SHOOT REGENERATION FROM SAFFRON PROTOPLASTS IMMOBILIZED IN Ca-ALGINATE BEADS

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Abstract

Saffron (*Crocus sativus* L.) protoplasts were isolated from the cells of a suspension culture or calli with a solution of Cellulase, Pectinase and Hemicellulase and embedded in Ca-alginate beads. They were cultured with or without nurse cells in MS medium supplemented with 2,4-D and 6-benzylaminopurine at 25°C. After several changes of medium, cell-clusters appeared on the surface of the Ca-alginate beads. The protoplasts without immobilization in Ca-alginate beads did not display cell division. Furthermore, growth of cell clusters in the medium with nurse cells was much better than in the medium without nurse cells. Then, the beads were transferred onto MS agar medium supplemented with 1-naphtalene acetic acid and 6-benzylaminopurine and cultured at 15°C or 20°C. After 3-4 months of culture, great calli were observed on the surface of the beads. The regenerated shoots were obtained approximately 6-7 months after protoplast isolation. The critical factors seem to be associated with the quality of the culture, enzyme composition and concentration used for protoplast isolation.

Introduction

Attempts to cultivate the saffron protoplasts were not successful. Alginate entrapment of plant protoplast has been subsequently reported as an effective method of protoplast cultured [6,11]. A nurse culture approach [7,9] on Ca-alginate beads, which is also an effective method of protoplast culture, is experimented to improve saffron protoplast culture. Saffron protoplast culture and regeneration in Ca-alginate beads at a very high frequency have been developed from calli and cell suspension culture [4]. This paper shows cultivation of saffron protoplasts by this method which leads to callus and shoot formation. The isolated protoplasts may be used for fusion or DNA transformation experiments to overcome sterility of saffron in future studies.

Keywords: Alginate beads; *Crocus sativus* L.; Protoplast culture; Protoplast immobilization; Shoot regeneration

Materials and Methods

Calli were derived from saffron (*Crocus sativus* L.)

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corms cultured on MS medium [8] supplemented with 0.5 mg/l of each of 2,4-D and BAP (abbreviated as MS D 0.5, B 0.5) at 25°C under dark conditions. For isolation of protoplasts, about 5 to 10 g of saffron callus or liquid cultured cells were collected [3], and treated with an enzyme solution consisting of MS basal medium with 1% (w/v) Cellulase (from *Tricoderma viride*, Sigma), 1% (w/v) Hemicellulase (from *Aspergillus niger*, Sigma), 1% (w/v) Pectinase (from *Rhizopus sp.*, Sigma) and 0.3 M Mannitol by shaking for about 1 h at 20±2°C. The isolated protoplasts were filtered and washed in 0.3 M Mannitol by 3 successive centrifugations (1500 rpm for 5 min). The prepared protoplasts (at the density of 5×10^4 protoplasts/ml) were mixed gently with a sterile Na-alginate solution (2% in 0.3 M Mannitol solution). These alginate solutions together with the protoplasts were added drop by drop with a sterile pasteur pipette into a MS basal medium containing 1% $CaCl_2$ and 0.3 M Mannitol. Each droplet immediately formed an alginate bead. The beads were left in this solution for 20 min to complete gelation, then washed twice with the MS D 0.5, B 0.5 medium containing 0.3 M Mannitol. They were cultured in MS D 0.5, B 0.5 medium with nurse cells (high density of cells of the same species in suspension culture) and without nurse culture at the above mentioned cell density. Isolated protoplasts were also cultured in the same nutrient medium without embedding in Ca-alginate gel as control [4]. Osmolarity of the nutrient media decreased when half of the original medium was replaced successively by a medium with a lower osmotic pressure every 7 to 10 days. Growth rate was estimated by counting the fractions of cells which divided inside the beads under the microscope. After 6 to 10 weeks of culture, the microcalli appeared on the surface of the alginate beads. The beads were transferred to MS agar-medium supplemented with 0.1 mg/l 1-naphtalene acetic acid and 1.0 mg/l 6-benzylaminopurine (abbreviated as MS N 0.1, B 1), and cultured at 15 or 20°C under dark conditions.

Results and Discussion

Growth rates of saffron protoplasts are shown in Table 1. Although control protoplasts without embedding in Ca-alginate gel did not show any cell-division, immobilized protoplasts were able to divide and form cell colonies. Immobilized protoplasts with nurse cells grew much better in comparison with immobilized cells being able to divide and form a microcallus; this suggests that protoplasts were protected by embedding during the early period of culture after protoplast isolation. Immobilization may offer improved protection for fragile cells such as plant protoplasts. Entrapment in Ca-alginate gel is one of the mildest immobilization procedures known, because the gelling agent itself is not toxic and the gelling process is themo independent [1]. The nurse beads showed a high growth rate and a callus was quickly formed suggesting that the mother cells promoted the growth of protoplasts in the nurse beads (Fig. 1, A-I). To decrease osmolarity in this experiment, half of the original medium was replaced successively by fresh medium with a lower osmotic pressure. Protoplast culture is usually successful when medium changes are frequently performed to allow a gradual decrease of the osmotic pressure and concentration of growth regulators [2,12]. Through the use of Ca-alginate beads, this objective could be attained easily and quickly. A density of 5×10^4 protoplasts/ml gave a higher growth rate than other densities suggesting that 5×10^4 protoplasts/ml was the best density for growth continuation. The microcalli appeared on the surface of the alginate beads after 6-10 weeks. After 3-4 months of culture, the calli were observed in some of the alginate beads (Fig. 1, E-I). After 6-7 months, shoot regeneration from the callus was achieved (Fig. 1, J). Plant regeneration from the callus of saffron protoplasts was achieved based on the methods reported previously [3]. It seems therefore, that the type and concentration of growth regulators, enzyme solution and Na-alginate solution and stability of temperature may be critical in achieving the optimum response reported previously [4]. The results of this study may be used for the isolation of totipotent protoplasts from embryogenic callus or cell suspension as well as the development of artificial seed technology [5,10,13].

Table 1. Effect of Ca-alginate and nurse culture on the growth rate of saffron protoplasts

Protoplast culture	2 weeks	2-4 weeks	6-10 weeks	3-4 months	6-7 months
Control (liquid cultured protoplasts)	No division	No division	No division	No division	No division
Ca-alginate beads	Cell division 2%	Cell colony 2%	Cell colony 2%	Cell colony 2%	Cell colony 2%
Ca-alginate beads + nurse cells	Cell division 6%	Cell colony 10%	Microcalli 10%	Calli 8%	Shoot regeneration 6%

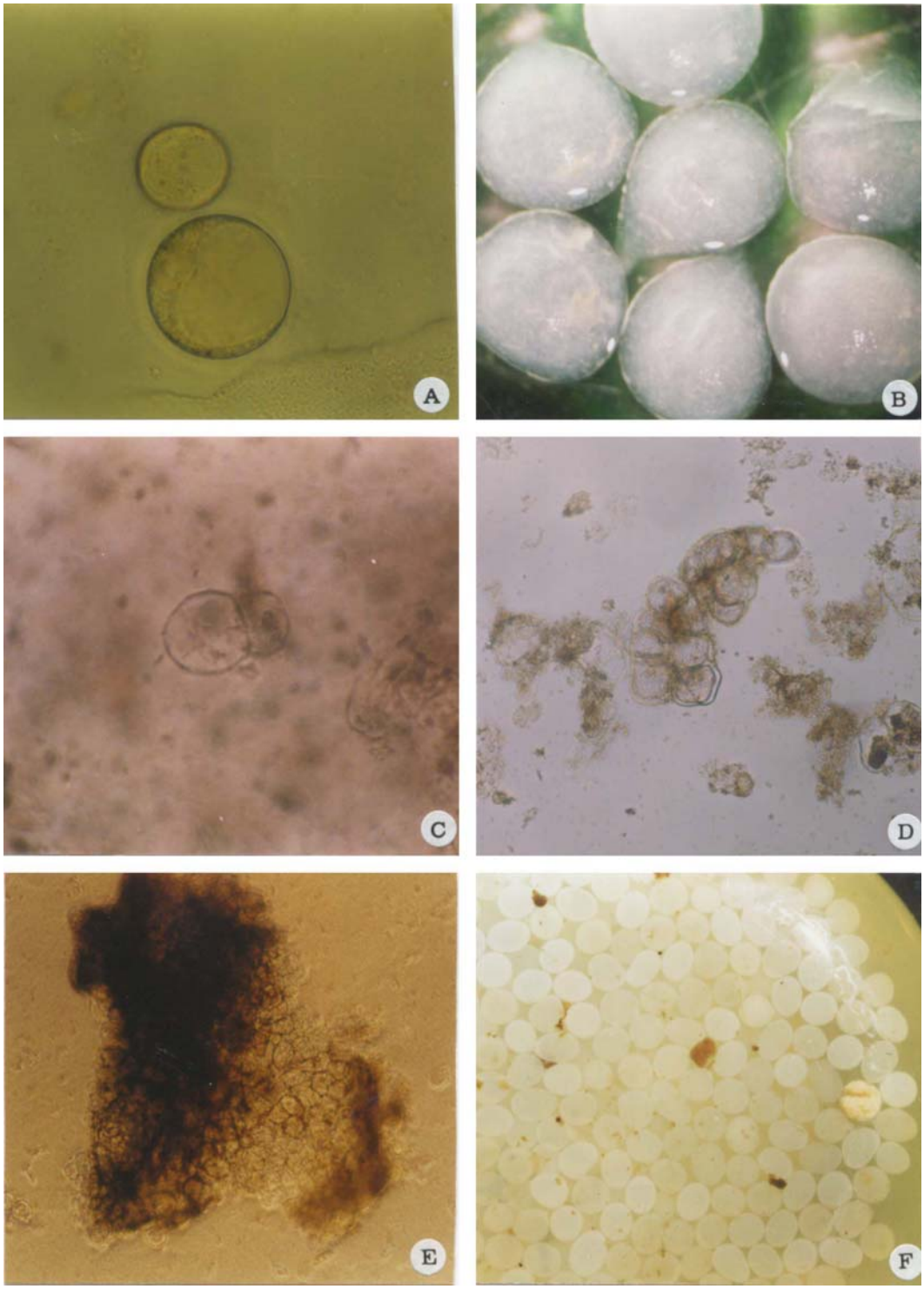

Figure 1. Development of immobilized protoplasts of saffron. *A:* isolated saffron protoplasts, *B:* nursed Ca-alginate beads, *C:* first cell division, *D:* cell colony, *E:* appearance of cell clusters on the beads, *F:* appearance of callus through beads, *G, H:* callus after 3 months, *I:* callus after 4 months, *J:* shoot regeneration on the callus after 6-7 months on MS: N 0.1, B 1 medium.

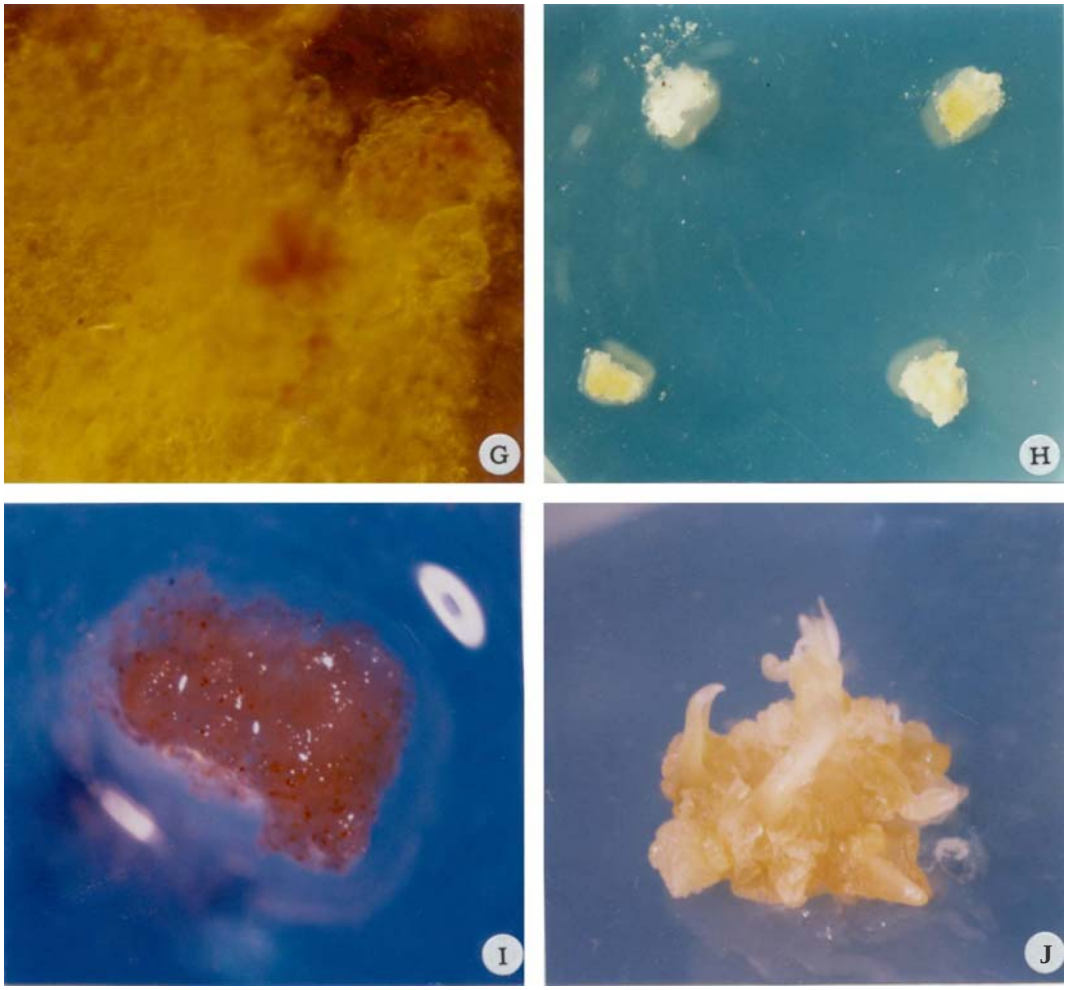

Figure 1. Continued.

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