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Comparison of methods for the assessment of growth of food spoilage moulds in solid substrates

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Abstract

The objective of the study was to evaluate the general suitability of ergosterol content, CFU, and colony diameters determinations for a range of fungi representing food spoilage moulds for the assessment of their growth on solid substrates, in particular intermediate moisture foods. Sixteen food-borne mould species were inoculated onto DG18 agar overlaid with cellophane, allowing determination of a direct measurement of biomass density weighing. The samples were also evaluated with regard to visible and microscopic colony diameters, total ergosterol content, Thoma counts, viable CFU counts, and so on. The same parameters were assayed in the spore suspensions obtained from those cultures. Data were evaluated by multivariate data analysis using projection methods such as principal component analysis (PCA), showing some groupings among the measured variables, mainly linked to the sporulating/nonsporulating nature of the different species tested. Ratios among the different variables were obtained, compared among the species, and evaluated along time (2–10 days). It was concluded that, as a general rule for all the species, ergosterol content and colony diameters were better correlated to fungal biomass dry weight than CFU counts were. Conversion factors were 0.3–3 μg ergosterol mg^{-1} biomass dry weight and 76–227 mg biomass dry weight cm^{-2}, depending on the species.

Keywords: Food; Fungal biomass; Colony diameter; Ergosterol; Biomass dry weight

1. Introduction

Fungi are important spoiling agents of foods of intermediate moisture content. Some moulds are capable of producing toxic metabolites that may be carcinogenic and therefore constitute a public health risk, whereas the growth of spoilage fungi results in nutritional and chemical changes and poor appearance and food flavour development, leading to consumer rejection (Gibson and Hocking, 1997).

Predictive modelling of filamentous fungal growth has not received the same level of attention as that of bacterial growth. This may be well because of the inherent complexities associated with the quantification of fungal growth. There is no rapid or simple

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method of gaining estimates of growth with respect to time. Measurement of hyphal extension rate, usually reported as radial growth rate (μm h^{-1}, mm d^{-1}), is probably the simplest and most direct measure. In the case of filamentous fungi, estimation of growth is more complicated owing to the formation of surface colonies and also of hyphae throughout the food; a cell count is not appropriate. Mould spoilage is often visible in the form of surface colonies; consequently, mould growth has been studied by measuring colony diameters on agar plates over time. A growth rate function can be derived by plotting colony diameter against time and measuring the slope of the straight part of the line. The colony or radial growth rate obtained under various conditions can then be modelled (Gibson and Hocking, 1997).

When developing new methods for fungal quantification, the target is having a good correlation with fungal biomass, which is impossible to be directly quantified in food systems.

Ergosterol is a fungal-specific membrane lipid (Weete, 1980). Seitz et al. (1977) pioneered an HPLC method for quantification of ergosterol that has become widely used for estimating the degree of fungal infection in grain and other plant materials. A number of studies have been made of the relationship of ergosterol to dry weight of mycelia (Seitz et al., 1979; Gessner and Chauvet, 1993; Schnurer, 1993; Newell, 1994). Ergosterol content has been mainly assayed in cereal samples as an indicator of both fungal growth and mycotoxins presence. Different investigators proposed a relationship between ergosterol content of grains and the presence of moulds (Seitz and Pomeranz, 1983; Schwadorf and Muller, 1989; Torres et al., 1992). The determination of ergosterol is also valuable in correlating fungal activity to synthesis of fungal secondary metabolites such as aflatoxins and ochratoxin A (OTA) (Gourama and Bullerman, 1995; Saxena et al., 2001). Good positive correlation has been observed among moisture content of barley, ergosterol content, OTA, and total CFU and CFU of *Aspergillus*, *Penicillium*, and *Eurotium* but not with CFU of *Fusarium* and deoxynivalenol (DON) content in stored barley samples (Olsson et al., 2002).

To date, comparisons between fungal quantification methods have rarely taken into account the effect of the different fungal species present in the convenience of using one method or another. Moreover, few efforts have been devoted to try and explain the results obtained by each method as a function of the fungal development stage. The present study aimed to evaluate the general suitability of ergosterol content, CFU, and colony diameters determinations for a range of fungi representing food spoilage moulds for the assessment of their growth in solid substrates. Complementary parameters were determined to help understand the impact of physiology and time in the usefulness of such determinations.

2. Material and methods

2.1. Fungal strains

The strains belonged to 16 fungal species representing general common food contaminants found in intermediate moisture foods: *Alternaria alternata* (Fr.) Keissl. CECT2662, *Aspergillus carbonarius* (Bainier) Thom CECT2086, *Aspergillus flavus* Link CECT2687, *Aspergillus ochraceus* K. Wilh. NRRL3174, *A. parasiticus* Speare CECT2688, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries CECT2110, *Eurotium amstelodami* L. Mangin CECT2586, *E. herbariorum* Link CECT2922, *Fusarium graminearum* Schwabe CECT2150, *F. verticillioides* (Sacc.) Nirenberg 25N, *Mucor racemosus* Fresen. CECT, *Penicillium chrysogenum* Thom CECT2802, *P. expansum* Thom CECT2278, *P. griseofulvum* Dierckx CECT2605, *P. verrucosum* Dierckx CECT2906, and *Rhizopus oryzae* Went and Prins. Geerl. (CECT: Spanish Type Culture Collection).

2.2. Preparation of medium, inoculation of the strains, and incubation

The medium used in this study was DG18 (glucose, 10 g; peptone, 5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; glycerol, 220 g; agar, 15 g; dichloran, 2 mg; chloramphenicol, 100 mg; distilled water, 1 l), and it was chosen because of its relatively low water activity (0.955), nearer to that of intermediate moisture foods. Sterilised cellophane membranes were placed on top of the DG18 medium (20 ml per 9-cm Petri plate). It has been shown that cellophane allows the fungus to

obtain nutrients from the substrate. Previous studies have shown that the growth rate is very similar on media with and without the cellophane layer (Ramos et al., 1999).

The strains were grown on DG18 without cellophane for 14 days, and suspensions (10^6 CFU ml^{-1}) were prepared in 0.005% Tween 80 solutions. Twenty-four cellophane-covered agar Petri plates were single-point inoculated (10^2–10^3 CFU) in the middle of each for each fungal suspension. Thus, a total number of 408 plates were incubated at 25 °C. After 2, 4, 7, and 10 days of incubation, six plates for each strain were taken, numbered at random from 1 to 6, and processed as follows.

Plates 1 and 2 were separately analysed for:

- Visible colony diameters.
- Microscopic colony diameters, using a binocular magnifier.
- Fungal biomass. The colony was separated from the cellophane layer, collected in a preweighed and predried filter paper, and weighed.
- Dry fungal biomass. The filter paper plus the colony were dried at 105 °C for 2.5 h (Pasanen et al., 1999), allowed to cool in a desiccator, and weighed.
- Ergosterol content. The dry colony on the filter paper was analysed for ergosterol content as described later.

Plates 3 and 4 were separately analysed for:

- Colony forming units from spores. The colony was separated from the cellophane layer and collected in a sterile flask containing 10 ml of 0.005% Tween 80 and stirred for 10 min. The suspension was passed through a cotton wool layer to retain hyphae and washed with further 0.005% Tween 80 until collection of up to 10-ml spores suspension. A 1-ml aliquot was taken for suitable dilutions and surface plating (0.1 ml) on DG18 plates, distributed evenly by stirring with a Drigalski spatula, and incubated at 25 °C for 7–10 days to assess the concentration of colony forming units.
- Spores dry weight. The remaining 9-ml spores suspension was vacuum-filtered through a preweighed membrane filter (pore size 0.45 μm), dried at 105 °C for 2.5 h (Pasanen et al., 1999), allowed to cool in a desiccator, and weighed.
- Ergosterol content of the spores. The dry spores on the membrane filter were analysed for ergosterol content as described below.

Plates 5 and 6 were separately analysed for:

- Spores count in Thoma chamber. The colony was separated from the cellophane layer and collected in a sterile flask containing 10 ml of 0.005% Tween 80 and stirred for 10 min. A drop of the resulting suspension was used for spores count in a Thoma chamber.
- Colony forming units. Suitable dilutions (estimated from the Thoma chamber counts) were surface-plated (0.1 ml) on DG18 plates, spread, and incubated at 25 °C for 7–10 days to assess the concentration of colony forming units.

2.3. Determination of ergosterol content

A modification of the method by Gourama and Bullerman (1995) was applied. Recovery rates were around 84% for the concentrations found in the study. The filters plus the fungal material were made into smaller pieces and collected in 100-ml Erlenmeyer flasks. Forty millilitres of 10% KOH in methanol were added and magnetically stirred for 30 min. A 10-ml aliquot was transferred to a screw cap tube and placed in a hot water bath (55–60 °C) for 20 min. The tubes were then allowed to cool to room temperature. Three millilitres of water and 2 ml of hexane were added to the tubes, which were then agitated in a Vortex mixer for 1 min. After separation of layers, the upper layer (hexane) was transferred to a 10-ml vial. Hexane extraction was repeated twice using 2 ml each time. The extracts were combined and evaporated to dryness under a stream of nitrogen. The dry extracts were dissolved in 2 ml of methanol and forced through 0.45-μm acetate filters. The HPLC equipment consisted of a Waters 515 isocratic pump (Waters Associated, Milford, MA), a Waters 717plus autoinjector, and a Waters Spherisorb ODS2 C18 column (4.6×250 mm). The Waters 2487 variable wavelength UV detector was set at 282 nm. The mobile phase was methanol at 1 ml min^{-1}. Ergosterol standard was purchased from Sigma (St. Louis, Mo).

2.4. Statistical analyses of the results

The Unscrambler® version 7.6 was used for the multivariate analysis of the resulting data matrix. Principal component analysis (PCA) allowed for an overview of the results and for establishing correlations among the different variables as a function of the fungal species tested. These techniques extract information from data with many variables (*m* variables) using all the variables simultaneously. In PCA, *m* axes will define an *m* dimensional space in which each sample (object) can be described by a point. The whole set of samples will be defined as a swarm of points in the *m* dimensional space. The first principal component (PC) describes the direction through the swarm that explains the largest variation of the data. The second PC is orthogonal to the first one and explains the second largest variation, etc. The main results from PCA are the score and loading plots. The score plot shows how the samples are related to each other, while the loading plot shows how the variable relate to each other (Olsson et al., 2000).

Analysis of variance (ANOVA) for the ratios obtained and Duncan tests were performed by using SAS version 8.02 (SAS Institute, Cary, NC, USA). Statistical significance was judged at the 5% level. Finally, correlations among variables and the associated plots were done in Microsoft Excel 2000.

3. Results

3.1. Visual observations of fungal growth on DG18

Colonies grown on DG18 may differ morphologically from those typically grown on MEA or CYA. Characteristics such as the degree of sporulation, development of dense mycelia, or growth rate (Table 1) are critical for the interpretation of the results of this study.

Table 1
Morphological observations of growing colonies on DG18

	Degree of sporulation	Mycelium development	Growth rate
A. alternata	−	+	−
A. carbonarius	+	+	−
A. flavus	++	+	++
A. ochraceus	++	+	++
A. parasiticus	+	+	++
C. cladosporioides	+	+	−
E. amstelodami	+	+	−
E. herbariorum	−	+	+
F. graminearum	−	−	−
F. verticillioides	+	+	+
M. racemosus	+	−	++
P. chrysogenum	−	−	+
P. expansum	−	−	+
P. griseofulvum	−	−	−
P. verrucosum	−	−	−
R. oryzae	+	−	++

Degree of sporulation: ++, heavy sporulated; +, sporulated; −, nonsporulated.
Mycelium development: +, dense and floccose; −, scarce.
Growth rate: ++, fast; +, medium; −, slow.

3.2. Correlated variables and sample grouping

A first PCA overview of the results, including all sampling times and fungal species, showed a high significance of PC1 that explained 73% of the total data variance. The scores of the samples on this PC increased from negative to positive values with increasing sampling times. Most of the samples taken after 2 days of incubation had negative scores and were quite grouped together, with no structure being observed among them as a function of fungal species. As for day 2, there were many null values, further analysis was carried out with the remaining sampling times: 4, 7, and 10 days. In this way, similar trends were observed, but the plots looked much clearer.

All the measured variables had positive scores in PC1 (Fig. 1a), meaning that this PC, which explained 63% of the total variance, was oriented from slow growing to less slow growing samples, in terms of the whole set of 'growth variables.' PC2 (18%), however, divided the variables into two groups, the first one containing Thoma counts, spores dry weight, number of CFU, and ergosterol content of the spores ('sporulation' variables); on the other hand, fungal biomass (either dry or not) and colony diameters were highly correlated among them, forming a second group. Total ergosterol was not explained at all by PC2. As a general rule, the first group of variables represented growth of all *Penicillium* species plus *F. verticillioides*, *A. ochraceus*, and *A. flavus*, while the

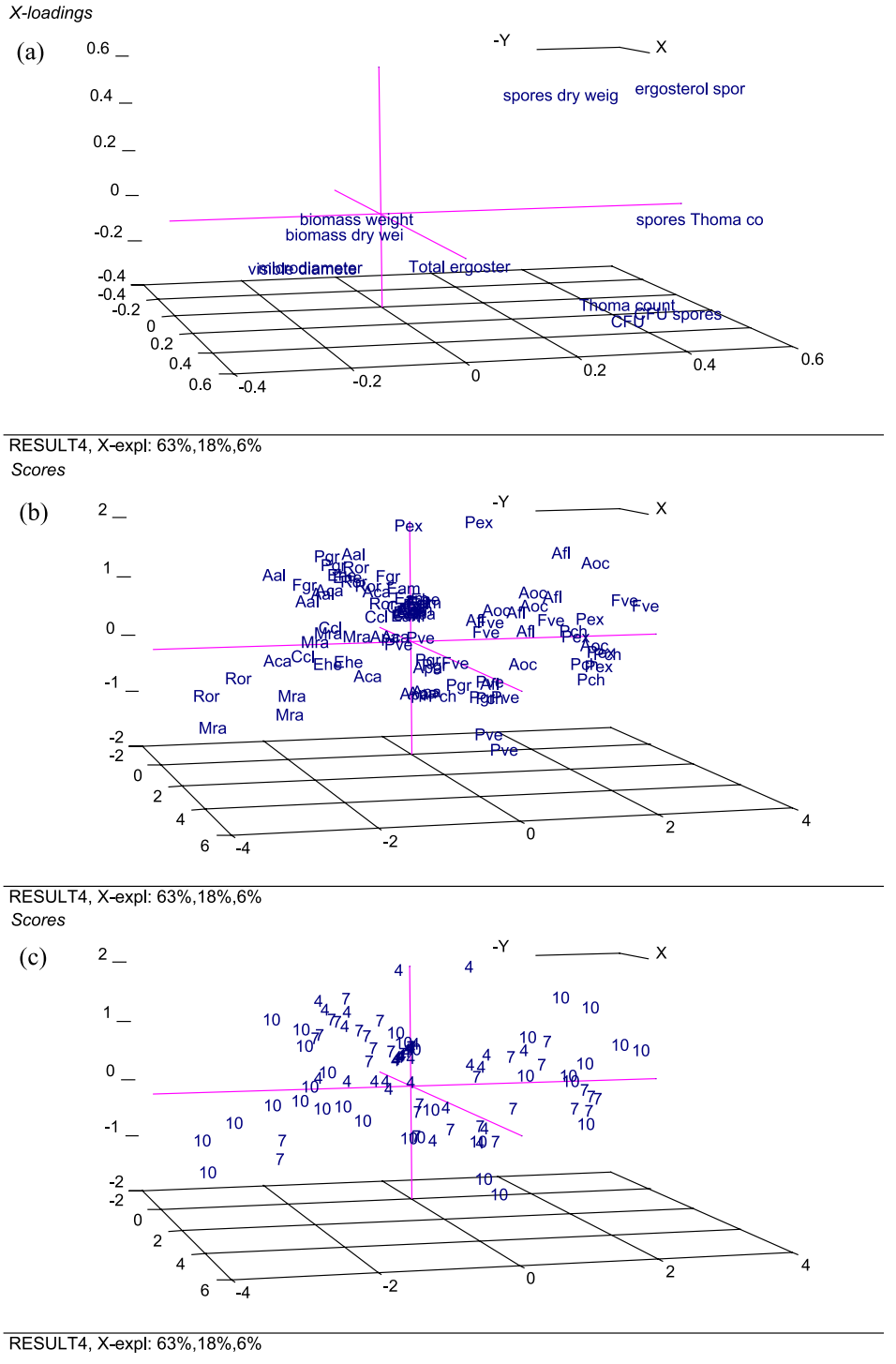

Fig. 1. Loadings (a) and scores (b, c) plots resulting from the PCA of the growth variables for the range of 17 fungi, considering incubation times of 4, 7, and 10 days.

second group included the less sporulating species: *M. racemosus*, *R. oryzae*, *A. carbonarius*, *A. alternata*, *E. herbariorum*, *A. parasiticus*, and *C. cladosporioides* (Fig. 1b). The remaining species were not either predominantly best represented by any of the 'growth variables' used or their growth was not significant. The third PC (6%) differentiated among the sporulating species, those with high CFU and Thoma counts from those with higher spores dry weight and ergosterol content in the spores. Higher counts corresponded, in general, to *Penicillium* species, while *A. ochraceus*, *A. flavus*, *F. verticillioides*, and *P. expansum* showed high spores dry weights and ergosterol content with no such high counts. Samples with low scores in all PCs were in general those of day 4, while samples of day 10 had higher scores, either positive or negative depending on their growth characteristics (Fig. 1c). The samples which were not explained by the model (scores close to 0) may have been explained by some of the variables if they had grown significantly (*E. amstelodami* <10 mm colonies in 10 days).

Table 2 shows the actual values for biomass dry weight, ergosterol content, and CFU, all increasing with time. Higher biomass dry weight values corresponded to *M. racemosus*, *A. ochraceus*, *A. flavus*, and *P.chrysogenum*, the higher CFU counts to *A. flavus*, *A. ochraceus*, *F. verticillioides*, and *P. chrysogenum*, and, finally, higher ergosterol contents were found for *A. flavus*, *A. ochraceus*, *F. verticillioides*, *P. chrysogenum*, *P. expansum*, and *M. racemosus*.

3.3. Ratios among measured variables

The significance of the measured variables for the different species, as given in the previous section, was obviously biased by the differences in growth rates. In this section, ratios are reported instead of absolute values for direct comparison.

Table 3 shows some of the mean ratios among variables studied for incubation times from 4 to 10 days. Time had a significant effect in most of the ratios, but the means for the whole period have been used in the table for summarizing purposes. The first five columns may help in the interpretation of the remaining ones.

As a general rule, those species observed in the previous section to be more related to sporulation were those with smaller spores. From the total dry weight of the colonies, spores accounted for were from 1% to 15%, being *A. alternata* the one with the 1% as most of its biomass corresponded to mycelium in those early stages; on the other hand, *Eurotium* species and some other *Aspergillus* species had the highest percentages, suggesting for these genera either a higher concentration of spores or heavier ones or both things simultaneously. Similarly, column 4 gives an idea of the relative contribution of spores/hyphal fragments to CFU. Those species with ratios near 1 are those in which the CFU technique is mainly a measurement of sporulation (*A. flavus*, *A. ochraceus*, *P.chrysogenum*, *P. griseofulvum*, *P. verrucosum*, and *F. verticillioides*), while those species that have values minor than 1 have sources other than spores for colony formation such as hyphal fragments (*A. alternata*, *R. oryzae*, and *F. graminearum*). In the former species, the colonies resulting from hyphal fragments are negligible in front of those from spores.

Significant differences were found only for a few species in terms of their biomass/diameter ratio; as shown before, both variables are well correlated. Those species showing a high ratio must have denser growth structures. A high concentration of spores per millimetre could be the reason for the slightly higher ratios for *A. ochraceus*, *P. chrysogenum*, and *P. expansum*.

Regarding the ratio CFU/diameter, the situation was quite different as there were highly significant differences among the different species. Thus, the heavy sporulating species suggested in the previous section, such as *A. flavus*, *A. ochraceus*, *F. verticillioides*, *P. chrysogenum*, *P. expansum*, and *P. verrucosum* showed higher ratios, and those with a poor ratio spores/mycelium such as *F. graminearum*, *A. alternata*, and *Eurotium* species showed lower ratios; this suggests that most of the CFU observed on the plates came from spores and not from hyphal fragments. However, regarding the ratio CFU/biomass, besides the differences in biomass density, the fact that *R. oryzae* and *C. cladosporioides* have relatively high ratios may imply their ability to produce new colonies from hyphal fragments.

Ergosterol content showed quite constant rates to biomass weight for the different species (about

Table 2
Effect of incubation time on biomass dry weight, CFU, and ergosterol content attained by the 16 species tested

Incubation time (d)	2			4			7			10			*R*	
	Biomass dry wt (mg)	Log CFU	Ergosterol (μg)	Biomass dry wt. (mg)	Log CFU	Ergosterol (μg)	Biomass dry wt. (mg)	Log CFU	Ergosterol (μg)	Biomass dry wt. (mg)	Log CFU	Ergosterol (μg)	Ergosterol/ biomass dry wt.	CFU/ biomass dry wt.
A. alternata	–	–	–	2.3±2.6	–	–	3.4±4.8	2.8±1.1	2.3±3.3	29.0±1.3	3.1±1.6	4.7±1.2	0.68	0.62
A. carbonarius	–	–	–	6.0±3.5	2.0±0.0	3.3±4.7	13.9±6.2	3.1±1.1	11.4±9.3	49.3±17.8	5.7±1.6	70.3±34.5	0.99	0.79
A. flavus	–	–	–	7.9±3.7	7.3±0.4	21.6±1.7	40.4±6.6	7.6±0.1	25.7±3.5	66.0±12.3	8.5±0.1	41.5±8.3	0.91	0.71
A. ochraceus	–	–	38.3±0.7	3.4±0.8	6.6±0.0	18.6±0.0	40.7±23.2	8.2±0.2	41.4±11.6	73.1±22.9	8.3±0.1	54.9±24.2	0.82	0.66
A. parasiticus	–	2.4±0.3	–	1.1±1.5	4.9±0.1	4.1±0.1	17.9±1.3	7.1±1.0	12.3±1.3	56.9±18.8	7.1±0.1	32.0±12.5	0.99	0.67
C. cladosporioides	–	–	–	–	–	–	5.5±3.6	4.6±0.1	18.9±0.02	37.4±23.8	4.6±0.2	25.8±5.2	0.79	0.61
E. amstelodami	–	–	–	4.6±5.0	–	–	6.1±5.7	3.0±0.0	–	6.3±0.8	4.0±0.0	9.3±0.3	0.32	0.60
E. herbariorum	–	–	–	3.9±4.1	–	–	5.4±1.7	3.2±0.2	5.7±0.2	41.7±7.9	6.0±0.1	14.6±2.1	0.94	0.90
F. graminearum	–	–	–	–	–	–	–	2.5±0.8	–	15.3±7.6	2.1±0.2	2.6±3.6	0.89	0.40
F. verticillioides	–	1.3±0.4	–	3.5±0.9	6.4±0.4	18.8±0.0	20.2±8.2	7.6±0.4	25.7±4.1	45.7±14.7	8.7±0.1	34.0±5.2	0.86	0.72
M. racemosus	7.8±0.1	1.6±0.1	–	8.3±0.6	5.1±0.0	19.4±0.5	44.1±5.8	6.2±0.1	22.3±1.0	92.4±13.2	6.8±0.4	33.1±1.0	0.78	0.74
P. chrysogenum	9.3±6.4	1.4±0.1	–	8.5±6.2	7.2±0.3	20.0±0.4	24.8±3.3	7.5±0.2	29.2±1.6	58.7±25.7	8.3±0.3	42.4±6.0	0.71	0.50
P. expansum	–	–	18.6±0.0	–	5.5±0.5	20.7±1.8	17.6±5.7	8.8±0.4	26.4±5.3	31.0±6.4	7.7±0.4	35.9±0.8	0.96	0.73
P. griseofulvum	4.8±1.6	–	–	4.6±2.3	2.3±0.5	3.9±0.2	6.9±0.6	6.4±0.5	11.0±7.6	24.3±4.2	7.1±0.2	18.1±14.7	0.75	0.70
P. verrucosum	–	1.2±0.2	2.1±2.9	2.6±1.4	5.6±0.0	5.7±0.7	14.7±6.0	7.2±0.6	18.2±7.9	32.5±1.8	8.0±0.5	49.5±6.5	0.99	0.78
R. oryzae	–	–	–	3.8±4.3	3.9±0.0	–	5.1±5.9	4.2±0.3	3.0±4.2	73.8±15.6	–	48.6±2.5	0.97	0.67

R, correlation coefficient; –, <limit of detection.

Table 3
Ratios between some of the different growth variables assayed for the 16 species

	Conidial size (μm)	Spores dry wt./biomass dry wt.	Log CFU spores/log CFU	Biomass/ diameter (mg mm^{-1})	Biomass density (mg cm^{-2})	CFU/ diameter (mm^{-1})	CFU/ biomass (mg^{-1})	Ergosterol/ diameter (μg mm^{-1})	Ergosterol/ biomass (μg mg^{-1})	Ergosterol/ CFU (μg)	Ergosterol spores/spores dry wt. (μg mg^{-1})	Ergosterol spores/CFU spores (μg)	Ergosterol spores/ ergosterol
A. alternata	8–12× 20–40	0.01^{f}	0.71^{ef}	1.66^{cde}	3.4	1.47^{fg}	0.18^{cd}	0.20^{g}	0.25^{c}	1.86^{b}	–	–	–
A. carbonarius	4–5	0.03^{def}	0.80^{cde}	2.48^{abcde}	8.5	2.46^{de}	0.16^{cd}	1.45^{abcd}	0.48^{bc}	7.46^{a}	–	–	–
A. flavus	3.5–5	0.12^{abc}	0.95^{abc}	2.75^{abcd}	2.3	6.18^{a}	0.13^{d}	0.75^{defg}	0.41^{bc}	3.76^{ab}	10.18^{bc}	2.67^{bcd}	0.66^{bc}
A. ochraceus	2.5–3.5	0.12^{abcd}	0.97^{ab}	3.21^{a}	2.9	6.27^{a}	0.14^{d}	1.24^{bcdef}	0.50^{bc}	4.83^{ab}	5.45^{bc}	2.84^{b}	0.69^{b}
A. parasiticus	4–6	0.05^{bcdef}	0.92^{abc}	2.10^{abcde}	3.3	4.95^{b}	0.25^{bcd}	0.50^{fg}	0.31^{bc}	2.37^{b}	0.94^{c}	0.69^{f}	0.51^{bcd}
C. cladosporioides	2–4×10–30	0.06^{bcdef}	0.91^{abc}	1.89^{bcde}	6.2	2.34^{e}	0.46^{abc}	1.83^{abc}	1.94^{a}	4.82^{ab}	–	–	–
E. amstelodami	4–5	0.15^{a}	–	1.54^{de}	15.6	1.95^{ef}	0.39^{abcd}	1.33^{abcde}	1.00^{abc}	2.32^{b}	–	–	–
E. herbariorum	6–7	0.13^{ab}	0.87^{abcd}	1.93^{bcde}	4.4	2.23^{e}	0.15^{cd}	0.46^{fg}	0.27^{c}	2.12^{b}	–	–	–
F. graminearum	No microconidia	–	0.76^{def}	2.14^{abcde}	4.2	0.90^{g}	0.12^{d}	0.21^{g}	0.10^{c}	2.23^{b}	–	–	–
F. verticillioides	1.5–4.5× 4.3–19	0.12^{abc}	0.93^{abc}	2.04^{abcde}	3.5	6.23^{a}	0.52^{ab}	1.09^{cdef}	1.60^{ab}	3.40^{b}	26.22^{a}	2.90^{b}	0.76^{b}
M. racemosus	5–8	0.04^{cdef}	0.88^{abcd}	1.93^{bcde}	1.4	4.32^{bc}	0.10^{d}	0.48^{fg}	0.39^{bc}	4.09^{ab}	4.63^{bc}	2.72^{bc}	0.59^{bc}
P. chrysogenum	2.5–4	0.06^{bcdef}	1.01^{a}	3.05^{ab}	3.7	6.27^{a}	0.14^{d}	1.18^{bcdef}	0.48^{bc}	3.93^{ab}	9.33^{bc}	2.31^{cd}	0.56^{bcd}
P. expansum	3–3.5	0.11^{abcde}	0.82^{bcde}	2.83^{abc}	5.9	6.22^{a}	0.30^{bcd}	2.05^{a}	1.10^{abc}	3.81^{ab}	8.73^{bc}	3.95^{a}	0.80^{b}
P. griseofulvum	3–3.5	0.02^{ef}	0.99^{a}	1.39^{e}	6.4	4.14^{c}	0.29^{bcd}	0.65^{efg}	0.55^{bc}	1.99^{b}	15.67^{ab}	1.63^{e}	0.31^{cd}
P. verrucosum	2.5–3	0.03^{def}	0.97^{ab}	1.98^{bcde}	5.2	6.78^{a}	0.39^{abcd}	1.34^{abcde}	0.70^{bc}	3.24^{b}	15.60^{ab}	0.75^{f}	0.22^{d}
R. oryzae	5–8	0.07^{bcdef}	0.76^{def}	1.61^{de}	2.8	3.17^{d}	0.68^{a}	0.55^{efg}	0.27^{c}	1.36^{b}	25.15^{a}	2.27^{d}	1.41^{a}

Different letters within a column mean significant differences among values.

0.3–1.1 μg ergosterol mg^{-1} biomass). Only *C. cladosporioides* seemed to have an ergosterol/biomass higher than the other species (1.94), while *A. alternata*, *E. herbariorum*, *F. graminearum*, and *R. oryzae* had ratios slightly lower. The trend was the same for ergosterol/diameter ratios, but the differences were wider. Interestingly, small differences were also found among the species in their ratios ergosterol/CFU, suggesting that ergosterol may provide a rough estimation of both aspects of fungal growth (mycelium and sporulation).

It was difficult to detect ergosterol in the spores because the total weight of harvested spores was low, thus no results could be collected for the slow-growing or poor-sporulating species *A. alternata*, *A. carbonarius*, *C. cladosporioides*, *A. amstelodami*, *E. herbariorum*, and *F. graminearum*. For the remaining ones, spores of *F. verticillioides* or *R. oryzae* seemed to be richer in ergosterol than the others, and those from *A. parasiticus* poorer than the mean values, but the differences were not high. Interestingly, although the ratio ergosterol/CFU was quite constant, the ratio obtained between the ergosterol found in the spores and the CFU obtained from those spores was quite variable, probably suggesting that the mycelia account for a significant part of the ergosterol. *P. expansum*, *F. verticillioides*, and *A. ochraceus* had values higher than mean, and *A. parasiticus* and *P. verrucosum*, lower. Finally, the total ergosterol that contained in the spores was low if compared with the high percentage of CFU from spores in the total CFU numbers.

3.4. Ergosterol/dry mass weight, CFU/dry mass weight, and colony diameter/dry mass weight ratios

Pearson correlation coefficients were higher for ergosterol/biomass dry weight correlations than for CFU/biomass dry weights ones, except for *E. amstelodami* which had no detectable amounts of ergosterol until day 10 (Table 2). CFU, although in low levels, could be counted in the plates at the seventh day for this species. The best correlations for both variables were found in the seventh day (data not shown). The mean variation coefficients for biomass dry weight, log CFU, and ergosterol analysis were 46%, 7%, and 35%, respectively. While, for biomass dry weight, the replicates were quite different in general (the colonies grew quite differently from one replicate to another), the standard deviation of ergosterol analysis was low, except for some abnormal cases. Fig. 2 shows the correlation between biomass dry weight and ergosterol content for the different species; the ratio varied for most of the species from 0.6 to 1.8 mg dry biomass

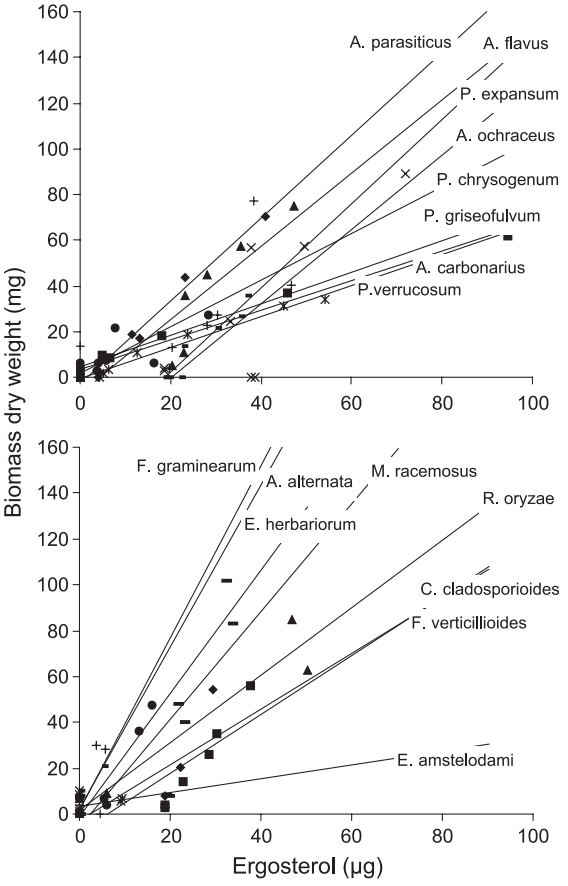

Fig. 2. Correlations between ergosterol content and biomass dry weight *A. parasiticus* $y=1.8144x-3.012$ $R^2=0.9826$; *A. carbonarius* $y=0.6428x+3.6494$ $R^2=0.9811$; *A. flavus* $y=1.6034x-7.031$ $R^2=0.8194$; *A. ochraceus* $y=1.6283x-33.056$ $R^2=0.6679$; *P. verrucosum* $y=0.6776x-0.3253$ $R^2=0.9763$; *P. griseofulvum* $y=0.693x+4.4606$ $R^2=0.5552$; *P. chrysogenum* $y=1.0179x+1.9565$ $R^2=0.4981$; *P. expansum* $y=1.8402x-34.667$ $R^2=0.93$; *F. graminearum* $y=3.7487x+1.4286$ $R^2=0.7913$; *A. alternata* $y=3.5155x+2.5117$ $R^2=0.4614$; *E. herbariorum* $y=2.6803x-0.8759$ $R^2=0.8884$; *R. oryzae* $y=1.4705x+1.7305$ $R^2=0.9481$; *C. cladosporioides* $y=1.2147x-2.8386$ $R^2=0.6295$; *F. verticillioides* $y=1.2783x-7.7185$ $R^2=0.7344$; *E. amstelodami* $y=0.2998x+3.5792$ $R^2=0.1043$; *M. racemosus* $y=2.3418x-5.6586$ $R^2=0.6407$.

μg^{-1} ergosterol. Slow-growing species had variable rates, such as 1.6–3.7 for *F. graminearum*, *A. alternata*, and *E. herbariorum* and about 0.3 for *E. amstelodami*. Similar Pearson correlation coefficients were found between biomass dry weight and colony diameters (Fig. 3).

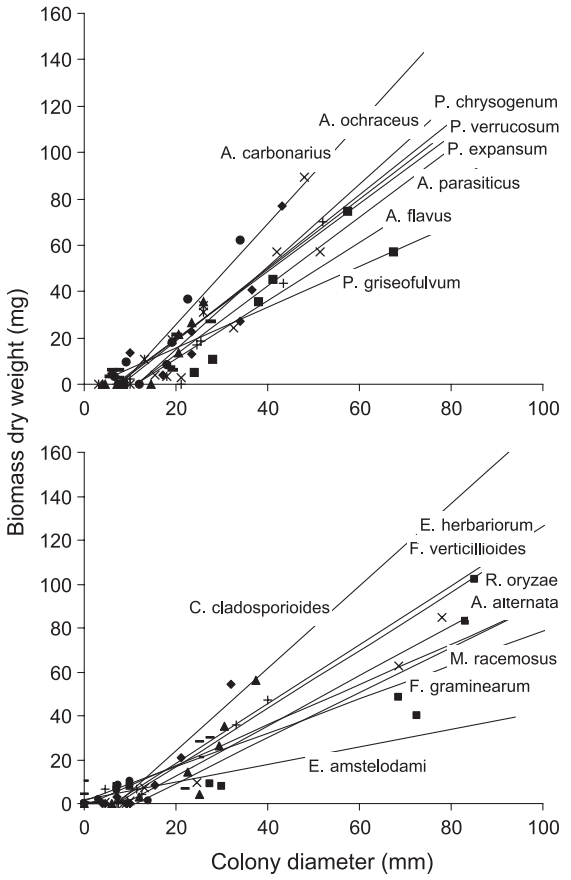

Fig. 3. Correlations between diameter under magnifier and biomass dry weight *A. Parasiticus* $y=1.4786x-16.406$ $R^2=0.9097$; *A. carbonarius* $y=2.1724x-17.582$ $R^2=0.867$; *A. flavus* $y=1.256x-14.286$ $R^2=0.8671$; *A. ochraceus* $y=1.78x-20.959$ $R^2=0.8455$; *P. verrucosum* $y=1.5141x-10.898$ $R^2=0.808$; *P. griseofulvum* $y=0.8809x-2.0343$ $R^2=0.671$; *P. chrysogenum* $y=1.5821x-12.943$ $R^2=0.7306$; *P. expansum* $y=1.4616x-9.8777$ $R^2=0.8189$; *F. graminearum* $y=0.7709x+1.4286$ $R^2=0.7913$; *A. alternata* $y=0.9232x-1.1665$ $R^2=0.7534$; *E. herbariorum* $y=1.3488x-8.4087$ $R^2=0.9487$; *R. oryzae* $y=1.1327x-9.8694$ $R^2=0.9778$; *C. cladosporioides* $y=1.8741x-13.379$ $R^2=0.8984$; *F. verticillioides* $y=1.3225x-9.5702$ $R^2=0.7221$; *E. amstelodami* $y=0.4023x+1.8362$ $R^2=0.2397$; *M. racemosus* $y=1.0319x-11.316$ $R^2=0.8138$.

4. Discussion

This is quite a basic study: the response of a range of 16 food-borne fungi to different classical methods and parameters to estimate their growth on solid substrates, either for research purposes or for food quality control at an industrial scale. Its aim is providing explanation to some of the conclusions drawn by several researchers for a restricted number of species (Schnurer, 1993; Gourama and Bullerman, 1995; Wyatt et al., 1995). Other studies exist on the comparison among some of these methods; however, most of them were carried out under optimal growth conditions (Schnurer, 1993; Gourama and Bullerman, 1995; Reeslev and Kjoller, 1995). In the present one, emphasis was put in the fact that fungi usually grow in intermediate moisture foods (when nonacidic), thus, under suboptimal water requirements, DG18 (a_w=0.955) was chosen for the experiments. From our experience, the water activity of the medium determines the physiology of the moulds, affecting the mycelia growth/sporulation balance expected for a certain fungus in a reference medium such as MEA, for example, *A. carbonarius* or *C. cladosporioides* in our study. Still, there is a drawback in this study, and this is the composition and structure of the medium, which are obviously different from those of food products. However, synthetic medium was chosen in a first step for two reasons: (i) we were interested in working with a wide range of fungal species to allow comparison; not all food substrates support growth of all these species, so there was no point in choosing a certain food substrate and characterizing their growth in that substrate, and (ii) some of the methods to be tested in this first step could not be applied to food samples, especially biomass determination, which is the reference one, so it could not be omitted. Moreover, some authors chose long incubation times for comparison of methods, but, in our case, short incubation times were used as the aim was to compare the techniques in the early stages of fungal growth, before the moulds in a food are so visible that the product would be immediately rejected. The drawback was that some of the fungi attained little growth in those short periods of time, and no results could be obtained for some of them because they did not sporulate at all or only at the longer times tested (*E. amstelodami*, *F. graminearum*, and so on).

This study highlights the dependence of the results of the methods of analysis not only on the fungal species tested but also on their physiological state, especially on the balance between vegetative growth and sporulation. Biomass dry weight is considered the target value to be indirectly estimated by the different methods (Schnurer, 1993; Bermingham et al., 1995; Reeslev et al., 2003). A good correlation was found in this study between colony diameters and biomass dry weight for the different species (Pearson correlation coefficients=0.67–0.99). This suggests that the widespread use of colony diameters as fungal growth estimator for research purposes in single cultures is a good choice as it is easier to be obtained (Reeslev and Kjoller, 1995) and more accurate than CFU counts. Some inconsistent correlations between the spread rate and dry weights may be caused by fungi producing thicker more dense colonial morphologies (Wyatt et al., 1995). Moreover, in those foods in which spoilage fungi do not involve the production of mycotoxins, such as low-moisture bakery products (Abellana et al., 1997a,b), the rejection will be caused by the visible colonies on their surface; this means a colony diameter resulting in a visible colony. In fungal analysis for the food industry, however, CFU counts are still used, although CFU results depend more on whether the fungi have sporulated than on actual biomass production (Schnurer, 1993), as shown in this study. Finally, ergosterol was a better estimation of biomass dry weight than CFU were. Ergosterol analysis has been successfully applied to cereals for estimation of fungal spoilage (Olsson et al., 2000, 2002; Saxena et al., 2001).

It has been found that a high percentage of the resulting ergosterol belongs to the mycelium (25–80%); this means that mycelium is better represented than in a CFU analysis in which colonies from hyphal fragments were only about 0–30% of the total CFU. Similarly, ergosterol analysis has been suggested for quantitative monitoring of fungi in solid substrates because of the good agreement between hyphal length and ergosterol content and between total ergosterol concentration and mycelial mass, even with more than one fungal species present (Schnurer, 1993; Newell, 1994). Moreover, a correlation between ergosterol content and hyphal length and CFU for nonsporulating fungi on synthetic agar substrate (Schnurer, 1993), as well as between ergosterol and CFU in grain, has been reported (Schnurer and Johnsson, 1992). The drawback is that ergosterol cannot be used to identify species. Ergosterol assay has been found suitable for early detection of mould activity (Gourama and Bullerman, 1995) when compared to microbiological methods such as a serial dilution and plate count technique. However, some authors have criticised as a drawback that ergosterol content does not increase in relation to sporulation, whereas the number of CFU does (Seitz et al., 1977; Schnurer, 1993); CFU, however, should not be regarded as a reference method. It has been stated that a correlation should be done between mycelial weight and ergosterol (Gourama and Bullerman, 1995).

In general, no constant ratios among parameters for each species could be obtained as they varied with time. Ratios such as ergosterol content/colony diameter did not change with time, while, for example, ergosterol content/biomass, ergosterol content/CFU, biomass/colony diameter, and CFU/colony diameter increased with time, and CFU/biomass decreased with it. Similarly, two-way ANOVA revealed statistically significant interspecific variation in ergosterol/biomass content in the mycelium for several aquatic hyphomycetes which was dependent upon incubation period (Bermingham et al., 1995); the rank order of a particular species being dependent upon time.

Ergosterol content ranged from 0.3 to 3.3 μg ergosterol mg^{-1} dry biomass, lower values than those observed by other authors for similar periods of time but at higher water availabilities.

Seitz et al. (1979) found an ergosterol content of 2.3 to 5.9 μg mg^{-1} of dry weight for *A. flavus*, *A. alternata*, and *A. amstelodami* in liquid cultures in malt extract medium for 2 to 10 days. Using a gravimetric method for quantifying fungal mass, Nout et al. (1987) determined that the ergosterol content of *Rhizopus oligosporus* when grown in synthetic laboratory liquid medium varied with age from 2 μg mg^{-1} in young cultures to 24 μg mg^{-1} for old ones. Values of 2.6–14 μg ergosterol mg^{-1} fungal dry mass have been found in liquid medium for wood-isolated species (Pasanen et al., 1999). Conversions to dry weights for *Fusarium culmorum*, *Penicillium rugulosum*, and *Rhizopus stolonifer* showed that spores contribute 3% to 5% to total fungal mass. Ergosterol contents were between 0.4 and 1.4 μg mg^{-1} of the calculated fungal mass in 2% malt extract agar for 3

days (Schnurer, 1993). Ergosterol concentrations have been reported as 2 to 14 μg mg^{-1} for *Candida albicans*, *Aspergillus fumigatus*, *A. flavus*, *Aspergillus niger*, *Alternaria* spp., *Cladosporium* spp., and *Penicillium* spp. cultured on Sabouraud dextrose agar for 2 to 4 days (Axelsson et al., 1995).

The standard deviation from the mean ergosterol content/biomass dry weight (1.74) was 0.96 (without taking into account *E. amstelodami* results) due to the interspecific variation for the range of 15 fungi. There must be low interspecific variation in ergosterol content, otherwise measures changes in ergosterol may reflect shifts in the balance of species rather than total biomass. More accurate estimates would be obtained with species-specific factors. Using these in combination with estimates of the proportion of the dominant species has been proposed for estimation of naturally established fungal communities on leaves (Gessner and Chauvet, 1993).

The conversion factors found in the present study were established on agar medium and may not be representative when fungi are growing on different food substrates. Further studies will be carried out involving different food substrates and species to validate the results of the present study. Moreover, correlation between ergosterol and mycotoxin production should be evaluated as it has been found for aflatoxins and OTA (Gourama and Bullerman, 1995; Saxena et al., 2001). The fact that it has been shown that fungal biomass is not necessarily correlated to mycotoxin accumulation in substrates adds more complexity to the problem. It is thus interesting to find among the existing methods which ones have better correlation with mycotoxin presence.

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