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https://doi.org/10.5109/4616

出版情報：九州大学大学院農学研究院紀要. 50 (1), pp.11-18, 2005-02-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

KYUSHU UNIVERSITY

J. Fac. Agr., Kyushu Univ., **50** (1), 11–18 (2005)

In Vitro Morphogenesis from Rhizomes of *Cymbidium sinense*

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(*Received September 10, 2004 and accepted November 11, 2004*)

Effects of N^6–benzyladenine (BA) and naphthyl acetic acid (NAA) and of nitrogen salt concentration in MS medium on *in vitro* morphogenesis from the rhizomes of *Cymbidium sinense* cv. Shue Pai Tsoa were investigated. Shoot formation was promoted with higher concentrations of BA irrespective of the presence of NAA. The plantlets with large number of leaves (>5) were obtained with higher concentrations of both BA and NAA. Reduction of ammonium and potassium nitrate concentrations to 1/4 or 1/2 from the original concentrations enhanced shoot formation. The responsiveness to these treatments was suggested to be common in a wide range of terrestrial *Cymbidium* species.

INTRODUCTION

In vitro shoot formation from the rhizomes of terrestrial *Cymbidium* species with various concentrations and combinations of cytokinins and auxins in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) has been demonstrated in *C. goeringii* (Ueda and Torikata, 1969; Hasegawa, 1987), *C. faberi* (Hasegawa *et al.*, 1985), *C. kanran* (Shimasaki and Uemoto, 1990), *C. forrestii* (Paek and Yeung, 1991) and *C. ensifolium* (Ogura and Okubo, 2003). Reduction of ammonium and potassium nitrate concentrations in MS medium from the original concentrations without an addition of any exogenous plant hormone was proved to be effective in promotion of shoot formation in *C. kanran* (Shimasaki and Uemoto, 1990; Ogura and Okubo, 2003) and *C. ensifolium* (Ogura and Okubo, 2003). Common effects of ethylene inhibitors to these species have also been reported. Shoot formation rate and number of shoots were increased from the rhizomes by silver thiosulfate (STS) and aminoethoxyvinyl glycine (AVG) in *C. kanran* (Shimasaki, 1992) and by $AgNO_3$ in *C. ensifolium* (Ogura, 2003). The results suggest that the responsiveness is universal among terrestrial *Cymbidium* species.

Cymbidium sinese, another terrestrial *Cymbidium* species that grows in China, Hong Kong, Taiwan, northeast India, Myanmar, northern Thailand and Amami and Yakushima Islands, Japan, showed the similar response to N^6–benzyladenine (BA) and naphthyl acetic acid (NAA) in shoot formation from the rhizomes to other terrestrial *Cymbidium* species as mentioned above, but it did not to the reduction of nitrogen salt concentrations (Ogura, 2003).

In this study, effects of cytokinin and auxin and of nitrogen salt concentration in MS medium on morphogenesis from the rhizomes of *C. sinense* were investigated in detail.

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MATERIALS AND METHODS

Plant materials and culture conditions

Five mm long apices of the rhizomes of *C. sinense* cv. Shue Pai Tsoa were prepared aseptically from seed-derived and one or two year old plants and cultured *in vitro* in Horticultural Science, National Chiayi University, Chiyai, Taiwan. Well-grown and branched rhizomes were cut into 1.5 cm long segments and used for the experiments.

An MS medium supplemented with 30 gl^{-1} sucrose and solidified with 2.7 gl^{-1} Gelrite was used as a basal 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.

All cultures were incubated at 25±2 °C in 16 h daylength (28–36 $\mu molsec^{-1}m^{-2}$).

Effects of BA and NAA on morphogenesis

The rhizome explants were cultured with various combinations of concentrations of BA and NAA. Ten explants were cultured in one 500 ml culture bottle containing 70 ml medium. Each treatment consisted of 50 explants with three replicates. Measurement took place after 90 days of culture on morphogenesis, and 150 days after culture on growth.

Effects of ammonium and potassium nitrate concentrations in MS medium on morphogenesis

The rhizome explants were cultured on the MS medium with various combinations and strength of ammonium nitrate and potassium nitrate (Table 1). Seven explants were

Table 1. Effects of BA and NAA on morphogenesis from rhizomes of *Cymbidium sinense*.

Treatment (mgl^{-1})		Number of	Number of	Browning
BA	NAA	rhizomes	shoots	(%)
0	0	6.7 d*	0	0
0	0.1	6.5 d	0	7
0	1.0	8.0 b	0	13
0	10	7.2 c	0	7
0.1	0	3.7 f	0	0
0.1	0.1	10.5 a	0	20
0.1	1.0	3.2 h	0	13
0.1	10	4.7 e	0	7
1.0	0	2.8 i	3.0 c	0
1.0	0.1	3.5 g	0.5 e	0
1.0	1.0	8.2 b	0.4 e	0
1.0	10	4.0 e	0.5 e	0
10	0	4.0 e	3.8 a	0
10	0.1	0.2 k	3.2 bc	0
10	1.0	1.3 k	3.3 b	0
10	10	2.0 j	2.3 d	0

Measured after 90 days of culture.

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

cultured in one 500 ml culture bottle containing 70 ml medium. Each treatment consisted of 21 explants with three replicates. Morphogenesis and plantlet sizes were recorded after 90 and 150 days of culture, respectively.

RESULTS AND DISCUSSION

Effects of BA and NAA on morphogenesis

Number of rhizomes 90 days after culture was more than four when the concentrations of NAA were the same as or higher than those of BA and when the treatments with 0.1 mgl^{-1} BA+1.0 mgl^{-1} NAA and 10 mgl^{-1} BA+10 mgl^{-1} NAA were given (Table 1, Fig. 1A). Among the treatments, the largest number of rhizomes was 10.5 with 0.1 mgl^{-1} BA+0.1 mgl^{-1} NAA, followed by 8.2 and 8.0 with 1.0 mgl^{-1} BA+1.0 mgl^{-1} NAA and 1.0 mgl^{-1} NAA only, respectively.

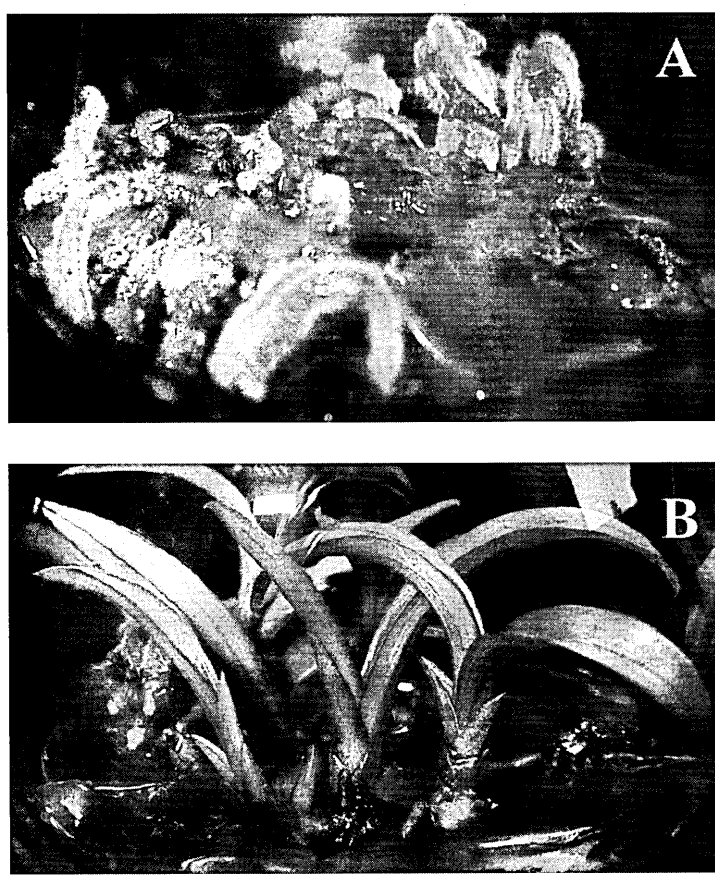

Fig. 1. Typical morphogenesis from rhizomes of *Cymbidium sinense*. (A); multiplication of rhizomes with BA 0.1 mgl^{-1}+NAA 0.1 mgl^{-1}, (B); differentiation of shoots with BA 1.0 mgl^{-1}+NAA 0 mgl^{-1}.

Shoot formation occurred when BA concentration was 1.0 mgl^{-1} or above with or without NAA, among which 10 mgl^{-1} BA only brought the largest number of shoots (3.8) (Table 1, Fig. 1B). No shoots were obtained with lower ($\leqq$0.1 mgl^{-1}) BA irrespective of the NAA concentrations by 90 days of culture. No browning of the rhizomes was observed with higher BA concentrations ($\geqq$1.0 mgl^{-1}).

The plantlets with large number of leaves (>5), when counted 150 days of culture, were obtained with higher concentrations of both BA and NAA (1.0 mgl^{-1} BA+1.0 mgl^{-1} NAA, 1.0 mgl^{-1} BA+10 mgl^{-1} NAA, 10 mgl^{-1} BA+10 mgl^{-1} NAA and 10 mgl^{-1} BA+1.0 mgl^{-1} NAA) (Table 2). No roots developed without NAA. BA did not affect the length and number of roots.

The results in *C. sinense* in this study are in agreement with the previous reports on other terrestrial *Cymbidium* species (Ueda and Torikata, 1969; Hasegawa *et al.*, 1985; Hasegawa, 1987; Shimasaki and Uemoto, 1990; Paek and Yeung, 1991; Ogura and Okubo, 2003). Response to cytokinins and auxins on *in vitro* shoot formation from rhizome explants seems to be general in terrestrial *Cymbidium* species.

Table 2. Effects of BA and NAA on growth of the cultures from rhizomes of *Cymbidium sinense*.

Treatment (mgl^{-1})		Shoot		Root	
BA	NAA	Leaf length (cm)	Number of leaves	Length (cm)	Number
0	0	0	0	0	0
0	0.1	0	0	0	0
0	1.0	0	0	0	0
0	10	2.8 def*	2.8 d	1.4 bc	1.5 b
0.1	0	2.4 ef	2.9 d	0	0
0.1	0.1	6.5 a	4.0 bc	2.4 ab	2.3 a
0.1	1.0	4.3 bc	3.9 bc	2.5 ab	2.1 a
0.1	10	3.3 cde	3.6 bcd	3.5 a	2.3 a
1.0	0	1.5 fg	4.1 bc	0	0
1.0	0.1	2.1 f	4.3 b	0.9 bc	1.3 b
1.0	1.0	4.6 b	5.8 a	1.6 ab	2.2 a
1.0	10	3.6 cd	5.7 a	0.7 c	1.9 ab
10	0	1.4 g	4.2 bc	0	0
10	0.1	2.4 ef	3.5 cd	0.8 bc	1.3 b
10	1.0	3.5 cd	5.1 a	1.2 bc	1.4 b
10	10	2.0 f	5.6 a	2.2 ab	1.7 ab

Measured after 150 days of culture.

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

Effects of ammonium and potassium nitrate concentrations in MS medium on morphogenseis

Reduction of ammonium and potassium nitrate strengths to 1/4 and 1/4, 1/4 and 1/2, and 1/2 and 1/4, respectively from the original concentrations brought 100% shoot forma-

tion after 90 days of culture (Table 3). The highest number of shoots obtained was 3.4 with 1/4 ammonium and 1/4 potassium nitrates, followed by 3.3 with 1/4 ammonium and 1/2 potassium nitrates. Half strength of ammonium nitrate also gave the higher number of shoots, 2.9 and 2.7, with 1/4 and 1/2 potassium nitrate, respectively. Number of rhizomes was not affected by the reduction of the concentrations.

Table 3. Effects of reduction of ammonium nitrate and potassium nitrate in MS medium on morphogenesis from rhizomes of *Cymbidium sinense*.

Strength of nitrogen salts in MS media		% of shoot formation	Number		Browning of rhizome (%)
NH_4NO_3	KNO_3		Rhizome	Shoot	
0	0	87	2.1 o*	2.5 bc	7
0	1/8	47	1.5 p	0.2 d	20
0	1/4	0	5.7 c	0	7
0	1/2	0	3.0 lm	0	0
0	1	40	3.6 ij	0.2 d	7
0	2	47	2.9 lmn	0.2 d	7
1/8	0	40	1.6 p	0.2 d	7
1/8	1/8	0	3.6 ij	0	7
1/8	1/4	0	4.3 g	0	13
1/8	1/2	0	1.4 p	0	28
1/8	1	0	3.4 jk	0	20
1/8	2	0	1.4 p	0	20
1/4	0	0	5.0 gh	0	0
1/4	1/8	40	5.0 gh	0.3 d	0
1/4	1/4	100	3.0 lm	3.4 a	0
1/4	1/2	100	5.6 cd	3.3 a	0
1/4	1	0	6.6 b	0	0
1/4	2	0	3.5 j	0	0
1/2	0	47	4.1 gh	0.3 d	13
1/2	1/8	0	4.3 g	0	13
1/2	1/4	100	1.7 p	2.9 ab	0
1/2	1/2	80	2.8 mn	2.7 abc	20
1/2	1	0	6.6 b	0	13
1/2	2	33	12.5 a	0.2 d	0
1	0	40	1.7 p	0.2 d	28
1	1/8	53	2.6 n	0.3 d	13
1	1/4	47	4.9 f	0.2 d	13
1	1/2	53	2.7 n	0.3 d	7
1	1	73	4.9 f	1.8 c	13
1	2	20	0.8 q	0.2 d	48
2	0	0	5.3 de	0	13
2	1/8	40	3.9 hi	0.2 d	7
2	1/4	40	2.6 n	0.2 d	7
2	1/2	0	3.5 j	0	20
2	1	40	4.0 gh	0.2 d	13
2	2	0	3.2 kl	0	13

Measured after 90 days of culture.

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

Number of leaves was not so much affected by the reduction, but among the treatments, combination of 1/2 strength of ammonium nitrate and 1/4 strength of potassium nitrate produced the plantlets having longest leaf (6.3 cm) and the largest number of leaves (4.5), 150 days after culture (Table 4). The weaker the ammonium nitrate strength (1/2 to 0) was, the longer the roots were. The higher the potassium nitrate con-

Table 4. Effects of reduction of ammonium nitrate and potassium nitrate in MS mdedium on growth of the cultures from rhizomes of *Cymbidium sinense*.

Strength of nitrogen salts in MS media		Shoot		Root	
NH_4NO_3	KNO_3	Leaf length (cm)	Number of leaves	Length (cm)	Number
0	0	2.1 j*	2.9 h	4.4 cd	1.0 o
0	1/8	1.6 j	2.9 h	5.3 ab	1.0 o
0	1/4	4.6 hi	4.1 bcd	5.8 a	1.9 klmn
0	1/2	4.5 i	3.8 def	4.8 bc	1.8 lmn
0	1	5.0 defghi	3.9 cdef	4.8 bc	2.2 ijklmn
0	2	5.2 cdefghi	4.0 bcde	4.5 c	2.9 efgh
1/8	0	5.2 cdefghi	4.0 bcde	2.0 hijklmn	2.4 ghijkl
1/8	1/8	5.2 cdefghi	3.7 efg	2.7 efg	2.3 hijklm
1/8	1/4	5.2 cdefghi	4.0 bcde	2.3 ghijk	1.6 no
1/8	1/2	4.9 efghi	4.0 bcde	2.9 ef	2.2 ijklmn
1/8	1	5.6 abcdefg	4.0 bcde	3.8 d	2.6 ghij
1/8	2	4.6 hi	3.8 def	3.1 e	2.1 jklmn
1/4	0	5.2 cdefghi	4.0 bcde	1.5 mno	2.7 fghij
1/4	1/8	5.0 defghi	3.7 efg	1.6 klmno	2.7 fghij
1/4	1/4	5.3 bcdefgh	4.0 bcde	1.4 no	2.9 efgh
1/4	1/2	6.1 ab	4.1 bcd	2.0 hijklmn	3.3 cdef
1/4	1	5.9 abc	4.3 ab	2.0 hijklmn	3.8 abc
1/4	2	4.9 fghi	3.9 cdef	2.1 ghijkl	3.0 defg
1/2	0	5.8 abcd	4.0 bcd	1.6 lmno	3.7 abc
1/2	1/8	5.7 abcde	4.3 ab	1.7 klmno	3.7 abc
1/2	1/4	6.3 a	4.5 a	1.8 mno	4.1 ab
1/2	1/2	6.1 ab	4.2 abc	1.9 ijklmn	3.5 bcde
1/2	1	5.9 abc	4.5 a	2.4 ghij	4.2 a
1/2	2	5.6 abcdef	4.2 abc	1.9 ijklmn	3.6 abcd
1	0	5.0 defghi	4.0 bcde	1.8 jklmno	2.5 ghijk
1	1/8	5.3 bcdefgh	4.0 bcde	3.0 ef	3.0 defg
1	1/4	5.5 bcdefg	3.7 efg	2.1 ghijklm	2.9 efgh
1	1/2	4.6 hi	3.9 cdef	1.8 klmno	1.7 mn
1	1	5.0 defghi	3.6 fg	2.5 efghi	2.1 jklmn
1	2	4.5 hi	3.4 g	2.6 efgh	2.4 ghijkl
2	0	4.8 fghi	3.4 g	1.2 o	1.6 no
2	1/8	5.1 cdefghi	3.9 cdef	2.0 hijklmn	2.5 ghijk
2	1/4	4.7 ghi	3.7 efg	1.5 mno	2.5 ghijk
2	1/2	5.0 defghi	3.8 def	1.8 jklmn	2.5 ghijk
2	1	5.0 defghi	3.9 cdef	2.0 hijklmn	2.8 fghi
2	2	4.9 fghi	3.6 fg	1.5 mno	2.3 hijklm

Measured after 150 days of culture.

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

centrations within the same ammonium nitrate concentrations, the longer the roots were. The antagonism of the two nitrogen salts is suggested in root growth. Ammonium ions were found to dramatically inhibit *Arabidopsis thaliana* seedling root growth in the absence of potassium, and this inhibition could be reversed by including in the growth medium low levels of potassium (Cao *et al.*, 1993).

It is clarified that *C. sinense* shows the similar response not only to the manipulation with BA and NAA but also with the reduction of nitrate salt concentrations of MS medium to other *Cymbidium* species. No promotional effect on shoot formation was, however, reported previously in *C. sinense* by the reduction of ammonium and potassium nitrate concentrations (Ogura, 2003). One possible reason of the difference in the results between two experiments may be due to the difference of the explant sources and ages. Due to long history of cultivation and cultivar development in different places in this species, there may be wide variation in physiological as well as in morphological characters. Difference by the ages of the rhizomes and seasonal changes in responsiveness to the treatments may exist. Another possibility is the positions and length of the explants used. Only the rhizome apices were used and the length was 0.5 cm in Ogura's experiment (Ogura, 2003), whereas all the positions of the rhizomes of 1.5 cm in length, including apices, were used here.

Cymbidium sinense is one of the most southern terrestrial species among other terrestrial *Cymbidium* species such as *C. goeringii, C. faberi, C. kanran, C. forrestii and C. ensifolium*, and less tolerant to low temperature. It means that *C. sinense* has weaker dormancy than others, and it is more sensitive to the treatments. This may be the reason that the shoot formation was observed without auxin and cytokinin and without the reduction of nitrate salt strength (Tables 3 and 4), although no shoots were formed in the first experiment of this study (Tables 1 and 2).

It is concluded that the responsiveness to the plant hormones and nitrate salt concentration is universal among terrestrial *Cymbidium* species.

Ogura (2003) showed the additive effects of the combined treatment with reduction of nitrate salt concentrations and with 1 mgl^{-1} BA + 0.1 mgl^{-1} NAA on shoot formation rate and number of shoots in *C. kanran*. The treatment may be applicable to *C. sinense*. Addition of AVG together with the reduction treatment of nitrate salts also gave an excellent number of shoots in *C. kanran*, although the response to $AgNO_3$ was weak (Ogura, 2003). It should be also taken into consideration in rhizome culture of *C. sinense*.

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