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Somatic Embryogenesis Induction in *Narcissus papyraceus* cv. Shirazi

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Abstract

Explants from bulb scales of *Narcissus papyraceus* cv. Shirazi cultured in MS and Nitsch media containing different concentrations of BAP, 2,4-D, GA_3, Kn, IBA and NAA produced highest number of regenerated bulblets in Nitsch medium containing BAP (2.2 mg/l) and 1.1 mg/l 2,4-D. The highest number of direct somatic embryos were observed in MS containing 0.5 mg/l GA_3, 1.6 mg/l BAP and 1.6 mg/l 2,4-D. Induced somatic embryos were multiplied by transferring them to hormone free MS.

Introduction

Genus *Narcissus* belongs to the family Amaryllidaceae (Dobson et al. 1997). *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 antitumor properties (Weniger et al. 1995, Moraes et al. 1997). Propagation of *Narcissus* (Daffodil) through vegetative methods (chipping and twin scales) is not efficient because of the low speed of their propagation. Introduction of newly bred cultivars through conventional methods usually take several years. Furthermore, because of a large number of propagation cycles in the field, conventionally produced bulbs are easily infected (Hanks 1986, Squires and Langton 1998, Lin et al. 1997). Therefore, the application of tissue culture techniques allow rapid and large-scale propagation of uniform plants for field culture (Staikidou et al. 2005, Riera et al. 1998, Merel and Langens. 1999). 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 in a modified MS supplemented with

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IBA (1 mg/l), BA (1.99 mg/l) and NAA (0.12 mg/l), BA (5.99 mg/l). Both media were found suitable 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 protocol for rapid and economical propagation using bioreactors.

There are some cultivars of *Narcissus* which possess 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 *Narcissus* cultivars of commercial value from interspecific hybrids. Somatic embryogenesis and regeneration of whole plants 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 supposedly transformed plants that remain stable in succeeding regenerations (Qayyum et al. 2005).

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

Materials and Methods

Bulb scales derived from terminal bulb**s** were surface sterilized in Ridomil (15 mg/l) for 15 min and commercial bleach solution 1.5% (v/v) for 20 min followed by shaking for 4 min in 0.1% $HgCl_2$ solution on a rotary shaker. The explants were then rinsed with sterile distilled water for at least three times and were cut into 10 mm long segments. Then they were transferred to the culture media for induction bulblets and somatic embryos. The media have been described in Tables 1 and 2.

MS salts supplemented with Miyo-inositol (100 mg/l), nicotinic acid (5 mg/l), pyridoxine HCl (0.5 mg/l) and thiamine HCl (0.5 mg/l) were used in one set of experiments and in the other set Nitsch salts supplemented with biotin (0.05 mg/l), folic acid (0.5 mg/l), glycine (2 mg/l), myo-inositol (100 mg/l), nicotinic acid (5 mg/l), pyridoxine (0.5 mg/l) and thiamin (0.5 mg/l).

Regeneration medium for induction of somatic embryos comprised MS without growth regulators; while the rooting medium for root initiation in the bulblet was MS containing IBA (1 mg/l).

In order to induce somatic embryos, cultures were maintained under dark conditions for two weeks at 24ºC, before their transfer to light conditions with 16 hr daily photoperiod and 8 hr dark keeping the temperature same in both light and dark conditions. 0.7% agar was used to solidify the medium keeping the pH at 5.6; and growing explants were sub-cultured every four weeks. The number of induced embryos and bublets per explant was recorded after ten weeks of initial culture. This experiment was designed in a completely randomized with three replicates each consisting of four explants. The conversion of $Y = (X + 0.04 \times 0.5)$ was used for data adjustment and all data were analyzed by Statistical Analysis Systems (SAS).

Table 1. Media for induction of somatic embryogenesis.

Treatment	Combination
A	MS with 2,4-D (0.5 mg/l)+ BAP (0.5 mg/l) + GA_3 (0.5 mg/l)
B	MS with 2,4-D (1.6 mg/l)+ BAP (1.6 mg/l) + GA_3 (0.5 mg/l)
C	MS with IBA (0.6 mg/l) + BAP (1.23 mg/l)
D	MS with IBA (0.2 mg/l) + BAP (0.9 mg/l)
E	Nitsch with 2,4-D (1.1 mg/l) + BAP (1.12 mg/l)

Table 2. Media for bulblet regeneration.

Treatment	Combination
G	MS with 2,4-D (0.2 mg/l), BAP (0.2 mg/l) + GA_3 (0.5 mg/l)
H	MS with 2,4-D (1.1 mg/l), BAP (1.1 mg/l)+GA_3 (0.5 mg/l)
I	MS with IBA (0.2 mg/l), BAP(1.23 mg/l)
J	MS with NAA (0.93 mg/l), Kn (0.1 mg/l)
K	MS with NAA (0.93 mg/l), Kn (1 mg/l)
R	MS with NAA (0.093 mg/l) and Kn (1 mg/l)
L	MS with IBA (0.3 mg/l), Kn (0.43 mg/l)
M	MS with IBA (1 mg/l), Kn (0.1 mg/l)
N	Nitsch with 2,4-D (0.22 mg/l), BAP (1.12 mg/l)
P	Nitsch with 2,4-D (1.1mg/l), BAP (2.2 mg/l)

Results and Discussion

Induction of somatic embryogenesis : Results of analysis of variance of the effect of 2,4-D and BAP in combination with GA_3 on induction of somatic embryos are shown in Table 3. Significant differences were observed between treatment A [2,4-D (0.5 mg/l) + BAP (0.5 mg/l) in combination with GA_3 (0.5 mg/l] and treatment B [2,4-D (1.6 mg/l) + BAP (1.6 mg/l) in combination with GA_3 (0.5 mg/l)]. The results at 5% showed that treatment B produced more embryos per

explant in comparison to treatment A (Fig. 2a). Explants in treatment A were swollen with translucent structures that were similar to early somatic embryos. The color of tissues changed to yellow, then after three weeks, proembryos changed to scutellar embryos without the intervention of globular embryos (Fig. 1A). While In treatment B, the proembryos changed to globular embryos (Fig. 1B). Previous studies showed that 2,4-D in combination with BAP proved to be suitable for induction of somatic embryogenesis in *Narcissus* and other genera of this family. Sage et al. (2005) 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 induction of somatic embryogenesis in *Nerin*, another bulbous plant of the family Amaryllidaceae.

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

Source of variation	D.F.	Mean squares	F	CV
Treatment	3	4.1	4.45*	5.7
Error	8	0.92	-	-

The analysis of variance on the effect of IBA and BAP on regeneration of *Narcissus papyraceus* was not significant. However, in the treatment D [IBA (0.2 mg/l) and BAP (0.9 mg/l)], there were more embryos; i.e., 3.5 embryos in each explant (at %5) than those in treatment C [IBA (0.6 mg/l) and BAP (1.23 mg/l)], where the number of embryos was 2.6 per explant.

These results were in agreement with those of Salema et al. (1998) in that they obtained somatic embryos in the 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 sections were cultured on half strength of MS fortified with IBA (4 mg/l) and BAP(2 mg/l). After 60 days an average of 41 shoota were recorded per explant. This treatment, namely, treatment E [2,4-D (1.1 mg/l) and BAP (1.12 mg/l)] yielded scutellar embryos after eight weeks of culture initiation.

Results indicated that equal concentrations of 2,4-D and BAP were necessary to induce somatic embryogenesis in *Narcissus papyraceus* cv. Shirazi, but bulblets replaced somatic embryogenesis when the two regulators were added to the medium in different concentrations.

Bulblet regeneration: The results of analysis of variance of the combined effects of BAP, 2,4-D and GA_3 on regeneration via bulbet formation (Table 4) showed that there were significant differences between treatment H [2,4-D (1.1

mg/l), BAP (1.1 mg/l) and GA_3 (0.5 mg/l)] and treatment G [2,4-D (0.2 mg/l), BAP (0.2 mg/l) and GA_3 (0.5 mg/l).

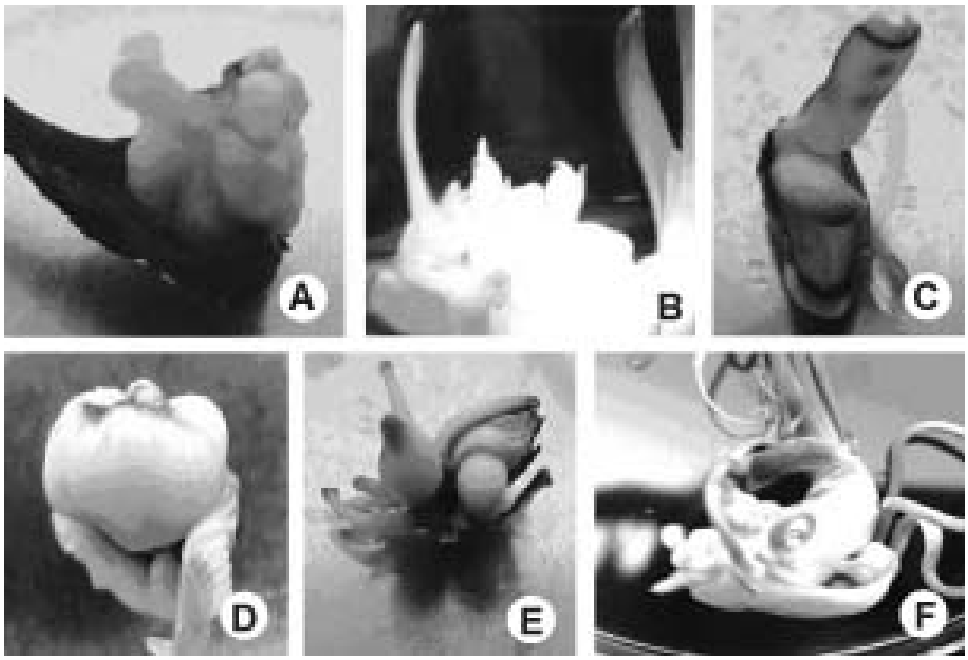

Fig. 1. A. Formation of scutellar embryos after ten weeks of culture. B. Formation of circular embryos three months after culture. C. Formation of bulblets after eight weeks of culture. D. swelling of tissues. E. Formation of whole plantlets from induced embryos. F. Formation of whole plantlets from regenerated bulblets.

The results indicated that treatment G produced 1. 33 bulblets per explant, whereas treatment H, produced 0.61 bulblet per explant (Fig. 2b). Treatment I [IBA (0.2 mg/l) and BAP (1.23 mg/l)] caused direct regeneration via bulbet formation. After eight weeks of culture initiation, explants were swollen giving rise to bulblets on the base of scale explants. The comparison of means (at 5%) between treatment J [NAA (0.93 mg/l) and Kn (0.1 mg/l)] and treatment K [NAA (0.93 mg/l) and Kn (1 mg/l)] showed that there were no significant differences between two treatments and both the treatments yielded 1.3 bulblets per explant. These 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 Kn, BA, zeatin and 2ip on production of bulbs. Their results showed that bulbet production was better when the medium was supplemented with NAA and 2ip. On the other hand, in the same combination Bansude et al. (2003) in *Agave* (Amaryllidaceae) somatic embryos. In treatment L [IBA (0.3 mg/l) and Kn (0.43 mg/l)] and treatment M [IBA (1 mg/l) and Kn (0.1 mg/l)] after six weeks of culture bulblets were initiated. The comparison of

means between these treatments at 5% showed that treatment L produced more bulblets, although no significant differences were observed between them. The results on the effects of BAP and 2,4-D on regeneration via bulbet formation (Table 5) indicated that treatment N [2,4-D (0.22 mg/l) and BAP (1.12 mg/l)] and treatment P [2,4-D (1.1mg/l) and BAP (2.2 mg/l)] caused regeneration via bulbet formation.

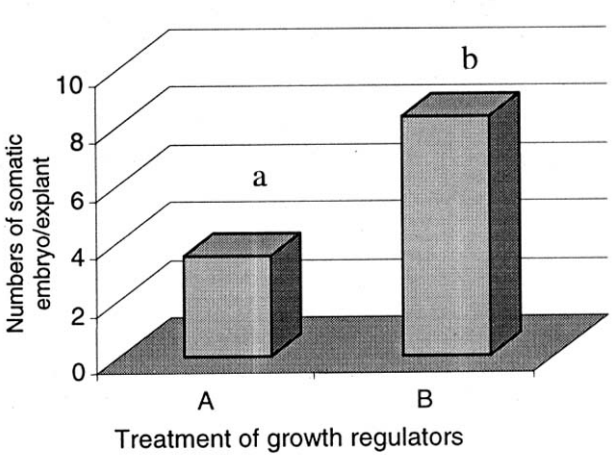

Fig. 2a. Means of number of somatic embryos in response to GA_3, 2,4-D and BAP supplements on somatic embryogenesis.

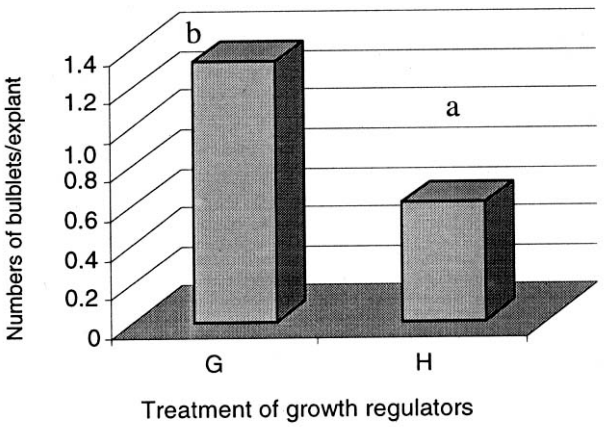

Fig. 2b. Means of number of bulblet embryos in response to GA_3, 2,4-D and BAP supplements on bulblet production.

In treatment R [NAA (0.093 mg/l) and Kn (1 mg/l)], explants were swollen after two weeks (Fig. 1D).

Germination and plantlet formation : Somatic embryos were transferred to MS for their germination and plantlet formation. The observation showed that

germination of somatic embryos and rooting occurred, respectively two - three and 12 weeks after transfer to the regeneration medium. The bulblets were transferred to half strength of MS containing IBA (1 mg/l) and roots developed at the base of bulblets after eight weeks (1E).

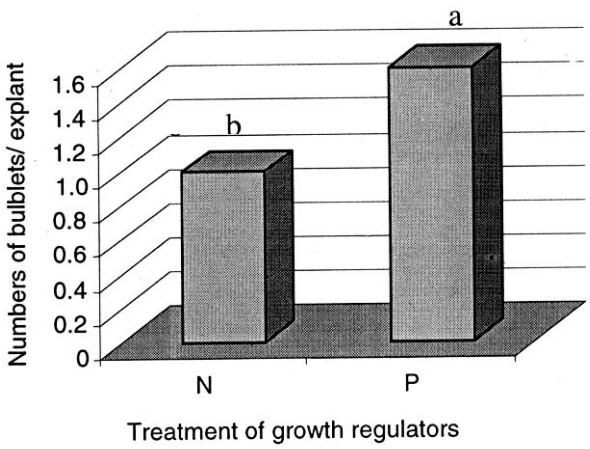

Fig. 3. Means of number of bulblet embryos in response to GA_3, 2,4-D and BAP supplements on bulblet production.

Somatic embryogenesis can provide a novel method for production of new plants at reduced costs (Sage and Hammatt 2005, Langens and Grrits 1996, Manuel et al. 1986). The process of somatic embryogenesis involves the formation, development and maturity of embryos and finally, development of plantlets. There are hardly any phenomenon in plants where growth regulators do not play any regulatory role. Morphogenesis involves two stages, namely, differentiation and elongation; both are influenced by plant growth regulators. Hence, it is possible to induce somatic embryogenesis in bulb scale explants by adjusting levels of plant growth regulators. Relatively high levels of plant growth regulators have inhibitory effects on the 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 regulators, did not only induce somatic embryogenesis in this daffodil variety, but also stimulated the production of bulblets. However, this did not hold true in all bulb producing species of Amaryllidaceae. Use of Kn (1-2 mg/l) and NAA (0.5 mg/l) induced somatic embryogenesis in Agave (Bansude et al. 2003), whereas smaller amount of Kn (0.5 mg/l) with the same amount NAA produced shoots in this plant species (Binh et al. 1990). In *Camellia japonica*, application of IBA (2 mg/l) and BAP (4 mg/l) did not induce somatic embryogenesis, whereas the

lower concentration caused production of somatic embryos (Vietez and Barciela 1990). The response of different species and genera to growth regulators for induction of somatic embryogenesis and regeneration is different. The combination of BAP and IBA in *Narcissus papyraceus* cv. Shirazi yielded bulblets,

Table 4. Analysis of variance for the effect of 2,4-D, BAP and GA3 on regeneration via bulbet formation of bulblets.

Source of variation	DF	Mean squares	F	CV
Treatment	3	0.64	9.27**	45
Error	8	0.06	-	-

Table 5. Analysis of variance on the effect of 2,4-D and BAP on regeneration via bulbet formation.

Source of variation	DF	Mean squares	F	CV
Treatment	2	0.96	52.29**	45
Error	6	0.01	-	-

whereas the same treatment produced somatic embryos in *Narcissus bulbocodium* (Salema and Salemak 2000). The treatment in which Kn was used in combination with IBA produced bulblets in *Narcissus papyraceus* cv. Shirazi, whereas in MS containing 2% sucrose, 10% coconut water, 0.8% agar and IBA (0.1 mg/l) and Kn (0.5 mg/l) shoots developed in *Agave cantala, A. fourcroydes* and *A. sisalana* (Binh et al. 1990). Different plant parts have different 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 explants but also the position of explants on the surface of culture medium is important to determine for induction of somatic embryogenesis. The explants that were cultured with their abaxial surface in contact with the medium produced more embryos compared to other surfaces (Sage 2005). Leshem et al. (1986) showed that scale sections, planted with their ventral side down regenerated a fewer and smaller bulbs, less roots, but much more callus than those with their dorsal side down. The most effective treatment was found to be MS containing GA_3 (0.5 mg/l), BAP (1.6 mg/l) and 2,4-D (1.6 mg/l). Although our data provide an efficient system for rapid and economical production of new and improved *Narcissus* varieties, it leaves room to further investigate the effects of other media on the maturity of induced somatic embryos, the effects of type of explants, the position of explants on the surface of the medium and the effects of different types of growth regulators and their concentrations on induction of somatic embryogenesis.

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