A novel method to produce *Anabaena*-free *Azolla* by in vitro fertilization of micromanipulated megasporocarps

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Received May 18, 1988/Revised version received August 2, 1988 – Communicated by G. C. Phillips

**ABSTRACT**

In the *Azolla-Anabaena azollae* symbiotic system, *Anabaena* akinetes get entrapped between the indusium and the apical cap of the megasporangial apparatus during megasporangial development, thus maintaining the continuity of the cyanobacterial association throughout the life cycle of the fern. The entrapped akinetes serve as a source of inoculum for infecting the new sporophyte when it is emerging from the megasporangial apparatus. A procedure to generate *Anabaena*-free *Azolla* was developed by utilizing the germinating megasporocarps in which the indusium along with the akinetes were removed by micromanipulation. This method has the advantage of not requiring drastic treatments of *Azolla* with antibiotics to eliminate the endosymbiotic cyanobacterial cells. Details of this new method and its usefulness in studies aimed at recombination of *Azolla* with *Anabaena azollae* are discussed.

**INTRODUCTION**

*Azolla* is a heterosporous aquatic fern which harbors the cyanobacterium *Anabaena azollae* Stras. as an endosymbiont in its leaf cavities. *Anabaena* is able to fix atmospheric nitrogen and can provide both itself and its host *Azolla* with their total nitrogen requirement (Peters and Mayne 1974 b). Agronomically, the *Azolla-Anabaena* symbiosis has been recognized as an important biofertilizer in lowland rice cultivation (Lumpkin and Plucknett 1982, Silver and Schroder 1984).

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**MATERIAL AND METHODS**

**Azolla Culture and Isolation of Sporocarps:**

Plants of *Azolla mexicana* Presl. were routinely grown in the greenhouse in plastic buckets containing IRRI medium (Yoshida et al. 1971) devoid of combined nitrogen (I− medium). About 30 d old greenhouse grown cultures were used to obtain sporocarps for this study. To remove contaminating epiphytic algae, sporulating cultures were subjected to washing under running tap water for 30 min, followed by surface sterilization with I− medium containing 1.5 % Clorox (v/v; 0.08% sodium hypochlorite) and 0.1 % SDS (w/v) for 2 min under aseptic conditions. This Clorox solution was quickly drained using a millipore filter unit connected to a vacuum pump and the fronds were washed repeatedly with large volumes of sterile I− medium for 30 min. Matured mega- and microsporocarps from the washed fronds were manually dissected out under the dissecting microscope in a laminar flow hood. Fresh mega- and microsporocarps were collected separately whenever needed. The isolated microsporocarps were crushed with forceps and the released microsporangia were washed once with the Clorox solution for 1 min and repeatedly with sterile I− medium isolation prior to making any attempts at recombining them. Until now, the methods employed to achieve cyanobacterium-free *Azolla* involved subjecting the vegetative plants to drastic treatments of a multitude of antibiotics for prolonged periods in culture (Peters and Mayne 1974 a, Ashton and Walsley 1976). However, such treatments may result in permanent changes in the fern at the molecular level. In this communication, we present a simple procedure to generate *Anabaena*-free *Azolla* from micromanipulated megasporocarps. The advantage of this procedure in reinfection studies is also discussed.
for 20 min before they were employed in the study.

Micromanipulation of Megasporocarps and in vitro Fertilization:

Isolated megasporocarps (Fig. 1A) were transferred to Petri dishes containing I−-medium plus agar (0.8 %; w/v) which was overlaid with a thin film of liquid I−-medium, and incubated at low light intensity in environmental growth chambers. Generally, after 3 d, greening of the megaspore and opening of the floats due to swelling of the megaspore apparatus inside the megasporocarp were observed which indicated the initial stages of germination (Fig. 1B). Such megasporocarps, before the emergence of the female gametophyte, were picked up and the indusium with underlying apical membrane together with its contents and sporocarp wall were removed (Fig. 1C) with the help of a fine stainless steel needle and forceps under the dissecting microscope kept in a laminar flow hood. The indusium-excised germinating megasporocarps (Fig. 1D) were quickly passed once through the Clorox solution for 1 min and then repeatedly through vigorously agitating sterile I−-medium for 20 min to remove the attached cyanobacterial akinetes, if any. Later these naked megasporocarps were returned to the Petri dishes containing fresh I−-medium overlaid with liquid I−-medium as above. To these Petri dishes, after 1 d (when the female gametophyte started emerging; Fig. 1E), were added massulae (obtained by crushing washed microsporangia with forceps) containing microspores and thoroughly mixed by shaking. Mixing enabled the massulae to get attached to the naked megasporocarps particularly at apical regions and/or on the perine (Fig. 1F). The mixed cultures were incubated at low light intensity in environmental chambers for the fertilization to take place. After fertilization, when the sporophytes