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The relationship between soil inoculum density and plant infection as a basis for a quantitative bioassay of *Verticillium dahliae*

M.P.M. Nagtzaam, A.J. Termorshuizen* and G.J. Bollen
*Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands (Fax: +31 317483412); *Author for correspondence*

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Abstract

Using potato, eggplant and thorn apple as test plants, the relationship between soil inoculum density and plant infection was studied as a basis for the development of a quantitative bioassay of *Verticillium dahliae*. A linear relationship was demonstrated ($P < 0.05$) between soil inoculum density and population density on roots for all three test plants and for soil inoculum density and population density in sap extracted from stems for eggplant. Correlation coefficients were higher with densities on or in roots (R^2 varying from 0.45 to 0.99) than with densities in stems (R^2 varying from 0.04 to 0.26). With eggplant, population densities on/in root and in sap extracted from stems were significantly correlated at 20 and 25 °C with Pearson's correlation coefficients of 0.41 and 0.53, respectively. For potato, root colonization was higher at 15 than at 20 °C, whereas the reverse applied to eggplant. Stems of potato were less densely colonized than stems of eggplant. The pathozone sensu Gilligan (1985) was calculated to be <300 μm, indicating that infection was caused by microsclerotia which were located close to the roots. To assess the density of *V. dahliae* in plant tissue pipetting infested plant sap on solidified ethanol agar medium without salts yielded higher densities than using pectate medium or mixing sap with molten agar. A bioassay for determining effects of (a)biotic factors on development of *V. dahliae* in the plant is recommended with eggplants as a test plant, grown in soil infested with 300 single, viable microsclerotia g^{-1} soil at a matric potential of -6.2 kPa, and incubated at 20 °C for 8 weeks.

Introduction

Verticillium dahliae Kleb. causes early dying of potato resulting in considerable yield losses in the Netherlands (Bollen et al., 1989; Scholte, 1989). Soil inoculum consists of microsclerotia that occur free or embedded in plant debris (Schreiber and Green, 1962) and may survive for many years (Wilhelm, 1955). In some studies disease incidence was shown to be related to soil inoculum density (e.g. Nicot and Rouse, 1987; Pullman and DeVay, 1982; Wheeler et al., 1992), whereas in other studies such a relation could not be established (Ashworth et al., 1972; Davis and Everson, 1986; DeVay et al., 1974). The main reason for this discrepancy may be the effects of the environment on processes that occur over the long period between germination of microsclerotia in soil and the appearance of disease symptoms, usually late in the growing season (Termorshuizen and Mol, 1995).

Research on introduced biocontrol agents and other environmental factors on the dynamics of *V. dahliae* requires a reliable quantitative bioassay. Plant bioassays have been used in ecological studies on *V. dahliae* (e.g. Evans et al., 1974) and for selection for resistance to the pathogen in host plants (Palloix et al., 1990; Zeise, 1992). These tests are generally based on symptom evaluation. Because symptom expression is strongly dependent on environmental factors, some of which are unknown, estimation of presence of the pathogen in the host plant provides absolute proof on infection of the plant. Evans et al. (1974) were the first to develop a quantitative bioassay based on root colonization by *V. dahliae*. Estimation of inoculum density of *V. dahliae* in stem tissue in addition to that

in or on the root would again add extra information. In studies where severity of symptom expression, yield and susceptibility of the host plant were shown to be associated with vascular colonization, methods were described to quantify colonization of the stem by *V. dahliae* (e.g. Pegg and Jonglaekha, 1981; Ordentlich et al., 1990; Hoyos et al., 1991; Johnson and Miliczky, 1993). However, thus far the degree of colonization of the stem has not been used in a quantitative bioassay. Moreover, experimental evidence for a relation between numbers of microsclerotia in soil and colony forming units (CFU) in stem sap is lacking. In field experiments, Davis et al. (1983 and 1996) found a significant correlation between estimated inoculum density of *V. dahliae* in naturally infested soils and inoculum density in dried potato stems.

The objective of the present investigation was to evaluate the usefulness of the techniques that are used for estimating the population densities on or in plant roots and in stems for determining effects of biotic and abiotic factors on development of *V. dahliae* in the plant in growth chamber bioassays.

Materials and methods

Preparation of inoculum

Microsclerotia were obtained either from potato stems collected from an infested field or artificially produced on autoclaved rye seeds as described by Kotcon et al. (1984). The inoculum was air dried in a sterile flow cabinet to eliminate conidia and mycelial fragments (Green, 1969). After chopping the potato stems or grinding the rye seeds with pestle and mortar, the inoculum was blended in tap water. The resulting suspension was poured through nested 150, 75 and 20 μm mesh sieves. Material retained on the 20 μm sieve was rinsed with additional water and resuspended in tap water. A drop was pipetted on a microscope slide and the separation of microsclerotia from stem material was verified by examination under a dissecting microscope (50×). The retained material was resuspended in 0.08% water agar (Slattery, 1981) to lessen sedimentation of the microsclerotia in the suspension. The density of the suspension was estimated by direct assessment of the number of microsclerotia in at least 10 drops of 10–20 μl.

Germination percentage of the microsclerotia was assessed by plating 0.2 ml of suspension on ethanol agar medium (EA) (Nadakavukaren and Horner, 1959), or by transferring single microsclerotia to modified soil extract medium (MSEA) (Harris et al., 1993) as described by Hawke and Lazarovits (1994). Instead of streptomycin, chloramphenicol and chlortetracycline, the bacteriostatic antibiotic oxytetracycline (50 μg/ml) was added to these media.

Preparation of soil and seed

An autoclaved (4 h, 115 °C and 50 kPa) sandy soil (pH-KCl 7.0, 0.3% organic matter, fraction particles $\geq$16 μm 97.4%, density 1.23 g cm^{-3}) was used in all experiments. Recolonization and microbial activity were standardized by growing the soil with wheat (*Triticum aestivum* L. cv Okapi) before use. Ten days after sowing, the wheat was cut at soil level and the soil was air-dried and sieved through a screen of 2 mm mesh to remove roots.

Seeds of eggplant (*Solanum melongena* L.) of the susceptible cv Black Beauty (Ferrandino and Elmer, 1993) and thorn apple (*Datura stramonium* L.) were surface-sterilized as described by Fahima and Henis (1990). Potato minitubers (Lommen and Struik, 1992), diameter 12–17 mm, of cv Element, shown to be highly susceptible to *V. dahliae* (Scholte, 1990), were washed with tap water, surface-sterilized for 10 min in 1% NaOCl, washed twice with sterile water and dried on sterilized filter paper. The tubers were incubated in the dark at 20 °C until sprouts started to grow, and then used for the bioassay.

Bioassay

A suspension of microsclerotia was applied to air-dried soil to obtain the required inoculum density. Differences in germinability between inoculum preparations were adjusted for by applying different quantities of inoculum. In the experiments with eggplant grown at 15 and 20 °C, microsclerotia were used that originated from potato stems collected from an infested field. In other experiments artificially produced microsclerotia were applied. The level of colonization of the roots can be affected by both the density and distribution pattern of microsclerotia in soil (Ashworth et al., 1972; Wheeler et al., 1994). In the field, part of the microsclerotia population is associated with plant debris, especially in the year after cultivating a susceptible crop (Ashworth et al., 1974, Slattery, 1981). The distribution pattern of microsclerotia depends on decay of the infected plant residues, which is strongly influenced by environmental conditions. For this reason we

applied microsclerotia, rather than incorporating plant residues infected by *V. dahliae*. The soil was moistened to a 10 and 15% moisture level corresponding with a matric potential of -6.2 and -4.2 kPa, respectively. Soil matric potential was measured by mini-tensiometers. Pots ($4 \times 4 \times 12$ cm) with parallel sides and a bottom of 22.4 μm mesh polyamide screen were filled with the soil. Three eggplant or thorn apple seeds or one potato minituber were placed in each pot. Pots were covered with plastic to maintain a high air humidity and incubated in growth chambers at a light intensity of 29 W m^{-2} 16 h day^{-1} and a relative air humidity of 80%. After emergence of the seedlings, the plastic was removed and the number of plants per pot was reduced to one. Soil temperatures at a depth of 5 cm below soil level were 1–2 °C higher than air temperatures. Soil moisture level was maintained by compensating for moisture losses and plant growth by adding half strength Hoagland's nutrient solution (about four times per week) after weighing the pots.

Detection of V. dahliae *in shoot tissue*

Stems were cut at the soil line, defoliated, washed in tap water, surface-disinfected in 1% NaOCl for 1 min, washed twice in sterile water, and blotted dry on sterilized filter paper. In order to estimate the extent of penetration of the disinfectant, stems were soaked in a solution of equal volumes of fluorescein isothiocyanate and TRIS-buffer (pH 6.8) for 1 and 30 min, respectively. Fluorescence microscopy showed that the fluorescence suspension penetrated 1–2 mm into the stem ends for both soaking durations. Five mm long segments were cut off at both ends to remove tissue that had absorbed NaOCl.

Stem infection was assessed by plating one 5 mm portion of the stem base on EA. Plates were incubated at 20 °C and examined for growth of *V. dahliae* after 1 week. In a preliminary experiment, methods were compared to quantify colonization of stem tissue. Sap was extracted from a 5 cm segment of the basal portion of a surface sterilized stem of potato using a plant press. If the first extraction yielded less than 0.1 ml of sap, the extraction procedure was repeated by further pressing after addition of a measured volume of sterile water. Extracted plant sap was mixed with molten agar medium according to Hoyos et al. (1991), or pipetted on solidified medium as described by Ordentlich et al. (1990). Between processing the samples, the press was cleaned with hot water, 95% ethanol and cold water, respectively. Aliquots of 0.1 or 0.2 ml were pipetted into plastic Petri dishes (Greiner, diam. 8.5 cm, with ridges) and 20 ml of molten medium (45–50 °C) added while the dish was agitated gently. Alternatively, sap was spread over the solidified medium with a surface-sterilized glass rod. The plates were incubated in the dark at 20–22 °C. Zilberstein et al. (1983) found that germination of microsclerotia was inhibited at lowered oxygen concentrations. Therefore, after 3 days the lids of the plates were moved to remove water barriers that possibly prevent inward oxygen diffusion. Starting at 7 days up to 3 weeks after preparing the plates, the number of CFU per ml sap was determined by counting the number of colonies that produced microsclerotia.

Three media were compared for their suitability to quantify *V. dahliae* in plant sap: pectate medium (PM) (Huisman and Ashworth, 1974; slightly modified by Bollen et al., 1989), EA (Nadakavukaren and Horner, 1959) and EA amended with salts but without PCNB (EA+) (Ausher et al., 1975). For all media, 50 mg l^{-1} oxytetracycline was added instead of streptomycin and chloramphenicol. All data were transformed to compensate for the possibility of overlapping colonies, using Gregory's (1948) multiple infection transformation. A mean colony size of 0.028 cm^2 at evaluation time was assumed, based on the ability to discriminate colonies on the agar plate. The transformed number of colonies $N_{s,t}$, with a Petri plate area of 56.7 cm^2 and N_s, the number of counted colonies per Petri plate, was calculated as $N_{s,t} = (-\ln(1 - 0.028 * N_s/56.7) * N_s) + N_s$.

Detection in root tissue

To assess root colonization, soil was washed off the roots under a gentle flow of tap water on a sieve with 0.25 mm pore size. Subsequently the roots were washed for 20 min in running tap water, rinsed twice in sterile water and blotted dry on sterile filter paper. The root system was cut into three parts, viz. the regions 0–3, 3–6 and >6 cm below the stem base, respectively. Each part was transferred to a plastic EA plate (diameter 8.5 cm). The roots were placed apart using preparation needles and pressed into the medium using a spoon. Root length was determined by automatic image analysis using a Quantimet 570 (Leica). Roots were then incubated for at least 7 days in the dark at 20–22 °C and regularly examined for growth of *V. dahliae*. The number of colonies obtained was adjusted for possibility of overlapping colonies assuming a mean colony length at evaluation of 2.5 mm. This length was based on observation of the plates and corresponds with

the lengths of 2 and 2–3 mm reported by Evans and Gleeson (1973) and Huisman (1988b), respectively. The transformed number of colonies on the root, $N_{r,t}$, is given by $(L/0.0025) * (-\ln(1-0.0025 * (N_r/L)))$, with root length L (m) and number of observed colonies of *V. dahliae* N_r on the root system.

Statistical design and data analysis

All experiments were designed as randomized blocks with 10 to 12 replicates and each pot representing an experimental unit. Data of stem and root colonization were analyzed after $\log_{10}(N_t + 1)$ transformation. Root and stem colonization were regressed on $\log_{10}$ (inoculum density g^{-1} soil + 1) and homogeneity of regression slopes was tested. Stem colonization was also regressed on root colonization and Pearson correlation coefficients calculated between root and stem colonization. In all analyses zero's were included.

Results

Assays for V. dahliae *in plant tissue*

Two methods and three media were evaluated to assess the density of *V. dahliae* in stems. Colonies developed on all media. Pipetting infested plant sap on EA (solidified ethanol agar medium) without salts, on EA with salts, on pectate medium or mixing the sap with molten EA yielded 67, 7, 14 and 5 CFU ml^{-1} sap, respectively (N = 10). With pipetting sap on EA without salts the highest density ($P < 0.05$) was obtained. This method was used in all further experiments.

Relationship between inoculum density and colonization of stems and roots

The relationship between inoculum density in soil inoculated with viable microsclerotia and root colonization is shown in Figure 1. In all experiments, a significant linear relationship was found between $\log_{10}$-transformed numbers of microsclerotia g^{-1} soil and $\log_{10}$-transformed numbers of CFU m^{-1} root, for all test plant species and all temperature regimes. Slopes of the lines varied between 0.42 and 1.11 (Figure 1). At densities ≥ 1 microsclerotium g^{-1} soil root systems were colonized in all pots. For potato, slopes of the lines were higher at 15 °C than at 20 °C ($P < 0.05$).

The relation between soil inoculum density and stem colonization is shown in Figure 2. Log_{10}-transformed numbers of *V. dahliae* ml^{-1} sap showed a linear relation with $\log_{10}$-transformed numbers of microsclerotia g^{-1} soil ($P < 0.01$) except at 15 °C with potato. The coefficient of determination increased with increasing temperature.

With eggplant, root and stem colonization were significantly correlated at 20 and 25 °C ($P < 0.01$) with Pearson's correlation coefficients of 0.41 and 0.53, respectively, but not at 15 °C. Regression analysis of density in the stem on soil inoculum density resulted in lower coefficients of determination than regression of root colonization on soil inoculum density (Figures 1 and 2).

Stem colonization generally increased with increasing soil inoculum density (Table 1). Occasionally, typical symptoms of disease developed as unilateral chlorosis and/or wilting of leaves. In the series with eggplant at 25 °C, contamination with *V. dahliae* resulted in a low level of infection in the noninoculated control. Most probably this occurred by dispersal of verticillium propagules present in water or soil during handling processes in the growth chambers.

The pathozone width was calculated for eggplant at 15 and 20 °C on the basis of the data on inoculum densities and root colonization, using the displacement model (Gilligan, 1985), excluding the mean radius of the microsclerotia and assuming an average root diameter of 0.34 mm. The pathozone width ranged between 0.1 mm and 0.3 mm and did not differ between inoculum densities and temperature ($P < 0.05$).

Influence of soil temperature and moisture level on colonization of roots and stems

At 20 °C and a soil moisture level of 10%, the basal part of the stem was colonized by *V. dahliae* in 100% of the plants examined 8 weeks after sowing (Table 2). Infection tended to be faster at the low soil moisture level. Root colonization remained at the same level during the experimental period at 20 °C, but decreased from 7 weeks after sowing at 25 °C (Figure 3).

Discussion

Differences in root colonization among plant species observed in the present research and elsewhere (Huisman and Gerik, 1989; Evans et al., 1974) can be due to various factors, e.g. root morphology, growth rate and growth pattern of the roots, phytoalexin production and the amount and composition of root exudates. The

Table 1. Proportion of stems of eggplants and potatoes infected by *Verticillium dahliae* in relation to inoculum density in soil

Experimental conditions				Inoculum density in soil (number of microsclerotia g^{-1} soil)						
Test plant	Temperature	Experimental period (weeks)	Detection method	0	1	10	30	100	300	1000
Eggplant	15 °C	8	sap	0[1]	–[2]	0.20	0.20	0.56	–	–
			segment	0	–	0	0	0.40	–	–
	20 °C	8	sap	0	0	0.20	0.50	0.60	0.60	0.60
			segment	0	0	0.70	0.70	0.40	0.70	1.00
	25 °C	7	sap	0.11	–	0.18	0.50	0.58	0.83	–
			segment	0.11	–	0.10	0.40	0.66	0.92	–
Potato	15 °C	8	sap	0	0	0	0	0	0.10	–
			segment	0	0	0	0	0.10	0.30	–
	18 °C	8	sap	0	0	0	0	0	0	0
			segment	0	0	0	0	0	0	0.10
	20 °C	8	sap	0	0	0	0.10	0.30	0.20	0.33
			segment	0	0	0	0	0.50	0.50	0.80

[1]Each number represents the proportion of infected stem portions found among 10–15 plants examined.
[2]Inoculum density not included.

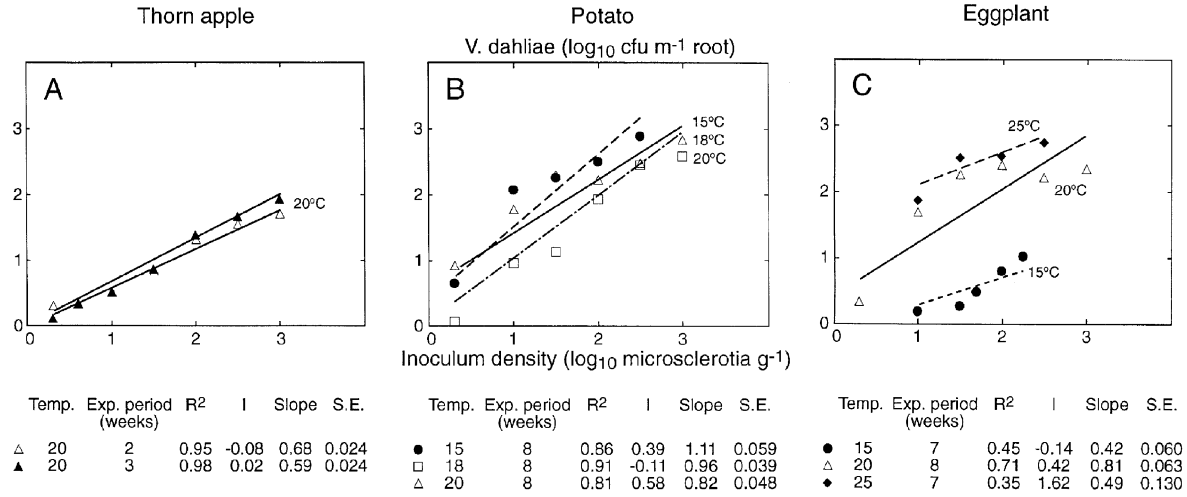
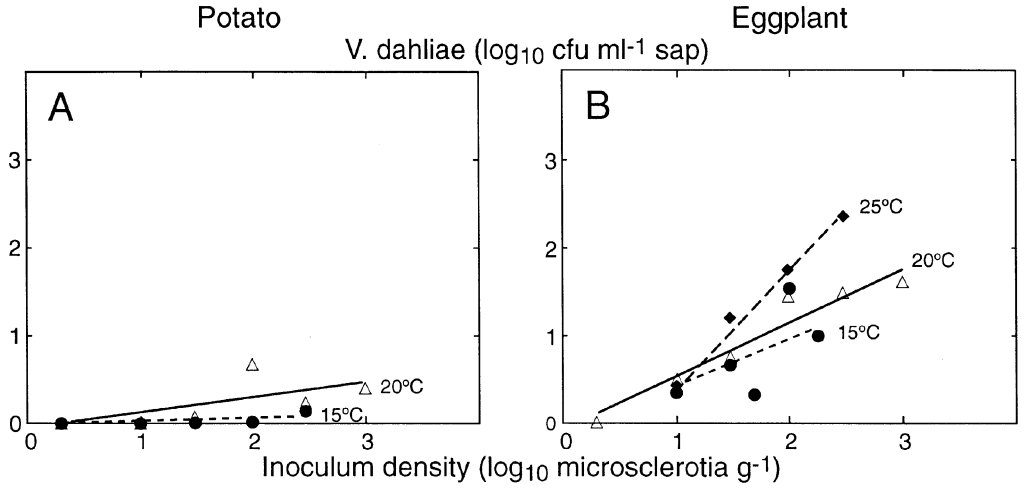


Figure 1. Log-log relationships between inoculum densities of *Verticillium dahliae* in soil and colony forming units per unit root length at different temperatures (°C) and experimental periods (weeks) for thorn apple (A), potato (B) and eggplant (C). Data were analyzed by regression analysis which was statistically significant in all experiments ($P < 0.001$). R^2, intercept (I), slope and standard error of slope (SE) are presented.

latter is probably the most important factor. Exudation depends not only on plant species but also on environmental conditions and on microbial activity in the rhizosphere (Schreiber and Green, 1963). We aimed to standardize the recolonization and microbial activity of soil by cropping the steamed soil with wheat. However, random deposition of airborne microbes on the freshly steamed soil can then still affect the process of recolonization. In most experiments (15 and 20 °C with potato and 20 °C with eggplant), the same batch of recolonized soil was used and consequently the factors mentioned above could not have been major sources of variance. The densities for root colonization in our experiments were in the same range as those reported by Evans et al. (1974) and Gaudreault et al. (1995) who performed their experiments also under controlled conditions. Root colonization densities in the field are often lower (Huisman, 1988b; Nagtzaam,



	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	8	0.04	-0.02	0.04	0.027
△	20	8	0.07	-0.04	0.17	0.071

	Temp.	Exp. period (weeks)	R^2	I	Slope	S.E.
●	15	7	0.11	-0.11	0.53	0.19
△	20	8	0.26	-0.07	0.61	0.13
◆	25	7	0.26	-1.00	1.37	0.34

Figure 2. Log-log relationships between inoculum densities of *Verticillium dahliae* in soil and colony forming units per ml plant sap at different temperatures (°C) and experimental periods (weeks) for potato (A) and eggplant (B). Data were analyzed by regression analysis which was significant in all experiments ($P < 0.01$) except at 15 °C with potato. R^2, intercept (I), slope (Sl) and standard error of slope (SE) are presented.

Table 2. Proportion of eggplant stem portions infected by *Verticillium dahliae* at four experimental periods, two temperatures and two soil moisture levels at an inoculum density of 300 microsclerotia g^{-1} soil

Experimental period (weeks)	Temperature and soil moisture level			
	20 °C		25 °C	
	10% –6.2	15% –4.2	10% –6.2	15% moisture content –4.2 kPa
6	0.7[1]	0.5	0.8	0.5
7	0.8	0.6	1.0	0.6
8	1.0	0.9	0.9	0.9

[1]Each number represents the proportion of infected stems among 10 plants examined.

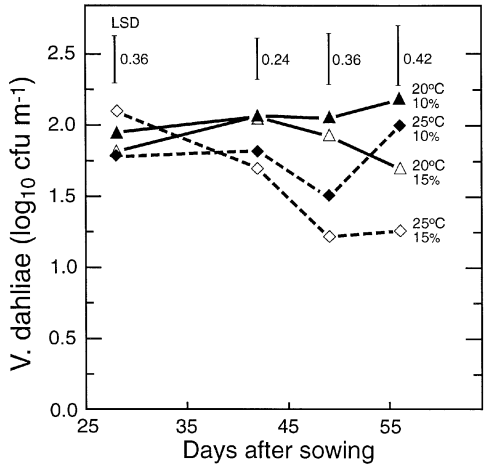


Figure 3. Colony density of *Verticillium dahliae* per unit root length at two temperatures (°C) and two soil moisture levels (%). Least significant difference ($P < 0.05$) is shown.

1995). This may be related to the interruption of the germination and infection processes due to different temporary biotic and abiotic conditions in the field.

Soil inoculum density and the population density in or on roots showed a $\log_{10}$-$\log_{10}$ relationship. This is in agreement with the observations of Evans et al. (1974) and Huisman (1988a) who found that the number of colonies on the root was directly proportional to inoculum density in soil. The linear relationship on $\log_{10}$-$\log_{10}$ scales does not support occurrence of extensive multiplication of *V. dahliae* neither on or in roots or in soil, nor does it support systemic colonization of a large part of the root system in soil or extensive growth in the root cortex, as this would lead to deviations from a linear relation on $\log_{10}$-$\log_{10}$ scale (Huisman and Gerik, 1989).

Several authors demonstrated a correlation between symptom expression and stem colonization by *V. dahliae* (Busch and Schooley, 1970; Hoyos et al., 1991; Ordentlich et al., 1990; Schreiber, 1992). Results of field experiments conducted by Ordentlich et al. (1990) indicated a correlation between stem colonization and yield of potato tubers. In the present study, the relationship between inoculum density in soil and population density of *V. dahliae* in stem sap was linear on a $\log_{10}$-$\log_{10}$ scale. Also significant correlations were obtained between population density on/in roots and in sap extracted from stems. We emphasize that our results are based on only one type of soil and with inoculum added under controlled conditions. Therefore, the significance of our results for other conditions is difficult to infer. Estimating population densities of *V. dahliae* in stem sap in addition to densities on the root provides extra information in studies on effects of environmental factors on the dynamics of *V. dahliae* in the soil or in the plant. The population densities in stem sap of the test plants were similar to those reported for potato by Hoyos et al. (1991). Although colonization of the roots was higher with potato than with eggplants, the stems were not more colonized and infected. Apparently, in potato the ascent and proliferation of the pathogen in stems is more limited by physical or chemical barriers than in eggplant.

The frequency of stem colonization never reached 100%, and in some cases it remained lower than expected at an inoculum density of 300 microsclerotia g^{-1} soil. In field soil, disease incidence of $>$90% has been reported from 5 ms g^{-1} soil onwards (e.g. Nicot and Rouse, 1987). The absence of nematodes in the soil used for our bioassays might have played a role. Moreover, we applied individual microsclerotia instead of aggregates of microsclerotia in plant residues which probably have a higher infection capacity.

Correlations of soil inoculum density were higher with root colonization than with stem colonization. This is easily explained as root colonization is one of the processes involved between germination of microsclerotia and stem colonization. It could be argued that the lower correlation coefficients observed at suboptimal temperatures for *V. dahliae* (Figures 1 and 2) reflect the lesser activity of *V. dahliae* at these temperatures.

Among the plant species, eggplant had the highest infection level of stems. Therefore, we recommend a bioassay with eggplant at a temperature of 20 °C, at a matric potential of –6.2 kPA and an experimental period of 8 weeks. A temperature of 20 °C is preferred as colonization of the root system was constant over time (Figure 3), whereas variation in stem and root colonization increased at lower temperatures (Figures 1 and 2). An experimental period of at least 8 weeks and a soil inoculum density of 300 microsclerotia g^{-1} soil is recommended to assure a sufficiently high infection percentage (Tables 1 and 2). At first sight this density seems rather high compared with population densities in infested soils. For example, Bollen et al. (1989), Davis et al. (1996), Melero-Vara et al. (1995) and Tjamos and Paplomatas (1987) reported soil inoculum densities of 17–27, 40–70, 2–85, 1–28 microsclerotia g^{-1} field soil, respectively. However, under a highly susceptible crop, populations can mount up to 1500 propagules g^{-1} soil (Jordan, 1971). In cotton fields in California with an incidence of verticillium wilt, densities usually range between 100 and 200 viable propagules g^{-1} soil (Schnathorst, 1981). Moreover, estimates of numbers of propagules based on recovery from soil should be considered with caution. They often lead to underestimation of the actual population since recovery ranges from 1–60% dependent on the isolation methods (Nicot and Rouse, 1987; Termorshuizen, 1995).

Root colonization by *V. dahliae* depends on the ability of the microsclerotia to germinate, grow and infect. These processes are affected by the secretion of exudates by the host plant. Our calculations of the pathozone width, indicating that microsclerotia must be present within a distance of 0.1–0.3 mm from the root surface in order to infect the root, support the stimulatory role of root exudates for germination of microsclerotia. The estimated pathozone width corresponds with data available in the literature for *V. dahliae*. Huisman and Gerik (1989) calculated a pathozone width of 0.13–0.43 mm for different host plants. Olsson and Nordbring-Hertz (1985) and Mol and Van Riesen (1995) found that microsclerotia germination percentages decreased rapidly to background levels at distances of 5 mm and 1 mm from the root, respectively.

In summary, a bioassay for determining effects of (a)biotic factors on development of *V. dahliae* in the plant is recommended with eggplants as a test plant grown in soil infested with 300 single, viable microsclerotia g^{-1} soil at a matric potential of -6.2 kPa and incubated at 20 °C for 8 weeks.

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