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EFFECT OF CO_2 ENRICHMENT ON *IN VITRO* PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS IN CYCLAMEN (*CYCLAMEN PERSICUM Mill.*)

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Abstract

The effect of CO_2 enrichment (3000 ppm) on somatic embryogenesis in cyclamen was investigated. No significant difference between the growth of callus under CO_2 enriched and normal conditions was observed. However, the calli induced under a CO_2 enriched condition formed the largest number of somatic embryos after subculture under the same CO_2 condition. The effect of CO_2 enrichment on *in vitro* germination of somatic embryos was also examined. Larger number of plantlets with leaves and roots were observed in somatic embryos cultured under a CO_2 enriched condition than under a natural condition. These results suggest the possibility of CO_2 enrichment being one of the useful treatments for *in vitro* plant regeneration through somatic embryogenesis in cyclamen.

Key words: callus, CO_2 enrichment, cyclamen, embryoid, *in vitro* germination, somatic embryo, somatic embryogenesis.

Introduction

In usual, commercial cyclamen cultivars are propagated by seeds. However, development of effective vegetative propagation methods has been desired, since it is difficult to fix their genetic characteristics by inbreeding due to inbreeding depression [1]. Because cyclamens do not form daughter tubers, micropropagation has been considered as a moderate and effective method of vegetative propagation. In addition, somatic embryogenesis is one of the effective methods for micropropagation, because of the great potential of multiplication [2].

Many reports on the somatic embryogenesis of cyclamens are available [3-16]. Berardi and Marino [3] reported the effect of CO_2, O_2 and ethylene concentrations in culture vessels on somatic embryogenesis of cyclamens on a solidified medium. Hohe *et al*. [5] reported the effect of CO_2 accumulation on the somatic embryogenesis in the suspension culture of cyclamen. However, the effect of CO_2 enrichment by using gas-permeable culture vessels on somatic embryogenesis of cyclamens has not yet been examined. The effect of CO_2 enrichment on plant regeneration through somatic embryogenesis in cyclamens was examined in the present study.

Materials and Methods

Effect of CO_2 enrichment on somatic embryogenesis

In vitro plantlets of cyclamen (*Cyclamen persicum* Mill.) were used as plant materials. All plantlets were obtained from a F_1 plant ('Anneke' × 'Pure White') through somatic embryogenesis. Cube-shaped sections (about 2 mm in length) were resected from tubers of plantlets, and were used as explants. Murashige and Skoog (MS) medium [17] with 5.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 μM kinetin, 6.0% (w/v) sucrose, and 0.2% (w/v) gellan gum was used for the callus induction from explants, and the medium without 2,4-D and kinetin was used for embryoid formation from callus. All media were adjusted to pH 5.8 before autoclaving. Explants or calli were cultured on the media in 200 mL glass bottles with caps made of polymethyl pentene with a 3 mm diameter hole fitted with a filter (MiliSeal, Japan Milipore Ltd., Japan). These cultures were placed in CO_2-enriched (3000 ppm) or non-enriched (natural; control) culture rooms, and were maintained at 25°C in the dark for both callus induction and embryo formation. Explants were cultured for four weeks for the callus induction, and then the calli were transferred to the medium for the embryoid formation and were cultured for eight weeks. Before transferring, the calli were weighed. Twenty explants were used for each treatment, and two replications of the experiment were carried out.

Effect of CO_2 enrichment on germination of somatic embryos

Somatic embryos (about 1 mm in diameter) obtained from *in vitro* clone plantlets of a F_1 plant ('Anneke' × 'Pure White') were used as explants. Embryos were derived from the same plant. Explants were cultured on a 1/2 MS medium with 3.0% (w/v) sucrose and 0.25% (w/v) gellan gum in 500 mL glass bottles with caps with a 3 mm diameter hole fitted with a filter (MiliSeal, Japan Milipore Ltd., Japan). The media were adjusted to pH 5.8 before autoclaving. These cultures were placed in chambers with or without CO_2 enrichment (3000 ppm). In a chamber with CO_2 enrichment, CO_2 concentration was maintained with an infrared CO_2 controller as reported by Tanaka *et al*. [18] Both chambers with and without CO_2 enrichment were placed in a culture room at 20°C. Eighty explants were cultured in the dark for four weeks, and then were cultured under a 16-h photoperiod (about 34 μmol m^{-2} s^{-1}).

Results and Discussion

Almost all explants both with and without CO_2 enrichment formed calli, whereas the size of calli varied depending on explants (data not shown). No significant difference of callus growth on a solidified medium between explants cultured with CO_2 enrichment and without CO_2 enrichment was recorded (Fig. 1), while Hohe *et al*. [5] reported CO_2 accumulation inhibited the cell growth in a suspension culture of cyclamen. This difference may be due to the difference of culture methods as well as the genotype of explants, as explants in the present study were exposed to CO_2-enriched air on a solidified medium at all times.

The effect of CO_2 enrichment on embryoid formation was obvious (Table 1). The calli induced under a CO_2-enriched condition formed larger number of somatic embryos than calli induced without CO_2 enrichment, as Hohe *et al*. [5] reported that regeneration ability of cell suspensions in cyclamens after a culture in bioreactors with CO_2 accumulation was better than cell suspensions after culture in bioreactors without CO_2 accumulation. The greatest number of somatic embryos were recorded when calli induced under a CO_2-enriched condition were cultured with CO_2 enrichment in subcultures.

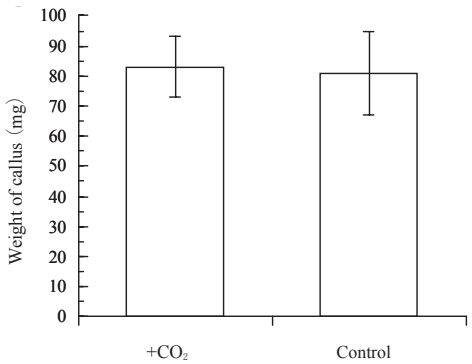

Fig. 1 Effect of CO_2 enrichment on the callus growth of cyclamen F_1 ('Anneke' × 'Pure White') after four weeks of a primary culture. Bars indicate standard error (n=2).

Table 1. Effect of CO_2 enrichment on the formation of somatic embryos in F_1 ('Anneke' × 'Pure White') cyclamen.

CO_2 enrichment		Percent explants forming somatic embryos	No. of somatic embryos per explant[z]
Primary culture	Subculture		
+	+	90	190.3 ± 33.0
+	−	70	101.4 ± 22.7
−	+	75	87.2 ± 18.0
−	−	65	65.1 ± 5.1

[z] Mean number ± Standard error (n=2).

The CO_2 enrichment was also effective for germination of somatic embryos (Table 2). It is natural for embryoids to form both shoots and roots for the normal development and growth of the plant. Larger number of plantlets with leaves and roots were formed from somatic embryos cultured under a CO_2-enriched condition than those cultured without CO_2 enrichment.

Table 2. Effect of CO_2 enrichment on the germination and growth of somatic embryos in F_1 ('Anneke' × 'Pure White') cyclamen.

CO_2 enrichment	No. of explants	No. of plants with		
		Leaves and roots	Leaves	Roots
+	80	14	14	57
−	80	4	7	62

Berardi and Marino [3] reported that growth of embryogenic calli and formation of embryoids from calli in tightly-closed culture vessels, which accumulate CO_2 and ethylene, were inhibited somatic embryogenesis of cyclamens on a solidified medium. They also reported that CO_2 and ethylene did not accumulate in gas permeable culture vessels. Culture vessels used in the present study had gas-permeability by a filter, whereas CO_2 concentration around vessels was enriched to 3000 ppm. Therefore, results of the present study indicate a possibility of ethylene in culture vessels being a main inhibitor of callus growth and embryoid formation in somatic

embryogenesis of the cyclamen.

Results of the present study suggest that CO_2 enrichment is useful for *in vitro* plant regeneration through somatic embryogenesis on a solidified medium in cyclamens, whereas it is not clear if the CO_2 enrichment affected somatic embryogenesis of cyclamens directly or not. It is probable that CO_2 enrichment affected the effect of other gases, especially ethylene, on somatic embryogenesis.

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シクラメンの体細胞胚形成による*in vitro*での植物体再生に及ぼすCO_2施用の影響

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要　　約

シクラメンの体細胞胚形成に及ぼすCO_2施用（3000 ppm）の影響を調査した結果、カルスの成長にはCO_2施用の有意な影響は認められなかったが、初代培養および継代培養のいずれにおいてもCO_2施用を行うことにより、最も多くの体細胞胚が形成された。また、体細胞胚の発芽に及ぼすCO_2施用の影響を調査したところ、CO_2施用下でより多くの体細胞胚が葉と根の両方を有する正常な小植物体に成長した。これらの結果により、CO_2施用がシクラメンの体細胞胚形成による*in vitro*での植物体再生に有効な手段となり得ることが示唆された。