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Somatic Embryogenesis Induction in *Narcissus papyraceus* cv. Shirazi

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Abstract: In order to investigate somatic embryogenesis in daffodil (*Narcissus papyraceus* cv. Shirazi) several experiments were carried. Explants were excised from the bulb scales and were transferred to MS and Nitsch media containing different concentrations of growth regulators such as BAP, 2,4-D, GA_3, Kin, IBA and NAA. The results showed that the highest number of regenerated bulblets were produced in Nitsch medium containing BAP (2.2 mg L^{-1}) and 2,4-D (1.1 mg L^{-1}). The highest rate of direct somatic embryos were observed on MS medium containing GA_3 (0.5 mg L^{-1}), BAP (1.6 mg L^{-1}) and 2,4-D (1.6 mg L^{-1}). To propagate the induced somatic embryos, they were transferred to hormone free MS medium. Then the produced bulblets were transferred to half MS medium containing IBA (1 mg L^{-1}) for root induction.

Key words: *Narcissus*, somatic embryogenesis, regeneration

INTRODUCTION

Narcisus is spring-flowering bulb which belongs to the family Amaryllidaceae plant family (Graham and Barrett, 2004). *Narcissus* plants are well known not only for their ornamental value, but also for their alkaloids, some of which exhibit various pharmacological properties. The Amaryllidaceae -type alkaloids possess antiviral and anti tumor properties (Colque *et al*., 2001). The natural propagation rate of *Narcissus* is very slow and propagation of *Narcissus* (Daffodil) through vegetative methods (chipping and twin scales) is not efficient. Introduction of newly bred cultivars through conventional methods may take several years. *In vitro* micropropagation of *Narcissus* is more efficient than conventional propagation for rapidly building up aseptic stocks of varieties, especially for the establishment of new cultivars and the production of pathogen-free stock material.

Furthermore, because of the large number of propagation cycles in the field, conventionally produced bulbs may become easily infected (Chen *et al*., 2005; Sochacki and Orlikowska, 2005; Gang *et al*., 2007). Therefore, the application of tissue culture techniques allows rapid and large-scale propagation of uniform plants for field culture (Ziv and Chen, 2003; Staikidou *et al*., 2005).

Santos *et al*. (2002) investigated the cultural conditions for *in vitro* production of bulbs of *Narcisssus asturiensis*. They used twin -scales as primary explants cultured on a modified MS supplemented with IBA (1 mg L^{-1}), BA (1.99 mg L^{-1}) and NAA (0.12 mg L^{-1}), BA (5.99 mg L^{-1}). Both media were appropriate for shoot induction and proliferation, although the multiplication rate of leafy shoots was higher with NAA and BA. After 60 days of culture on both media, tiny bulb-like structures were formed at the base of the leaves. Jiao *et al*. (2005) suggested that *in vitro* anther culture can provide an efficient new micropropagation technique for callus induction and plant regeneration in Chinese *Narcissus* (*Narcissus tazetta* var Chnensis Roem). Sage (2005) established a new strategy for rapid and economical propagation using bioreactors.

There are some cultivars of *Narcissus* which possese genes for resistance to Smoulder (caused by *Botrytis narcissicold*) (Hanks *et al*., 2004). Transfer of these resistant genes into other *Narcissus* cultivars via conventional breeding will take a long time and it is rather difficult to breed disease and insect pest resistant. Somatic embryogenesis and regeneration of whole plant is an important step in plant transformation method. Gene transfer into plants initially requires optimization of tissue culture system for regeneration of transformed cells. Successful production of transformed plants depends on regeneration of numerous supposed transformed plants that are independent and their subsequent evaluation and screening (Qayyum *et al*., 2005).

The purpose of this research was to find out the best treatment for induction of somatic embryogenesis and regeneration in *Narcissus papyraceus* cv. Shirazi.

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MATERIALS AND METHODS

These Experiments were conducted in the Liboratory of Tissue Culture of the University of Imam Khomeini in April 2006. Bulb scales of *Narcissus papyraceus* cv. shirazi were collected from the field around of Karaj (the central of Iran). Bulb scales derived from terminal bulb units were surface sterilized in Ridomil (15 mg L^{-1}) for 15 min and commercial bleach solution 1.5% (v/v) for 20 min followed by 0.1% $HgCl_2$ solution for 4 min shaking on rotary shaker. The explants were then rinsed with sterile distilled water for at least 3 times and were cut into segments with 10 mm length. Then they were transferred to the culture media to induce bulblets and somatic embryos. The media was described in Table 1 and 2.

MS salts supplemented with miyo-inositol (100 mg L^{-1}), nicotinic acid (5 mg L^{-1}), pyridoxine HCl (0.5 mg L^{-1}) and thiamine HCl (0.5 mg L^{-1}).

Nitsch salts supplemented with biotin (0.05 mg L^{-1}), folic acid (0.5 mg L^{-1}), glycine (2 mg L^{-1}), miyo - inositol (100 mg L^{-1}), nicotinic acid (5 mg L^{-1}), pyridoxine (0.5 mg L^{-1}) and thiamin (0.5 mg L^{-1}).

Regeneration medium for induced somatic embryos included MS medium without growth regulator and rooting medium for root initiation in the bulblet produced included MS medium containing IBA (1 mg L^{-1}).

Table 1: Media for induction of somatic embryogenesis

Treatments	Combination
A	MS medium supplemented with 2,4-D (0.5 mg L^{-1})+ BAP (0.5 mg L^{-1})+GA_3 (0.5 mg L^{-1}).
B	MS medium supplemented with 2,4-D (1.6 mg L^{-1})+ BAP (1.6 mg L^{-1})+GA_3 (0.5 mg L^{-1})
C	MS medium supplemented with IBA (0.6 mg L^{-1})+ BAP (1.23 mg L^{-1})
D	MS medium supplemented with IBA (0.2 mg L^{-1})+ BAP (0.9 mg L^{-1})
E	Nitsch medium supplemented with 2,4-D (1.1 mg L^{-1})+ BAP (1.12 mg L^{-1})

Table 2: Media for bulblet regeneration

Treatments	Combination
G	MS medium supplemented with 2,4-D (0.2 mg L^{-1}), BAP (0.2 mg L^{-1})+GA_3 (0.5 mg L^{-1})
H	MS medium supplemented with 2,4-D (1.1 mg L^{-1}), BAP (1.1 mg L^{-1})+GA_3 (0.5 mg L^{-1})
I	MS medium supplemented with IBA (0.2 mg L^{-1}), BAP (1.23 mg L^{-1})
J	MS medium supplemented with NAA (0.93 mg L^{-1}), Kin (0.1 mg L^{-1})
K	MS medium supplemented with NAA (0.93 mg L^{-1}), Kin (1 mg L^{-1})
R	MS medium supplemented with NAA (0.093 mg L^{-1}) and Kin (1 mg L^{-1})
L	MS medium supplemented with IBA (0.3 mg L^{-1}), Kin (0.43 mg L^{-1})
M	MS medium supplemented with IBA (1 mg L^{-1}), Kin (0.1 mg L^{-1})
N	Nitsch medium supplemented with 2,4-D (0.22 mg L^{-1}), BAP (1.12 mg L^{-1})
P	Nitsch medium supplemented with 2,4-D (1.1 mg L^{-1}), BAP (2.2 mg L^{-1})

In order to induce somatic embryos, cultures were maintained under dark conditions for two weeks at 24°C, before transferred to light conditions with 16 h daily photoperiod and 8 h dark at the same temperature. In all media was used agar (0.7 %) with pH = 5.6. Explants were subcultured every 4 weeks. The number of induced embryos and bublets per explant was recorded after 10 weeks of initial culture. This experiment was designed in a completely randomized with 3 replicates (each replication consisted of 4 explants). The conversion of $Y = (X+0.04 \times 0.5)$ were used for data adjustment and statistical analysis was carried out using Statistical Analysis Systems (SAS).

RESULTS AND DISCUSSION

Induction of somatic embryogenesis: Significant differences were observed between A treatment [2,4-D (0.5 mg L^{-1}) + BAP (0.5 mg L^{-1}) in combination with GA_3 (0.5 mg L^{-1})] and B treatment [2,4-D (1.6 mg L^{-1}) + BAP (1.6 mg L^{-1}) in combination with GA_3 (0.5 mg L^{-1})] (Table 3). The results (at 5%) showed that B treatment produced more embryos per explant as comparison to A treatment (Table 4).

Explants in A treatment were swollen with translucent structures were produced which resembled early somatic embryos. The colour of tissues changed to yellow, then after 3 weeks of culture initiation, proembryos changed to scutellar embryos without formation of globular embryos (Fig. 1A). While in B treatment, the proembryos were changed to globular embryos (Fig. 1B). In previous research, the combination of 2,4-D and BAP was used as the appropriate treatment for induction of somatic embryogenesis in *Narcissus* and other gena of this family. Sage *et al*. (2000) used these combinations as the most efficient combination for induction of somatic embryogenesis in *Narcissus pseudonarcissus* cv. St. Keverne and Golden Harvest. The findings of Zive *et al*. (1995) confirmed the positive effect of this combination on

Table 3: Analysis of variance for the effect of 2,4-D, BAP and GA_3 on induction somatic embryos

Source of variation	df	Mean squares (MS)	F	CV
Treatment	3	4.10	4.45*	5.7
Error	8	0.92		

*Significant at the 0.05 probability level

Table 4: Means of number somatic embryos related to effect of GA_3, 2,4-D and BAP on somatic embryogenesis

Treatment of growth regulator	No. of somatic embryos per explant
A	3.6[a]
B	8.3[b]

[a]: Difference was significant at 0.05 by using mean comparison test (LSD), [b]: Difference was significant at 0.05 by using mean comparison test (LSD)

Fig.1: A: Formation of scutellar embryos after 10 weeks of culture, B: Formation of circular embryos 3 months after culture, C: Formation of bulblets after 8 weeks of culture, D: Swelling of tissues. E: Formation of whole plantlets from induced embryos and F. Formation of whole plantlets from regenerated bulblets

induction of somatic embryogenesis in bulb plant of *Nerin* (Amaryllidaceae).

Although, the analysis of variance of effect of IBA and BAP on regeneration of daffodil was no significant, but the results (at 5%) between C treatment [IBA (0.6 mg L^{-1}) and BAP (1.23 mg L^{-1})] and D treatment [IBA (0.2 mg L^{-1}) and BAP (0.9 mg L^{-1})] showed that D treatment produced more embryos (3.5 embryos in each explant) in comparison with C treatment (2.6 embryos in each explant).

This results were in agreement with that of Salema *et al.* (1998) that could produce somatic embryos in medium containing BAP and IBA in *Narcissus bulbocodium*. Nhut *et al.* (2001) developed an efficient system for *in vitro* plant regeneration of *Lilium longiflorum* Thunb by flower buds. The explants were cultured on half MS having IBA (4 mg L^{-1}) and BAP (2 mg L^{-1}). After 60 days an average of 41 shoot were formed per explant.

The various cultivar have different ability to regeneration via somatic embryogenesis. Perhaps the main reason of obvious difference between this genus and *Narcissus* may be various genotypes.

In this study E treatment [2,4-D (1.1 mg L^{-1}) and BAP (1.12 mg L^{-1})] produced scutellar embryo after 8 weeks of culture initiation.

Riebas-Vargas *et al.* (2003) obtained somatic embryos by the culture of pedicel and stem explants on semisolid MS supplemented with combination of 2,4-D and BAP, at 0, 0.5, 1.0, 2.0 or 5.0 mg L^{-1}. Although these results were in agreement with results but present results showed that to use equal concentrations of these plant growth regulators in *Narcissus papyraceus* cv. Shirazi could cause to induce somatic embryogenesis. Whereas different concentration of both of them produced bulblets.

Bulblet regeneration: The results of analysis of variance of effects of BAP and 2,4-D in combination with GA_3 on regeneration via formation of bulblet (Table 5) showed that there were significant differences between H treatment [2,4-D (1.1 mg L^{-1}), BAP (1.1 mg L^{-1}) in combination with GA_3 (0.5 mg L^{-1})] and G treatment [2,4-D (0.2 mg L^{-1}), BAP (0.2 mg L^{-1}) in combination with GA_3 (0.5 mg L^{-1})].

The results obtained indicated that G treatment produced 1. 33 bulblets per explant whereas treatment H, produced 0.61 bulblet per explant (Table 6). I treatment [IBA (0.2 mg L^{-1}) and BAP (1.23 mg L^{-1}).] was caused direct regeneration via formation of bulblet. After 8 weeks of culture initiation, explants were swollen and bulblets were produced on the base of scale explant. The comparison of means (at 5%) between J treatment [NAA (0.93 mg L^{-1}) and Kin (0.1 mg L^{-1})] and K treatment [NAA (0.93 mg L^{-1}) and Kin (1 mg L^{-1})] showed that there were no significant differences between two treatments and both of them produced 1.3 bulblets per explant. This results were in agreement with that of Maesato *et al.* (1994) on bulb plant of *Lilium juponicum*. They investigated the effect of interaction between NAA and Kin, BA, Zeatin and 2ip on production bulbs. Their results showed that the combination of NAA and 2ip had better results in production bulblet, whereas, Bansude *et al.* (2003) by using of this combination in *Agave* (*Amaryllidaceae*) achieved somatic embryos.

Table 5: Analysis of variance for the effect of 2,4-D, BAP and GA_3 on regeneration via formation of bulblet

Source of variation	df	Mean squares (MS)	F-value	CV
Treatment	3	0.64	9.27**	45
Error	8	0.06		

**Significant at the 0.01 probability level

Table 6: Means of number of bulblets related to effect of GA3, 2,4-D and BAP bulblet production

Treatment of growth regulator	No. of bulblets per explant
G	1.3[b]
H	0.61[a]

[a]: Difference was significant at 0.01 by using mean comparison test (LSD), [b]: Difference was significant at 0.01 by using mean comparison test (LSD)

Table 7: Analysis of variance for the effect of 2,4-D, BAP on regeneration via formation of bulblet

Source of variation	df	Mean squares (MS)	F-value	CV
Treatment	2	0.96	52.29**	45
Error	6	0.01		

**Significant at the 0.01 probability level

Table 8: Means of number of bulblets related to effect of 2,4-D and BAP bulblet production

Treatment of growth regulator	No. of bulblets per explant
N	1[b]
P	1.6[a]

[a]: Difference was significant at 0.01 by using mean comparison test (LSD), [b]: Difference was significant at 0.01 by using mean comparison test (LSD)

In L treatment [IBA (0.3 mg L^{-1}) and Kin (0.43 mg L^{-1})] and M treatment [IBA (1 mg L^{-1}) and Kin (0.1 mg L^{-1})] after 6 weeks of culture initiation bulblets were produced. The comparison of means between these treatments (at 5%) showed that although significant differences were not observed, but L treatment produced more bulblets. The result of effects of BAP and 2,4-D on regeneration via formation of bulblet (Table 7) indicated that N treatment [2,4-D (0.22 mg L^{-1}) and BAP (1.12 mg L^{-1})] and P treatment [2,4-D (1.1 mg L^{-1}) and BAP (2.2 mg L^{-1})] caused regeneration via formation of bulblet (Table 8).

In R treatment [NAA (0.093 mg L^{-1}) and Kin (1 mg L^{-1})], after 2 weeks explants were swollen (Fig. 1D).

Germination and plantlet formation: Somatic embryos were transferred to MS medium for germination and

plantlet formation. The observation showed that germination of somatic embryos and rooting occurred respectively two- three and 12 weeks after transfer to regeneration medium. The produced bulblets were transferred to half MS medium containing IBA (1 mg L^{-1}) and roots were formed in the base of bulblets after 8 weeks (Fig. 1E). Somatic embryogenesis can provide a novel method for production new plant at reduced cost (Manuel *et al.*, 1986; Langens and Grrits, 1996; Sage and Hammatt, 2005). The process of somatic embryogenesis involves the formation of embryo, development of embryo, maturity of embryo and finally, formation of plantlet. There are a few phenomena in plants that plant growth regulators have no regulative effect on them.

The morphogenesis involves two stages to include differentiation and elongation that are influenced by plant growth regulators. Hence, it is possible to induce somatic embryogenesis in bulb scale explants via the change of levels of plant growth regulators. Relatively high levels of plant growth regulators have the effect of inhibitory on establishment and subsequent development of somatic embryos (De-Klerk *et al.*, 1997).

The results of usage of various concentrations of 2,4-D and BAP in *Narcissus papyraceus* cv. Shirazi showed that higher concentrations of both of them in comparison with less concentrations, not only did n't induce somatic embryogenesis, but also stimulated the production of bulblet, but this result didn't hold true in all cases as in bulb plant *Agave* (Amaryllidaceae) to use Kin (1-2 mg L^{-1}) and NAA (0.5 mg L^{-1}) caused to induce somatic embryogenesis (Bansude *et al.*, 2003), whereas using less amount of Kin (0.5 mg L^{-1}) with the same amount NAA caused to produce shoot in this plant (Binh *et al.*, 1990). In *Camellia japonica*, application of IBA (2 mg L^{-1}) and BAP (4 mg L^{-1}) was unable to induce somatic embryogenesis whereas the lower concentration caused production of somatic embryos (Vietez and Barciela, 1990). The response of different species and genia to somatic embryogenesis and regeneration is different. The combination of BAP and IBA in *Narcissus papyraceus* cv. Shirazi caused to produce bulblet, whereas the same treatment in *Narcissus bulbocodium* produced somatic embryos (Salema and Salemak, 2000). The using of Kin in combination with IBA in *Narcissus papyraceus* cv. Shirazi caused to produce bulblet whereas application MS medium containing 2% sucrose, 10% coconut water, 0.8% agar and IBA (0.1 mg L^{-1}) and Kin (0.5 mg L^{-1}) in *Agave cantala*, *A. fourcroydes*, *A sisalana*, produced shoot (Binh *et al.*, 1990). Although, the genotype maybe the main reason of obvious difference between this genus with *Narcissus papyraceus* cv. Shirazi but it is impossible to ignore the effect of less concentrations of sucrose and also the nutrient substance of coconut water. This result impresses on the effect of the combination of medium on organogenesis as one of the main factors. The different parts of plant have various abilities for regeneration. In *Amaryllis* (Amaryllidaceae) only twin scales and immature scape explants regenerated to plantlets (Brun *et al.*, 1991). Not only the type of explant but also position of explant on surface of culture medium is effective in induction of somatic embryogenesis. As the explants that were cultured with their abaxial surface in contact with the medium were produced more embryos compared to other surfaces (Sage, 2005). Leshem *et al.* (1986) showed that scale sections planted with their ventral side down regenerated fewer and smaller bulbs, less root, but much more callus than those with their dorsal side down. The results presented here showed that the different combinations were used in this research such as the combination of IBA, BAP and BAP plus 2,4-D in combination with GA_3 or without GA_3 could induce somatic embryogenesis in *Narcissus* and the most effective treatment between them was MS containing GA_3 (0.5 mg L^{-1}), BAP (1.6 mg L^{-1}) and 2,4-D (1.6 mg L^{-1}). Somatic Embryogenesis (SE), the most promising technology to multiply from advanced breeding and genetic program, is excepted to play an important role in increasing productivity. SE offers the potential of efficient clonal germplasm storage, since embryos can be kept under seed repository condition for long period time. Plants derived from SE appear to originate from single cells, thus, manipulation of embryo genetic cell cultures can yield genetically modified non-chimeric plants. Techniques for genetic modification using SE includes transformation and *in vitro* selection. on the other hand, SE is new technique for breeding of plants and transfer benefit gene from wild species to cultivar and investigation of genetic diversity (Hadgins and Barrett, 2007) but The most important problem for achieving to this propose is the lake of an efficient system. In order to induction of somatic embryogenesis, we investigated several treatment. Present finding showed that the highest rate of direct somatic embryos were observed on MS medium containing GA_3 (0.5 mg L^{-1}), BAP (1.6 mg L^{-1}) and 2,4-D (1.6 mg L^{-1}). Therefore the using of this treatment can develop a high frequency somatic embryogenesis and can help to provide this wanted for further investigation.

Because of shortage of data relate to this plant, it seems that it is necessary to investigate the effect of other media on the maturity of induced somatic embryos, the effect of type of explants, the position of explants on the

surface of medium and the effect of type and different concentrations of polysucarics on induction of somatic embryogenesis.

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