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Effect of plant roots on the germination of microsclerotia of *Verticillium dahliae*

I. Use of root observation boxes to assess differences among crops

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Abstract

Root observation boxes were used to study the effects of hosts and non-hosts on the germination of microsclerotia of *V. dahliae*. The effects of roots on microsclerotia were examined within a radius of 1 mm around the root tip. Host plants such as potato and field bean induced a higher percentage of germination of the microsclerotia than a non-host such as barley. A susceptible potato cultivar stimulated germination more than a resistant cultivar. The germination percentage and the number of hyphae per microsclerotium decreased with distance from the root surface regardless of the plant species or cultivar.

Abbreviations: MS = microsclerotium, microsclerotia.

Introduction

Microsclerotia (MS) of *Verticillium dahliae* (Kleb.) remain dormant in the soil but are stimulated to germinate by root exudates. The infectious hyphae that emerge from MS penetrate roots mainly in the areas of cell differentiation and in the root hair zone [Schnathorst, 1981]. Only a very small proportion of the hyphae will successfully infect the root. If a species or cultivar can be systematically infected by *V. dahliae* it is called a host. However, large numbers of colonies per unit root length have been found in non-host plants as well in host plants [Evans and Gleeson, 1973]. *Verticillium dahliae* primarily colonises the root cortex near the root tip; the maximum density of penetration is 1 cm from the root apex [Gerik and Huisman, 1988].

Microsclerotia of *V. dahliae* can germinate more than once, but eventually become exhausted [Farley *et al.*, 1971]. Since both hosts and non-hosts can induce germination of MS [Schreiber and Green, 1963], a possible strategy to control *V. dahliae* might be to grow non-hosts. Indeed, inducing MS to germinate without producing abundant propagules after plants have been infected may be a major factor in the *Verticillium* wilt control, that has often been reported when non-hosts are used in rotations [Schnathorst, 1981].

The reasons for the root's greater stimulating effect on microsclerotia around its tip compared with other sites along the root axis may be that exudates are excreted from the zone of root elongation [Curl and Truelove, 1986; Rovira and Davey, 1974] and that there are relatively low densities of organisms competing for nutrients here [Olsson *et al.*, 1987]. Fitzell *et al.* [1980] found that the density of root colonisation by *V. dahliae* increased up to 20 mm from a growing root tip in wheat (*Triticum aestivum*) and thornapple (*Datura stramonium*), but Gerik and Huisman [1988] showed that in cotton the occurrence of colonies of *V. dahliae* did not increase beyond 5 mm from the root tip. Both these studies [Fitzell *et al.*, 1980; Gerik and Huisman, 1988] give only an indication of the number of colonies at the root surface. Because of possible inter-

actions between MS, root exudates, and rhizosphere microorganisms, the colonization of the root does not necessarily give a good indication of the effect of a root on the germination of MS in the soil. After germination most of the hyphae of the MS stop growing and die.

To understand the contribution of the induction of MS germination by plant roots to the control of the pathogen, quantitative information on the influence of crop roots on the germination of MS in the soil is needed. This paper reports on research to address this by measuring the effect of single root tips on MS in soil non-destructively. The results of two experiments in which the germination of MS was measured as influenced by host plants and non-host plants are presented.

Materials and methods

Production of microsclerotia

Green potato stems from the field were cut so they were long enough to be contained upright in an Erlenmyer flask. The stems were autoclaved for 20 min. at 120 °C. Two-week old sporulating *V. dahliae* culture on PDA slants, isolated from potato, was blended with sterile water.

The stems were dipped in the solution, and were incubated under sterile conditions for four weeks at 22 °C. By the end of this period the stems were completely covered with microsclerotia. The stems were air-dried, ground and kept in a dry place at room temperature until used.

Root observation boxes

To study the effect of roots on the germination of MS, wooden root observation boxes (24×18.5×5 cm) with one removable transparent acrylate side were constructed (Fig. 1). Their bases were perforated to allow free passage of water.

Ground potato stem material containing MS of *V. dahliae* was wet-sieved with tap-water through a 125 μm and a 38 μm sieve. The residue on the 38 μm sieve was collected in a minimum amount of water, and the concentration of the MS suspension was calculated by counting the MS under a microscope. The MS were added to water-agar (7.5 g L^{-1}) maintained at a temperature between 35 and 40 °C, and the mixture was mixed thoroughly. Using a syringe, an agar layer

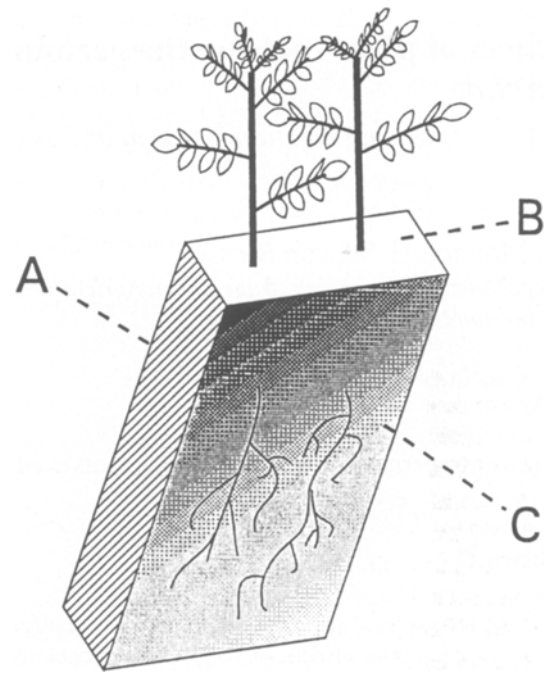

Fig. 1. A root observation box (24×18.5×5 cm) constructed of wooden sides (A), with one transparent acrylate plate (C). The box is filled with unsterilised soil (B).

2 mm thick containing the MS (on average 15 MS mm^{-2}) was built up on the transparent plate.

Before the transparent plates were screwed back onto the boxes, unsterilised moistened sandy soil (pH: 5.8, 9.1% organic matter) was put into the boxes to within 3 mm of the top. This uppermost 3 mm was then filled in with ground dry soil, to ensure good contact (without air spaces) with the agar layer containing the MS. An unsterilised soil was used, to maintain the effect of microbial activity on MS [Emmatty and Green, 1969]. The boxes were placed on a slant, to ensure contact between plant roots and the agar layer. Light penetration through the transparent wall was prevented.

The prepared boxes were pre-incubated for 7 days under the conditions described under 'Experiments', to allow an equilibrium to be reached between the microlife in the box and the agar layer. During the incubation and the experiments, the boxes were moistened by placing them on a bed of wet sand and by watering from above.

Optimisation of the method

The method described above was developed and refined in accordance with various preliminary experiments. In one such experiments, the plant material containing the MS was not sieved and there appeared to be many aggregates of MS and extensive growth of microorganisms from plant tissue particles. This made it difficult to observe the germination of MS. Tests showed that the problem of adherent microorganisms could be decreased by wet sieving the plant material. This technique also removed small MS and large aggregates of MS.

A soft water-agar (7.5 g L^{-1}) was used to fix the MS to the inner side of the transparent plate. We experimented in Petri dishes to find out if the germination of MS was affected by the initial temperature of the agar. We tested agar at two temperatures: 33 °C or 40 °C and found no difference in the germination of the MS.

In another preliminary experiment, four methods of adding the MS were tested in Petri dishes: A) pouring the liquid agar over the MS on the base of the dish; B) putting the MS on the top of the solidified agar; C) putting the MS on the top of the liquid medium, and D) pouring the agar after suspending the MS. Treatment D showed the highest germination percentage of the MS, and had the best distribution of the MS over the surface of the dish. Propagules near the surface had a higher germination percentage than MS deeper in the agar.

After the observations on germination, Petri dishes were filled with a sandy soil and the treatments were tested to ascertain the visibility of fungal hyphae and germination of MS. It appeared that hyphae and germinated MS were very visible in treatments B and D. Some space between the MS and the soil background was necessary to enable the MS to be distinguished from soil particles. Therefore, it was decided to use treatment D as the standard method for the preparation of the agar layer inside the boxes.

Experiments

Twenty boxes were placed in a growth chamber at 22/15 °C day/night with a 14 h thermo- and photophase. After the pre-incubation, two 10-day-old seedlings or two pre-rooted potato sprouts were planted per box. Four plant taxa were grown in four replications: potato (*Solanum tuberosum*) cv. Element (a sensitive cultivar), potato cv. Mirka (a tolerant cultivar) [Scholte and s'Jacob, 1990], barley (*Hordeum vulgare*) cv. Prisma (a non-host), and field bean (*Vicia faba*) cv. Victor (a host). Four boxes without a crop were used as a control. Two weeks after planting, the root system had reached the bottom of the box and measurements were started. The experiments was repeated in five replications with a different observer (Experiments 1 and 2, respectively).

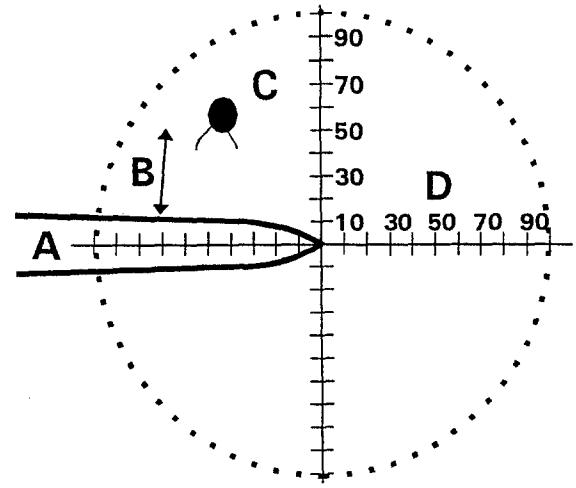

Fig. 2. Schematic representation of the view through the microscope. The centre of the measuring eyepiece is set at the root tip (A). In a circle of radius 1 mm, the distance of the MS (C) to the root surface (B) was measured in units of 10 μm (D). The number of hyphae per microsclerotium was counted.

Non-destructive observation of roots and microsclerotia

Five root tips were examined per box. To avoid the influence of excessive moisture at the bottom of the box, and the influence of fluctuations of moisture near the top of the box, roots in the centre of the box were selected for examination. Because it was known that the largest effects would be measured close to the root surface, a zone of only 1 mm in radium around a root tip was examined. In the control, measurements were taken at five spots with a radium of 1 mm, randomly in the middle of the box.

A binocular-microscope (magnification 100×) was used to count the number of hyphae per MS and to measure the distance between a germination or a non-germinated MS and the root surface (Fig. 2). Per root tip 45 MS were examined. Distances were measured with a measuring eyepiece in units of 10 μm. A long distance object-lens was required to be able to focus at the roots and MS under the 3 mm thick acrylate plate.

Results

The two experiments produced very comparable results (Table 1). The highest levels of germination were found for potato cv. Element and field bean, followed by potato cv. Mirka and barley, and with the control lagging far behind. The most hyphae per germination MS were found in potato cv. Element and field bean. The control had the fewest hyphae per MS, but for this parameter differences between treatments were not large. The number of hyphae per MS, indicating the overall effect of the root, was more than three times higher in potato cv. Element than in the control. Field bean, potato cv. Mirka, and barley also stimulated the number of hyphae per MS, but the effect was least in barley.

Linear regression on the distance of the root surface showed that both the percentage of germinated MS and the number of hyphae per MS decreased significantly ($p < 0.01$) with distance from the root tip (Figs. 3a and b). The intercepts of the lines for the crops were significantly different ($p < 0.025$), but there were no statistically significant differences among the regression coefficients of the four crops.

Discussion

From the above it is clear that this experimental method using root observation boxes gives a quantitatively reproducible description of the influence of plant roots on MS of *V. dahliae*. The method has two shortcomings: the MS do not make actual contact with the soil, and roots next to the transparent acrylate side are not fully covered with soil. Because of this, one cannot be sure that the effect as measured is similar in nature and of comparable level to that occurring in real life in the soil [Lockwood, 1964]. However, the method has the advantage that the influence of the roots on the MS can be easily and directly measured in space and time.

In the control treatment, with no plant, some germination was observed, though this was very much lower than that in all treatments with plants, however, germination was much higher. In accordance with other authors [Emmatty and Green, 1969; Fitzell *et al.*, 1980; Schnathorst, 1981; Schreiber and Green, 1963; Zilberstein *et al.*, 1983b], we found that the stimulation of germination of MS of *V. dahliae* by plant roots is to some extent unspecific for crops. This may be related to the demonstrated effect of various carbohydrates [Emmatty and Green, 1969; Zilberstein *et al.*, 1983b] and amino acids [Emmatty and Green, 1969] on the induction of germination.

The germination in the control treatment in our experiments was high, but is comparable with the results obtained by Schreiber and Green [1963] who used an agar disc technique. No data are available about the germination of MS in the soil under field conditions without the influence of a plant. The germination we found in the control could have been caused by components from the agar layer, by a lack of antagonistic organisms in the agar, or by soluble compounds of the soil solution, but not by fresh specific exudates from plant roots.

Roots of host crops (potato, field bean) showed a stronger stimulating effect on germination of MS per root tip than the roots of the known non-host crop (barley). This confirms the results of Schreiber and Green [1963] and Fitzell *et al.* [1980]. There were clear differences between the potato cvs Element and Mirka. Part of the sensitivity may be expressed at the level of the stimulation of the germination of the MS. The composition of the exudates might be a determining factor. Genetic research has shown that the host genotype may play a major role in determining the characteristics of the rhizosphere populations of bacteria in wheat, largely through control of the quality of the root exudates [Curl and Truelove, 1986]. Differences between crops are even clearer when the effect of crop roots (or of their exudates) over distance are considered (Figs. 3a and b). Although the level of germination at the root surface of barley was rather high, at a distance of 1 mm it had fallen to that of the control treatment. In the Verticillium-susceptible crops potato cv. Element and field bean the germination at a distance of 1 mm from the root surface was still more than double that in barley and in the control. So, the influence of these crops will be perceptible at a distance of more than 1 mm. From this we infer that the results of germination and the number of hyphae per MS of potato cvs Element and Mirka, and field bean underestimate the influence per root tip compared with barley or the control. An effect over a certain distance will be important for the reduction of the MS population in the soil caused by the plant roots. Hyphae beyond a critical distance are likely to lyse before they reach the root surface [Fitzell *et al.*, 1980], because *Verticillium* spp. have shown to be very poor saprophytes in the soil [Schnathorst, 1981]. If the concentration of the exudates is the determinant in stimulating MS to germinate, the relation should be non-linear. It is remarkable that we did not get a better fit with a function relating the effect on the MS

Table 1. Effects of roots on the germination of microsclerotia (MS) within a radius of 1 mm around the root tip in Experiments 1 and 2

	Potato		Field bean	Barley	Control	LSD ($p = 0.05$)
	cv. Element	cv. Mirka				
Ms germinated tip^{-1} *(%)*						
Exp. 1	37.9	62.2	38.7	29.7	15.3	10.7
Exp. 2	48.7	37.6	43.6	35.4	20.5	10.4
Mean	43.9	32.7	41.4	32.9	18.2	6.8*
Number of hyphae (germinated MS)$^{-1}$						
Exp. 1	1.61	1.52	1.43	1.39	1.40	0.28
Exp. 2	1.44	1.53	1.31	1.13	1.15	0.15
Mean	1.52	1.53	1.36	1.25	1.27	0.14*
Number of hyphae MS^{-1}						
Exp. 1	0.66	0.44	0.56	0.43	0.23	0.26
Exp. 2	0.71	0.58	0.57	0.40	0.24	0.16
Mean	0.69	0.52	0.56	0.41	0.23	0.13*

* LSD values for the means are calculated from an analysis of variance based on the combined data of Experiments 1 and 2.

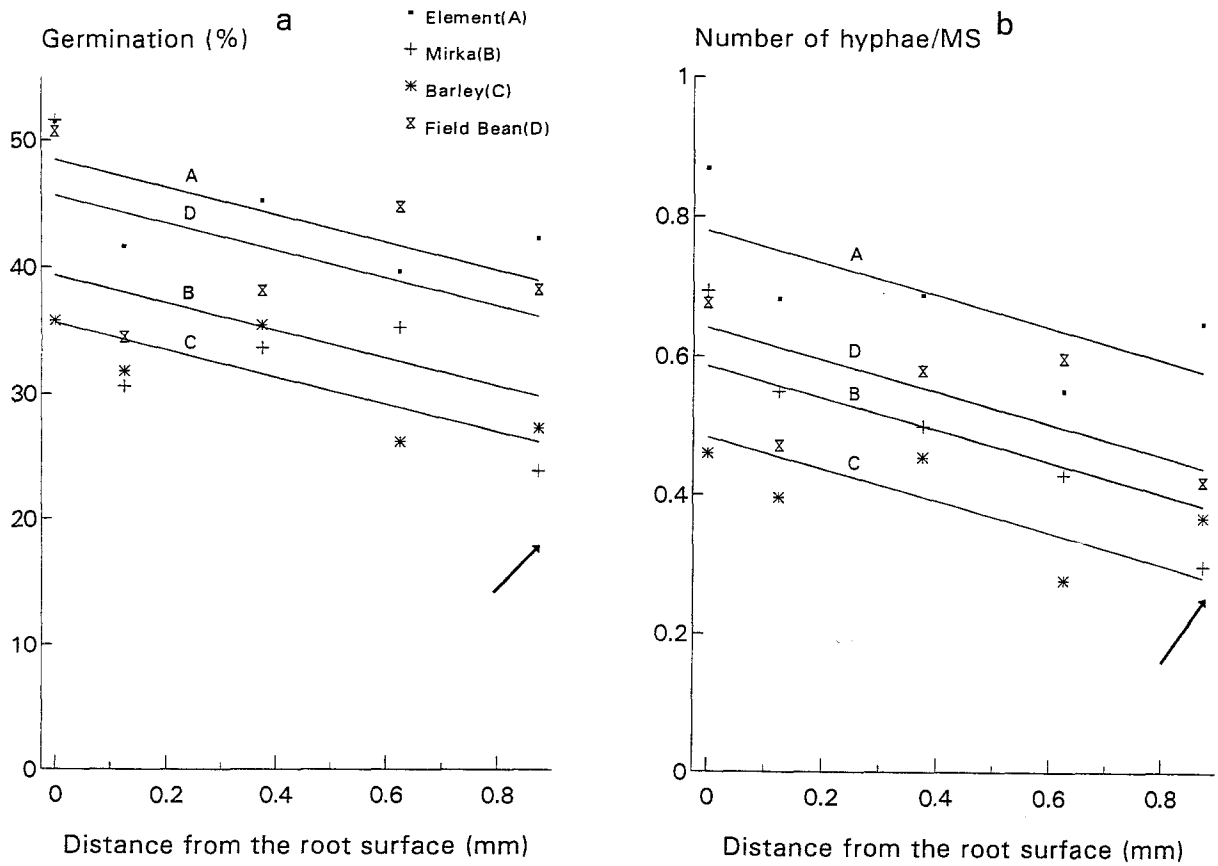

Fig. 3. Relation between the distance from the root and the percentage germinated microsclerotia (a) or the number of hyphae per microsclerotia (b) for the four crops tested. Before the regression was calculated, MS were grouped into five distance classes from the root: 0, 0.01–0.25, 0.26–0.50, 0.51–0.75, and 0.76–1.00 mm. The medians of the classes were taken as the independent variable. There was a statistically significant decrease over the distance ($p < 0.01$). Intercepts also differed statistically significantly among crops ($p < 0.025$), but slopes did not. The arrows indicate the level of the control.

to the inverse distance from the root surface. Perhaps, a high microbial activity reduced the effect of the exudates close to the root, leading to a flattening of the line. Additionally, the area observed around the root tip may have been too small to yield sufficient data to establish a non-linear relationship. The correlation for each single crop was not very high and a change of one data point may have a considerable effect on the shape of the line.

Microsclerotia require no exogenous source of nutrients for germination *in vitro* [Green, 1971]. Furthermore, it has been shown that germination can be inhibited by high CO_2 concentration, low O_2 concentration [Zilberstein *et al.*, 1983a], and a relatively high soil temperature [Dutta and Isaac, 1979]. It is also difficult to draw general conclusions, because just one type of soil is used in the experiments. Research done by Rovira and Davey [1974] suggests that the age and stage of development of the plant, the light intensity, temperature, pH, soil moisture, and O_2 and CO_2 concentrations may indirectly influence the stimulation of the MS qualitatively and quantitatively via their influence on the exudation of plants [Rovira and Davey, 1974]. However, from our experiments it is clear that crops differ in their stimulation of the germination of MS of *V. dahliae*; this aspect deserves to be followed up and clarified.

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