Regeneration of Saffron Protoplasts Immobilized in Ca-alginate Beads

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A methods for growing of saffron (*Crocus sativus* L.) protoplasts immobilized in Ca-alginate is presented in detail. Protoplasts were isolated from the cells of the suspension culture (ISA and OGASAWARA 1988) with a solution of Cellulase "Onozuka" RS, Pectolyase Y-23 and Driselase, and embedded in Ca-alginate beads. They were cultured with or without nurse cells in MS medium (MURASHIGE and SKOOG 1962) supplemented with 2,4-D and Zeatin at 25°C. After several changes of medium, cell-clusters appeared on the surface of the Ca-alginate beads, although the protoplasts without immobilization in Ca-alginate beads did not display cell division. Growth of the cell clusters in the medium with nurse cells was much better than that in the medium without nurse cells. Then, the beads were transferred onto MS agar medium supplemented with 1-naphtaleneacetic acid and 6-benzylaminopurine, and subcultured at 15°C or 20°C. After 2 to 3 months of culture, shoots and roots regenerated from the callus tissue.

KEY WORDS: *Crocus sativus*, saffron, protoplast, Ca-alginate, nurse-culture, plant-regeneration.

Introduction

In an earlier report, we described an efficient method of culture for plant regeneration through saffron cell culture. However attempts to cultivate the saffron protoplasts were not successful. Alginate entrapment of plant protoplasts has been recently reported as an effective method of protoplast culture (SCHNABL and YOUNGMAN 1983, LARKIN *et. al.* 1988). We tried to use this method for the culture of saffron protoplasts. A nurse culture approach (RHODES *et. al.* 1988, LAUDENIR and SONDAHL 1989) on Ca-alginate beads, which is also an effective method of protoplast culture was executed to improve saffron protoplast culture. Based on these studies, we demonstrate here for the first time that saffron protoplasts are able to divide and regenerate in Ca-alginate beads at a very high frequency.

Materials and Methods

Calli were derived from saffron (*Crocus sativus* L.) bulbs cultured on MS medium supplemented with 0.5mg/l 2,4-D and 0.3mg/l Zeatin (abbreviated as MS DO.5, ZO.3) at 25°C under dark conditions as reported previously (ISA and OGASAWARA 1987). The method for the isolation of protoplasts is as follows: About 5 to 10g of saffron callus or liquid cultured cells were collected, and treated with an enzyme solution consisting of MS basal medium with 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co.), 1% Cellulase "Onozuka" RS (Yakuruto HONSHA Co.), 1% Driselase (KYOWA HAKKO KOGYO Co.) and 0.3M Mannitol (KANTO CHEMICAL Co.) by shaking for about 1hr at 25°C. The isolated protoplasts were sieved through 150 mesh nylon-cloth, collected by centrifugation at 100 × g for 2 min, and washed in a 0.3M Mannitol solution three times.

The prepared protoplasts were mixed gently with a sterile Na-alginate solution (WAKO

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PURE CHEMICAL INDUSTRIES Co., 2% in 0.3M Mannitol solution) at the density of $5 \times 10^4/\text{ml}$ and $1 \times 10^5/\text{ml}$. These alginate solutions with the protoplasts were added drop by drop with a sterile Pasteur pipette into a MS basal medium containing 1% CaCl_2 (KANTO CHEMICAL Co.) and 0.3M 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 DO.3, ZO.2 medium containing 0.3 M Mannitol. They were cultured in MS DO.3, ZO.2 medium with and without a high density of cells of the same species in suspension culture. Isolated protoplasts were also cultured in the same nutrient medium without embedding in ca-alginate gel as controls.

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 of the beads under the microscope. After 6 to 10 weeks of culture, the microcalli appeared on the surface of the alginate beads. Some of the beads were transferred to MS agar-medium supplemented with 0.1mg/l 1-naphtaleneacetic acid and 1.0mg/l 6-benzylaminopurine, and cultured at 15°C 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 protoplasts without nurse cells.

The fact that immobilized protoplasts without nurse cells could divide and form a callus 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 proce-

Table 1. Effects of Ca-alginate, nurse-culture and plating density on the growth rate of saffron protoplasts

	1 week	2 weeks	1 month	2 months
Control (liquid cultured protoplasts) $5 \times 10^4/\text{ml}$	no division	no division	no division	no division (swell, red)
Ca-alginate beads $5 \times 10^4/\text{ml}$	no division	no division	second division 2.0 %	cell colony 3.0 %
Ca-alginate nurse beads $1 \times 10^5/\text{ml}$	no division	first division 3.0 %	second division 5.0 %	cell colony 6.0 %
Ca-alginate nurse beads $5 \times 10^4/\text{ml}$	first division 2.0 %	Second division 9.0 %	cell colony 1.0 %	cell colony 15.0 %

Fig. 1. Development of immobilized protoplasts of saffron. A: nursed Ca-alginate beads, B: first cell division in the beads, C: cell colony, D: appearance of cell cluster on the beads, E: breakage of the beads, F: appearance of callus through beads, G: shoot regeneration on the callus, H: regenerated plantlet.

dures known, because the gelling agent itself is not toxic and the gelling process is thermo independent (BRODELIUS and NILSSON 1980). The nurse beads showed a high growth rate and a callus was quickly formed (Fig. 1, A~F), suggesting that the mother cells promoted the growth of protoplasts in the nurse beads.

In this experiment, we frequently replaced the original medium with a 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 the growth regulators (DRAGET *et. al.* 1988, THOMAS and ROSE 1988). Through the use of Calcium alginate beads this objective could be attained easily and quickly.

A density of 5×10^4 protoplasts/ml gave a higher growth rate than 1×10^5 protoplasts/ml suggesting that the former cell density was better than the latter.

Plant regeneration (Fig. 1, G~H) from the callus of saffron protoplasts was achieved based on the methods reported previously (ISA and OGASAWARA 1988). Since the saffron plant grows from late autumn to early winter in nature, callus culture under low-temperature-conditions was attempted. It was observed that a 15°C or 20°C temperature was suitable for the regeneration of saffron callus. After 2 to 3 months of culture, about 80% of the calli regenerated.

As mentioned above, we report for the first time a method of successful cultivation of saffron protoplasts by alginate entrapment and nurse culture.

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アルギン酸カルシウムゲルに包埋したサフランプロトプラストからの個体再生

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アルギン酸カルシウムゲル包埋の技術は、従来、細胞の固定化とそれに引き続くバイオリアクターとしての利用が主であった。しかし最近、植物のプロトプラストをこれに包埋して培養することによりプロトプラストの培養に成功した例が報告された。我々は、先にサフランの培養系を確立したが未だプロトプラスト培養系の確立には至っていなかった。今後、サフランを用いた細胞工学的育種及び研究を行うに際して、プロトプラストの培養系確立は非常に重要である。そこで、アルギン酸カルシウムゲル包埋の技術を用いてサフランプロトプラストの培養を試みた。

サフランカルの誘導は前報(伊佐ら 1988)の方法に従った。誘導後の継代が3～4代目のカルスもしくは液体培養細胞を5～10グラム用意し、これを次の方法でプロトプラスト化した。MS基本培地にセルラーゼオノズカRS 1.0%, ドリセラゼ 1.0%, ペクトリアーゼ Y-23 0.1%を加え、マンニトールで浸透圧を0.3 Mに調製した酵素液に細胞を加えた。25℃でゆるやかに約1時間振盪し、検鏡しながらプロトプラスト化の確認をした。単離調製したプロトプラストは、別に準備したアルギン酸溶液(2%)に所定の密度で懸濁した後、塩化カルシウム溶液(1%)に順次滴下した。そのまま20分放置してゲルを形成させた後、培養液(MS基本培地に2,4-D 0.3 mg/ℓとゼアチンを0.2 mg/ℓ、及びマンニトール0.3 M添加)で2回洗浄し液交換した。このゲルを、同じ組成の培地で一方はそのまま、他方は高密度の液体培養細胞で保護培養した。浸透圧を順次下げつつ、7～10日間隔で数回培養液交換を行いながら培養した。

その結果、ゲル包埋せずにそのまま液体培養したプロトプラストは全く分裂に至らなかったが、ゲル包埋を行い、かつ保護培養した場合には、ゲル包埋のみで保護培養しなかったものに比べて、より旺盛な細胞分裂とカルス形成が認められた(Table 1.)。

前報の方法に従い、形成されたカルスを再分化培地に移植した結果、カルス当たり80%の頻度で植物体が再生した(Fig. 1.)。以上のように、従来の普通の培養方法では全く培養できなかったサフランプロトプラストを、アルギン酸ゲル包埋と保護培養によって、かなり高率に分裂および再生させることに成功した。