, to a poor agar medium to improve germination of the microsclerotia, indicated that the media do not lack a stimulant (unpublished data). Rather, germination or growth of *V. dahliae* into the agar medium may be inhibited by competing micro-organisms. To favour growth of *V. dahliae*, semi-selective carbon sources, such as pectate (polygalacturonic acid) (Green and Papavizas, 1968; Zehsasian, 1966) or ethanol (Nadakavukaren and Horner, 1959), that can only be metabolised by relatively few other fungi, are added to the agar medium. In addition, Tergitol NP-10 (NPX) has been used to restrict fungal colony size (Huisman and Ashworth, 1974a) and antibiotics to prevent bacterial growth. Data obtained in our own laboratory show that the brand

of agar used can greatly affect quantification. When Oxoid technical No-3 agar (Oxoid, UK) was replaced with BDH technical agar (BDH, UK), significantly lower counts were obtained. Similar problems were observed with sodium polypectate (Sorensen et al., 1991). Currently the use of polypectate is problematic as the most frequently used brands (Bulmer, UK and Sigma, USA) have become unavailable recently, and other brands need to be optimised for pH before use (R. Hooftman, pers. comm.; Kabir et al., 2001).

Dry plating

All published dry plating methods use an Andersen Air Sampler (Thermo Andersen, Smyrna, Georgia, USA) to distribute the soil on the medium (Butterfield and DeVay, 1977). The sampler was designed for sampling and subsequent analysing the microbial community of air (Andersen, 1958). It was first used by Buxton and Kendrick (1963) for analyses of soil and later modified for quantification of *V. dahliae* propagules in soil (Butterfield and DeVay, 1977; DeVay et al., 1974; Harrison and Livingston, 1966). Soil is drawn by an air current through two sieve plates with 400 pores of 1.18 (top) and 0.83 mm (bottom), and impacted in 400 regular spots on Petri dishes placed below the sieve plates. The top Petri dish is changed after each subsample and the bottom 'catcher' Petri dish is changed after the final subsample. Several researchers prefer to use one Petri dish (one-tiered), placed beneath the sieve plate with 1.18 mm pores, which is removed after each subsample (e.g. Nicot and Rouse, 1987a; Paplomatas et al., 1992).

As an alternative to the Andersen Air Sampler, another dry plating method was used by us, viz., sprinkling by hand. A known amount of air dry soil is put on a watch glass and carefully sprinkled randomly over a Petri dish from approximately 10 cm height by ticking against the watch glass. A sheet of white paper under the Petri dish catches any soil that falls outside the Petri dish. No significant differences were observed between the number of CFU g^{-1} soil detected by the Andersen Air Sampler method and the dry sprinkling method, but the sprinkling method took five times longer.

Wet plating

Previously published wet plating methods can be divided into two procedures. The first procedure spreads the entire sieved (see Soil handling and refinement procedures) soil sample over a number of agar plates, The second procedure suspends the soil in water and spreads small aliquots of this suspension over a number of agar plates. The first procedure is often confusingly referred to as 'wet sieving' (Ashworth et al., 1972b; Huisman and Ashworth, 1974a), and the second procedure is often erroneously referred to as 'soil dilution' (Nicot and Rouse, 1987a), even though no dilution series is made (Isaac et al., 1971; Rush et al., 1992). The main difference between the two methods is the amount of soil (equivalents) plated per Petri dish, which is about 1.0 g for the wet sieving method (Ashworth et al., 1972b; Huisman and Ashworth, 1974a; Nicot and Rouse, 1987a) and about 0.1–0.5 g for the soil dilution method (Nicot and Rouse, 1987a; Harris et al., 1993).

Both wet and dry plating require basic laboratory facilities to prepare the medium, an incubator and a dissecting microscope. Time needed for plating the soil sample and counting the colonies varies between methods, but usually around 30 samples or more can be plated in one day and counted during several days to 1 week. Time needed for incubation is 2–6 weeks (Termorshuizen et al., 1998).

Soil handling and refinement procedures

Milling and mixing. Milling of the soil sample to release microsclerotia from soil particles is necessary for clay-type soils. Severe milling, however, may break microsclerotia into smaller fragments, resulting in inoculum densities that are up to five times greater than the actual densities in the field. Prolonged drying of the soil before milling may even aggravate this effect (Ashworth et al., 1974). Milling procedures that led to the highest amount of microsclerotium breakage (as assessed by plating different size fractions) were mortar and pestle (e.g. Schnathorst and Fogle, 1973), followed by high speed (20,000 rpm) micromill (Harrison and Livingston, 1966) and low speed (<200 rpm) milling to 2 mm size (Ashworth et al., 1974; Harris et al., 1993). Butterfield and DeVay (1977) used a revolving jar mill to reduce soil particle size, which did not increase apparent inoculum levels. An extra advantage of this latter technique was that the samples became well mixed, which is very important when working with small subsample sizes necessary for dry plating. Wet plating methods use larger subsamples and mixing is less critical than with dry plating methods. Breaking soil aggregates with a 1% sodium hexametaphosphate solution before wet plating had no effect on quantification (Harris et al., 1993). Breaking down heavy clay soils with sodium pyrophosphate in

the soil suspension was used occasionally (Nagtzaam, 1998).

Sieving. Microsclerotia can be separated from larger or smaller organisms by wet or dry sieving. The diameter of microsclerotia in naturally infested soils varies between 11 and 125 μm, but larger aggregates may occur (Ashworth et al., 1972b; DeVay et al., 1974). Microsclerotial size distribution varies strongly between soils, probably as a result of cropping history. For example, the fraction smaller than 38 μm has been reported to be <2% in potato soils (Smith and Rowe, 1984), but 30–68% in cotton and sunflower soils (Butterfield and DeVay, 1977; Camporota and Rouxel, 1977).

Wet sieving with nested sieves (with pore sizes of e.g. 125 and 20 μm) consists of vigorous washing with tap water to break down soil aggregates, manually or with a shaking machine (Harris et al., 1993; Huisman and Ashworth, 1974a). Dry sieving of small amounts of soil is also possible (Ashworth et al., 1972b; Camporota and Rouxel, 1977), and larger amounts can be sieved with an Alpine air current dry sieving device (Hosokawa Alpine Inc., Augsburg, Germany), which creates a continuously moving air current on the sieve surface to prevent pore occlusion. The sieve is covered with a lid and only one sieve can be used at a time. Soil particles are not broken down by dry sieving, which makes the procedure less efficient than wet sieving: more microsclerotia were lost during dry sieving and they were concentrated in a larger volume of soil, viz., 25% (sand) to 33% (clay) of the original soil sample for dry sieving and 15% (sand and clay) for wet sieving (unpublished data).

Sedimentation and flotation techniques. Sedimentation and decanting have been used to separate microsclerotia from smaller soil particles and conidia of soil fungi (Evans et al., 1967). Flotation techniques use the specific density of the microsclerotia to separate them from the bulk soil. A soil sample is suspended in a 65% (w/w) or saturated solution of sucrose (Harris et al., 1993; Huisman and Ashworth, 1974b) and centrifuged. The supernatant containing most of the microsclerotia is decanted, sieved, resuspended in tap water and plated. The flotation step can be repeated to minimise losses (Huisman and Ashworth, 1974b), though Harris et al. (1993) observed no significant improvement after this second step. Ben-Yephet and Pinkas (1976) described a flotation technique for loess soil, using 50% (w/v) caesium chloride and a separatory funnel. This eliminated centrifugation and resulted in a recovery of 55%, with low variation.

Sedimentation and flotation techniques can easily be combined with other refinement procedures such as wet sieving (Huisman and Ashworth, 1974b). An advantage of flotation techniques is that counting the *V. dahliae* colonies is quicker, because the colonies formed are often larger due to less competition from other fungi as a result of low amounts of soil being plated and/or a nutrition effect of the sucrose (Harris et al., 1993). A disadvantage of flotation techniques is that they are time consuming and do not always lead to improved detection percentages or detection limits (Harris et al., 1993; Termorshuizen et al., 1998).

Breaking of dormancy. Kapulnik et al. (1985) described the use of methionine to break fungistasis and to significantly improve detection percentages of dry plating. Ten gram of dry soil was incubated for 1 week at 33 °C with 15 mg of dl-methionine (Paplomatas et al., 1992). Rinsing for 10 s in 0.5% NaOCl was used to relieve microsclerotia from copper-induced fungistasis (Ashworth et al., 1976), but this appeared not to improve detection in soils without copper-induced fungistasis (Harris et al., 1993).

Bioassays

Bioassays for quantification of *V. dahliae* in soil have not been used on a regular basis and have remained at an experimental stage (Evans et al., 1974; Soesanto, 2000). Sometimes, they have been used for special purposes, such as selection of plants resistant to the pathogen (Palloix et al., 1990) or the evaluation of biocontrol agents (e.g. Nagtzaam et al., 1997). However, bioassays are not truly quantitative, unless serial dilutions of the soil are made with clean soil, and disease or infection is analysed using most probable number statistics (Maloy and Alexander, 1958). During bioassays, soil is planted with susceptible plants (e.g. eggplant (*S. melongena*) or *Arabidopsis thaliana*) or resistant plants (thorn apple (*Datura stramonium*)). After growing these plants at standardised conditions for 3–8 weeks, roots are washed and plated on a semi-selective medium. After incubation, the percentage of the root length infected (Soesanto, 2000) or number of colonies per unit root length (Evans et al., 1974; Nagtzaam et al., 1997) can be counted. Alternatively, on susceptible plants, disease can be scored or the amount of systemic infection can be assessed by plating

stem pieces or xylem sap, but these measurements are less sensitive (Nagtzaam et al., 1997; Soesanto, 2000). Assessment of the amount of newly-formed microsclerotia in senescing shoot tissue was the most sensitive measure, but also more variable than root plating (Soesanto, 2000).

The bioassays of Soesanto (2000) and Nagtzaam et al. (1998) had the lowest published detection limits (viz. 1 microsclerotium g^{-1} soil), but have only been tested with artificially infested semi-sterile soils and not with field soil. The method of Evans et al. (1974) was claimed to have a detection limit of 2–3 microsclerotia g^{-1} dry field soil, but it was probably higher because of underestimation of the natural infestation level, which was estimated by plating.

Bioassays require standardised growing conditions (temperature, soil humidity) and expertise in executing and standardising all steps involved (transplanting, disease assessment, root plating).

Other approaches use measurements of infection in the crops of interest as a measure for soil infestation. Davis et al. (1983) assessed the number of *V. dahliae* microsclerotia in dried stem tissue of potato by plating ground potato stems on semi-selective medium. The increase rate of microsclerotia in the potato stem during the growing season (the slope of the line) was linearly correlated with soil inoculum levels. Similar relationships were found by other authors (Bollen et al., 1989; Nagtzaam, 1998; Nicot and Rouse, 1987b). Alternatively, microsclerotia can be released from potato stems by grinding and dry sieving (Isaac et al., 1971), blending and wet sieving (Soesanto, 2000) or enzymatically with cellulase (unpublished data). Some authors plated potato stem sap (Hoyos et al., 1991; Nagtzaam et al., 1997) on agar media, or assessed the concentration of cotton microsclerotia in a suspension by means of light transmittance at 400 nm (Tsai and Erwin, 1975). A similar approach of focusing on disease in the crop of interest is chosen by some tree nursery growers in the Netherlands. When renting land they include a clause in the contract allowing them to leave the land whenever verticillium wilt is encountered in their first growing season. During this first season they plant throughout the field the highly susceptible *Acer ginnala*. They regard this method as being more reliable and indicative for disease than plating methods (W. Bijvoet, pers. comm.). Probably, data obtained are a good indicator for disease prediction in future crops. However, such methods are essentially post-planting and therefore suboptimal for high value or perennial crops. Moreover, stem infection of potato is cultivar-dependent (Davis et al., 1983). Because stem colonisation increases during the growing season, multiple samples are necessary to assess the increase rate (Bollen et al., 1989; Davis et al., 1983).

Immunoassays

Enzyme-linked immunosorbent assays (ELISA) have not frequently been used for quantification of *V. dahliae* in soil. Heppner and Heitefuss (1997) developed a double-antibody sandwich (DAS) ELISA. This method uses a specific monoclonal antibody, together with a polyclonal antiserum, which react with soluble proteins from microsclerotia. The method includes making a soil suspension, wet sieving, air drying the 20–125 μm fraction, sonification in an extraction buffer, centrifugation, incubation of the supernatant in microtiter plates and measuring the optical density at 405 nm. The detection limit was 2.4 μg of microsclerotia g^{-1} soil equalling about 1–2 microsclerotia g^{-1} soil. A negative relation with yield of oilseed rape was observed in field plots in the range of 0–240 μg of microsclerotia g^{-1} soil (Heppner and Heitefuss, 1997).

Molecular methods

Molecular methods for quantification of *V. dahliae* in soil are based on detection of species-specific nucleotide sequences, using the polymerase chain reaction (PCR) with specific primers. Most of the methods described in literature use sequence differences in the intervening transcribed spacer (ITS) regions of the rDNA. They were initially developed for pure cultures (Carder et al., 1994; Nazar et al., 1991; Robb et al., 1993) and for detection and quantification (Aspromougos and Schlösser, 2000; Hu et al., 1993; Robb et al., 1994) *in planta* and were adapted for detection (Mahuku et al., 1999; Pérez-Artés et al., 2001; Platt et al., 2000) and quantification (Krishnamurthy et al., 2001; Mahuku and Platt, 2002; Robb and Nazar, 1997) in soil. Other methods use sequence differences in the mitochondrial small rDNA (Li et al., 1994) or genomic DNA (Li et al., 1999). The method of Li et al. (1999) was used for quantification of *V. dahliae* in potato and in silica sand. During quantitative assays, different amounts of a competitor DNA are added, which are amplified by the same primers as the target DNA, resulting in an amplified fragment of a different size (competitive PCR). The two fragments are

visible on the gel as two bands. The different amounts of competitor DNA result in different ratios of band intensities between the competitor and the target DNA fragment. Ratios close to 1.0 are used to estimate the concentration of the target DNA (Mahuku and Platt, 2002).

Mahuku and Platt (2002) observed that the log of the estimated amount of target DNA in the range of 10 ng–100 fg was linearly correlated with the log of the number of microsclerotia in the range of 5–100 microsclerotia. The log of the estimated amount of target DNA in the range of 10 ng–1 pg was linearly correlated with the log of the number of conidia in the range of 10^2–10^7 conidia. Higher amounts of microsclerotia or higher conidium densities resulted in the saturation of the PCR reaction and a new range of competitor DNA or dilutions were needed to accurately quantify target DNA. The method of Krishnamurthy et al. (2001) accurately detected DNA in the range of 2–200 pg, with 2 pg approximately equalling 60 genomes of *V. dahliae*. The detection limit observed by Li et al. (1999) was 100 pg of pure genomic DNA (50 copies of target DNA). The lowest numbers of fungal structures they were able to detect were 450 conidia and 17–165 microsclerotia. However, microsclerotium size and consequently number of genomes may vary considerably and the consequences for epidemiological interpretation of PCR-based results need further investigation. Most experiments were performed on microsclerotia or conidia added to sterile soil, but Mahuku et al. (1999) and Mahuku and Platt (2002) quantified *V. dahliae* in field soil. They compared their PCR assay with a plating method (wet sieving over a 38 μm screen followed by sucrose flotation and plating onto NPX-medium). The PCR assay generally detected more microsclerotia than the plating method.

Molecular methods can discriminate between *V. dahliae* and *V. tricorpus* (Heinz and Platt, 2000), and can be adapted to detect other plant pathogenic *Verticillium* species (Robb et al., 1994) or groups within *V. dahliae*, such as the cotton defoliating and non-defoliating strains (Mercado-Blanco et al., 2001; Pérez-Artés et al., 2001). PCR assays deliver results in 1–2 days which is fast compared to plating techniques, as lengthened incubation steps are not necessary. Time consuming plate counts are not needed, but molecular methods need larger initial investments on equipment and chemicals. Molecular quantification methods need to be compared with different plating methods, preferably in an inter-laboratory comparison, to obtain insight in their relative performance. More data is needed on the relation between amount of DNA detected in soil and disease in the field.

Biological relevance of quantification methods

Biological relevance is the most important criterion for the quality of a detection method. Does the result of pre-planting quantification correlate with the amount of disease in the crops of interest? At first sight, biological relevance seems greatest in bioassays, where only those microsclerotia are detected that are viable, germinable and capable of infecting the root or the root cortex. Moreover, *Verticillium* is quantified in the presence of many other soil organisms that can have strong competitive (Evans and Gleeson, 1973; Isaac, 1967), interactive (Martin et al., 1982; Wheeler et al., 1994) or antagonistic (Berg and Ballin, 1994; Marois et al., 1982) effects, thus giving insight in the conduciveness or suppressiveness of a soil. The detection percentage might be negatively affected, but the result may be biologically relevant. However, inoculum density–disease incidence relationships are host-dependent, experimental conditions may affect the results, and care should be taken whenever the test plant is another species or cultivar than the crop of interest. When roots are plated after the bioassay, the colonisation of the root cortex may not be related to pathogenicity (Evans et al., 1974; Huisman, 1988). Colonisation of the vascular system is usually related to disease (Huisman, 1988; Soesanto, 2000).

Quantitative detection through soil plating is also biologically relevant, because only those microsclerotia that are viable, germinable and able to form colonies containing new microsclerotia are counted. Competitive and fungistatic effects are partly taken into account on the semi-selective nutrient media, but are likely to be different than their effects in the soil. All agar media described as semi-selective for *V. dahliae* still sustain the growth of a considerable number of other organisms, though bacterial activity is strongly reduced. The importance of competitors and antagonists on agar plates is illustrated in Figure 1, showing that the more soil is put on a Petri dish, the less colonies of *V. dahliae* are observed, both relative to the amount of soil (CFU g^{-1} soil) and (partly) in an absolute sense (numbers of colonies per plate). Measurements on artificially infested, partially sterilised soil are not biologically relevant. In the expression of inoculum density as CFU g^{-1} soil, biological

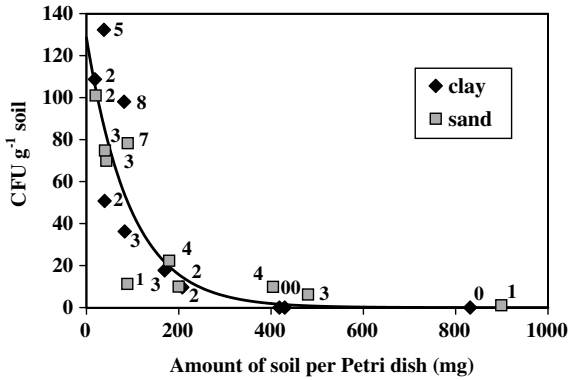

Figure 1. Relation between the detected inoculum density of *V. dahliae* (CFU g^{-1} soil) and the amount of soil per Petri dish. One sandy and one clay soil were used, which were naturally infested with *V. dahliae*. Soils were dry-sieved and only the 20–106 μm fraction was dry plated by hand sprinkling different amounts of soil on modified soil extract agar (Harris et al., 1993), with 50 ppm oxytetracyclin as the single bacteriostatic agent. Each point represents one Petri dish. Figures next to the data points indicate the absolute amount of colonies on that Petri dish. The fitted line shows a significant ($P < 0.0001$) exponential decline model describing the experimental data: the detected inoculum density (CFU g^{-1} soil) $= 129 \exp(-0.01 \times$ the amount of soil per Petri dish (mg)). Data for clay and sand were pooled, because 95% confidence limits for separate parameter estimates overlapped.

relevant information is lost, because large microsclerotia form larger colonies, which are more clearly visible than small microsclerotia (Ben-Yephet and Pinkas, 1977; Hawke and Lazarovits, 1994). Chemical soil characteristics can also influence detection. Ashworth et al. (1976) observed a sudden failure of their detection method because of copper-induced fungistasis of the microsclerotia. However, the infection of cotton in the field was also strongly reduced, so detection was still meaningful. In general, all frequently-used plating methods, such as dry plating, wet sieving and sucrose flotation, have given significant correlations with observed disease (e.g. Ashworth et al., 1972a; Nicot and Rouse, 1987b; Paplomatas et al., 1992), so in principle all are biologically meaningful for specific soils and crops of interest. DeVay et al. (1974) found no significant correlation between inoculum densities assessed by dry plating on soil extract agar with a layer of cellophane and disease in cotton, but possibly many more unpublished negative results exist.

Molecular quantification methods do not take the soil microbial community into account, though it can interfere during DNA isolation. Primers are only useful when they are *V. dahliae*-specific and therefore quantification depends only on the amount of DNA of *V. dahliae* present. This unique advantage can result in detection of dead microsclerotia (Mahuku and Platt, 2002), and could be biologically irrelevant. It has been hypothesised that this problem could be overcome by quantification of mRNA, because those represent recently transcribed genes, and thus represent the active microsclerotia. More data is needed on the relation between PCR-based quantification in the soil and observed disease, to assess the bias due to detection of dead microsclerotia.

Quality of performance of plating methods

The quality of performance of a method depends on two characteristics, viz., the detection percentage and the detection limit. The detection percentage of a method can only be determined for artificially infested soils. Care should be taken to use different soil types and microsclerotia from different origins, because of presence of a method × soil interaction, as observed by Termorshuizen et al. (1998). They included in their study, one soil which had been artificially infested at 5 and 60 microsclerotia g^{-1} soil. The best methods detected 30–50% of the microsclerotia in this soil. However, because of the observed soil-type dependency of methods, it seems better not to focus on performance of methods with this particular soil type. Rather, the performance of a method tested on all soils (in this particular study 12 naturally and 2 artificially infested) should be emphasised and compared with the performance of the best overall method (Table 1). Methods differed much in their performance (Table 1). Dry plating methods generally had a higher overall mean detection percentage than wet plating methods. Dry plating methods performed best at the lowest amounts of soil (25 mg) per Petri dish and performance decreased with increasing amounts of soil (Figure 2), which is in agreement with our own findings (Figure 1), but contradict earlier findings of Butterfield and DeVay (1977). For the wet plating method this relation was unclear, a finding which is in agreement with those of Harris et al. (1993). The detection limit is a measure describing the lowest concentration of CFUs in the soil that can be detected. This means 1 colony in the total number of Petri dishes, and is calculated as 1 divided by the total amount of soil on all Petri dishes. Whenever microsclerotia are concentrated in a smaller volume of soil by refinement procedures, 1 is divided by the original amount of soil that is represented by

Table 1. Detection percentages and detection limits derived from an inter-laboratory comparison of methods (a–k) to quantify *V. dahliae* in soil (Termorshuizen et al., 1998; 1999)

	Plating method										
	Wet plating					Dry plating					
	a[1]	b	d	e	f1	f2	g	h	i	j	k
Amount of soil or soil equivalents per Petri dish (g)	1.50	0.20	0.20	0.10	0.27	0.09	0.10	0.09	0.05	0.05	0.025
Number of plates	10	10	10	6	5	5	4	$9+1^2$	5	3	10
Total amount of soil plated (g)	15	2.0	2.0	0.6	1.35	0.45	0.4	0.81	0.25	0.15	0.25
Theoretical detection limit[3]	0.067	0.5	0.5	1.7	0.74	2.2	2.5	1.23	4.0	6.7	4.0
Observed detection (CFU g^{-1} soil)[4]	0.57	6.4	17	26	18	36	16	36	41	54	67
Detection percentage relative to the best method[5]	0.85	9.6	25	39	27	54	24	54	61	81	100
Relative detection limit (CFU g^{-1} soil)[6]	7.88	5.25	1.97	4.38	2.75	4.09	10.5	2.29	6.5	8.3	4.0
Relative detection limit per Petri dish	78.8	52.5	19.7	26.3	13.8	20.5	42.0	22.9	32.5	24.9	40.0
Variability (%)[7]	30	4.4	1.9	1.7	2.0	1.4	1.6	1.3	1.4	1.0	1.0
Ranking detection percentage	11	10	8	6	7	4.5	9	4.5	3	2	1
Ranking relative detection limit per Petri dish	11	10	2	6	1	3	9	4	7	5	8
Ranking variability	11	10	8	7	9	4	6	3	5	2	1

[1]Methods as described in Termorshuizen et al. (1998). In brief: method a: wet sieving (38–130 μm) followed by sucrose flotation, method d: wet sieving (20–130 μm), other methods applied no refinement steps. Method h used a two-tiered Andersen Sampler, other dry plating methods used a one-tiered Andersen Sampler. Methods h and j used an ethanol–sucrose medium, other methods used pectate-based media.
[2]Nine Petri dishes and 1 catcher Petri dish.
[3]Calculated from 1 colony observed on the total number of plates.
[4]Overall average of 12 naturally infested soils and 2 (one soil type at 2 densities) artificially infested soils.
[5]Method k had the highest amount of CFU g^{-1} soil and was set to 100%.
[6]The theoretical detection limit divided by the detection percentage relative to the best method.
[7]Standard deviation among Petri dishes of the same soil sample ($\sqrt{s_P^2}$ as described in Termorshuizen et al. (1998) multiplied by 100) divided by the observed detection (CFU g^{-1} soil).

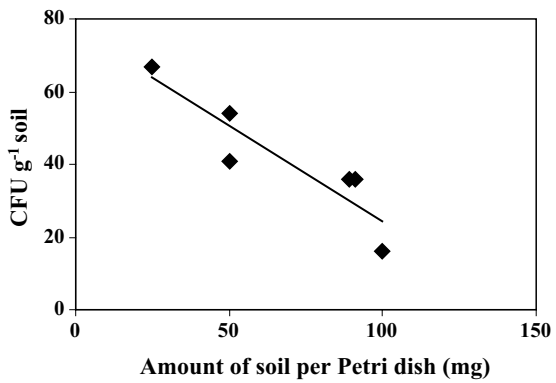

Figure 2. Quantification of *V. dahliae* (CFU g^{-1} soil) in relation to amount of soil per Petri dish. Soil was dry plated with an Andersen Sampler. Data obtained from Termorshuizen et al. (1998; 1999). The fitted line describes a significant ($P = 0.01$) linear regression of the data.

the concentrated soil on the Petri dishes. In this way, methods that use larger amounts of soil (or after refinement: more soil equivalents) have lower theoretical detection limits than methods that use smaller amounts of soil (or soil equivalents). However, these published theoretical detection limits are of no use in practice because they do not take into account the detection percentage. If a certain method is said to have a theoretical detection limit of 0.5 CFU g^{-1} soil, but a detection percentage of 30% (Termorshuizen et al., 1998), the real detection limit would be $0.5/0.3 = 1.67$ CFU g^{-1} soil. Because the real detection percentage is unknown (see above), detection percentages relative to the best method can be used to calculate the relative detection limit of a method (Table 1). Expressed per Petri dish (Table 1) this figure can be used to compare methods properly. By doing so, it becomes clear that, despite of what is generally thought, wet plating methods do not have better detection limits than dry plating methods.

Since observing the colonies on the plates is the most time consuming step (about 5 min per plate), the desired amount of plates and thus the detection limit can be varied depending on the sensitivity of the crop of interest without affecting the performance of a method. However, our suggestion is to use at least 5 Petri dishes, since Termorshuizen et al. (1998) observed that the greatest variation did not occur among soil samples, but among dishes within the same soil samples. Dry plating methods are generally less variable than wet plating methods (Table 1), but at low inoculum densities (approx. smaller than 10 CFU g^{-1} soil) dry plating methods are more variable than wet plating methods (Termorshuizen et al., 1998).

In summary, when deciding which method to use, focus should be placed on (1) the relative detection percentage and (2) the relative detection limit per Petri dish. By doing so (Table 1), it appears that none of the methods listed in Termorshuizen et al. (1998) combines a high detection percentage with a low relative detection limit per Petri dish, but dry plating methods generally combine the two characteristics better than wet plating methods. Whenever artificially infested soils are included, they have to be different origins, infested with microsclerotia from different sources. To compare variability, methods should use the same number of Petri dishes. To gain more insight in real detection limits, the inclusion of a number of soils with very low inoculum levels is necessary, as was done by Harris et al. (1993).

Conclusions

One may wonder why the impressive research efforts have not yet led to a robust and reliable detection technique. One reason may be that the semi-selective polypectate medium used for *V. dahliae* is not as selective as those developed for other soil-borne pathogens such as *Fusarium oxysporum* (Komada, 1975) and *Pythium* spp. (e.g. Stanghellini et al., 1982). Another reason is that *V. dahliae* is able to incite damage to plants at relatively low inoculum densities. Inoculum densities as low as 1 microsclerotium g^{-1} (artificially infested) soil have resulted in symptom development during climate chamber experiments (Soesanto, 2000; unpublished data). Significant symptoms have occurred in the field at detected inoculum levels around 1 CFU g^{-1} naturally infested soil in cotton (Paplomatas et al., 1992), strawberry (Harris and Yang, 1996) and Norway maple (Goud et al., 2000). However, actual densities were probably two to three times higher, because plating methods detect only part of the microsclerotia (Termorshuizen et al., 1998).

The observation that the largest variation in detection of *V. dahliae* occurs at the Petri dish level and not at the subsample level (Termorshuizen et al., 1998), may indicate that the mechanism that explains variability in detection of *V. dahliae* has a stochastic behaviour. For example, the absolute distance between the germinating microsclerotium and other organisms, capable to grow on the medium, could be the decisive factor whether or not *V. dahliae* will form a visible colony. Possibly the best approach to test whether colony formation is inhibited, is by concentrating microsclerotia onto agar media, and quantifying the microsclerotia using molecular methods. After incubation, molecular methods could target at the germinated microsclerotia, and results of molecular quantification and CFU counts could be compared. Insight into the mechanism could well yield information that can be extrapolated to the microsclerotium behaviour in rhizosphere soil.

Alternatives to plating methods include bioassays and molecular methods, but bioassays are very time-consuming and prone to variation according to growing conditions, and molecular methods focusing on direct DNA-isolation presume that a known, same amount of DNA is present in each microsclerotium and that a fixed fraction of the DNA is viable. Until molecular quantification methods have been tested for correlations with disease on a larger scale, plating methods will remain the most reliable means of quantification in soil. However, due to soil type × method interactions it is questionable whether a worldwide standardisation of methods is possible. Interlaboratory comparison of plating and molecular methods will remain important to assess relative performances.

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References

Andersen AA (1958) A new sampler for the collection, sizing and enumeration of viable air-borne particles. Journal of Bacteriology 76: 471

Ashworth LJ, Harper DM and Andris HL (1974) The influence of milling air-dry soil upon apparent inoculum density and propagule size of *Verticillium albo-atrum*. Phytopathology 64: 629–632

Ashworth LJ, Huisman OC, Grogan RG and Harper DM (1976) Copper induced fungistasis of microsclerotia of *Verticillium albo-atrum* and its influence on infection of cotton in the field. Phytopathology 66: 970–977

Ashworth LJ, McCutcheon OD and George AG (1972a) *Verticillium albo-atrum*: The quantitative relationship between inoculum density and infection of cotton. Phytopathology 62: 901–903

Ashworth LJ, Waters JE, George AG and McCutcheon OD (1972b) Assessment of microsclerotia of *Verticillium albo-atrum* in field soils. Phytopathology 62: 715–719

Aspromougos I and Schlösser E (2000) A competitive PCR assay to quantify *Verticillium dahliae* in infected, resistant and susceptible tomato hybrids with HPLC. In: Tjamos EC, Rowe RC, Heale JB and Fravel DR (eds) Advances in *Verticillium*: Research and Disease Management (pp 44–47) APS Press, St. Paul

Ben-Yephet Y and Pinkas Y (1976) A cesium chloride flotation technique for the isolation of *Verticillium dahliae* microsclerotia from soil. Phytopathology 66: 1252–1254

Ben-Yephet Y and Pinkas Y (1977) Germination of individual microsclerotia of *Verticillium dahliae*. Phytoparasitica 5: 159–166

Berg G and Ballin G (1994) Bacterial antagonists to *Verticillium dahliae* Kleb. Journal of Phytopathology 141: 99–110

Bollen GJ, Hoekstra O, Scholte K, Hofman TW, Celetti MJ and Schirring A (1989) Incidence of soilborne pathogens in potato related to the frequency of potato growing on a clay loam. In: Vos J, van Loon CD and Bollen GJ (eds) Effects of Crop Rotation on Potato Production in the Temperate Zones (pp 203–222) Kluwer Academic Publishers, Dordrecht

Butterfield EJ and DeVay JE (1977) Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67: 1073–1078

Buxton EW and Kendrick JB (1963) A method of isolating *Pythium* and *Fusarium* from soil. Annals of Applied Biology 51: 215–221

Camporota P and Rouxel F (1977) Intérêt du broyage et du tamisage à sec dans les techniques d'isolement du *Verticillium dahliae* à partir du sol. Annales de Phytopathologie 9: 77–81

Carder JH, Morton AA, Tabrett AM and Barbara DJ (1994) Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. In: Schots A, Dewey FM and Oliver R (eds) Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification (pp 91–97) CAB International, Wallingford

Davis JR, Pavek JJ and Corsini DL (1983) A sensitive method for quantifying *Verticillium dahliae* colonization in plant tissue and evaluating resistance among potato genotypes. Phytopathology 73: 1009–1014

DeVay JE, Forrester LL, Garber RH and Butterfield EJ (1974) Characteristics and concentration of propagules of *Verticillium dahliae* in air-dried field soils in relation to the prevalence of verticillium wilt in cotton. Phytopathology 64: 22–29

Emmatty DA and Green RJ (1969) Fungistasis and the behavior of the microsclerotia of *Verticillium albo-atrum* in soil. Phytopathology 59: 1590–1595

Evans G and Gleeson AC (1973) Observations on the origin and nature of *Verticillium dahliae* colonizing plant roots. Australian Journal of Biological Sciences 26: 151–161

Evans G, McKeen CD and Gleeson AC (1974) A quantitative bioassay for determining low numbers of microsclerotia of *Verticillium dahliae* in field soils. Canadian Journal of Microbiology 20: 119–124

Evans G, Wilhelm S and Snyder WC (1967) Quantitative studies by plate counts of propagules of the *Verticillium* wilt fungus in cotton field soils. Phytopathology 57: 1250–1255

Goud JC, Termorshuizen AJ, Blok WJ and Coenen GCM (2000) Biologische grondontsmetting en de invloed van initiële inoculumdichtheid op de ontwikkeling van *Verticillium dahliae* in Noorse esdoorn en trompetboom. Gewasbescherming 31: 13

Green RJ and Papavizas GC (1968) The effect of carbon source, carbon to nitrogen ratios, and organic amendments on survival of propagules of *Verticillium albo-atrum* in soil. Phytopathology 58: 567–570

Harris DC (1998) An introduction to verticillium wilts. In: Hiemstra JA and Harris DC (eds) A Compendium of Verticillium Wilts in Tree Species (pp 1–4) CPRO-DLO/HRI-EM, Wageningen/West Malling

Harris DC and Yang YR (1996) The relationship between the amount of *Verticillium dahliae* in soil and the incidence of strawberry wilt as a basis for disease risk prediction. Plant Pathology 45: 106–114

Harris DC, Yang YR and Ridout MS (1993) The detection and estimation of *Verticillium dahliae* in naturally infested soil. Plant Pathology 42: 238–250

Harrison HD and Livingston CH (1966) A method for isolating *Verticillium* from field soil. Plant Disease Reporter 50: 897–899

Haverkort AJ, Vos J, Groenwold J and Hoekstra G (1989) Crop characteristics and yield reduction of potato due to biotic stress in short crop rotations. In: Vos J, van Loon CD and Bollen GJ (eds) Effects of Crop Rotation on Potato Production in the Temperate Zones (pp 273–290) Kluwer Academic Publishers, Dordrecht

Hawke MA and Lazarovits G (1994) Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. Phytopathology 84: 883–890

Heinz RA and Platt HW (2000) A competitive PCR-based assay to quantify *Verticillium tricorpus* propagules in soil. Canadian Journal of Plant Pathology 22: 122–130

Heppner C and Heitefuss R (1997) Development of an enzyme-linked immunosorbent assay (ELISA) for *Verticillium dahliae* detection in soil. In: Dehne HW, Adam G, Diekmann M, Frahm J, Mauler-Machnik A and van Halteren P (eds) Diagnosis and Identification of Plant Pathogens. Proceedings of the 4th International Symposium of the European Foundation for Plant Pathology, Bonn, Germany, 9–12 September 1996 (pp 105–108) Kluwer Academic Publishers, Dordrecht

Hoyos GP, Zambino PJ and Anderson NA (1991) An assay to quantify vascular colonization of potato by *Verticillium dahliae*. American Potato Journal 68: 727–742

Hu X, Nazar RN and Robb J (1993) Quantification of *Verticillium* biomass in wilt disease development. Physiological and Molecular Plant Pathology 42: 23–36

Huisman OC (1988) Seasonal colonization of roots of field-grown cotton by *Verticillium dahliae* and *V. tricorpus*. Phytopathology 78: 708–716

Huisman OC and Ashworth LJ (1974a) Quantitative assessment of *Verticillium albo-atrum* in field soils: Procedural and substrate improvements. Phytopathology 64: 1043–1044

Huisman OC and Ashworth LJ (1974b) *Verticillium albo-atrum*: Quantitative isolation of microsclerotia from field soils. Phytopathology 64: 1159–1163

Isaac I (1967) Speciation in *Verticillium*. Annual Review of Phytopathology 5: 201–222

Isaac I, Fletcher P and Harrison JAC (1971) Quantitative isolation of *Verticillium* spp. from soil and moribund potato haulm. Annals of Applied Biology 67: 177–183

Jiménez-Díaz RM, Tjamos EC and Cirulli M (1998) Verticillium wilts of major tree hosts. Olive. In: Hiemstra JA and Harris DC (eds) A Compendium of Verticillium Wilts in Tree Species (pp 13–16) CPRO-DLO/HRI-EM, Wageningen/West Malling

Kabir Z, Bhat RG and Subbarao KV (2001) Optimization of polygalacturonic acid with NaOH in NP-10 medium to improve *Verticillium dahliae* recovery from soil. Book of Abstracts, 8th International *Verticillium* Symposium, Cordoba, Spain, 5–8 November 2001, p 71

Kapulnik E, Quick J and DeVay JE (1975) Germination of propagules of *Verticillium dahliae* in soil treated with methionine and other substances affecting ethylene production. Phytopathology 75: 1348

Komada H (1975) Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research 8: 114–124

Krishnamurthy V, Barbara D and Price A (2001) PCR-based quantification of *Verticillium dahliae* in soil. Book of Abstracts, 8th International *Verticillium* Symposium, Cordoba, Spain, 5–8 November 2001, p 73

Li KN, Rouse DI, Eyestone EJ and German TL (1999) The generation of specific DNA primers using random amplified polymorphic DNA and its application to *Verticillium dahliae*. Mycological Research 103: 1361–1368

Li KN, Rouse DI and German TL (1994) PCR primers that allow intergeneric differentiation of Ascomycetes and their application to *Verticillium* spp. Applied and Environmental Microbiology 60: 4324–4331

Locke T (2001) The effect of *V. dahliae* on potato production in England and Wales. Book of Abstracts, 8th International *Verticillium* Symposium, Cordoba, Spain, 5–8 November 2001, p 77

Mahuku GS and Platt HW (2002) Quantifying *Verticillium dahliae* in soils collected from potato fields using a competitive PCR assay. American Journal of Potato Research 79: 107–117

Mahuku GS, Platt HW and Maxwell P (1999) Comparison of polymerase chain reaction methods with plating on media to detect and identify *Verticillium* wilt pathogens on potato. Canadian Journal of Plant Pathology 21: 125–131

Maloy OC and Alexander M (1958) The 'most probable number' method for estimating populations of plant pathogenic organisms in the soil. Phytopathology 48: 126–128

Marois JJ, Johnston SA, Dunn MT and Papavizas GC (1982) Biological control of verticillium wilt of eggplant in the field. Plant Disease 66: 1166–1168

Martin MJ, Riedel RM and Rowe RC (1982) *Verticillium dahliae* and *Pratylenchus penetrans*: Interactions in the early dying complex of potatoes in Ohio. Phytopathology 72: 640–644

Menzies JD and Griebel GE (1967) Survival and saprophytic growth of *Verticillium dahliae* in uncropped soil. Phytopathology 57: 703–709

Mercado-Blanco J, Rodríguez-Jurado D, Pérez-Artés E and Jimenez-Díaz RM (2001) Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. Plant Pathology 50: 609–619

Nadakavukaren MJ and Horner CE (1959) An alcohol agar medium selective for determining *Verticillium microsclerotia* in soil. Phytopathology 49: 527–528

Nagtzaam MPM (1998) Biological Control of *Verticillium dahliae* by *Talaromyces flavus*. PhD Thesis, Wageningen University, Wageningen

Nagtzaam MPM, Termorshuizen AJ and Bollen GJ (1997) The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*. European Journal of Plant Pathology 103: 597–605

Nazar RN, Hu X, Schmidt D, Culham D and Robb J (1991) Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of verticillium wilt pathogens. Physiological and Molecular Plant Pathology 39: 1–11

Nicot PC and Rouse DI (1987a) Precision and bias of three quantitative soil assays for *Verticillium dahliae*. Phytopathology 77: 875–881

Nicot PC and Rouse DI (1987b) Relationship between soil inoculum density of *Verticillium dahliae* and systemic colonization of potato stems in commercial fields over time. Phytopathology 77: 1346–1355

Olsson S and Nordbring-Hertz B (1985) Microsclerotial germination of *Verticillium dahliae* as affected by rape rhizosphere. FEMS Microbiology Ecology 31: 293–299

Palloix A, Pochard E, Phaly T and Daubèze AM (1990) Recurrent selection for resistance to *Verticillium dahliae* in pepper. Euphytica 47: 79–89

Paplomatas EJ, Bassett DM, Broome JC and DeVay JE (1992) Incidence of *Verticillium* wilt and yield losses of cotton cultivars (*Gossypium hirsutum*) based on soil inoculum density of *Verticillium dahliae*. Phytopathology 82: 1417–1420

Pegg GF and Brady BL (2002) Verticillium Wilts. CABI Publishing, Wallingford

Pérez-Artés E, Ruz Carrillo A and Jiménez-Díaz RM (2001) PCR-based detection of defoliating and nondefoliating pathotypes of *Verticillium dahliae* in soil. Book of Abstracts, 8th International *Verticillium* Symposium, Cordoba, Spain, 5–8 November 2001, p 60

Platt HW, Mahuku GS, Maxwell P and MacLean V (2000) Detection techniques for research on *Verticillium* species in potato soils. In: Tjamos EC, Rowe RC, Heale JB and Fravel DR (eds) Advances in *Verticillium*: Research and Disease Management (pp 140–143) APS Press, St. Paul

Powelson RL (1966) Availability of diffusable nutrients for germination and growth of *Verticillium dahliae* in soils amended with oat and alfalfa residues. Phytopathology 56: 895

Robb J and Nazar RN (1997) Application of PCR-based diagnostics to monitor *Verticillium*–nematode disease complexes in the field. Book of Abstracts, 7th International *Verticillium*

Symposium, Cape Sounion, Athens, Greece, 6–10 October 1997, p 16

Robb J, Moukhamedov R, Hu X, Platt HW and Nazar RN (1993) Putative subgroups of *Verticillium albo-atrum* distinguishable by PCR-based assays. Physiological and Molecular Plant Pathology 43: 423–436

Robb J, Hu X, Platt HW and Nazar RN (1994) PCR-based assays for the detection and quantification of *Verticillium* species in potato. In: Schots A, Dewey FM and Oliver R (eds) Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification (pp 83–90) CAB International, Wallingford

Rush CM, Mihail JD and Singleton LL (1992) Introduction. In: Singleton LL, Mihail JD and Rush CM (eds) Methods for Research on Soilborne Phytopathogenic Fungi (pp 3–6) APS Press, St. Paul

Schnathorst WC and Fogle D (1973) Survival structures of *Verticillium dahliae* in naturally infested field soils. Proceedings of the Beltwide Cotton Production Research Conference, Phoenix, Arizona, 9–10 January, 1973, p 24

Schreiber LR and Green RJ (1963) Effect of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. Phytopathology 53: 260–264

Smith VL and Rowe RC (1984) Characteristics and distribution of propagules of *Verticillium dahliae* in Ohio potato field soils and assessment of two assay methods. Phytopathology 74: 553–556

Soesanto L (2000) Ecology and Biological Control of *Verticillium dahliae*. PhD Thesis, Wageningen University, Wageningen

Sorensen LH, Schneider AT and Davis JR (1991) Influence of sodium polygalacturonate sources and improved recovery of *Verticillium* spp. from soil. Phytopathology 81: 1347

Stanghellini ME, von Bretzel P, Kronland WC and Jenkins AD (1982) Inoculum densities of *Pythium aphanidermatum* in soils of irrigated sugar beet fields in Arizona. Phytopathology 72: 1481–1485

Taylor JB (1969) Fluctuations in natural soil populations of *Verticillium tricorpus*. Canadian Journal of Botany 47: 737–740

Termorshuizen AJ, Davis JR, Gort G, Harris DC, Huisman OC, Lazarovits G, Locke T, Melero Vara JM, Mol L, Paplomatas EJ, Platt HW, Powelson M, Rouse DI, Rowe RC and Tsror L (1998) Interlaboratory comparison of methods to quantify microsclerotia of *Verticillium dahliae* in soil. Applied and Environmental Microbiology 64: 3846–3853

Termorshuizen AJ, Davis JR, Gort G, Harris DC, Huisman OC, Lazarovits G, Locke T, Melero Vara JM, Mol L, Paplomatas EJ, Platt HW, Powelson M, Rouse DI, Rowe RC and Tsror L (1999) Interlaboratory comparison of methods to quantify microsclerotia of *Verticillium dahliae* in soil. Applied and Environmental Microbiology 65: 2805 (Erratum)

Tsai SD and Erwin DC (1975) A method of quantifying numbers of microsclerotia of *Verticillium albo-atrum* in cotton plant tissue and in pure culture. Phytopathology 65: 1027–1028

Wheeler TA, Madden LV, Riedel RM and Rowe RC (1994) Distribution and yield-loss relations of *Verticillium dahliae*, *Pratylenchus penetrans*, *P. scribneri*, *P. crenatus*, and *Meloidogyne hapla* in commercial potato fields. Phytopathology 84: 843–852

Zehsazian H (1966) An Improved Selective Medium for the Isolation of *Verticillium dahliae* Kleb. MSc Thesis, Oregon State University, Corvallis