SHORT COMMUNICATIONS

Sexual Differentiation under Submerged Conditions in *Aspergillus nidulans*

By R. K. SAXENA AND U. SINHA

Department of Botany, University of Delhi, Delhi 110007, India

(Received 25 April 1977)

INTRODUCTION

Microbes provide a good model for the study of differentiation because morphogenesis can be controlled by adjusting physical and chemical factors in the environment. In solid or surface cultures the physical nature of the mycelial mat of filamentous fungi creates more physiological variation than that found in pellets formed in submerged shake cultures. This is one of the reasons for using submerged cultures for biochemical and molecular studies. Under such cultural conditions, most of the filamentous fungi remain entirely vegetative (Cochrane, 1958). However, asexual differentiation in various filamentous fungi can be induced and increased under submerged shake culture conditions by manipulating the physiological factors and media constituents (reviewed by Vezena & Singh, 1975).

Asexual sporulation observed in stirred batch cultures of *Aspergillus nidulans* is associated with maximal operation of the hexose monophosphate pathway (Carter & Bull, 1969). However, Bainbridge *et al.* (1971) have reported that under glucose maintenance or starvation conditions, there is no marked sporulative morphogenesis in *A. nidulans*. Conidiation in shaken batch cultures in this fungus has been reported by Martinelli (1972) and Saxena & Sinha (1973). Saxena (1976) and Martinelli (1976) have worked out the optimal conditions for conidiation of the wild-type mycelium and the conditions which prevent conidiation.

However, there are limited studies on the control of sexual differentiation in *A. nidulans*. Zonneveld (1972) observed that the omission of trace elements prevents cleistothecium formation in this fungus. He also showed (Zonneveld, 1976*a, b*) that manganese is the essential element for fructification and that its deficiency results in an impairment of phosphoglucomutase activity. Zonneveld's studies were made on solid cultures. In this paper we report for the first time that sexual differentiation can be achieved in submerged shake cultures and that the frequency of cleistothecia can be controlled by changing the cultural conditions of media constituents.

METHODS

Organism. The strain of *Aspergillus nidulans* (*riboA1*, *biA1*, requiring riboflavin and biotin and having green conidia) was from the departmental stock (initially obtained from Glasgow).

Media and cultural conditions. These have been described previously (Sinha, 1969; Saxena & Sinha, 1973). Minimal (MM) and complete (CM) media both contained 10 g D-glucose l⁻¹, unless otherwise stated. Liquid shake cultures were grown in 50 ml medium in 250 ml flasks, seeded with 5×10^7 conidia. Cultures were incubated at 37 °C at 150 rev. min⁻¹ (unless otherwise mentioned) on a New Brunswick gyrotory shaker.

Microscopy. One hundred randomly chosen pellets were examined per treatment to determine the frequency of cleistothecium formation. Anatomical details of the cleistothecia produced were examined after fixing mycelial pellets in formalin/glacial acetic acid/70% (v/v) ethanol (1:1:18, by vol.). Usual methods of dehydration and paraffin embedding were followed (Johansen, 1940). Serial sections were cut (15 µm thick) and stained with safranin [0.5% (w/v) in 50% (v/v) ethanol] and fast green [0.5% (w/v) in ethanol].

Fig. 1. Sexual differentiation of the *riboA1*, *biA1* strain of *Aspergillus nidulans* under submerged conditions. (a) Cleistothecia with hülle cells. (b) Surface view of a maturing cleistothecium. (c) Section of cleistothecium. (d) Section of a mature cleistothecium showing inner wall (IW), outer wall (OW) and central contents.

RESULTS AND DISCUSSION

Aspergillus nidulans, under submerged shake culture conditions, conidiates within 36 h of incubation (Saxena & Sinha, 1973). When conidiating cultures of the *riboA1*, *biA1* strain were incubated further in minimal or in complete media, a yellowish-brown pigment, perhaps melanin, was secreted by the mycelium into the medium after 48 h of incubation. This was accompanied by an increase in the pH of the culture filtrate and a change in the colour of pellets. Hülle cells started differentiating after 80 h of incubation and were intercalary as well as terminal in the hyphae. They were large, double-walled and almost circular with dense cytoplasmic contents. The mean diameter of mature hülle cells ranged from 6 to 8 µm.

Table 1. *Effect of D-glucose concentration on differentiation of hülle cells and cleistothecia*

Glucose concn (%, w/v)	Time of appearance (h) of:	
	Hülle cells	Cleistothecia
1.0	80	165
1.5	96	180
2.0	120	210

These cells were separated from their subtending hyphae by a septum. By this time, the pH of the culture filtrate had increased to 7.4 from the initial pH of 6.5. In both media (MM and CM), cleistothecia (the sexual fruiting bodies) started differentiating after 120 h of growth, and well-developed mature cleistothecia, surrounded by a large number of loosely arranged hülle cells, were formed within 160 to 165 h of incubation (Fig. 1*a, b*). Initially, cleistothecia were soft and light orange, but later they became less soft and brown to black. They were formed towards the centres of the pellets and the average mean diameter of mature cleistothecia ranged from 25 to 50 μm . Diameters of cleistothecia formed on solid media range from 200 to 500 μm . The pH of the culture filtrate increased to 8.4. Immature ascospores were observed within the cleistothecia (Fig. 1*c*). In MM only about 5 % of the pellets contained cleistothecia and there were 3 to 5 cleistothecia per pellet. In CM about 8 % of the pellets contained cleistothecia and there were 6 to 8 cleistothecia per pellet.

Effect of aeration and glucose concentration on submerged sexual reproduction

Aeration and the concentration of glucose were varied to investigate their influence on the frequency of cleistothecia. First, cultures were grown in minimal medium at 50, 100, 125, 150 and 200 rev. min^{-1} . Observations taken from random samples showed that the frequency of the cleistothecia produced was 5 to 8 per pellet at 100 rev. min^{-1} ; this was approximately double the value obtained at 150 rev. min^{-1} (3 to 5 per pellet). In both cases only about 5 % of the pellets bore cleistothecia. The fungus grew on the surface of the medium at 50 rev. min^{-1} ; at 200 rev. min^{-1} the pellets were too small, perhaps due to vigorous shaking, and cleistothecia were not differentiated.

As D-glucose is the best carbon source for growth of *A. nidulans* (Martinelli, 1976), cultures were grown in minimal medium containing 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0 % (w/v) D-glucose. Only 1.0, 1.5 and 2.0 % D-glucose could support cleistothecia formation. However, sexual differentiation was gradually delayed with an increase in D-glucose concentration (Table 1). The maximum numbers (6 to 8) of cleistothecia per pellet were produced with 2 % glucose. Concentrations of 0.5, 3.0 and 5.0 % did not support cleistothecia formation up to 165 to 170 h of incubation. This might be due either to the low initial concentration, for 0.5 %, or to an excess of glucose, which supported more vegetative growth, for the other concentrations.

Anatomy of cleistothecia

Serial sections (15 μm thick) of a large number of mycelial pellets were cut and stained with safranin and fast green. Observations revealed (Fig. 1*d*) that a mature cleistothecium formed in liquid shake cultures was a double-walled structure. The outer wall of the cleistothecium was made up of loosely arranged hülle cells, while the inner wall was one to two cells thick and was parenchymatous. The centre of the cleistothecium contained a mass of immature ascospores.

Besides being the first report of its kind amongst filamentous fungi, the present study of submerged sexual differentiation in *A. nidulans* is also of great practical importance to those working with the hormonal mechanisms and controls involved in the completion of this morphogenetic event.

The authors have great pleasure in thanking Professor B. M. Johri for his valuable suggestions. One of us (R.K.S.) acknowledges with thanks the receipt of a senior research fellowship from the Council of Scientific and Industrial Research, New Delhi, India.

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