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Somatic embryogenesis of *Cyclamen persicum* Mill. 'Anneke' from aseptic seedlings

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Summary. In *Cyclamen persicum* 'Anneke', explants from the various vegetative organs of aseptic seedling formed embryoids. The optimal responses were recorded in Murashige and Skoog (MS) medium enriched with 5.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 μM kinetin and 3-6% sucrose. Embryogenesis was enhanced at higher temperature of 25-30°C. On the other hand, light inhibited embryogenesis. Histological and morphological studies confirmed that the embryoids were indeed somatic embryos.

Abbreviations : MS = Murashige and Skoog, 2,4-D = 2,4-dichlorophenoxyacetic acid.

Introduction

Cyclamen (*Cyclamen persicum* Mill.) is commercially propagated by seeds. The breeding programme has been successful to produce many different cultivars. Nevertheless, vegetative propagation is desirable because there is variation in the seed-derived cultivars. In addition, F_1 seeds are relatively expensive. Conventional micropropagation through axillary or adventitious systems is slow and not economical although there are many reports on *in vitro* multiplication of cyclamen (Geier *et al.*, 1990).

Somatic embryogenesis is one of the common methods for micropropagation since embryogenic culture potentially can produce large number of somatic embryos per culture flask, much more than the multiple shoots generated adventitiously via organogenesis (Ammirato, 1982). Some earlier reports on somatic embryogenesis in cyclamen (Fersing *et al.*, 1982; Wicart *et al.*, 1984; Otani and Shimada, 1991; Kiviharju *et al.*,1992; Oohashi *et al.*, 1992) indicated a wide difference in embryogenic potential among the different cultivars. Few studies were made on the culture conditions for the successful production of embryoids.

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In this study, we examined the effects of temperature and light as well as plant growth regulators and sucrose on the embryogenesis of the seedling explants of *C. persicum* 'Anneke'.

Materials and methods

Culture for embryogenesis.. The dry seeds of *C. persicum* 'Anneke' were soaked in 20% sodium hypochlorite solution (about 2% available chlorine) containing a few drops of detergent for 10 minutes, and rinsed three times with sterile distilled water. The disinfected seeds were sown on 1/3 MS (Murashige and Skoog, 1962) medium with 3% sucrose and 0.3% gellan gum. They were incubated at 20°C in the dark for 3 weeks followed by 16 h photoperiod (day light, about 30 μmol m^{-2} s^{-1}) for another 4 weeks. The 7 week-old aseptic seedlings were then divided into cotyledons, petioles, tubers and roots. The cotyledons, petioles and tubers were sectioned into 5, 4 and 8 segments, respectively, and the roots were cut into about 5 mm length and used as explants.

Inorganic and organic nutrient of MS solidified with 0.2% gellan gum was used as basic medium. For investigations on the effects of plant growth regulators and sucrose on embryogenesis, 2,4-D at 0, 5.0 and 50.0 μM and kinetin at 0, 0.5, 5.0 and 50.0 μM were tested in each basic medium with 6% sucrose. Sucrose concentrations ranging from 0-9% were tested in medium containing 5.0 μM 2,4-D and 0.5 μM kinetin. Cultures were maintained at 25°C in the dark.

To investigate the effects of temperature and light on embryogenesis, the explants were cultured on MS medium with 5.0 μM 2,4-D, 0.5 μM kinetin, 6% sucrose and 0.2% gellan gum. Cultures were maintained at 20, 25 and 30°C in the dark, or cultured in the dark and 24 h photoperiod (day light, about 30 μmol m^{-2} s^{-1}) at 25°C.

Ten seedlings were used in each experiments and 3 replication of experiment was carried out. In all experiments, the media were adjusted to pH 5.8 and autoclaved at 121°C for 20 minutes. The number of explants forming embryoids and the number of embryoids per organ were recorded after 6 weeks of culture. The cultures produced were then transferred to MS medium without plant growth regulator for further development.

After 5 weeks of subculture, the number of cultures forming embryoids and the number of embryoids per organ were also recorded. Somatic embryos were transferred to MS medium with 3% sucrose, 0.1 μM kinetin and 0.2% gellan gum and regenerated plantlets were grown in the greenhouse after acclimatization.

Histology. Calli and somatic embryoids were fixed in FAA solution (formalin: acetic acid: 70% ethanol, 1:1:18, V/V). They were dehydrated in tertiary butyl alcohol series and embedded in paraffin (melting point 58-60°C). Paraffin embedded materials were sectioned at 10 μm and stained with 0.25% Hidenhein's iron hematoxylin for microscopic examination.

Results and discussion

Effects of plant growth regulators

Callus formation and embryoid formation were observed in all four types of explants, namely, cotyledon, petiole, tuber and root, from aseptic seedlings (Fig.1). Earlier, Wainwright and Harwood (1985) had not been able to induced somatic embryos from the aseptic seedling tissues of *C. persicum* 'Rosamunde' and Oohashi *et al.* (1992) have reported that somatic embryos were formed only from the root explants of the young seedlings via organogenesis of *C. persicum* 'Chopin'.

All types of explants showed much higher number of embryoids and greater percentage of explants forming embryoids in the media with 2,4-D and kinetin at 10 : 1 concentration ratio. The best response appeared to be at 5.0 μM 2,4-D with 0.5 μM kinetin. Kiviharju *et al.* (1992) reported that a higher percentage of somatic embryo production was observed in the medium with 2,4-D and coconut milk than without coconut milk; Otani and Shimada (1991) also reported that kinetin at a low concentration (0.1 mg/*l*) stimulated embryogenic callus formation in the medium with 1.0 mg/*l* 2,4-D. These findings suggest that the presence of auxin (2,4-D) and cytokinin-like substance in the medium, and the relative ratio of auxin to cytokinin were important factors for the embryogenesis of cyclamen.

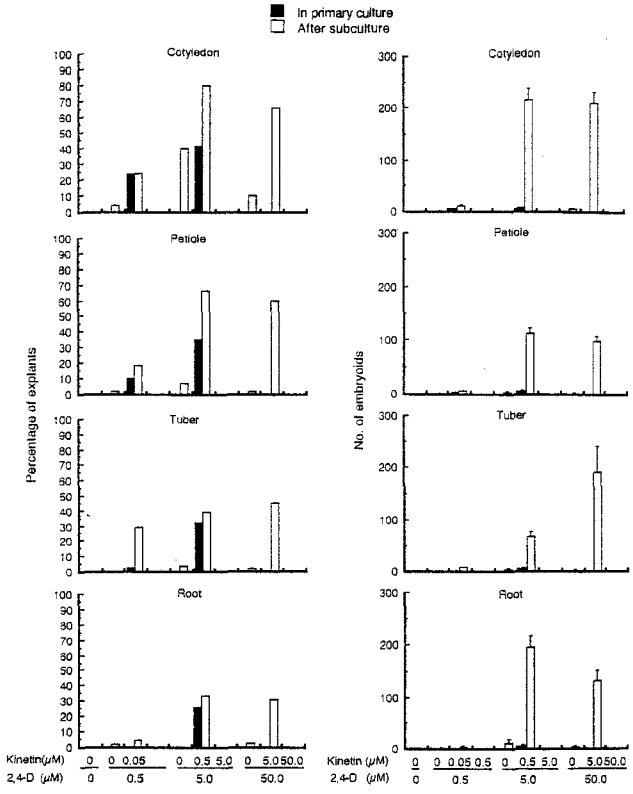

Fig. 1. Effects of plant growth regulators on somatic embryogenesis from aseptic seedling tissues in cyclamen 'Anneke'. (Left) Percent explants forming embryoids. (Right) Number of embryoids per organ.

Embryogenesis was observed in primary culture on the medium with 5.0 μM 2,4-D and 0.5 μM kinetin although no embryoid was produced in primary culture on the medium with 50.0 μM 2,4-D and 5.0 μM kinetin (Fig.1). It indicates that embryogenesis was inhibited or delayed by high concentration of plant growth regulators, perhaps especially auxin.

The optimal concentrations of plant growth regulators in the medium varied according to explant types. Tuber explants showed the highest embryoid number in the medium with 50.0 μM 2,4-D and 5.0 μM kinetin, while the medium with 5.0 μM 2,4-D and 0.5 μM kinetin was optimal for root explants.

Effects of sucrose

The optimal concentration of sucrose in the medium varied according to explants types (Fig. 2). Explants from cotyledons and roots showed the highest embryoid number in the medium with 6% sucrose, while 3% sucrose was optimal for explants from the petioles and tubers.

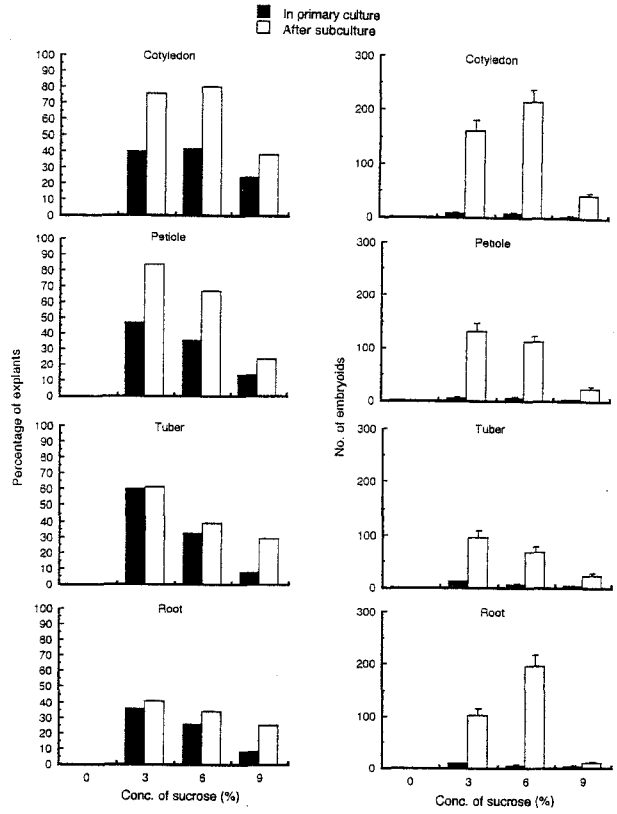

Fig. 2. Effects of sucrose on somatic embryogenesis of cyclamen 'Anneke'. (Left) Percent explants forming embryoids. (Right) Number of embryoids per organ.

Effects of temperature

Increasing the temperature from 20°C to 30°C enhanced embryogenesis in all types of explants (Fig.3). Many explants were forming embryoids in primary cultures

when incubated at 30°C. After subculture, the explants from the cotyledons and petioles, however, showed the highest embryoid number and percentage of explants forming embryoids at 25°C. Other explants did not show significant difference in the number of embryoids produced between 25°C and 30°C incubation. These results suggested that higher temperature stimulated embryogenesis although it was not always effective in some types of explants.

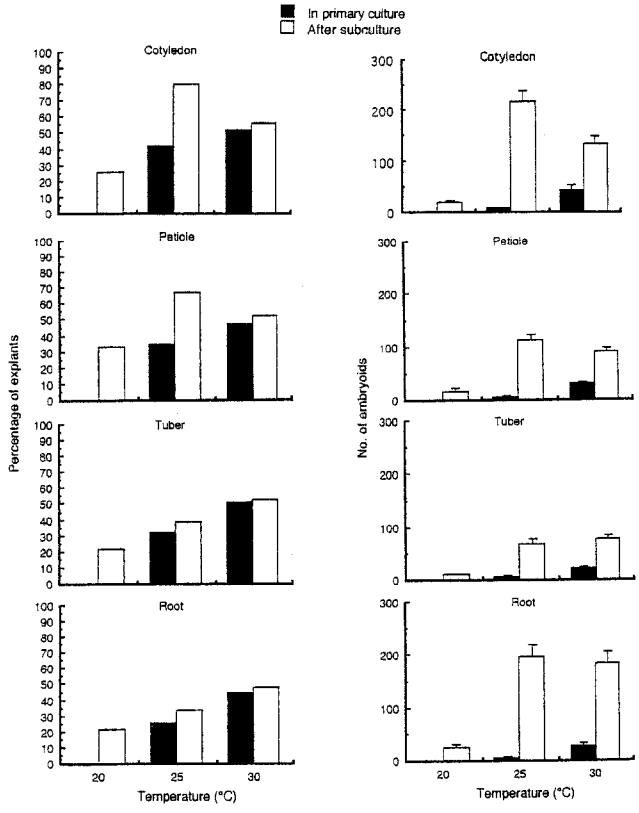

Fig. 3. Effects of temperature on somatic embryogenesis of cyclamen 'Anneke'. (Left) Percent explants forming embryoids. (Right) Number of embryoids per organ.

Effects of light

Much higher number of embryoids and greater percentage of explants forming embryoids were observed in the dark than in the light (24 h photoperiod) in all types of explants (Fig.4). In *Daucus* (Ammirato and Steward, 1971) and caraway (Ammirato, 1974), somatic embryo maturation also proceeded more normally in complete darkness. In cyclamen, Kiviharju *et al.* (1992) reported that the germination of somatic embryo was significantly better in the dark than in the light, but the effects of light on somatic embryo formation was not studied.

These results suggested that light inhibited embryoid differentiation as well as the germination of embryoids in cyclamen. The effects of other environmental conditions, for example, humidity, O_2, CO_2, etc., on somatic embryogenesis in cyclamen should also be investigated.

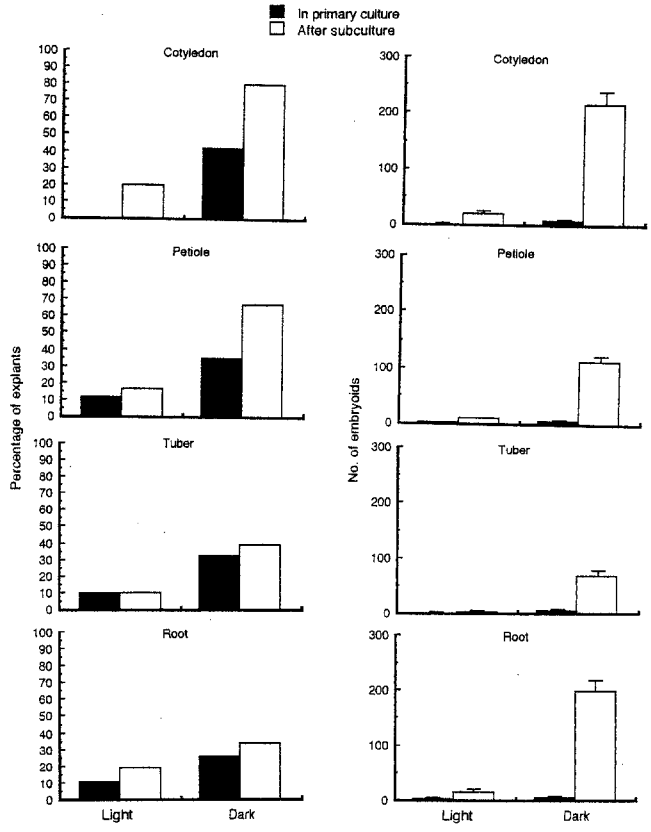

Fig. 4. Effects of light on somatic embryogenesis of cyclamen 'Anneke'. (Left) Percent explants forming embryoids. (Right) Number of embryoids per organ.

Morphological observation of embryo differentiation and growth

In all experiments, embryoids were white and formed usually from transparent and friable callus (Fig. 5A). From histological examination, it was observed that embryoids arose from globular through heart-shaped stage as in zygotic embryos. These embryoids had no vascular connection to the embryogenic callus (Fig. 5B). Embryoids cultured on MS medium with 1 μM kinetin showed close similarity to seedlings (Fig. 6). From these observations, it was confirmed that embryoids obtained in this study conformed to the general definition of somatic embryos. Approximately 60% of plantlets derived from somatic embryos grew well and flowered in the greenhouse (Fig. 7).

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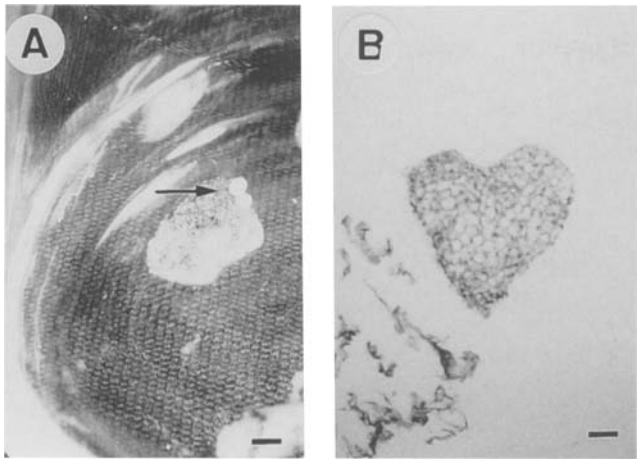

Fig. 5. Embryoids from aseptic seedling tissues. A) Somatic embryos (arrow) from tansparent and friable callus (in primary culture). Bar=2 mm. B) A herat-shaped somatic embryo (10 μm section). Bar = 100 μm.

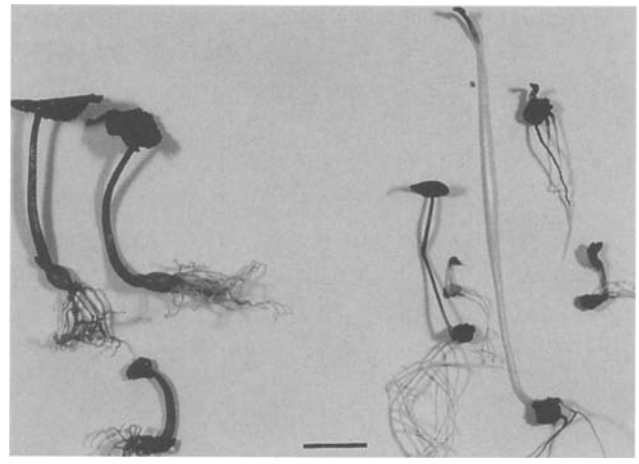

Fig. 6. Plantlets from somatic embryos and seeds. Bar = 1 cm. (Left) Seedlings from seeds. (Right) Young plantlets from somatic embryos.

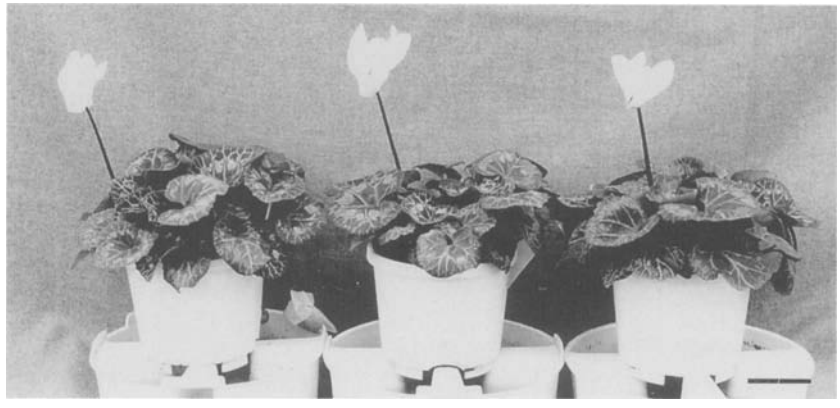

Fig. 7. Flowering plants derived from somatic embryos of *C. persicum* 'Anneke'. Bar = 5 cm

References

Ammirato, P.V. (1974) Bot. Gaz. 135: 328-337

Ammirato, P.V. (1982) In: Evans, D.A., Sharp, W.R., Ammirato, P.V., Yamada, Y. (eds) Handbook of Plant Cell Culture. Macmillan Publishing Co., New York, pp 82-113

Ammirato, P.V., Steward, F.C. (1971) Bot. Gaz. 132: 149-158

Fersing, J., Mouras, A., Lutz, A. (1982) Repin Hort. Maraichars 224: 27-30

Geier, T., Kohlenbach, H.W., Reuther, G. (1990) In: Ammirato, P.V., Evans, D.A., Sharp, W.R., Bajaj, Y.P.S. (eds) Handbook of Plant Cell Culture, vol. 5, Ornamental species. Macmillan Publishing Co., New York, pp 352-374

Kiviharju, E., Tuominen, U., Törmälä, T. (1992) Plant Cell, Tissue and Organ Culture 28: 187-194

Murashige, T., Skoog, F. (1962) Physiol. Plant. 15: 473-497

Oohashi, K., Wakui, T., Yonai, S. (1992) Bull. Tochigi Agr. Exp. Stn. 39: 57-76 (in Japanese)

Otani, M., Shimada, T. (1991) Plant Tissue Cult. Lett. 8: 121-123

Wainwright, H., Harwood, A.C. (1985) Journal of Horticultural Science 60: 397-403

Wicart, G.A., Mouras, A., Lutz, A. (1984) Protoplasma 119: 159-167