Effects of Different Hormone Treatments on Nonembryogenic and Embryogenic Callus Induction and Time-Term Enzyme Treatments on Number and Viability of isolated Protoplasts in Saffron (*Crocus sativus* L.)

E. Darvishi  
Agricultural Biotechnology research Institute  
Karaj  
Iran

R. Zarghami, C.A. Mishani, M. Omidi  
Department of Plant Breeding  
College of Agriculture and Natural Resources  
University of Tehran  
Karaj 31587  
Iran

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**Abstract**

In order to determine the best hormone treatment for nonembryogenic and embryogenic calli induction, apical meristem of young corms derived from saffron (*Crocus sativus* L.) were cultured in solid LS medium supplemented with different concentration of two auxin (NAA and 2,4-D) and three cytokinin (BAP, Kin and 2ip) at 22°C in darkness. Statistical analysis using Kruskal-Wallis Test showed that treatment containing 2 mg/L NAA and BAP with highest Mean Rank had the beat effect on induction of nonembryogenic callus and treatment containing 1 mg/L 2, 4-D and BAP had the best effect on induction of embryogenic callus. The results obtained from counting number and viability of protoplasts isolated from embryogenic calli with homeocytometer and Trypan Blue under the enzyme solution consisting of MS medium with 0.1 % (w/v) 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 on a rotary shaker (200 rpm) at 25 °C in darkness for 4 time-term treatment (1.5, 3, 4 & 5 hour) indicated that after 3 h enzyme treatment, $40 \times 10^5$ 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 viability of fragile protoplasts, Calsium-alginats beads were used for their immobilization.

**INTRODUCTION**

*Crocus sativus* L. is a triploid and sterile crop that propagates vegetatively by corm. Disease factors such as fungi, bacteria and viruses that exist in corms usually result in decrease of growth and flowering in this plant. Tissue culture is a useful method for large scale production of pathogen-free plants (Ding et al., 1981; Chrugoo et al., 1987). Reported that in LS medium containing NAA and BAP induction of nonembryogenic calli were highest (Ahuja et al., 1994; Homes et al., 1987). Showed that synthetic auxins such as 2,4-D in low concentration were essential for embryogenesis in some monocots (Plessner et al., 1990; Vasil et al., 1992). Ebrahimzadeh et al., (2000) used meristem culture of saffron in LS medium containing different concentration of NAA, 2,4-D and Kin. They observed different stages of embryogenesis and plantlet formation. 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
protoplasts derived from cell-suspension often have failed to produced green plants (Maddock, 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 this research, effect of different hormone treatments on nonembryogenic and embryogenic callus induction and time-term enzyme treatment on number and viability of isolated protoplasts derived from embryogenic callus were studied.

MATERIALS AND METHODS
Materials, Sterilization, Medium and Culture Condition

Saffron corms were collected from the Province of Mashhad, Iran in June 2004. In order to sterilize the corms, after removing the fibers, were rinsed in running tap water for 30 min, then dipped in 70 % ethanol. They surface-sterilized in 2 % HgCl₂ for 30 min and rinsed 3 times in sterile water. Apical meristem of corms together with little part of the corm tissue attached to them, were removed as explants. In order to prevent browning of the tissue before culture, explants were soaked for 10 min in 1 % ascorbic acid. Sterilized explants were cultured on LS (Linsmaier and Skoog, 1965) medium containing 3 % sucrose and different concentration of two auxin: NAA (0, 5, 10, 15 mg/L) and 2,4-D(0, 1, 2, 10 mg/L) and three cytokinin: BAP, Kin, 2ip (0, 1, 2 mg/L). All media were solidified by 0.7 % agar after adjusting the pH to 5.7 and autoclaved for 15 min at 1.2 kg/cm². Cultures were incubated at 22±2 °C in darkness. All cultures were subcultured every 1 month in the same medium. The experiment was repeated four times using three explants per treatment. Data obtained from tissue culture experiments were statistically analyzed using non-parametric Kruskal-Wallis Test with SPSS program.

Proliferation and Maintenance of Embryogenic Calli

Precise selection of embryogenic calli which were compact with shining globular regions and transferring them to glassware with 2 cm width and 10 cm height carried out due to proliferation and maturation of embryogenic calli. In order to maintain embryogenesis property, embryogenic calli together with globular embryoids were transferred to a maintenance MS (Murashig and Skoog, 1962) medium supplemented with 3 % sucrose, 100 mg/l ascorbic acid, 2 mg/l BAP and 1 mg/l 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 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:

Number of protoplasts per 1 ml of suspension = \frac{\text{Counted area (cm}^2\times\text{chamber depth (cm)}\times\text{dilution} \times \text{The first volume of the protoplast suspension}}{\text{Total number of protoplast}} - \text{Number of blue-color protoplast}

Viability of protoplast = \frac{\text{Total number of protoplast}}{\text{Total number 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. This investigation performed from July 2004 until May 2005 in the Agricultural Biotechnology Research Institute of Iran (ABRII).

**RESULTS AND DISCUSSION**

**Induction of Nonembryogenic and Embryogenic Calli**

After 4.5 month of culture, nonembryogenic calli formed from apical meristem on LS medium containing different growth regulators. Results obtained from statistical analysis using Kruskal-Wallis Test indicated that there was significant difference among treatments at 0.01 probability level from view of nonembryogenic and embryogenic callus induction. (Table 1, Figs. 1 and 2).

The treatment containing 2 mg/L NAA and 2 mg/L BAP had the highest Mean Rank of nonembryogenic callus induction (Fig. 3a). This result is similar to the Ahuja et al., (1994) study on *C. sativus*. LS medium containing 2 mg/L 2, 4-D and 1 mg/L Kin is in secondary importance which is corresponded to George et al., (1992) studies. After 6 month of cultures, the highest Mean Rank of embryogenic callus induction was recorded with LS media contained 1 mg/L 2, 4-D and BAP (Fig. 3b). This result is similar to Sano and Himeno (1987) and Vasil and Vasil (1992) studies. During initial stages of callus development, white to yellowish and soft calli grew from cultured explants, which after 4 transfers were visually distinguishable to be of two types: off-white, friable and nodular embryogenic calli and yellowish and soft nonembryogenic calli. This asymmetric development that resulted in nonembryogenic and embryogenic callus formation (Figure 3c) is distinguishable property of numbers of monocots (Vasil et al., 1988). Careful selection of embryogenic regions and transferring them into glassware with 2 cm width and 10 cm height resulted in notable growth of embryogenic calli (Figure 3d). As noted embryo formation in vitro condition is a stress response, hence limitation of this glassware in supplying sufficient space for growth and nutrient exchange, increased embryogenesis (Williamson et al., 1985; Morris et al., 1990).
Isolation and Immobilization of Protoplasts

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. 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. 3e-f). The results indicated that after 3 h enzyme treatment, number of protoplasts and their viability were highest (Fig. 3g). 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, Calsium-alginats beads were used for their immobilization (Fig. 3h). This method is an effective tool because the gelling 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).

Literature Cited


Table 1. The result of Kruskal-Wallis Test in measured characters

<table>
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Fig. 1. Ranking of different hormone treatment effects in nonembryogenic callus induction
Fig. 2. Ranking of different hormone treatment effects in embryogenic callus induction
Fig. 3. Induction of nonembryogenic and embryogenic callus and isolation of protoplast derived from embryogenic callus of *C. sativus* (a) Nonembryogenic callus (b) Embryogenic callus (c) Asymmetric growth of calli (d) Transferring of embryogenic callus into glassware with 2 cm width and 10 cm height. (e) Counting number of isolated protoplast using homeocytometer (f) Staining of dead protoplast using Trypan Blue solution (g) freshly isolated protoplast after 3 h enzyme treatment (h) Ca-alginate beads