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Germination of chlamydospores of Fusarium oxysporum f. sp. pisi race 1 in the rhizosphere, and penetration of the pathogen into roots of a susceptible and a resistant pea cultivar

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Abstract

Germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in the rhizosphere of pea seedlings and red clover seedlings grown in natural soil heavily infested with the pathogen, was highest in percentage along the actively growing parts of the roots. At these sites, exudation of ninhydrin-positive substances and reducing sugars was most intense with seedlings grown in vitro.

No significant difference in the percentage of germinating chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 were observed in the rhizosphere soil and on the root surface of homologous parts of roots of seedlings and mature plants of a susceptible 'Rondo' and a resistant 'Rovar' pea cultivar grown in natural soil heavily infested with the pathogen. Differences in the growth of mycelium of the pathogen on the root surface, or in the attachment of the mycelium to the root surface of both cultivars were not observed. Epidermis and cortex cells of roots of both cultivars reacted on penetration by the pathogen by producing a cellulose thickening of the cell wall, which later became infiltrated with a ligning-like material. A selective effect on the activities of the pathogen in the rhizosphere, on the root surface and in the epidermis and cortex in relation to resistance thus could not be demonstrated. Formation of new chlamydospores from germ tubes of germinating chlamydospores was frequently observed in the rhizosphere of the susceptible and resistant pea cultivar and in the rhizosphere of red clover seedlings.

Introduction

The important role plant exudates play in the pathogenesis by soilborne plant pathogens has become manifest. The extensive literature on this subject is discussed critically by Schroth and Hildebrand (1964). Good examples have been given of the influence of the amount of exudates released in soil on severity of a soil-borne disease (Flentje, 1947, 1959; Flentje and Saksena, 1964; Husain and McKeen, 1963; Rajagopalan and Bhuvaneswari, 1964; Schroth and Cook, 1964; Schroth et al., 1966). An indirect influence of exudates on the incidence of disease seems to exist in those cases where, in the rhizosphere, a high number of organisms antagonistic to the pathogen, is associated with a lower incidence of disease (Schroth and Hildebrand, 1964).

However, no incontrovertible evidence has been presented so far, for the occurrence of specific compounds or of a specific composition of exudates of underground plant parts, as a major factor contributing to disease resistance, though such a selective effect of root exudates in relation to resistance was questioned by Buxton (1957a). Exudates collected from each of three cultivars of *Pisum sativum.* grown in sterile liquid cultures, which were differentially susceptible to three physiological races of

Fusarium oxysporum f. sp. *pisi* specificially depressed conidium germination and mycelial growth in vitro of the races to which the cultivars appeared to be resistant, but mycelial growth was stimulated by exudates from susceptible cultivars. The spore germination of the three races was affected by water extracts of the three rhizosphere soils in the same way as by root exudates from the three cultivars (Buxton, 1957b). These experiences together with observed quantitative differences in the composition of the rhizosphere microflora of the three cultivars led Buxton to suggest that substances exuded by roots of cultivars resistant to race 1, together with the effect of altering the soil microflora, prevent race 1 from germinating, thus lowering the amount of effective inoculum and delaying the onset of wilt. In 1960, Buxton concluded that although the results of his experiments suggest that part of the resistance of peas to soil-borne *F. oxysporum* may act outside the roots in the rhizosphere, under natural conditions root exudates are perhaps unlikely to be dominant factors in the rhizosphere environment. Kommedahl (1966), who used chlamydospores formed in vitro in his experiments on germination of spores of *F. oxysporum* f. sp. *pisi* race 1 and race 2 in response to root exudates of three near-isogenic lines of *Pisum sativum* 'Greenfast' that differentiate between the two physiologic races, could not confirm the findings of Buxton. Chlamydospores and microconidia of both races germinated alike either in exudates or on excised roots of peas of the three lines. Kommedahl's observations of the pathogen in autoclaved soil agree with those of Buxton (1957a), that mycelium invaded only the outer cortex of roots of cultivars inoculated with the race to which they were resistant.

The observations of Buxton and Kommedahl, which are mainly based on experiments under aseptic conditions, raise the question of what are the activities of *F. oxysporum* f. sp. *pisi* in the rhizosphere of a susceptible and a resistant variety in terms of chlamydospore germination, germtube and mycelial growth, attachment to the root surface and penetration into the roots. Chlamydospores formed in nonsterile soil and influenced in their activities by rhizosphere microorganisms may react differently to exudates released from roots grown in nonsterile soil, than microconidia and chlamydospores produced in vitro to exudates from the root system of pea seedlings grown in sterile media.

Observations on these activities of *F. oxysporum* f. sp. *pisi* race 1 in the rhizosphere and on the root surface of a susceptible and a resistant pea cultivar (Schippers, 1967) and in the rhizosphere of a non-host are presented in this paper.

Material and methods

Soil, plant, and pathogen

The soils used were a clay loam from the Noord Oost Polder (NOP) and a clay loam from the Eempolder, both with a pH of 7.9–8.2.

Both clay loams were sieved through a 14 mesh sieve and thoroughly mixed with sieved river sand one to one on a dry weight basis. The soil was then stored at 5 °C in a polyethylene bag and transferred to 20 °C, five days before being used for the preparation of "chlamydospore soil".

The isolate of *Fusarium oxysporum* Sch. f. sp. *pisi* race 1 Snyder and Hansen used, was kindly provided by Ir N. Hubbeling from the IPO, Wageningen. Two pea cultivars (*Pisum sativum*) were used: 'Rondo', susceptible to race 1 and 2; 'Rovar'

resistant to race 1 but susceptible to race 2. Red clover, cv. 'Kuhn', was used as a non-host. The pathogen grown on PDA (potato dextrose agar) kept its virulence during the two year of research. 'Rondo' seedlings, sown in the greenhouse in non-sterile "NOP-clay" inoculated with a microconidial suspension, part of which was used for the final experiment described in this paper, developed severe wilt symptoms within 10 days. 'Rovar' seedlings sown in this soil did not show wilting although they were somewhat stunted in comparison with plants grown in uninoculated clay. The pathogen could be reisolated from the main stem of 'Rondo' and 'Rovar' seedlings 12 days after sowing.

Preparation of "chlamydospore soil"

About 22 liters of Czapek Dox solution, distributed among 200 conical flasks of 300 ml and inoculated with microconidia of the pathogen were shaken on a shaker at 22 °C. When started with 0.02×10^6 microconidia per ml, the concentration of microconidia was highest at the 5th day of shaking, and was more than 5×10^6 conidia per ml. Thereafter the concentration leveled off quickly. The shake cultures were sieved over nylon cloth to get rid of the sparse mycelium. The microconidia were centrifuged, resuspended once in sterilized deionized water and concentrated by continuous flow centrifugation at 10,000 g. The microconidia were thoroughly mixed with about 50 g of soil. The water content was then lowered by allowing evaporation of soil in an air current to the original water contents of approximately 20%. The soil was then stored at least 18 days at 20 °C. After this incubation period, in thin smears of this soil on an object slide, stained with acid fuchsin, 5 to 10 chlamydospores could usually be found in one high-power field ($\times$ 450).

Estimation of the percentage of germinating chlamydospores in rhizosphere soil

Seeds of the two pea cultivars were soaked for 16 h in tap water. Thereafter they were introduced into the chlamydospore soil or allowed to germinate and grow for 3 or 6 days on moistened filter paper, or, they were allowed to germinate and grew for 6 days in non-treated clay loam before being introduced into the chlamydospore soil. Seeds to be germinated and grown on moist filter paper, were surface disinfected for 10 min in a 5% "Halamid"-solution and rinsed for 2 h in running tap water.

Two layers of chlamydospore soil were obtained by filling both halves of plastic boxes of $10 \times 2 \times 1$ cm with an approximately 0.4 mm thick layer of chlamydospore soil, so that when the halves were put together, the two layers of soil stuck to each other. The seedlings were enclosed with their roots between the two layers of chlamydospore soil and kept in an incubator for 24 h at 20 °C or in a greenhouse at 20°–24 °C; when kept in the greenhouse, the seedlings were incubated for a longer period up to 10 days. When grown on moist filter paper, the roots were rinsed in tap water after which water adhering to the roots was removed in an air current, just before they were introduced into the chlamydospore soil. For studying the pathogen in the rhizosphere soil of older plants, two roots of plants grown for 3 weeks in NOP clay in plastic pots in the greenhouse, were led through holes in the pot wall between two layers of chlamydospore soil in plastic boxes. The boxes fitted into the holes in the pot wall.

At the end of the incubation periods, the two layers of chlamydospore soil were separated, and the roots were carefully removed leaving a root print in both layers of

soil. Pin point samples of soil were taken from the root prints at tip, middle and base of the root within 1 mm distance of the root surface using a dissecting microscope at × 40 magnification (see Fig. 2). The last 10 to 15 mm of the root developed during the incubation period was considered as the tip of the main root, a part over 10–15 mm at about equal distances from the utmost root tip and the cotyledons as the middle. The base of the root extends over 10–15 mm and is about 10 mm below the cotyledons. At this part secondary roots start to develop about 1 week after germination of the seed.

The 5 to 10 pin point samples of rhizosphere soil taken from the prints of tip, middle, and base of the roots in both halves of the boxes, were mixed thoroughly in a drop of tap water, smeared in a thin layer on an object slide and dried immediately by an air current. After removal of the bigger sand particles, the smears were stained with 0.1 % acid-fuchsin in 85 % lactophenol, covered with a cover slip and examined with the microscope for germinating chlamydospores. The percentage of germinating chlamydospores was calculated for 500 chlamydospores, 250 in each of two smears made of the rhizosphere soil samples from the tip, middle, and base of the main root of each seedling.

A chlamydospore was considered to be germinated when a germtube was clearly visible at a × 1000 magnification (Fig. 3).

Observation of the pathogen on the root surface and in the root tissue

Roots were fixed in FAA (Johansen, 1940) after incubation in chlamydospore soil. Cross and tangential sections were cut from root pieces embedded in paraffine. Epidermis strips and microtome sections of fixed roots were stained with thionine and differentiated with Orange G according to Stoughton (1929) thereafter embedded in Caedax and examined with the microscope.

Detection of regions of root exudation in vitro

Seeds of 'Rondo' and 'Rovar' were disinfected in a solution of 5 % Halamid and 2 % ethanol for 3 minutes and rinsed with sterile tap water. Two- and 5-day-old seedlings of both cultivars grown from disinfected seeds were incubated for 48 hr between sheets of sterile moist Whatmann paper no. 3, according to the method of Schroth and Snyder (1961). After incubation the roots were tested for contamination by placing them on a potato-dextrose agar (PDA). Using the method of Porter et al. (1957), Whatman filter paper was sprayed with ninhydrin color reagent to detect exudation of acids, amides and amines. Silver nitrate was sprayed after the method of Partridge (1946) to detect exudation of reducing sugars.

Experiments and results

Formation of chlamydospores in soil

Microconidia developed in the Czapek Dox shake cultures, were of the one and two-celled type. They did not germinate for more than 1 % after being introduced into the natural soil. The transformation of cells of these microconidia into chlamydospores in the clay loams could be noticed from the 5th day of incubation on. After 18 days of incubation, most of the microconidia had turned into chlamydospores, while others disappeared by lysis, just as did the few mycelial fragments. Some of the one and two-

Fig. 1. Chlamydospores originating from microconidia of *Fusarium oxysporum* f. sp. *pisi* race 1 in natural soil. Some retain the microconidial shape (b), others round off (a).

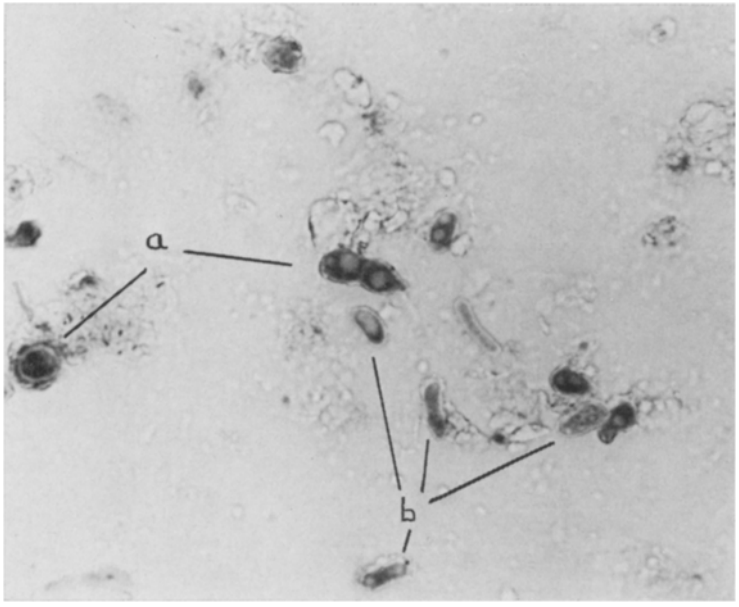

Fig. 1. Chlamydosporen gevormd uit microconidiën van Fusarium oxysporum f. sp. pisi ras 1 in niet steriele grond. Sommige behouden ongeveer de vorm van het microconidium (b), andere ronden zich tijdens hun vorming af (a).

celled microconidia hardly changed their original shape whereas in others the cells rounded off (Fig. 1). The cells of both types became double walled and their contents stained more deeply with acid fuchsin than the contents of microconidia. No differences in percentage germination were observed between these two types of chlamydospores 24 h after 0.2 ml of a 2% glucose – 2% asparagine solution was added to 0.5 g air-dried soil. No discrimination was made between these different types of chlamydospores in experiments on chlamydospore germination.

Germination of chlamydospores in the rhizosphere

The percentages of germinating chlamydospores in the rhizosphere soil of tip, middle and base of the main roots of the susceptible 'Rondo' and resistant 'Rovar' seedlings, were estimated and compared with each other. As the composition of root exudates depends on the age of the pea plants, according to Rovira (1959), plants of different ages were used in these experiments. Germination of chlamydospores in the rhizotsphere of red clover seedlings was also examined.

Roots were incubated for only 24 h in chlamydospore soil to obtain a clear picture of the stimulating or inhibiting influence of substances exuded by the different parts of the roots on the chlamydospore germination, apart from the lytic activity of soil microorganisms.

Fig. 2. Pea seedling 'Rondo' of 4 days old, after 24 h incubation in "NOP chlamydospore soil". b (base), m (middle) and t (tip) mark the root regions in the rootprint from where soil samples were taken.

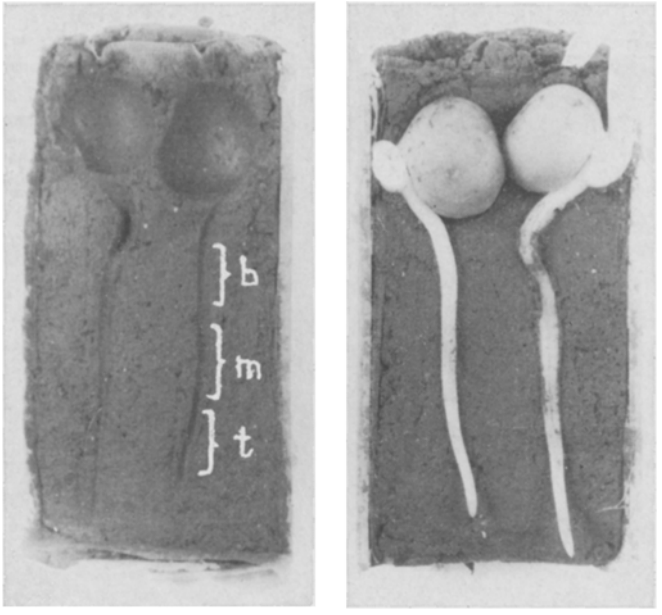

Fig. 2. Vier dagen oude erwtekiemplant 'Rondo' na 24 uur incubatie in "NOP chlamydosporengrond" b (basis), m (midden) en t (top) markeren de delen van de wortelafdruk in de grond waarvan grondmonsterstjes werden genomen waarin de kieming van chlamydosporen werd bestudeerd.

The complex influence over a longer period of time of exudates and of rhizosphere microflora, lysis of germ tubes included, on the percentage of germinated chlamydospores, was studied by incubating seedlings of both cultivars for longer than 24 h and at a maximum of 10 days in chlamydospore soil.

Germination of chlamydospores after 24 h in the rhizosphere

Five susceptible 'Rondo' and four resistant 'Rovar' seedlings, germinated and grown for 3 days on moist filter paper were incubated for 24 h in 35-day-old "NOP chlamydospore soil", an equal number of other seedlings of each cultivar in 52-day-old "Eempolder chlamydospore soil". The lengths of the roots of the seedlings varied from 35 to 45 mm at the end of the incubation period (Fig. 2). Neither significant difference in percentage of germinating chlamydospores was noted between tip, middle, and base of the root of each seedling nor between the homologous parts of susceptible and resistant seedlings in NOP soil as well as in Eempolder soil (Table 1). Five seedlings of both varieties were germinated and grown for 6 days on Hoagland solution, while an equal number of seedlings of both varieties were germinated and grown for 6 days in non-sterile NOP clay. Thereafter the seedlings were transferred to the NOP chlamydospore soil and incubated for 24 h. The length of the roots varied from 7 to 9 cm at the end of the incubation period.

Table 1. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in the rhizosphere of 3–4 day-old seedlings of a susceptible 'Rondo' and a resistant 'Rovar' pea cultivar, 24 h after the seedlings were transferred to the chlamydospore soil. The pea seedlings were germinated and grown on moist filter paper.

Chlamydospore soil	Varietal reaction	Chlamydospore germination per root region		
		base (%)	middle (%)	tip (%)
NOP[1]	susceptible	5	7	9.5
		10.5	11	3
		2.5	9	6.5
		14	4	5.5
		6	10	6
	resistant	3.5	8	6
		3.5	2	9.5
		10	7	8.5
		3.5	5	7.5
Eempolder[2]	susceptible	7.5	7	10
		8.7	6.7	9
		7	6.5	6.7
		8.5	9	11.2
		4	5.2	5.2
	resistant	11.7	6.2	8.2
		10.5	3.7	6.2
		7.2	8	7
		12	9.5	10.2

[1] Soil was 35 days old.
[2] Soil was 52 days old.

Tabel 1. Kieming van chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 in de rhizosfeer van 3–4 dagen oude kiemplanten van een vatbaar 'Rondo'- en een resistent 'Rovar' erwteras, 24 uur na het overbrengen van de zaailingen in de chlamydosporengrond. De kiemplanten werden na kieming van het zaad, 2–3 dagen op vochtig filtreerpapier geïncubeerd.

Both susceptible and resistant cultivars grown for 6 days on Hoagland solution revealed a percentage germinating chlamydospores that was higher at the tip and base of the roots, than at the middle at the end of the incubation period (significant, Wilcoxon's two sample test; $\alpha = 0.01$: Table 2).

When comparing homologous parts of roots of both cultivars, a significant difference in percentage germinating chlamydospores is noticed only at the base of the roots of the susceptible and the resistant cultivars ($\alpha = 0.01$). The percentage germinating chlamydospores, however, is highest for the resistant seedlings.

Seedlings of both cultivars, germinated and grown in NOP soil and then incubated in chlamydospore soil, showed a significantly higher percentage of germinating chlamydospores at the tip, than at middle and base of the roots ($\alpha = 0.01$), whereas no significant difference was noticed between middle and base (Table 2). No signi-

Table 2. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in "NOP chlamydospore soil" in the rhizosphere of 6–7 day-old seedlings of a susceptible 'Rondo' and a resistant 'Rovar' pea cultivar 24 h after the seedlings were transferred to the chlamydospore soil.

Substrate for seedlings before transfer to chlamydospore soil	Varietal reaction	Chlamydospore germination per root region		
		base (%)	middle (%)	tip (%)
Hoagland solution[1]	susceptible	12.6	2.4	6.4
		8	4	10
		9	3	11
		14.4	2.6	10
		9.6	3.6	9
	resistant	19	4.2	10
		20.6	3.4	7.6
		18.8	4.2	15
		17.2	4	9.6
NOP soil[2]	susceptible	1.8	1.2	7.2
		1.8	1	7.2
		1.4	0.8	5.6
		0.6	0.6	5.2
		1.2	1.2	5.8
	resistant	1.6	0.2	2.8
		2.2	1.2	4.4
		0.8	0.6	6.1
		1.8	1.2	6.4

[1] Chlamydospore soil was 29 days old.
[2] Chlamydospore soil was 37 days old.

Tabel 2. Kieming van chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 in "NOP chlamydosporengrond" in de rhizosfeer van 6–7 dagen oude kiemplanten van een vatbaar 'Rondo' en een resistent 'Rovar' erwteras, 24 uur na het overbrengen van de zaailingen in de chlamydosporengrond.

ficant difference in chlamydospore germination exists between homologous parts of both cultivars. Comparable results were obtained with duplicate experiments.

The percentage of germinating chlamydospores after 24 h in the rhizosphere of 24-day-old susceptible and resistant pea plants grown in pots in the greenhouse is presented in Table 3. At the middle of these roots was chosen a 10-mm part of the roots, at a distance of 30 to 45 mm from the utmost root tip. No significant difference in percentage of germinating chlamydospores between tip and middle of the roots nor between the homologous parts of roots of the susceptible and resistant cultivars was observed.

From the data presented in Table 1, 2 and 3, it is concluded that no specific stimulating or inhibiting substance that could influence chlamydospore germination in the rhizosphere soil is released from the roots of the susceptible or resistant pea cultivar. At the base of the roots of both cultivars, where secondary roots start to develop with those seedlings grown for 6 days on Hoagland solution, and at the tip of the roots,

Table 3. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in NOP chlamydospore soil[1] in the rhizoshpere of secundary roots of pea plant sof a susceptible 'Rondo' and a resistant 'Rovar' cultivar, 24 h after these roots were transferred to chlamydospore soil. The pea plants had been germinated and grown for 24 days in natural NOP soil.

Varietal reactions	Chlamydospore germination per root region	
	middle (%)	tip (%)
Susceptible	2.6	6.4
	8.4	3.8
	1.4	9.2
	6.4	7.4
	3	16
	2.6	1
	5.2	5.8
	7.8	7.2
Resistant	4.4	3
	4.2	1.6
	11.6	10.1
	10	7.8
	4.4	5.2
	9.6	5.4
	4.8	4.2
	4.2	6

[1] Chlamydospore soil was 18 days old.

Tabel 3. Kieming van chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 in "NOP chlamydosporengrond" in de rhizosfeer van zijwortels van erwteplanten van een vatbaar 'Rondo' en een resistent 'Rovar' ras, 24 uur na het overbrengen van deze wortels in chlamydosporengrond. De erwteplanten waren 24 dagen oud en gegroeid in "NOP grond".

chlamydospore germination is significantly higher than at the middle. The absence of a higher percentage of germinating chlamydospores along the base of the root, than at the tip of seedlings grown for 6 days in "NOP soil", is ascribed to a delayed onset of the development of secondary roots with seedlings grown in soil (Table 2).

The behavior of chlamydospores of the pathogen in the rhizosphere of a nonhost was studied with five red clover seedlings, germinated and grown for 7 days on a Hoagland solution and incubated for 24 h with their roots in chlamydospore soil. At the end of the incubation period, the root of the seedlings that had not yet developed secondary roots were about 10 cm long. The mean percentage of germinating chlamydospores in the rhizosphere soil of base, middle and tip of the roots amounted to 12, 3 and 17%, respectively. The stimulation of the germination of chlamydospores of *F. oxysporum* f. sp. *pisi* in rhizosphere soil seems to be a non-specific phenomenon. Germination of chlamydospores and formation of chlamydospores from germinating chlamydospores were seen in smears of rhizosphere soil of red clover seedlings that were germinated and grown for 6 days in chlamydospore soil.

Fig. 3. Germinating chalmydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in rhizosphere soil of *Pisum sativum* 'Rovar'.

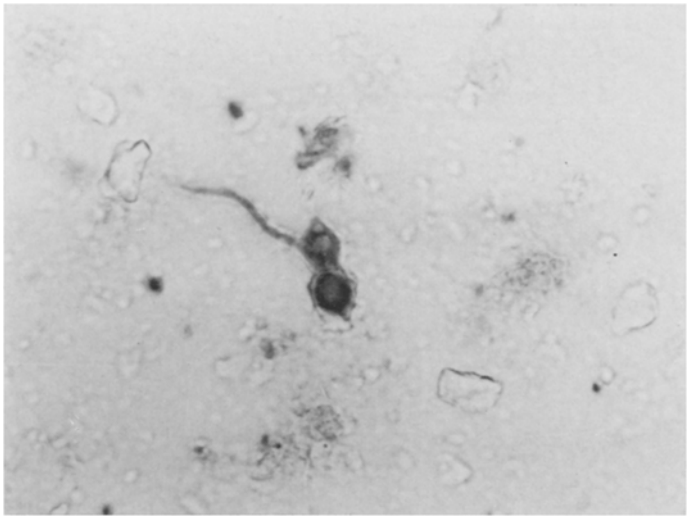

Fig. 3. Gekiemde chlamydospore van Fusarium oxysporum f. sp. pisi ras 1 in rhizosfeergrond van een erwtekiemplant 'Rovar'.

Germination of chlamydospores in the rhizosphere of seedlings grown for longer than 24 h in "chlamydospore soil"

The possibility of a differential change in percentage of germinating chlamydospores in the rhizosphere of the susceptible or resistant cultivar when incubated for longer than 24 h in the chlamydospore soil, was studied.

Each 24 h, for a 10-day period, the percentage of germinating chlamydospores at tip, middle and base of the root of two of 20 seedlings of both cultivars, growing in NOP chlamydospore soil, was estimated. The percentage of germinating chlamydospores in no case exceeded 8.8%. Random tests on chlamydospore germination in the rhizosphere soil of seedlings of both cultivars incubated up to 10 days in chlamydospore soil also did not reveal any indication of a differential change in percentage germinated chlamydospores in the rhizosphere of both cultivars.

Six seedlings of both cultivars were then germinated and grown for 6 days in NOP chlamydospore soil. At the end of this period there was no significant difference between the percentage of germinating chlamydospores at tip and middle of each root nor between homologous parts of the roots of both cultivars (Table 4). At the base of the roots of both cultivars, the percentage was lower than at the middle and tip of the roots (significant, Wilcoxon's two sample test, $\alpha = 0.01$). This lower percentage of germinating chlamydospores along the base of seedlings is ascribed to a delayed onset of development of secondary roots with seedlings grown in soil. The germ tubes of chlamydospores germinated along the base of the roots during the first days of growth of these seedlings, were cleared away by lytic action after 6 days in chlamydospore soil. The equal percentages of germinating chlamydospores along the tip and middle

Table 4. Germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 in the rhizosphere of a susceptible 'Rondo' and resistant 'Rovar' pea cultivar that have been germinated and grown for 6 days in NOP chlamydospore soil[1].

Varietal reaction	Chlamydospore germination per root region		
	base (%)	middle (%)	tip (%)
Susceptible	1	4.6	4.6
	2.8	5	1.8
	2.8	5.4	3.9
	1.4	1.6	1
	2.2	3.6	4.2
	3.2	5	5.6
Resistant	2.2	4.4	4.6
	2.2	5.4	2.9
	0.4	4.8	5.2
	1	3	1.4
	3.8	4.4	4.6
	1.2	5.4	0

[1] Chlamydospore soil was 22 days old.

Tabel 4. Kieming van chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 in de rhizosfeer van kiemplanten van een vatbaar 'Rondo' en een resistent 'Rovar' erwteras, welke gekiemd zijn en zich vervolgens gedurende 6 dagen ontwikkeld hadden in "NOP chlamydosporengrond".

of roots of these plants is ascribed to the stimulation of germination by the root tip. Soil, along the root tip had become the rhizosphere soil along the middle part of the root 1 or 2 days later, when soil samples were taken. The germ tubes had not been lysed within these two days.

Formation of new chlamydospores and thick-walled mycelia from germ tubes, as well as lysing germ tubes, were frequently found in the rhizosphere soil at middle and base of the roots of both cultivars.

In all experiments the percentage of germinating chlamydospores in the chlamydospore soil was less than 1 % in soil samples taken at a distance of about 10 mm from the root surface.

The activities of the pathogen on the root surface and in the epidermis and cortex

Seedlings of both cultivars germinated and grown for 3 days on moist filter paper were incubated for at most 10 days in NOP chlamydospore soil. Each 24 h roots of two seedlings of each cultivar were freed from soil and fixed in FAA (Johansen, 1940). Epidermis strips and cross and tangential sections of tip, middle and base of the roots stained with thionine were examined with the microscope. Chlamydospores, germ tubes and mycelia of the pathogen, but also other microorganisms on the epidermal surface or inside the root tissue, became purple stained and clearly distinguishable from the tissues. No significant difference in percentage germinating chlamydospores on the root surface between tip, middle and base of each cultivar, nor between the

Fig. 4. Thickenings of cell walls in an epidermis strip of a root of a resistant pea seedling 'Rovar' that has been incubated in NOP clay, highly infested with chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1. A: after 24 h, B: after 48 h of incubation.

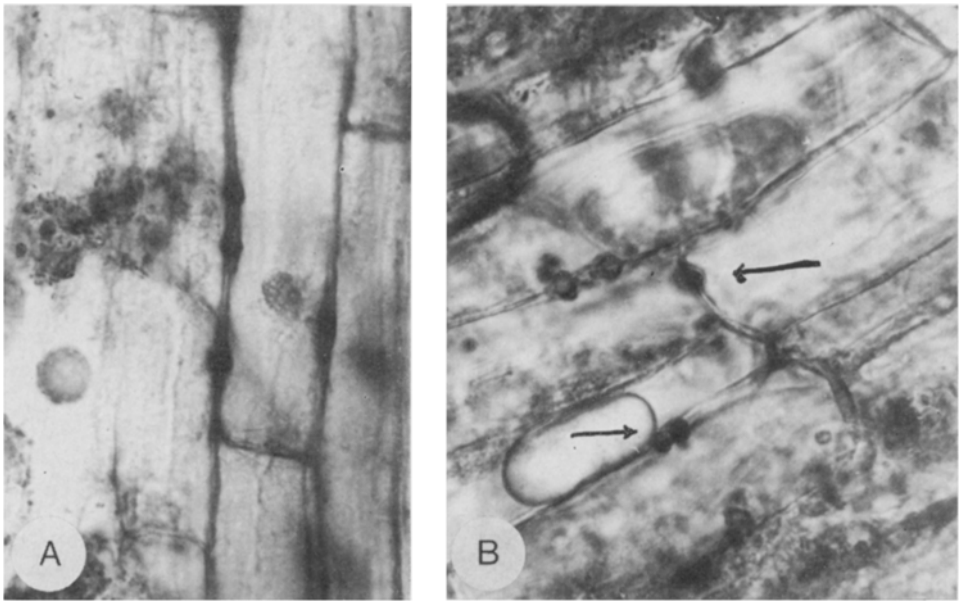

Fig. 4. Verdikkingen in celwanden van de epidermis van een wortel van een resistente erwtekiemplant 'Rovar' na incubatie in NOP grond met een hoge concentratie chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1. A. na 24 uur incubatie, B. na 48 uur incubatie.

homologous parts of the two cultivars could be observed. The percentage germinating chlamydospores amounted to 25–30% after 24 h, 49–55% after 48 h and up to 100% after 72 h of incubation. No differences were noticed in development of mycelium on the root surface, nor in attachment to the epidermal surface nor in penetration of the pathogen into the epidermis of roots of both cultivars.

The utmost tip of the roots appeared to be free of microorganisms. Small thickenings of the radial walls of the epidermis at tip, middle and base of the roots of both cultivars were present after 24 h of incubation (Fig. 4A). The green color of these thickenings indicated their cellulose nature according to Stoughton (1929). Germ tubes or hyphae were often seen terminating in these cellwall thickenings which were found mainly in radial cellwalls (Fig. 5). After 48 h 9f incubation, the thickenings had enlarged (Fig. 4B). Mycelium had developed abundantly all over the root surfaces of both cultivars, especially along the longitudinal walls of the epidermis cells. 24 h later an opening was observed in some of the thickenings, the site apparently through which the hyphae penetrate the cell wall. Where cellwalls were thickened over their full length, the epidermis cells set free from each other, and the pathogen had penetrated into the outer cortex cells. Intercellular spaces in the cortex became filled with a green cellulosic substance. The amount of chlamydospores and mycelia on the root

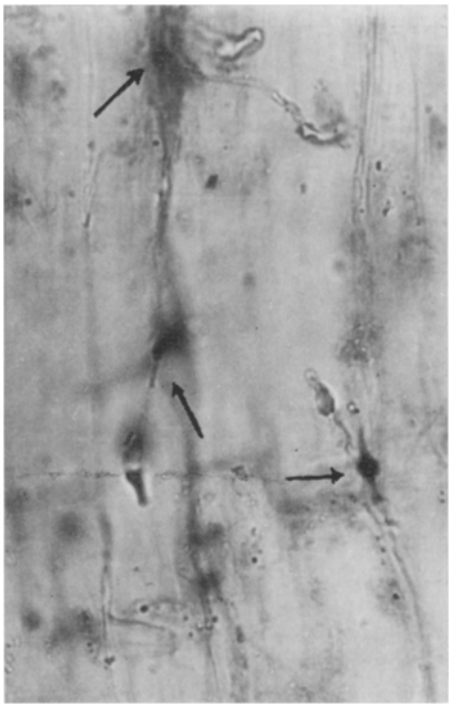

Fig. 5. Germinating chlamydospores of *Fusarium oxysporum* f. sp. *pisi* race 1 terminating with their germ tubes in cell wall thickenings of the epidermis of a root of a 'Rondo' seedling that has been incubated for 48 h in "NOP chlamydospore soil".

Fig. 5. Gekiemde chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 die celwand verdikkingen veroorzaken waar zij de epidermis van de wortel binnendringen van een erwtekiemplant 'Rondo' na 48 uur incubatie in "NOP chlamydosporen grond".

surfaces of base and middle of the roots of both cultivars had obviously decreased after 96 h. The color of the cellwall thickenings at these sites had changed to blue which is an indication of ligning formation according to Stoughton (1929). New chlamydospores formed at germ tubes were found on the root surface and in the rhizosphere soil along base and middle of the roots.

After 168 h of incubation, little mycelium is left on the root surfaces at base and middle of roots. Spherical cell wall thickenings were now also present in the cell walls of the outer cortex. Tiny, blue-stained tubes can be found running from such a thickening in the outer epidermal cell wall to the opposite wall of the cell. After 216 h epidermis and cortex cells became loosened at some sites. Germinating chlamydospores with their germ tubes stuck to the center of round cell wall thickenings were found in the younger root tip regions after 240 h. These thickenings stained green (cellulosic) while those in older parts of the roots, where no more germ tubes or mycelia could be observed, were blue (lignified). Cell wall thickenings were exceptionally frequent along cracks in the root tissue. In a section through the root of a susceptible seedling only once were cell wall thickenings, attended with intercellular spaces filled with a greenish substance, found close to the young vascular system. In these regions, cells were present with both a course and a fine granulation as earlier described by Schroeder and Walker (1942).

Regions of root exudation

Exudation of ninhydrin-reacting materials was demonstrated over the full length of

Fig. 6. Regions of exudation of ninhydrin-positive substances from the root of a 3–4 day old (A) and a 5–6 day old (B) 'Rovar' seedling grown on moist Whatman paper for 48 h.

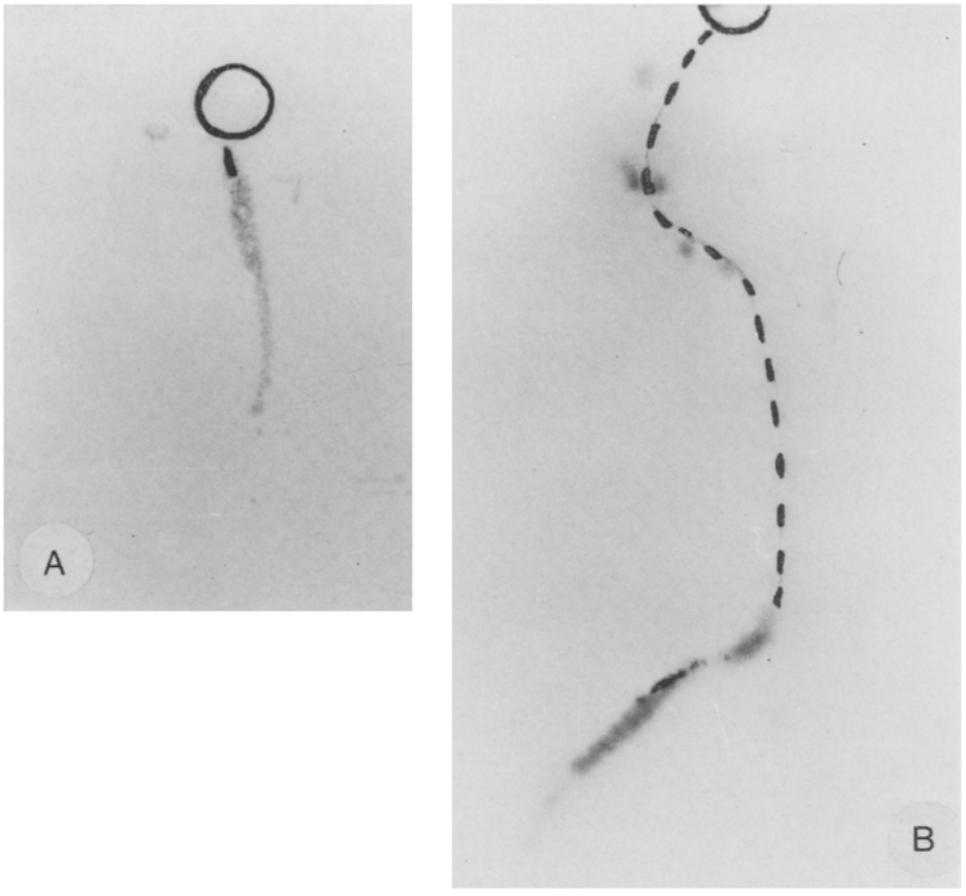

Fig. 6. Ninhydrine-positieve stoffen afgescheiden uit de wortel van een 3 tot 4 dagen oude (A) en een 5 tot 6 dagen oude (B) erwtekiemplant 'Rovar' na 48 uur incubatie op vochtig Whatman papier.

the roots of seedlings incubated between Whatman paper from the 3rd to the 5th day after germination. With seedlings incubated from the 5th to the 7th day after germination, exudation was only demonstrated along the base of the roots where secondary roots were about to be formed, and along the growing root tip (Fig. 6A and B). Similar, though faint patterns of exudation were obtained when the Whatman paper was developed with a color reagent for sugars. Patterns of exudation regions of the susceptible cultivar were similar to those of the resistant ones.

Exudate patterns of seedlings that appeared to be contaminated after incubation did not differ obviously from those that were non-contaminated. Exudation from the germinating seeds was only evident, when the seed-coats had not been removed before incubation.

Discussion

The almost complete inhibition of germination of chlamydospores of *Fusarium oxysporum* f. sp. *pisi* formed in non-sterile NOP soil, was partly overcome in the rhizosphere of seedlings and mature plants of the susceptible 'Rondo' and the resistant 'Rovar' pea cultivar and in the rhizosphere of red clover seedlings.

This non-specific phenomenon has been described for a number of other soilborne pathogens (Schroth and Hildebrand, 1964).

The percentage of germinating chlamydospores was highest in the rhizosphere of actively growing root tissue, namely along root tips, and along the base of the main root of seedlings germinated and grown for 6 days on Hoagland solution and incubated thereafter for 24 h in chlamydospore soil. With these seedlings secondary roots were about to start their development from the base of the main root during the incubation in chlamydospore soil.

The greater amount of ninhydrin-positive substances and of reducing sugars exuded from the active root tissue, may be responsible for the differences in percentage of germinating chlamydospores, but does not exclude an important role of other organic substances released from these sites of the root. A similar correlation between germination of chlamydospores and root exudates was reported by Schroth and Snyder (1961) for *Fusarium solani* f. sp. *phaseoli* in the rhizosphere of beans.

No significant differences in percentages of germinating chlamydospores were observed in the rhizosphere soil and on the root surface between homologous parts of roots of the susceptible 'Rondo' and resistant 'Rovar' seedlings, except in rhizosphere soil along the base of roots of seedlings grown for 6 days on Hoagland solution (Table 3). A delay in the onset of formation of secondary roots at the root base of susceptible seedlings, might be responsible for the low percentage of germination at the base. The percentage of germinating chlamydospores dit not surpass 8.8 in the rhizosphere of seedlings of both cultivars in one experiment where seedlings were incubated for longer then 24 h up to 10 days in chlamydospore soil. On the root surface of these seedlings, germination reached almost 100% within 3 days. A much higher concentration of amino acids and sugars on the root surface than in the rhizosphere soil may account for this, although other substances exuded in smaller amounts and therefore hardly available in the rhizosphere soil, may be responsible for this phenomenon.

Formation of new chlamydospores and thick-walled mycelia from germ tubes in the rhizosphere and on the root surface of pea seedlings and red clover seedlings indicated that susceptible hosts, but also resistant host plants and non-hosts support the survival of the pathogen in the soil. This phenomenon has been described for *F. solani* f. sp. *phaseoli* by Schroth and Hendrix (1962).

Differences in the behavior of germ tubes and mycelia on the root surface as noticed by Buxton (1957a) and Kommedahl (1966) with susceptible and resistant pea cultivars grown under aseptic conditions, were not observed on roots of seedlings of the 'Rondo' and 'Rovar' cultivars grown in natural soil.

The activities of *F. oxysporum* f. sp. *pisi* as observed in the rhizosphere of the susceptible 'Rondo' and resistant 'Rovar' pea cultivar grown in natural soil, do not support the view that differences in chlamydospore germination, in germ tube- and mycelial growth or in attachment of mycelium to the root surface are factors in the resistance of pea cultivars to this pathogen.

The observed reaction of pea roots to invasion by *F. oxysporum* f. sp. *pisi* race 1 by the formation of cellulose-thickenings and deposition of lignin-like substances, has not been reported before. The cell wall reactions were observed with the susceptible as well as the resistant cultivar. Additional experiments are required to demonstrate a possible qualitative or quantitative differentiation of the cellwall reactions of the susceptible and resistant pea cultivar.

Griffiths and Lim (1964) reported that root hairs of some hosts react to invasion by certain vascular wilt fungi by the deposition of lignin-like substances in the cellulose lamellae and by development of lignitubers around the penetrating hyphae. The results of their investigations suggest a tentative correlation between the hosts reaction to invasion by *Verticillium* sp. and host resistance in the field.

Hijwegen (1963) observed lignification of cortex-parenchyma cells of the hypocotyl in cucumber seedlings of a cultivar resistant to *Cladosporium cucumerinum* when inoculated with this pathogen. Such a lignification could not be observed in a susceptible cultivar inoculated with the fungus.

In recent studies on physiology and biochemistry of plant–pathogen interactions, lignin, among other aromatic compounds has been suspected of being involved in resistant and hypersensitive reactions (Rohringer and Samborski, 1967; Fuchs et al., 1967; Fuchs and de Vries, 1969). In view of these considerations, lignification as a reaction to invasion by a pathogen, deserves further attention as a factor in disease resistance.

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Samenvatting

Kieming van chlamydosporen van Fusarium oxysporum f. sp. pisi ras 1 in de rhizosfeer en binnendringing van het pathogeen in wortels van een vatbaar en een resistent erwteras.

Microconidiën van *Fusarium oxysporum* f. sp. *pisi* ras 1, gevormd in schudcultuur, werden na inbrengen in niet steriele grond afkomstig uit de Noord-Oost Polder (NOP) binnen 3 weken omgevormd tot chlamydosporen (Fig. 1) of verdwenen tengevolge van lysis.

Het percentage kiemende chlamydosporen in de rhizosfeer van erwtezaailingen en rode klaver zaailingen in met het pathogeen zwaar geïnfecteerde grond was het hoogst bij de actief groeiende delen van de wortels (Tabel 2). Uit deze delen van de wortels van in vitro gekweekte zaailingen bleek de hoeveelheid afgescheiden ninhydrinpositieve stoffen en reducerende suikers het grootst te zijn (Fig. 6). Geen significante verschillen konden worden aangetoond tussen het percentage kiemende chlamydosporen in rhizosfeergrond van homologe delen van de wortels van zaailingen en volwassen planten van een vatbare ('Rondo') en een resistente ('Rovar') erwteras in niet

steriele NOP-grond en Eempoldergrond (Tabellen 1, 2, 3 en 4).

Met het lichtmicroscoop kon geen verschil in groei van mycelium van het pathogeen en aanhechting van het mycelium aan het worteloppervlak bij wortels van zaailingen van beide cultivars worden waargenomen. Epidermis- en buitenste cortexcellen van de wortels van beide cultivars reageren op het binnendringen van de kiembuis van de chlamydosporen van het pathogeen met een celluloseverdikking welke later wordt geïnfiltreerd met een lignine-achtige substantie (Fig. 4 en 5). Een selektief effect op de activiteit van het pathogeen in de rhizosfeer, in de epidermis en buitenste cortexcellagen in relatie tot resistentie voor het pathogeen kon derhalve niet worden aangetoond.

De vorming van nieuwe chlamydosporen uit gekiemde chlamydosporen bleek plaats te vinden in de rhizosfeer van de vatbare en resistente erwterassen en in de rhizosfeer van zaailingen van rode klaver.

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