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https://doi.org/10.5109/4617

出版情報：九州大学大学院農学研究院紀要. 50 (1), pp.19-25, 2005-02-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **50** (1), 19–25 (2005)

In Vitro Morphogenesis from Ovaries of *Hippeastrum* x *hybridum*

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(Received October 18, 2004 and accepted November 11, 2004)

In vitro morphogenensis from ovary-slices of *Hippeastrum* x *hybridum* cv. Hermitage was examined. Bulblet formation rates were high with 1 mgl^{-1} and higher concentrations of naphthyl acetic acid (NAA) together with high (1, 2 or 5 mgl^{-1}) concentrations of N^6-benzyladenine (BA), and the highest number of bulblets was 5.3 with 5 mgl^{-1} NAA+5 mgl^{-1} BA. Somatic embryogenesis was histologically confirmed. High frequency of embryogenesis was obtained with higher concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1–5 mgl^{-1})+ thidiazuron (TDZ) (0.02–2 mgl^{-1}), which lead the high rates and number of bulblet formation.

INTRODUCTION

Natural asexual propagation rate of *Hippeastrum* x *hybridum* through offsets is low. Twin scaling is the most advanced technique on the propagation of *Hippeastrum*, *Narcissus* and other Amaryllidaceae. Since Mii *et al.* (1974) first achieved the *in vitro* organogenesis in *Hippeastrum* x *hybridum* from scale explants, various organs have been used for *in vitro* propagation of this species to speed up vegetative propagation. They were scale (Seabrook and Cumming, 1977; Yanagawa and Sakanishi 1977, 1980; Huang *et al.*, 1990; Blakesley and Constantine, 1992), twin scales (Hussey 1975a, 1976a; Huang *et al.*, 1990), flower stem (Hussey 1975a, 1976a; Seabrook and Cumming, 1977), leaf (Hussey, 1976a; Seabrook and Cumming, 1977), ovary (Seabrook and Cumming, 1977) and peduncle (Seabrook and Cumming, 1977).

Ovaries have been often used for micropropagation successfully in many other bulbous species such as *Hyacinthus* (Hussey, 1975a, b), *Lilium* (Novak and Petru, 1981), *Muscari* (Hussey, 1975a), *Narcissus* (Hussey, 1975a), *Ornithogalum* (Hussey, 1975a, 1976b) and *Scilla* (Hussey, 1975a).

Not so many reports are available on somatic embryogenesis in bulbs (Chakravarty and Sen 1987; Gude and Dijkema, 1997; Sage *et al.*, 2000), although it has been pointed out that somatic embryogenesis has the potential as the most effective means of micropropagation of plants (Tisserat *et al.*, 1979). No plantlets were obtained through somatic embryogenesis in *Hippeastrum*.

In this study, we examined the *in vitro* morphogenensis from ovary-slices of *Hippeastrum* x *hybridum* to search for more efficient micropropagation methods in this species.

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MATERIALS AND METHODS

Plant materials

Bulbs of *Hippeastrum* x *hybridum* cv. Hermitage grown in the fields at National Chiayi University, Chiayi, Taiwan were used. The inflorescences of 15–20 cm in length were prepared during 6 and 13 April 2004 and surface sterilized with 70% ethanol for 30 seconds. Ovaries were aseptically taken out and sterilized with 1.5% NaOCl for 15 minutes. After rinsing them with sterile distilled water three times, explants were prepared by cutting them in round slices with 0.8–1.0 mm thickness.

Culture medium

The explants were placed on MS medium (Murashige and Skoog, 1962) with 30 gl^{-1} sucrose and 2.7 gl^{-1} Gelrite but modified by reducing the concentrations of ammonium nitrate and potassium nitrate to 1/4 and 1/2, respectively from their original concentrations of MS medium. The pH was adjusted to 5.5–5.7 before adding the Gelrite, and the medium was autoclaved at 121 °C for 15 minutes.

Experiments

Effects of naphthyl acetic acid (NAA) and N^6–benzyladenine (BA) and effects of 2,4–dichlorophenoxyacetic acid (2,4–D) and thidiazuron (TDZ) with various combinations of concentrations on morphogenesis and growth were examined.

All cultures were incubated at 25±2 °C in darkness for the first one month and then in 16 h daylength (28–36 $\mu molsec^{-1} m^{-2}$). Each treatment consisted of 15 explants in five glass tubes (30 mm in diameter × 150 mm in height) filled with 10 ml of the medium. Histological observations of the explants took place every two weeks.

RESULTS AND DISCUSSION

Effects of NAA and BA

Protuberances were produced in all the treatments, among which the rates were higher with 1 mgl^{-1} and higher concentrations of NAA irrespective of the concentrations of BA (Table 1). Rates of bulblet formation were high with 1 mgl^{-1} and higher concentrations of NAA+1, 2 or 5 mgl^{-1} BA. No bulblets were obtained with lower (0.1 or 0 mgl^{-1}) NAA concentration except when 5 mgl^{-1} BA was added. The highest rates of root formation were observed with 5 mgl^{-1} NAA with or without the addition of BA. Initiation of protuberances, shoot formation, root initiation and growth are shown in Fig. 1.

Number of bulblets was greatest (5.3) with 5 mgl^{-1} NAA+5 mgl^{-1} BA followed by 4.9 in the second position with 5 mgl^{-1} NAA+1 mgl^{-1} BA or 5 mgl^{-1} NAA+2 mgl^{-1} BA, but it was not completely reflected the number of protuberances initiated (Table 2). The treatments that resulted in large number of bulblets also brought larger number of leaves and longer leaves. Larger number of roots was obtained with higher NAA concentrations.

The yield of bulblets obtained from ovaries in this study is higher than those of bulblets from twin scales or those of protocorm–like bodies from single scales when compared with our previous results (Huang *et al.*, 1990). Ovaries are considered to be superior explant sources in *Hippeastrum* to scales. Seabrook and Cumming (1977)

Table 1. Effect of NAA and BA on morphogenesis from ovaries of *Hippeastrum* x *hybridum* cv. Hermitage.

Treatment (mgl^{-1})		Protuberance	Bulblet	Root	Browning
NAA	BA	formation (%)	formation (%)	formation (%)	(%)
0	0	60	0	0	47
0	0.1	60	0	0	33
0	1	60	0	0	27
0	2	67	0	0	40
0	5	87	20	0	13
0.1	0	40	0	0	60
0.1	0.1	73	0	0	27
0.1	1	87	0	0	13
0.1	2	73	0	0	27
0.1	5	67	0	0	13
1	0	80	47	20	13
1	0.1	87	40	20	7
1	1	67	100	0	0
1	2	100	93	40	0
1	5	100	100	7	0
2	0	100	7	27	0
2	0.1	93	13	60	0
2	1	80	53	27	0
2	2	100	100	46	0
2	5	100	100	0	0
5	0	100	47	100	0
5	0.1	83	40	60	0
5	1	100	100	46	0
5	2	100	93	100	0
5	5	100	100	67	0

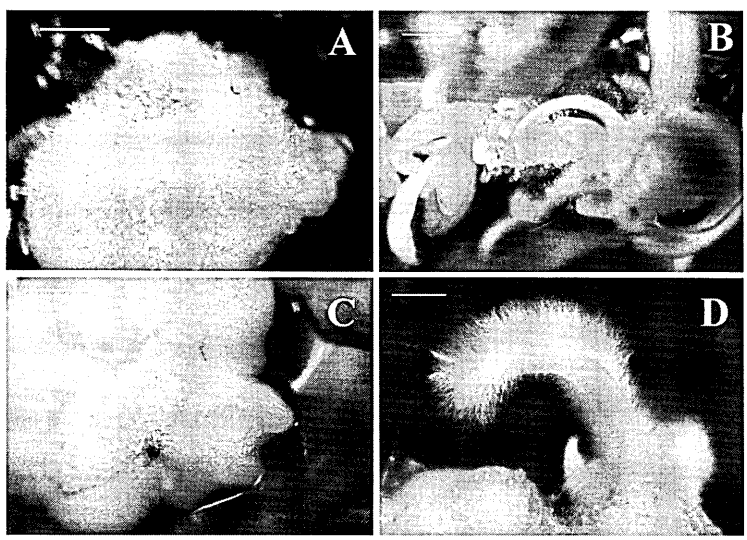

Fig. 1. Morphogenesis from ovary explant of *Hippeastrum* x *hybridum* cv. Hermitage with NAA and BA. A; protuberances, B; shoots, C; root primordia, D; roots. Bars=3 mm.

Table 2. Effect of NAA and BA on morphogenesis from ovaries of *Hippeastrum* x *hybridum* cv. Hermitage.

Treatment (mgl^{-1})		No. of protuberances/ explant	No. of bulblets/ explant	No. of leaves/ explant	Length of leaves (mm)	No. of roots/ explant
NAA	BA					
0	0	1.9 fgh*	0	0	–	0
0	0.1	1.9 fgh	0	0	–	0
0	1	1.8 gh	0	0	–	0
0	2	3.5 defg	0	0	–	0
0	5	4.1 cde	0.3 g	0	–	0
0.1	0	1.6 h	0	0	–	0
0.1	0.1	2.9 efgh	0	0	–	0
0.1	1	1.8 fgh	0	0	–	0
0.1	2	2.9 efgh	0	0	–	0
0.1	5	2.5 efgh	0	0	–	0
1	0	4.1 cde	0.9 fg	0.7 cde	0.1 d	0.6 ef
1	0.1	3.2 efgh	1.5 efg	0.8 cde	0.1 d	1.1 def
1	1	6.9 ab	2.7 cde	1.8 bcd	0.2 cd	0
1	2	5.1 bcd	2.2 def	1.9 bc	0.6 b	2.8 bc
1	5	6.8 ab	3.8 abc	2.8 ab	0.3 c	0.1 f
2	0	2.8 efgh	0.1 g	0.1 e	0.1 d	2.5 bcd
2	0.1	1.9 fgh	0.1 g	0.1 e	0.1 d	2.9 bc
2	1	3.2 efgh	1.3 efg	0.6 de	0.1 d	1.3 cdef
2	2	5.8 abc	3.6 bcd	2.7 b	1.2 a	1.9 cde
2	5	3.9 de	3.9 abc	2.7 ab	0.7 b	0
5	0	6.0 ab	1.2 efg	0.5 de	0.1 d	3.7 b
5	0.1	3.7 def	0.6 g	0.3 e	0.1 de	2.1 bcde
5	1	7.5 a	4.9 ab	3.0 ab	0.2 cd	1.1 def
5	2	2.5 efgh	4.9 ab	0.5 e	0.1 d	5.7 a
5	5	6.1 ab	5.3 a	3.9 a	0.7 b	1.8 cde

* Values followed by different letters are significantly different at 5% level.

reported the shoot formation from ovary tissues of *Hippeastrum* at 4.0 mgl^{-1} BA+2.0 (or 2.4; 2.0 in the text, but 2.4 in Table) mgl^{-1} NAA or 4.0 mgl^{-1} BA+4.0 mgl^{-1} NAA, similarly to our results. In their results, callus formation was also observed from the ovaries with 4.0 mgl^{-1} BA+2.0 mgl^{-1} NAA, whereas there was not in the current experiment.

Effects of 2,4–D and TDZ

Embryogenesis occurred with high rates with higher concentrations of 2,4–D (1–5 mgl^{-1}) and TDZ (0.02–2 mgl^{-1}) although shoot primordium formation was observed in any treatment (Tables 3 and 4). Accordingly the formation rates and number of bulblets were also higher with higher concentrations of 2,4–D and TDZ. However, higher concentrations of TDZ or lower concentrations of 2,4–D inhibited root formation and growth.

The developmental process from somatic embryo initiation to bulblets through globular and cotyledonous embryos is shown in Fig. 2. Somatic embryogenesis was confirmed by the histological observation. Vascular bundle connection was observed between the explant and newly formed organs in adventitious shoot initiation (Fig. 3A),

Table 3. Effect of 2,4–D and TDZ on morphogenesis from ovaries of *Hippeastrum* x *hybridum* cv. Hermitage.

Treatment (mgl^{-1})		Shoot primordium formation (%)	Embryogenesis (%)	Bulblet formation (%)	Root formation (%)	Browning (%)
2,4–D	TDZ					
0	0	53	0	33	0	33
0	0.002	80	0	27	0	13
0	0.02	53	0	0	0	33
0	0.2	87	80	93	0	13
0	2	93	27	60	0	7
0.1	0	80	0	20	0	20
0.1	0.002	87	0	0	0	13
0.1	0.02	93	0	0	0	7
0.1	0.2	93	0	0	0	7
0.1	2	100	53	87	0	0
1	0	93	53	7	40	7
1	0.002	100	67	33	40	0
1	0.02	100	60	87	33	0
1	0.2	100	80	80	0	0
1	2	93	100	100	0	0
2	0	93	0	27	20	7
2	0.002	87	27	33	13	0
2	0.02	87	33	20	40	0
2	0.2	87	100	100	0	0
2	2	100	73	93	0	0
5	0	93	67	93	13	7
5	0.002	93	0	33	0	13
5	0.02	100	73	27	0	0
5	0.2	100	100	53	27	0
5	2	100	33	60	7	0

but not in somatic embryogenesis (Fig. 3B).

The possibility of plantlet regeneration through somatic embryos from ovaries of *Hippeastrum* was shown in this study, although the bulblet yields were not so higher from somatic embryos than from adventitious bulblet formation. Improvement of the methods for embryogenesis is required.

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Table 4. Effect of 2,4–D and TDZ on morphogenesis from ovaries of *Hippeastrum* x *hybridum* cv. Hermitage.

Treatment (mgl^{-1})		No. of shoot primordia/ explant	No. of somatic embryos/ explant	No. of bulblets/ explant	No. of leaves/ explant	No. of roots/ explant
2,4–D	TDZ					
0	0	2.2 j*	0	0.5 defg	0	0
0	0.002	3.2 hij	0	0.1 g	0	0
0	0.02	2.5 j	0	0	0	0
0	0.2	4.4 efghij	4.4 abc	3.9 ab	0.9 ab	0
0	2	3.3 hij	0.3 f	1.0 cdefg	0	0
0.1	0	2.7 ij	0	0.3 fg	0	0
0.1	0.002	2.9 ij	0	0	0	0
0.1	0.02	4.2 fghij	0	0	0	0
0.1	0.2	3.1 ij	0	0	0	0
0.1	2	4.7 efghi	1.9 def	1.3 cdefg	0	0
1	0	3.0 ij	1.0 ef	0.1 g	0	1.7 ab
1	0.002	3.6 ghij	2.4 cde	0.6 defg	0	2.6 a
1	0.02	6.2 cde	1.3 def	2.1 bcdef	0	1.1 bc
1	0.2	7.7 bcd	3.1 bcd	1.6 cdefg	0	0
1	2	5.5 efg	5.3 a	2.4 abcd	0	0
2	0	3.3 hij	0	0.3 fg	0	0.6 cde
2	0.002	4.0 fghij	0.4 ef	0.5 defg	0	0.3 cde
2	0.02	5.9 def	0.5 ef	0.4 efg	0	0.9 bcde
2	0.2	8.5 ab	4.9 ab	4.1 a	0.8 ab	0
2	2	8.7 ab	1.5 def	2.8 abc	0.9 ab	0
5	0	5.1 efgh	1.9 def	2.3 abcd	0	0.2 cde
5	0.002	2.6 j	0	0.5 defg	0	0
5	0.02	3.8 ghij	2.0 def	0.1 g	1.3 a	0
5	0.2	10.2 a	4.1 abc	1.1 cdefg	0.3 bc	0.9 bcd
5	2	8.0 bc	0.5 ef	1.3 cdefg	0.8 ab	0.1 de

* Values followed by different letters are significantly different at 5% level.

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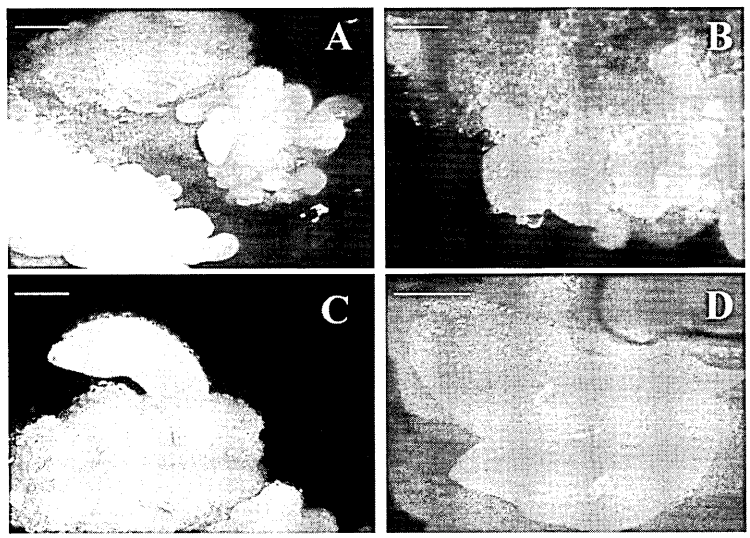

Fig. 2. Bulblet formation from somatic embryos from ovary explant of *Hippeastrum* x *hybridum* cv. Hermitage with 2,4–D and TDZ. A; somatic embryos, B; globular shaped embryos, C; cotyledonous embryos, D; bulblet formation. Bars = 1 mm.

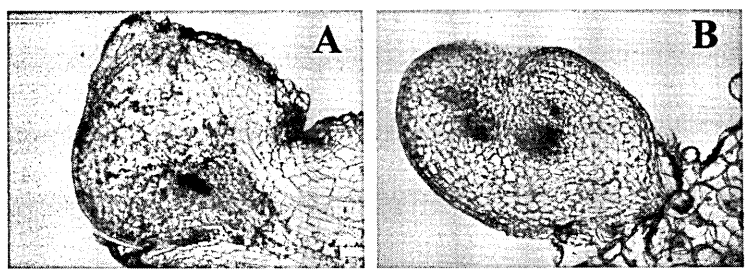

Fig. 3. Histological observation of adventitious shoot differentiation (A) and somatic embryogenesis (B) from ovary explants of *Hippeastrum* x *hybridum* cv. Hermitage. Vascular bundle connection was seen in A, whereas it was not in B. Bars = 300 μm.