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Indirect Somatic Embryogenesis from Petal Explant of Endangered Wild Population of *Fritillaria imperialis*

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Abstract: *Fritillaria imperialis* is an endangered bulbous plant and therefore *in vitro* micropropagation of this plant will have a great importance for germplasm conservation and commercial production. Petal explants, for the first time, were cultured on media containing various concentrations of plant growth regulators. In addition, the effects of cold pretreatment and light on induction and regeneration of somatic embryogenesis trough callus were studied in detail. Cold pretreatment had inhibitory effects on somatic embryogenesis pathway. Among the different combinations of 6-Bnzylaminopurine (BAP), α-naphthaleneacetic acid (NAA) and indole-3-aceticacid (IAA) tested, B5 medium supplemented with 0.1 mg L^{-1} BAP + 0.6 mg L^{-1} NAA + 0.4 mg L^{-1} IAA was the best treatment for bulblet production (6 bulblets per somatic embryogenesis callus). This research presents petal as a reliable material for micropropagation and germplasm conservation of *Fritillaria imperialis*.

Key words: Bulblet regeneration, cold pretreatment, endangered plant, *Fritillaria*, petal, plant conservation

INTRODUCTION

Fritillaria imperialis, belonging to the Liliaceae family, is an important ornamental and medicinal plant. This plant has been used either as pot plant for designing landscape or cut flower. Because of attractive red and yellow flowers which open in early spring, this plant reveals a great commercial potential (De Hertogh and Lenard, 1993).

The bulbs of *F. imperialis* have been used as one of the most important anti-tussive, expectorant and anti-hypertensive drugs in traditional Chinese medicine (Wang *et al*., 2005). Alkalids and non-alkaloid constituents have been isolated from *Fritillaria* bulbs (Mir and Ghatak, 1965; Gilani, *et al*., 1997; Li *et al*., 1999, 2000). As known in recent studies, *Fritillaria* species has high starch content and can be considered as new starch sources for the food and medicine industry (Wang *et al*., 2005).

The fascinating *Fritillaria* genus including 100 species, of which 14 species are native to Iran (Parsa, 1959; De Hertogh and Lenard, 1993). Iran is center of origin and genetic diversity of *F. imperialia*. Unfortunately in recent years, wild populations of this plant are at the risk of extinction in Iran, because of pastures destruction, pests overflow and continual grazing (Ebrahimie *et al*., 2006).

The natural propagation rate of most geophytes is relatively low. This often hampers the large-scale cultivation of these plants. Previous studies have shown that *F. imperialis* can not rapidly and efficiently propagate by traditional methods like bulb scaling and bulb cutting (De Hertogh and Lenard, 1993). Low propagation rate by bulb scaling or bulb cutting is related to small numbers of scales (3-5) per bulb and consequently, the restricted amount of meristematic cells (Bryan, 1989). *F. imperialia* can be propagated from seed; however, it takes five to seven years from seed to develop plant capable of flower production. In addition, produced plants by seeds are not true to type; because of cross-pollinate nature of *Fritillaria* (De Hertogh and Lenard, 1993).

In vitro propagation of endangered plants can offer considerable benefits for the rapid cultivation of species that are at risk, that have limited reproductive capacity and exist in threatened habitats (Fay, 1992). *In vitro* propagation methods are essential components of plant genetic resources management and they are becoming increasingly important for conservation of rare and endangered plant species (Sudha *et al*., 1998; Benson *et al*., 2002; Almeida *et al*., 2005). The application of tissue culture techniques might allow rapid and large-scale propagation of uniform plants for field culture.

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Bulb scales are the most common used explants for micropropagation of bulbous plants including Liliaceae family (Mirici *et al*., 2005). *In vitro* micropropagation by using bulb scale sections has been reported previously for defined cultivar of *F. imperialis* (Witomska and Lukaszewska, 1997; Witomska, 2000) and *F. thunbergii* (Paek and Murthy, 2002). The use of bulbs as a source of explant is often associated with heavy bacterial and fungal contamination (Langens-Gerrits *et al*., 1998; Mirici *et al*., 2005). In the case of *F. imperialis*, because of a limited number of scales per bulb, the problem is more serious (Witomska and Lukaszewska 1997; Witomska, 2000). In addition, the use of bulb scale pieces can result in destruction of endangered parent plant. As a result, finding the other sources of explants, especially foliar explants, is necessary. Each plant of *F. imperialis*, commonly, produces 7-8 big flowers, so it seems that petal can be considered as an adequate and ready available explant. In this study, for the first time, petal has been used in tissue culture of wild population of *F. imperialis*. In this study, using petal explant, a protocol for somatic embryogenesis through callus and bulblet regeneration of *F. imperialis* has been presented after the investigation the effects of different plant growth regulators, light and cold pretreatment.

MATERIALS AND METHODS

Plant material and explants preparation: Wild populations of *Fritillaria imperialis* are mostly found in high altitudes (> 2000 meters) of western parts of Iran such as Gholam Abad (Chahar Mahalo Bakhtiari province of Iran). Green flower buds on 25-30 cm long branches were collected in end of April from this site and rapidly transferred to the lab.

Two types of explants utilized in this study: explant type I (petal without cold pretreatment) and explant type II (petal with cold pretreatment).

Explant type I (petal without cold pretreatment): Green flower buds were excised from each branch and surface-sterilized with 1.5% (w/v) sodium hypochlorite for 15 min and washed 3-4 times with sterile distilled water. Following the separation of petal from surface-disinfested flower bud, it used as explant type I (Fig. 1) and cultured on plant growth regulator containing media.

Explant type II (Petal with cold pretreatment): To study the effect of cold pretreatment, at first, some of the flower branches were held in 4°C cold for 4 weeks. Then, petals were taken from surface-disinfested flower buds and cultured on plant growth regulator containing media.

Factorial experimentation of plant growth regulators based on completely randomized block design (RCBD): The factorial experimentation of plant growth regulators based on completely randomized block design (RCBD) was conducted with 3 replications (3 Petri dishes) and 3 explants in each Petri dish (90×15 mm). Different intensities of light radiations were applied as blocks: b1 = 40 μmol $m^{-2}s^{-1}$ (high light intensity), b2 = 20 μmol $m^{-2}s^{-1}$ (low light intensity), b3 = darkness for 1 month, then transferring to 20 μmol m^{-2} s^{-1}. For each explant (with and without cold per-treatment), the B5 medium (Gamborg *et al*., 1968) was supplemented with three growth regulators (3 factors) supplied at different concentrations (levels): BAP (0, 0.1 and 1 mg L^{-1}), NAA (0, 0.3 and 0.6 mg L^{-1}) and IAA (0, 0.4 and 0.8 mg L^{-1}). As a result, twenty seven different combinations were tested for each explant type. These combinations included the use of each growth regulator supplied alone at different concentrations, mixture of the three growth regulators at different concentrations and control (no growth regulator). For each explant type, the experiment was repeated twice.

Since the homogeneity of variance between two experiments of each explant type was observed, the use of combined analysis of variance was allowed. Based on this result, mean of the two experiments for each treatment at each explant type was calculated and used for mean comparisons. Mean comparisons and separation were conducted using Duncan's multiple range test at 0.05 probability level (Gomez and Gomez, 1984).

The basal medium contained full-strength B5 macro- and micro-elements, vitamins and sucrose (30 g L^{-1}). Prior to autoclaving at 121°C for 15 min, pH was adjusted to 5.8. The media were solidified with 7% (w/v) agar (plant agar, Duchefa). The petal explants were placed horizontally on the surface of the medium (20 mL) in Petri dishes (90×15 mm), sealed with parafilm and maintained at 19±1°C.

Computed traits and statistical evaluation

Evaluation of explant survival: Percentage of surviving explant was measured by dividing the number of survived explants per the number of cultured explants, 45 days after petal culture. This trait determined the effects of plant growth regulators on explant remaining.

Callus formation: For callus formation, 90 days after petal culture, percentage of callus formation (callus %) was measured by dividing the number of explants producing callus per the number of cultured explants.

Induction of embryogenic callus: To evaluate the induction of indirect somatic embryogenesis pathway, percentage of somatic embryogenic callus induction was calculated by dividing the number of callus showing somatic embryogenesis pathway per the number of cultured explants, 120 days after petal culture.

Bulblet production (regeneration of produced somatic embrogenic callus): It should be mentioned that in bulbous plants, regeneration of somatic embryos reveals as bulblets. Percentage of bulblet production was measured by dividing the number of regenerated bulblets per the number of somatic embryogenic callus.

Bulblet enlargement: Produced bulblets were transferred to half-strength MS basal salts supplemented with 3% (w/v) sucrose without growth regulators and kept under 20 μmol $m^{-2} s^{-1}$ light intensity and 19±1°C temperature.

RESULTS AND DISCUSSION

Explants responses

Explant type I (petal explant without cold pretreatment): After culture of explant type I on B5 medium supplemented with different plant growth regulators combinations, two types of responses were observed (Table 1): (1) non-embryogenic callus in treatments containing auxins or auxins in combination with high level of BAP (1 mg L^{-1}) and (2) embryogenic callus in treatments containing high level of auxins and low level of BAP (0.1 mg L^{-1}) (Fig. 1).

Explant type II (petal explant with cold pretreatment): After culture of explant type II, in both experiments (repeated experiment), neither embryogenic nor non-embryogenic callus was observed. It clearly demonstrated the inhibitory effects of cold pretreatment on callus formation and somatic embryogenesis pathway via callus.

Explant survival was affected by plant growth regulators of culture medium: BAP (cytokinin) dramatically increased surviving of petal explants of *F. imperialis* (Table 1). Explants cultured in treatments containing BAP, either in low (0.1 mg L^{-1}) or high (1 mg L^{-1}) concentration, survived much better than others. Petal could not survive on medium without plant growth regulators or supplemented with weak auxin (IAA) (Table 1), whereas NAA had positive effect on survival of petals. Generally cytokinins have critical role in delaying senescence. It has been shown that exogenous application of cytokinins by increasing the internal cytokinin level inhibits the degradation of chlorophyll and photosynthetic proteins (Richmond and Lang, 1957; Badenoch-Jones *et al*., 1996). Senescence is also delayed in transgenic plants producing cytokinin by expression of a bacterial gene encoding ipt, the enzyme catalyzing the first step of

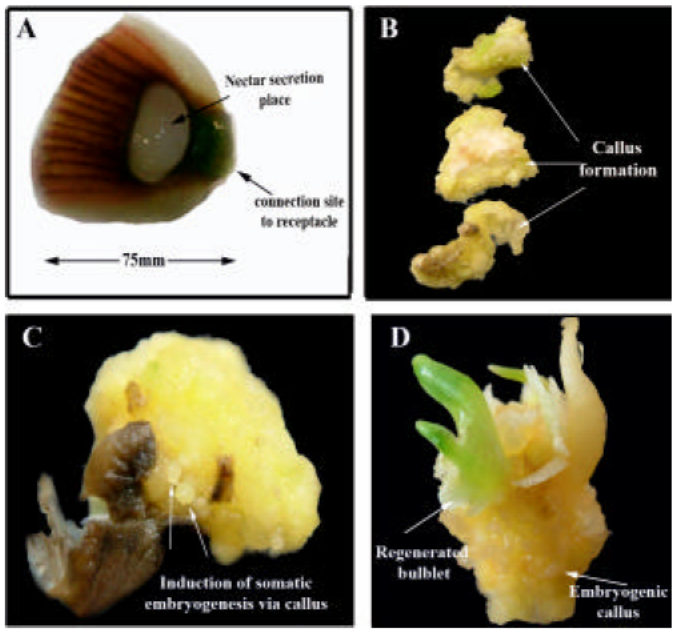

Fig. 1: Embryogenic and non-embryogenic callus formation and bulblet regeneration from petal explant of *Fritillaria imperialis* on B5 medium containing 0.1 mg L^{-1} BAP + 0.6 mg L^{-1} NAA + 0.4 mg L^{-1} IAA. A: Petal explant, B: Non-embryogenic callus, C: Induction of globular embryos on callus and D: Regeneration of bulblets from embryogenic calluss

Table 1: Effects of some of plant growth regulator combinations on petal explant *Fritillaria imperialis* without cold pretreatment (explant type I)

Some of the plant growth regulators combinations	Survived explant (%), 45 days after culture	Callus formation (%), 90 days after culture	Embryogenic callus (%), 120 days after culture	Bublllet production (%), 150 days after culture
Control	0c	0.0b	0	0
1 BAP+0.3 NAA + 0.4 IAA	83a	16.2b	0	0
0.1 BAP+0.6 NAA + 0.4 IAA	83a	56.0a	56	6
0.1 BAP	55b	0.0b	0	0
1 BAP	80a	0.0b	0	0
0.6 NAA	34b	15.5b	0	0
0.4 IAA	0c	0.0b	0	0
0.6 NAA+0.4 IAA	38b	19.5b	0	0

Means followed by the same letter(s) within columns are not significantly different at 0.05 probability

cytokinin synthesis (Smart *et al*., 1991; Gan and Amasino, 1995). In consistent with previous observations, in the present study, we observed that BAP had critical effect on explant survival. Moreover, it has been observed that externally applied NAA can rise the internal cytokinin content (Mercier *et al*., 2003). It seems that uptake of NAA from the petal culture medium of *Fritillaria* by increasing endogenous cytokinin content is involved in explant survival.

The role of growth regulators in indirect somatic embryogenesis from petal explant type I (without cold pretreatment): Sole application of BAP blocked formation of both embryogenic and non-embryogenic callus. Table 1 shows that 0.1 or 1 mg L^{-1} BAP could not result any callus. Cytokinins have been effectively used to induce callus-free organogenesis in many dicots (Zapata *et al*., 1999) and monocots (Lin *et al*., 1997).

Auxins (IAA and NAA) produced non-embryogenic callus: Table 1 shows that although NAA and IAA significantly accelerated callus production, transition of callus to somatic embryogenesis phase obviously needed extra hormones, other than NAA or IAA. As example, 19.5 % of explants produced callus under the control of 0.6 mg L^{-1} NAA + 0.4 mg L^{-1} IAA. However, none of them could convert to embryogenic callus.

Low concentration of BAP in combination with auxins induced and regenerated embryogenic callus: As it can be seen in Table 1, all of the produced callus in treatment containing 0.1 mg L^{-1} BAP + 0.6 mg L^{-1} NAA + 0.4 mg L^{-1} IAA converted to embryogenic callus. Later on, this treatment could regenerate 6 bulblets per embryogenic callus with 4 subcultures and revealed the best performance in somatic embryogenesis through callus in petal culture of *F. imperialis*. The results of this experiment showed that low concentration of BAP in combination with auxins is critical in induction and regeneration of indirect somatic embryos and overcoming the genotype dependency in heterogeneous and heterozygote population of *F. imperialis*.

Effect of light on induction of indirect somatic embryogenesis: Analysis of variance showed that somatic embryogenesis through callus, derived from petal explant of *F. imperialis*, was not affected by light (data not shown).

The importance of conservation of genetic resources cannot be overemphasized (Ford-lloyd and Jackson, 1986; Frankel and Hawkes, 1975; Arora and Bhojwani, 1989). Wild populations of *Fritillaria imperialis* which mostly found in places with more than 2000 meters height in Iran are at the risk of complete extinction. With respect to the low natural propagation rate of this valuable plant, success in induction of somatic embryogenesis and raising bulblets through this pathway has opened up the possibility for large-scale clonal propagation and germplasm conservation through artificial seeds and might help in the genetic improvement of this species for commercial use. The results obtained in the present study could be of enormous significance, since there is no data on propagation of endangered wild population of *Fritillaria imperialis*.

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