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Chlamydospore formation and lysis of macroconidia of *Fusarium solani* f. *cucurbitae* in chitin-amended soil.

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Abstract

Addition of chitin to soil reduced chlamydospore formation from macroconidia of *F. solani f. cucurbitae* in soil. It also inhibited the lysis of some cells of the macroconidia, which resulted in significantly higher numbers of viable although partly lysed propagules of the pathogen in chitin-amended, than in untreated soil over a period of 6 weeks. Thereafter the numbers of viable propagules dropped below those in untreated soil. This decrease is due to the complete lysis of the macroconidia. It is suggested that the initial inhibition both of macroconidial lysis and of chlamydospore formation is due to inhibition of germination of conidia. The importance of partly lysed macroconidia as survival structures is discussed.

Introduction

Preferential stimulation by soil amendments of a soil microflora antagonistic to soil-borne pathogens has interested many plant pathologists as a possible means of biological control. Mitchell and Alexander (1961 a and b) demonstrated that the addition of chitin to soil, naturally infected with *Fusarium solani* f. *phaseoli* or *F. oxysporum* f. *conglutinans*, significantly decreased the incidence of root rot in bean plants and radish, respectively, meanwhile stimulating a chitinoclastic microflora. A similar effect has been demonstrated for other host-pathogen combinations (Buxton et al., 1965; Khalifa, 1965; Mitchell, 1963). A decrease in disease incidence in most cases correlated with a decrease in population density of the pathogen. In some cases different effects of chitin amendment were found. Peterson et al. (1965) did not succeed in demonstrating any effect of chitin amendment to soil on disease incidence of flax caused by *F. oxysporum* f. *lini*. Maurer and Baker (1964) even noted an increase of bean root rot caused by *F. solani* f. *phaseoli* in chitin-amended soil.

The correlation between the increase in a chitinoclastic microflora after addition of chitin to soil and the specific suppression of fungal pathogens gave rise to the hypothesis that heterolytic mechanisms play an important role in the breakdown of these pathogens (Mitchell, 1963; Mitchell and Alexander, 1962 and 1963).

The hypothesis of heterolysis is supported by the fact that most of the mycolytic actinomycetes and bacteria examined produce chitinase (Lloyd et al., 1965; Mitchell

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and Alexander, 1962). Some of them have been shown to produce chitinase capable of degrading living mycelium (Horikoshi and Iida, 1959; Skujins et al., 1965). Chitinase producing strains of *Pseudomonas* and *Rhizopus*, however, failed to lyse mycelium of *F. oxysporum* nor was mycelium of this pathogen lysed by crude chitinase prepared from cultivated mushroom (Mitchell and Alexander, 1963). Moreover, two bacterial isolates that did not produce extracellular chitinase were able to restore the mycolytic property of soil destroyed by sterilization (Lloyd and Lockwood, 1966). It is still doubtful whether lytic activity of chitinase produced by a chitinoclastic microflora plays any role in decreasing the population density of pathogenic fungi in chitin-amended soil. It is still uncertain what other mechanisms act in controlling fungal pathogens in chitin-amended soil.

The present study deals with the fate of macroconidia of *Fusarium solani* f. *cucurbitae* in soil with and without chitin amendment. Lysis and chlamydospore formation from macroconidia in the soils has been observed by light microscopy while simultaneously changes in population density of the pathogen have been studied by agar plate counts. A brief report of some of these results has been published earlier (Van Vuurden and Schippers, 1971).

Materials and methods

The soil. Chitin amendments. Two soils were used: 'Lienden sandy loam' was collected at the experimental agricultural fields of the Institute of Phytopathological Research (IPO), Wageningen, near Lienden, and 'Vleuten sandy loam' was collected in a greenhouse at 'Vleuten's Proeftuin' at Vleuten.

Characteristics of the soils used:

	Lienden soil	Vleuten soil	
organic matter	4.1%	5%	
$CaCO_3$	6.6%	7.9%	
soluble salts	0.24 mmho/cm	0.82 mmho/cm	soil: water ratio $^1/_5$
pH	7.4	7.7	
P_2O_5	110 ppm	32 ppm	soil: water ratio $^1/_5$
K_2O	140 ppm	125 ppm	soil: water ratio $^1/_5$
MgO	119 ppm	409 ppm	soil: water ratio $^1/_5$
sand > 1.0 mm	0.1%	0.3%	
sand 0.2 –1.0 mm	8%	10%	
sand 0.075–0.2 mm	30%	14%	
silt 0.016–0.002 mm	12%	14%	
clay < 0.002 mm	15%	20%	

The soils were passed first through a 2-mm, then a 1-mm-mesh stainless steel sieve, mixed thoroughly by rotating and 50 g of each was transferred to 225-ml beakers. Samples of 50 g were either left untreated, or mixed thoroughly with 0.5 g chitin

(BDH). The beakers were then covered with polyethylene sheet which allowed CO_2 and O_2 diffusion but inhibited moisture changes.

The pathogen. Soil inoculation techniques. A suspension of macroconidia of a Dutch strain (Schippers and Snyder, 1967) of *Fusarium solani* (Mart.) Appel & Wr. f. *cucurbitae* Snyder & Hansen was obtained by gently washing macroconidia from 7-day-old monospore cultures grown on potato-dextrose agar (PDA). The macroconidia were then washed by centrifuging twice in distilled tap water. Seven days after chitin addition, a suspension of these macroconidia in distilled water was evenly distributed through soil with an atomizer to reach a final concentration of 10^6 spores/g moist soil.

Soil moisture content was 30–33 % on a dry-weight basis in all experiments. Soils were stored in the beakers in an incubator at 20 °C. Experiments were done in duplicate and repeated at least three times.

Assessment of the pathogen population in soil. Plate counts. The numbers of viable propagules of the pathogen in soil were determined at various intervals over two months by agar plate counts: samples of 0.5 g were composed by mixing 10 random aliquots of 50 mg. They were suspended in 10 ml tap water on a magnetic stirrer at high speed for 10 min (1:20 suspension). Subsequently, 0.2 ml was pipetted during slowly stirring the soil suspension and diluted $\times$ 400. Of this final suspension, 0.2 ml containing approximately 0.25×10^{-4} g of soil was spread over plates with modified Fusarium selective agar medium (Papavizas, 1967). Platings were done in 10-fold. Numbers of viable propagules in soil are presented as the mean number of colonies of the pathogen obtained from 0.25×10^{-4}g of soil, which is also the mean number of colonies per plate.

Microscopic examination of soil smear preparations. Counts of morphologically distinguishable propagules derived from macroconidia in soil were made at various intervals by microscopic examination of soil smear preparations: 0.2 ml of the 1:20 suspension was evenly spread over 26 $\times$ 40 mm on a slide and dried. After removal of the larger mineral fragments, the smears were stained with acid fuchsin and covered with a cover slip. Each time, 4 soil smears of a chitin-amended and 4 of non-amended soil were examined at $\times$ 1000 magn., 2 per beaker. Final counts are presented as the average number of propagules seen in 0.25×10^{-4}g of soil, for comparison with the plate counts.

The pH of chitin-amended and non-amended soil was equal on day 0, 1, 2, 5 and 14 after introduction of the macroconidia, being 7.2.

Results

Influence of chitin amendments on numbers of viable propagules of the pathogen in soil as determined by plate counts

Fig. 1 gives a representative example of the changes in numbers of viable (colony-producing) propagules of the pathogen in chitin-amended and non-amended soil over a period of 70 days after the introduction of macroconidia. In both soils, num-

Fig. 1. Changes in the total numbers of viable propagules of *F. solani* f. *cucurbitae* after introduction of macroconidia of the pathogen into chitin-amended and non-amended 'Vleuten' soil determined by agar plate counts. Macroconidia were introduced in both soils at day O, one week after addition of chitin to soil. Changes in the number of propagules have been distinghuished in 5 periods (I–V).

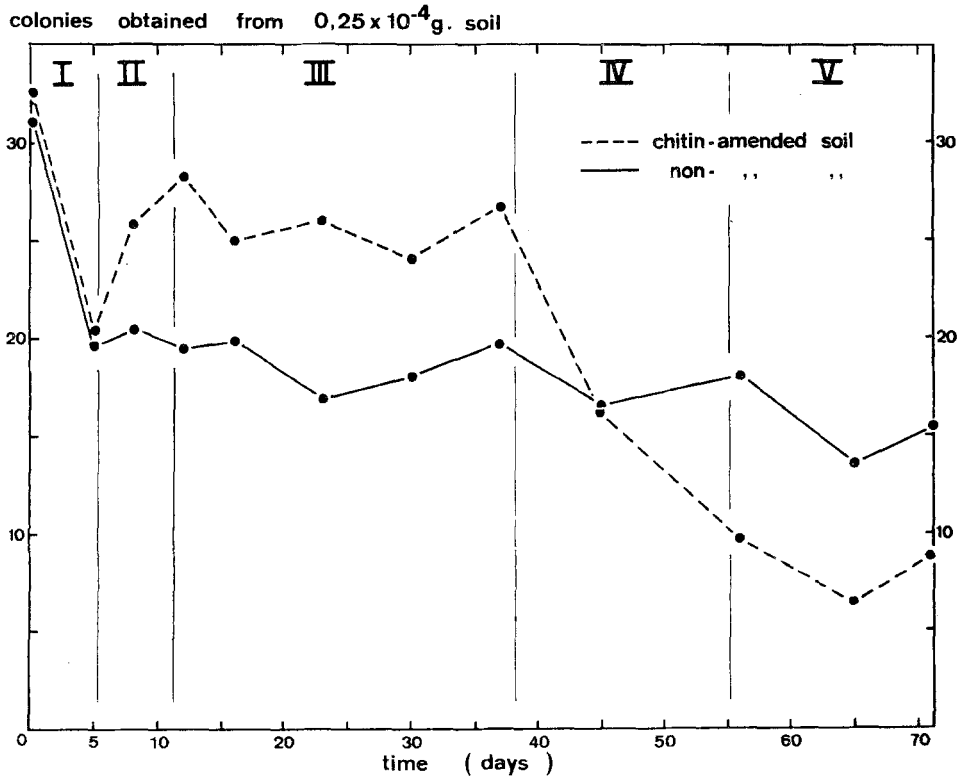

Fig. 1. Veranderingen in het totaal aantal kiemkrachtige sporen van F. solani f. cucurbitae na toedienen van macroconidiën aan chitine behandelde en onbehandelde 'Vleuten' grond, bepaald met agar-telplaten. Macroconidiën werden aan grond toegevoegd op dag O, 1 week na toedienen van chitine aan grond. Veranderingen in het aantal sporen werden onderscheiden in 5 perioden (I–V).

bers of propagules dropped from about 32 (100%) per 0.25 $\times$ 10^{-4}g soil to 20 (65%) within 5 days (Period I). In non-amended soil, numbers slowly decreased thereafter to about 15 (50%) at the end of the incubation period. In chitin-amended soil, however, numbers increased again to about 27 (90%) at day 12 (Period II). Numbers stayed at this level for about 30 days (Period III), and decreased rapidly during the following 25 days (Period IV) to 10 (30%). Thereafter they slowly decreased parallel to those in non-amended soil to about 8 (25%) during Period V. During Period III, numbers of viable propagules in chitin-amended soil stayed 30% above, then during Period IV, dropped to about 50% below those in non-amended soil (differences are significant, according to Wilcoxon's two sample test; $\alpha < 0.01$). In some replicate experiments, this decrease in Period IV in numbers of viable propagules in chitin-amended soils started one week earlier. Similar results were obtained with both 'Lienden'- and 'Vleuten' soils.

Influence of chitin amendments on lysis and chlamydospore formation determined by microscopic examination of soil smear preparations

Five types of propagules derived form macroconidia added to soils were distinguished (Fig. 2): non-lysed conidia (A); partly lysed conidia (B) i.e. macroconidia of which some cells had lost their contents and may have completely disappeared; non- or partly lysed conidia containing chlamydospores (C); non- or partly lysed conidia that have germinated and formed chlamydospores outside conidia at the end of a short germ tube or directly adjacent to a conidial cell (D); one- or more-celled loose chlamydospores (E).

To compare the course of lysis of conidia and formation of chlamydospores in amended with that in non-amended soils, average numbers of propagules from group A and B were bulked and considered as 'macroconidial cells', and those from C, D and E as 'chlamydospore-like structures'. The course of lysis of conidia and of chlamydospore formation until 38 days after introduction of macroconidia in soil is presented in Fig. 3, while Table 1 presents the percentages of the different types of propagules formed in soils and also the total numbers of all propagules per g soil. In duplicate experiments the course of lysis and chlamydospore formation was determined from the 15th day on until the 75th day after introduction of the macroconidia in soils. Representative data of these experiments are given in Fig. 4. In non-amended soil, numbers of chlamydospore-like structures seen in 0.25×10^{-4}g of soil increased to 18 (85%) within 12 days after addition of macroconidia to soil (Fig. 3, Table 1). In chitin-amended soil, however, numbers by then have risen to 10 (33%) only, about half

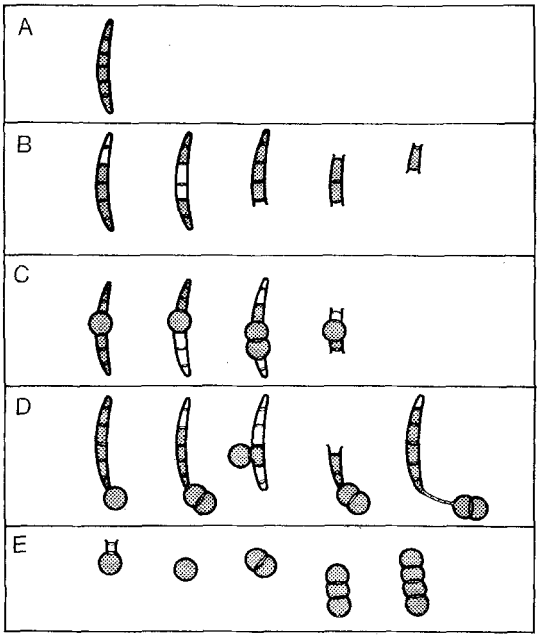

Fig. 2. Different types of propagules of *F. solani* f. *cucurbitae* as distinguished after introduction of macroconidia of the pathogen in soil.
Macroconidial cells: A. Non-lysed conidium; B. Partly lysed conidia.
Chlamydospore-like cells: C. Chlamydospores inside conidia; D. Chlamydospores outside conidia; E. Loose chlamydospores.

Fig. 2. Verschillende typen sporen van F. solani f. cucurbitae onderscheiden na introductie van de macroconidiën van het pathogeen in grond.
Macroconidium-achtige cellen: A. Nietgelyseerd conidium; B. Deels gelyseerde conidia.
Chlamydospore-achtige cellen: C. Chlamydosporen binnen conidia; D. Chlamydosporen buiten conidia; E. Losse chlamydosporen.

Fig. 3 and 4. Changes in the total numbers of macroconidial cells and chlamydospore-like structures of *F. solani* f. *cucurbitae* seen in chitin-amended and non-amended 'Vleuten' soil by microscopic examination of soil smear preparations. Macroconidia were introduced at day O, one week after addition of chitin to soil. Data in Fig. 3 and 4 were obtained from 2 different experiments.

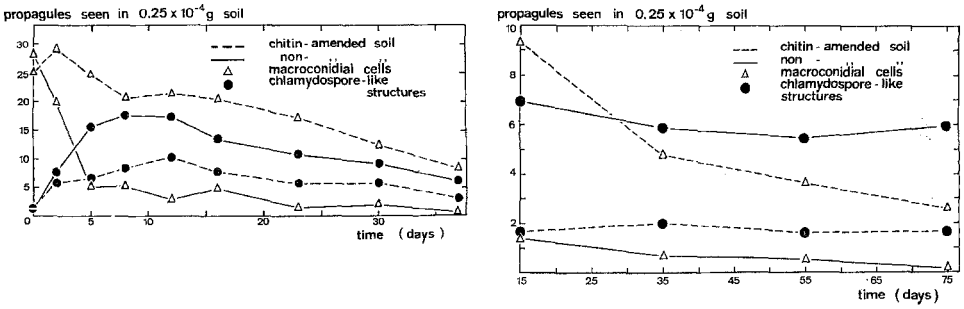

Fig. 3 en 4. Veranderingen in het totaal aantal macroconidium-achtige cellen en chlamydosporen-achtige structuren van F. solani f. cucurbitae waargenomen in chitine behandelde en onbehandelde 'Vleuten' grond door microscopische analyse van grond-uitstrijkpreparaten. Macroconidiën werden aan grond toegevoegd op dag O, 1 week na toedienen van chitine aan grond. De gegevens in Fig. 3 en 4 zijn afkomstig uit verschillende experimenten.

of those in non-amended soil. In contrast, numbers of macroconidial cells rapidly decreased in non-amended soil from 28 to 4 (15%), whilst in chitin-amended soil they stayed at 22 (67%) after 12 days of incubation. Numbers of macroconidial cells and of chlamydospores slowly decreased in parallel in both soils during the next 25 days. In both soils, macroconidial cells were then mainly represented by partly lysed macroconidia (Fig. 2, B), most of which were two-celled, while chlamydospore-like structures were mainly represented by loose chlamydospores with one, two, or more cells. The decrease of total numbers of viable propagules in chitin-amended soil during Period IV and V (Fig. 1) seems to be mainly due to a decrease in the numbers of partly lysed conidia as shown in Table 1. Partly lysed conidia (B) drop from 62% after 45 days, to 35% of the total numbers of propagules, after 71 days of incubation.

A similar course of events is demonstrated with data of the duplicate experiments presented in Fig. 4. The decrease of partly lysed structures in these experiments, however, starts earlier.

It is more difficult to explain the sudden decrease during Period I and increase during Period II of viable propagules in chitin-amended soil (Fig. 1). The decrease was thought to be due to complete lysis of part of the macroconidia and the increase to a splitting up of partly lysed macroconidia into two- or more separate macroconidial cells. There is, however, no sudden decrease or increase of the total numbers of propagules of the pathogen seen in soil-smear preparations as can be calculated from Fig. 3 en Table 1. Similar results were obtained with both 'Lienden'- and 'Vleuten'-soil.

Table 1. Percentage of different types of propagules (A–E, Fig. 2) of the mean total number of propagules of *F. solani* f. *cucurbitae* seen in 1 g soil by microscopic examination of soil smear preparations of chitin-amended (+) and non-amended (−) soil. Soil smear preparations were made at intervals after introduction of macroconidia to the soils at day O = one week after the addition of chitin.

Days after introduction of macr. in soil	Different types of propagules(%)														Total number	
	A		B		C		D		E		A+B		C+D+E		A+B+C+D+E[1]	
	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
0	86	86	11	10	–	–	–	–	3	4	97	96	3	4	118	106
2	44	24	27	59	4	4	15	8	10	5	71	83	29	17	111	140
5	6	2	18	77	5	3	27	11	44	7	24	79	76	21	83	125
8	0	1	23	70	4	9	10	7	63	13	23	71	77	29	92	117
12	–	–	15	67	2	2	6	12	77	19	15	67	85	33	82	128
16	–	–	27	73	1	2	15	9	57	16	27	73	73	27	74	113
23	–	–	12	75	1	3	15	8	72	14	12	75	88	25	49	95
30	–	–	18	68	2	3	7	7	73	22	18	68	82	32	45	73
37	–	–	7	75	1	0	10	9	82	16	7	75	93	25	30	44
45	–	–	6	60	–	–	10	10	84	30	6	60	94	40	35	35
56	–	–	12	40	–	–	14	12	74	48	12	40	88	60	32	18
71	–	–	7	35	–	–	11	16	82	49	7	35	93	65	29	16

[1] Figures represent the mean total numbers of propagules × 10^4 seen in 1 g soil.

Tabel 1. Het percentage verschillende typen propagula (A-E, Fig. 2) van het gemiddelde totaal aantal propagula van F. solani f. cucurbitae in 1 g grond waargenomen door microscopische analyse van grond-uitstrijkpreparaten, die met tussenpozen werden gemaakt na toedienen van macroconidiën aan grond op dag O, 1 week na chitine-toediening aan grond.

Discussion

The experiments clearly demonstrate that complete lysis of macroconidia is delayed and formation of chlamydospores is strongly inhibited in chitin-amended soil. During the first 6 weeks of incubation this results in significantly higher numbers of viable propagules of the pathogen in this soil than in non-amended soil. During this period, most propagules in chitin-amended soil are partly lysed macroconidia, in non-amended soil, however, they are mostly chlamydospores. The partly lysed macroconidia, however, do not seem to be as persistent as the chlamydospores. Their numbers rapidly decrease during the seventh and eighth week, resulting in half as many viable propagules in chitin-amended as in non-amended soil. Some of the partly lysed macroconidia stay viable for much longer periods. Their importance for survival in soil besides chlamydospores, therefore should not be ignored.

There is ample evidence that addition of chitin to soils stimulates the development of a chitinoclastic microflora (Mitchell and Alexander, 1961a and 1963). For the 'Vleuten'soil a significant increase in numbers of chitinoclastic bacteria (30 ×) and of actinomycetes (5 ×) within one week after chitin-addition has been demonstrated in plate counts.

Thus, an increase in a chitinoclastic microflora does apparently not promote lysis of the macroconidia. On the contrary, lysis initially is less rapid in chitin-amended than in non-amended soil, supporting the view that autolysis rather than heterolysis is the main mechanism responsible for mycolysis in soil (Ko and Lockwood, 1970).

The lower concentration in chitin-amended soil of viable propagules after 10 weeks therefore has to be ascribed to the inhibition of chlamydospore formation.

Formation of chlamydospore inside the macroconidium is rather unimportant in these experiments and about equal in both soils (Table 1C). Most chlamydospores are formed after germination of the macroconidium, directly adjacent to it or from short germ tubes. Numbers of this type of propagule are about twice as high in non-amended as in chitin-amended soil 2 and 5 days after inoculation (Table 1D). The numbers of these structures observed in both soils are, however, only an estimate of the real numbers that have been formed because germ tubes are lysed soon after their formation. Low numbers of chlamydospores produced in chitin-amended soil are therefore likely to be due to a stronger inhibition of germination in this soil than in non-amended soil. Also the inhibition of complete lysis of macroconidia in chitin-amended soil may be due to inhibition of germination, as germinated spores are more rapidly lysed than non-germinated ones.

Hora and Baker (1970) noted that frequency of germination of spores of various fungi was much lower in alkaline than in acid or neutral soils. A sharp decrease in germination was demonstrated over the range of pH 7 to 7.5. Inhibition of germination in chitin-amended soil in our experiments can not be ascribed to the pH, because the pH was equal in both chitin-amended and untreated soil.

Soluble carbohydrates are necessary for germination of propagules of *F. solani* f. *phaseoli* (Griffin, 1964). Lignin – chitin amendments increased the rate of utilization of glucose added to soil, in comparison with untreated soil (Benson and Baker, 1970). Chitin-amendment decreases the C/N ratio in soil. The inhibition of germination in our chitin-amended soils therefore may be due to competition by other micro-organisms for simple carbon compounds. Lower germination in its turn may account for less lysis of macroconidia and less formation of chlamydospores. A volatile factor has been shown to inhibit spore germination of various fungi, particularly in alkaline clay loam (Hora and Baker, 1970). Chitin-amended soil has a marked characteristic actinomycete-like smell, which suggests that more volatile compounds are present in such treated than in normal soil which is comparatively odourless. Activity of volatiles will have to be considered as an alternative or supplementary explanation for germination inhibition and its consequences for lysis and chlamydospore formation in chitin-amended soil.

Samenvatting

De invloed van de toevoeging van chitine aan grond op de vorming van chlamydosporen en de lysis van macroconidiën van Fusarium solani f. cucurbitae

Toevoeging van chitine aan grond verminderde de vorming van chlamydosporen uit macroconidiën van *Fusarium solani* f. *cucurbitae*. Eveneens werd de volledige afbraak van de macroconidiën geremd, waardoor veel gedeeltelijk gelyseerde, veelal tweecellige structuren ontstonden. Dit leidde tot een significant hogere concentratie

kiemkrachtige sporen van het pathogeen gedurende de eerste 6 weken na introductie van de macroconidiën in grond. Na deze periode nam het aantal gedeeltelijk gelyseerde macroconidiën snel af, als gevolg van voortgaande lysis, waardoor het totaal aantal kiemkrachtige propagula in chitine-grond na ongeveer 2 maanden, significant lager werd dan in grond zonder chitine-toevoeging. De geringere chlamydosporenvorming en de geringere lysis in chitine-grond lijkt het gevolg te zijn van de kiemremming van de macroconidiën. Deze kiemremming kan zijn veroorzaakt door de verlaging van de C/N verhouding in de grond. Een toename van kiemremmende vluchtige stoffen door afbraak van chitine is misschien mede van betekenis. Naast chlamydosporen spelen ook gedeeltelijk gelyseerde macroconidiën een zekere rol bij de overleving van het pathogeen in grond.

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