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A one-hit model for the infection of clubroot-susceptible cabbage (*Brassica oleracea* var. *capitata*) by *Plasmodiophora brassicae* at various inoculum densities

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Abstract

In two glasshouse and three phytotron experiments, clubroot-susceptible cabbage (*Brassica oleracea* var *capitata*) cv Septa was inoculated with clubroot resting spores at inoculum densities ranging from 0 to $2 \cdot 10^7$ spores·plant^{-1}. At densities of 10^5 spores·plant^{-1} and higher all plants developed clubroot symptoms, except in one glasshouse experiment conducted in winter. The proportion of plants developing symptoms plotted against inoculum density showed a sigmoid curve. Although the shape of the curve was similar in all experiments, the inoculum densities required to induce 50% disease incidence varied from 10^3 to 10^5 resting spores·plant^{-1}. The data of all five experiments could be well described by a generalized one-hit model which involves variation between plants with regard to the probability of infection.

Abbreviations: cv = cultivar; ECD = European Clubroot Differential set.

Introduction

For studies on the control of clubroot, caused by the fungus *Plasmodiophora brassicae* Wor., or on the resistance of various host species to this disease, a reproducible test method is essential. The results of field tests are often difficult to reproduce, due to variation in inoculum density, temperature and soil moisture within and between experiments. Therefore several methods have been devised to inoculate seedlings in a glasshouse environment with resting spores of *P. brassicae*, offering a better control of climate and soil conditions and inoculum source.

Generally, the inoculum for glasshouse tests is prepared by macerating fresh or frozen clubs, the swollen roots characteristic of the disease, in water using an electric blender. The currently used inoculation methods are all variations of the root dip, slurry or pipette methods. In the root dip method [Johnston, 1968], roots of seedlings are dipped in a suspension of resting spores before planting in potting compost. In the slurry method [Toxopeus and Janssen, 1975], a resting spore suspension is mixed with potting compost. The resulting slurry is placed in holes in compost, and seedlings are planted in the slurry. In the pipette method [Lamers and Toxopeus, 1977; Voorrips and Visser, 1993], seeds are sown in potting compost, one seed per pot, and a defined quantity of a resting spore suspension is pipetted in each pot. After inoculation the plants are grown for six to eight weeks at high soil moisture. The symptoms are generally assessed visually and classified in grades. A widely used grading system is described by Buczacki *et al.* [1975].

In most studies rather high inoculum densities have been used. In his review concerning the root dip and slurry methods, Dixon [1976] asserted that the inoculum densities used by different researchers varied from 10^5 to about 10^8 resting spores per plant. Voorrips and Visser [1993] applied $2 \cdot 10^7$ spores per plant using the pipette method, and Robak and Gabrielson [1988] considered 10^5-10^8 spores per plant necessary for consistent results using a similar test method. It has long been

recognized that high inoculum densities are required to ensure consistent symptom development on susceptible plants under varying environmental conditions [e.g. Colhoun, 1958].

This report describes a series of experiments directed to the question as to whether the infection of clubroot-susceptible host plants by spores of *P. brassicae* can be described by a one-hit model, as originally formulated by Ridout *et al.* [1993] to describe infection of insects by viruses. The basic assumptions underlying one-hit models are that pathogen individuals act independently (i.e. without significant interaction during infection and pathogenesis) and that infection by one individual suffices to cause disease symptoms. Although interaction does occur between zoospores during clubroot pathogenesis [Tommerup and Ingram, 1971], the frequency of this interaction is not known. In the experiments described here, inoculations were performed with varying numbers of resting spores per plant. The number of resting spores available per plant appeared to be most precisely controlled with the pipette inoculation method which was therefore utilized in this study.

Materials and methods

Cabbage cv Septa (Bejo Seeds b.v., Warmenhuizen, the Netherlands) was used as clubroot-susceptible host in all experiments. The inoculum used was a *P. brassicae* isolate obtained from an infested field at Brabant Experimental Station in the Netherlands and characterized as ECD 16/3/30 [Buczacki *et al.*, 1975; Voorrips and Visser, 1993]. Resting spore suspensions were prepared from clubs stored at −20 °C for up to 27 months as described by Voorrips and Visser [1993], and used for inoculation on the same day.

Two inoculation experiments (G1 and G2) were performed in a glasshouse as described by Voorrips and Visser [1993] in early spring (G1) and in winter (G2). Daylight was supplemented by 400 W SON-T lamps (45 $\mu E \cdot m^{-2} \cdot s^{-1}$, 14 $h \cdot day^{-1}$). Three similar experiments (P1, P2 and P3) were carried out in a phytotron chamber, at 22 °C, with a photoperiod of 16 h at 110 $\mu E \cdot m^{-2} \cdot s^{-1}$ HPI-T illumination.

Each inoculation experiment consisted of a different series of inoculum densities, always including a control treatment with zero resting spores per pot and a treatment with at least 10^5 resting spores per pot; one seed was sown in each pot. The experiments were laid out in a randomized complete block design with two,

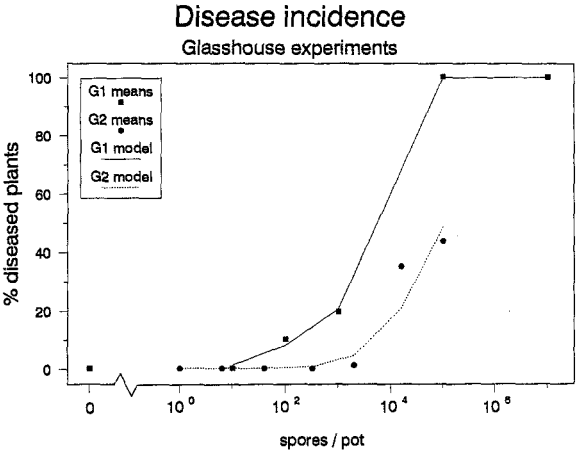

Fig. 1. Observed disease incidence means and values predicted by the generalized one-hit model for the infection of cabbage plants inoculated with varying numbers of resting spores of *P. brassicae* in two glasshouse experiments: G1 (spring) and G2 (winter).

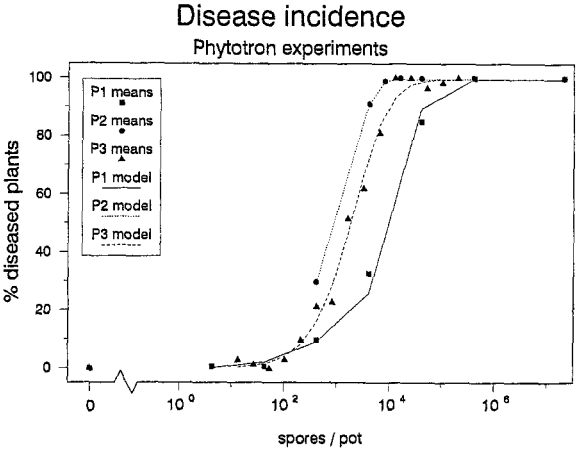

Fig. 2. Observed disease incidence means and values predicted by the generalized one-hit model for the infection of cabbage plants inoculated with varying numbers of resting spores of *P. brassicae* in three phytotron experiments: P1, P2 and P3.

three or four blocks per experiment and one replicate of each treatment per block. Replicates consisted of 42 pots each. Six weeks after inoculation the disease incidence was determined for each replicate as the percentage of well-developed plants showing symptoms, disregarding the variation in disease severity.

The validity of the basic and generalized one-hit model [Ridout *et al.*, 1993] describing the relation between the mean number of inoculated resting spores and the proportion of diseased plants was examined. The Genstat5 command FITNONLINEAR [Genstat 5 Committee, 1987] was used to find parameter values

yielding a minimum residual deviance for each experiment. The difference between the residual deviance of the one-hit models and of a general model without constraints on the mean probability of infection at each inoculum density was used to test the goodness of fit of the one-hit models. This difference of residual deviances follows approximately a χ^2_d distribution, where d is the difference in residual degrees of freedom between the one-hit and the general model [McCullagh and Nelder, 1989].

One of the assumptions implicit in the one-hit models is that resting spores in suspensions can be considered to be randomly distributed, resulting in a Poisson distribution of the number of resting spores inoculated per pot. This was verified in three freshly prepared suspensions by counting the number of resting spores in each of 100 or 200 unit cells of a Thoma haemocytometer. The volume of individual cells was 0.25 nl. The deviation of the counts from a Poisson distribution was tested by the Index of Dispersion test, with $(N-1) \times s^2/\bar{x}$ having approximately a χ^2-distribution with $(N-1)$ degrees of freedom.

Results and discussion

Large between-test variation

The disease incidence treatment means of all inoculation experiments are shown in Figs. 1 and 2. Since no significant block effects were detected, the means were calculated as the total number of diseased plants divided by the total number of plants per treatment. Five replicates with less than 20 well-developed plants were rejected.

Among the experiments a large variation was observed in the incidence at similar inoculum densities (Figs. 1 and 2). The inoculum densities required to induce 50 % disease incidence varied from less than 10^3 resting spores·plant^{-1} in phytotron experiment P2 to approximately 10^5 spores·plant^{-1} in glasshouse experiment G2.

The low disease incidence in experiment G2 was possibly caused in part by more adverse conditions in the glasshouse in winter, even though minimum air and soil temperature were kept at 18 °C and 23 °C respectively, and daylight was supplemented with artificial light. However, probably not all variation between experiments was due to culture conditions. Differences in condition of the inoculum may also have been of importance. Since only small amounts of inoculum were prepared for each experiment, differences between individual clubs may have had a significant effect. This is supported by the fact that the differences between phytotron experiments were comparable to those between the glasshouse experiments.

Random distribution of resting spores in suspensions

If resting spores are randomly distributed in suspensions, as assumed in the one-hit models, the number of spores in a volume of suspension should follow a Poisson distribution. This was investigated using a haemocytometer. In three freshly prepared suspensions the number of resting spores per haemocytometer cell closely followed a Poisson distribution (Table 1). Also, in the freshly prepared suspensions resting spores did not adhere to each other. Therefore, the number of resting spores inoculated per pot using the pipette method may be considered to follow a Poisson distribution.

Validity of the basic one-hit model

The results were used to test the validity of the basic one-hit model, describing the relation between inoculum density and proportion of diseased plants. The expected proportion of infected plants (P_{inf}) is related to the mean number of resting spores inoculated per plant (n) as

$$P_{inf} = 1 - e^{-n \cdot \omega} \qquad (1)$$

where ω is the (constant) probability of a spore infecting the host. This model is based on three assumptions: (i) the actual number of resting spores inoculated per plant follows a Poisson distribution; (ii) the probability that a spore infects the host plant is constant within each block in an experiment, and is not influenced by the inoculum density; and (iii) a plant will develop symptoms when infected by at least one spore. Assumption (i) was verified separately (Table 1). In this simple model, the effects of production and fusion of secondary zoospores and other aspects of the pathogenesis of clubroot are disregarded.

Most of the experiments did not fit the basic one-hit model (Table 2). Only in experiment P2 was a non-significant ($P > 0.05$) residual deviance obtained. In this experiment however, only few data were obtained in the most informative range of inoculum density. In general, the disease incidence appeared to increase more gradually than predicted by the basic one-hit model.

Table 1. Frequency distribution of resting spores of *P. brassicae* in three freshly prepared resting spore suspensions in unit haemocytometer cells, and test for deviation from a Poisson distribution

Suspension		Number of spores per cell															Mean	d.f.[1]	I.D.[2]
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14			
A	obs.[3]	0	0	1	6	14	15	13	12	7	13	7	6	3	3	0	6.97	99	103.4 n.s.
	fit.[3]	0.1	0.7	2.3	5.3	9.2	12.9	15.0	14.9	13.0	10.1	7.0	4.4	2.6	1.4	0.7			
B	obs.	0	4	22	21	32	41	29	26	11	6	3	2	1	2	0	5.16	199	198.6 n.s.
	fit.	1.1	5.9	15.3	26.3	33.9	35.0	30.1	22.2	14.3	8.2	4.2	2.0	0.9	0.3	0.1			
C	obs.	30	68	53	27	17	4	1	0	0	0	0	0	0	0	0	1.74	199	184.5 n.s.
	fit.	34.9	61.0	53.2	30.9	13.5	4.7	1.4	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0			

[1] d.f.: degrees of freedom
[2] I.D.: Index of Dispersion; n.s.: deviation from Poisson distribution not significant at P=0.05
[3] obs. and fit.: observed and fitted frequencies of number of spores per haemocytometer cell (the fitted frequencies were obtained using the sample mean as Poisson parameter)

Table 2. Fit of basic one-hit model in two glasshouse (G1 and G2) and three phytotron (P1-P3) experiments with varying numbers of resting spores of *P. brassicae* inoculated per cabbage plant

Test	ω^1	Degrees of freedom[2]	Residual deviance[2,3]
G1	2.95×10^{-4}	5	15.08 *
G2	7.97×10^{-6}	7	31.48 **
P1	6.44×10^{-5}	7	49.98 **
P2	6.67×10^{-4}	7	3.53 n.s.
P3	3.50×10^{-4}	15	69.61 **

[1] ω: estimate of the proportion of spores successfully infecting the host.
[2] differences of degrees of freedom and of residual deviance between the basic one-hit model and a model without constraints on the expected proportion of diseased plants at each inoculum density
[3] significance of lack of fit: $P \geq 0.05$ (n.s.), $P < 0.05$ (*), $P < 0.01$ (**).

Table 3. Fit of generalized one-hit model in two glasshouse (G1 and G2) and three phytotron (P1-P3) experiments with varying numbers of resting spores of *P. brassicae* inoculated per cabbage plant

Test	ω^1(s.e.)	ψ^1(s.e.)	Degrees freedom[2]	Residual deviance[2,3]
G1	1.07×10^{-4} (6.60×10^{-7})	5.23×10^{-8} (9.76×10^{-9})	4	3.77 n.s.
G2	2.50×10^{-5} (2.47×10^{-8})	8.60×10^{-5} (6.93×10^{-8})	6	12.54 n.s.
P1	5.53×10^{-5} (2.00×10^{-8})	1.40×10^{-8} (4.6×10^{-11})	6	8.56 n.s.
P2	6.15×10^{-4} (8.34×10^{-5})	9.70×10^{-6} (8.56×10^{-6})	6	2.34 n.s.
P3	4.63×10^{-4} (7.50×10^{-6})	2.15×10^{-4} (2.67×10^{-5})	14	21.28 n.s.

[1] ω: estimate of the expected proportion of spores successfully infecting the host. ψ: estimate of magnitude of variation of ω between plants. s.e.: standard error of estimates
[2] differences of degrees of freedom and of residual deviance between the generalized one-hit model and a model without constraints on the expected proportion of diseased plants at each inoculum density
[3] n.s.: non-significant lack of fit ($P \geq 0.05$)

Fitting the generalized one-hit model

In the generalized one-hit model considered here, assumption (ii) of the basic one-hit model was relaxed, to allow variation in susceptibility among host plants. The probability that a host plant is infected by an individual spore was considered to vary between plants according to a Beta distribution [Ridout *et al.*, 1993]. The probability θ_j of a plant being infected when inoculated with j resting spores is given by Ridout *et al.* [1993] as

$$\theta_j = 1 - \frac{\Pi_{i=1}^{j}[1 - \omega + (i-1)\psi]}{\Pi_{i=1}^{j}[1 + (i-1)\omega]}, j = 1, 2, \ldots \quad (2)$$

where ω is the mean probability that a plant is infected by an individual spore and ψ is a measure of the variability of ω between plants. If $\psi = 0$, no variation in susceptibility between plants is present and the basic one-hit model is obtained. If $\psi > 0$, variation in susceptibility occurs, and P_{inf} will increase more slowly with inoculum density than is predicted by the basic one-hit model.

Formula (2) and the Poisson distribution of the number of resting spores inoculated in each pot are not easy to compute for large numbers of spores. The Poisson distribution was therefore approximated by a Normal distribution with the same expectation and variance for expectations ≥ 56. Formula (2) was rewritten as

$$\theta_j = 1 - (1-\omega) \cdot \exp(\sum_{i=2}^{j} \ln[1-\omega+ (i-1)\psi] - \ln[1+(i-1)\psi]) \quad (3)$$

and approximated by

$$\theta_j = 1 - (1-\omega) \cdot \exp(\int_{1.5}^{j+0.5} ln[1-\omega+ (i-1)\psi] - \ln[1+(i-1)\psi]di), j = 1,2,\ldots \quad (4)$$

which is easily computed for any number of inoculated resting spores. The probability of a plant being infected (P_{inf}) was calculated by dividing the range of inoculated number of resting spores between $n-3\sqrt{n}$ and $n+3\sqrt{n}$ into 80 steps, multiplying the probability of each step by the θ_j for the midpoint of the step and summing over all the steps.

The generalized one-hit model fits the data well (Table 3, Figs. 1 and 2), in contrast to the basic one-hit model. This indicates that variation in the probability of infection between plants is an important factor in these experiments. The host, cv Septa, is part of the European Clubroot differential set (ECD, Buczacki *et al.*, 1975) and generally considered to be susceptible to clubroot. Also in ECD tests with the clubroot isolate used in this study, cv Septa was always classified as susceptible [Voorrips and Visser, 1993]. However, all those tests were performed with high inoculum densities. It is possible that in cv Septa, an open-pollinated cultivar, a low level of resistance is segregating which becomes evident at low inoculum densities. However, the effects of possible genetic variation for susceptibility cannot be distinguished from those of variation in other factors, such as differences in soil humidity and soil packing between pots and in the spatial distribution of resting spores in the soil, and physiological differences between plants in the first weeks after germination.

The estimated mean probability of a spore infecting the host (ω) was remarkably low in all experiments (Table 3). In most experiments with single-spore inoculations [Buczacki, 1977; Haji Tinggal and Webster, 1981; Jones *et al.*, 1982; Scott, 1985], about one percent of the inoculations resulted in an infected plant, whereas in the work presented here the estimation of the proportion infecting resting spores was generally much lower. The higher infection frequency in single-spore inoculations was probably caused by the fact that the resting spore was placed in direct contact with the seedling root. The low infection rate is therefore in this case a consequence of the inoculation method, and not indicative of synergistic spore action [Garrett, 1970].

The good fit of the generalized one-hit model in each of the five experiments shows that the interaction between *P. brassicae* and its host can be modelled without assuming synergistic or competitive interactions among pathogen individuals. This suggests that interactions between pathogen individuals do not have a large influence on the probability of infection. Fusion of secondary zoospores has been observed [Tommerup and Ingram, 1971], although it is not known if this is a necessary condition for secondary infection. The results presented here suggest that either fusion is not necessary for infection, or that the number of dikaryotic spores formed by fusion is proportional to the number of resting spores inoculated.

If pathogen individuals do indeed act largely independently a new way to obtain genetically uniform pathogen isolates can be envisaged. At inoculum densities where only a small proportion of plants becomes diseased, the probability that those plants are infected by only one spore is large. Instead of manually isolating single resting spores, the much less laborious procedure of inoculating large numbers of plants with very dilute resting spore suspensions and harvesting the few diseased plants can be used.

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