PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF *MUSCARI ARMENIACUM*

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ABSTRACT

A system of plant regeneration from leaf explants of Muscari armeniacum via somatic embryogenesis was established. The process of somatic embryogenesis was studied, and histological observation of this process was made. The results showed that: somatic embryos could be obtained from direct embryogenesis or indirect embryogenesis, and less abnormal embryos were achieved through the direct approach, i.e. direct somatic embryos were more suitable for plant propagation and genetic transformation; MS basal medium containing 0.5 mg·L^{-1} 2,4-D and 0.1 mg·L^{-1} TDZ showed a high frequency of indirect somatic embryo production, while MS basal medium supplement with 0.1 mg·L^{-1} 6-BA and 0.1 mg·L^{-1} TDZ exhibited a high frequency of direct somatic embryogenesis from cut leaf explants; the process of somatic embryogenesis of M. armeniacum was similar to that of monocotyledons, which included three stages: globular embryo, partial pear-shaped embryo and club-shaped embryo, and finally development into a plantlet.

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Abbreviations: 2,4-D: 2,4-benzylaminopurine; 6-BA: N6-benzyladenine; BM: basal medium; MS: Murashige and Skoog; PGRs: plant growth regulators; SE: standard error; TDZ: thidiazuron

Introduction

Muscari armeniacum, a member of the family Hyacinthaceae and also called grape hyacinths, is characterized with sapphire blue flowers of a cup-shaped crown with a white rim. Among about 200 cultivated taxons of *Muscari*, *M. armeniacum* is one of the most often cultivated taxons (17). It is widely used as pot and garden ornamentals throughout the temperate regions of the world (15). *M. armeniacum* is conventionally propagated by either seed or bulb division; however, seedlings usually exhibit large phenotypic variations (8). As the seeds are not always true to type, plants from seeds grow very slowly. It takes about 3–5 years to mature and flower under natural conditions (11).

To supply a large number of uniform plants and target tissue for genetic transformation, *M. armeniacum* can be propagated by *in vitro* culture, including two pathways: organogenesis and somatic embryogenesis. Somatic embryogenesis has several advantages over regeneration by organogenesis, e.g. the assumed single-cell origin by which chimeras are avoided in the regenerated plants (14). Previous research on somatic embryogenesis of *Muscari* has mainly focused on plant regeneration through indirect somatic embryogenesis (6, 8, 15). However, direct embryogenesis is beneficial with its reduced time for plant propagation as well as with minimized culture-induced genetic changes (5).

Suzuki and Nakano (15) analyzed four types of explants (leaf, flower stalk, bulb scale and root) of *M. armeniacum* Leichtl. ex Bak., and found that the percentage of explants producing calli was highest in leaf explants. It was reported by Mori and Nakano (8) that, for *M. armeniacum* genotypes, flower bud-derived calli have high potential to produce somatic embryos compared with leaf-derived calli; however, for *M. armeniacum* cv. Blue Spike, the percentage of leaf-derived calli (46.7 %) to produce somatic embryos was not far from that of flower-bud-derived calli (63.3 %). Moreover, leaves are available in abundant numbers throughout the year from potted plants grown in greenhouse conditions, while flower buds could only be obtained in a small number at specific times. Therefore, leaves are a better choice as explants for somatic embryo induction.

The objective of the present study was to describe a protocol for direct somatic embryogenesis of *M. armeniacum*, and to make a comparison between direct and indirect somatic embryogenesis. In addition, histological observations were performed in order to understand the process of somatic embryogenesis.

TABLE 1

Effects of plant growth regulators (PGRs) on induction of direct somatic embryos from leaf segments of Muscari armeniacum

PGRs (mg·L^{-1})			No. of samples tested	No. of segments producing direct SM*	Percentage of segments producing direct SM*	No. of direct SM*/ segment (mean ± SE)
2,4-D	6-BA	TDZ				
0.2	0	0	92	33	35.89 ± 0.41 b	14.00 ± 2.52 cd
0.2	0.1	0.1	71	28	39.84 ± 1.79 b	16.33 ± 0.88 c
0.2	0.5	0.5	88	35	39.92 ± 3.78 b	14.67 ± 1.76 c
0.5	0	0.1	94	21	22.17 ± 3.07 c	5.00 ± 1.53 f
0.5	0.1	0.5	96	17	17.78 ± 0.41 c	9.33 ± 1.45 e
0.5	0.5	0	81	14	17.55 ± 1.70 c	10.00 ± 1.15 de
1.0	0	0.5	76	0	0 d	0 g
1.0	0.1	0	73	0	0 d	0 g
1.0	0.5	0.1	75	13	18.17 ± 9.12 c	1.67 ± 0.88 fg
0	0.1	0.1	83	73	**88.16 ± 1.80 a**	**29.67 ± 0.88 a**
0	0.5	0.5	89	79	88.74 ± 2.30 a	24.00 ± 2.08 b

*SM: somatic embryo scored after 30 days of culture. Results are presented as means ± SE. Data with different letters in the same column are significantly different at $P < 0.05$ by Duncan's multiple range test.

TABLE 2

Effects of plant growth regulators (PGRs) on the induction of embryogenic calli and indirect somatic embryos from leaf explants of *Muscari armeniacum*

PGRs (mg·L^{-1})			No. of samples tested	NO. of EC* producing indirect SM*	Percentage of EC* producing indirect SM*	No. of indirect SM*/EC* (mean ± SE)
2,4-D	6-BA	TDZ				
0.2	0	0	83	65	77.27 ± 9.00 c	80.00 ± 4.58 cd
0.2	0.1	0.1	62	59	95.06 ± 3.27 ab	93.67 ± 4.26 bc
0.2	0.5	0.5	79	75	95.06 ± 3.27 ab	84.67 ± 5.90 cd
0.5	0	0.1	85	85	**100.00 ± 0.00 a**	**113.33 ± 5.24 a**
0.5	0.1	0.5	87	79	90.49 ± 46.1 abc	101.67 ± 5.21 ab
0.5	0.5	0	72	67	92.54 ± 3.44 abc	92.67 ± 6.12 bc
1.0	0	0.5	67	37	55.41 ± 1.24 d	72.67 ± 4.33 de
1.0	0.1	0	64	21	31.71 ± 7.53 e	60.33 ± 4.06 ef
1.0	0.5	0.1	66	52	79.63 ± 6.68 bc	50.00 ± 3.79 fg
0	0.1	0.1	74	38	51.06 ± 1.78 d	40.33 ± 1.45 g
0	0.5	0.5	80	48	60.07 ± 7.94 d	47.00 ± 2.31 fg

*EC: embryogenic callus scored after 60 days of culture; *SM: somatic embryo scored after 60 days of culture. Results are presented as means ± SE. Data with different letters in the same column are significantly different at $P < 0.05$ by Duncan's multiple range test.

Materials and Methods

Plant material and explants preparation

Field-grown 3-year-old plants of *M. armeniacum* 'Blue Spike' were obtained from Xi'an botanical garden, Shaanxi Province, China. Leaves from the plants were excised and used as explants. Middle parts of the leaves were first cut transversely into pieces (0.5 cm ×1.5–2 cm), and then the pieces were surface-sterilized with 70 % ethanol for 30 s, followed by 0.1 % mercuric chloride for 10 min, and finally were rinsed three times with sterile distilled water.

Culture media

The BM consisted of Murashige and Skoog (MS) medium (9), 3 % (w/v) sucrose and 0.6 % (w/v) agar. The pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 21 min. Several PGRs with various combinations (**Table 1**) were added to BM for induction of somatic embryogenesis.

Experiments were repeated three times with 30 explants per treatment in each experiment.

Experimental procedures

Cultures were incubated at 25 °C ± 2 °C in the dark for 4 weeks and then the percentage of leaf segments producing direct somatic embryos, and the number of somatic embryos formed on each responding explant were scored using a microscope. After 8 weeks of culture, the number of embryogenic calli and the number of somatic embryos formed on each callus were scored. According to our preliminary experiments, calli with a compacted structure and nodular appearance were recorded as embryogenic calli. All leaf segments and calli were subcultured every 30 days under the same conditions as those for somatic embryo induction.

To obtain plantlets, direct somatic embryos at the club-shaped stage were removed from the explants and transferred to BM, and somatic embryos developed into plantlets after 30 days of culture. However, to get plantlets via indirect somatic embryogenesis, embryogenic calli from leaves were transferred to BM, and indirect somatic embryos via calli were observed in 30 days. When these somatic embryos elongated into club-shaped embryos, they were separated from calli and cultured on BM; then plantlets were obtained from somatic embryos 30 days later.

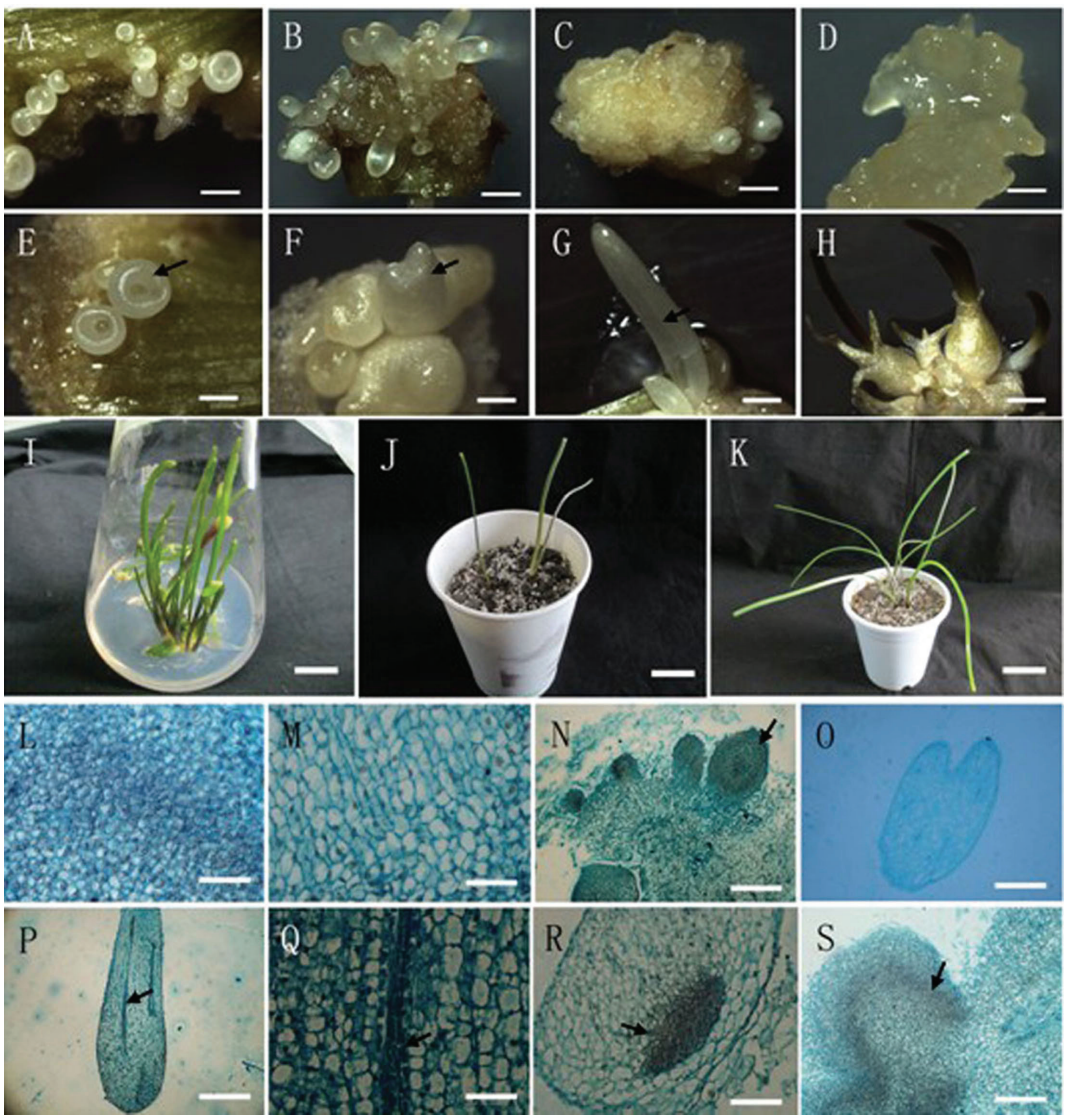

Fig. 1. Plant regeneration via somatic embryogenesis from leaf explants and histological observation of somatic embryogenesis of *M. armeniacum* cv. Blue Spike. Direct embryogenesis on the surface of leaf (**A**). Indirect embryogenesis on the surface of callus from leaf (**B**). Embryogenic (**C**) and non-embryogenic (**D**) callus induced from leaf. Globular embryo (**E**, arrow), partial pear-shaped embryo (**F**, arrow), club-shaped embryo (**G**, arrow). Mature plantlets (**H**). Plantlet in basal medium (**I**). Primarily acclimatized plant in paper cup (**J**). Secondarily acclimatized plants in plastic pots (**K**). Embryogenic (**L**) and non-embryogenic (**M**) callus. Globular embryos (arrow) derived from callus surface (**N**). Partial pear-shaped embryo (**O**). Club-shaped embryo showing procambium (**P**, arrow). Procambium with tracheid (arrow) inside (**Q**). Embryonic cells (arrow) derived from callus inside (**R**) or from callus surface (**S**). Bars: 1 mm (**A–D**, **G–H**, **N–P**); 0.5 mm (**E–F**); 2 cm (**I–J**); 5 cm (**K**); 0.1 mm (**L–M**); 0.2 mm (**Q**); 0.4 mm (**R–S**).

Histological analysis

The progress of somatic embryogenesis was recorded constantly under a stereo microscope (Olympus BX 60, Tokyo, Japan). Callus and various somatic embryos were fixed in a solution of 70 % ethanol: formalin: acetic acid (FAA) (90:5:5), dehydrated in an ethanol series (70 %, 85 %, 95 % and 100 %), and embedded in paraffin. Sections (8 μm to 10 μm thick) were cut using a rotary microtome (Leica RM2016, Leica Microsystems, Germany), stained with safranin and fast green, according to Bancroft and Cook (1), and observed under a light microscope.

Acclimatization and transfer of plantlets into soil

Young plantlets, after removal from culture medium, were washed with sterile distilled water and were then, planted in perforated paper cups containing two types of sterilized potting mixture of perlite: turf (3:1). The plantlets were incubated in a closed growth chamber at 20 °C to 25 °C and a relative humidity of 50 % to 70 %; the light was provided with cool fluorescent tube lights under a 16 h photoperiod for 60 days. Then, the hardened plants were transferred to greenhouse conditions in 10 cm plastic pots, the plants were removed gently from the paper cups without hampering the bulb and potted in the same mixture.

Statistical analysis

Statistical analysis was done using the data obtained from three repeated experiments. Analysis of variance (ANOVA) was performed and the significant level was determined with Duncan's multiple range test (DMRT) at ($P < 0.05$)

Results and Discussion

Somatic embryogenesis

In vitro somatic embryos can develop either from callus or directly from the explants without an intermediate callus stage (5). In this experiment, somatic embryos were produced directly from the leaf explants (**Fig. 1A**) after 2–3 weeks of culture. Leaf explants cultured on BM supplemented with 1.0 mg·L^{-1} 2,4-D and 0.5 mg·L^{-1} TDZ or 0.1 mg·L^{-1} 6-BA did not initiate direct somatic embryo production. The other nine types of media showed variable percentages of explants producing direct somatic embryos after 30 days of culture (**Table 1**). Number of somatic embryos per responding leaf explants varied with different media.

The obtained results (**Table 1**) showed that no or less direct somatic embryos were induced on the media with a high concentration of 2,4-D (1.0 mg·L^{-1}). Clearly, 6-BA in combination with TDZ induced high percentages of direct somatic embryo production on the leaf explants of *M. armeniacum* cv. Blue Spike. The percentage of responding explants showing differentiation of plantlets *in vitro* was significantly high on media containing 6-BA (0.1 mg·L, 0.5 mg·L^{-1}) and TDZ (0.1 mg·L^{-1}, 0.5 mg·L^{-1}). Among the tested media, the best one for direct somatic embryogenesis of leaf explants was MS medium containing 0.1 mg·L^{-1} 6-BA and 0.1 mg·L^{-1} TDZ, since 88.16 % of the cultured explants produced an average of 29.67 embryos.

After 60 days of culture, two types of calli were produced: white compact calli (**Fig. 1C**) and yellow loose calli (**Fig. 1D**). The results given in **Table 2** show that more embryogenic calli were produced on the media with lower concentrations of 2,4-D (0.2 mg·L^{-1} or 0.5 mg·L^{-1}). The best medium for embryogenic callus production was MS medium with 0.5 mg·L^{-1} 2,4-D and 0.1 mg·L^{-1} TDZ, since all leaf explants on the medium turned into embryogenic calli, and each piece of embryogenic callus produced an average of 113.33 embryos. White compact calli, which are proved to be embryogenic, were selected and transferred to BM. After 30 days of dark cultivation, somatic embryos (**Fig. 1B**) were obtained on the callus surface.

Auxins are considered as the best inducers for obtaining embryogenic cells (4). However, induction of embryogenesis by cytokinins alone has been reported in a number of plant species (3, 7, 10). In this experiment, it was found that the frequency of indirect somatic embryogenesis was regulated by both cytokinins and auxin, while the cytokinins individually were associated with a high frequency of direct somatic embryogenesis. It is clearly shown in **Table 1** and **Table 2** that lower concentrations of 2,4-D resulted in a high frequency of direct somatic embryo production, and moderate concentrations of 2,4-D led to indirect somatic embryogenesis.

In this experiment, somatic embryos were firstly produced directly from leaf explants, in other words, via direct somatic embryogenesis. However, with the increased callus derived from leaf explants, somatic embryos were mainly induced from callus, which means it changed into indirect somatic embryogenesis. The results (**Table 1** and **Table 2**) indicated that indirect somatic embryogenesis induced more somatic embryos than direct somatic embryogenesis. However, less abnormal embryos were achieved via direct embryogenesis in *M. armeniacum*, for the avoidance of an intermediate callus may reduce the frequency of gene and chromosomal changes in regenerated plants (2). Therefore, direct somatic embryos could be considered a better choice for plant propagation and genetic transformation.

Development of somatic embryo and plantlet regeneration

For both direct somatic embryogenesis and indirect somatic embryogenesis, a mature somatic embryo was formed through three stages: globular embryo (**Fig. 1E**), partial pear-shaped embryo (**Fig. 1F**) and club-shaped embryo (**Fig. 1G**), then a mature somatic embryo could develop into a mature plantlet (**Fig. 1H**).

Histology

Embryogenic cells were small and closely packed. These cells contained a thick cell wall, dense cytoplasm and a large nucleus, which was located in the center of the cells (**Fig. 1L**), while non-embryogenic cells were big and out of shape (**Fig. 1M**). Somatic embryos were induced from the internal (**Fig. 1R**) or surface (**Fig. 1S**) of the callus. The bipolarity of somatic embryos was shown at the partial pear-shaped stage and club-

shaped stage. The club-shaped embryos showed initiation of vascularisation, which is consistent with banana (12). The vascular tissue was a procambium with tracheid (**Fig. 1Q**, arrow), indicating that conducting tissue of a somatic embryo was formed.

One or two layers of outer cells (**Fig. 1N**, arrow) of somatic embryos were dyed deeper than the internal cells at all stages, forming a globular embryo (**Fig. 1N**), partial pear-shaped embryo (**Fig. 1O**) and club-shaped embryo (**Fig. 1P**), the 1–2 layers of cells might be an isolation layer of the somatic embryo, which could keep embryos apart from surrounding cells. Therefore, embryos arose as independent structures without vascular connections among them or with the initial explants or derived calli, similar to findings reported in several species (13, 16, 18).

Acclimatization and transfer of plantlets into soil

Direct or indirect somatic embryos after conversion into plantlets were further cultured in BM for rooting and forming bulbs (**Fig. 1I**). After 4 weeks, the rooted plants were transferred to a mixture of perlite and turf (3:1) in paper cups for primary acclimatization in growth chamber conditions (**Fig. 1J**). The mean survival percentage of plants after 30 days of primary acclimatization was 92 %. Three months later, the plantlets were transferred to plastic pots containing the same mixture under greenhouse conditions for secondary acclimatization. About 90 % of the plants subjected to secondary acclimatization survived after 90 days and could be grown in pots kept in a green house (**Fig. 1K**).

Conclusions

Two simple, efficient and cost effective protocols for induction of both direct and indirect somatic embryogenesis of *M. armeniacum* cv. Blue Spike from leaf explants *in vitro* have been presented, in which different morphological stages of somatic embryos were recognized. Through this research, a system of quick production of *M. armeniacum* by embryogenic pathway was achieved. Also, it is the initial experiment for genetic transformation, cryopreservation and many other practical applications of this ornamental species.

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