Neth. J. Pl. Path. 95 (1989) 37-49

Application of a mathematical function for a temperature optimum curve to establish differences in growth between isolates of a fungus

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Accepted 4 November 1988

Abstract

A function that approximates the curve of the growth rate of the mycelium of the imperfect fungus *Sphaeropsis sapinea* in relation to temperature is proposed. This function contains three free parameters, representing maximum growth rate, optimum temperature and shape of the curve. It was applied to data from an experiment with 27 isolates, in which the growth rate was measured in two replications at ten temperatures ranging from −2 °C to 45 °C.

Fitting the function to data from each isolate in each replication resulted in estimates of three parameters in which the information about the curve contained in the ten original observations is compressed. The estimates of the optimum temperature and of the shape were used in a further statistical analysis aimed at comparing the isolates and at ascertaining whether they could be divided into a few distinct groups, possibly related to different strains. The latter proved not to be the case.

Additional keywords: multivariate analysis of variance, *Sphaeropsis sapinea*.

Introduction

Strains of a fungus may differ in many respects. One physiological feature of a fungus is the in-vitro growth of the mycelium under defined experimental conditions. In this paper we consider the situation in which there is one well-defined and meaningful summary of growth: the growth rate.

A complete picture of the in-vitro growth of the mycelium in relation to temperature would be obtained by plotting the curve of true growth rate against temperature. Such a curve is commonly unimodal and asymmetric. Henceforth we shall refer to it as 'optimum curve' or merely 'curve'. Arguably the most relevant features of the relation between growth rate and temperature are the optimum temperature for growth and the

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growth rate at one or more fixed temperatures. Brasier et al. (1981) demonstrated the feasibility of using these growth features for distinguishing strains of a fungus. However, general characteristics of the optimum curve may be more satisfactory as a description of in-vitro growth than the growth rate at a few arbitrarily chosen temperatures. Cohen and Yarwood (1952) used empirical optimum curves for different fungi. They transformed the scale for the growth rates in such a way that the relation with temperature was approximately linear, thus reducing the problem of comparing optimum curves to comparing straight lines. Based on a mathematical function that approximates the optimum curves, Schrödter (1965) developed a nomogram in order to perform the transformations to straight lines in a simple way. He used this model when discussing the problems of relating growth rate to mean temperature in situations with changing temperatures. It is unclear how the transformation should be used for comparing different optimum curves objectively. In this paper we describe how a mathematical function that approximates the optimum curves can be used to compare curves of different isolates of a fungus. The function contains three free parameters, representing the height of the curve, the optimum temperature and a shape parameter, respectively. Different curves have different values for one or more of these parameters. The aim of this paper is to explain how to obtain estimates of parameters and how to use these in a statistical analysis aimed at comparing optimum curves. In our opinion this approach is very useful. The statistical techniques are not new, but as we have found no example of their use in microbiological research they are certainly not applied routinely in this area.

The data we use for illustrating the methodology originate from an experiment with 27 isolates of the fungus *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, collected in the Netherlands and six other countries. In-vitro colonial radial growth was measured at a number of fixed temperatures. The experiment was designed in order to investigate whether the 27 isolates of the fungus could be classified into one group or into different groups on the basis of their optimum curves.

Materials and methods

Growth experiment. From each of the 27 isolates of *Sphaeropsis sapinea* ten punches of 5 mm diameter were taken and distributed between ten Petri dishes containing 'Merck'-PDA culture medium. The ten Petri dishes were themselves distributed over ten temperatures: -2, 5, 10, 15, 20, 25, 30, 35, 40 and 45 °C. The growth rate observed was the average increase in diameter in two horizontal perpendicular directions between 24 and 69 hours for the temperatures that induced the mycelium to grow rapidly (20, 25, 30 and 35 °C) and between 24 and 212 hours for the other temperatures. A replication was obtained by repeating the procedure four hours later. In order to check that the growth rate was constant with time, the size of the mycelium in all the Petri dishes of two isolates was measured at intermediate times as well as after 69 or 212 hours.

The statistical analysis. The statistical analysis was carried out in two steps. In the first step, the function describing the curve was fitted to the observed growth rates at the different temperatures, for each separate unit. As the function contained three unknown parameters the ten observations were thus summarized into three new 'observations' that describe the curve. In the second step the isolates were compared using the parameter estimates as observations of new variables.

The function describing the optimum curve. The common basic form of the optimum curve is that the growth rate is zero below a minimum temperature T_{min} and above a maximum temperature T_{max}. Steadily increasing the temperature from T_{min} causes the growth rate to increase sigmoidally until the maximum is reached at the optimum temperature T_{opt}. Above T_{opt} the growth rate decreases rapidly to zero at T_{max}. Define, with T for temperature:

$$x = \frac{T - T_{min}}{T_{max} - T_{min}} \tag{1}$$

$$u = a + bx + cx^2 \tag{2}$$

The function introduced by Schrödter (1965) as an approximation to an optimum curve is:

$$f(x) = \begin{cases} y_{max} \sin^2(ux) & ; \text{if } 0 < x < 1 \\ 0 & ; \text{elsewhere} \end{cases} \tag{3}$$

Note that f(x) is a function of T because of Eqn 1. This function can be generalized, replacing the power 2 by an unknown parameter, resulting in:

$$f(x) = \begin{cases} y_{max} \sin^p(ux) & ; \text{if } 0 < x < 1 \\ 0 & ; \text{elsewhere} \end{cases} \tag{4}$$

with u and x defined in Eqn 1 and 2. Because $ux = 0$ if $x = 0$, then $f(T_{min})$ must equal zero. $f(T_{max}) = 0$ implies that $ux = \pi$ if $x = 1$, so one of the parameters a, b and c can be expressed as a function of the others, for example:

$$a = \pi - b - c \tag{5}$$

The maximum of the function is y_{max}, attained at $x = x_{opt}$ if $ux = \pi/2$. The optimum temperature T_{opt} can be derived from x_{opt} by Eqn 1. From $ux_{opt} = \pi/2$ it follows:

$$b = \pi \frac{x_{opt} - 0.5}{x_{opt}(1 - x_{opt})} - c(1 + x_{opt}) \tag{6}$$

So the general function can be expressed in six parameters: y_{max}, T_{min}, T_{opt}, T_{max}, c and p. Parameters y_{max}, T_{min}, T_{opt} and T_{max} have a direct and meaningful interpretation, whereas c and p are shape parameters. As illustrated in Fig. 1, p controls the general width of the function including the sharpness of the peak (the larger the p, the sharper the peak), whereas c controls the width for low growth rates.

In order to guarantee that the function is unimodal, u in Eqn 2 must be non-negative. Theoretically, this means that a restriction has to be imposed on the parameters. In practice, however, this problem can be solved by changing all negative values for u into zero.

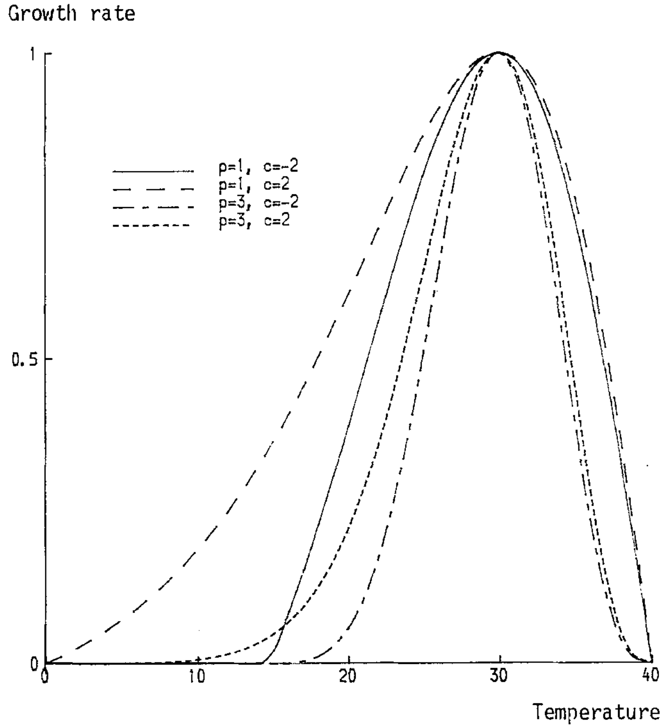

Fig. 1. Examples of function (4) with T_{min} = 0 °C, T_{max} = 40 °C, T_{opt} = 30 °C and y_{max} = 1 (mm/h).

Fitting the function. Function (4) with T_{min} = 4 °C, T_{max} = 40 °C and c = 0 was fitted to the data of each unit separately. The three free parameters y_{max}, T_{opt} and p were estimated using the modified Gauss-Newton method, by applying Genstat (Payne et al., 1987). The resulting parameter estimates are called $\hat{y}_{max}$, $\hat{T}_{opt}$ and $\hat{p}$. We specified the Poisson distribution instead of the normal distribution as the error distribution. This specification implies that weights were chosen according to the assumption that the variance is proportional to the mean. Furthermore, we made use of the facility to handle y_{max} as a linear parameter, leaving two nonlinear parameters to be estimated. Estimating nonlinear parameters is an iterative procedure in which, starting with initial guesses for the parameter values, the parameter estimates finally obtained are such that the discrepancy between the data and the function is minimized. The minimal discrepancy is called the deviance. The starting values used for the nonlinear parameters were 30 for $\hat{T}_{opt}$ and 2 for $\hat{p}$. The overall goodness of fit was examined by visual inspection of the fitted curves in relation to the data, using the deviance as a relative measure of goodness of fit.

Testing and interpreting differences between isolates. Because height is suspected to be a very unstable feature of a temperature optimum curve for growth rate the isolates were compared using $\hat{T}_{opt}$ and $\hat{p}$ only, and not $\hat{y}_{max}$.

Standard statistical analyses (e.g. multivariate analysis of variance (MANOVA)) require constant variation and approximately normal distribution for the variables. Therefore, first the kind of variation between replicates in relation to the scale of measurement was examined. Transformations to remove dependence of the variation on the mean were carried out if necessary.

Differences between isolates were tested by applying MANOVA to the standardized and possibly transformed variates $\hat{T}_{opt}$ and $\hat{p}$. The test statistic used was Wilks' lambda. The corresponding P-values were obtained from tables in Kres (1975). Canonical variates were derived in order to examine whether or not one linear combination of the two variates explained all differences between isolates. If this is so, the differences between isolates can be summarized in one essential feature of the curves. The first canonical variate is the linear combination of the variates with largest possible F-value (the test statistic for testing differences between isolates by applying univariate analysis of variance (ANOVA)). In this case, because there are just two variates, the second canonical variate was the linear combination of the variates with the smallest possible F-value. The number of canonical variates needed to describe the differences between isolates was deduced from the F-values informally.

The values of the linear combination defining a canonical variate are called loadings. The loadings indicate which variate is mainly responsible for the canonical variate.

Results

Goodness of fit. The result of fitting Eqn 4 with T_{min} = 4 °C, T_{max} = 40 °C and c = 0 to the data of each unit has been summarized in Table 1. Convergence was obtained for each unit. The last column of Table 1 shows the deviance. Three units have relatively large (though not extreme) deviances (> 0.6) compared with the others (which were between 0 and 0.4). This may indicate outliers for these units. To check the goodness of fit visually, the data and fitted curves for six units were plotted against temperature, see Fig. 2. The six units are the three with largest deviance (units 41, 3 and 19) and the three with smallest deviance (units 2, 48 and 52). From Fig. 2 the general conclusion is that the fitted function approximates the data well enough for all units. The large deviances for units 41, 3 and 19 are probably caused by outliers, although parameter estimates do not seem to be influenced substantially.

Variation and transformations. Fig. 3 is a plot of $\hat{T}_{opt}$ against $\hat{p}$. Variation can be observed from the differences between replicates. From plots of the variation against the mean for $\hat{T}_{opt}$ and $\hat{p}$ (not shown) it is concluded that for both variates the variation is related to the mean and that the relationship can be approximated by a straight line through 30.5 for $\hat{T}_{opt}$ and through 0.75 for $\hat{p}$. One reason that there is a relation between variation and mean could be that parameters are restricted: T_{opt} must lie between 0 and 40 °C, whereas p must be larger than 0. Near the borders of the parameter space a small change in parameter value has a large effect on the curve. The following transformations were used to remove much of the relation between variation and mean:

for $\hat{T}_{opt}$: $-\log(30.5 - \hat{T}_{opt})$
for $\hat{p}$: $\log(\hat{p} - 0.75)$

Table 1. Parameter estimates, obtained by fitting Eqn 4 with T_{min} = 4 °C, T_{max} = 40 °C and c = 0 to the data of each unit separately.

Unit	Isolate	Replicate	$\hat{T}_{opt}$	$\hat{y}_{max}$	$\hat{p}$	Deviance
1	1	1	27.2	0.78	1.26	0.221
2	1	2	27.2	0.89	1.51	0.013
3	2	1	29.3	1.27	2.50	0.705
4	2	2	28.6	1.21	3.20	0.145
5	3	1	29.9	1.28	1.27	0.210
6	3	2	29.6	1.06	1.22	0.091
7	4	1	29.4	1.38	1.50	0.200
8	4	2	29.4	1.47	1.21	0.112
9	5	1	29.3	1.27	1.05	0.294
10	5	2	29.3	1.37	1.06	0.206
11	6	1	29.5	1.54	1.71	0.353
12	6	2	29.4	1.39	1.53	0.101
13	7	1	29.5	1.32	1.00	0.148
14	7	2	28.2	1.29	1.40	0.137
15	8	1	27.4	1.10	2.43	0.180
16	8	2	25.4	0.92	2.35	0.089
17	9	1	29.5	1.64	1.40	0.215
18	9	2	29.0	1.39	1.65	0.099
19	10	1	29.4	1.45	1.61	0.637
20	10	2	29.3	1.30	1.16	0.146
21	11	1	29.4	1.74	1.35	0.212
22	11	2	29.3	1.62	1.09	0.198
23	12	1	29.4	1.41	1.32	0.398
24	12	2	29.4	1.24	1.12	0.127
25	13	1	29.6	0.89	0.82	0.258
26	13	2	29.5	1.03	0.81	0.321
27	14	1	29.4	1.49	1.50	0.337
28	14	2	29.1	1.34	1.25	0.061
29	15	1	29.3	1.12	1.08	0.137
30	15	2	29.0	1.20	1.31	0.066
31	16	1	29.3	1.60	1.42	0.067
32	16	2	29.2	1.32	1.29	0.108
33	17	1	29.5	1.54	1.47	0.278
34	17	2	29.5	1.40	1.30	0.154
35	18	1	29.4	1.34	1.41	0.134
36	18	2	29.3	1.18	1.11	0.175
37	19	1	29.5	1.37	1.63	0.221
38	19	2	29.4	1.39	1.48	0.252
39	20	1	29.5	1.22	1.25	0.156
40	20	2	29.5	1.11	1.38	0.093
41	21	1	30.4	1.11	3.27	1.001
42	21	2	30.3	1.01	2.07	0.208
43	22	1	29.5	1.39	1.29	0.134
44	22	2	29.4	1.38	1.26	0.163
45	23	1	29.3	1.37	1.33	0.208
46	23	2	29.4	1.27	1.17	0.158

Table 1. (Continued).

Unit	Isolate	Replicate	$\hat{T}_{opt}$	$\hat{y}_{max}$	$\hat{p}$	Deviance
47	24	1	28.8	0.38	2.10	0.080
48	24	2	28.0	0.39	4.85	0.032
49	25	1	30.1	0.26	1.63	0.211
50	25	2	26.9	0.32	1.97	0.098
51	26	1	27.5	0.55	2.23	0.127
52	26	2	28.2	0.67	1.69	0.048
53	27	1	29.7	0.47	1.91	0.250
54	27	2	29.6	0.58	2.03	0.072

The result of these transformations is shown in the plot of $\hat{T}_{opt}$ against $\hat{p}$ on these transformed scales, presented in Fig. 4. Note the difference between Figures 3 and 4, not only with respect to variation, but also with respect to the mutual distances between isolates. After transformation, only the large variation in $\hat{T}_{opt}$ for isolate 25 is extreme, whereas for other isolates, e.g. 24, the transformation has removed the seemingly extreme variation on the original scale. Re-examining the original observations, the replicate of isolate 25 with $\hat{T}_{opt}$ 30 °C seems to contain an outlier. A dramatic change in mutual distances between isolates as a result of the transformation can be observed for isolate 13.

Comparison of isolates using $-\log(30.5 - \hat{T}_{opt})$ and $\log(\hat{p} - 0.75)$. From Fig. 4 it can be seen that many isolates are clustered and that isolates 1, 2, 8, 13, 21, 24, 25, 26 and 27 seem to lie outside this main group. First it was investigated if all isolates could be considered to have the same curve. Applying MANOVA to $-\log(30.5 - \hat{T}_{opt})$ and $\log(\hat{p} - 0.75)$ resulted in the value 0.012 for Wilks' lambda, which indicates significant differences between isolates at the 1% level. F-values and loadings for the two canonical variates, summarized in Table 2, show that the differences between isolates are related to differences in both $\hat{T}_{opt}$ and $\hat{p}$, not to just one of these or to one linear combination of $-\log(30.5 - \hat{T}_{opt})$ and $\log(\hat{p} - 0.75)$. Five or six groups are needed in order to explain the differences between isolates. A possible arrangement into groups is: isolates 1, 8, 13 and 21 each in a separate group; isolates 2, 24, 25, 26 and 27 in one group; and a group with the remaining isolates. It is not possible to arrange the isolates in two or three groups such that the variation within groups is in agreement with the variation between replicates.

Discussion

Growth rate. According to Brancato and Golding (1953) the diameter of a colony lends itself satisfactorily for the measurement of the growth rate, because there is no acceleration of the growth rate with time. For another fast growing fungus Trinci (1969) established that the period preceding the phase of constant growth rate lasted approximately 18 hours. He concluded that colonial radial growth is a reliable parameter for determining the optimum temperature for the growth of a fungus. Our observations confirmed that the growth rate was constant with time, at least after 24 hours.

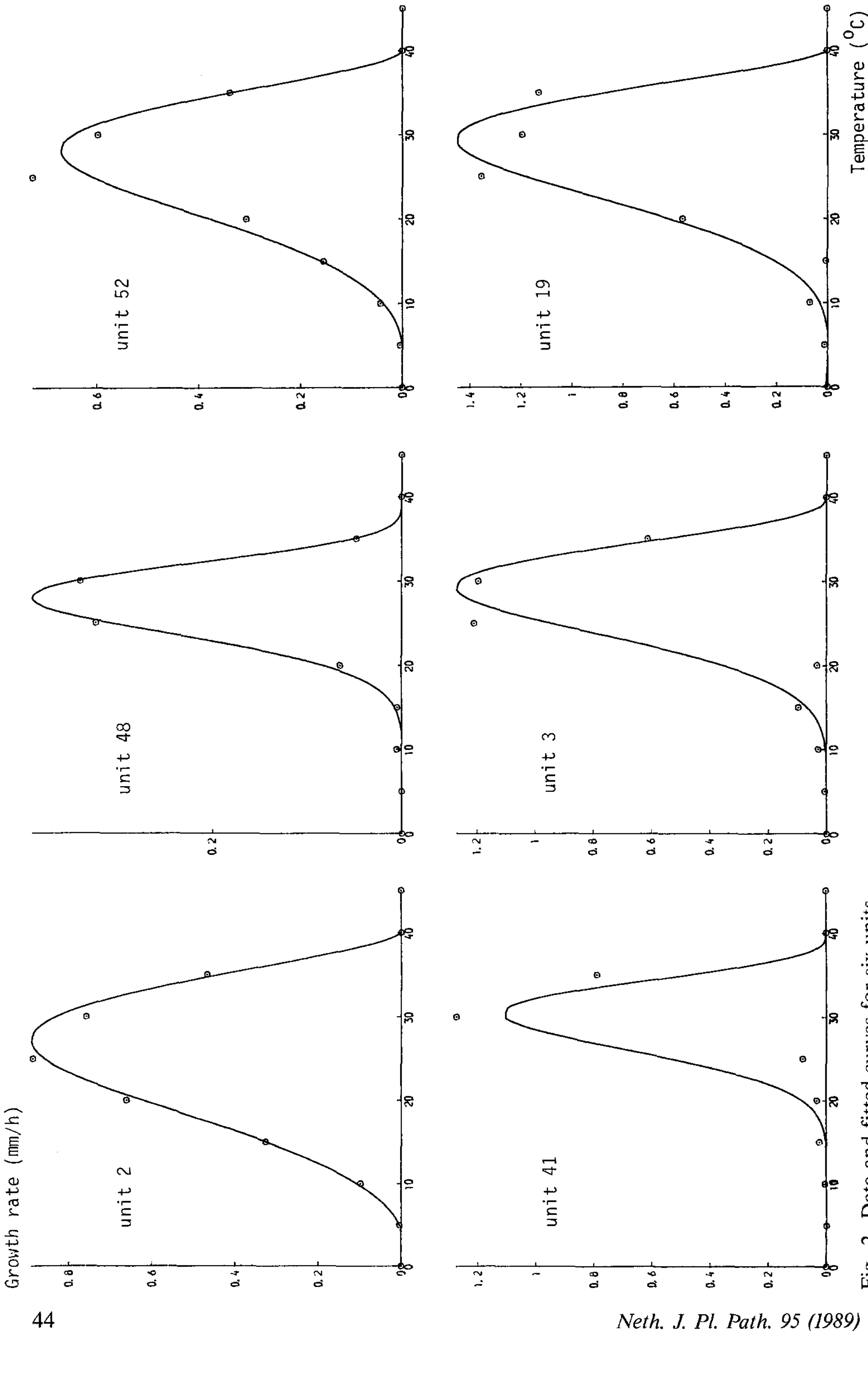

Fig. 2. Data and fitted curves for six units.

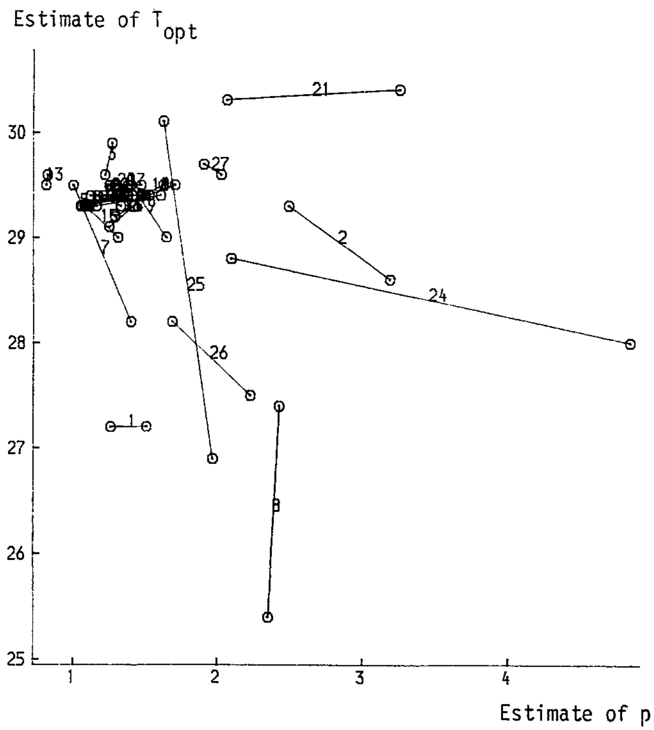

Fig. 3. Plot of $\widehat{T}_{opt}$ (°C) against $\hat{p}$. The estimates have been obtained by fitting Eqn 4 with T_{min} = 4 °C, T_{max} = 40 °C and c = 0 to each isolate-replication combination. Points for the same isolate have been connected.

The statistical analysis. In experimental design terminology the isolate-replicate combinations are the experimental units and the measurements at the different temperatures are repeated measurements of the response variable 'growth rate'. Therefore the experimental design is analogous to a repeated measurements design or growth curve design, with temperature replacing time. For a discussion on models and analyses for observations from such designs, see Keen et al. (1986).

The function applied. The difference between Schrödter's function (Eqn 3) and ours is the choice of one of the free parameters in the general expression (Eqn 4). Our free parameter is p, fixing c at the value 0; Schrödter's free parameter is c, fixing p at the value 2. Our choice was based on two arguments. Firstly our function fitted our data better than Schrödter's function (which possibly fitted better to the type of curve he was faced with). For our data p = 2 induced too sharp a peak for the function. This could have been a reason for fixing p at the value 1 for example, with c as free shape parameter. The main advantage of taking parameter p as free instead of c is, however, that then the iteration process perfoms better: fewer iterations are necessary for convergence. The probable reason for this is that p affects the whole curve whereas c merely affects a part of the curve, so that the data contains more information about p than about c.

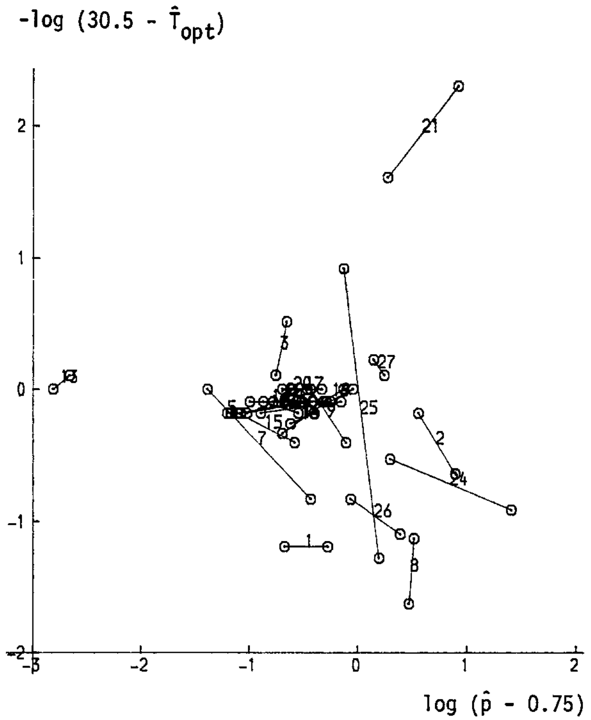

Fig. 4. Plot of $-\log(30.5 - \hat{T}_{opt})$ against $\log(\hat{p} - 0.75)$. The estimates have been obtained by fitting Equation 4 with $T_{min} = 4$ °C, $T_{max} = 40$ °C and $c = 0$ to each isolate-replication combination. Points for the same isolate have been connected.

Table 2. F-values and loadings for the canonical variates obtained with MANOVA applied to $-\log(30.5 - \hat{T}_{opt})$ and $\log(\hat{p} - 0.75)$.

		Canonical variate	
		1	2
F-values		11.8	5.6
loadings for	$-\log(30.5 - \hat{T}_{opt})$	0.27	0.31
	$\log(\hat{p} - 0.75)$	0.44	−0.16

Fitting the function. In this study a few scattered observations were available for fitting the function. In this situation problems of convergence can be expected, so some precautions for preventing these have to be taken. Firstly, good starting values for the nonlinear parameters are required. This turned out not to be critical. A sufficiently accurate initial value for $\hat{T}_{opt}$ can be obtained by examining the plot of observed growth rates against temperature. For $\hat{p}$, a value of 1 or 2 usually suffices. Secondly, care has to be taken that the function values can be calculated accurately for every possible combination of parameter values. This requires careful examination of the function and adaptation of the mathematical expression for extreme parameter settings. Thirdly, it is essential to specify that the variance is proportional to some power of the mean. This forces the fit through the lower growth rates. The exact relationship between variance and mean can only be guessed, but fortunately it is not crucial. For a discussion on models with non-normal error distributions, see McCullagh and Nelder (1983).

Note that standard errors of parameter estimates obtained by the fitting procedure are not relevant, because in these standard errors only within-unit variation is included and not between-unit variation.

Using the function; advantages and warnings. The most important advantage of using a function is that all information in the data about the curve is compressed in the fewest possible relevant features in the best possible way, making allowance for heterogeneity of variance. The fitting procedure is more objective than hand drawn curves and allows a sound statistical analysis for comparing curves. It is more effective than using the observations at the temperatures in the experiment as variates, and the results of a statistical analysis are better interpretable in terms of the global features of the curves.

Generally, estimates of nonlinear parameters are biased because of measurement errors. This is rarely a serious problem, especially not for comparisons. Serious bias can be caused by differences between the curve and the best fitting function, i.e. by approximation error. In our experiment the data contain little information about the shape of the curve, particularly near the optimum temperature, so the function may not be a good approximation of the true curve there. Therefore, although $\hat{T}_{opt}$, ranging from 26.9 to 30.4 °C agrees well with published values for the optimum temperature (28 °C) (Brookhauser and Peterson, 1971; Johnson et al., 1985), it may be a biased estimate of the optimum temperature. Because isolates may differ in shape of the curve, that is not caused by p, the bias may even be specific to the isolate. Consequently, univariate analysis of $\hat{T}_{opt}$ may reveal differences that result from differences in the shapes of the curves and not from differences in optimum temperature. This is one reason why we prefer a multivariate analysis of the parameter estimates, despite the fact that the parameters have a well defined meaning in the function. The second reason for preferring a multivariate analysis is that estimates of nonlinear parameters are always dependent.

The choice of MANOVA as the multivariate technique for comparing isolates is the logical extension of ANOVA that would have been applied if there had been one variate for each unit. MANOVA is the technique that searches for differences between isolates that are large relative to differences between replicates; therefore, the canonical variates seem to be the most relevant properties of the isolates for identifying groups. The transformations used for $\hat{T}_{opt}$ and $\hat{p}$ were chosen on a purely empirical basis, just because these performed well in removing the dependence of the variation on the mean. Standardization was applied because in the comparison of isolates this gives equal importance to $\hat{T}_{opt}$ and $\hat{p}$.

Differences between isolates. Except for isolates 22 to 27 (which originated from the United States of America) all isolates were collected in the same period. However, they have different histories. Although the isolates were kept under identical conditions for more than a year, their growth rates at a given temperature will not be equal, even if they belong to the same strain. It is an empirical fact that the growth rate at a given temperature changes with time of year, not necessarily in the same way for different isolates. This is reflected mainly in parameter y_{max}. However, the ratio of growth rates at two fixed temperatures may also change, and such changes are reflected in the parameters p and T_{opt}. Therefore, it cannot be irrefutably concluded that the differences between isolates compared with the variation between replicates indicate different strains. The differences could be explained as random differences that exist between curves at each moment in time. These random differences were made systematic here, because the replicates were obtained almost at the same time. Conclusions about the existence of strains require additional hypotheses about the size of the random differences between curves at one moment in time.

It may be hypothesized that differences between strains are large compared with the random differences between isolates of the same strain at a fixed point in time. Obviously, this is a somewhat subjective and not very satisfactory criterion for deciding how many strains exist. The only way to avoid this subjectivity would be to quantify the size of the random differences between isolates by choosing replicates at points in time that are far apart. Our subjective 'conclusion' on the basis of Fig. 4 and the results of MANOVA is, that there is little evidence supporting the existence of different strains. Note that the subjectivity has nothing to do with the first step of the statistical analysis: fitting a function and using the parameter estimates in a further analysis. It is related solely to how this further analysis is carried out; and in turn this depends on how the problem has been defined and whether or not the experiment contains the necessary information for solving the problem. In this case in our opinion this experiment contains useful information about differences between isolates in growth, but for definite conclusions about the existence of strains the information supplied by the data is insufficient.

Acknowledgements

For their constructive comments we wish to thank A.A.M. Jansen, J.Th.N.M. Thissen and C.J.F. ter Braak (Agricultural Mathematics Group), M. de Kam (Research Institute for Forestry and Landscape Planning 'De Dorschkamp'), R.A. Daamen (Research Institute for Plant Protection) and W.A.H. Rossing (Wageningen Agricultural University, Department of Theoretical Production Ecology). We also thank the referees for their critical remarks and for drawing our attention to some very useful papers.

Samenvatting

Toepassing van een functie die de temperatuurcurve van een schimmel benadert, ter vaststelling van verschillen in groei tussen isolaten

Een functie is voorgesteld die de groeisnelheid van de imperfecte schimmel *Sphaeropsis sapinea* in relatie tot de temperatuur beschrijft. De functie bevat drie vrije parameters,

die de maximale groeisnelheid, de optimum temperatuur en de vorm van de curve representeren. De functie is toegepast op gegevens afkomstig van een experiment met 27 isolaten, aangelegd in twee herhalingen, waarin de groeisnelheid is gemeten bij tien temperaturen. Aanpassen van de functie aan de gegevens per isolaat per herhaling levert schattingen voor de drie vrije parameters die de informatie over de curve in de tien oorspronkelijke waarnemingen comprimeren. De schattingen van de optimum temperatuur en de vorm zijn gebruikt in een statistische vervolganalyse, gericht op het vergelijken van de isolaten. De specifieke vraag hierbij was of de isolaten in enkele duidelijke gescheiden groepen waren in te delen. Dit bleek niet erg aannemelijk.

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