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Investigation of the Best Time of Enzyme Treatment in Order to Isolate the Protoplast from Embryogenic Callus of Saffron (*Crocus sativus* L.)

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Abstract: *Crocus sativus* L. is a triploid and sterile crop that propagates vegetatively by corm. Therefore, its improvement via traditional breeding method is difficult. Protoplast culture is one of the suitable tools for breeding of this plant. Protoplasts with a high viability are potentially interesting materials, especially for somatic hybridization, transformation and selection studies. In this research, In order to extract and isolate the protoplast, embryogenic calli were suspended in enzyme solution consisting of MS medium with 0.1% Pectolyase Y-23, 1% Cellulose R-10, 1% Deriselase, 0.1% MES (2-N-morpholino ethane sulfonic acid) and 0.3 M mannitol at pH 5.7. The mixture was placed on a rotary shaker (200 rpm) at 25°C in darkness under 4 time-term treatment (1.5, 3, 4 and 5 h). Counting number and viability of protoplasts isolated from embryogenic calli with homeocytometer and Trypan Blue after 1.5, 3, 4 and 5 h of enzyme treatment indicated that after 3 h enzyme treatment, 40×10^5 protoplasts per 1 mL of suspension with 98% viability obtained that were the best time-term treatment. Finally, in order to protect and maintain the fragile protoplast, Calcium-alginats beads were used for their immobilization.

Key words: Embryogenic callus, protoplast, viability, homeocytometer, trypan blue, immobilization

INTRODUCTION

Protoplast with a high viability is potentially interesting material, especially for somatic hybridization, transformation and selection studies. In monocots, culture of protoplast isolated directly from the plant's organs is difficult (Vasil and Vasil, 1992; Ebrahimzadeh *et al*., 1996) and protoplast derived from cell-suspension often have failed to produced green plants (Maddok, 1987; Lee *et al.,* 1988). Indeed, plant regeneration has been usually successful when embryogenic calli were used as the source of protoplast extraction (Vasil, 1988; Ebrahimzadeh *et al*., 1996). In the present study, effect of time-term enzyme treatment on number and viability of isolated protoplasts derived from embryogenic callus were studied.

MATERIALS AND METHODS

Maintenance of embryogenic calli: Embryogenic calli obtained from meristem culture of *Crocus sativus* together with globular embryoids were transferred to a maintenance MS (Murashig and Skoog, 1962) medium supplemented with 3% sucrose, 100 mg L^{-1} ascorbic acid, 2 mg L^{-1} BAP and 1 mg L^{-1} NAA. Cultures were incubated at 22±2°C in darkness, subcultured at 15 day intervals for 2-3 months. (Ebrahimzadeh and Karamian, 2001).

Isolation of protoplasts: Produced embryogenic calli were transferred to centrifuge tubes and were suspended in enzyme solution consisted of MS medium with 0.1% Pectolyase Y-23, 1% Cellulose R-10, 1% Deriselase, 0.1% MES (2-N-morpholino ethane sulfonic acid) and 0.3 M

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mannitol at pH 5.7. The mixture was placed on a rotary shaker (200 rpm) at 25°C in darkness under 4 time-term treatments (1.5, 3, 4 and 5 h). In order to remove cell debris, the incubated mixture was filtered through a 45 μm nylon mesh. The filtrate was then centrifuged for 3 min at 500 g. The supernatants was discarded and the pellet was resuspended in a washing solution (0.1% MES in 0.3 M Mannitol at pH 5.7) and centrifuged for 3 min at 500 g. After another washing following the above procedure, the centrifugation was repeated.

Counting number and determine viability of isolated protoplast: Ten microliter of protoplast suspension mixed with 90 μL of Trypan Blue solution and a little amount of this mixture laid on homeocytometer. Then total number of protoplasts and blue-color protoplasts were counted under the light microscope. Finally, number of protoplasts per 1 mL of suspension and their viability were calculated using following formulas:

$$\text{No. of protoplasts per 1 mL of suspension} = \frac{\text{Total No. of protoplast}}{\text{Counted area (cm}^2\text{)} \times \text{chamber depth (cm)} \times \text{dilution} \times \text{the first volume of the protoplast suspension}}$$

$$\text{Viability of protoplast} = \frac{\text{Total No. of protoplast} - \text{No. of blue protoplast}}{\text{Total No. of protoplast}}$$

Immobilization of protoplasts: The prepared protoplasts were mixed gently with 2% sterile Na-alginate solution (Riedel-de Haën) in 0.3 M mannitol at the density of 1×10^5 protoplasts mL^{-1} (Isa *et al.*,1990).These alginate solution with the protoplasts were added drop by drop with a sterile Pasteur pipette into MS medium containing 1% $CaCl_2$ and 0.3 M mannitol. Each drop immediately formed an alginate bead.

RESULTS AND DISCUSSION

Maintenance of embryogenic calli: Quality and quantity of the isolated protoplasts were primarily depended on the condition of embryogenic calli and the duration of subcultured. Due to existence of essential material for the growth and division of cells in maintenance medium, cells have the high growth ability and high amount of protoplasts with high growth ability have produced from them.

Isolation and Immobilization of protoplasts: Counting number and viability of protoplasts isolated from embryogenic calli with homeocytometer and Trypan Blue after 1.5, 3, 4 and 5 h of enzyme treatment indicated that 22×10^5, 40×10^5, 32×10^5 and 26×10^5 protoplasts per 1 mL of suspension with 100, 98, 91 and 84% viability were produced, respectively (Fig. 1-3). The results indicated that after 3 h enzyme treatment, number of protoplasts and their viability were highest (Fig. 3c). Increase in time term of enzyme treatment resulted in high contacting of isolated protoplasts to the centrifuge tubes walls and decreasing number of viable protoplasts. In order to protect and maintain the fragile protoplast, Calcium-alginats beads were used for their immobilization (Fig. 3d). This method is an effective tool because the gelling

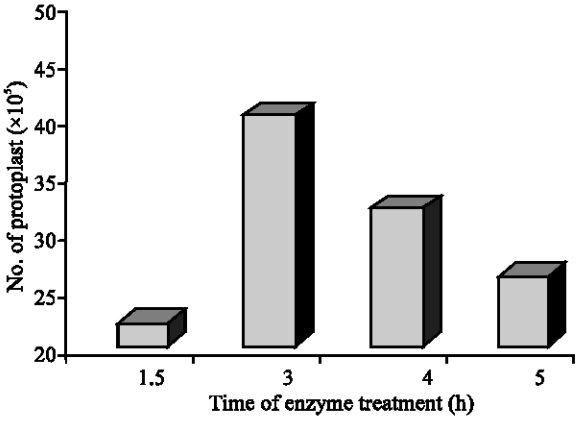

Fig. 1: Effect of different times of enzyme treatment on number of protoplast

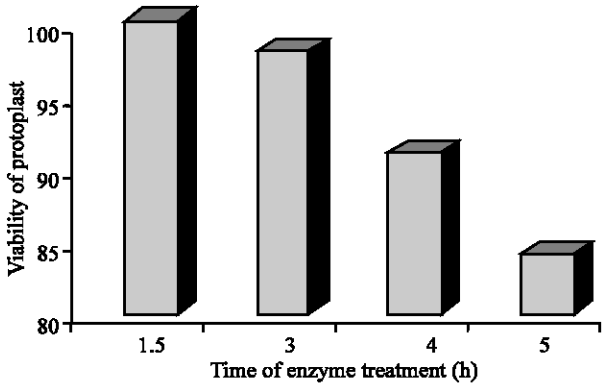

Fig. 2: Effect of different times of enzyme treatment on viability of protoplast

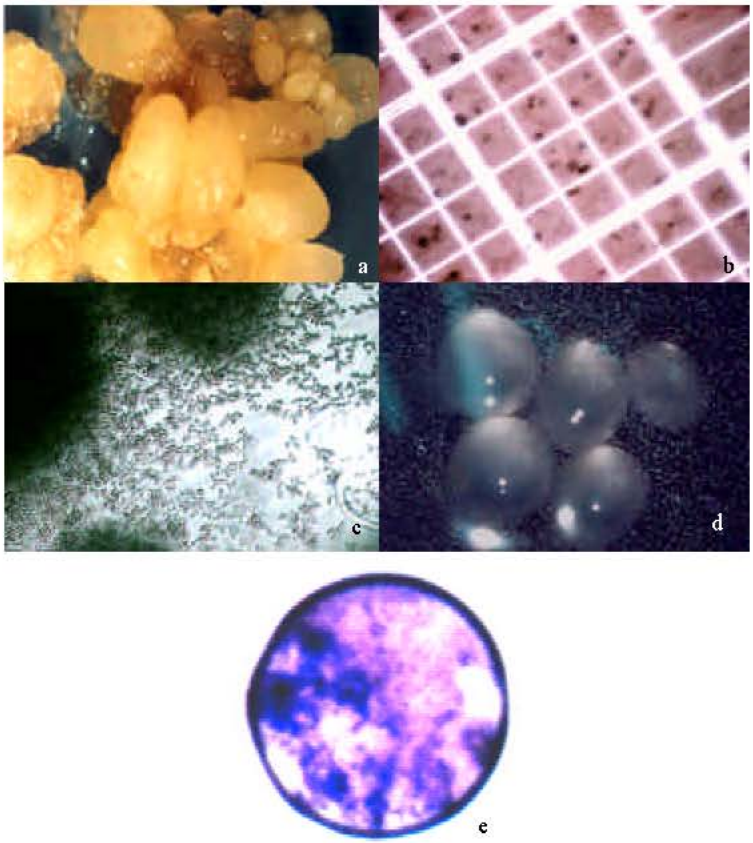

Fig. 3a-e: Isolation of protoplast derived from embryogenic callus of *C. sativus* (a) embryogenic callus together with globular embryoids (b) Counting number of isolated protoplasts using homeocytometer (c) Freshly isolated protoplasts after 3-hour enzyme treatment (d) Ca-alginate beads (f) Staining of dead protoplasts using Trypan Blue solution

agent itself is not toxic and gelling process is thermo independent which improved protection for fragile cells such as plant protoplasts (Brodelius and Nilsson, 1980).

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