Mycol. Res. **99** (3): 325–329 (1995) *Printed in Great Britain*

Induction of precocious fruiting by a diffusible factor in the polypore *Phellinus contiguus*

GILLIAN M. BUTLER

School of Biological Sciences, University of Birmingham, Birmingham B15 2TT, U.K.

Malt extract agar in diffusion contact with growing colonies of *Phellinus contiguus* developed the capacity to induce precocious fruiting in new colonies of the same fungus. Spent agar from beneath colonies of *P. contiguus* growing on top of Cellophane, when incorporated into 1% malt extract agar, significantly enhanced the extent of macroscopically visible poroid hymenophore tissue on 10 mm assay discs after incubation for approximately 14 d. The factor was heat stable and its activity increased with increasing concentration, over the range × 0·125 to × 0·5 of the *in situ* concentration. The factor was produced by both dark- and light-incubated colonies but light was required for expression of the fruiting response. The significantly lower activity present in diffusates from 6-d-old than 21-d-old colonies suggests that this factor is responsible for the minimum colony size required for competence to fruit.

The techniques of molecular biology have enabled good progress to be made in characterizing fruiting specific genes and their protein products in the most studied basidiomycetous fungi, especially *Schizophyllum commune* (Horton & Raper, 1991; Wessels, 1991). However, the biochemical and physiological mechanisms involved in the developmental processes leading to the mature structure of the basidiome remain poorly understood (Moore, 1994; Watling & Moore, 1994). In fungi in general there is growing evidence that chemical sex factors are involved in the regulation of sexual differentiation (Dyer, Ingram & Johnstone, 1992). Fungal extracts which initiate fruiting or control morphologenesis of the fruit bodies have been described in a taxonomic diversity of fungi. However, Dyer *et al.* (1992) report that the few studies in which the chemicals have been purified and identified have implicated a variety of classes. This chemical diversity occurs even within the Basidiomycotina (Manachère, 1988). Most studies in this group have concentrated on species with a mushroom form of fruiting body, with a pileus and gills, and there is little information on the role of chemical factors in basidiomycetes with other forms of fruit body (Rayner & Boddy, 1988). These latter taxa include many economically important wood-inhabitating fungi and *Phellinus contiguus* (Pers.:Fr.) Pat. is a member of one such genus. In *P. contiguus* the resupinate polyporous basidiome is formed in culture (Butler & Wood, 1988) and information on the role of chemical factors in morphogenesis in this species would be useful.

Butler & Wood (1988) showed that even in the light and in conditions of aeration and temperature favourable to fruiting, colonies of *P. contiguus* growing on 2% malt extract agar tended to have a central area of vegetative mycelium around which the centrifugally-extending fruiting tissue developed. However, in this strain of *P. contiguus* it has been observed that chance satellite colonies growing in the same agar as well established colonies begin to fruit at a smaller size. This suggested the hypothesis that fruiting is induced by a diffusible factor produced by the growing colony. Experiments designed to test this hypothesis are described in this paper.

MATERIALS AND METHODS

The strain of *P. contiguus* and the cultural conditions were as used in previous work (Butler & Wood, 1988; Butler 1988, 1992*a*, *b*). The culture was initially isolated as mycelium from decayed wood. Except where otherwise stated all cultures were grown on 2% malt extract agar (MA) and incubated at 25 °C in a controlled environment room with light for 18 h d^{-1}. Dark-grown cultures were incubated in black paper envelopes and light-grown ones in similar envelopes with transparent acetate windows, or in plastic boxes with aeration holes.

Extract production system

Extracts were prepared from the underlying agar of 'sandwich' cultures by growing *P. contiguus* from a central inoculum into 10 ml 2% MA on top of thick Cellophane disks (British Cellophane PT 600), 80 mm diam. which overlaid 15 ml 2% MA in 90 mm Petri dishes. Cultures were grown for up to 4 wk before removal of the Cellophane together with the surface colonies. The underlying spent agar was removed, chopped with a scalpel and mixed thoroughly. Except where otherwise stated, dilutions were prepared by weight using malt extract and water agar to give a range of from × 0·5 to × 0·0625 of the *in situ* concentration in the spent agar, all with

a final added malt extract concentration of 1%. Six g of each extract were sterilized by autoclaving (15 min at 120°) and poured into a 50 mm Petri dish. In experiments in which activities of different ages of producer colony were compared, fresh and dry weights of spent agar samples were determined and water was added to the older extracts in order to equalize water contents.

Assay system

Disks of test extracts in agar, 10 mm diam., were each placed centrally in a well 15 mm diam. in water agar in 90 mm Petri dishes, with 4–8 wells per dish. Each disk was inoculated centrally with a 'mini' disk, 1 mm diam. from the margin of a 5–6 d-old dark-grown colony on 2% MA. In each experiment there were 6–8 blocks of dishes, each containing one replicate of every treatment. The assay plates were incubated upside down in the light for up to 28 d.

Assessment of fruiting

In all except the first experiment a quantitative measure of fruiting was obtained by recording the proportion (on a scale of 0 to 4, estimated by eye) of the disk radius from the central inoculum, in four sample directions at 90° to each other, which was occupied by fruiting tissue, i.e. with recognizable dissepiment tissue, either as island initials (Butler, 1988) or as poroid hymenophore. The mean score per disk was used for analysis. Since the scores were proportions, which are not normally distributed towards the ends of their range, arcsin transformation of the data was used both for presentation of results and in analysis of variance.

RESULTS

Development of assay procedure

The initial observation of the fruiting-inducing activity of agar around established colonies of *P. contiguus* was confirmed by comparing the growth from mini-inocula placed on the peripheral agar around intact colonies with that in dishes from which the colony had been excised or containing uninoculated 2% MA. The established colonies had been grown in the dark for 21 d. In the treatment in which this established colony was excised, moisture levels within dishes were maintained by substitution of uninoculated 2% MA, separated from the test peripheral agar by an agar-free diffusion barrier. After incubation for 13 d in the light, macroscopically visible basidiome initials were common to abundant on the new colonies not only around the intact producer colonies but also where the colonies had been removed, whilst fruiting was sparse on uninoculated agar. Thus it appears that a persistent change promoting fruiting takes place in agar which has been in diffusion contact with a growing colony of *P. contiguus*.

The effect of autoclaving on fruiting-inducing capacity of peripheral agar from around 28-d-old colonies was tested by using disks, 10 mm diam. and explanted from the peripheral agar, either directly, or after reconstituting to produce disks of similar dimensions following autoclaving. Seventeen days after inoculation with 'mini' inocula, island dissepiment initials were present over most of the surface of disks containing both directly-used and autoclaved peripheral agar (Fig. 1). In all treatments fruit body tissue was usually developed on the 'mini' inoculum but on assay disks containing control 2% MA further fruit body tissue was sparse and mostly restricted to close to the inoculum. Growth on the control disks mostly took the form of submerged mycelium associated with dark brown pigmentation. Thus the change occurring in peripheral spent agar is stable on autoclaving and fruiting-inducing capacity can be tested on isolated disks.

Colonies of *P. contiguus* growing in a thin layer of agar on top of a thick Cellophane membrane overlying agar only slowly penetrated the membrane. After incubation for 21 d, no penetration was detected by growth tests following removal of the Cellophane, although the underlying medium was capable of supporting growth from fresh inoculum of *P. contiguus*. However after 28 d penetration at a small number of sites was detected but there was hardly any submerged growth whilst the top colonies were *in situ*. When fruiting was assayed on 15 mm disks of 2% MA placed on top of the spent agar after removal of 28-d-old colonies on Cellophane, there was a significantly greater extent of fruiting after 12 d on disks over spent agar than over uninoculated control 2% MA (mean arcsin proportion of radius fruiting $1{\cdot}38 \pm 0{\cdot}14$ and

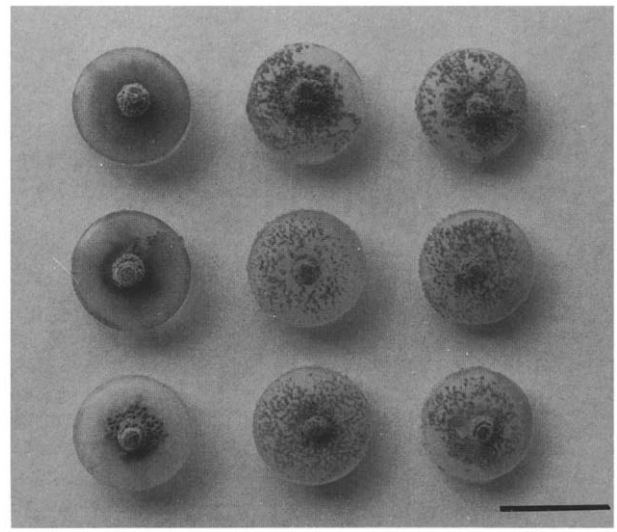

Fig. 1. Fruit body initials of *P. contiguus* after growth for 17 d on agar disks containing malt extract (left column) and spent agar without (centre column), and after (right column) autoclaving (bar = 10 mm).

Table 1. Effect removal of volatiles on fruiting-inducing activity of $\times 0{\cdot}41$ concentration of spent agar from beneath colonies on Cellophane after incubation of assay disks for 14 d

Treatment	Extent of fruiting (mean arcsin proportion of radius ± S.E.M., $n = 6$)
Direct spent agar	$0{\cdot}71 \pm 0{\cdot}14$
Spent agar after removal of volatiles	$0{\cdot}62 \pm 0{\cdot}14$
2% malt extract control	$0{\cdot}25 \pm 0{\cdot}09$
1% malt extract control	$0{\cdot}12 \pm 0{\cdot}07$

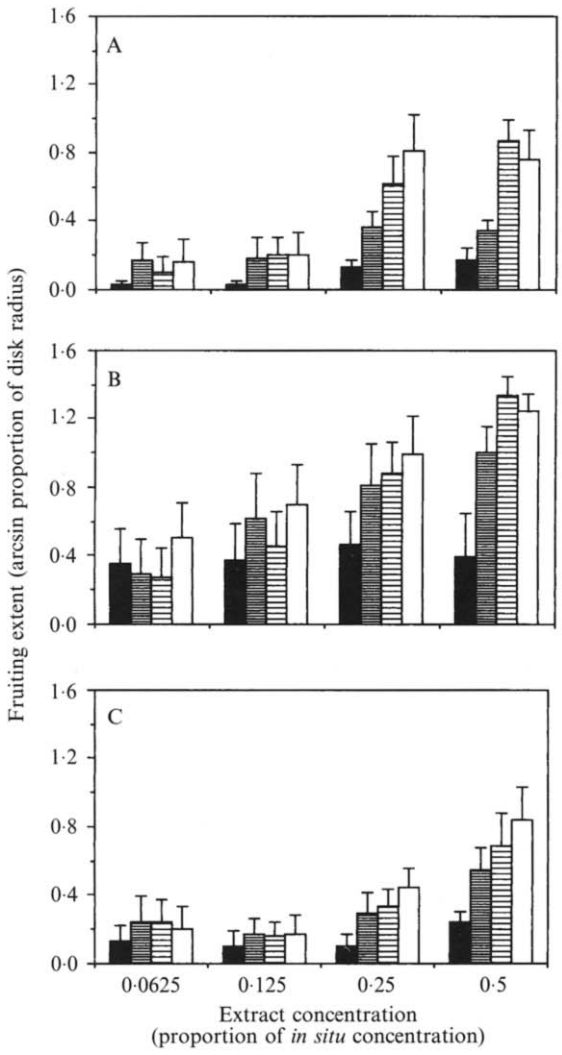

Fig. 2. Extent of fruiting (mean ± S.E.M., $n = 8$) on assay disks containing four concentrations of spent agar extract from different locations under producer colonies of different ages and grown in the dark (A, B) or light (C). Assay disks were incubated for 13 (A), 28 (B) and 14 (C) d. Spent agar extracts from beneath the margin of 28–31 (□) and 18–20 (▤) d-old producer colonies, beyond the colony margin of 18–20 d old (▤) producer colonies and without producer colonies (■).

$0{\cdot}26 \pm 0{\cdot}12$ respectively, $P < 0{\cdot}001$ where $n = 8$). The fruiting-inducing activity produced during colony growth is present in the spent agar beneath the Cellophane.

The importance of volatiles in the effect of spent agar was evaluated by chopping up samples of spent agar from beneath 32-d-old Cellophane colonies and allowing volatiles to escape for 18 h at 30°. The samples were made up to the original fresh weight with distilled water and fruiting-inducing activity was compared with direct spent agar samples after dilution of each to × 0·41 of the *in situ* concentration and addition of 1% malt extract. When assayed after 14 d (Table 1) fruiting-inducing activity was much higher on both spent agars than on control malt extract agar. The difference between spent agar before and after allowing volatiles to escape was not significant. Most, if not all, of the active agent is not volatile.

The importance of the diffusion barrier around the assay disks was evaluated by placing disks, containing × 0·5 concentration of spent agar from beneath 29-d-old Cellophane cultures with 1% added malt extract, either isolated in wells or directly on the surface of 1% MA. In this experiment there was no fruiting on the latter disks after 15 d whereas there was substantial fruiting where diffusion was prevented (mean arcsin proportion of radius fruiting $0{\cdot}41 \pm 0{\cdot}15$, $n = 8$). The fruiting-inducing activity is dissipated where diffusion can occur.

All assay disks were set up in the light. The effect of light during subsequent incubation was tested using three spent agar extracts from beneath 20-, 31- and 42-d-old colonies, all at × 0·5 concentration. In all cases there was no fruiting in the dark after 15 d, regardless of the presence or absence of fruiting-inducing extract. This contrasted with the abundant fruiting, covering on average 55% of the disk radius, in the light, but only in the presence of fruiting-inducing extract.

Effects of growth time of producer colony and light during growth on fruiting inducing activity of extracts

The activities of extracts from specific locations in the agar beneath 18- and 31-d-old dark-grown producer colonies on Cellophane were compared using spent agar samples obtained as sets of disks, 10 mm diam. from each location beneath 10 replicate producer colonies. The locations were beneath the margins, 14 and 26 mm from the centres of young and old producer colonies respectively, and from beyond the margin of the younger colonies, 26 mm from their centres. After 14 d (Fig. 2A) there were significant effects of both source of extract and concentration ($P < 0{\cdot}001$ in both cases). There was little fruiting on any dilution of control malt extract and the greatest fruiting extent occurred on the two highest concentrations of spent agar extract, × 0·5 and × 0·25 of the *in situ* concentration. At × 0·5 concentration the extract collected from beyond the margin of the younger producer colonies had significantly less fruiting inducing activity than equivalent samples from beneath the margins of both younger and older colonies ($P < 0{\cdot}01$ and $< 0{\cdot}05$ respectively). During incubation for a further 14 d both the depth of the hymenophore and the extent of fruiting increased. Analysis of variance of the results after 28 d (Fig. 2B) reinforced the previous findings of the significant enhancing effects on extent of fruiting of presence of spent agar extracts and of extract concentration ($P < 0{\cdot}05$ and $< 0{\cdot}001$ respectively). However, by this stage, although the trends in the extent of fruiting induced by extracts derived from locations beyond and within the colony margin were similar to those at 14 d, differences were not significant.

Similar enhancement of fruiting was obtained when this experiment was repeated using spent agar samples from colonies grown in daily light (Fig. 2C). The highest concentration of extract from beneath colonies of both ages produced a significantly greater extent of fruiting after 14 d than the same concentration of control malt extract ($P < 0{\cdot}05$). Also a sample of the extract from the older dark-grown colonies in the previous experiment supported in this experiment a similar amount of fruiting at × 0·5 concentration (arcsin proportion of radius fruiting $0{\cdot}89 \pm 0{\cdot}11$) as the equivalent light-grown culture extract. However, in this

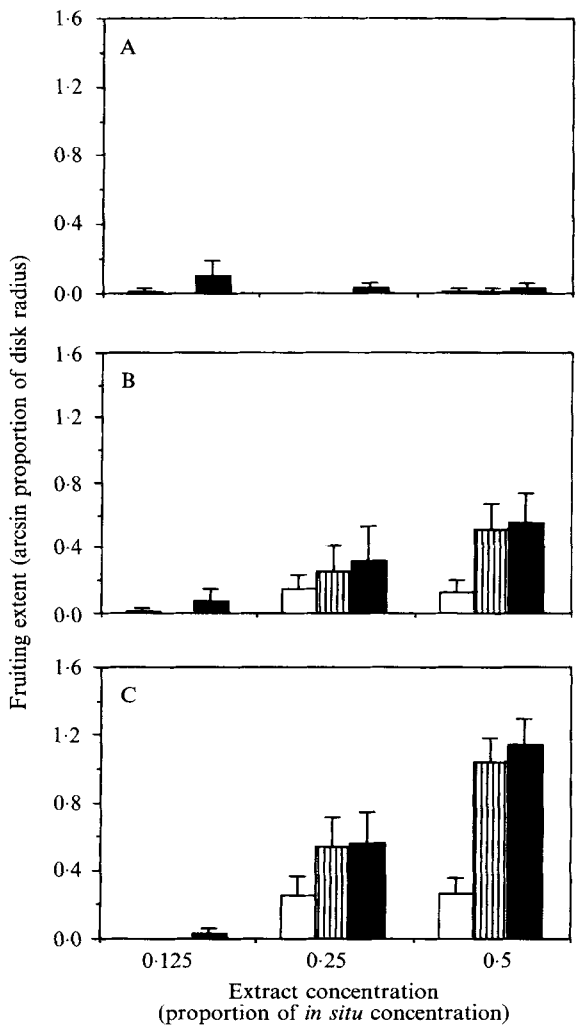

Fig 3. Extent of fruiting (mean ± S.E.M., $n = 8$) after incubation for different lengths of time on assay disks containing three concentrations of (A) malt extract agar, (B) spent agar from beneath the margin of 6-d-old and (C) spent agar from beneath the margin of 21-d-old dark-grown colonies of *P. contiguus* (disks incubated for □ 13, ▥ 21 and ■ 28 d).

experiment, in which the extracts were prepared from 20- and 28-d-old producer colonies, the activity of the extract derived from beyond the margin of the younger producer colonies was not significantly different from that of extracts obtained from beneath colonies.

The fruiting-inducing activity of spent agar extract from very young producer colonies, 6-d-old, was compared with that from 21-d-old colonies. Spent agar samples were prepared from 3 mm diam. disks removed from beneath the margins of 56 younger and 14 older colonies. In this experiment (Fig. 3), although fruiting-inducing activity was not yet significantly detectable in any treatment after 13 d incubation, most of the fruiting area was defined after growth for 21 d. As before control malt concentrations supported very little fruiting. However, not only increasing concentration of spent agar extract but also increasing age of source colony enhanced the extent of the fruiting response ($P < 0{\cdot}01$ and $< 0{\cdot}05$ respectively from an analysis of variance excluding control malt extracts). At the highest tested concentration, × 0·5, and after incubation for 21 d or more, fruiting occurred over more than 80% of the radius on disks containing extract from older colonies, in comparison with less than 50% on those containing extract from beneath very young colonies. Very young colonies had produced fruiting-inducing activity but significantly less per unit volume than older, larger colonies ($P < 0{\cdot}05$).

DISCUSSION

The extracts used in these experiments were not purified. They thus contain remaining constituents of the malt extract agar medium together with all diffusible and heat stable extracellular metabolites produced during colony growth of *P. contiguus*. Addition of 1% MA to all test assays means that, together with residual nutrients in the spent agar (used at not more than half strength), the maximum and minimum possible nutrient contents in assay disks are × 1 and × 0·5 respectively of those in the original 2% MA. This range of concentration of the medium constituents had virtually no differential effect on fruiting extent on test disks in the absence of colony diffusates. The conclusion that fruiting is promoted by one or more metabolic products rather than by nutrient depletion, i.e. disappearance of metabolically active medium constituent(s) which prevent fruiting, is supported by the positive relationship between the extent of basidiome tissue formed on assay disks and the concentration of extract (over the range between × 0·125 and × 0·5 of the *in situ* concentration beneath fruiting-size colonies).

Light is required for development of basidiome tissue in *P. contiguus* (Butler & Wood, 1988; Butler, 1992*b*). However, the fruiting-inducing factor is produced in physiologically active concentrations by colonies of fruiting size which have been grown in either light or dark. Production of the factor does not require differentiated fruiting tissue and its concentration is maintained at physiologically active levels during formation of basidiome tissue. Moreover, the factor does not replace the requirement for light. The concentration of fruiting-inducing factor below the margins of small, 6-d-old, colonies is significantly lower than from an equivalent position below larger, fruiting-size colonies. This suggests that production of one or more constituents of the fruiting-inducing factor is responsible for the minimum size of colony capable of fruiting in the light. If this is so, then accumulation of this constituent of the fruiting-inducing factor is a necessary prerequisite for the light response. In developmental terms, in the presence of sufficient fruiting-inducing factor, colonies are competent to fruit in response to light. The requirement for a period of vegetative growth before the colony is competent to initiate fruit body differentiation in response to light is of general occurrence in the basidiomycetes (Ross, 1985). Although the adaptive significance of this pre-fruiting assmilative phase is recognized, ideas about underlying mechanisms have remained speculative (Ross, 1985; Wessels, 1992).

The terminology of chemical factors involved in sexual differentiation is confused by the different usages of different workers. In a recent review Dyer *et al.* (1992) used the term chemical sex factor to cover all chemicals controlling sexual morphogenesis and subdivided this into sex hormones, which act as triggers at low concentrations and sexual growth

substances, which are active at higher concentrations, for example as nutrients having a direct effect on metabolism. In the former they included both pheromones, concerned with compatibility and zygote formation, and sex morphogens, which initiate and coordinate fruit body differentiation. In *P. contiguus* the fruiting-inducing factor induces precocious fruiting in a fertile culture. Thus it is not a pheromone. However, the distinction between activity as a sex growth substance and a sex morphogen cannot yet be made since not only the chemical nature(s) but also the concentration(s) of the physiologically active substance(s) await further investigation.

Many of the chemical factors controlling sexual differentiation which have been reported in basidiomycetes have been detected by induction of sexual differentiation in homokaryons, which do not normally fruit alone (Manachère, 1988). However, in some examples, e.g. *Schizophyllum commune* (Kawai, 1987), the same extracts have been shown to enhance fruiting in fertile cultures, e.g. compatible matings or dikaryons, indicating a similarity with the factor in *P. contiguus*. However, this similarity does not point to a particular type of active chemical amongst the diversity which have been implicated in basidiomycetes (Manachère, 1988; Dyer *et al.*, 1992). Even within one fungus, *S. commune*, there is evidence that more than one type of compound is implicated. In this fungus Kawai (1987) believes that the fruiting inducing substance (FIS) reported by Leonard & Dick (1968) is different from his acetone extract from fruit bodies, which has been shown to contain morphogenetically active cerebrosides (Kawai & Ikeda, 1982). The extract from *P. contiguus* resembles the FIS of Leonard & Dick (1968) in its diffusibility in water and heat stability. FIS has not been characterized and, unlike the factor in *P. contiguus*, only a low level of activity was detected in extracts from vegetative mycelium, in comparison with those from fruiting bodies.

There is increasing awareness of the probable morphogenetic importance of extracellular substances which modify the immediate environment of differentiating hyphae (Moore, 1994). The diffusibility of the *P. contiguus* factor means that it is affecting the general environment. This is consistent with having an effect on an early stage in the developmental sequence leading to the differentiated basidiome. The existence of this fruiting-inducing factor in *P. contiguus* provides a useful tool for further analysis of the developmental processes which lead to fruit body differentiation.

(Accepted 20 June 1994)

REFERENCES

Butler, G. M. (1988). Pattern of pore morphogenesis in the resupinate basidiome of *Phellinus contiguus*. *Transactions of the British Mycological Society* **91**, 677–686.

Butler, G. M. (1992*a*). Location of hyphal differentiation in the agar pore field of the basidiome of *Phellinus contiguus*. *Mycological Research* **96**, 313–317.

Butler, G. M. (1992*b*). Capacity for differentiation of setae and other hyphal types of the basidiome in explants from cultures of the polypore *Phellinus contiguus*. *Mycological Research* **96**, 949–955.

Butler, G. M. & Wood, A. E. (1988). Effects of environmental factors on basidiome development in the resupinate polypore *Phellinus contiguus*. *Transactions of the British Mycological Society* **90**, 75–83.

Dyer, P. S., Ingram, D. S. & Johnstone, K. (1992). The control of sexual morphogenesis in the Ascomycotina. *Biological Reviews* **67**, 421–458.

Horton, J. S. & Raper, C. A. (1991). A mushroom-inducing DNA sequence isolated from the basidiomycete, *Schizophyllum commune*. *Genetics* **129**, 707–716.

Kawai, G. (1987). Biological and scanning electron microscopic analysis of the inducing effect of a cerebroside on fruiting of *Schizophyllum commune*. *Transactions of the Mycological Society of Japan* **28**, 107–119.

Kawai, G. & Ikeda, Y. (1982). Fruiting-inducing activity of cerebrosides observed with *Schizophyllum commune*. *Biochimica et Biophysica Acta* **719**, 612–618.

Leonard, T. J. & Dick, S. (1968). Chemical induction of haploid fruiting bodies in *Schizophyllum commune*. *Proceedings of the National Academy of Sciences* **59**, 745–751.

Manachère, G. (1988). Regulation of sporophore differentiation in some macromycetes, particulary in coprini: an overview of some experimental studies, from fruiting initiation to sporogenesis. *Cryptogamie, Mycologie* **9**, 291–323.

Moore, D. (1994). Tissue formation. In *The Growing Fungus* (ed. N. A. R. Gow & G. M. Gadd) in press. Chapman & Hall: London, U.K.

Rayner, A. D. M. & Boddy, L. (1988). *Fungal Decomposition of Wood: its Biology and Ecology*. John Wiley: Chichester, U.K.

Ross, I. K. (1985). Determination of the initial steps in differentiation in *Coprinus congregatus*. In *Developmental Biology of Higher Fungi* (ed. D. Moore, L. A. Casselton, D. A. Wood & J. C. Frankland), pp. 353–373. Cambridge University Press: Cambridge, U.K.

Watling, R. & Moore, D. (1944). Moulding moulds into mushrooms: shape and form in the higher fungi. In *Shape and Form in Plants and Fungi* (ed. D. S. Ingram & A. Hudson). pp. 270–290. Academic Press: London, U.K.

Wessels, J. G. (1992). Gene expression during fruiting in *Schizophyllum commune*. *Mycological Research* **96**, 609–620.