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Effects of temperature on *Phytophthora porri in vitro, in planta*, and in soil

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Abstract

Cardinal temperatures for mycelial growth of *Phytophthora porri* on corn-meal agar were <5 (minimum), 15–20 (optimum) and just above 25 °C (maximum). The number of infections after zoospore inoculation of young leaf plants was relatively low at supra-optimal temperatures, but was not affected by sub-optimal temperatures. Even at 0 °C plants were infected. The incubation periods needed for symptom formation were 36–57 d at 0 °C, 13–18 d at 5 °C, and 4–11 d at >11 °C, and were fitted to temperature between 0 and 24 °C with a hyperbolical model (1/p = 0.00812*T + 0.0243). Oospore germination, reported for the first time for *P. porri*, was strongly reduced after 5 h at 45 °C, and totally absent after 5 h at 55 °C. Soil solarization for six weeks during an exceptionally warm period in May-June 1992 in The Netherlands raised the soil temperature at 5 cm depth for 17 h above 45 °C, but did not reduce the initial level of disease in August significantly.

Introduction

Phytophthora porri Foister is a serious disease of winter leek in Europe (Smilde et al., submitted). The disease causes white, lozenge-shaped local lesions of ca. 3×5 cm cm on leaves, sometimes surrounded by a green water-logged zone. Sporangia are formed readily in water-logged lesions under wet conditions, and may release 10–30 zoospores. Epidemics may destroy the crop before January-April, when winter leek is harvested. The fungus is homothallic. Oospores, and possibly chlamydospores which are very similar in shape, formed in infected leaves, may enter the soil with leaf debris and survive the crop-free period from February till July, and probably longer. In autumn, some oospores may germinate to form immediately sporangia, which may release zoospores. The zoospores may be transported to leek leaves by rain splash, thus triggering a new epidemic.

Species of *Phytophthora* may be seen as groups of isolates, brought together by taxonomic conventions, but not necessarily separated from other species by natural barriers (Waterhouse, 1983). Non-morphological characters such as host specialization, cardinal (minimum, optimum, and maximum) or lethal temperatures, are not considered to be important for the definition of species. Yet these characters are ecologically relevant and may provide important clues for disease control.

Cardinal temperatures for mycelial growth *in vitro* of *P. porri* were determined by several authors (Table 1). In real life, however, the fungus is growing in living tissue, so it is interesting to compare the temperature effects on growth *in vitro* and development *in planta*.

Lethal temperatures of several *Phytophthora* species have been determined in various studies, mainly in relation to disease control based on thermotherapy (Table 2). Temperatures associated with solar heating of soil in subtropical climates (Juarez-Palacios, 1991) or composting (Bollen, 1985) appeared to be lethal to several *Phytophthora* spp. The most heat resistant structure of *P. capsici* was the oospore (Bollen et al., 1989). We assume that the same is true for *P. porri*.

Table 1. Minimum, optimum and maximum temperatures (°C) reported for *in vitro* growth of *P. porri* isolates from various countries and host plants

Reference	Temperature			Country[1]	Host
	Min	Opt	Max		
Foister, 1931	<8	<25	<35	UK	Leek
Leonian, 1934			27	UK	Leek
Legge, 1951			27	UK	*Campanula*
Tomlinson, 1951			27	UK	Onion
Waterhouse, 1963	<5	25	33–35	UK	Leek
Van Hoof, 1959	3	15–19	26	NL	Leek
Katsura et al., 1969	0	15–20	27	JA	*Allium* spp.
Noviello et al., 1971	<5	20	27	IT	Onion
Semb, 1971	−3	15–22	25	NO	Cabbage
Geeson, 1976	0	15–20	25	UK	Cabbage
Yokoyama, 1976	<5	15–20	28	JA	*Allium* spp.
Kouyeas, 1977			27	GR	*Gladiolus*
					Carnation
Ho, 1983	0	15–20	31–32	CA	Carrot
Von Maltitz et al., 1983		20		SA	Onion
Kröber, 1985	2	20	23–26	GE	Leek
					Cabbage
Kiewnick, 1985	<5	20	30	GE	Cabbage
Luo et al., 1988	<5	25–30		CA	Leek, Carrot
De Cock et al., 1992	<3	18–21	24–27	various	Leek & others

[1] CA = Canada, GE = Germany, GR = Greece, IT = Italy, JA = Japan, NO = Norway, SA = South Africa, UK = United Kingdom.

Table 2. Comparison of lethal temperatures and corresponding exposure periods for oospores, chlamydospores or mycelium of several *Phytophthora* species

Phytophthora species	Fungal structure[1]	Lethal temp. + exposure time		Optimum growth temp. (°C)	Reference[4]
		(°C)	(min; d)		
P. cinnamomi	c	38	30 min	24–28[3]	a
P. capsici	c	42.5–45	30 min[2]	28[3]	b
P. capsici	o	47.5–50	30 min[2]	28[3]	b
P. cryptogea	o	40–45	30 min[2]	22–24[3]	b
P. infestans	o	40	2 d[2]	20[3]	c
P. megasperma	o	45	30 min	24	d
P. megasperma	o	>45	30 min	30	d
P. cactorum	o	45	30 min	25[3]	d
P. fragariae	o	30	40 d	20–22[3]	e
P. porri	m	45	10 min	15–20	f
P. porri	m	40	20 min	15–20	f
P. porri	m	25	90 d	15–20	f

[1] c = chlamydospore, o = oospore, m = mycelium
[2] no other exposure times tested
[3] data from Waterhouse (1963)
[4] a = Barbercheck and von Broembsen, 1986; b = Bollen, 1985; c = Drenth et al., 1995; d = Juarez-Palacios et al., 1991; e = Duncan, 1985; f = Yokoyama, 1976.

The aims of this study were, first, to study the effect of temperature on *P. porri* growth *in vitro* and development *in planta*. For measurement of *in planta* development a new inoculation method was developed, and the results of the *in planta* development experiment are described by a degree-day model which may be applied to field data. Secondly, the prospects for thermotherapy were explored by determining the lethal temperature for *P. porri* oospores *in vitro* and in a field experiment. As no germination of *P. porri* oospores has been reported in earlier studies, a new germination assay had to be developed for the *in vitro* test. In the field experiment, an attempt was made to eliminate natural inoculum in soil by soil solarization.

Materials and methods

General

Origin of isolates. All four isolates were obtained from commercial leek crops in The Netherlands. Isolate 1 originates from Gelderland (Lienden), 1991; isolate 2 originates from Noord-Brabant (Rijsbergen), 1987, and is stored at the Centraal Bureau voor Schimmelculture as CBS 141.87; isolate 3 and 4 originate from Limburg (Blerick and Horst-Meterik, respectively), 1992.

Isolation, identification and maintenance of P. porri. Isolates were obtained from light-green, water-logged margins of fresh lesions. Excised leaf pieces were rinsed for 2 min in 1% NaOCl and cut into pieces of 1×0.5 cm which were placed on Petri-dishes with cornmeal agar (17 $g.L^{-1}$; Oxoid, code CM103), amended with ca. 200 ppm Vancomycin (Tsao, 1983) at 15 °C. The fungus was identified using the most recent morphological key (Stamps et al., 1990). The fungus was subcultured at 15 °C in Petri-dishes on 1.2% agar (Oxoid Technical Agar No. 3) mixed with 20% leek extract, or occasionally on 1.2% agar mixed with 20% V8 broth. Leek extract was made from 200 g leaf tissue, mixed and boiled with 10 g saccharose in 1 L demineralized water, and then sieved through a plastic sieve (mesh width 0.5 mm).

Mass production of sporangia. Fresh axenic sporangia were obtained by growing the fungus on ca. 30 agar pieces of 5 mm^2 in diluted (2%) leek extract in Petri dishes, with notches to improve aeration, at 15 °C in the dark. The leek extract was decanted after 2–3 d, and sterile soil-extract was added. Sporangia developed on fresh mycelium after another 2–3 days. Then, the plates were used for harvesting zoospores or transferred to 4 °C for later zoospore harvesting. Sporangia remained viable for several months at 4 °C.

Sterile soil-extract was made by mixing 500 g steamed Trio 17 peat soil with 1 L demineralized water. The mixture was decanted and sieved. The filtrate was autoclaved the next day. Tap water and materials containing copper were avoided, as traces of copper may inhibit sporangium formation (Ribeiro, 1983).

Inoculation of plantlets by submersion in zoospore suspension. Zoospores were harvested shortly before inoculation by decanting the soil extract and adding cold (4 °C) sterile demineralized water (SDW) to the dishes. Two to three hours later the dishes contained a suspension of 1000–5000 active zoospores per ml. This suspension was decanted and poured into sterilized capped glass bottles which were placed on ice for some hours, before transfer to nonsterile plastic containers ($40 \times 25 \times 15$ cm) and tenfold dilution with SDW of 4 °C. Each container was filled with 4–5 L of the diluted suspension. For inoculation, 3 months old plants (5–7 leaves) were uprooted and their roots were shortly rinsed to remove most of the adhering soil and to check for the absence of root diseases. Thirty to seventy healthy plants were placed horizontally into the container for ca. 20 h, at 15 °C in a dark climate chamber. The plants were completely submerged during incubation.

Oospore germination assay. Oospores were harvested from *P. porri* cultures in a nutrient-rich liquid medium (20% leek extract or 20% V8) in Petri-dishes (⌀ 9 cm) after at least one month of incubation at 18–20 °C. A semi-sterile enzyme mixture (NovoZym; Sigma L-2265; 0.5 % w/v, incubated overnight at 18–22 °C) was used to digest the mycelium and sporangia. NovoZym was removed by three cycles of centrifugation at low speed (3000 rpm), discarding the supernatant and adding SDW (Spielman et al., 1989). Germination was tested in SDW at 20 °C and dimmed artificial light (6 $W.m^{-2}$) in Microtiter plates (Greiner, ⌀ 1 cm wells).

Experiments

Experiment 1. In vitro *growth.* To compare the cardinal temperatures of the Dutch isolates to those given in Table 1, radial growth of two isolates was measured in

Petri-dishes (⌀ 9 cm) with 13 ± 0.1 ml corn-meal agar (CMA), incubated in six dark cabinets with constant temperatures (5, 10, 15, 20, 25, 30 ± 0.2 °C). Fungal colonies grew from agar pieces (⌀ 5 mm) taken from the advancing margin of a *P. porri* colony on CMA, and placed upside down on the agar plates. During the first 24 h Petri dishes were placed at 15 °C to let the fungus grow into CMA. After this first day the Petri dishes were distributed over the five temperature cabinets in six replicates. Radial growth rates were calculated as the mean of four radii measured along two perpendicular lines, divided by the incubation period, including the first day of growth at 15 °C.

Experiment 2. In planta *development.* Repeat 1: On 27 December 1993, 70 leek plantlets were inoculated by submersion at 15 °C and divided over eight rooms with constant temperatures at 0, 5, 11, 14, 18, 22, 24, 30 ± 1 °C. Ten plants per treatment were used. After inoculation the plants were placed horizontally in a plastic container in a closed polythene bag, with wetted cotton plugs covering the root system. At 0 and 5 °C the plantlets were incubated in dark rooms. At ≥11 °C plants were incubated in shadowed glasshouses with natural daylight (20–40 $W.m^{-2}$) for ca. 8 h per day.

Repeat 2: On 17 Jan 1994, 35 plantlets were inoculated at 15 °C and submitted to the same seven incubation treatments. Only five plants per treatment were available.

Repeat 3: On 12 Jan 1995, 18 plants were divided into three groups, which were inoculated by submersion at 0 °C, 5 °C (dark rooms) and 9 °C (glasshouse), and subsequently incubated at these temperatures in the described way. This experiment was designed to prove that not only colonization, but also infection of leaves may occur at low temperatures.

Plants were inspected daily. Lesions were counted as soon as they appeared and marked by a plastic peg to prevent double counts. The total numbers of lesions per leaf, and the longest, shortest and average incubation periods were determined.

Experiment 3. Oospore germination. Repeat 1: Oospores of isolate 1 were harvested as described above from 20% leek-broth cultures that were first incubated at 15–17 °C for five months, and at 4 °C for another five months. The oospores were divided over three temperature treatments. Aliquots of 0.02 ml SDW, containing ca. 280 oospores, were pipetted into wells of three Microtiter plates. Twelve wells per plate were used. A varying volume of additional SDW was pipetted into each well, as the volume of SDW affected the germination rate in preliminary experiments. The volume of additional SDW was 0.3, 0.6 or 0.9 ml. Each SDW treatment was applied to four wells per plate. The three Microtiter plates were incubated at 20, 45 or 55 °C for five hours. Numbers of germinated oospores per well were counted at 100× magnification with a Zeiss Axiovert light microscope.

Repeat 2: Oospores of isolates 3 and 4 were harvested from 20% leek broth cultures incubated at 15–17 °C for four months. Temperature and SDW treatments were as described for repeat 1. Ca. 350 oospores were pipetted into each well. Two wells were used for each temperature × SDW volume treatment.

Experiment 4. Solarization. Four plots (5×5 m) of a field (20×5 m) with natural infestation by *P. porri* were used for a solarization experiment. The field was located at CPRO-DLO, 'De Goor', Wageningen. On May 27, 1992, the soil was drenched to field capacity and in two plots the soil was covered with a transparent polythene sheet. These plots were solarized for six weeks. Temperature at 5 cm depth was measured automatically every hour by three thermocouples at 5 cm depth in a solarized plot and one in a control plot. Soil tillage after solarization was restricted to the topmost 2 cm-layer the soil. The four plots were planted on 7 July 1992 with three months old plants of cv. Carina. In each plot, 180 plants were planted at 12 cm within rows and 50 cm between rows. The plots were separated from each other by placing straw bales on the borders between plots. On 25 August and 18 September, all diseased leaves were counted per plant.

Results

Morphological identification. Phytophthora-isolates from leek were readily identified as *P. porri* by coiling hyphae in solid media. This character distinguishes *P. porri* from all other *Phytophthora* species. The presence of semi-papillate sporangia and of a mixture of paragynous and amphigynous antheridia leads to the same conclusion concerning the species name (Stamps et al., 1990). The sporangia were ellipsoid or ovoid, seldomly obpyriform, noncaducous, with length/breadth ratios of ca. 1.3 and sizes of 45–75 μ. Twin apices, tapered bases, lateral attachments and distorted shapes were noted occasionally. Oospores were formed readily in host and in nutrient-rich culture media, were markedly aplerotic, had thick

Table 3. Experiment 1. Radial growth rates (× 0.01 $mm.d^{-1}$) of 2 isolates of *P. porri* on corn meal agar at 6 temperatures. Means and their standard deviations are given ($n = 6$)

°C	Isolate 1	Isolate 2
5	5.0 ± 0.8	3.1 ± 0.5
10	4.6 ± 0.9	4.5 ± 0.3
15	14.9 ± 2.3	8.6 ± 1.3
20	9.3 ± 4.3	5.8 ± 1.4
25	0.3 ± 0.2	0.4 ± 0.1
30	0	0

walls (2–5 μ) occasionally and varied in size from 25–35 μ. Chains of intercalary hyphal swellings were often observed in water. These characters are in accordance with Stamps's key. Intercalary attached sporangia and aerial sporangiophores on agar, mentioned in the key, were not found. Coralloid hyphal swellings were observed in some isolates, but not mentioned in the key. These coralloid structures survived the digestion of mycelium with NovoZym which was used for cleaning the oospore culture.

Experiment 1. In vitro *growth.* (Table 3) The optimum temperature for *in vitro* growth of *P. porri* was ca. 15 °C for isolates 1 and 2. The temperature minimum is below 5 °C. The variance in growth rates was relatively high at supra-optimal temperatures. The temperature maximum is between 25 °C and 30 °C, and colonies died at 30 °C.

Experiment 2. In planta *development.* (Figure 1) The mean incubation period ranged from 5 d at 24 °C to 43 d at 0 °C. The inverse of the mean incubation period was fitted to the average temperature during the incubation period, including the first day of inoculation at 15 °C for repeats 1 and 2. The equation of the fitted hyperbolic line is given in Figure 1. This equation implies that, on average, a lesion appears at 120 degree-days (base temperature −3.0 °C) after infection.

The time between appearance of the first and last lesion was 2 days at 24 °C and ca. 20 d at 0 °C. In Figure 1 the considerable variation in incubation period is shown by the hyperboles fitted through the observed shortest and longest incubation periods. The shortest incubation period corresponded with on average 92 degree-days, and the longest with 154 degree-days (base temperature −3.0 °C).

The total number of lesions per leaf at the end of the experiment was 1.5–2.5 at temperatures below 18 °C

Table 4. Mean numbers of germinated oospores per well in microtiterplates after temperature treatments of 5 h. Differences between 20 and 45 °C in each column are statistically significant ($P<0.05$)

Isolate	1[1]	3[2]	4[2]
Oospores.well^{-1}	280	360	350
d after inoculation	32	43	43
20 °C	62	62	20
45 °C	29	14	2
55 °C	0	0	1[3]

[1] averaged over 12 wells (4 replicates at 0.3, 0.6 and 0.9 ml SDW)
[2] averaged over 4 wells (2 replicates at 0.6 and 0.9 ml SDW)
[3] short, abortive germination tubes.

(Figure 2). The low temperature during inoculation in repeat 3 did not affect the number of lesions strongly. Temperatures above 18 °C were apparently supra-optimal for lesion development. No lesions appeared at 30 °C. At all temperatures, lesions were typically 1–3 cm long and increased slowly in size. A semi-permanent kind of latent infections was discovered in plants that were incubated at 24 °C for 10 d. When these plants were transferred from 24 °C to 15 °C, new lesions were observed after 6–8 d.

Experiment 3. Oospore germination. Treatment at 45 °C for 5 h reduced germination of all isolates, compared with the control at 20 °C (Table 4). The contrast between 45 °C and control treatment was statistically significant ($P<0.05$, F-test). Treatment at 55 °C prevented germination in both repeats, except for a few abortive germination tubes from oospores of isolate 4.

An inhibitory effect of high water volumes on germination at 20 and 45 °C was found in repeat 1 ($P < 0.01$, F-test). In repeat 2 the control (0.3 ml SDW) failed due to stronger evaporation during incubation.

The first germinated oospore was observed 27 days after incubation in both repeats. The highest numbers of germinated oospores were counted 1–2 weeks later. Later countings were impossible because of mycelium growth and lysis. Oospores often formed a sporangium immediately after germination (Figure 3).

Experiment 4. Solarization. During the solarization treatment the weather was exceptionally warm for Dutch conditions. June 1992 belonged to the warmest six months of June since 1900. In solarized plots, at 5 cm depth, the average daily maximum temperature was 37 °C. The temperature exceeded 40 °C during 59 h,

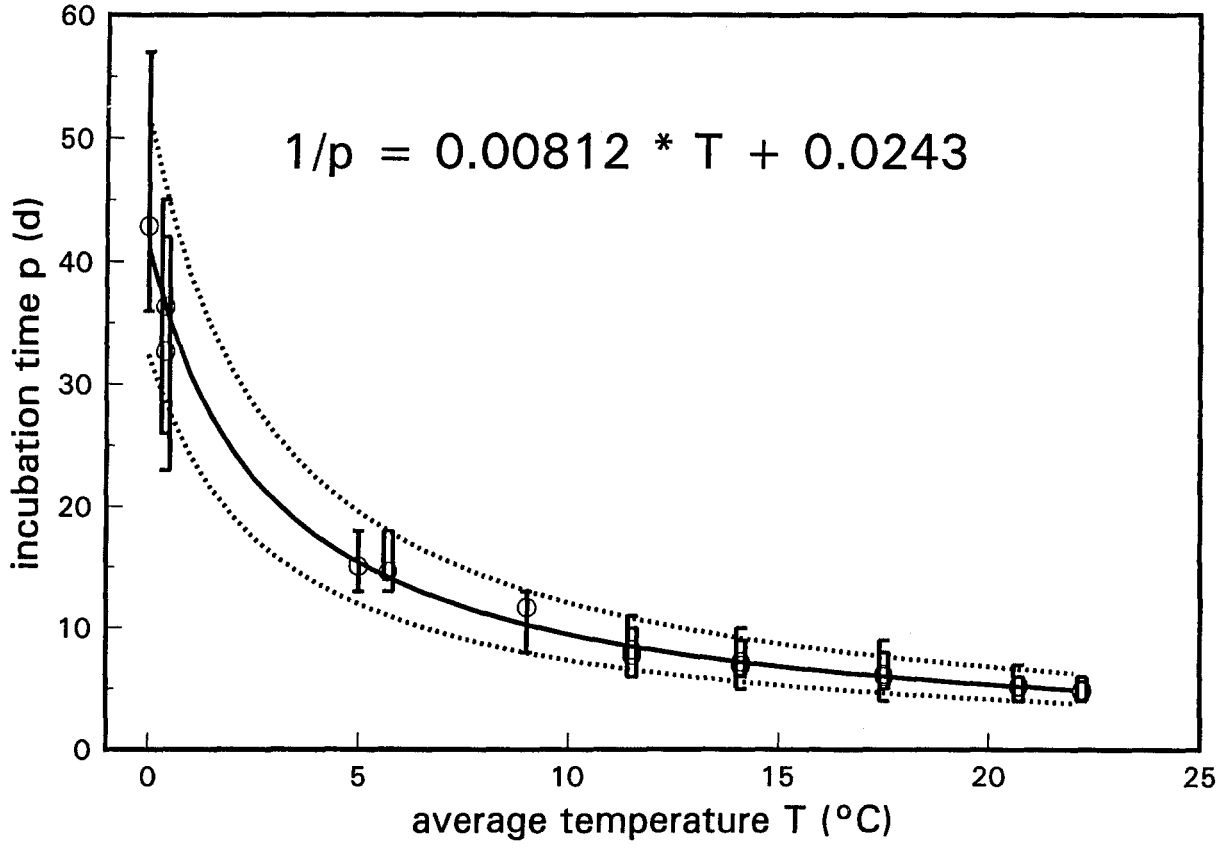

Figure 1. Incubation period (p) of *P. porri* on leek plantlets at various temperatures after submersion-inoculation with zoospores. ○ = mean p, – shortest p, longest p. Vertical lines represent the ranges for p within each experiment. Hyperboles were fitted by means of linear regression of the inverse of p on temperature. __ mean p, ... shortest p, longest p. Mean p as a function of temperature T is given above the graphs.

45 °C during 17 h, and never exceeded 48 °C. In control plots the average daily maximum was 28 °C and never exceeded 34 °C. The average number of diseased leaves was 0.6 and 1.4 in solarized and control plots, respectively. The solarization effect was, however, not statistically significant ($0.1 > P > 0.15$), which may be partially ascribed to the low number of degrees of freedom. The weak solarization effect had disappeared 3 weeks later, when the number of diseased leaves was 2.5 and 2.2 in solarized and control plots, respectively.

Discussion

The maximum temperature for mycelial growth of *P. porri* mentioned by Foister (1931) in his original description of the fungus was 'below 35 °C'. Leonian (1934), using Foister's isolate, published a more precise maximum of 27 °, which was confirmed by several authors (Table 1) and in the present work, but not by Waterhouse (1963) and Waterhouse et al. (1983) after examining 'authentic material', and Ribeiro (1978), relying on Waterhouse's data. Kouyeas (1977) and Ho (1983) pointed out that Waterhouse's isolate is an extreme variant. De Cock et al. (1992) suggest that aberrant isolates do not belong to *P. porri* sensu stricto, as he showed that a *Phytophthora* isolate from leek, morphologically similar to *P. porri* but with higher cardinal temperatures, had mtDNA restriction patterns which were similar to *P. nicotiana*, a species with relatively high cardinal temperatures. The *P. porri* isolate from carrot described by Ho (1983) with maximum temperature above 27 °C also had a mtDNA restriction pattern that differed from *P. porri sensu stricto*.

The optimum temperature for *in vitro* growth of *P. porri* is between 15 and 20 °C according to most authors listed in Table 1. This is confirmed in the present work. The *in planta* development rate seems to increase with temperature, possibly even beyond 24 °C, which was the highest temperature used in this

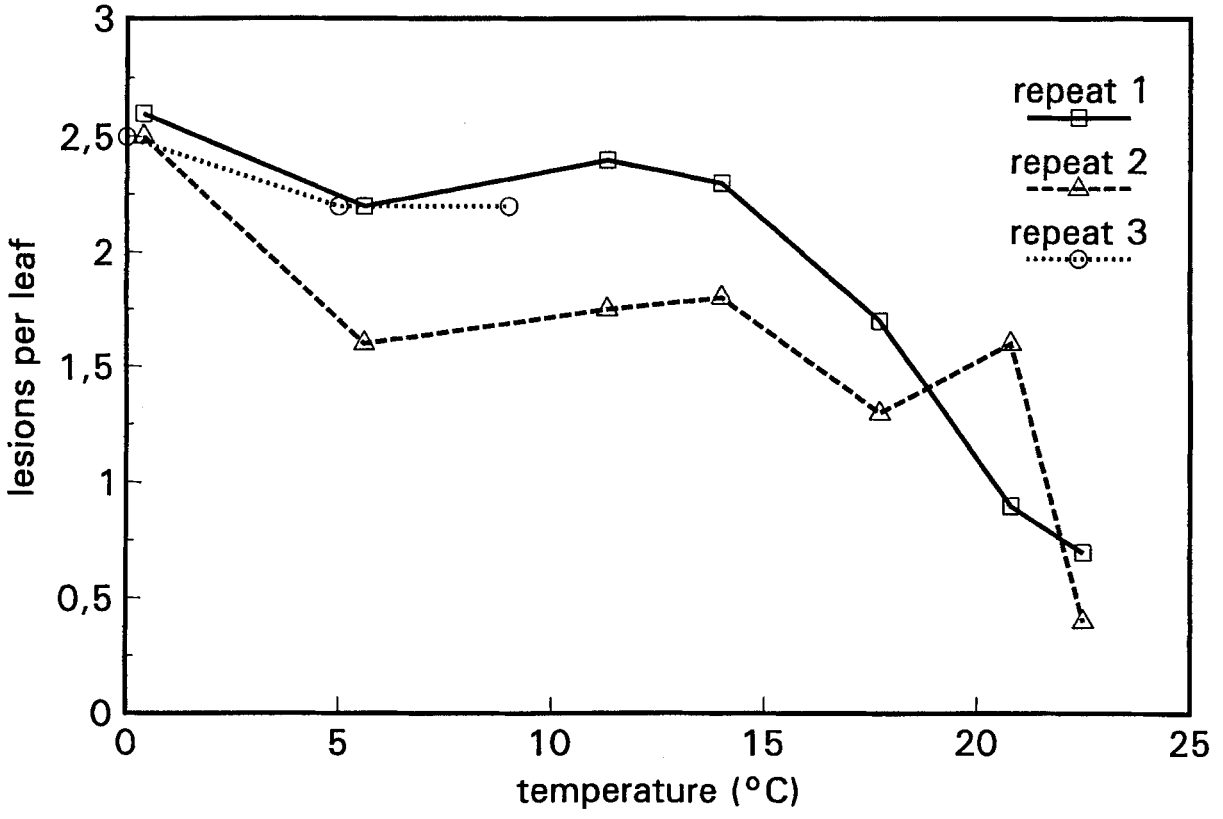

Figure 2. Total number of lesions per leaf developing after submersion-inoculation of leek plantlets, followed by incubation at various temperatures.

study. However, the number of lesions in inoculated plantlets appears to be relatively low at temperatures above 20 °C. It may be speculated that at temperatures above 20 °C young *P. porri* colonies may have two different fates, if they do not die: they either develop very fast or they remain latent until lower temperatures return. A similar kind of semi-permanent latent infection was reported by Sutton (1989) for *Botrytis squamosa* on onion.

The extrapolated minimum temperature of *P. porri* development *in planta* is −3 °C, which is in accordance with Semb (1971). It may be assumed that ice formation limits the infection capacity of zoospores, and therefore the minimum temperature for infection is probably 0 °C. Remarkably, the infection capacity of zoospores is not reduced at 0 °C, as was convincingly shown in experiment 2, repeat 3.

Low temperatures apparently slow down infection and colonization in a regular, predictable way, in spite of the relatively large variation of incubation periods at each treatment. To develop the degree-day model, it was assumed that light conditions have no effect on the development rate. This assumption remains to be proven. The base temperature for adult leek plant development is 0 °C (Hay and Brown, 1988). Leek leaves appear at intervals of 132 degree-days. Thus, the development rate of the pathogen at low temperatures

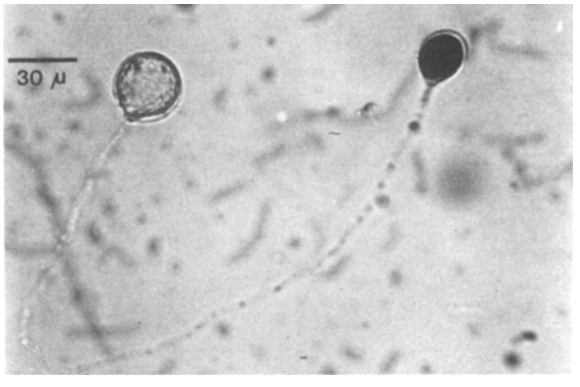

Figure 3. Germinated oospore of *P. porri* with an immature sporangium at the end of the germination tube.

is relatively fast in comparison to the development rate of the crop.

Oospores of *P. porri* did not germinate until they were 4–5 months old, although they were visually mature after 1 month. After the 4–5 month period, they still needed an incubation period of ca. 1 month in the liquid medium, although in some instances (data not shown) spores germinated in 1–2 weeks. These results indicate that oospores may become internally dormant. External dormancy may further regulate a timely germination in the field. It may be speculated that oospore germination was inhibited in the wells with more water, because of reduced aeration of the medium. If this is true, oospores may be activated by rain, as rain will improve the degree of aeration of soil water. Similar effects of aeration have been reported for formation and germination of sporangia of various *Phytophthora* species (Ribeiro, 1983).

The lethal temperature for oospores of *P. porri* is between 45 and 55 °C, which is higher than for mycelium of *P. porri* (Yokoyama, 1976), and comparable to a *P. megasperma* isolate with a temperature optimum at 30 °C (Table 2). This indicates that *P. porri* is adapted to high temperatures for oospore survival, in spite of the relatively low optimum and maximum temperatures for mycelial growth.

P. porri of leek is one of the few soil-borne *Phytophthora* species that do not infect roots. Therefore, the inoculum in the topmost layer of soil is probably indispensable for the initiation of a new infection cycle. Solar heating will mainly affect the top layer. In spite of this, soil solarization is not a useful control option in The Netherlands, because the climate is too cold. Nevertheless, soil-solarization may be useful in a temperate climate if it can be combined with other disinfecting treatments, and if it can be targeted to infested leaf debris, as proposed by Entwistle (1990) in England for white rot of onion. Composting of leaf debris may be a more effective thermotherapy. Composting temperatures are 50–70 °C (Bollen et al., 1989), and may therefore kill oospores of *P. porri*. A more radical solution is mentioned by Foister (1961) who speculates that the recommendation to burn or bury crop debris instead of ploughing it into the land has contributed to the gradual decrease in importance of the disease in Scotland after 1931.

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