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Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil

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The morphology of two soil-borne *Verticillium* species, *V. dahliae* and *V. tricorpus*, was studied on two semi-selective agar media, in the absence and presence of soil. Morphology of the fungi differed considerably between the media, with respect to presence and shape of microsclerotia, dark hyphae (i.e. short melanised hyphae attached to the microsclerotia) and dark mycelium (i.e. melanised mycelium throughout the colony). On modified soil extract agar (MSEA), a pectate based agar, *V. dahliae* always had globose to elongate microsclerotia, without dark hyphae or dark mycelium, whereas *V. tricorpus* always had dark hyphae or dark mycelium, and microsclerotia, whenever present, were globose to irregular in shape. On ethanol agar (EA), *V. dahliae* had large microsclerotia and abundant dark hyphae, whereas *V. tricorpus* did not form microsclerotia, but always abundant dark mycelium. For the first time we observed the formation of dark hyphae by *V. dahliae* to a great extent. In the presence of soil, most characteristics were less pronounced, and *V. dahliae* microsclerotia were smaller, but *V. tricorpus* produced large microsclerotia, even when they were absent in pure culture. Morphological characteristics suitable for discrimination between the two species on MSEA plates in the presence of soil were selected and tested with fresh isolates from agricultural fields. The two fungi could be distinguished using qualitative characteristics and microsclerotial size. Molecular analysis and morphology on potato dextrose agar confirmed all identifications made on soil dilution plates.

INTRODUCTION

Representatives of the form-genus *Verticillium* Nees 1816 in the strict sense (anamorphs of the *Phyllachorales* (Messner *et al*. 1996), are commonly found in agricultural soils (Domsch, Gams & Anderson 1980). The genus includes the virulent plant pathogenic species *V. dahliae* Kleb. 1913, *V. longisporum* (Stark) Karapapa, Bainbridge & Heale 1997 and *V. albo-atrum* Reinke & Berth. 1879, which have low saprotrophic abilities, *V. nubilum* Pethybr. 1918 and *V. nigrescens* Pethybr. 1919 which are saprobes and weak pathogens, and *V. tricorpus* Isaac 1953 which has intermediate saprotrophic ability and is pathogenic to a limited number of crops. All these species can be associated with the same crop, e.g. potato or tomato (e.g. Isaac 1967, Skotland 1971, Domsch *et al*. 1980, Pegg & Brady 2002).

The six species can be differentiated morphologically by the types of resting structures they form in and on the surface of plant material, and on many artificial agar media, such as potato dextrose agar (PDA). *V. dahliae* and *V. longisporum* form microsclerotia, which are black melanised clumps, formed by budding of mycelial cells; *V. albo-atrum* forms melanised resting mycelium; *V. nigrescens* and *V. nubilum* form chlamydospores, and *V. tricorpus* forms microsclerotia, resting mycelium and chlamydospores. The morphological differences of the microsclerotia of *V. dahliae* and *V. tricorpus* are pronounced on PDA, as described by Isaac (1949, 1953) and Smith (1965): *V. tricorpus* forms large and irregularly shaped microsclerotia, usually with melanised hyphae growing from them, and basally pigmented conidiophores, whereas *V. dahliae* forms smaller and oval to elongate microsclerotia which are sharply differentiated from the hyaline mycelium and hyaline conidiophores. Moreover, *V. tricorpus* often causes a yellow discoloration of the PDA medium upon first isolation. *V. longisporum* is known to be a heterodiploid between *V. dahliae* and *V. albo-atrum*. Morphologically *V. longisporum* is most similar to *V. dahliae* but it can be differentiated from *V. dahliae* in pure culture by the shape of the microsclerotia, the number of phialides per node and, particularly, its larger conidia, 8×2.5 µm. *V. longisporum* is mainly known from cruciferous hosts (Karapapa *et al*. 1997).

Table 1. Origin of isolates of *Verticillium dahliae* and *V. tricorpus* used in Experiment 1.

Species	Isolate[a]	Host of origin	Year of isolation	Location	Isolation by
V. dahliae	A59 mc-1	Blackberry (*Rubus fruticosus*)	1996	Spijk, The Netherlands	J. W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A45 mc-1	Strawberry (*Fragaria* sp.)	1996	Elst, The Netherlands	J. W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A60 mc-1	Maple (*Acer* sp.)	1993	Swolgen, The Netherlands	Auf'm Keller, Plant Protection Service
V. dahliae	A56 mc-1	Rose (*Rosa* sp.)	1996	Elst, The Netherlands	J. W. Veenbaas-Rijks, Plant Protection Service
V. dahliae	A63 mc-1	*Forsythia* sp.	1997	Aalsmeer, The Netherlands	B. Wessels, Plant Protection Service
V. tricorpus	A36 mc-1	*Alstroemeria* sp.	1996	Rijnsburg, The Netherlands	J. W. Veenbaas-Rijks, Plant Protection Service
V. tricorpus	127.79A mc-1	Tomato (*Lycopersicon esculentum*)	1979	Roxborough, New Zealand	B. Taylor[b]
V. tricorpus	384.84 mc-1	Potato (*Solanum tuberosum*)	1984	Wageningen, The Netherlands	J. van der Spek, Institute for Plant Protection Research
V. tricorpus	227.84 mc-1	Potato (*Solanum tuberosum*)	1984	Dronten, The Netherlands	T. Hofman, Wageningen University
V. tricorpus	Vtx mc-1	–	–	–	–

[a] Voucher cultures are preserved in CBS (Utrecht).
[b] Unknown.

Because of the resemblance of *V. dahliae* and *V. tricorpus* on poor isolation media, our study is mainly concerned with these two species.

In a number of field crops, but also many woody species, *V. dahliae* can cause serious wilt disease, whereas *V. tricorpus* is generally harmless (Hiemstra 1998). *V. tricorpus* has even been recommended for the biocontrol of *Rhizoctonia solani* in cotton seedlings (DeVay *et al.* 1988, Paplomatas, Tzalavaras & DeVay 2000) and *V. dahliae* in potato (Davis *et al.* 2000). There is a need for the reliable assessment of soil population densities of *V. dahliae* in order to predict possible problems in tree nurseries and agricultural crops. Plating soil samples on semi-selective media is currently the most reliable way to assess population densities of *V. dahliae* in soil (Termorshuizen *et al.* 1998). Microsclerotia are detected indirectly by giving rise to characteristic new colonies with microsclerotia on a semi-selective agar medium. However, microsclerotia of *V. tricorpus*, when present in these soil samples, can also form similar colonies with microsclerotia. Rich media, like PDA, cannot be used during quantification of *V. dahliae* in soil, because rapidly growing and sporulating fungi, present in all soils, will overgrow *V. dahliae*, resulting in failure of quantification. While morphological differences are pronounced on rich media, on (poor) semi-selective media *V. dahliae* and *V. tricorpus* are easily confused, even by experienced researchers (Termorshuizen *et al.* 1998). Isolated colonies can be characterised using molecular techniques (Robb *et al.* 1994). However, the method of transferring colonies from semi-selective media to PDA, and purification for identification of species is too laborious to be employed as a standard procedure. Therefore it is important for disease prediction to compare the morphology of the two species on the original semi-selective media directly, and describe differential characteristics of the two species.

In this study we: (1) compared the morphology of *V. dahliae* and *V. tricorpus* on semi-selective media in pure culture and in the presence of insterile soil; (2) distinguish characteristics that can be used to separate the two species on semi-selective media in the presence of soil; and (3) test these characteristics on colonies from soil samples of agricultural fields. The identity of isolates was always verified by molecular analysis.

MATERIALS AND METHODS

In Experiment 1, five single-spore isolates of both *Verticillium dahliae* and *V. tricorpus* were studied. In Table 1 these isolates and their origin are listed. Morphology was first studied in pure culture on PDA to determine the species: isolates with irregularly shaped microsclerotia, chlamydospores, melanised mycelium, and yellow discoloration of the medium were classified as *V. tricorpus*. Isolates with globose, oval to elongate microsclerotia and without chlamydospores or melanised mycelium were classified as *V. dahliae* (Isaac 1949, 1953, Smith 1965). All isolates fitted these descriptions, except for the *V. tricorpus* isolates 384.84 mc-1 and Vtx mc-1 (Table 1) which did not form microsclerotia on PDA, and did not show yellow discoloration of the medium.

As experimental treatment, two semi-selective media were compared: modified soil extract agar (MSEA) (Harris, Yang & Ridout 1993), which is a pectate-based medium often used for quantitative detection in soil, and ethanol agar medium (EA) (Nadakavukaren & Horner 1959), often used for isolating *V. dahliae* from plant material. Each semi-selective medium was amended with $50\ \mathrm{mg\,l^{-1}}$ oxytetracycline after

autoclaving as the single bacteriostatic agent. Media for Experiment 1 were produced once, to avoid batch to batch variation. Ten microsclerotia per plate, produced on PDA covered with cellophane to stimulate formation of microsclerotia (DeVay *et al.* 1974), were placed individually on the two media.

Colony growth of *Verticillium* species was studied with and without the presence of soil. For the latter treatment, 0.8 ml a suspension of non-sterile, *Verticillium*-free sandy soil was added to the surface of the inoculated agar plates. The soil had been steamed and cropped with wheat for 6 wk before the start of the experiment. During the experiment, the soil was treated in the same way as a soil sample is treated during the protocol for quantitative detection of *V. dahliae* (Harris *et al.* 1993): 12.5 g of dry soil was sieved, the 20–106 μm fraction was suspended in 0.08% water agar, and 0.8 ml of this suspension was spread over the agar surface in a 9.4 cm diam. Petri dish, after which the plates were air dried for 15 min. The inoculated Petri dishes, with or without soil, were incubated upside down in closed plastic bags for 6 wk at 20 °C. Five Petri dishes were incubated per treatment per isolate with and without soil.

Numbers of microsclerotia and their shape were recorded for each colony. Length and width of ten microsclerotia per colony were measured on three colonies of each combination. The microsclerotia to be measured were randomly chosen from the area halfway between the centre and the edge of the colony. Presence and intensity of dark melanised mycelium, growing radially from the centre throughout the colony was recorded (Figs 2, 6). The intensity was estimated as the percentage of the colony covered by this type of mycelium. In our study, this type of mycelium will be referred to as 'dark mycelium'. The term 'dark hyphae' will be used for short portions (shorter than 120 μm) of melanised hyphae. Dark hyphae usually arise from microsclerotia (Figs 4, 7), but they can also occur singly or several together without connection to a microsclerotium. Dark hyphae cannot be observed properly when dark mycelium is abundant. Colony characteristics like the pattern in which microsclerotia are formed, radial (Fig. 1), scattered (Figs 5–6), or intermediate, and yellow discoloration of the agar medium, were recorded. Chlamydospores were not scored because they were regarded unsuitable for discrimination, for two reasons: (1) chlamydospores are mostly hyaline and therefore difficult to spot under a dissecting microscope; and (2) similar chlamydospores can be formed by various soil-borne fungi and therefore usually occur abundantly throughout semi-selective soil plates. Size of individual microsclerotial cells was not included in the study, because: (1) individual cells cannot be observed properly through a dissecting microscope; and (2) preliminary observations revealed overlap between cell sizes of *V. dahliae* and *V. tricorpus*.

In Experiment 2, ten individual colonies on MSEA soil plates surface-inoculated with a 0.8 ml soil suspension from agricultural fields were examined. Only colonies with microsclerotia were taken into account. Mainly small colonies were chosen, with fewer than 25 microsclerotia, as they are the most difficult to classify as either *V. dahliae* or *V. tricorpus*. Length and width of ten microsclerotia were measured, the pattern in which the microsclerotia were formed, and the presence or absence of connected dark hyphae or dark mycelium was scored. Based on the morphological characteristics of Experiment 1, isolates of Experiment 2 were classified as *V. dahliae* or *V. tricorpus*. Then, pure isolates of these colonies were obtained by retrieving individual microsclerotia from these colonies, washing them several times in sterile demineralised water, and re-plating them on MSEA. Colonies were transferred to clean MSEA plates until they were pure, after which they were grown on PDA for species identification, using the morphological characteristics described above (Isaac 1949, 1953). Microsclerotia of some (later proven to be *V. tricorpus*) colonies were sporulating, and those isolates could be put onto PDA directly, by touching the conidiophore with a sterile needle.

The identity of the ten soil isolates was checked with species-specific primers (Robb *et al.* 1994). In brief, DNA was extracted from conidia and mycelium and purified, followed by a PCR using a primer pair specific for amplification of either *V. dahliae* or *V. tricorpus* rDNA of the ITS region. When amplification of the DNA occurs, this is visible as a 334 bp band when the PCR product is run through a gel.

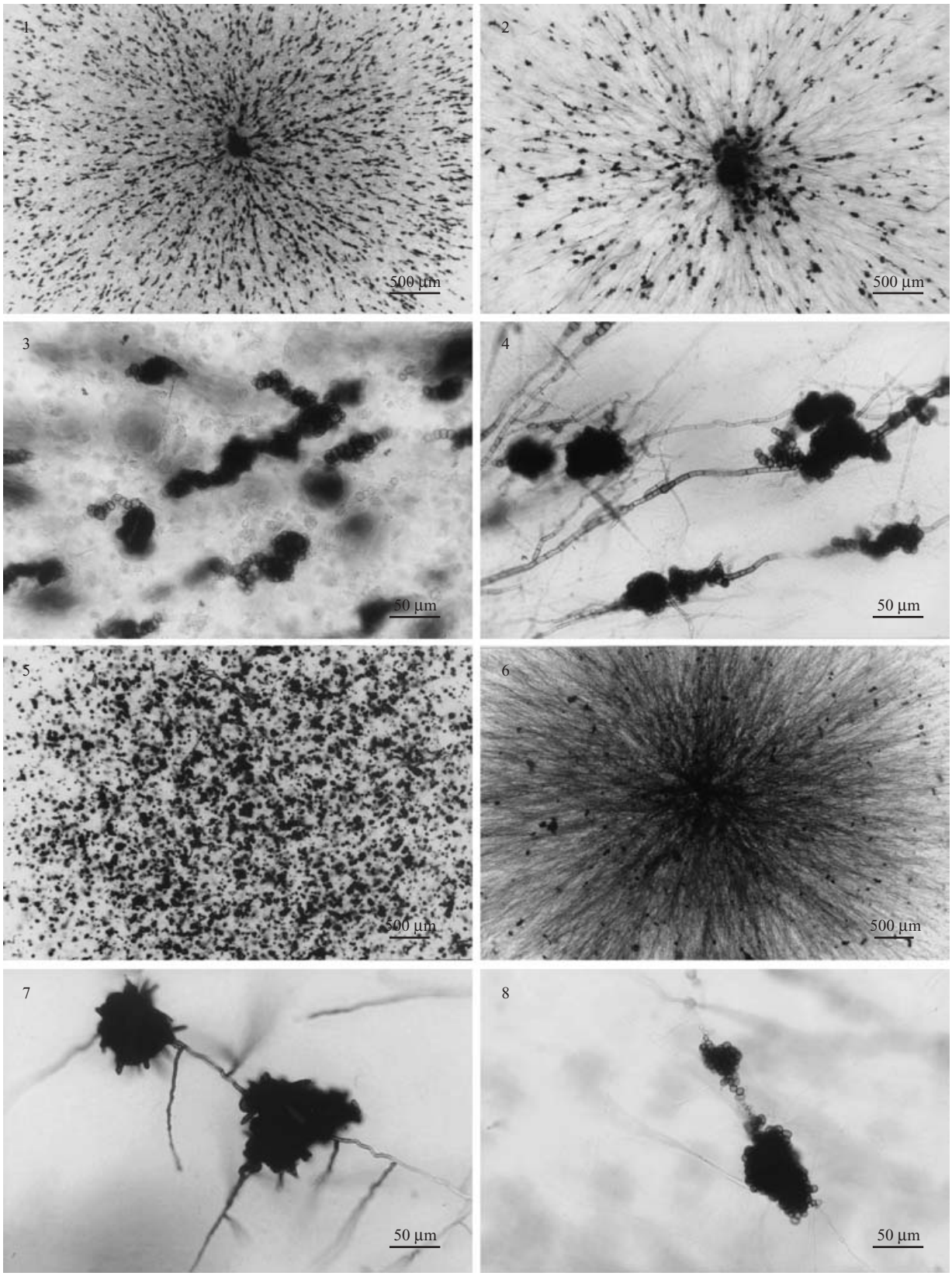
Data on microsclerotial size were analysed using the GLM procedure of SAS version 8.0 (SAS Institute, Cary, NC). Specific research questions were tested with *t*-tests (mentioned directly in the text) or using contrast analyses (presented in the tables).

RESULTS

Experiment 1a. Morphology in the absence of soil

Verticillium dahliae on MSEA: Microsclerotia ($n = 150$) abundant (250–3000 per colony), shape usually elongate to oval (Fig. 3), sometimes globose (A45 mc-1) (Table 1), (30–)32–113(–190) × (10–)15–45(–55) μm (the 5th and 95th percentiles between brackets) (average 62 × 29 μm; length-width ratio (l/w) (1–)1.1–4.9 (–10.5), average 2.3), radially distributed through the colonies (Fig. 1). Dark hyphae usually absent, though 1 isolate (A60 mc-1) exhibited dark hyphae connected to some (1%) of the microsclerotia or unconnected to microsclerotia in several (4%) of the colonies. Dark mycelium throughout the colony absent. Yellow discoloration of the medium absent.

V. tricorpus on MSEA: Microsclerotia ($n = 90$), present only in isolates A36 mc-1 (1000–1500 per colony), 227.84 mc-1 (1000–2500 per colony), and in 24% of the colonies of isolate 127.79A mc-1 (0–50 per colony), irregularly shaped (Fig. 4), (15–)25–85



Figs 1–8. Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media without soil. **Fig. 1.** *V. dahliae* colony on MSEA. The microsclerotia are formed in a radial pattern, which is typical for *V. dahliae*, although a scattered pattern can also occur. **Fig. 2.** *V. tricorpus* colony on MSEA, with dark mycelium in a radial pattern and microsclerotia in a scattered to intermediate between radial and scattered pattern. **Fig. 3.** Globose to elongate *V. dahliae* microsclerotia on MSEA. **Fig. 4.** Globose to irregularly shaped *V. tricorpus* microsclerotia on MSEA. **Fig. 5.** *V. dahliae* colony on EA, with microsclerotia in a scattered pattern. **Fig. 6.** *V. tricorpus* colony on EA, with microsclerotia in a scattered pattern and dark mycelium in a radial pattern. **Fig. 7.** Large, globose to irregularly shaped *V. dahliae* microsclerotia with connected dark hyphae on EA. **Fig. 8.** Globose to oval (to irregularly shaped, not shown) *V. tricorpus* microsclerotia on EA. Bars Figs 1–2 and 5–6 = 500 µm; Figs 3–4 and 7–8 = 50 µm.

Table 2. Significance levels[a] of type III sums of squares of overall effects in Experiment 1.

Factor	Variable		
	Length of microsclerotia	Width of microsclerotia	Visible surface of microsclerotia
Species[b]	<0.0001	<0.0001	0.0001
Medium[c]	<0.0001	<0.0001	<0.0001
Soil[d]	0.0006	<0.0001	0.0001
Species × medium interaction	0.0006	<0.0001	<0.0001
Species × soil interaction	0.39	0.0048	0.26
Medium × soil interaction	0.57	<0.0001	0.0096
Species × medium × soil interaction	0.16	0.22	0.86

[a] Calculated using the GLM procedure of SAS version 8.0.
[b] *Verticillium dahliae* or *V. tricorpus*.
[c] Modified soil extract agar or ethanol agar.
[d] Present or absent.

(–270) × (10–)20–50(–70) μm (average 50 × 31 μm, l/w = 1.0–2.1(–5.4), average 1.7), usually scattered through the colony, but sometimes intermediate between radial and scattered (Fig. 2). Dark hyphae were present and connected to all microsclerotia in every colony of isolates A36 mc-1 and 227.84 mc-1 (Fig. 4), but occurred mostly separate from the microsclerotia in 52% of the colonies in isolate 127.79A mc-1. Dark hyphae that were not connected to microsclerotia could not be scored in colonies with abundant presence of dark mycelium. Dark mycelium usually present and abundant (covering 80–100% of the colony area), but absent in 127.79A mc-1 and scarce in A36 mc-1 (present in 44% of the colonies and on average covering 5% of the colony area) (Fig. 2). Yellow discoloration of the medium present in all colonies of isolate 227.84 mc-1 and several colonies of isolate A36 mc-1, and absent in all other colonies.

V. dahliae on EA: Microsclerotia ($n = 150$) mostly abundant (1000–6000 per colony), but variable in A63 mc-1 (several–2000 per colony), globose to elongate or irregular in shape (Fig. 7), (30–)40–156(–220) × (25–)30–90(–170) μm (average 86 × 56 μm, l/w = 1.0–2.3(–4), average 1.5), scattered through the medium (Fig. 5), except for isolate A59 mc-1 which shows a radial distribution and isolate A63 mc-1 which shows a scattered distribution in colonies containing few (< 50) and a random distribution in colonies containing more microsclerotia. Dark hyphae connected or close to most (80–95%) of the microsclerotia in most isolates (Fig. 7), but less frequent in A63 mc-1 (50%) and A56 mc-1 (10%), some dark hyphae also occurring unconnected to microsclerotia. Dark mycelium throughout the colony mostly absent, but rare in A60 mc-1 and A63 mc-1, covering 5% of the colony diameter. Yellow discoloration of the medium absent.

V. tricorpus on EA: Microsclerotia absent after 6 wk of incubation. Dark mycelium abundant, covering 100% of the colony diameter (Fig. 6). Dark hyphae that were unconnected to microsclerotia could not be scored properly, because of the dark mycelium. After scoring and leaving the Petri dishes at room temperature for another 6 wk, microsclerotia with dark hyphae were formed in some colonies of isolate A36 mc-1 (0–200 per colony). Microsclerotia ($n = 30$) globose (Fig. 8) to irregular in shape, (20–)30–141(–160) × 20–70(–120) (average 62 × 39 μm, l/w = 1.0–2.7(–3.2), average 1.6), formed in a radial to scattered pattern (Fig. 6). Yellow discoloration of the medium absent, except for one colony of isolate 227.84 mc-1 and visible only during the second week of incubation.

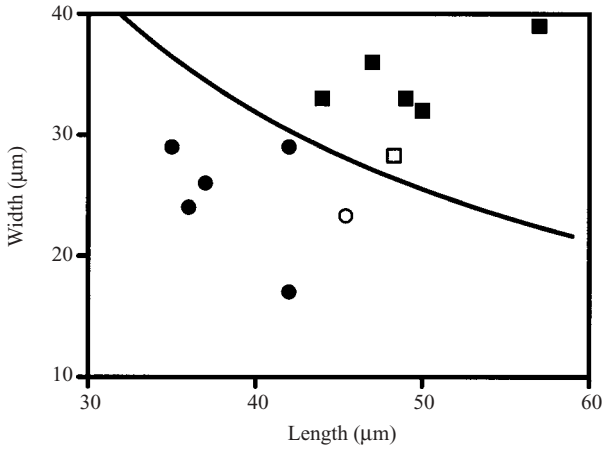


Fig. 9. Average length and width of microsclerotia of the ten colonies from MSEA soil plates from agricultural fields (Experiment 2), together with the average length and width of microsclerotia of *Verticillium dahliae* and *V. tricorpus* reference isolates (Experiment 1). ●, *V. dahliae* field isolates; ■, *V. tricorpus* field isolates; ○, average of *V. dahliae* reference isolates; □, average of *V. tricorpus* reference isolates; the line indicates a length × width of microsclerotia, i.e. the visible microsclerotial surface, which was iteratively set at 1275 μm².

Experiment 1b. Morphology in the presence of soil

Verticillium dahliae on MSEA: Microsclerotia ($n = 150$), usually present (around 200 per colony; less abundant or sometimes absent in A59 mc-1 (0–100 per colony)),

globose to elongate, 20–80(–100) × 10–50(–60) μm (average 45 × 23 μm, l/w = 1.0–4.8(–10.0), average 2.3), radially distributed except for A59 mc-1 which showed a scattered distribution and A45 mc-1 which showed a scattered distribution in the colony centre and radial distribution near the colony edges. Dark hyphae or dark mycelium absent. No discoloration of the medium.

V. tricorpus on MSEA: Microsclerotia ($n = 90$) present in about half of the colonies (isolates A36mc-1 and 127.79A mc-1 always showed microsclerotia (10–100 per colony)), usually (around 90%) with dark hyphae connected to them, except for isolates 384.84 mc-1 and Vtx mc-1 without, and 227.84 mc-1 with few microsclerotia (0–50 per colony), often irregularly shaped, sometimes globose to oval, (10–)20–116(–260) × (10–)15–50(–100) μm (average 48 × 28 μm, l/w = 1.0–2.9(–6.5), average 1.7), usually scattered through the colony, but sometimes intermediate between radial and scattered. Dark mycelium present in about half of the colonies, covering about 5% of the total colony area. Isolate 227.84 mc-1 showed numerous dark hyphae that were not connected to microsclerotia. No discoloration of the medium.

V. dahliae on EA: Microsclerotia ($n = 90$) present in about half of the colonies, usually not abundant (0–100 per colony) except for isolate A45 mc-1 (around 400 per colony), globose to elongate, (20–)30–140(–700) × (10–)15–61(–120) μm (average 75 × 37 μm, l/w = (1.0–)1.2–4.2(–14.0), average 2.2), scattered through the colony. Dark hyphae or dark mycelium absent. No discoloration of the medium.

V. tricorpus on EA: Microsclerotia ($n = 90$) present in about half of the colonies, but never abundant (0–30 per colony), irregular in shape, small and elongate in 127.79A mc-1 and large and globose in A36 mc-1, (20–)23–83(–170) × 10–50(–80) μm (average 47 × 28 μm, l/w = (1.0–)1.1–3.0(–5.3), average 1.8), usually scattered through the colony, but radially distributed in 227.84 mc-1. Dark hyphae and dark mycelium present in about half of the colonies, covering about 40% of the colony area. No discoloration of the medium.

Experiment 1. Overall effects on microsclerotial size

Microsclerotial size is generally different for the two species (larger for *V. dahliae*) and is severely affected by choice of the medium (larger on EA) and presence or absence of soil (larger if absent) (Table 2). The actual size, the surface that is visible through a microscope, is probably most clearly expressed in the factor length × width. The significant medium × soil interaction for the factors width and visible surface (Table 2) illustrates that microsclerotial size is more stable on MSEA. Significance levels of the species × soil interaction and the three-way interaction are misleading, because microsclerotia were often not formed.

The effect of adding soil on morphology: Presence of a mixed soil microflora can alter the morphology of the two species considerably. On MSEA, the presence of soil resulted in shorter and narrower ($P < 0.001$) *V. dahliae* microsclerotia, but had no significant effect on the size of *V. tricorpus* microsclerotia. On EA, the presence of soil resulted in shorter ($P = 0.036$) and narrower ($P < 0.001$) *V. dahliae* microsclerotia, whereas in *V. tricorpus*, soil induced formation of microsclerotia in two isolates, and accelerated formation of microsclerotia in isolate A36 mc-1, without affecting the size.

On MSEA without soil, *V. dahliae* microsclerotia were longer ($P = 0.037$) but not wider than those of *V. tricorpus*, whereas in the presence of soil, *V. tricorpus* microsclerotia were wider ($P = 0.015$) and slightly, but not significantly, longer than those of *V. dahliae* (Fig. 9). On EA, *V. dahliae* microsclerotia were longer and wider than those of *V. tricorpus*, both without and with soil ($P < 0.004$). Despite these consistent differences, EA is less suited for quantification of *V. dahliae* in soil, because *V. dahliae* microsclerotia are not always formed (see above). Morphological characteristics suitable to separate *V. dahliae* and *V. tricorpus* on MSEA in the presence of soil are summarised in Table 3.

Experiment 2. Classification of colonies of soil samples from agricultural fields

On the basis of the differential features recognised on MSEA with added soil (Experiment 1b), five of ten field soil colonies were scored as *Verticillium dahliae* and five as *V. tricorpus* (Table 3). In colony no. 2, microsclerotia were oval to elongate and arranged in a radial pattern, classifying it as *V. dahliae*. Colony no. 6 had globose to elongate microsclerotia in a more irregular pattern, and was also classified as *V. dahliae*, based on the elongate shape of the microsclerotia. Colonies nos 1, 3 and 7 had dark hyphae attached to the microsclerotia, classifying them as *V. tricorpus*. This identification was confirmed for colonies nos 1 and 3, which contained irregularly shaped microsclerotia. A comparison of the visible surface (length × width) of the microsclerotia of each field soil colony against the reference isolates of *V. dahliae* and *V. tricorpus* (Experiment 1) confirmed these identifications, and identified three more *V. dahliae* and two more *V. tricorpus* isolates (Table 3). Checking the identity of the purified isolate by PCR with specific primers and morphology on PDA confirmed the original identification based on morphological characteristics in the MSEA soil plates (Table 3).

DISCUSSION

Morphology of *Verticillium* species can be highly variable on different semi-selective agar media. Shape of microsclerotia and abundance of dark hyphae of *V. dahliae* on EA resembles morphology of *V. tricorpus* on MSEA. Moreover, the distribution of microsclerotia through the colony on EA is scattered, whereas on

Table 3. Morphological characteristics distinguishing *Verticillium dahliae* and *V. tricorpus* on MSEA in the presence of soil.

Isolate(s)	Colony pattern[a]	Microsclerotial shape	Approx. % of microsclerotia with connected dark hyphae	Dark mycelium	Average microsclerotial surface (length × width) (μm^2)	Probability[b] based on microsclerotial size of the field isolates being		Species identification of isolates based on morphology on MSEA in the presence of soil	Check of identity of purified isolate by	
						V. dahliae	*V. tricorpus*		PCR specific primers[c]	Morphology on PDA[d]
Reference isolates (Exp. 1b)										
V. dahliae	Radial, scattered or intermediate	Globose, oval to elongate	0	Absent	1190	NA[e]	NA	NA	*V. dahliae*	*V. dahliae*
V. tricorpus	Scattered or intermediate	Globose, oval to irregular	90	Absent or present	1725	NA	NA	NA	*V. tricorpus*	*V. tricorpus*
Field isolates (Exp. 2)										
1	Intermediate	Irregular	50	Absent	1690	0.24[f]	0.94[f]	*V. tricorpus*	*V. tricorpus*	*V. tricorpus*
2	Radial	Oval-elongate	0	Absent	720	0.26	0.02	*V. dahliae*	*V. dahliae*	*V. dahliae*
3	Scattered	Globose-irregular	80	Absent	1520	0.43	0.66	*V. tricorpus*	*V. tricorpus*	*V. tricorpus*[g]
4	Scattered	Globose-oval	0	Absent	2240	0.01	0.23	*V. tricorpus*	*V. tricorpus*	*V. tricorpus*
5	Intermediate	Globose-oval	0	Absent	1020	0.69	0.10	*V. dahliae*	*V. dahliae*	*V. dahliae*
6	Intermediate	Globose-elongate	0	Absent	880	0.46	0.05	*V. dahliae*	*V. dahliae*	*V. dahliae*
7	Intermediate	Globose-oval	70	Absent	1710	0.22	0.97	*V. tricorpus*	?[h]	*V. tricorpus*[g]
8	Scattered	Globose-oval	0	Absent	1810	0.14	0.84	*V. tricorpus*	*V. tricorpus*	*V. tricorpus*
9	Intermediate	Globose-oval	0	Absent	1030	0.70	0.11	*V. dahliae*	*V. dahliae*	*V. dahliae*
10	Scattered	Globose-oval	0	Absent	1260	0.87	0.28	*V. dahliae*	*V. dahliae*	*V. dahliae*

[a] Colony pattern is unclear when few microsclerotia are present.

[b] Contrast analysis of each isolate of Experiment 2 against the average of all isolates of *V. dahliae* and all microsclerotia forming isolates of *V. tricorpus*, respectively, of Experiment 1b on MSEA in the presence of soil (GLM procedure, SAS version 8.0, SAS, Cary, NC).

[c] Robb *et al.* (1994).

[d] Isaac (1949, 1953).

[e] Not applicable.

[f] The higher probability value (either *V. dahliae* or *V. tricorpus*) indicates the most likely possibility.

[g] These isolates caused yellow coloration of the PDA medium.

[h] No signal with either *V. dahliae* or *V. tricorpus* primers.

MSEA it is usually (but not always) radial. This is the first report of the occurrence of dark hyphae of *V. dahliae in vitro*. *In vivo* production of dark hyphae has sometimes been reported in cotton (Garber & Houston 1966) and olive (Rodríguez-Jurado 1993). Researchers only familiar with *V. dahliae* on pectate-based agars can easily be confused when occasionally working with EA.

On MSEA, microsclerotia are the most important structures to recognise *Verticillium* colonies in the presence of soil, because many other fungi present in the soil are able to form melanised mycelium or chlamydospores (Domsch *et al.* 1980). Therefore, presence of dark hyphae is a useful characteristic only, when the dark hyphae are connected to the microsclerotia. Because this is the case with most of the *V. tricorpus* microsclerotia and never occurs in *V. dahliae*, this characteristic is very important for discrimination, and easy to observe. Shape of the microsclerotia is a useful characteristic, when the colony contains elongate (*V. dahliae*) or irregularly shaped (*V. tricorpus*) microsclerotia, which is also easy to observe. The pattern in which microsclerotia are formed discriminates only when it is clearly radial. When microsclerotia are scarce, the pattern is often unclear. Dark mycelium, when present, identifies a colony as *V. tricorpus*. However, dark mycelium did not occur in any of the *V. tricorpus* field colonies tested, so this characteristic does not seem to be useful for practice.

All previously mentioned qualitative characteristics fail to discriminate between *V. dahliae* and *V. tricorpus* when dark hyphae and dark mycelium are absent, and microsclerotia are globose to oval and arranged in an irregular pattern. Size of the microsclerotia is a useful character for discrimination, despite the overlap in length and width of the field isolates with the reference isolates (Fig. 9). Size, however, is more clearly expressed as the average length × width of the microsclerotia, i.e. the visible surface. The visible surface differentiates clearly to the experienced observer, even without measuring the microsclerotia on a standard basis. Using both qualitative and quantitative characteristics, it is possible to discriminate *V. dahliae* and *V. tricorpus* on MSEA in the presence of soil.

These observations provide a more practical (yet reliable) method to characterise *Verticillium* colonies on MSEA soil dilution plates. The alternatives, to purify individual colonies and identify them using morphological or molecular characteristics, are too laborious and costly if large numbers of colonies need to be identified.

These observations in part confirm those of Davis & McDole (1979) and Davis *et al.* (2000) on the pectate based agar NPX (Butterfield & DeVay 1977) and of Huisman (1988) on a similar pectate medium. All authors mentioned the radially grouped *V. dahliae*, and scattered, dark hyphae bearing *V. tricorpus* microsclerotia, as well as the microsclerotial size difference. However, they did not mention any deviations from these typical characteristics.

MSEA is used for quantification of *V. dahliae* in research (Termorshuizen *et al.* 1998) and commercially (Anon. 1997) in soils with the two species present. Quantitative detection using MSEA has given a good prediction of disease for *V. dahliae*, e.g. in *Acer platanoides* (Goud *et al.* 2001), but for *V. tricorpus* the method was not conclusive because some isolates did not form microsclerotia. EA cannot be used for quantification of *V. dahliae* in soil. EA is suitable for working in the absence of soil, e.g. during plating of stem pieces of crops that can suffer from both species, like potato (e.g. Nagtzaam, Termorshuizen & Bollen 1997).

The addition of soil to MSEA Petri dishes affected microsclerotial size in *V. dahliae* more than in *V. tricorpus* isolates, which could be explained by the nature of the two species. *V. dahliae* is regarded as a plant pathogen, with low saprobic ability, whereas *V. tricorpus* is regarded as being more a soil inhabitant (Isaac 1967). The formation of microsclerotia by *V. tricorpus* on EA in the presence of soil but not in pure culture, or only after prolonged incubation, might be induced by competing microorganisms. Probably, microsclerotia are more durable resting structures than melanised mycelium, and are more frequently formed in the presence of other soil organisms. This hypothesis needs further testing.

In conclusion, the morphological characters used to identify pure cultures of dark *Verticillium* species cannot be used uncritically for soil dilution plates. For distinguishing *V. dahliae* and *V. tricorpus* on MSEA soil dilution plates we recommend the following key:

***Key to distinguish* Verticillium dahliae *and* V. tricorpus**

1	Dark hyphae or dark mycelium present	**tricorpus**
	Dark hyphae and dark mycelium absent	2
2(1)	Microsclerotia irregularly shaped	**tricorpus**
	Microsclerotia not irregularly shaped; globose to oval to elongate	3
3(2)	Microsclerotia elongate	**dahliae**
	Microsclerotia globose to oval	4
4(3)	Distribution of microsclerotia within medium clearly radial	**dahliae**
	Distribution of microsclerotia within medium not clearly radial	5
5(4)	Average surface (average length × average width) of microsclerotia ($n = 10$) < 1275 μm²	**dahliae**
	Average surface (average length × average width) of microsclerotia ($n = 10$) > 1275 μm²	**tricorpus**

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