

## Somatic Embryogenesis from the Etiolated Petiole of Cyclamen (*Cyclamen persicum* Mill.)

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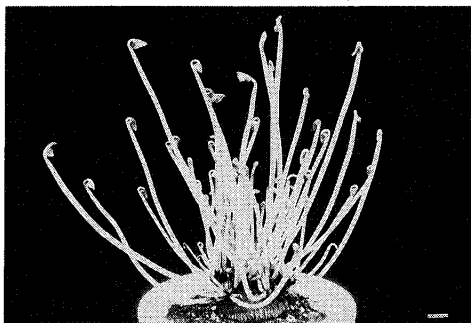
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The induction of somatic embryos from etiolated petioles was examined in five commercial cyclamen (*Cyclamen persicum* Mill.) cultivars. Somatic embryogenesis was only observed in 'Anneke'. The optimal medium was Murashige and Skoog medium with 5.0  $\mu\text{M}$  2, 4-D and 0.5  $\mu\text{M}$  kinetin, and the optimal incubation condition was complete dark at 25°C. A much higher percentage of explants forming embryoids and also larger number of embryoids were recorded in the explants from a third distal end of 5-10 cm petioles than from other explants. This study suggests that somatic embryogenesis from etiolated petiole tissues was effectively utilized for the micropropagation of cyclamen.

### Introduction

Cyclamen (*Cyclamen persicum* Mill.) is one of the most popular pot plants and it is commercially propagated by seeds. In horticultural practice, vegetative propagation is desirable because of the heterogeneity in seedlings of the most cultivars. It is, however, difficult to propagate cyclamen by division, cutting and grafting. Consequently, the micropropagation of cyclamen has been expected.

One of the serious problems encountered in the micropropagation of cyclamen is microbial contamination. Furthermore, conventional micropropagation methods through axillary or adventitious bud systems has not been economical in cyclamen<sup>1)</sup> because of the low multiplication rate. Therefore, micropropagation via somatic embryogenesis without microbial contamination are desired. Though it has already been reported that microbial contamination was prevented by the use of etiolated petioles (Fig. 1) in the organogenesis of cyclamen<sup>2,3)</sup>, the somatic embryogenesis from the etiolated petiole tissues has never been reported. We, therefore, examined the induction



**Fig. 1** Etiolated petioles of cyclamen after incubation at 20°C in the dark for 42 days.  
Bar = 2 cm

of somatic embryos from the etiolated petioles of mature plants in commercial cyclamen cultivars.

### Materials and Methods

Five cyclamen cultivars, *i. e.* 'Anneke', 'Bonfire', 'Golden Boy', 'Lilac' and 'Pure White', were used as the plant materials. The etiolation treatment on the mother plants and the surface sterilization procedures were in accordance with Ando and Murasaki<sup>2)</sup>.

Effects of plant growth regulators were initially examined. After surface sterilization, the distal quarters of etiolated petioles which had elongated to 10 cm in length were resected and about 5 mm of the both ends were removed. Then, they were cut into 2 mm lengths and used as explants. In this experiment, 40 explants were used for each treatment. Murashige and Skoog (MS) medium<sup>4)</sup> with 6.0% sucrose and 0.2% gellan gum was used as a basal medium, to which different concentrations of 2, 4-D and kinetin were added. For comparison of the frequency of contaminated explants, 40 sterilized leaf-segments in each cultivar were collected before the etiolation treatment as reported by Otani and Shimada<sup>5)</sup> and cultured on the basic media with 5.0  $\mu\text{M}$  2, 4-D and 0.5  $\mu\text{M}$  kinetin. The cultures were maintained at 25°C in the dark. The effects of temperature and light were secondly examined. The explants plated on the basic media with 5.0  $\mu\text{M}$  2, 4-D and 0.5  $\mu\text{M}$  kinetin were cultured in the dark at 20, 25 and 30°C, and 16 h photoperiod (daylight, about 20  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 25°C.

The effects of the length and position of etiolated petioles were also investigated. The etiolated petioles of 5, 10 and 20 cm long, were trisected and then sterilized. Each of the disinfected petioles were cut into 2 mm lengths after resecting both ends about 5 mm. The number of explants used for 5, 10 and 20 cm petioles was 40, 72 and 168, respectively. MS medium with 5.0  $\mu\text{M}$  2, 4-D, 0.5  $\mu\text{M}$  kinetin, 6.0% sucrose and 0.2% gellan gum was used for the culture and the cultures were maintained at 25°C in the dark.

The experiments were repeated at least twice. In all experiments, the media were adjusted to pH 5.8 and autoclaved at 121°C for 20 minutes. The cultures were transferred to MS medium without plant growth regulator after 6 weeks of culture. After 5 weeks of subculture, the number of cultures forming embryoids and the number of embryoids formed were recorded. Fifty plantlets obtained in the optimal condition for somatic embryogenesis were planted in the greenhouse and allowed to flower.

Some calli and embryoids were collected and then embedded in paraffin (melting point 58–60°C) as in previous reports<sup>6)</sup>. The materials embedded in paraffin blocks were sectioned at 10  $\mu\text{m}$  and stained with 0.25% Hidenhein's iron hematoxylin for microscopic examination.

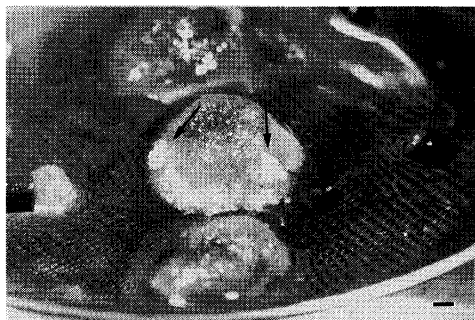
### Results and Discussion

Microbial contamination was prevented in all the explants of etiolated petioles except three explants in 'Lilac', whereas 12.5 to 85% of explants were contaminated in the explants of leaf segments (Table 1). The three contaminated etiolate petiole explants in 'Lilac' might be due to the infection with bacterium in the mother plant, since contamination percentage of the leaf segments in 'Lilac' were also much higher than that in the other cultivars and the 'Lilac' plant died of a bacterial disease during the cultivation after the etiolated treatment.

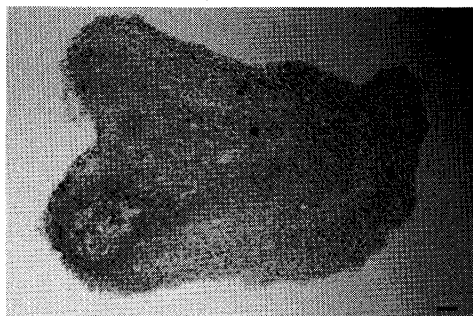
In every experiment, somatic embryogenesis was observed only in 'Anneke'. Two types of calli were formed in the petiole culture of this cultivar, one was transparent and friable and the other was opaque white and compact. The former produced somatic embryoids (Fig. 2) as reported by Otani and Shimada<sup>5)</sup>. The somatic embryoids developed through the heart- and torpedo-shaped

**Table 1.** Frequency of contaminated explants in the cultures of etiolated petiole and leaf-segment explants on basal medium with 5  $\mu\text{M}$  2, 4-D and 0.5  $\mu\text{M}$  kinetin.

Cultivar	No. of explants observed	No. of explants contaminated	Percent explants contaminated
Etiolated petiole			
'Anneke'	40	0	0
'Bonfire'	40	0	0
'Golden boy'	40	0	0
'Lilac'	40	3	7.5
'Pure White'	40	0	0
Leaf segments			
'Anneke'	40	14	35
'Bonfire'	40	5	12.5
'Golden boy'	40	8	20
'Lilac'	40	34	85
'Pure White'	40	15	37.5



**Fig. 2** Somatic embryoids (arrows) on the transparent and friable callus derived from the etiolated petiole explant of cyclamen 'Anneke'.  
Bar=3 mm



**Fig. 3** Ten  $\mu\text{m}$  section of the torpedo-shaped somatic embryos, which have no vascular connection to other tissues, derived from the etiolated petiole explant of cyclamen 'Anneke'.  
Bar=50  $\mu\text{m}$

stages as zygotic embryos, but they had no vascular connection to the other tissues (**Fig. 3**).

Though embryogenesis was observed on the media with 5.0  $\mu\text{M}$  2, 4-D, 5.0  $\mu\text{M}$  2, 4-D plus 0.5  $\mu\text{M}$  kinetin, and 50.0  $\mu\text{M}$  2, 4-D plus 5.0  $\mu\text{M}$  kinetin, the optimal concentration of plant growth regulator for embryogenesis from etiolated petioles was 5.0  $\mu\text{M}$  2, 4-D plus 0.5  $\mu\text{M}$  kinetin (**Table 2**).

**Table 2.** Effects of plant growth regulators on embryogenesis in the etiolated petiole explants of *C. persicum* 'Anneke'.

Concentration ( $\mu\text{M}$ )		No. of explants cultured	Percent explants forming embryoids	No. of embryoids per explant forming embryoids
2, 4-D	kinetin			
0	0	40	0	—
5.0	0	40	7.5	5.7 $\pm$ 1.5
5.0	0.5	40	45.0	36.6 $\pm$ 4.2
5.0	5.0	40	0	—
0.5	0.05	40	0	—
50.0	5.0	40	20.0	26.4 $\pm$ 11.2

**Table 3.** Effects of temperature and light on embryogenesis in the etiolated petiole explants of *C. persicum* 'Anneke'.

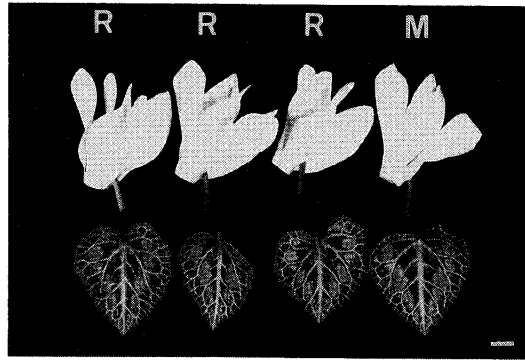
Condition	No. of explants cultured	Percent explants forming embryoids	No. of embryoids per explant forming embryoids
20°C Dark	40	30.0	6.3±1.8
25°C Dark	40	42.5	31.2±6.4
30°C Dark	40	22.5	26.6±9.3
25°C Light	40	5.0	9.5±3.9

In embryogenesis from the petiole tissues of cyclamen seedling, it has been reported that the number of somatic embryoid and percentage of explants forming embryoids were higher at 25°C than at 20°C or 30°C<sup>6</sup>). Similarly, 25°C was the optimal temperature for the embryogenesis from the etiolated petioles (**Table 3**). It was reported that the maturation of somatic embryos of *Daucus* and caraway proceeded more normally in complete darkness than under light condition<sup>7,8</sup>). In seedling tissue culture of cyclamen, a much higher number of embryoids were obtained in the dark than under light condition<sup>6</sup>). In the etiolated petiole of cyclamen, a much higher number of embryoids and percentage of explants forming somatic embryoids occurred in the dark than under light condition.

It has been reported that the number of explants producing embryogenic callus was higher in the explants from the top portion of the hypocotyl than in the explants from the middle and bottom portions of hypocotyl in *Solanum melongena*<sup>9</sup>). In cyclamen, the percentage of explants forming embryoids and the number of embryoids produced varied according to the portions and length of etiolated petioles (**Table 4**). The somatic embryogenesis was not observed in the explants from the bottom portions of the etiolated petioles (bottom explants) and much higher percentage of explants forming embryoids and number of embryoids were recorded in the explants from the top portions of etiolated petioles (top explants) than the middle portions (middle explants). The highest percentage of explants forming embryoids was recorded in the top explants of 5 cm etiolated petioles and the highest number of embryoids was recorded in the top explants of 5 and 10 cm etiolated petioles. These results suggest the importance of petiole length and the portion used as explants, which has

**Table 4.** Somatic embryogenesis in various length and parts of etiolated petioles of *C. persicum* 'Anneke'.

Length of petiole (cm)	Portion of excised explant on petiole	No. of explants cultured	Percent explants forming embryoids	No. of embryoids per explant forming embryoids
5	Top	40	40.0	35.1±4.8
	Middle	40	10.0	14.0±5.0
	Bottom	40	0	—
10	Top	72	26.4	31.5±3.1
	Middle	72	2.8	7.5±3.5
	Bottom	72	0	—
20	Top	168	10.1	26.2±2.2
	Middle	168	0.6	1
	Bottom	168	0	—
<i>Significance</i>				
Length (L)			$p < 0.001$	$0.001 < p < 0.01$
Portion (P)			$p < 0.001$	$p < 0.001$
P × L			$p < 0.001$	Not significant



**Fig. 4** Comparison of the petal and leaf characteristics between regenerated plants(R) and mother plants(M) through somatic embryogenesis in etiolated-petiole tissue culture in cyclamen 'Anneke'.

Bar=8 mm

never been reported in the etiolated petiole culture of cyclamen<sup>2,3</sup>. From our observation, only the top portion of petioles elongated during the etiolation treatment of plants, suggesting that the cells with high potential of division and/or elongation were involved in this portion. This presumed high potential of the cells might affect the ability of somatic embryogenesis in the top explants.

At present, more than 80 plants regenerated from the top explants of 5 cm etiolated petioles through somatic embryogenesis flowered and no morphological mutation has been observed (**Fig. 4**).

The elimination of microbial contamination was hardly mentioned in the reports on the micropropagation of cyclamen via somatic embryogenesis<sup>5,10-13</sup>. In contrast, the use of aseptic seedling was beneficial for micropropagation through somatic embryogenesis without microbial contamination<sup>6</sup>. However, propagation of true-to-type plants may not be sure in the aseptic seedling tissue culture because of the heterogenous nature of the seedlings in many of the cultivars commercially available. In the present study, we confirmed that the etiolated petioles of cyclamen were scarcely infected by microorganisms. As we can obtain the etiolated petioles as the explants after observation of the characteristics of flowering plants, somatic embryogenesis from etiolated petiole tissues should be an effective method for the micropropagation of true-to-type cyclamen cultivars.

Varietal differences were previously reported in the efficiency of somatic embryogenesis from the aseptic seedlings of cyclamen<sup>14</sup>. Somatic embryogenesis was observed only in 'Anneke' although 5 cultivars were examined in this study. The studies of genetic effects on the somatic embryogenesis is required for further application of tissue culture for commercial micropropagation of cyclamen.

### Acknowledgements

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## 《和文要約》

### シクラメン (*Cyclamen persicum* Mill.) の黄化葉柄からの体細胞胚形成

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5品種のシクラメン (*Cyclamen persicum* Mill.) を用いて、黄化葉柄からの体細胞胚の誘導を試みた結果、'アンネッケ' のみで体細胞胚形成が認められた。

5.0  $\mu$ M の 2, 4-D と 0.5  $\mu$ M のカイネチンを添加した MS 培地を用いた 25°C 暗黒下での培養が体細胞胚形成に最も適しており、また、5-10 cm の葉柄の先端 1/3 の部分から得られた外植体において、より高い割合でより多くの体細胞胚が形成された。

本研究により、黄化葉柄からの体細胞胚の形成は、シクラメンのマイクロプロパゲーションにおける有効な手段の一つになり得ることが示唆された。