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Full Length Research Paper

Evidence of somatic embryogenesis for plantlet regeneration in *Muscari neglectum* Guss.

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***Muscari neglectum* Guss. is one of the ornamental species from Liliaceae family and has been used as well as other species of the genus *Muscari*, for its medicinal properties. The root is anti-inflammatory, antiallergic, aphrodisiac and pectoral stimulant. Somatic embryogenesis in *M. neglectum* Guss. was induced from bulb explants cultured in Murashige and Skoog (MS) medium plant growth regulator free or supplemented with different concentrations of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA). The best results were obtained on plant growth regulator free medium or with lower NAA and BA concentrations and 30 g/l sucrose. Somatic embryo development was observed on 1/2 MS medium plant growth regulator free under continuous illumination with fluorescent light. Matured somatic embryos germinated and converted to plantlets in the same medium or containing 1 mg/l BA after 3-4 months. The plantlets left in the medium especially containing BA produced bulbs after 5 months.**

Key words: Bulb explant, *Muscari neglectum*, plantlet regeneration, somatic embryogenesis.

INTODUCTION

The genus Muscari Miller belongs to Liliaceace family, some members of which are cultivated in some countries as garden plants. *Muscari neglectum* Guss. is one of the ornamental species from the genus and has been used as well as other species of Muscari, for its diuretic and stimulant properties. The root is anti-inflammatory, antiallergic, aphrodisiac and pectoral stimulant (Usher, 1974). This bulbous plant has several attractive traits such as dark blue flower colour and vigorous growth, which are desired to be incorporated via somatic hybridization into the other liliaceous ornamental plants (Nakano et al., 2005). Somatic embryogenesis potentially offers a promising system for plant regeneration because of the high proliferation capacity and probable single cell origin, which may avoid the risk of chimeric plants and facilitate the application for mutant selection and recombinant DNA technology (Neves et al., 1999). There have been some reports of *in vitro* plant regeneration in different ornamental species of Liliaceae and Iridaceae such as Muscari (Suzuki and Nakano, 2001; Suzuki and Nakano, 2003; Moris and Nakano, 2004), Lilium (Nakano et al., 2000; Moris et al., 2005) and Crocus (George et al., 1992; Ahuja et al., 1994; Ebrahimzadeh et al., 2000; Karamian and Ebrahimzadeh, 2001; Karamian, 2007). Somatic embryogenesis of *M. neglectum* was achieved from leaf-derived calli by Moris and Nakano (2004) for the first time. We describe here plantlet regeneration and somatic embryogenesis from bulb-derived calli of *M. neglectum*.

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Abbreviations: BA, 6-Benzylaminopurine; **FAA,** formalin-glacial acetic acid-ethanol; **MS,** Murashige and Skoog; **NAA,** 1-naphthaleneacetic acid; **PGR,** plant growth regulators; **DNA,** deoxyribonucleic acid.

MATERIALS AND METHODS

Induction of embryogenic callus

Bulb explants were excised from *M. neglectum* plants collected from Hamedan Province, Iran. They were surface-sterilized in 5% calcium hypochlorite solution for 15 min followed by three rinses with sterile distilled water. Then they were cultured on MS basal medium (Murashige and Skoog, 1962) plant growth regulators (PGR)-free or supplemented with NAA and BA in different combinations, 3% (w/v) sucrose and 0.8% (w/v) agar (Table 1). Twenty five explants per treatment were cultured in Petri dishes (6 cm diameter) containing 20 ml medium and covered with parafilm. Each treatment was done in triplicates. All media were adjusted to

Table 1. The effect of different concentrations of NAA and BA on somatic embryo induction of *M. neglectum* after 8 weeks.

Plant growth regulator (mg/l)	Explants producing somatic embryo* (%)
0 NAA+0 BA	88.2^{e}
0 NAA+0.1 BA	62.3^{de}
0 NAA+0.5 BA	58.2^{de}
0 NAA+1 BA	42.1^{d}
0.1 NAA+0 BA	40.3^{d}
0.1 NAA+0.1 BA	39.8^{d}
0.1 NAA+0.5 BA	32.7^{cd}
0.1 NAA+1 BA	26.6^{bc}
0.5 NAA+0 BA	25.8^{bc}
0.5 NAA+0.1 BA	23.3^{bc}
0.5 NAA+0.5 BA	16.2^{ab}
0.5 NAA+1 BA	14.5^{ab}
1 NAA+0 BA	13.8^{ab}
1 NAA+0.1 BA	13.1^{ab}
1 NAA+0.5 BA	12.3^{ab}
1 NAA+1 BA	6.3^{a}

* Values are the mean for three experiments each of which consisted of 25 replicates. Means in the same column followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range tests. BA, 6-Benzylaminopurine; NAA, 1-naphthaleneacetic acid.

pH 5.7-5.8 prior to autoclaving at 121°C for 15 min. For embryogenic callus production, all cultures were maintained at 25 ± 2°C in the dark for nearly 4 weeks.

Selection of embryogenic calli and somatic embryo formation

After 6-7 weeks of culture, some embryogenic regions were organized from soft calli. The embryogenic regions obtained in different treatments were excised and transferred on MS medium PGR-free or containing lower NAA and BA concentrations for differentiation of globular somatic embryos. Cultures were incubated under continuous illumination with fluorescent light (50 µmol m^{-2} s^{-1}). Data on the frequency of callus producing somatic embryos were recorded after 8 weeks (Table 1).

Plantlet regeneration

For somatic embryo development, embryogenic calli were transferred to 1/2 MS medium PGR-free and incubated at 25 ± 2°C under continuous illumination with fluorescent light (50 µmol m^{-2} s^{-1}). Somatic embryo germination and plantlet regeneration were achieved in 1/2 MS medium PGR-free as well as in 1/2 MS medium containing 1 mg/l BA at 25 ± 2°C under continuous illumination with fluorescent light (50 µmol m^{-2} s^{-1}) after 3-4 months.

Histological studies

For histological investigation, calli with somatic embryos were fixed in FAA (formalin-glacial acetic acid-ethanol, 5:5:90, v/v) for 24 h, dehydrated with a graded series of alcohol, and then embedded in paraffin. Embedded tissues were cut into 10 µm thick sections with a rotary microtome. Sections were stained with hematoxylin and observed under a light microscope.

Statistical analysis

Twenty pieces of calli were used for each experiment and repeated three times. Data were analyzed using a SAS program (1999) and separated by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Induction of embryogenic callus

After 4 weeks of culture, off-white soft calli were initiated on bulb explants nearly in all treatments (Figure 1A). These calli were soft and friable with no morphogenetic potential at first, but then small granular structures organized from some regions of the calli (Figure 1B).

Selection of embryogenic calli and somatic embryo formation

After nearly 5-6 weeks of culture, some organized parts

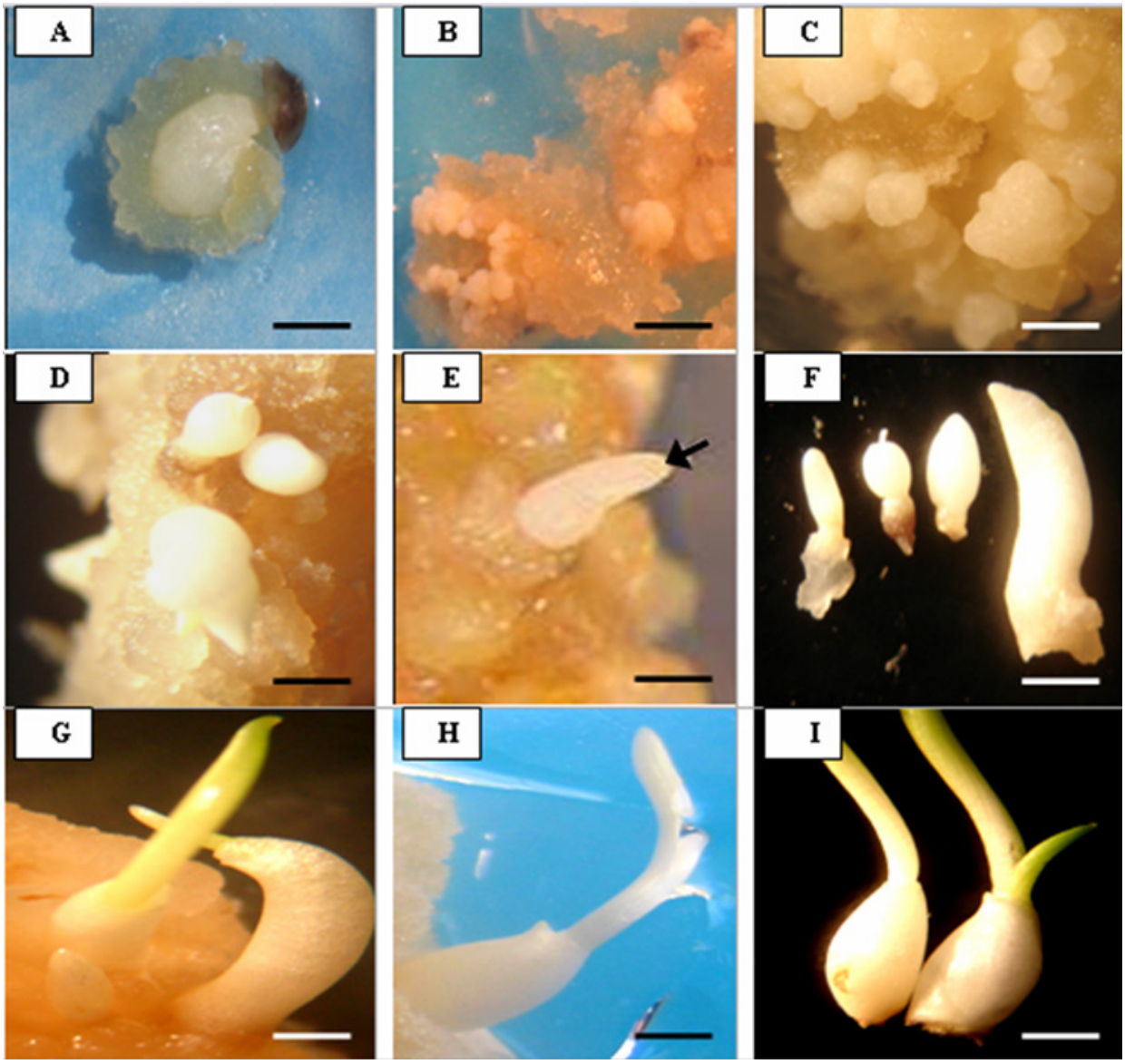

Figure 1. Somatic embryogenesis in *M. neglectum*. A, Non-embryogenic callus induced from bulb explants on MS medium supplemented with 0.1 mg/l NAA, 0.1 mg/l BA and 30 g/l sucrose in darkness after 4 weeks (Bar = 2 mm); B and C, embryogenic calli induced explants on MS medium PGR-free after 6-7 weeks (Bar = 2 mm); D, globular somatic embryos induced under continuous illumination after 8-9 weeks (Bar = 1 mm); E, torpedo somatic embryo showing bipolarity in half strength MS medium PGR-free (Bar = 1 mm); F, different stages of somatic embryo development (Bar = 1 mm); G, somatic embryo germination in 1/2 MS medium PGR-free under continuous illumination after 11 weeks (Bar = 5 mm); H, Bulb formation in 1/2 MS medium supplemented with 1 mg/l BA and 30 g/l sucrose under continuous illumination after 4 months (Bar = 5 mm); I, regenerated plantlets with bulbs after 5 months (Bar = 5 mm).

could be recognized in the calli (Figure 1C). These parts had morphogenetic potential and produced hard and white globular somatic embryos on MS medium PGR-free or containing lower NAA and BA concentrations under continuous illumination (Figures 1D and E). Production of globular somatic embryos was initially slow but careful selection of embryogenic regions at subculturing and incubation under continuous illumination, resulted in vigorous proliferation. Somatic embryo production was greatly inhibited by increasing BA and NAA concentrations in the media. However, inclusion of higher concentrations of BA negatively affected somatic embryo production more than NAA (Table 1).

Plantlet regeneration

Somatic embryo maturation and germination were observed on 1/2 MS medium PGR-free after 3-4 months under continuous illumination (Figures 1F and G). Globular

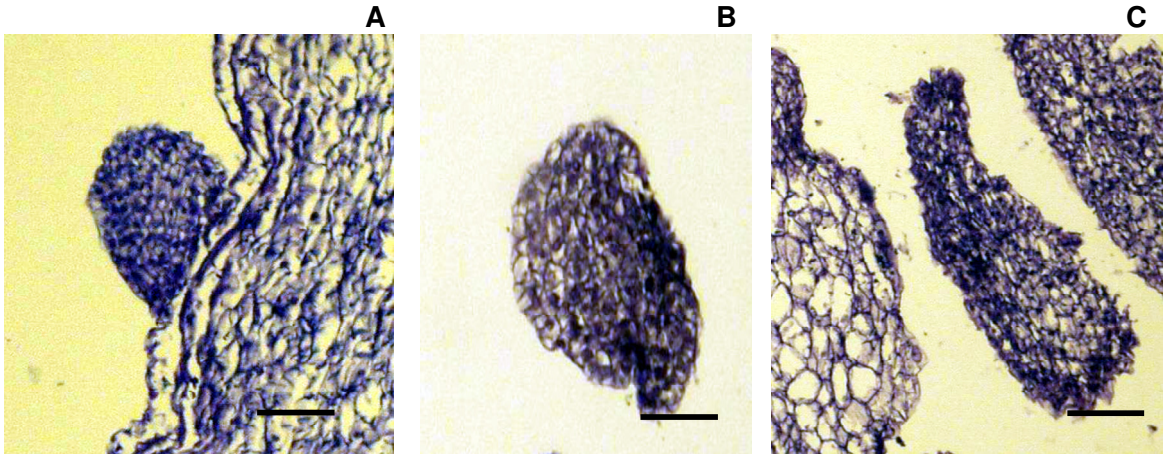

Figure 2. Histological sections of somatic embryogenesis in *M. neglectum*. A) Globular somatic embryo; B) heart-shaped somatic embryo; C) torpedo somatic embryo showing bipolarity (Bar = 500 µm).

Table 2. The effect of different concentration of BA on conversion of somatic embryos into plantlets in *M. neglectum* after 3-4 months.

BA (mg/l)	Embryo conversion* (%)
0.00	22.3 [a]
0.1	24.6 [a]
0.5	28.8 [b]
1	38.2 [c]
2	30.5 [b]

* Values are the mean for three experiments. Means in the same column followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range tests. BA, 6-Benzylaminopurine.

embryos developed further into heart-shaped and torpedo-like embryos in 1/2 MS medium PGR-free (Figures 2A, B and C). Somatic embryo development in the present case was asynchronous and various stages of globular, heart-shaped and torpedo-like embryos could be observed simultaneously in the same embryogenic calli, as previously described for some members of Liliaceae and Iridaceae (Ebrahimzadeh et al., 2000; Karamian and Ebrahimzadeh, 2001; Suzuki and Nakano, 2001; Karamian, 2007). It seems that increasing of NAA and BA concentrations in media negatively affected somatic embryo production and development. In this case, BA showed higher negative effect than NAA. Then, a significantly higher ($p < 0.05$) percentage of somatic embryo production was obtained in MS medium PGR-free. The positive effects of PGR-free medium on somatic embryo development has been reported frequently, especially for the members of Liliaceae and Iridaceae families (Ebrahimzadeh et al., 2000; Karamian and Ebrahimzadeh, 2001; Suzuki and Nakano, 2001; Nakano et al., 2005; Karamian, 2007). Many aspects can affect the maturation and germination of somatic embryos such as temperature and light conditions (Tremblay and Tremblay, 1991; Firoozabady and DeBoer, 1993), age of explants (Iida et al., 1992) and concentration of growth regulators (Tremblay and Tremblay, 1991; Karamian, 2007).

Plantlet regeneration was achieved in the same medium (Figure 1H). However, transferring of germinated somatic embryos to 1/2 MS medium containing 1 mg/l BA increased plant conversion efficiency. The percentage of conversion of somatic embryos into plantlets, according to the BA concentration in the medium, is presented in Table 2. The addition of BA up to 1 mg/l increased conversion efficiency with 0-1 mg/l BA, the plantlets presented a normal development, but higher concentrations (2 mg/l) promoted abnormalities such as curled and dark green leaves or multiple shooting. The positive effect of lower concentrations of BA (0.1-0.4 mg/l) on conversion efficiency has been reported in *Allium sativum* previously (Fereol et al., 2002). The plantlets left in the medium especially containing BA produced bulbs after 5 months (Figure 1I).

The data reported here demonstrated for the first time the somatic embryogenesis and plantlet regeneration from bulb-derived calli of *M. neglectum*. This effective approach offers the possibility to mass multiply material that has been improved by genetic manipulation experiments.

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