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Organogenesis and Somatic Embryogenesis from Young Flower Buds of *Agapanthus africanus* Hoffmanns.

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Abstract

Adventitious shoots and roots were directly formed from young flower bud explants of *Agapanthus africanus* on Murashige and Skoog (MS) medium containing 30 g•L^{-1} sucrose, 2 g•L^{-1} gellan gum, and kinetin or thidiazuron at 1 mg•L^{-1} in combination with 1 mg•L^{-1} NAA. Friable calli with a high rate of plant regeneration were also induced by culturing the explants on medium containing NAA, picloram or 2, 4-D at concentrations varying from 1 to 10 mg•L^{-1}, especially at low concentrations of picloram or 2, 4-D. Shoots were readily induced from these calli when transferred onto plant growth regulator-free or 1 mg•L^{-1} zeatin-containing MS medium. Nodular compact calli were also formed with friable calli on medium supplemented with 2, 4-D or picloram. The compact calli induced with 1 mg•L^{-1} picloram developed into somatic embryos. In both shoot and embryo regeneration systems, complete plantlets were obtained when they were transferred onto a medium containing no plant growth regulator.

1. Introduction

The genus *Agapanthus,* a member of family Liliaceae, contains several ornamental species such as *A. africanus, A. inaperthus,* and *A. praecox.* These species have flowers with dark blue-violet to milky-white colors and commonly called 'agapanthus' or 'lily of the Nile'. The agapanthus has recently become popular in the florist trade as a potted plant, for landscaping, and as cut flowers because of its beautiful flowers [1]. In agapanthus, the seedlings with desirable characteristics have been selected and propagated vegetatively by the division of rhizomes as clonal cultivars. However, commercially available plants consist mostly of heterozygous seedlings due to the lack of a highly efficient vegetative propagation method of the cultivars. Therefore, it is expected that an efficient micropropagation method of agapanthus will be established. To date, however, there has been no report on tissue culture of agapanthus.

It is also expected that an efficient system for plant regeneration from tissue cultures will be utilized for improving the important traits of this plant such as flower color through genetic transformation techniques. The present study demonstrates the successful establishment of a plant regeneration system through organogenesis and embryogenesis in *in vitro* culture of young flower bud explants of *Agapanthus africanus.*

2. Materials and Methods

2. 1 Plant material

Young inflorescences were collected from several plants of *Agapanthus africanus* vegetatively propagated from a seedling with pale blue flowers at the stage when the flower heads were still enfolded in the green bract. The pollen in the flower buds was at the early binucleate stage. After washing for a few minutes with running water, the flower heads with adjacent flower stalk were dipped into 70% ethanol for 10 seconds, surface-sterilized with sodium hypochlorite solution (1% active chlorine) with a few drops of Tween 20 for 15 minutes, and washed 3 times with sterile distilled water. After removal of bracts and cutting away most of the flower stalk, clusters of flower buds were separated into portions so that each contained a flower bud with pedicel and a part of flower head tissue using a razor blade.

2. 2 Culture medium and culture condition

Basal medium used throughout this study was 2 g•L^{-1} gellan gum (Kelco, Division of Merck & Co. Inc., SanDiego, CA)-solidified MS [2] medium containing 30 g•L^{-1} sucrose, to which plant growth regulators were added. The media were adjusted to pH 5.7-5.8 prior to autoclaving and 35 m*l* aliquot was plated to each sterile plastic Petri dish (90×20 mm). The cultures were incubated under continuous illumination with day light fluorescent lamps (22 μmol m^{-2}s^{-1}) at 20± 1°C.

2. 3 Induction of direct organogenesis

Flower bud explants, each of which included a young floret, pedicel and a part of flower head tissue, were cultured on the basal media containing various combinations of cytokinins; N^6-benzylaminopurine (BA), 6-furfuryl aminopurine (kinetin), or N-(1, 2, 3-thiadiazol-5-yl)-N'-phenylurea (thidiazuron, TDZ; Wako, Japan) and auxins; (α-naphthaleneacetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2, 4-D) or 4-amino-3, 4, 6-trichloro picolinic acid (picloram)) each at 1 mg•L^{-1} as shown in **Table 1.** The experiment was repeated 3 times with 3 plates for each treatment, each with 5-8 flower bud explants. The cultures in each medium were transferred to the same medium after 30 days of culture. Shoot and root formation and other morphological characteristics were observed 60 days after initiation of the culture. The number of shoots per flower bud was classified into 4 categories; 0: no shoot, 1: 1-2 shoots, 2: 3-5 shoots, and 3: more than 5 shoots per flower bud, and the average was used as the shoot regeneration efficiency.

2. 4 Callus induction

The efficiency of callus induction from flower bud was compared among 3 auxins (NAA, 2, 4-D and picloram), each at 1, 5, and 10 mg•L^{-1}. No cytokinin was added to these media. For each treatment, 2 plates, each containing 6 flower buds were made and the experiment was repeated 3 times. Callus formation was observed after 60 days of culture.

2. 5 Shoot initiation from friable callus

To establish the shoot regeneration system from callus cultures, friable pale yellow calli induced from flower buds were isolated after 2 months of culture and routinely subcultured at 1 month-intervals in medium containing 1 mg•L^{-1} picloram. The calli proliferated well on this medium and became aggregated during the subculture. After 3 subcultures, the calli were cultured for testing the regeneration ability on MS media supplemented with 0, 1, or 2 mg•L^{-1} of cytokinin (BA, kinetin, TDZ, or [9R] Z, 9-β-D-ribofuranosylzeatin (zeatin)) in combination with NAA at 0, 0.5, or 1 mg•L^{-1}. Six plates were made per treatment, each with 4 pieces of 0.5 g calli and the experiment was repeated twice. The culture medium was replaced by fresh one after 30 days of culture and the regeneration of shoots was observed 65 days after culture. Shoot regeneration ability was expressed both as the percentage of calli producing shoots and as the shoot regeneration index. The intensity of shoot regeneration was roughly classified into 4 categories according to the area of shoot formation on the upper surface of the callus 0: no shoot, 1: <10%, 2: 10-50% and 3: >50% of shoot developing area per callus, and the average was expressed as the shoot regeneration index.

2. 6 Induction of embryogenesis

Compact creamy-yellow calli that were occasionally formed together with the friable calli were selected and multiplied on MS medium containing 1 mg•L^{-1} picloram. Embryogenic calli with embryo-like structures produced on these compact calli were further transferred onto plant growth regulator-free medium for plantlet regeneration.

Table 1.
Effects of cytokinins and auxins on direct organogenesis from young flower buds of *Agapanthus africanus*.

Cytokinin (1 mg•L^{-1})	Auxin (1 mg•L^{-1})	Shoot induction (%)	Shoot regeneration Index*2	Root induction (%)
BA	NAA	27.0 b*1	1.7	21.4 bc*1
	2, 4-D	21.7 b	1.2	6.7 d
	Picloram	12.5 b	1.0	8.3 bc
Kinetin	NAA	36.1 ab	1.7	69.4 a
	2, 4-D	21.7 b	1.5	47.5 ab
	Picloram	17.8 b	1.5	41.1 abc
TDZ	NAA	62.0 a	2.6	30.5 bc
	2, 4-D	30.2 ab	1.8	28.6 bc
	Picloram	32.2 ab	1.2	24.4 bc

*1 Different letters within the column show significant difference by Duncan's multiple range test at $p=0.05$ and the data were expressed as the averages of 3 repeated experiments.

*2 Number of shoots per explant was classified into following 4 categories and the average was used as 'shoot regeneration index'; 0: no shoot, 1: 1-2 shoots, 2: 3-5 shoots, 3: more than 5 shoots.

3. Results

3. 1 Direct organogenesis from flower bud explants

In most of the plant growth regulator combinations tested, each at 1 mg•L^{-1}, flower buds showed the ability to induce direct organogenesis (**Table 1** and **Fig. 1A**). The explants initially expanded entirely, and then shoot and root were directly regenerated from the explants. Friable green callus was partially formed in some cultures in which 2, 4-D or picloram was added (data not shown). Both the percentage of flower buds with directly induced shoots (62.0%) and the shoot regeneration efficiency (2.6) were the highest on medium containing TDZ and NAA both at 1 mg•L^{-1}, on which the clustered shoots were predominantly produced (**Fig. 1A**). Among the cytokinins tested, TDZ was more effective for shoot induction than kinetin and BA, whereas NAA was more suitable than 2, 4-D and picloram when used in combination with any kind of cytokinins tested. The shoots induced from flower buds could be multiplied *in vitro* (**Fig. 1B**) and success-

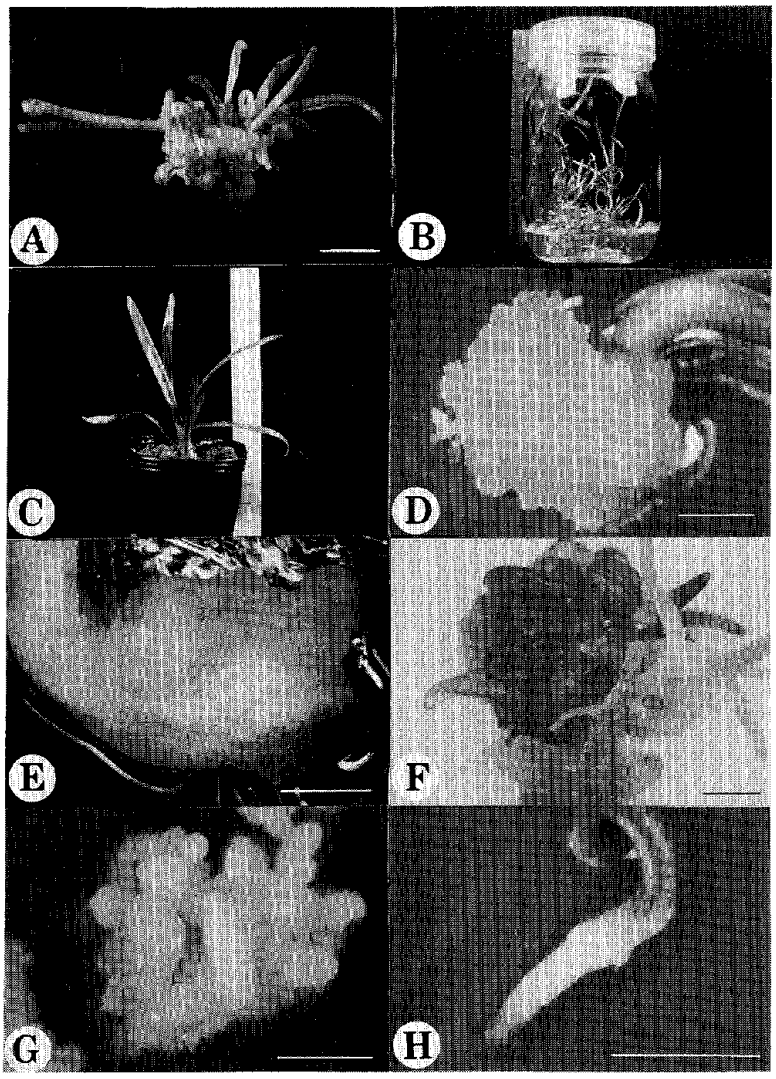

Fig. 1 Plant regeneration from flower bud explants of *Agapanthus africanus*.
(A) Shoot formation from flower head tissue on MS medium containing 1 mg•L^{-1} of both TDZ and NAA, bar=1 cm. (B) *In vitro*-proliferated plantlets after transferring the shoots onto MS medium supplemented with 0.5 mg•L^{-1} TDZ and 0.1 mg•L^{-1} NAA. (C) Flower bud-derived plant 50 days after transfer to soil in a pot. (D) Green friable callus formation from flower head tissue on MS medium containing 1mg•L^{-1} picloram. (E) Nodular compact calli produced on medium containing 1 mg•L^{-1} picloram. (F) Shoot regeneration from friable calli on MS medium supplemented with 1 mg•L^{-1} zeatin produced roots after transfer to MS medium containing 0.1 mg•L^{-1} NAA. (G) Development of somatic embryos from embryogenic calli after 2 months on MS medium containing 1 mg•L^{-1} picloram. (H) Germinating somatic embryos after transfer to basal MS medium; bar in (D), (E), (F), (G), and (H)=0.5 cm.

fully produced roots. The plantlets thus obtained were successfully transplanted to soil (**Fig. 1C**).

The combination of kinetin and NAA showed the highest percentage of root induction (69.4%) which occurred independently from shoot formation. The roots were initially white but rapidly turned green and further elongated. In media containing BA, growth and development of flower buds were inhibited and browning occured especially when 2, 4-D or picloram was applied.

3. 2 Callus induction

Calli were readily induced from flower buds in media containing any kind of auxins (NAA, 2, 4-D and picloram) at all concentrations tested (1-10 mg•L^{-1}). Especially, picloram at 1 mg•L^{-1}, 2, 4-D at 1-5 mg•L^{-1}, NAA at 5 mg•L^{-1} induced calli from more than 80% of the explants (**Fig. 2**). However, high concentrations of picloram and 2, 4-D; especially 2, 4-D at 10 mg•L^{-1} reduced the percentage of callus formation, caused tissue browning and inhibited the expansion of flower buds. Calli were initiated from all parts of the explants, but flower head tissue attaching at the base of pedicel produced more calli than other parts. Most of the calli initially formed were soft and friable and had pale yellow or bright green color (**Fig. 1D**). White and fluffy calli were occasionally found in the cultures, but they grew poorly and finally died. On the other hand, nodular compact calli were partially produced from flower bud on media containing 2, 4-D or picloram (**Fig. 1E** and **Fig. 2**). The optimum concentration for the induction of compact callus was 1 to 5 mg•L^{-1} in both auxins. The compact calli were readily multiplied by subculturing on medium containing 2, 4-D or picloram at 1 mg•L^{-1}.

3. 3 Shoot regeneration

Shoot regeneration ability of flower bud-derived calli is shown in **Table 2**. Shoots were easily regenerated on plant growth regulator-free MS medium in which 75% of the calli regenerated shoots. Mostly, the supplementation of cytokinins and/or NAA to the culture media acted inhibitorily for shoot regeneration especially when a high concentration (2 mg•L^{-1}) of cytokinins was combined with NAA (0.5 or 1 mg•L^{-1}). However, some plant growth regulator combinations such as 1 mg•L^{-1} zeatin and 1 mg•L^{-1} NAA gave high percentages of shoot regeneration (91.7%) and a high shoot regeneration index (2.6). The shoot growth and proliferation on this medium were also better than those on plant growth regulator-free or other cytokinin-added media. These shoots easily produced roots when transferred to medium containing 0.1 mg•L^{-1} NAA (**Fig. 1F**, data not shown) although no roots were formed on the shoot induction media. Occasionally, succulent transparent shoots were produced in cultures. These abnormal shoots showing hyperhydricity were poor in growth and finally turned brown.

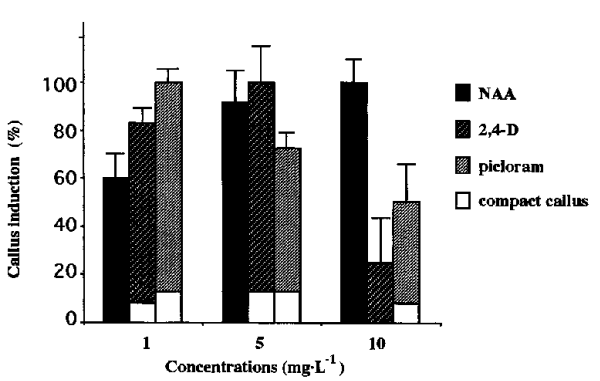

Fig. 2 Effect of auxins on callus induction from young flower buds of *Agapanthus africanus*.
The vertical bars represent standard errors of 3 independent experiments.

3. 4 Somatic embryogenesis from compact callus

Nodular compact calli produced with friable calli from flower buds on media containing either picloram or 2, 4-D at 1 mg•L^{-1} were successfully multiplied by subculturing on the same medium. However, the compact calli induced at 10 mg•L^{-1} 2, 4-D or picloram did not proliferate well when cultured continuously at this concentration level. The calli subcultured on medium containing 1 mg•L^{-1} picloram initiated to produce somatic embryos (**Fig. 1G**) but those on 2, 4-D medium only showed callus proliferation. The somatic embryos further developed into normal plantlets after transfer to the medium with no supplement of plant growth regulators (**Fig. 1H**). They were then successfully transplanted into soil after acclimatization.

4. Discussion

Explants from various parts of inflorescence have been recognized as suitable sources for micropropagation of Liliaceous plant species [3]. Adventitious shoot formation without an intermediate callus phase [4, 5] and callus-mediated somatic embryogenesis from these explants [6] have previously been reported. The present study also showed the usefulness of young flower bud explants for plant regeneration through direct organogenesis, and through shoot formation or embryogenesis indirectly from callus cultures in *Agapanthus africanus*. In some species in the related genera such as *Allium*, flower head regions were efficiently used for induction of organogenesis [7, 8]. In *Agapanthus africanus*., flower head tissue was also the most responsive part to the culture media. These results suggest that flower head tissue probably has the undifferentiated meristematic nature as previously suggested by Novâk and Havel [8]. In

Table 2.
Shoot regeneration from calli derived from young flower buds of *Agapanthus africanus*.

Cytokinin kind	Cytokinin conc.(mg·L^{-1})	NAA (mg·L^{-1})	Shoot (%) regeneration*1	Shoot regeneration index*2	Characteristics*3
None		0.0	75.0	2.0	GS, WS
		0.5	16.7	0.8	C
		1.0	0.0	0.0	C
BA	1	0.0	50.0	1.3	GS, B
		0.5	16.7	0.5	B, C
		1.0	16.7	1.3	WS
	2	0.0	16.7	0.8	B
		0.5	0.0	0.0	B
		1.0	25.0	0.8	GS, WS
Kinetin	1	0.0	83.3	1.8	GS
		0.5	41.7	1.4	GS, WS
		1.0	50.0	1.7	WS, B
	2	0.0	33.3	1.5	WS
		0.5	33.3	1.3	WS, B
		1.0	8.3	2.0	WS, B
TDZ	1	0.0	58.3	2.3	GS
		0.5	41.7	2.4	GS
		1.0	25.0	1.3	GS, B
	2	0.0	41.7	1.4	GS
		0.5	25.0	1.7	WS
		1.0	8.3	1.0	B
Zeatin	1	0.0	83.3	2.6	GS
		0.5	58.3	2.6	GS
		1.0	91.7	2.6	GS
	2	0.0	50.0	1.8	GS
		0.5	25.0	2.3	GS, B
		1.0	50.0	1.6	GS, WS

*1 In each treatment 24 pieces of calli (0.5 g each) were inoculated and the experiments were repeated twice. Data were expressed as averages.

*2 Intensity of shoot regeneration was classified into 4 categories based on the areas of shoot formation on the upper surface of the callus: 0: no shoot, 1: <10%, 2: 11-49% and 3: more than 50% of shoot developing area per callus, and the average was expressed as "shoot regeneration index".

*3 Characteristics of regenerated shoots and other responses of explants; GS: green shoots, WS: white or transparent succulent shoots, B: browning, C: callus.

monocotyledonous plants including Liliaceous plants, 2, 4-D was predominantly used for the induction of embryogenic callus [3, 9]. In *Agapanthus africanus*, however, somatic embryogenesis was not induced by 2, 4-D but by picloram. The effectiveness of picloram for inducing somatic embryogenesis has also been reported in some other monocotyledonous plants [10, 11] as well as some dicots such as pea [12] and chickpea [13].

Because of the high ability in regeneration and multiplication, the plant regeneration systems of agapanthus established in the present study will be efficiently used for rapid clonal propagation if the micropropagated plants have no aberrant somaclonal variations. Moreover, callus cultures with high regeneration ability may also be used for breeding of this crop through, for example, selection of useful somaclonal variations, polyploid production, somatic hybridization and genetic transformation. In some Liliaceous plant species such as *Asparagus officinalis* [14, 15], *Allium ampeloprasum* [16] and *Lilium* × *formolongi* [17, 18]. embryogenic cells or nodular cell clumps with high shoot regeneration ability have been utilized as the source of protoplasts which would regenerate into fertile plants. Establishment of a protoplast culture system in *Agapanthus* is now in progress by utilizing the embryogenic calli produced in the present study.

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