Ammonium ions and glutamine inhibit sporulation of Coprinus cinereus basidia assayed in vitro

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ABSTRACT

Basidia of *Coprinus cinereus* (Schaeff.: Fr) S. F. Gray are committed to their developmental pathway, continuing through meiosis and sporulation even when excised from their parental fruit body. A technique is described which permits this *in vitro* differentiation to be used as a rapid, small-scale bioassay for chemicals which interfere with these morphogenetic processes. Of a range of compounds tested, only ammonium and glutamine, and some structural analogues, were able to inhibit basidium differentiation. Growth was not inhibited; instead the differentiation inhibitors caused vegetative hyphal tips to grow out from regions of the basidial apparatus expected to be in active growth during sporulation. Depending on the stage reached at the time of exposure to the inhibitors, vegetative hyphal tips emerged from the four apical sites for sterigmata, from the tips of sterigmata, from partially formed or abnormal spores, and from the basal regions of the basidium from which paraphyses would be expected to arise. The experiments show that ammonium ions and glutamine halt meiocyte differentiation. Reports of similar effects in other organisms, animals and plants as well as fungi, may imply that sporulation events are generally sensitive to ammonium inhibition.

INTRODUCTION

Studies with both light and electron microscopes have demonstrated that the hymenium of the basidiomycete *Coprinus cinereus* (Schaeff.: Fr) S. F. Gray comprises four very distinct differentiated cell types: **basidia** (the meiocytes), **paraphyses, cystidia** and **cystesia.** Basidia and cystidia differentiate from the tips of branches which arise from the subhymenial tramal hyphae and together form the hymenium as an 'epidermal layer' of the gill plate (Fig. 1). A hymenial hyphal tip has a probability of about 40% of becoming a cystidium, but when a cystidium does arise, it inhibits formation of further cystidia in the same hymenium within a radius of about 30 μm. As a result, only about 8% of these tramal hyphal branches become cystidia; the rest become probasidia (Horner & Moore, 1987) which proceed to karyogamy and initiate the meiotic cycle ending with sporulation. Cystesia differentiate in response to a contact stimulus from a cystidium emerging from the opposing hymenium and then develop into cells which adhere strongly to the cystidial tip (Fig. 1). The cystesium-cystidium combinations may hold neighbouring gills in po-

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sition for co-ordinate development, and allow space for maturation of the basidia of opposing hymenia.

Paraphyses arise as branches of sub-basidial cells and insert into the hymenium (Fig. 1B). About 75% of the paraphyseal population has inserted by the time meiosis is completed; the rest insert at later stages of gill development to expand the gills for active discharge of basidiospores from basidia to take place (Rosin & Moore, 1985).

Of these four differentiated cell types, only basidia are committed to their particular pathway of differentiation. On explantation to agar medium paraphyses and cystidia revert to vegetative hyphal growth whereas basidia do not. Depending on the stage of meiosis reached at the time of explantation, basidia are either arrested or continue their development and sporulate normally (Chiu & Moore, 1988). This explantation technique has been developed into a rapid small-scale bioassay which can be used to study the effects of exogenous compounds on the progress of differentiation of basidia after removal from their parent fruit body. As we have demonstrated previously that NADP-linked glutamate dehydrogenase (NADP-GDH) and glutamine synthetase are co-ordinately derepressed during cap development (Moore, 1984; Moore *et al.*, 1987) we have concentrated attention on compounds related to the metabolism associated with these enzymes.

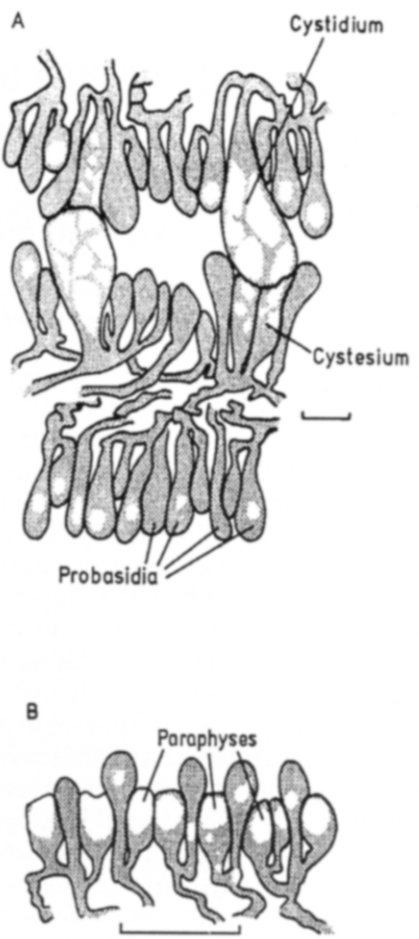

Fig. 1. *Structure of the* Coprinus *hymenium as seen in sectional view, (A) prior to meiosis and (B) at the later meiotic stage. Both diagrams are constructed from tracings of published micrographs from Rosin & Moore (1985) and Horner & Moore (1987). Scale bars = 20* μm.

MATERIALS AND METHODS

The Meathops strain of *Coprinus cinereus*, which was collected in Cumbria, was used for these experiments, cultures being grown and fruit bodies produced as described by Moore & Ewaze (1976). Gill lamellae were excised from a fruit body after the cells of the veil had been removed from the cap surface. A segment con-

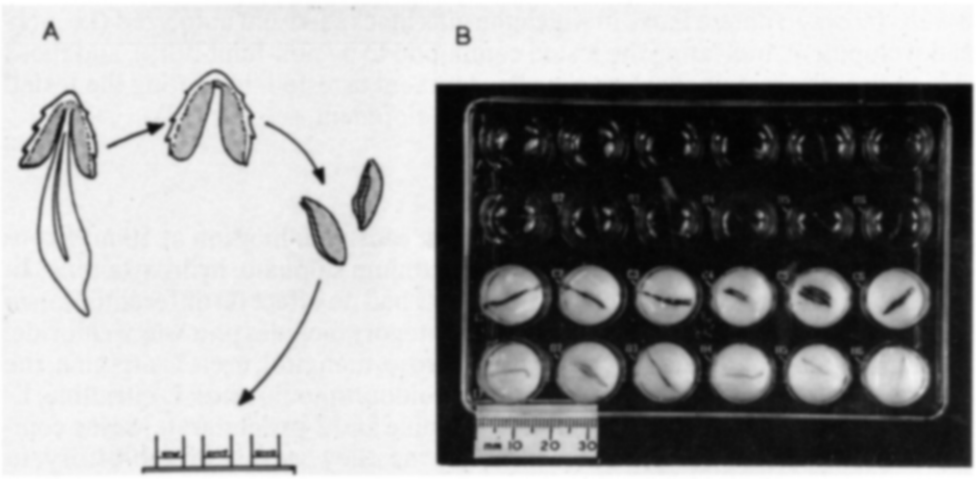

Fig. 2. The in vitro explantation technique. (A) in diagrammatic form, (B) photograph of a Multiwell plate 24 h after explantation of the gill segments. The top two rows of wells contain agar media and the bottom two liquid media, and in each case the lower of each pair of rows contains medium supplemented with 50 mM ammonium chloride. Note that the gills remain white in the ammonium-tests but become covered with pigmented spores in the control media.

sisting of two or three gills was explanted to Nunclon Delta SI 24-well 'Multidishes' which have 15 mm internal diam wells (Fig. 2). For tests on agar media, 0.35 ml of autoclaved agar (buffered to pH 7 with 67 mM Sørensen buffer except in experiments testing the effect of pH) was placed into each well, and covered with a sterile disk of cellophane. For tests with liquid media, 0.35 ml of the filter-sterilised solution (buffered to pH 7 as above) was placed in each well with two sterile 14 mm diam disks of Whatman GF/D glass microfibre filters and subsequently overlaid with a disk of cellophane.

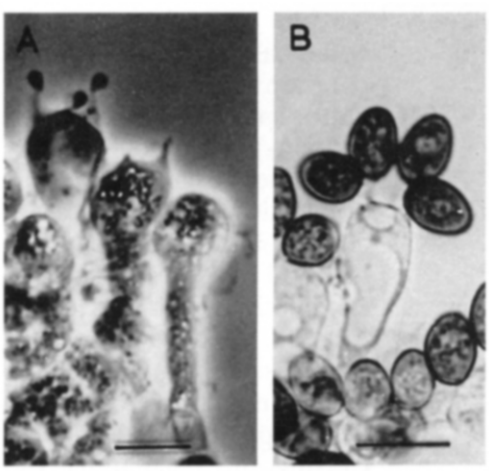

Fig. 3. Cytological states of the hymenial tissues. (A) at the time of explantation, the stage of development typically ranging from meiotic interphase II to early sporulation. (B) after 24 h incubation on buffer agar such tissue as is shown in Fig. 3A has completed sporulation, producing normally-pigmented basidiospores. Scale bars = 10 μm.

The developmental stage of the tissue at explantation was determined by microscopic examination of squash preparations stained with acridine orange (Chiu & Moore, 1988). For general purpose assays the gills were excised just at the conclusion of meiosis (i.e. the beginning of sporulation, see Fig. 3) so that, in control explants to plain buffer agar, spore pigmentation and gill autolysis occurred within 24 hr after explantation (Fig. 4). This permitted a range of compounds to be screened rapidly by simply scanning

the wells by eye to detect those in which the gills blackened and autolyzed (i.e. normal development, indicating the tested compound to be non-inhibitory), and those in which the gills remained white (i.e. development arrested, indicating the tested compound to be an inhibitor of hymenium development, see Fig. 2B).

RESULTS AND DISCUSSION

The most effective inhibitors of development, causing inhibition at 50 mM concentrations were ammonium chloride, ammonium sulphate, hydroxylamine, L-glutamine, and L-methionine. Many compounds had no effect on differentiation *in vitro* at concentrations less than 150 mM. This category includes potassium chloride, potassium sulphate, D-glucose, D-fructose, sucrose, mannitol, urea, L-arginine, the glutamine analogue albizziine (L-2-amino-3-ureidopropionic acid), L-citrulline, L-ornithine, L-glutamate, L-asparagine, D-glutamine and 2-oxoglutarate. Some compounds were classed as partially effective as they were not inhibitory to differentiation at 50 mM but inhibited at concentrations of 100 mM and above. This category includes potassium nitrate, L-proline, methylamine, and the glutamine analogues L-ethionine, L-glutamic acid monohydroxamate, L-methionine sulphoximine, azaserine, and 6-diazo-5-oxo-L-norleucine.

Tests at various pH values and ammonium (chloride and sulphate) concentrations shows that highly alkaline pH values inhibit gill development, but at permissive pH values (6 - 8) ammonium concentrations of 50 mM are inhibitory (Table 1). Neither potassium chloride nor potassium sulphate had any effect.

Table 1. Effects of ammonium salts on gill development *in vitro* at different pH values

	pH				
NH_4Cl (mM)	6	7	8	9	10
0	N	N	N	I	I
2	N	N	N	I	I
5	N	N	N	I	I
10	N	N	N	I	I
25	N	N	N	I	I
50	I	I	I	I	I
100	I	I	I	I	I
150	I	I	I	I	I

[N = gill development normal; I = sporulation inhibited]

Ammonium salts injected into the caps of young fruit bodies also terminated further development. Very young primordia (prekaryogamy) were not able to withstand the damage caused by injection and in most cases aborted. However, injections of 2.5 μl of 1 M ammonium salt solutions (buffered to pH 7) were effective in locally suppressing sporulation if injected in post-meiotic and early sporulation stages, causing the occurrence of white zones around the point of injection as the rest of the cap matured and produced its crop of blackened spores. Similar injections of water or buffer had no visible effect on fruit body maturation.

Ammonium inhibited the meiocyte development pathway *in vitro* when applied at any time during meiosis (stages prophase I through to the second meiotic division were tested). When applied at similar stages *in vivo*, ammonium retarded the rate of progress through meiosis but did not suppress sporulation. When applied at later sporulation stages (sterigma formation, spore formation, spore pigmentation),

ammonium arrested sporulation completely both *in vivo* and *in vitro*. Samples explanted to ammonium-containing medium remained white whereas neighbouring segments explanted to buffer agar blackened, due to pigmentation of spores, and showed signs of autolysis (Fig. 2B). Similarly, ammonium injections caused the zone around the injection point to remain white and undeveloped while the zones around injections of buffer blackened and developed normally. Thus, ammonium ions inhibit meiocyte development both *in vivo* when injected into otherwise undisturbed fruit bodies and *in vitro* when excised gills were exposed to ammonium in the explantation medium.

Cytological examination of gills excised at prophase I and explanted to ammonium-medium for 24 hr showed that some were arrested at prophase I, others continued to metaphase I and some even completed the meiotic division, but no sporulation was observed. Samples which were explanted at later stages suffered ammonium-arrest at correspondingly later meiotic stages and in early sporulation stages. However, tissue explanted during those early sporulation stages seemed to become arrested immediately so, while the meiotic process shows some sensitivity

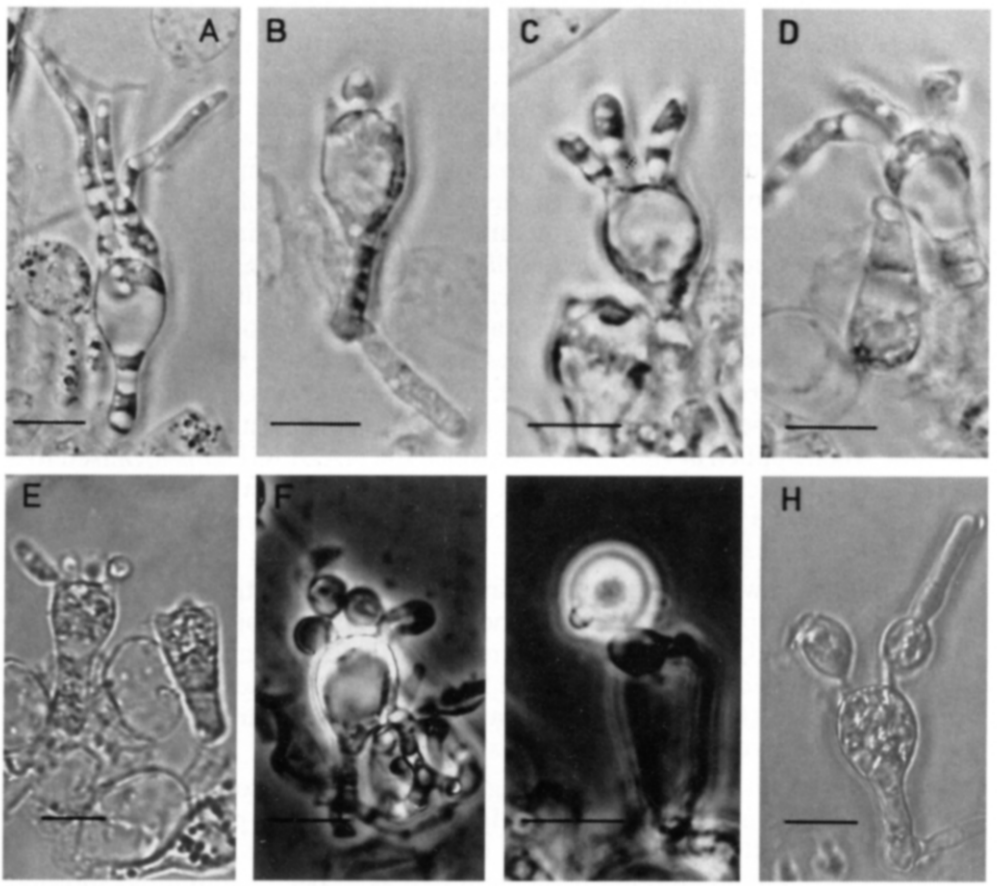

Fig. 4. Micrographs showing how explantation to ammonium-containing medium for 24 - 48 h causes cessation of sporulation and prompt outgrowth of vegetative hyphal tips from specific (actively growing) regions of basidia. Scale bars = 10 μm.

to ammonium-arrest, by far the most obvious ammonium-sensitive stages are the post-meiotic sporulation processes of sterigma and spore formation.

Some ammonium-treated basidia reverted to hyphal growth, behaviour quite unusual for basidia which characteristically continue sporulation if explanted to buffer agar after karyogamy (Fig. 4), and remain arrested but without reverting if explanted before karyogamy (Chiu & Moore, 1988). Thus a further direct effect of ammonium treatment is the rapid and regular promotion of reversion to hyphal tip growth among the basidial cells. This constitutes a breakdown of the commitment normally shown by these cells to their developmental pathway. The new vegetative hyphal apices were formed at sites involved in active wall synthesis during the normal progress of development (Fig. 4). When the tissue exposed to ammonium treatment was in post-meiotic and early sporulation stages the reversion hyphae grew out at the sites of sterigma formation (Fig. 4A); if the basidia had formed sterigmata, hyphae, instead of basidiospores, grew from their tips (Fig. 4B - E); if spores were in process of formation, exposure to ammonium caused termination of spore formation and outgrowth of hyphal apices (Fig. 4F - H). In addition, hyphae also emerged from basal regions of the basidium (Fig. 4B and F).

Sterile elements of the hymenium immediately revert to hyphal growth on explantation to agar media (Chiu & Moore, 1988). This implies that such reversion must be actively inhibited during development of the normal hymenium. As ammonium ions and L-glutamine cause basidia, the only committed cells of the hymenium, to abort sporulation and revert to hyphal growth normal sporulation may require some form of protection from the inhibitory effects of metabolic sources of these metabolites. Significantly, the ammonium assimilating enzyme NADP-GDH is derepresssed specifically in basidia, being localised in microvesicles associated with the cell periphery (Elhiti *et al.*, 1987) where it could serve as a detoxifying ammonium scavenger. Such a function might also be ascribed to the glutamine synthetase which is derepressed co-ordinately with NADP-GDH (Moore, 1984; Moore *et al.*, 1987) though enhanced synthesis of glutamine in the basidium seems to be inconsistent with the inhibition of sporulation caused by L-glutamine when applied *in vitro*. However, since the NADP-GDH is localised to a particular microvesicle, glutamine synthetase and/or its product may also be vesicular, so protecting the basidium from the sort of inhibitory effect caused by exogenous glutamine.

It is not known how ammonium or glutamine inhibit sporulation in *Coprinus*, but it is important to emphasise that though the impact of the inhibitors on sporulation is dramatic and rapid, there is no inhibition of growth. Rather, ammonium and glutamine cause diversion of effort from the highly regulated assembly of the sporulation architecture (basidium, sterigmata and spores) towards the more basic organization of the vegetative hyphal apex.

Initiation of sexual reproduction in fungi has often been associated with low nitrogen levels in the medium or substrate. In some cases, as for perithecium formation in the ascomycetes *Venturia* (Ross & Bremner, 1971) and *Sordaria* (Roure & Bouillant, 1986), ammonium has been identified as a specific inhibitor. Sporulation (i.e. meiosis and ascospore formation) in the yeast *Saccharomyces cerevisiae* can be induced by transferring diploid vegetative yeast cells to a nitrogen-free me-

dium (Fowell, 1969, 1975; Tingle *et al.*, 1973; Freese *et al.*, 1982). Yeast sporulation is inhibited by ammonium and glutamine (Miller, 1963; Piñon, 1977). Treatment with ammonium delays the protein degradation which normally occurs at the onset of meiosis and inhibits protein and DNA syntheses (Croes *et al.*, 1978). Dickinson & Dawes (1983) suggest there are two stages sensitive to ammonium inhibition in yeast, one early in meiosis (perhaps initiation) and the other later, concerned with organization and delimitation of spores. Piñon (1977) claims the most sensitive period in yeast is just at the end of premeiotic DNA synthesis and meiotic prophase. Although ammonium does not inhibit the initiation of premeiotic DNA synthesis, replication is arrested after initiation; continued incubation in the presence of ammonium leads to massive DNA degradation.

There are parallel phenomena, too, in other organisms. Ammonium inhibits reinitiation of meiosis in starfish oocytes when applied during the hormone (1-methyladenine) dependent period (Doree *et al.*, 1982). Also, gametogenesis in *Chlamydomonas reinhardtii* is induced by removal of ammonium from the culture medium but addition of ammonium at any time following induction causing the gametes to dedifferentiate to vegetative cells (Sager & Granick, 1954; Kates & Jones, 1964; Martin & Goodenough, 1975). Thus, ammonium inhibition of meiotic processes seems to be common to a wide range of eukaryotes. Some non-meiotic sporulation processes can also be affected. Ammonium has been identified as a morphogen determining choice of pathways in the morphological cycle of *Dictyostelium discoideum* (Schindler & Sussman, 1977) and L-glutamine specifically inhibits asexual sporulation of *Achlya* (LéJohn, 1983). In the latter case application of glutamine caused diminished synthesis of a protein, presumed to be actin, actively synthesised in sporulating cells; the causal effect was claimed to be exerted directly on gene expression. However, apart from this suggestion, and the claim by Doree *et al.* (1982) that ammonium inhibition was due to establishment of a proton sink within the starfish oocyte, the way (or ways) in which ammonium or glutamine cause inhibition of sporulation processes has not yet been established in any organism. Nevertheless, the frequent observation of such inhibitions may suggest action against evolutionarily conserved components of the cell. Though there are differences in detail, the common feature is that ammonium and glutamine cause a reproductive state to revert to a purely vegetative one. In view of the inhibitory effect on actin synthesis in *Achlya* and the inhibition of the close control over wall growth which characterises sporulation in *Coprinus*, these phenomena could be rationalized by the suggestion that the inhibitors affect cytoskeletal elements generally concerned with karyogamy and sporulation; the precise expression of the inhibition depending upon the consequential effects in a given organism of disruption of such cytoskeletal architecture.

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