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# The Separation of Viable Rye Microspores from Mixed Populations and Their Development in Culture

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*Summary.* A technique is described to isolate rye microspores from anthers precultured on agar and from intact spikes. Highly purified fractions consisting predominantly of viable late uninucleate or early binucleate microspores are obtained by filtration and centrifugation techniques. The preparations form an excellent starting material to attempt to increase the frequency of microspore plantlet formation above that normally obtained by anther culture. Microspores isolated from anthers precultured for five or six days on agar, develop after three weeks culture in a liquid medium into multicellular structures, the largest of which burst through the microspore wall.

## Introduction

Microspores within cultured anthers of *Secale* give rise to multicellular structures some of which develop further producing calluses or embryoids and plantlets (Thomas and Wenzel, 1975a). However, the percentage of microspores which respond is extremely low partially because only a small number of microspores in any one anther are in the correct developmental stage for culture (immediately prior to, during or just after the first pollen mitosis). Even in cases where multicellular structures are obtained from microspores many later abort possibly due to competition between the structures for compounds necessary for further development. One possible way to increase the percentage success would be to attempt to obtain by physical separation techniques, fractions of high purity which contain only microspores in the correct developmental stage and to determine the conditions permitting their growth in isolation.

## Methods and Results

Spikes of winter rye $F_1$ hybrids were harvested still enclosed within their flag leaves. They were sterilised by swabbing with 70% ethanol, enclosed within sterile plastic bags and cultured in liquid Nitsch and Nitsch (1969; NN) medium until the ears emerged from the flag leaf (Thomas, Hoffmann, Wenzel, 1975). Microscopic examination showed that microspores in anthers from the middle of the spike were most likely to be in the correct developmental stage for culture. The microspores were either isolated directly from the spikes by cutting the spikes into 1 mm sections followed by gentle maceration in a washing medium contained in a glass mortar or they were isolated from anthers precultured on NN plus 0.25 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for five or six days at 25° C

in the dark. 1.5 g fresh weight spike segments or 30 precultured anthers were macerated in 15 ml of a washing medium, which contained 0.6 M mannitol, 50 mM $Ca(NO_3)_2$ at pH 7.0. Following filtration of the macerated tissue through a 500 μ filter the filtrate was centrifuged at 30 g for 3 min, and the pellet resuspended in washing medium. This procedure was repeated through a filter of 100 μ. The composition of such a preparation is shown in Fig. 1a. It can be observed that the suspension still contains high proportions of dead microspores in which the cytoplasm is coagulated. Such dead microspores are also present within fresh or precultured anthers before isolation. Their percentage varies with the $F_1$ hybrid type and with the cultural conditions used for plant growth. To separate the viable and non-viable microspores the suspension was placed on the surface of a 30% sucrose solution and centrifuged for 5 min at 1200 g. The active vacuolated microspores formed a band at the top of the sucrose solution while all dead and nonvacuolated structures and mature pollen grains sedimented. Examination of the resulting microspore fraction showed that predominantly late uninucleate spores were present; a small number were binucleate. The band was pipetted off, diluted with washing medium and sedimented by centrifugation at 500 g for 1 min. The quality of the final preparation is shown in Fig. 1b. A slightly modified technique also enables other stages of microspore development to be isolated (Wenzel, Hoffmann, Thomas, unpl.).

The microspores were inoculated at a concentration of $10^5$ microspores per ml into a liquid culture medium $P_2$ (Table 1) contained in 3 cm plastic petri dishes sealed with parafilm. The dishes were incubated at 25°C in the dark in a moist chamber. The survival rate of isolated spores increased with increasing mannitol concentrations (Fig. 2), but all microspores separated from spikes without agar preculture died between one and six days, even though they often showed very active plasmic streaming. However, many of the microspores precultured on the NN agar medium for five or six days remained alive and continued further development. After two to three days in liquid culture the cytoplasm of such microspores concentrated in the centre and after seven to nine days the first divisions could be observed (Fig. 1c–e). Although the stability of the spores increased with increasing mannitol concentrations, the percentage of divisions decreased drastically, when the molarity reached values higher than 0.5 M (Fig. 2). In Fig. 1c the nuclei are still attached to the newly formed wall. Subsequently further divisions take place (Fig. 1f) and after 20 days the structures burst through the microspore wall (Fig. 1g). Although medium containing 0.3 M mannitol supported the growth and cell division of a high percentage (up to 33%) of living microspores, medium lacking mannitol did allow the development of small numbers of spores. In such cases comparatively large callus-like structures composed of very thick walled cells were formed (Fig. 1f, g).

Some of the microspores undergo nuclear division without wall formation, an observation also reported in anther cultured microspores (Wenzel and Thomas, 1974). In $P_2$ culture medium, which completely lacks organic nutrition compounds, growth stops after 20 days; the walls become very thick and the whole structure gradually dies. Currently attempts are being made to modify the medium in a manner which may permit of further growth of the multicellular structures, after initial growth in $P_2$.

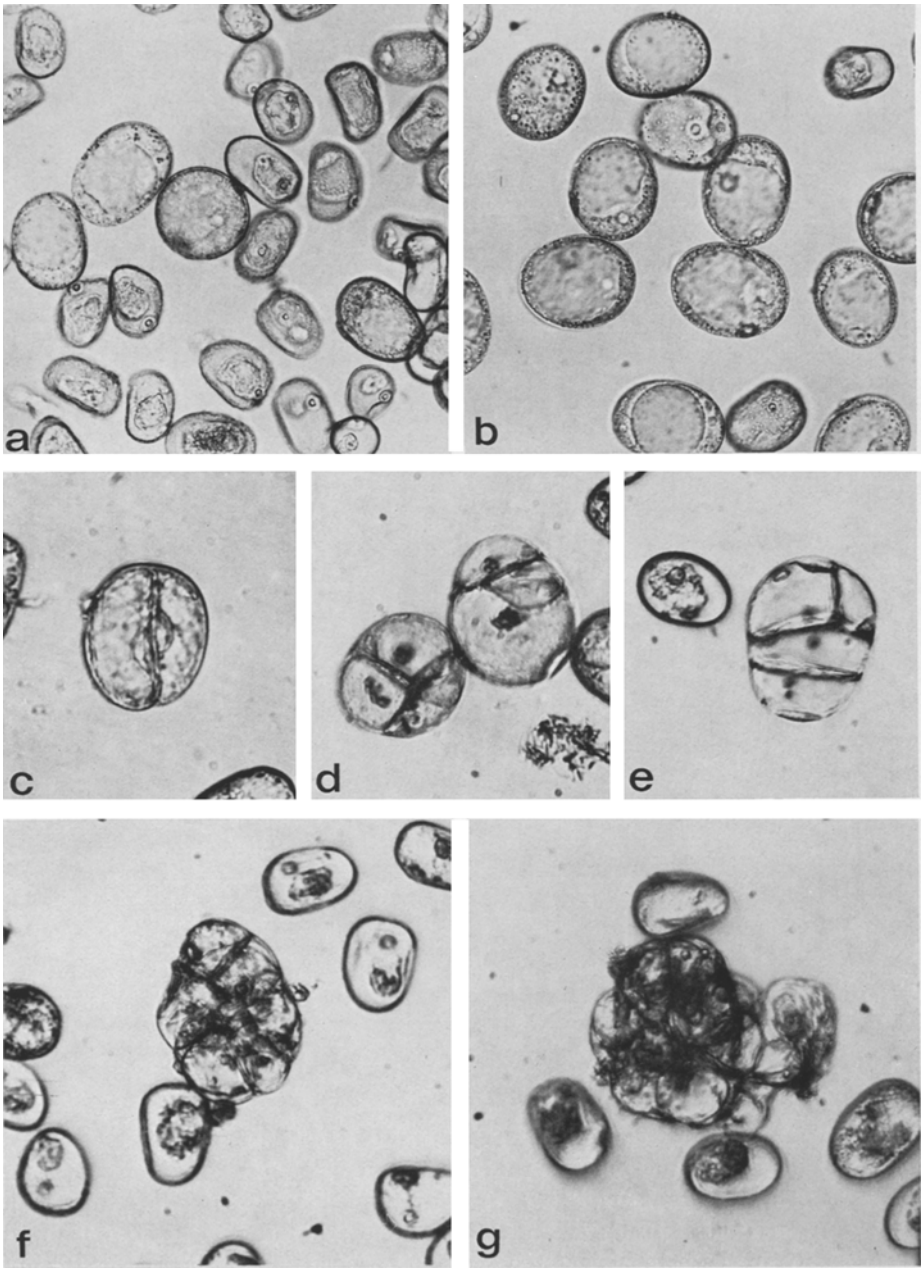


Fig. 1. (a) Microspore preparation before purification, containing numerous dead microspores; (b) Preparation after centrifugation over 30% sucrose; (c) bicellular structure with nuclei still attached to the new wall; (d, e) Early multicellular structures formed in $P_2$ medium containing 0.3 M mannitol; (f, g) Callus-like masses formed in $P_2$ medium without mannitol and which have burst through the exine

Table 1. Culture medium $P_2$ for isolated rye microspores

| | | | |
|---|---|---|---|
| $Ca(NO_3)_2$ | 10 mM | Fe-solution | 5 ml |
| $KNO_3$ | 5 mM | according to NN medium | |
| $CaCl_2$ | 5 mM | mannitol | 0.3 M |
| $MgSO_4$ | 1 mM | 2,4-D | 0.1 mg/l |
| $KH_2PO_4$ | 1 mM | pH | 6.0 |
| $NaH_2PO_4$ | 0.15 mM | | |

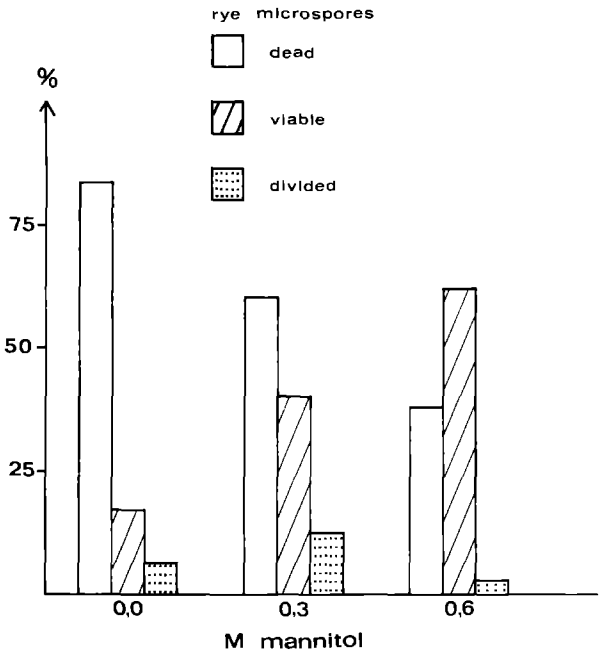


Fig. 2. The behavior of isolated rye microspores in response to different mannitol concentrations after 12 days of culture

## Discussion

At present greatest success in the culture of isolated microspores has been achieved in Solanaceous plants (Sharp, Raskin, Sommer, 1972; Nitsch and Norreel, 1973). So far attempts with other dicotyledonous plants have met with only limited success (Kameya and Hinata, 1970; Binding, 1972; Thomas and Wenzel, 1975b). In cereal plants there have been no reports on the culture of isolated microspores. The high success rates in Solanaceae may in part be due to the high synchrony of the developing microspores within the anther such that there is a greater possibility of obtaining large numbers of isolated microspores in the correct developmental stage for culture. The technique now described opens up the possibility of obtaining such microspore fractions in plants where pollen development is largely asynchronous. Our observations that microspores from

precultured anthers of rye give consistently better results than those from non-cultured anthers is in agreement with the observations in *Datura* cultures (Nitsch and Norreel, 1973).

The two patterns of microspore development observable in our isolated microspore cultures (normal cell division and nuclear division without wall formation) are very similar to those which occur within certain rye anthers. However, the advantages of this new technique over anther culture not only lie in the possibility of increasing the percentage of development above that obtained by anther culture technique but it also enables direct observation of the effects of changes in the physical and chemical environment. Currently we have experimented only with a very basic culture medium lacking organic compounds. Experiments are now in progress to develop a medium which allows the growth of multicellular structures into calluses or embryoids.

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