Mycelial differentiation of *Morchella esculenta* in pure culture

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The development of mycelia derived from single or several ascospores of *Morchella esculenta* was studied on two nutrient media and at different temperatures. The cultures displayed distinctive types and patterns of production of sclerotia, associated with variation in the production of aerial mycelium and expression of non-self-recognition reactions.

In nature, the mycelium of morels (*Morchella* spp.) undergoes several physiological and morphological changes before it produces fruit bodies. A sterile conidial stage has been observed (Costantin, 1936) and sometimes obtained in culture (Molliard, 1904a, b; Cailleux, 1968; Ower, 1982; Buscot, 1987). Buscot & Roux (1987) described subterranean networks of compact, mycelial masses formed in association with roots or tubers, and connected by mycelial strands to the fruit bodies. Ecophysiological and biochemical studies demonstrated that these subterranean structures form during summer and autumn, remain dormant over winter, and then give rise in spring to both ascomata and new associations with rootlets (Buscot, 1989; Buscot & Bernillon, 1991). The latter associations constitute the starting point of the next vegetative cycle, and with *Picea abies* Karst. they appear to be ectomycorrhizal (Buscot & Kottke, 1990).

Although there have been numerous cultural studies on morels, these have only recently concerned reproductive biology (Fron, 1905; Brock, 1951; William, Trzcinski & William-Engels, 1956; Gilbert, 1960; Litchfield, Overbeck & Davidson, 1963; Robbins & Hervey, 1965; Litchfield, 1967; Impens, 1972; Kaul, 1977; Sekahram, Narashimam & Alma, 1978; Kone, 1979). Hervey, Bistig & Leong (1978) observed segregation of two morphological phenotypes ('flat' and 'fluffy') among single ascospore cultures from a single fruit body, and noted that confrontation of these cultures in non-self-combination resulted in barrage-like lines of aerial mycelium at the interaction interface. Yolk & Leonard (1989, 1990) termed these barrages 'mycelial melds', and interpreted them as a secondary heterokaryotic mycelial phase with dikaryotic hyphal tips. The latter interpretation contradicts the cytological studies of Greiss (1940), which indicated that, as in typical discomycetes, morel dikaryons only form at the end of ascomatal development, are immediately isolated by septation, and are restricted to a few cells of the hypothecium. No evidence of mating types has been reported in morels, although this possibility was considered in a study of population biology (Gessner, Romano & Schultz, 1987).

Another cultural feature of morel mycelium, the formation of sclerotia in culture, has been reported by several authors. Molliard (1905) considered the sclerotia to be imperfectly developed fruit bodies. Ower (1982) and Ower, Mills & Malachowski (1986) showed that ascomata form using nutrients redistributed from sclerotia. Volk & Leonard (1990) correlated the occurrence of heterokaryotic sclerotia in subcultures from mycelial melds with the formation of ascomata.

Analysis of the morel life cycle remains difficult for three major reasons: (i) all stages are not permanently observable in nature (Buscot, 1987); (ii) the cultural mycelial forms often differ morphologically from the natural ones (William, Trzcinski & William-Engel, 1956); (iii) morel mycelium rapidly degenerates in prolonged culture (Hervey et al., 1978; Buscot, 1987). Few workers, therefore, have attempted to correlate laboratory and field results. However, by analysing mycospores in natural and cultured mycelial structures, Buscot & Bernillon (1991) indicated a relationship between morel sclerotia and fructifications, and also distinguished two kinds of sclerotia formerly recognized by Mayr (1982). One kind of sclerotia had biochemical affinities with very young fruit bodies, the other with the natural, subterranean, mycelial masses connected to ascomata.

This paper describes distinctive patterns of production of these two kinds of sclerotia under varied culture conditions.

**MATERIALS AND METHODS**

To avoid mycelial degeneration, all cultures were grown from freshly germinated ascospores; these were from a single fruit body of *Morchella esculenta* Pers.: St Amans (syn. *Morchella rotula* Pers.; Boudier), collected near Strasbourg (France).

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Two culture media were used. Medium A comprised (g l⁻¹):
Figs 1, 2. 6-wk-old polyspore cultures of *Morchella esculenta* on medium A. At 25° (1) early, encrusting sclerotia (EES) and late, isolated sclerotia (LIS) form; at 15° (2) only EES form. Fig. 3. Detail of an LIS (P, pigmented central part; M, unpigmented margin which remains growing). Fig. 4. 6-wk-old sectored polyspore culture of *Morchella esculenta* on medium B (S, three sectors with EES formed at different times of development; O, two sectors with no sclerotia; MM, mycelial meld). Figs 5, 6. Pairing of subcultures from single-ascospore cultures of *Morchella esculenta* on medium B. (5) Self-confrontation, the frontier (F) remains diffuse; (6) non-self-confrontation, the frontier is a ’mycelial meld’ (I, inoculum).
The spores were germinated overnight in medium B without agar, and transferred aseptically on to 90 or 140 mm diam. plates incubated in darkness at 25°, or (medium A only) at 17°, 10° and 4°. Both single and multiple (5–10 spores) ascospore cultures were grown.

The 6-wk-old single-ascospore cultures on medium B were used for subculture, and 30 of them for pairing tests. Plugs (6 mm diam.) of undifferentiated mycelium were cut out and transferred singly or in pairs on to new plates of medium B. To test the ability of sclerotia and mycelium to survive low temperature, these mycelial structures were transferred aseptically into sterile moist chambers and maintained either for 25 d at 25° (control), or successively 7 d at 0±1°, 2 d at −2±1°, again 7 d at 0±1° and 2 d at −2±1°, and finally 7 d at 0±1°. A freezing test (−17°/25 d) was also performed. The samples were then re-inoculated on medium A at 25° to determine the survival rate.

### RESULTS

#### Types of sclerotia

Two kinds of sclerotia were distinguished on the basis of their morphogenetic and other characteristics. The morphogenetic distinction between the two categories was clearest in ascospore cultures on medium A at 25°. Here the mycelia grew out homogeneously, and the first kind of sclerotia formed abundantly and synchronously at the colony margin 2 d after reaching the edge of the Petri dishes (respectively 6 and 8 d on 9 cm and 14 cm dishes). The sclerotia did not exceed 0.2–0.5 mm diam. individually but tended to aggregate into circular crusts, which rapidly became pigmented (Fig. 1). They were therefore termed 'early, encrusting sclerotia' (EES).

The second kind of sclerotia began to form, irrespective of dish size, up to the 12th day after inoculation (Fig. 2). They remained separate, could occur anywhere on the mycelium, and were few in number (7–8 per mycelium). They were, therefore, termed 'late, isolated sclerotia' (LIS). Their central part became pigmented while their margin remained growing (Fig. 3), attaining 5–8 mm diam.

Formation of LIS appeared to necessitate an incubation temperature > 20°, whereas EES were able to form at 10° (Tables 1, 2; Figs 1, 2). By contrast, LIS were the only structures able to regenerate mycelium after the low temperature or even the freezing treatments (Table 2). The resultant mycelia had a greater concentration of EES than the original ones.

Of 95 single ascospore cultures on medium A, none produced EES and only eight produced several LIS, which were initiated after 6 wk.

The patterns of sclerotium production on medium B differed from those on medium A. Most polyspore cultures on medium B developed radial lines of aerial mycelium delimiting from two to six irregular sectors on which both, only one, or even none of the two types of sclerotia formed (Table 1; Fig. 4). Sclerotium formation occurred after similar delays as on medium A. Nevertheless, it was not synchronous on the different sectors and the EES were not confined to the colony margins.

### Table 1. Morphological characteristics of polyspore cultures of *Morchella esculenta* on media A and B at different temperatures

<table>
<thead>
<tr>
<th>Medium</th>
<th>4°</th>
<th>10°</th>
<th>15°</th>
<th>25°</th>
<th>25°</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>94</td>
<td>98*</td>
</tr>
</tbody>
</table>

* On the sectored mycelia formed on medium B, sclerotia formation and fluffy or flat morphology varied depending on the sectors (sect. dep.). EES, early, encrusting sclerotia; LIS, late, isolated sclerotia.

### Table 2. Ability of mycelial structures of *Morchella esculenta* to regenerate after various temperature treatments

<table>
<thead>
<tr>
<th>No. of regenerated cultures*</th>
<th>Mycelium</th>
<th>EES</th>
<th>LIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 25°</td>
<td>17</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>Cold treatment</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Freezing (−17°)</td>
<td>0</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

* From 20 replicates.

### Table 3. Developmental characteristics of single-ascospore cultures of *Morchella esculenta* on medium B at 25° and their modification after subculture

<table>
<thead>
<tr>
<th>Parent cultures</th>
<th>Subcultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultures examined</td>
<td>100 (46+ 12− 30+ 30−)</td>
</tr>
<tr>
<td>Surviving cultures</td>
<td>88 46 12 30</td>
</tr>
<tr>
<td>Cultures with EES and LIS</td>
<td>46 4 0 0</td>
</tr>
<tr>
<td>Cultures with only LIS</td>
<td>42 (12+ 30+)</td>
</tr>
<tr>
<td>Cultures with sectors</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>Flat cultures</td>
<td>58 (46+ 12−)</td>
</tr>
<tr>
<td>Fluffy cultures</td>
<td>30 (30+)</td>
</tr>
</tbody>
</table>

EES, early, encrusting sclerotia; LIS, late, isolated sclerotia. Among the parents, 46 cultures formed both kinds of sclerotia (46+), whereas 42 only formed LIS (12 of them within 6 wk (12−) and 30 of them after 6 wk (30+)). The subcultures were divided into three groups, according to parental characteristics.

agar, 10; malt extract, 5; glucose, 10. Medium B comprised (g l−1): agar, 10; yeast extract, 4; starch, 15; glucose, 10; KH₂PO₄, 1; MgSO₄. 7H₂O, 0.5. The pH was adjusted to 6.5 before autoclaving (20 min./120° C).
Monospore cultures on medium B did not form sectors, and formation on them of both kinds of sclerotia was delayed relative to polyspore cultures. EES formed within 3 wk, whereas formation of LIS started after 3 wk and continued over several months. Additionally, the abundance of sclerotia was reduced and EES mostly did not form at the margin of the mycelia. Slightly more than half the cultures formed EES, whereas almost all of them produced at least one LIS (Table 3).

Cause of sectoring in polyspore cultures

Because sectoring only occurred in polyspore cultures and not on monosporal ones (Tables 1, 3), it was likely to be due to separation of individual genotypes. This was confirmed by pairing subcultures from 6-wk-old single-ascospore cultures. These subcultures were divided into three groups according to whether or not the parent culture had the following features as the subcultures were performed: (i) presence of EES; (ii) presence only of LIS; (iii) no sclerotia.

A line of aerial mycelium similar to that between sectors in polyspore cultures formed in all non-self pairings, even when subcultures from the same mycelial group were confronted (Figs 5, 6).

Relation between formation of sclerotia and aerial mycelium

In polyspore cultures on medium A, the mycelium formed a few secondary aerial hyphae but otherwise remained flat (Table 1). By contrast, all single-ascospore cultures on medium A produced an abundant secondary aerial mycelium and became fluffy.

In polyspore cultures on medium B, formation of aerial secondary hyphae was weak on most sectors with EES but abundant on sectors with only LIS or without sclerotia (Table 3). In single-ascospore cultures on medium B (Table 3) formation of secondary aerial hyphae was weak on all thalli with EES which remained flat. Single-ascospore cultures with only LIS also remained flat, if the sclerotia had formed within 6 wk, but produced abundant aerial hyphae if the sclerotia were formed later. All subcultures were fluffy, and few produced sclerotia.

DISCUSSION

The developmental variation in cultures grown from spores described here has not previously been reported, perhaps due to the rapid degeneration of morel mycelium during routine subculture (Mayr, 1982; Buscot, 1987; Meinhardt, personal communication). The most important finding is that two types of sclerotia exist, which differ with respect to the conditions affecting their initiation and distribution. Cultivation on dishes with different diameters shows that EES initiation is promoted by growth interruption, whereas LIS initiation is related to ageing of the culture.

Sclerotia similar to those in culture have not yet been observed in nature. However, the LIS have similar temperature requirements, cold resistance and biochemical properties (quantity and type of mycosporins) to the subterranean mycelial structures connected with ascocarps in nature. The latter function as storage and resting structures from which resources are redistributed to fructifications in the spring (Buscot, 1989; Buscot & Bernillon, 1991). By the same token, the formation of EES at low temperatures is a feature in common with young ascocarps (Jacquetant, 1984; Buscot, 1989), as are the types and quantities of mycosporins (Buscot & Bernillon, 1991). The morphological differences between the sclerotia formed in culture and natural forms may be due to the conditions of pure culture. The subterranean mycelial masses and ascocarps have an important associated microflora (Buscot & Kottke, 1990), and micro-organisms are known to modify both mycelial pigmentation and sclerotal formation in morel (Buscot, 1987).

LIS thus appear to represent cultural forms of the subterranean mycelial structures associated with ascocarps growing in nature. Since normal mycelium is not able to survive frost, and Schmidt (1983) demonstrated ascospores not to be resting propagules in morel, these structures are probably the only form able to over-winter. The biological significance of the EES remains uncertain, but it is likely that they represent imperfectly developed fruit bodies, as suggested by Mollard (1905) and Mayr (1982), or perhaps some sexual structure, as suggested by the fact that only one mycelial class produces EES.

In all cultures, formation of abundant aerial secondary hyphae only occurred when sclerotium production was reduced or delayed, suggesting that these processes are competitive and that distinction between 'flat' and 'fluffy' classes by Hervey et al. (1978) may not be absolute. Moreover, the observation that morel sclerotia only form after the total available nitrogen has been depleted from the medium (Mayr, 1982) would suggest that limitations on supply of this nutrient may fundamentally affect the balance between aerial mycelium formation and sclerotal formation. This could help to explain the differences between development and expression of recognition reaction on the different media.

On both media there was clear evidence that the presence of genetically different strains enhanced sclerotium formation. This effect was especially clear on medium A, where EES formation and low nitrogen content may have combined to suppress formation of aerial mycelium and discrete boundaries between individual genotypes.

The ability to shift from one pattern of development to another, dependent on nutrient availability, may be significant during natural colonization processes. Morels, which are poor competitors (Petersen, 1985), can establish either ephemeraly on disturbed soils (Carpenter et al., 1987; Turnau, 1987) or as perennial mycorrhizae in forests (Buscot, 1987; Buscot & Kottke, 1990; Buscot & Bernillon, 1991). Such fundamentally different niches would impose distinctive physiological requirements.

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REFERENCES


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