MICROSPORE CULTURE TECHNOLOGY

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I. INTRODUCTION

Pollen development in vivo follows a precise sequence from the postmeiotic release of microspores from tetrads to the formation of a male gametophyte (bicellular pollen, Fig. 1) and eventually to the dispersal and germination of mature pollen. However, when cultured in vitro, microspores or immature pollen (hereafter referred to as microspores, for simplicity) can be diverted from the normal developmental pathway to undergo repeated divisions leading to the formation of embryos or calli from which plants can be regenerated. This induced developmental pathway, which has been shown to occur in several taxonomic groups, will be referred to as microspore embryogenesis. Production of plants from microspores was first observed in *Datura innoxia* 25 yr ago (33,34), during a study of meiosis in vitro. Since then great interest has developed in using this technique to produce haploid and homozygous diploid plants for genetic and developmental studies as well as for plant breeding purposes (20-22,35,39,50). Microspore-derived plants (microspore-plants) have been produced in hundreds of species by anther culture (22). Isolated microspore culture, although less explored, has also yielded plants in a number of plant species, including tobacco (61-63), *Nicotiana rustica* (45), *Brassica carinata* (14), barley (77), rice (11), and maize (18,56). Microspore and anther cultures have been used by plant breeders to produce doubled haploid plants efficiently. Microspore embryogenesis also provides a valuable system for developmental as well as genetic studies. The use of microspore cultures as recipient systems for gene transfer is a relatively new concept. Similar to protoplast culture, isolated microspore culture provides a single-cell system suitable for studies of direct DNA uptake, either chemically or electrically mediated. Microspore cultures at different stages during development (from a single microspore to a young microspore-plant) can be used as recipient cells for introduction of foreign genes, either by *Agrobacterium*-mediated gene transfer (73) or by microinjection (52). In such cases the transformed cells are haploid, and chromosomal doubling, either spontaneous or colchicine-induced, will lead directly to the production of homozygous diploid transformants. One additional advantage of using microspore culture as a recipient system for foreign genes is the high efficiency of plant regeneration in some species, such as *Brassica napus* in which over 20% of the cultured microspore can develop into embryos and, subsequently, plants (39).

This paper will describe the methodologies of microspore culture that have been successfully developed in *Brassica napus* (rapeseed), *Zea mays* (maize), and *Hordeum vulgare* (barley), three major, important crop species. However, the protocols can be applied to other plant species effectively, perhaps with some testing and modification.

II. MATERIALS

A. Equipment

- Autoclave
- Laminar air flow hood
- Two incubators or growth rooms (25 ° and 32 ° C)
- Growth room, 16 h light, 20 ° and/or 25 ° C
- Microwave oven
- Clinical centrifuge and centrifuge tubes
- Growth chambers for donor plants (optional)
- Inverted microscope
- Domestic fridge and freezer
- Stereoscope (optional)
- Balance
- pH Meter
- Millipore filtration units (0.2 µm membrane)

B. Glassware, culture vessels, and miscellaneous items

- Glass beakers of various sizes
- Glass flasks of various sizes
- Glass funnels
- Lipshaw basket (or tea strainer)
- Tweezers
- Hemacytometer
- Nylon mesh (44 µm pores)
- Parafilm (American National Can)
- Pasteur pipette
- Plastic petri dishes, e.g., Falcon 1001 100 X 15 mm
- Falcon 1005 100 X 20 mm
- Falcon 1007 60 X 15 mm
- Falcon 1008 35 X 10 mm

C. Chemicals

- Agarose
- Agar
Colchicine, Sigma
Ca hypoehlorite
Ethanol
Acetocarmine stain (solution)
Chemicals used in culture medium (Table 1)
All the equipment, chemicals, and other materials listed above can be purchased from laboratory supply companies.

III. PROCEDURE

A. Preparations

Glassware and distilled water are sterilized by autoclaving at 120°C for 20 min.

For convenience, concentrated media stocks containing salts and organic supplements can be prepared at 5X strength and stored in a freezer for several months. Media can also be made days in advance and stored at 2 to 4°C until use. Media for washing and culture of microspore should be filter sterilized, whereas media for plant regeneration are normally autoclaved. Components for various commonly used tissue culture media are listed in Table 1.

B. Brassica napus (rapeseed) (17,14-17,25,30,41,42,48,49,55,60,72)

1. Growth of donor plants

Many genotypes, including Topas (Spring type) and Jet Neuf (winter type), have been shown to be good donor plants for microspore culture. Donor plants may be grown in the field or in a growth chamber (15°C to 20°C, 16 h light), with regular supply of fertilizers and routine watering.

When plants begin to bolt, approximately 5 wk after seed germination, it is best to lower the growth temperature (if a growth chamber is available) to 10°C/5°C (39).

2. Bud sterilization

Young flower buds (3 to 4 mm in length, containing late uninucleate to early bicellular microspores, or stages 3 to 5 as illustrated in Fig. 1) are collected from lateral recemes. Buds are placed in a small Lipshaw basket or a tea strainer (1 to 20 buds/cage) and immersed in 7% Ca hypochlorite (or commercial bleach) for 15 min. They are then given three 5-min rinses in sterile water.

3. Microspore stage

The developmental stage of microspores is determined by squashing an anther in a drop of lacto-propionic acid (1%, in 22.5% each lactic acid and propionic acid) and examining under a light microscope. Microspores from the late uninucleate to the early binucleate stage (stages 3 to 5, Fig. 1) are the most embryogenic (25,55).

4. Microspore isolation

The buds are placed in B5 medium (Table 1) containing 13% sucrose (B5-13) in a small beaker (1 to 10 buds/ml) and macerated with a glass rod or a syringe barrel against the wall of the beaker. The homogenate containing the released microspores is then passed through 44-μm nylon mesh and the filtrate is centrifuged at 100 Xg for 3 to 5 min. The