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In vitro Propagation of Bulblets and Elimination of Viruses by Bulb-scale Cultures of *Hippeastrum hybridum* Bulbs

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The effect of *in vitro* culture conditions on bulblet regeneration of bulb-scale explants excised from *Hippeastrum* bulbs and the possibility of virus elimination by this method were investigated. Explants containing the extreme base of bulb-scale produced bulblets at a high rate. Bulblet regeneration was stimulated on White's (1943) agar medium supplemented with 0.01 mg/l α-naphthaleneacetic acid (NAA) and 5.0 mg/l 6-benzylaminopurine (BA). In these cases, a high regeneration rate was found for explants with 2-mm cuts treatment on their proximal ends. Bulblet multiplication was stimulated by subculturing the bulblets formed on the bulb-scale base explants in liquid White's medium with shake-culture. Especially, in the subculture, the bulblets with notching treatment on their bases produced the largest number of new bulblets. Based on these results, a mass propagation scheme for *Hippeastrum* using a shake culture has been established.

In bulblets obtained from the bulb-scale base explant culture, almost all the bulblets were cucumber mosaic virus (CMV)-free by dot immunobinding assay (DIBA), and in 33% of the bulblets hippeastrum mosaic virus (HiMV) was detected by the direct negative staining method. However, all of the new bulblets obtained from the subculture of the bulblets were free of both viruses. These results indicate that the method developed in this study can be utilized as a novel method for the *in vitro* propagation of virus-free plants.

Introduction

Hippeastrum is an important horticultural ornamental in the Amaryllidaceae. Since the propagation rate of *Hippeastrum* through natural offsets is relatively low, scale-stem cutting[1,2] and twin-scaling[3,4] have been used for bulb propagation. Tissue culture has been investigated as an efficient tool for the rapid clonal propagation of *Hippeastrum*[5-12].

In Japan, cucumber mosaic virus (CMV) and hippeastrum mosaic virus (HiMV) are known to infect *Hippeastrum* plants. The infected plants reveal symptoms of mosaic on their leaves and flowers. Attempts at obtaining virus-free plants of *Hippeastrum* by subculture of mound-like tissue produced on twin-scale pieces[10] or meristem culture[12] have been reported. These experiments suggest the possibility of tissue culture propagation of *Hippeastrum* virus-free stocks.

In this paper, the effect of *in vitro* culture conditions on rapid clonal propagation using bulb-scale cultures and the possibility of obtaining virus-free plants using the bulb-scale culture methods are described.

Materials and Methods

Bulbs were taken from *Hippeastrum hybridum* (cultivar 'Dairin-akabana') plants grown in the

experimental farm at Kyoto University of Education. The bulbs were washed thoroughly with tap water and their apical parts, most of outer basal plates and 2 or 3 of their outer bulb-scales were removed. The remainder of the bulbs were surface-sterilized in 70% ethyl alcohol for 10 sec., followed by 10% calcium hypochlorite solution for 15 min., and finally rinsed twice in sterilized deionized water for 5 min. Sterilized bulbs were transferred to sterilized petri dishes and divided vertically into 8 parts. Each bulb segment was then dissected into single bulb-scale segments each with basal plate tissue. Square explants (5-mm × 5-mm) were excised from different parts of the dissected bulb-scale segments. In some experiments, the explants with 2-mm cuts treatment on their proximal ends were prepared.

The explants were vertically inoculated with their distal ends upwards into 18 × 140 mm test tubes containing 10 ml White's[13] agar medium containing 20 g/l sucrose and 8 g/l agar (primary culture). In some experiments, the medium was supplemented with 0.01 mg/l α-naphthaleneacetic acid (NAA) and 5.0 mg/l 6-benzylaminopurine (BA). Test tubes were capped with aluminum foil. The media were adjusted to pH 5.8 with 1 N HCl or 1 N KOH prior to autoclaving (15 min., 121°C, 1.2 kg/cm^2). The cultures were kept at 25 ± 1°C in a culture room under continuous fluorescent illumination of 2,000 lux provided by Homolux plant growth fluorescent tubes (manufactured by National Electric Co., Ltd.).

Bulblets formed on the explants were transferred for subculture into 50 ml Erlenmeyer flasks containing 10 ml liquid White's medium supplemented with NAA and BA. The cultures were shaken at 70 r.p.m. on a reciprocating shaker and incubated under the same culture conditions as used for the initial bulb-scale culture.

CMV was detected by dot immunobinding assay (DIBA)[14] in *Hippeastrum* plants grown in the experimental farm and bulblets obtained from both bulb-scale explants and subcultured bulblets. Particles of HiMV in these plants and bulblets were detected using the direct negative staining method under the electron microscope[15]. In these detections, extracts were made by grinding 5-mm squares of leaves excised from regenerated bulblets. Five-mm squares of bulb-scales and basal plates excised from different parts of the infected bulbs were ground in carbonate buffer and CMV was detected by enzyme-linked immunosorbent assay (ELISA)[16].

Results

Bulblet regeneration of square explants excised from different parts of *Hippeastrum* bulbs was initially investigated on White's agar medium without NAA and BA. Explants containing both bulb-scale base and basal plate tissues produced bulblets at a high rate. Explants of the extreme base of bulb-scale without the basal plate also produced bulblets at the same rate. However, the bulb-scale explants whose proximal end had been located 3 mm above the junction between bulb-scale and basal plate produced bulblets at the rate of 43% and no regeneration was found in those explants excised from 10 mm or more above the junction.

The effect of the combination of BA and NAA on bulblet regeneration was investigated on White's agar medium using explants excised from various parts of the bulb-scales. The addition of 0.01 mg/l NAA and 5.0 mg/l BA slightly increased the regeneration rate on the bulb-scale explants excised from 3-10 mm above the junction (**Table 1**). In a comparison of the position of bulb-scales in bulbs, a higher regeneration rate was found for the outer bulb-scale base explants without the basal plate than for the inner ones (**Table 2**). Number of regenerated bulblets in the bulb-scale base explants increased with the increase of the number of the 2-mm cuts treatment on their proximal ends and 3 cuts-treatment gave the highest number of bulblets (2.8-3.4) (**Table 3**). When

bulblets formed on the bulb-scale base explants were transferred to liquid White's medium for shake culture, new bulblet regeneration was stimulated, especially, in the bulblets with notching treatment on their bases (**Table 4**).

Virus infection rates in tested *Hippeastrum* plants in the experimental farm were 63% and 97% for CMV and HiMV, respectively. The concentration of CMV appeared to be higher in the bulb-scale parts located 8 mm or more above the junction of the basal plate in the inner part than in the extreme basal parts (0 mm) of the bulb-scale in the outer part of infected *Hippeastrum* bulbs (**Table 5**).

Table 1. Effects of plant growth regulators on bulblet regeneration of explants excised from various parts of bulb-scales.

Medium*[1]	Percentage of bulblet regeneration (Number of bulblets regenerated/explant)				
	Explant of bulb-scale base		Explant*[2] of bulb-scale		
	with basal plate	without basal plate	3 mm	10 mm	20 mm
Cont.	92 (1. 1)	93 (1. 1)	43 (1. 1)	0 (—)	0 (—)
NAA+BA	93 (1. 4)	92 (1. 1)	60 (1. 1)	5 (1. 0)	0 (—)

In each treatment, 22 to 29 explants were cultured on medium at 25°C under continuous fluorescent illumination of 2000 lux for 12 weeks.

*[1] Cont.: White's agar medium, NAA+BA: White's agar medium supplemented with 0. 01 mg/l NAA and 5. 0 mg/l BA.

*[2] Bulb-scale explants whose proximal ends had been located 3 mm, 10 mm and 20 mm above the junction between bulb-scale and basal plate were excised.

Table 2. Regenerative response of the bulb-scale base explants excised from various bulb-scales differed in their positions on a bulb.

Origin of explant	Position of bulb-scales	Percentage of bulblet regeneration
With basal plate	Outer	97
	Middle	98
	Inner	96
Without*[1] basal plate	Outer	98
	Middle	70
	Inner	42

In each treatment, 28 to 40 explants were cultured on White's agar medium for 12 weeks. Cultural conditions are the same as those described in **Table 1**.

*[1] Bulb-scale base explants whose proximal ends had bordered on the basal plate were excised without basal plate.

Table 3. Effects of cutting treatments on the proximal ends of bulb-scale base explants on bulblet regeneration.

Number of cut	Percentage of Bulblet regeneration (Number of bulblets regenerated/explant)	
	Explant of bulb-scale base with basal plate	Explant of bulb-scale base without basal plate
0	100 (1. 4)	94 (1. 2)
1	93 (1. 5)	89 (1. 5)
2	100 (2. 0)	89 (1. 9)
3	93 (2. 8)	94 (3. 4)

In each treatment, 22 to 31 explants were incubated on White's agar medium supplemented with 0. 01 mg/l NAA and 5. 0 mg/l BA for 12 weeks. Cultural conditions are the same as those described in **Table 1**.

Table 4. Effects of cutting and notching treatments to the bulblets obtained from the bulb-scale explants on new bulblet regeneration.

Treatment	Number of new bulblets regenerated/bulblet explant
Cont. (No treatment)	0
Longitudinal cutting treatment	2.0
Notching treatment on the base	3.0

In each treatment, 20 bulblets were shake-cultured using a reciprocating shaker in White's liquid medium supplemented with 0.01 mg/l NAA and 5.0 mg/l BA for 12 weeks. Cultural conditions are the same as those described in **Table 1**.

Table 5. Concentration of cucumber mosaic virus (CMV) in different parts of infected bulbs.

Organ	Position in bulb		
	Inner	Middle	Outer
Bulb-scale			
30 mm*1	0.421*2	0.302	0.188
15	0.472	0.480	0.230
8	0.421	0.477	0.211
0	0.410	0.263	0.070
Basal plate	0.023	0.015	0.013

Five infected bulbs were used in each detection.

*1 Distance from the junction between bulb-scale and basal plate.

*2 Values obtained by indirect ELISA test (410 nm). The high value shows high concentration of CMV. Values of foliage leaves in the infected plants by indirect ELISA test were 0.616.

Table 6. Elimination of cucumber mosaic virus (CMV) and hippeastrum mosaic virus (HiMV) in bulblets obtained from bulb-scale base explants in primary culture and from bulblets in subculture.

Virus*1	Bulblet from primary culture*2				Bulblet from subculture*3
	<3 mm*4	3-5 mm	5 mm<	Total	
CMV	21/21*5 (100)*6	16/16 (100)	4/5 (80)	41/42 (98)	44/44 (100)
HiMV	4/13 (31)	2/6 (33)	2/5 (40)	8/24 (33)	8/8 (100)

*1 CMV and HiMV were detected by DIBA and the direct negative staining method, respectively.

*2 Bulblets formed on the bulb-scale base explants in primary culture.

*3 New bulblets formed on bulblet explants in first subculture.

*4 Diameter of tested bulblets.

*5 Number of virus free bulblets/Number of tested bulblets.

*6 Percentage of virus free bulblets.

After the primary culture of bulb-scale base explants, CMV was eliminated from 98% of the regenerated bulblets regardless of the size of the tested bulblets. The percentage of HiMV elimination in bulblets obtained from the primary culture was 33%. However, HiMV was completely eliminated in the bulblets obtained from subculturing bulblets formed in the primary culture but infected with the virus. In the case of bulblets infected with CMV, the percentages of virus-free bulblets were 98% and 100% for bulblets from the primary culture and those from the subculture, respectively (**Table 6**).

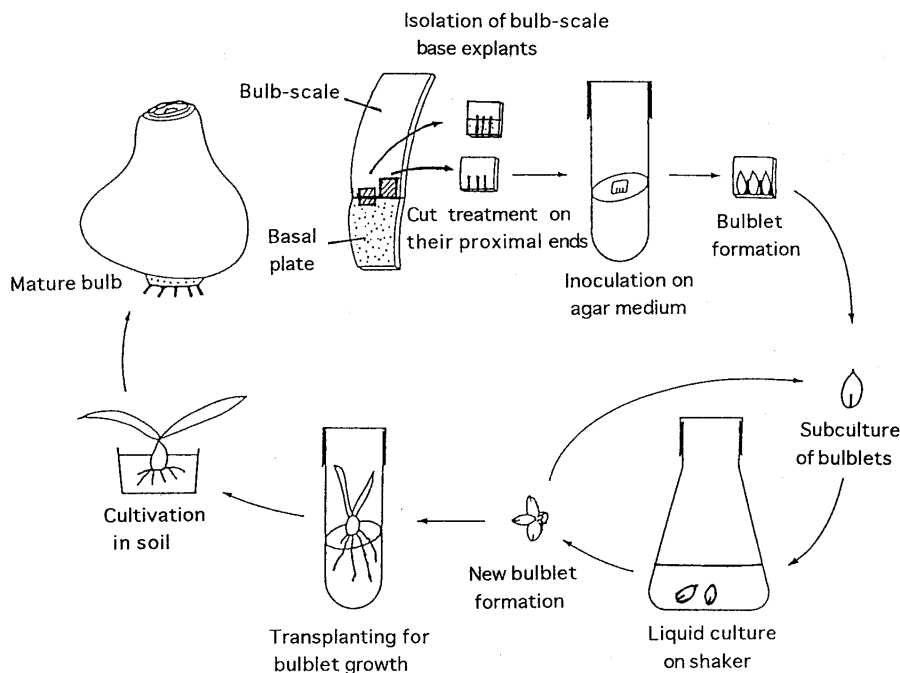

Fig. 1 Schematic diagram of the steps for rapid propagation of *Hippeastrum* by tissue culture.

Discussion

Bulblet regeneration was confined to occur on the explants from the basal portion of the bulb-scale near the basal plate in almost all of the genera belonging to the Amaryllidaceae, including *Crinum, Cyrtanthus, Habranthus, Hippeastrum, Hymenocallis, Lycoris, Sprekelia* and *Sternbergia*[8,17,18], *Nerine*[19], *Eucharis*[20] and *Amarcrinum*[21]. In the present study bulblet regeneration from the bulb-scale base explants of *Hippeastrum* was stimulated by cut treatment on the explants. Previous reports also revealed that cut treatments to segments for scale-stem cuttings of tunicated bulbs[4] and to scales for scale propagation of lilies[22] stimulated bulblet regeneration.

The results of the present study also indicate that numerous new bulblets can be obtained by repeating the liquid shake culture of the bulblets with notching treatment. The bulblets produced by shake culture were easily harvested from the culture vessel and transplanted into soil.

In CMV-infected bulbs, uneven distribution of CMV was found in bulb-scales or bulbs. Similar results were found with CMV and lily symptomless virus (LSV) in bulbs of *Lilium leichtlinii* var *maximowiczii*[23]. In *Hippeastrum* bulbs, the concentration of CMV in bulb-scale base explants, which showed a higher regenerative rate, appeared to be lower than that in parts in bulbs. Bulblets produced by the bulb-scale base explants were easily subcultured to the liquid medium. These results suggest that a low concentration of virus in the bulb-scale base explants may result in the production of virus-free bulblets.

The propagation scheme (**Fig. 1**) of virus-free bulblets established in this study is much easier for regenerating bulblets than shoot tip culture. The possibility for regenerating virus-free bulblets in *Lilium* and other tunicated bulbs will be described in a subsequent publication.

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《和文要約》

アマリリス球根のりん葉切片からの *in vitro* 増殖とウイルスの無毒化

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アマリリス(*Hippeastrum hybridum*)大輪赤花系の球根のりん葉基部切片を用いて，*in vitro* での子球の増殖条件を検討するとともに，得られた子球のウイルス保毒検定を行った．りん葉基部の切片に切れ目を入れてNAAとBAを添加した寒天培地で培養し，得られた子球に十字の切れ目を入れてさらに液体培地で振とう培養する方法により *in vitro* で効率的に子球を獲得できることがわかった．得られた子球のウイルス検定の結果，りん葉基部の切片に形成された子球では，キュウリモザイクウイルス(CMV)は98%除去されたが，アマリリスモザイクウイルス(HiMV)の除去率は33%であった．この子球をさらに再培養して得られた新子球のウイルス検定では，CMV，HiMVともに100%のウイルス除去率が得られ，これらの方法による子球の増殖とウイルス無毒株獲得の可能性が示された．