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S. Declerck · D. D'or · S. Cranenbrouck
E. Le Boulengé

Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture

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Abstract Spore production of arbuscular mycorrhizal fungi is important in inoculum production, and monoxenic culture is a promising way to produce large amounts of contaminant-free inoculum. Mass production of spores is therefore essential and mathematical models useful as descriptive and predictive tools of sporulation dynamics. We followed the sporulation dynamics of three *Glomus* strains i.e. *G. intraradices*, *G. proliferum* and *G. caledonium*, cultured monoxenically on a nutrient agar medium containing macro- and microelements, vitamins and sucrose. Three models (Schnute, logistic, and Gompertz) were fitted to the data and compared in order to select the most adequate model. The Schnute model was the reference against which the two other models were tested. For all three *Glomus* strains examined, the sporulation dynamics followed a sigmoidal curve with a lag, a log, and a plateau phase. Visually, all three models fitted the data very well, with R^2 values ranging from 0.9703 to 0.9995. They thus appeared adequate for describing the temporal dynamics of spore production. In most cases the Gompertz model described sporulation as accurately as the Schnute model, but the performance of the logistic model was seldom as good. The Gompertz model is thus convenient for modelling the sporulation dynamics of *Glomus* strains grown in a well-defined nutrient agar medium. As such, its use may facilitate and help improve exploitation of monoxenic culture systems.

Keywords Arbuscular mycorrhizal fungi · Gompertz · Monoxenic culture · Sporulation dynamic

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S. Declerck (✉)
Université catholique de Louvain,
Mycothèque de l'Université catholique de Louvain (MUCL),
Unité de microbiologie, 3 Place Croix du Sud,
1348 Louvain-la-Neuve, Belgium
e-mail: declerck@mbla.ucl.ac.be
Tel.: +32-10-474644, Fax: +32-10-451501

D. D'or · E. Le Boulengé
Université catholique de Louvain,
Unité de biométrie et d'analyse des données,
2 Place Croix du Sud Bte 16, 1348 Louvain-la-Neuve, Belgium

S. Cranenbrouck
Université catholique de Louvain, Unité de microbiologie,
3 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium

Introduction

Arbuscular mycorrhizal fungi (AMF) are obligate symbiotic soil microorganisms that cannot yet be cultured axenically. Little is known about the dynamics of hyphal growth (Gazey et al. 1992; Jakobsen et al. 1992) and practically nothing about the dynamics of sporulation (Gazey et al. 1992). Most information on these characteristics has been obtained from experimental microcosms or from the field. The usual methods of spore counting are generally destructive, consisting of successive rhizospheric samplings (Bago et al. 1998) that make undisturbed time-course observations impractical. Monoxenic culturing of AMF allows a non-destructive approach that should help describe sporulation dynamics without disturbing the intimate coexistence of the fungus and its host. The first attempt at such an approach was made with *Glomus versiforme* Karsten and Berch associated with transformed carrot roots on a synthetic nutrient agar medium (Declerck et al. 1996). In this closed sterile system, spore production was shown to follow sigmoidal dynamics, with a lag, a log, and a stationary phase. To our knowledge, this is the only study to have focused on the sporulation dynamics of AMF strains under monoxenic conditions. One reason may be the huge, time-consuming task of spore counting. Yet spore production is important in inoculum development, and monoxenic culturing a promising way to produce large amounts of contaminant-free AMF inoculum (St Arnaud et al. 1996). Hence, mathematical functions that adequately describe the sporulation dynamics of various AMF strains in monoxenic cultures may facilitate research in this field. Such models could: (1) facilitate time-course spore counting by decreasing the number of necessary

observations, (2) be used to determine the rate of spore production, and thus help select AMF strains with a high sporulating potential, and (3) show the impact upon sporulation dynamics of various factors (nutrients, pH, etc.) which characterize monoxenic culture systems, and thus help to improve these contaminant-free systems.

In this study, we monitored the sporulation of three AMF strains grown on transformed carrot roots in a defined standard growth medium. We chose the modified Strullu-Romand (MSR) medium, as it has been used in monoxenic cultures of several AMF strains (Strullu et al. 1997; Declerck et al. 1998). Our main objectives were: (1) to see whether the sporulation dynamics of AMF strains could be modelled, (2) to compare three commonly used growth models, i.e. the Gompertz (1825), Schnute (1981), and logistic models (Verhulst 1838) to see how adequately they describe sporulation dynamics and to select the most adequate growth model, and (3) to illustrate the use of the most adequate model in comparing sporulation dynamics in different strains.

Materials and methods

Biological material

We used three AMF strains: *Glomus intraradices* Schenck and Smith (MUCL 41833), *Glomus proliferum* Dalpé and Declerck (MUCL 41827), and *Glomus caledonium* (Nicol. and Gerd.) Trappe and Gerd. (BEG-20). *G. intraradices* and *G. proliferum* were purchased from the Mycothèque de l'Université catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium) and *G. caledonium* was provided by the Banque Européenne des Glomales (BEG, Dijon, France).

The strains were propagated in pot cultures of leek (*Allium porrum* var. bleu de Solaise) grown on Terra green (Agsorb 8/16 LVM-GA, Chicago, Ill.). The leek plants were grown under greenhouse conditions set at 24/20°C (day/night) under natural light intensity. The plants were fertilized at regular intervals (Hewitt 1966) and rainwater was delivered every 2–3 days. After 6 months of culture, the roots and spores were recovered from the pot cultures. For *G. intraradices* and *G. proliferum*, root pieces were used as starter inoculum and disinfected as described by Strullu and Romand (1986). For *G. caledonium*, spores were used as starter inoculum and disinfected as follows: spores were placed in the upper part of a sterilized (121°C for 1 h) 45-µm filter (Gelman Science, Mich.) holder apparatus (VEL, Leuven). They were soaked under vacuum for 10 min in 20 ml of a 2% (w/v) chloramine T solution containing 20 µl Tween 20, then for 10 min in 20 ml antibiotic solution (streptomycin 200 mg/l and gentamycin 100 mg/l). After disinfection, root pieces and spores were incubated in the dark at 27°C on the MSR medium (see Declerck et al. 1998) solidified with 4 g l^{-1} Gel Gro (ICN Biomedicals, Irvine, Calif.) instead of 8 g l^{-1} Bacto agar (Difco, Detroit, Mich.). When hyphal growth was apparent, one propagule, i.e. a mycorrhizal root piece or spore, was placed with a transformed carrot root (*Daucus carota* L.) on the MSR medium as previously described (Declerck et al. 1998).

Each experimental unit consisted of a Petri dish (diameter 90 mm) containing 30 ml MSR medium, a 5-mm mycorrhizal root piece or spore, and a 70-mm Ri T-DNA-transformed carrot root (MUCL). Petri dishes were incubated upside-down in the dark at 27°C. We used five experimental units for *G. intraradices* and *G. proliferum* and eight for *G. caledonium*.

A 10×10-mm grid was marked with a scalpel on the bottom of each Petri dish to facilitate spore counting (Declerck et al. 1996). Spores were counted individually on each cell, under a binocular microscope, and totalled over the entire Petri dish. Spores were counted weekly for each experimental unit from week 1 to 5 after initiation, then every 2 weeks until week 13. After these first nine measurements, the schedule was adapted so that each strain reached the plateau phase. This required two more measurements for *G. intraradices* (weeks 15 and 16) and *G. caledonium* (weeks 19 and 21), and five for *G. proliferum*. (weeks 15–19).

Table 1 Modified expressions (Zwietering et al. 1990) of the Schnute, Gompertz and logistic growth models

Model	Modified equation[a]
Schnute	$E(Yt)=\left(\mu_m \frac{1-b}{a}\right)\left[\frac{1-b\exp(a\lambda+1-b-at)}{1-b}\right.$
Gompertz	$E(Yt)=A\exp\left(-\exp\left[\frac{\mu_m e}{A}(\lambda-t)+1\right]\right)$
Logistic	$E(Yt)=\frac{A}{\left(1+\exp\left[\frac{4\mu m}{A}(\lambda-t)+2\right]\right)}$

[a] e=exp(1). In the present context, $E(Yt)$ is the expected number of spores counted in a culture at time t; A is the maximum number of spores; μ_m is the maximum rate of increase in spore number; λ is the lag time; b is peculiar to the Schnute model and has no simple biological meaning. In the Schnute model, $a=\frac{\mu_m e}{A}$

Growth models

From the counts for each Petri dish, a sporulation curve was drawn and the models were fitted to each curve. We thus obtained an estimate of each parameter for each experimental unit. Measuring, for each strain, only the mean of the spore counts for all replicates would have resulted in the loss of information on within-strain variability.

Three mathematical growth functions were fitted to the sporulation dynamics observed within each experimental unit. We concentrated on three functions frequently used in the literature to model fungal or bacterial growth, i.e. the Schnute (1981), Gompertz (1825), and logistic (Verhulst 1838) functions. We reparameterized these models as done by Zwietering et al. (1990) so as to express the three-parameter Gompertz and logistic functions as sub-models of the four-parameter Schnute model (Table 1).

The model parameters (Table 1) can be interpreted as follows: A is the cumulated number of spores produced when the plateau phase is reached; μ_m is the maximum spore production rate (tangent of the sporulation curve at the inflexion point); λ is a lag time (time to the intersection between the tangent at the inflexion point and the time axis). The last parameter, b, peculiar to the Schnute model, has no simple biological meaning. The measured feature, Yt, is the number of spores produced by time t.

Models were fitted to the data by nonlinear regression using a Nelder-Mead simplex optimization algorithm (Nelder and Mead 1965) minimizing the residual sum of squares (RSS). Computations were done with the mathematical software MATLAB (MATLAB 5.3; The MathWorks, Natick, Mass.).

Analysis of the residuals and computation of the R^2 were used to evaluate the quality of the fit between the models and the data.

Model comparisons

As stated in Zwietering et al. (1990), the logistic and Gompertz models are nested in the Schnute model. The Gompertz model results from setting parameter b of the Schnute model at 0, with $a>0$. With $a>0$ and b set at –1, Schnute simplifies to the logistic model. The Schnute model uses four parameters and will thus al-

ways fit the data better than the three-parameter models. It was thus chosen as the reference. The residual mean square (RMS) of the Schnute model, with t–4 df (where t=time), was used as an estimate of the measurement error. The adequacy of the three-parameter models was evaluated by testing for a significant reduction in the RSS when introducing a fourth parameter leading to the Schnute model (Fisher-Snedecor F-test with 1 and t–4 df) (Seber and Wild 1989):

$$F = \frac{(RSS_3 - RSS_4)}{RMS_4} \quad (1)$$

Small P-values indicate non-equivalence of the three-parameter and Schnute models. For non-linear models, the test is only asymptotically exact (Seber and Wild 1989). In our case these tests must be interpreted with caution because of the rather small number of observations.

An alternative test of model equivalence is based on the confidence intervals (CIs) that can be built around the parameter estimates of the Schnute model. For instance, if the CI for b contains the value 0 and the CI for a is in the positive range, then one may accept the Gompertz model as equivalent to the Schnute model. Here again, the procedure would only be formally correct (i.e. the risk underlying rejection of the equivalence would be 1 minus the P-level of the CI) if the parameter estimates were mutually independent, which they are not. As a result, sometimes the F and CI procedures may yield contradictory results.

Strain comparisons

In order to select the strain with the highest spore production potential (i.e. maximal A), the parameters of the Gompertz model for the three strains were analysed by multivariate ANOVA (MANOVA) with the strain as factor. Scheffé's test was used to compare the means of each parameter between the three strains. $P<0.05$ and $P<0.01$ indicate, respectively, a significant or highly significant difference between two strains for the considered parameter. The GLM procedure in the SAS software (SAS Institute, Cary, N.C.) was used to compute these tests (SAS Institute 1990).

Results

Sporulation dynamics and modelling

The three *Glomus* strains followed three-phase sporulation dynamics, characterized by a lag phase, a phase of rapid increase in spore production, and a plateau phase (Fig. 1).

Visually all three models, Schnute, Gompertz, and logistic, fit the data quite well. Differences between the Gompertz and Schnute models were generally negligible (Fig. 2a, for example). In the case of *G. caledonium*, the logistic model deviated more strongly than the Gompertz model for all but three replicates, but in the case of *G. proliferum*, only one replicate showed a clear difference between the logistic and Schnute models (Fig. 2b).

The existence of a trend in the residuals is a sign that the model inadequately fits the data. It results from non-integration into the model of one or more important factors. In this experiment, analysis of the residuals showed a typical, erratic pattern, without any significant trend in the residual values. All three models thus appear to describe the data adequately.

The coefficient of determination R^2 was computed for each experimental unit and each model. Its value ranged from 0.9703 to 0.9995 with a mean of 0.9943 and a SD of 0.0071. The R^2 histogram showed a strong asymmetry towards the higher values. All three models thus give an extremely good fit of the data.

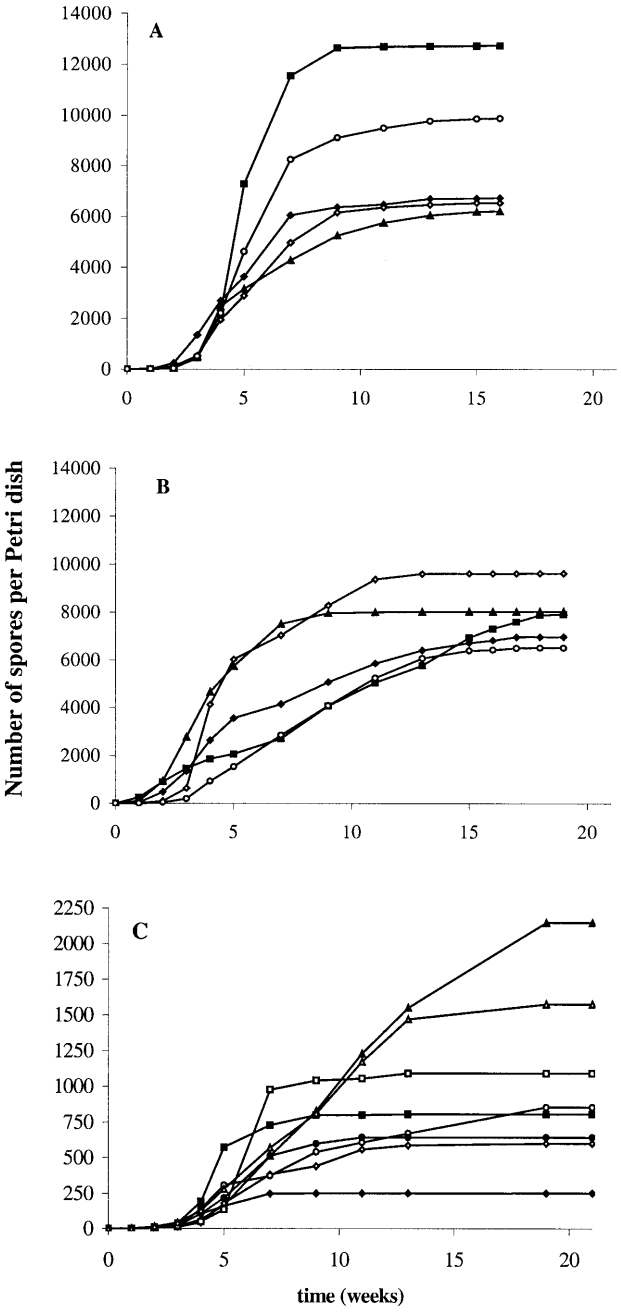

Fig. 1 Spore production of *Glomus intraradices* (**a**), *Glomus proliferum* (**b**) and *Glomus caledonium* (**c**) following association with a transformed carrot root on modified Strullu-Romand medium. Each curve illustrates the evolution of the number of spores in one replicate experiment. Note the different scale of the *y*-axis for *G. caledonium*

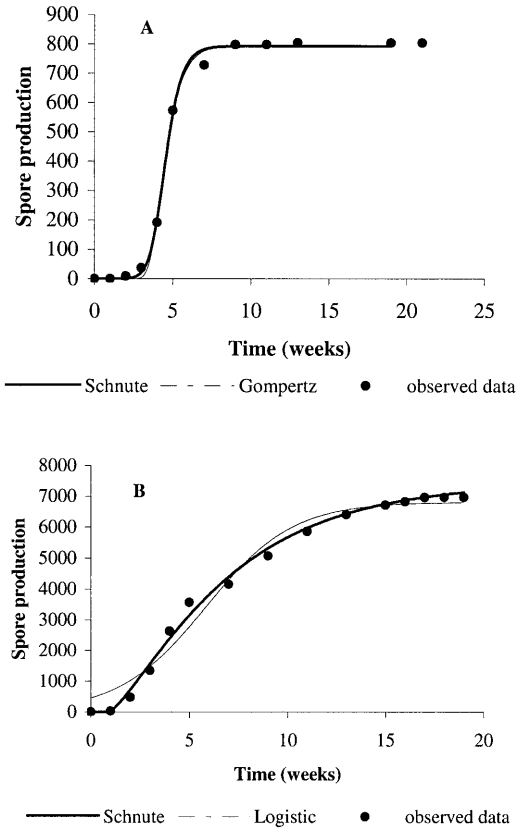

Fig. 2a, b Examples of sporulation curves fitted with the Schnute (*bold line*) and Gompertz or logistic models (*fine line*). **a** Typical case (see Table 2, *G. caledonium*, replicate 2); **b** worst case (see Table 2, *G. proliferum*, replicate 1)

Model comparisons

When the *F*-test for nested models (Table 2) was applied, the Gompertz model was never rejected for *G. intraradices*. It was rejected for only one out of five replicates of *G. proliferum* ($P<0.001$), and two out of eight replicates of *G. caledonium* (both Ps=0.021). The logistic model, on the other hand, was rejected for four out of five replicates of *G. intraradices*, all replicates of *G. proliferum* and five out of eight replicates of *G. caledonium*. The *P*-values for rejection ranged from $P<0.001$ to $P=0.026$, while the *P*-values for the five non-rejected replicates ranged from 0.097 to 0.674.

CI analysis (Table 2) shows whether the values of parameters *a* and *b* of the Schnute model allow simplification to either the Gompertz or the logistic model. When the CI for *a* contains positive values and the CI for *b* contains zero, the Schnute model simplifies to the Gompertz one. When the CI for *a* contains positive values and the CI for *b* contains –1, the Schnute model reduces to the logistic model. According to these rules, the Schnute and Gompertz models appear equivalent in 16 out of 18 cases. The logistic growth curve was accepted in only nine out of 18 cases. For replicate 7 of *G. caledonium* (see Table 2), the atypical sporulation pattern near the beginning of the plateau phase resulted in instability for the estimate of parameter *b*, and consequently in a very broad CI for this parameter. As *b* and *a* are strongly correlated, the CI for *a* is also extremely broad.

Table 2 Conclusions for the comparison of the Gompertz and logistic models vs. the Schnute model (*P*-values for the *F*-test and "*R*", for rejection of the model, for the *t*-test), and limits for the 95% confidence intervals to the *a* and *b* parameters of the model of Schnute. *t* Number of times spores are counted in each Petri dish, *min.* minimum, *max.* maximum

Strains	Replicate	Conclusion for the test				Limits for the 95% confidence intervals			
		Gompertz		Logistic		*a* min.	*a* max.	*b* min.	*b* max.
		F-test[a] (*P*-value)	*t*-test	*F*-test[a] (*P*-value)	*t*-test				
Glomus intraradices (t=12)	1	0.652		0.102	R	0.10	0.86	–0.39	0.92
	2	0.923		*0.008*		–1.52	5.11	–4.89	3.00
	3	0.256		*0.010*		–5.76	8.47	–11.64	10.74
	4	0.813		*0.003*		–0.28	1.91	–3.11	2.36
	5	0.700		*0.001*		–0.63	2.97	–3.71	2.63
Glomus proliferum (*t*=15)	1	*<0.001*	R	*<0.001*	R	0.09	0.29	0.49	0.92
	2	0.178		*0.012*	R	–0.12	0.25	–0.69	1.93
	3	0.337		*<0.001*	R	0.43	0.80	–0.46	0.71
	4	1.000		*0.014*	R	0.16	0.99	–0.48	0.48
	5	0.736		*0.001*	R	0.12	0.46	–0.81	0.95
Glomus caledonium (*t*=12)	1	0.406		0.180		0.001	1.98	–2.35	1.67
	2	0.517		0.611		–0.74	4.04	–3.89	2.87
	3	0.356		*<0.001*	R	0.07	0.35	–0.63	0.93
	4	0.342		*0.009*	R	0.20	0.68	–0.02	0.29
	5	0.072	R	*0.004*	R	0.03	0.38	0.09	0.77
	6	*0.021*		*0.026*		0.47	1.17	–1.38	0.47
	7	*0.021*		0.097		–272.54	292.58	–302.85	283.10
	8	0.799		*0.012*		0.01	0.51	–1.24	1.38

[a] Data in italics indicate rejection of the model with an *F*-test ($P<0.05$)

Table 3 Parameter values for the *A*, μ_m, and λ parameters fitted with the model of Gompertz. In *each column*, for *each species*, the *first row* gives the mean value and the *second row* gives the coefficient of variation. Values in the *same column* followed by the *same letter* are not different at the α=0.01 level according to the mean comparison test of Scheffé

	A	μ_m	λ
G. intraradices	8,426.2 a 34.03	2,190.3 a 67.00	2.693 a, b 21.06
G. proliferum	8,277.6 a 17.89	1,198.4 a, b 58.86	1.931 a 43.11
G. caledonium	1,020.1 b 64.4	222.5 b 82.11	3.65 b 23.81

Strain comparisons

For the mass production of an inoculum, it is important to identify and select strains with a maximal potential to produce spores by the end of the collecting time (end of the experiment). This production potential is estimated by parameter *A*.

The spore production potential differed among the three strains (MANOVA). Scheffé's comparison test showed that *G. caledonium* had a lower potential than the other two strains, but the latter did not seem to differ with respect to the data at hand (Table 3). To tell whether this lack of difference is real, or due to large within-strain variability of sporulation dynamics (coefficients of variation, Table 3), would require larger numbers of replicates to be examined.

G. caledonium appeared to differ from the two other strains by a longer lag phase, a slower growth rate, and lower maximum spore production. The sporulation patterns of *G. intraradices* and *G. proliferum* did not differ significantly.

These analytical results were strengthened by visual observations of mycelium and spore development. For both *G. intraradices* and *G. proliferum* the mycelia were composed primarily of long hyphae growing straight into the agar. Spores appeared at terminal or intercalary positions, sometimes single but more often in clusters of up to ten spores. The presence of large clusters of spores (>100) in *G. proliferum* could be used to differentiate this strain from *G. intraradices*. The mycelium of *G. caledonium* was composed of both long, straight hyphae growing into the agar and short, tangled, sinuous hyphae. Spores of *G. caledonium* never appeared in clusters but were always produced singly in the mycelium.

Discussion

We used sigmoidal mathematical functions with three or four parameters to describe the sporulation dynamics of AMF in monoxenic cultures. All three AMF strains followed three-step sporulation dynamics as previously described for *G. versiforme* (Declerck et al. 1996).

All three models fit the data extremely well. The coefficients of determination are very similar and the residuals show a typical erratic pattern, demonstrating the adequate parameterization of the models.

The Gompertz model, in most cases, was sufficient to describe the sporulation data for all strains. In 72% of cases it proved as adequate as the Schnute model for describing the sporulation dynamics, according to both the *t*-test and the *F*-test. Our results agree with those for *G. versiforme* (Declerck et al. 1996), which also showed a sigmoidal developmental pattern and the equivalence of the Gompertz and Schnute models (P=0.1), whereas the logistic model was rejected (P<0.001).

The Gompertz model is easier to use than a four-parameter model. It is more stable because the parameters are less correlated, and it yields estimates with more *df*s (Zwietering et al. 1990), which could be important when only a few points are measured. This may reduce the amount of data required, which can be difficult to obtain for AMF sporulation. Furthermore, the fourth parameter (*b*) of the four-parameter Schnute model is hard to interpret biologically; it introduces numerical instability during curve fitting when its value approaches zero and it correlates strongly with other parameters. This suggests that the Gompertz model is the most useful.

We further have shown that the Gompertz model is useful in selecting strains with a high sporulation potential. In this case, *G. intraradices* and *G. proliferum* were found to be the best strains. *G. caledonium* showed a lower plateau phase than these two strains, indicating a lower sporulation potential per production unit, a clear disadvantage when the goal is mass production of spores.

It is unclear whether these differences are linked to the AMF strain, the host plant, the growth conditions, or to a combination of these factors. Yet in experiments where *G. caledonium* strains of different origins were grown monoxenically on different media and different host plants, they consistently produced few spores (Karandashov et al. 1999), whereas sporulation of *G. intraradices* was high regardless of the host plant and growth medium (St Arnaud et al. 1996; Strullu et al. 1997; Declerck et al. 1998). *Glomus proliferum* was also shown to produce a high number of spores (Declerck et al. 2000). There are other examples of inter-species differences in sporulation, e.g. *Gigaspora margarita* produces only a few spores monoxenically (Diop et al. 1992), whereas *G. versiforme* produces abundant spores after only 1 month in monoxenic culture (Declerck et al. 1996). These observations suggest that in closed monoxenic cultivation systems with a determined life span in terms of nutrient availability and host growth, the strains which have a short lag time (λ) and high maximal growth rate (μ_m) would be the best adapted for sporulation and therefore for the mass production of spore inoculum.

Diop et al. (1992) observed a strong correlation between root infection and spore production in *Gigaspora margarita* grown in vitro on transformed carrot roots.

This was confirmed by Declerck et al. (1996) with *G. versiforme*. These authors observed that the extraradical spore production was strongly correlated with the root colonization, i.e. the amount of intraradical mycelium and vesicles, suggesting a relationship between the intraradical spread and development of the fungus and sporulation. Therefore, modelling the spore production, as developed in the present study, could be investigated as an indicator of the internal colonization of the root, i.e. the inoculum potential. Such a study should be conducted on various strains and on a time-course basis to decipher the relationships between the intra- and extra-radical phase of the fungus and to give an estimate of the total inoculum potential of an in vitro culture.

In conclusion, the sporulation dynamics of three *Glomus* strains grown monoxenically on a well-defined growth medium were found to follow a sigmoidal curve, as classically shown for non-symbiotic fungi (Griffin 1994). In most cases the Gompertz model described the sporulation dynamics as accurately as the four-parameter Schnute model, whatever the test used to compare them. This function thus represents an easy-to-use, robust, and adequate tool for the modelling of sporulation dynamics. Its use can facilitate spore counting, help select abundantly sporulating species, and help describe the impact of environmental factors.

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