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Tissue Culture Note

Plantlet regeneration from protoplasts of *Muscari armeniacum* Leichtl. ex Bak.

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Abstract Protoplasts were isolated from embryogenic calluses of *Muscari armeniacum* 'Blue Pearl', which had been subcultured for 3 years. Protoplasts started to divide after 5–7 days of culture, and colonies consisting of 50–100 cells were produced after one month. The highest plating efficiency (10.9%) was obtained by using a medium containing 5.4 μM NAA and 4.4 μM BA, 0.5 M glucose and 2 g l^{-1} gellan gum. Protoplast-derived calluses produced somatic embryos at frequencies of 4.3–89.6% on media containing 0 or 0.54 μM NAA in combination with 0, 4.4, 22 or 44 μM BA, but few embryos converted into plantlets. On the other hand, over 35% of the calluses produced adventitious shoots on media containing 4.4 μM BA or 0.54 μM NAA in combination with 44 μM BA, and some of these shoots developed into plantlets following transfer to a medium without PGRs.

Key words: Adventitious shoot, muscari, plantlet regeneration, protoplast culture, somatic embryo.

Liliaceous ornamental plant *Muscari armeniacum* Leichtl. ex Bak., generally called 'muscari' or 'grape hyacinths', is cultivated for pot or garden uses throughout the temperate regions of the world. This bulbous plant has several attractive traits such as blue flower color and vigorous growth, which are desired to be incorporated via somatic hybridization into the other Liliaceous ornamental plants. In order to apply somatic hybridization for genetic improvement, establishment of a protoplast culture system is prerequisite. Although plant regeneration from protoplasts has so far been reported for several Liliaceous ornamental plants such as *Agapanthus praecox* ssp. *orientalis* (Nakano et al. 2003), *Lilium* spp. (Mii et al. 1994; Godo et al. 1996; Horita et al. 2002) and *Hemerocallis hybrida* (Fitter and Krikorian 1981), no studies on protoplast culture have yet been reported for *M. armeniacum*. In the present study, we describe for the first time plantlet regeneration from protoplasts of *M. armeniacum*.

Leaf-derived embryogenic calluses of *M. armeniacum* 'Blue Pearl' (Suzuki and Nakano 2001; Mori and Nakano 2004) were used as a protoplast source. Before protoplast isolation, the calluses had been maintained for 3 years by monthly subculturing onto half-strength MS medium (Murashige and Skoog 1962) containing 54 μM NAA, 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum. All media used in the present study were adjusted to pH 5.6–5.8 prior to autoclaving at 121°C for 15 min. For isolating protoplasts, 1 g fresh weight of the calluses 2–4 weeks after subculture were transferred to 10 ml of a filter-sterilized enzyme solution containing half-strength MS salts, 5 mM MES, 0.6 M sorbitol, 20 g l^{-1} Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Japan), 5 g l^{-1} Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan) and 0.5 g l^{-1} Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), pH 5.8. After 4–5h of incubation at 25°C in the dark, the mixture was passed through a nylon sieve (50 μm pore size), and protoplasts were rinsed three times in a 0.6 M sorbitol solution by resuspension and centrifugation (120 *g* for 3 min). Protoplasts were routinely isolated from the embryogenic calluses with yields of about 1.5×10^6 protoplasts per g fresh weight of calluses (Figure 1A). They showed over 85% of viability as assessed with fluorescein diacetate staining (Widholm 1972).

Protoplasts were cultured at a density of 1×10^5 cells ml^{-1} in half-strength MS media containing 5 mM MES, 0.5 M glucose and 2 g l^{-1} gellan gum, and 5.4 or 54 μM NAA with or without 4.4 μM BA (Table 1). Embedding protoplasts in gellan gum-solidified media was performed as previously described (Nakano et al. 1995). Plastic Petri dishes (60×15 mm) each containing 2 ml of medium were used for protoplast culture, and they were maintained at 25°C in the dark. Protoplasts started to divide after 5–7 days of culture (Figure 1B), and protoplast-derived colonies consisting of 50–100 cells were produced after one month (Figure 1C). Table 1 shows the effects of NAA and BA on the plating

Abbreviations: BA, 6-benzyladenine; MES, 2-morpholinoethanesulfonic acid; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator.

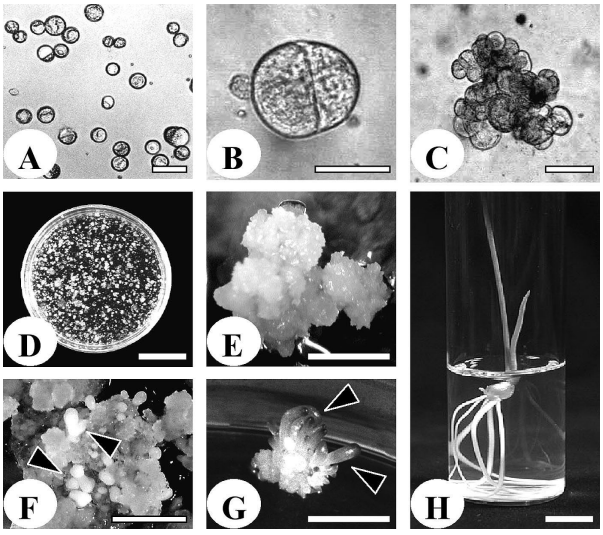

Figure 1. Plantlet regeneration from protoplasts of *Muscari armeniacum* 'Blue Pearl'. Protoplasts were cultured in a medium containing 5.4 μM NAA and 4.4 μM BA. (A) Freshly-isolated protoplasts. Bar=100 μm. (B) First protoplast division after 5 days of culture. Bar=30 μm. (C) A protoplast-derived colony after one month of culture. Bar=100 μm. (D) Protoplast-derived visible colonies after 2 months of culture. Bar=2 cm. (E) A protoplast-derived callus. Bar=2 mm. (F) Somatic embryos (arrowheads) produced from protoplast-derived callus on PGR-free medium. Bar=5 mm. (G) Adventitious shoots (arrowheads) produced from protoplast-derived callus on a medium containing 4.4 μM BA. Bar=5 mm. (H) A plantlet developed from protoplast-derived adventitious shoot. Bar=1 cm.

Table 1. Effects of NAA and BA on the plating efficiency in protoplast culture of *Muscari armeniacum* 'Blue Pearl'

NAA (μM)	BA (μM)	Plating efficiency (%)
5.4	0	3.7 a
5.4	4.4	10.9 b
54	0	4.5 a
54	4.4	6.3 a

Data were recorded after one month of culture. Values represent the mean of at least 3 independent experiments each of which consisted of 5 culture dishes. Means with the same letter are not significantly different at $p=0.05$ with LSD test.

efficiency (percentage of protoplasts which formed colonies) after one month of culture. The highest plating efficiency (10.9%) was obtained by using a medium containing 5.4 μM NAA and 4.4 μM BA, although embryogenic calluses used as a protoplast source had been subcultured on a medium only containing 54 μM NAA. In order to promote colony growth, 2 ml of the same medium but lacking gellan gum was added to each culture dish after one month of culture, and 2 ml of the same medium but lacking gellan gum and containing a lowered concentration (0.3 M) of glucose was added to each culture dish one month later. Some colonies sustained growth and became visible to the naked eye after 2–3 months of culture (Figure 1D). Visible colonies of 0.5–1 mm in diameter, which had been obtained in a medium containing 5.4 μM NAA and 4.4 μM BA after 3 months of protoplast culture, were transferred for callus proliferation onto half-strength MS medium containing 5.4 μM NAA, 4.4 μM BA, 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum. Cultures during and after callus proliferation were incubated at 25°C under continuous illumination with fluorescent light (50 μmol m^{-2} s^{-1}). Almost all of the colonies grew into calluses of over 5 mm in diameter after 2–3 months of transfer (Figure 1E).

Table 2. Effects of NAA and BA on the percentages of protoplast-derived calluses that produced somatic embryos or adventitious shoots in *Muscari armeniacum* 'Blue Pearl'

NAA (μM)	BA (μM)	Somatic embryos (%)	Adventitious shoots (%)
0	0	89.6 e	18.6 b
0	4.4	48.3 d	36.7 d
0	22	23.6 bc	25.8 bc
0	44	4.3 a	21.2 b
0.54	0	36.6 d	5.7 a
0.54	4.4	33.8 cd	6.7 a
0.54	22	16.2 ab	20.8 b
0.54	44	14.8 ab	35.8 d

Calluses obtained by culturing protoplasts in a medium containing 5.4 μM NAA and 4.4 μM BA were used. Data were recorded after 3 months of transfer to regeneration media. Values represent the mean of 3 independent experiments each of which consisted of 30 protoplast-derived calluses. Means in the same column followed by the same letter are not significantly different at $p=0.05$ with LSD test.

Protoplast-derived calluses of about 5 mm in diameter were further transferred for regeneration to half-strength MS media containing 30 g l^{-1} sucrose, 2 g l^{-1} gellan gum, and 5.4 or 54 μM NAA in combination with 0, 4.4, 22 or 44 μM BA (Table 2). After 1–2 months of transfer to regeneration media, protoplast-derived calluses developed somatic embryos (Figure 1F) and/or adventitious shoots (Figure 1G). Under continuous illumination, somatic embryos were white in color, while adventitious shoots were green as suggested by Suzuki and Nakano (2001). Table 2 shows the effects of NAA and BA on production of somatic embryos and adventitious shoots from protoplast-derived calluses after 3 months of transfer to regeneration media. A significantly higher percentage of calluses producing somatic embryos was obtained on PGR-free medium (89.6%). Somatic embryo production from protoplast-derived calluses was greatly inhibited on BA-containing media as in the case of embryogenic calluses used as a protoplast source (Suzuki and Nakano 2001). Inclusion of NAA in regeneration media also negatively affected somatic embryo production. In contrast, BA promoted adventitious shoot regeneration from protoplast-derived calluses, and significantly higher percentages of regeneration were obtained on media containing 4.4 μM BA (36.7%) or 0.54 μM NAA in combination with 44 μM BA (35.8%).

Regenerated somatic embryos and adventitious shoots were transferred for germination and rooting, respective-

ly, to half-strength MS medium lacking PGRs but with $30\,g\,l^{-1}$ sucrose and $2\,g\,l^{-1}$ gellan gum. However, few somatic embryos showed greening and germination on this medium. Treatments with gibberellic acid and cold treatments had no promotive effects on conversion of somatic embryos (data not shown). On the other hand, about 10% of protoplast-derived adventitious shoots produced roots and developed into plantlets after 2 months of transfer to PGR-free medium (Figure 1H). However, all of these plantlets died after transplantation to the greenhouse.

In the present study, we succeeded, for the first time, in plantlet regeneration from protoplasts of *Muscari* species. Protoplasts were isolated from 3-year-old embryogenic callus cultures of *M. armeniacum* 'Blue Pearl', and somatic embryos and adventitious shoots were produced from protoplast-derived calluses. However, these somatic embryos did not converted into plantlets. In addition, only a few protoplast-derived adventitious shoots developed into plantlets, all of which died following transplantation to the greenhouse. Such problems were not observed in our previous study: almost all of both somatic embryos and adventitious shoots, which had been regenerated from 6-month-old embryogenic callus cultures of *M. armeniacum* 'Blue Pearl', developed into plantlets, and almost all of these plantlets were successfully transplanted to the greenhouse (Suzuki and Nakano 2001). Thus, the failure in the conversion of somatic embryos and in the survival of plantlets following transplantation to the greenhouse in the present study may be partly due to some genetic and/or physiological changes of embryogenic callus cultures used as a protoplast source during long-term subculture (3 years). Further study should be directed to develop an efficient protoplast-to-plant system by isolating protoplasts from embryogenic calluses immediately after establishment of callus cultures.

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