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# HIGHLY-EFFICIENT SOMATIC EMBRYOGENESIS FROM CELL SUSPENSION CULTURES OF PHALAENOPSIS ORCHIDS BY ADJUSTING CARBOHYDRATE SOURCES

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## Summary

The influences of various carbohydrate sources, dried yeast (DY), and 6-benzylaminopurine (BA) were estimated on growth and development of shoot tip-derived suspension cells of phalaenopsis orchid. Among the carbohydrates tested on *Doriataenopsis* cultured on gelled medium, glucose at 58.4 m*M* gave the highest efficiency of protocorm-like body (PLB) formation. Maltose and sorbitol only induced PLB formation without callus proliferation. Sucrose induced comparable callus proliferation to glucose but without PLB formation. In contrast, fructose resulted in half the amount of callus proliferation as occurred with glucose. Lactose was an inadequate carbon source as neither PLB formation nor callus proliferation occurred. DY enhanced cell proliferation at 0.1–1 g $l^{-1}$ but inhibited both cell proliferation and PLB formation at 10 g $l^{-1}$. Low BA (0.4 μ*M*) slightly increased callus proliferation but inhibited PLB formation. Only one treatment, sucrose and 1 g $l^{-1}$ DY, yielded a small number of plants. For suspension cultures of *Phalaenopsis* Snow Parade and *P.* Wedding Promenade, PLB formation was most efficiently induced by sucrose at 29.2 m*M* for *P.* Snow Parade and 14.6 m*M* glucose for *P.* Wedding Promenade. Histological observation revealed that cells in suspension culture developed into plants through the same developmental process as germinating seeds.

*Key words:* PLB formation; embryogenesis; micropropagation; sugar; *Doritaenopsis*; orchid.

## Introduction

Phalaenopsis orchid, which includes cultivars of *Phalaenopsis* and its intergeneric hybrid with *Doritis*, *Doritaenopsis*, is one of the most important orchids, known for its beautiful flowers. Since Morel (1960) successfully propagated *Cymbidium* plants through protocorm-like bodies (PLB), by shoot-tip culture for obtaining virus-free plants, most of the commercially cultivated orchids, such as *Cattleya*, *Cymbidium*, *Dendrobium*, *Miltonia*, and *Oncidium*, have been propagated by shoot-tip culture through PLB formation. In *Phalaenopsis*, micropropagation of the plants through PLB formation has been reported using various kinds of explants (Arditti and Ernst, 1993) including shoot-tip culture of the flower stalk bud (Tokuhara and Mii, 1993). Recently, we established a highly efficient system of micropropagation for *Phalaenopsis* (Tokuhara and Mii, 2001). Embryogenic callus is induced from shoot-tip cultures mediated from flower stalk buds. Fine cell suspension cultures are developed from the callus. Plants are regenerated from callus and cell suspension cultures. These systems should facilitate commercial micropropagation of this orchid. Moreover, embryogenic callus is now considered to be a useful target material for *Agrobacterium*-mediated transformation, since transgenic plants have been regenerated from *Phalaenopsis* using an embryogenic cell suspension culture (Belarmino and Mii, 2000). For both breeding and micropropagation, therefore, establishment of a highly efficient plant regeneration system from embryogenic cell culture is important. In the present paper, we report efficient PLB formation from cell suspension culture by selecting appropriate carbohydrate sources and concentrations.

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## Materials and Methods

Shoot tips were excised from flower stalk buds of *Phalaenopsis* Snow Parade (PP1776), *P.* Wedding Promenade (PP1572), and *Doritaenopsis* New Toyohashi (DTP643), and cultured on New Dogashima medium (NDM) (Tokuhara and Mii, 1993) containing 0.5 μ*M* α-naphthaleneacetic acid (NAA), 4.4 μ*M* 6-benzylaminopurine (BA), 29.2 m*M* sucrose, and 2 g $l^{-1}$ gellan gum (Ina Food Industry Co., Japan) at 23 ± 1°C under 14 h of photoperiod with fluorescent light (Toshiba, FL40SS N/37) at 33 μmol $m^{-2}$ - $s^{-1}$ (Tokuhara and Mii, 2001). Each explant was inoculated onto 15 ml medium in a 24 mm × 100 mm test tube capped with aluminum foil. Four months after initiation of shoot-tip culture, they were transferred onto the same medium but with an increased concentration of sucrose (58.4 m*M*). These cultures were transferred to the same fresh medium at monthly intervals. After 3 mo. of culture, soft yellow calluses were isolated, sieved through a 600 μm stainless steel mesh and 100 mg fresh weight of cell clumps were transferred to a 100 ml Erlenmeyer flask containing 50 ml liquid NDM with the same concentrations of plant growth regulators (PGRs) and sucrose. The flasks were set on a rotary shaker at 80 rpm under the same environmental conditions used for callus production. The cells were

subcultured monthly, in the same medium, by transferring 1 g fresh weight of cells to 50 ml liquid NDM.

To test carbohydrate source on PLB formation, 100 mg fresh weight of cells of *D.* New Toyohashi (DTP643), which had been subcultured in NDM supplemented with 58.4 m*M* sucrose, were transferred into a 90 × 20 mm plastic Petri dish containing 25 ml PGR-free NDM supplemented with 58.4 m*M* of either fructose, glucose, sucrose, maltose, lactose, or sorbitol. The influences of dried yeast (DY; Wako Pure Chemical Industries Ltd., Japan) (0.1, 1.0, 10.0 $g\,l^{-1}$) and BA (0.4, 4.4 μ*M*) on PLB formation were also studied on NDM supplemented with 58.4 m*M* sucrose. Media were solidified with 2 $g\,l^{-1}$ gellan gum and adjusted to pH 5.4 prior to sterilization, then autoclaved for 15 min at 120°C and 105 kPa. After inoculation with cells, the dishes were sealed with Sealon film (Fuji Film Co. Ltd.) and cultured under the same conditions used for callus initiation. Five replicates were made for each treatment. After 4 mo. of culture without any subcultures, cells and PLBs were suspended in distilled water. Then the cell suspension was passed through a 600 μm stainless steel mesh, on which PLBs were collected. The remaining cell suspension was again passed through a filter unit (filter holder with filter bottle, Schleicher and Schuell) to collect the cell clumps on it. The fresh weight of the cells and PLBs thus separated were measured and the number of PLBs was counted under the microscope.

To determine the optimum concentrations of glucose and sucrose on PLB formation, 100 mg fresh weight of cells of *P.* Snow Parade (PP1776) and *P.* Wedding Promenade (PP1572) were cultured on PGR-free NDM supplemented with either carbohydrate source at 2.9, 14.8, 29.2, 58.4, or 87.6 m*M*. Five replicates were used for each treatment and all experiments were repeated three times. After 4 mo. of culture without subculture, the fresh weight of cells was measured and the number of PLBs was counted under the microscope.

For histological investigations, PLBs which were induced from calluses 4 mo. after transferring onto 2 $g\,l^{-1}$ gellan gum-solidified NDM supplemented with 29.2 m*M* sucrose, as well as seed-derived protocorms, were fixed with FAA solution (50% ethanol, 5% acetic acid, and 5% formaldehyde) for 30 min. After fixation, samples were dehydrated with a graded series of ethanol and butanol solutions and then embedded in paraffin. Ten-micrometer sections were made from the paraffin-embedded samples using a rotary microtome. Sections were stained with Delafield's hematoxylin (Mutou Pure Chemicals Ltd., Japan) and observed at ×40 magnification, under a light microscope.

## Results and Discussion

*Plantlet regeneration from cell suspension culture.* Cell suspension cultures could be readily established by transferring the calluses, after sieving with a stainless steel mesh (600 μm), into liquid NDM and culturing for 3 mo., irrespective of the carbohydrate sources (Fig. 1*A*, *B*). After transferring these cells onto gellan gum-solidified medium, some of the cell clumps (Fig. 1*C*) developed into plantlets through the protocorm stage, with the same apparent developmental process as seedlings of *Phalaenopsis* (Wang and Torikata, 1968) (Figs. 1 and 2). Eight weeks after transfer (Figs. 1*D* and 2*A*), the cell clumps developed into globular-shaped embryos, the size of which was almost comparable to that of the zygotic embryo in the seed. At this stage, embryos had no apparent meristematic region, as indicated by Tokuhara and Mii (2001). Subsequently, they developed into PLBs with rod-like structure 1 mo. after globular embryo formation (Fig. 1*E*). Then, they started to produce a depression near the tip of the elongated PLB, and the meristematic region was formed at the depression (Figs. 1*F* and 2*B*). Four months after globular embryo formation, leaves formed at the edge of the depression (Figs. 1*G* and 2*C*) and then roots were produced just below the base of the leaves. Three months later, they grew into plantlets with two to three leaves and three to four roots (Fig. 1*H*). After transferring to pots in the greenhouse at this stage, they successfully grew into almost mature plants about 2 yr after globular embryo formation, as already shown in the previous paper (Tokuhara and Mii, 2001) (Fig. 1*I*).

*Influence of carbohydrate type, DY, and BA on PLB formation.* PLB formation from suspension culture-derived cells of *D.* New Toyohashi (DTP643) was affected by the type of carbohydrate in the medium (Table 1). When the carbohydrate sources tested were added at 58.4 m*M*, glucose gave the highest efficiencies for PLB formation which started 8 wk after the transfer. On media containing maltose, most cells turned from yellow to green within 1 mo. of culture and produced PLBs without callus proliferation. However, the number of PLBs produced was only 8% of that obtained by glucose. Sorbitol resulted in little callus proliferation and a small amount of yellow PLBs which were first observed 8 wk after the transfer, as in the case of glucose. On the other hand, sucrose resulted in high callus proliferation, but almost no PLBs formed. Fructose gave almost the same effect as sucrose but half the proliferation of callus. Lactose was not favorable for either cell proliferation or PLB formation, and induced necrosis of the cells after 2 mo. of culture.

DY also affected both PLB formation and callus proliferation from the cells derived from suspension culture of *D.* New Toyohashi (DTP643) in the presence of 58.4 m*M* sucrose. DY at 0.1 $g\,l^{-1}$ gave twice the proliferation rate of callus as compared with the control, but it did not affect PLB formation (Table 1). DY at 1.0 $g\,l^{-1}$ produced yellow-green callus and the amount of callus was comparable to that obtained at 0.1 $g\,l^{-1}$. However, it slightly increased the number of PLBs as compared with the control and the size of the PLBs was slightly larger than those obtained with other media. DY at 1.0 $g\,l^{-1}$ resulted in the regeneration of several plantlets within 4 mo. of culture. DY at 10 $g\,l^{-1}$ completely inhibited callus growth and PLB formation.

Addition of BA at low concentration (0.4 μ*M*) to the medium containing 58.4 m*M* sucrose increased the proliferation of the callus but completely inhibited PLB formation (Table 1). At a higher concentration (4.4 μ*M*), it almost inhibited callus growth.

In conclusion, glucose was the best carbohydrate source for producing PLBs. In total, 3620 PLBs were induced from only 100 mg of cells after 4 mo. of culture on medium containing 58.4 m*M* glucose without subculturing.

Several reports have shown that the ability of the cells to induce somatic embryogenesis is influenced by the carbohydrate sources used for the culture and that each species should have suitable carbohydrate sources for somatic embryogenesis in *in vitro* culture (Parrott and Bailey, 1993; Sculler and Reuther, 1993). Recently Islam and Ichihashi (1999) examined the effect of three carbohydrate sources on PLB formation and callus growth from embryogenic callus in *Phalaenopsis* and demonstrated that sucrose was suitable for callus proliferation, maltose for PLB proliferation, and sorbitol for PLB growth. Although similar effects of sucrose and maltose were observed in the present study, glucose was shown to be the optimum carbohydrate source for somatic embryogenesis by PLB formation from cell suspension culture of phalaenopsis orchid.

For tissue culture of orchid, many kinds of natural substances, such as fruit juices and coconut milk, have been used for stimulating the growth of PLBs and plantlets *in vitro* (Arditti and Ernst, 1993). In the present study, growth of PLBs was stimulated by the low concentration (1 $g\,l^{-1}$) of DY added to the media (Table 1). DY is not same as yeast extract, which is commonly used for plant tissue cultures, because it is not hydrolyzed. However, DY

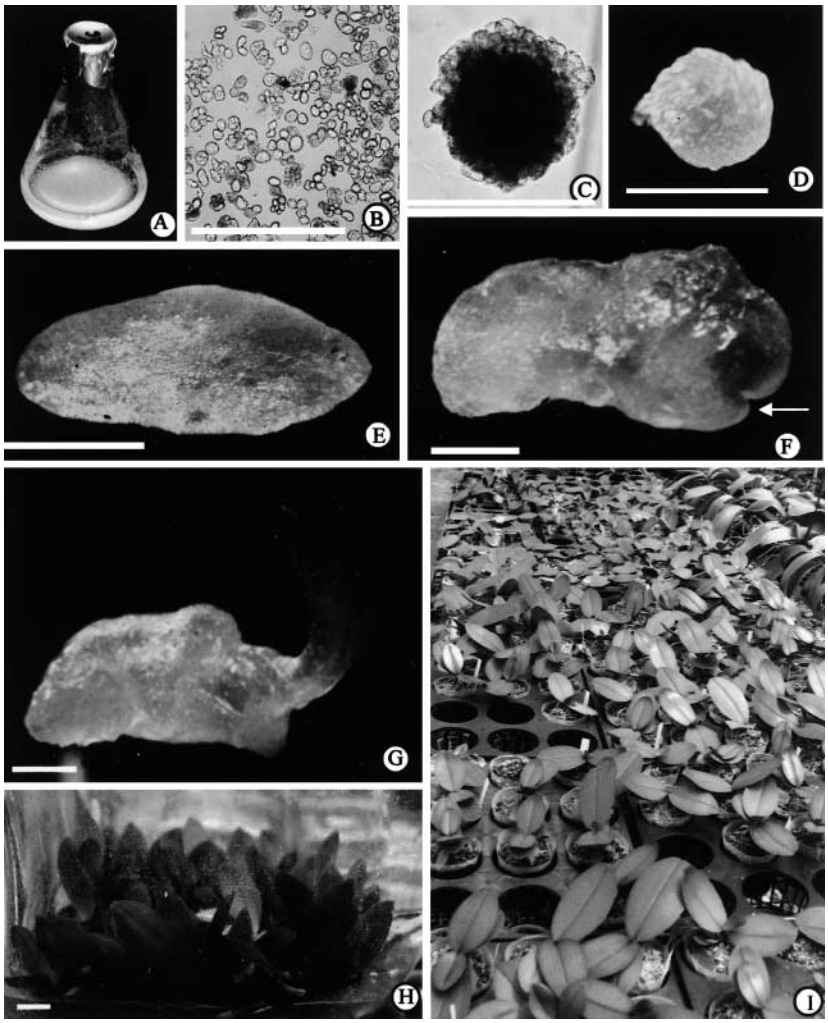


FIG. 1. Plant regeneration from suspension cells of phalaenopsis orchid. *A*, Suspension culture after 3 mo. *B*, Embryogenic cells. *C*, Cell clumps. *D*, PLB with globular-shaped embryo. *E*, PLB elongated like a rod. *F*, PLB with flat protuberance at the upper part and with a depression at the tip of the major axis (*arrow*). *G*, PLB with leaf development. *H*, Plants derived from embryogenic cells 3 mo. after emergence of the first leaf. *I*, Plants with leaves 10–15 cm long derived from embryogenic cells about 2 yr after globular embryo formation. *Bar* = 1 mm (*B–G*); 1 cm (*H*).

contains a comparable amount of undefined amino acids, peptides, vitamins, and plant growth substances which might have affected the development and growth of callus and PLBs, as suggested by George (1993).

*Effects of glucose and sucrose concentrations on callus proliferation and PLB formation.* Concentrations of sucrose and glucose affected both callus proliferation and PLB formation from the suspension culture-derived cells in both genotypes, *P.* Snow

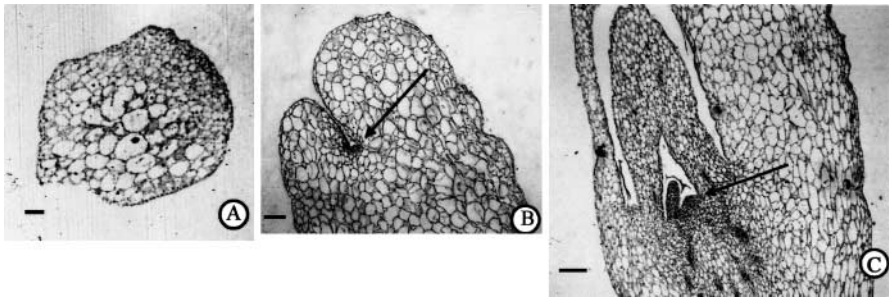


FIG. 2. Histological observation on shoot development from callus-derived PLB of phalaenopsis orchid. A, PLB at the stage shown in Fig. 1*D*. *B*, Elongated PLB at the stage shown in Fig. 1*F* (*arrow* shows merismatic region). *C*, Fully developed shoot tip with meristem (*arrow*) and some leaf primordia at the stage shown in Fig. 1*G*. *Bars* = 0.1 mm.

TABLE 1

EFFECT OF VARIOUS CARBOHYDRATES, DRIED YEAST, AND BA ON GROWTH AND PLB FORMATION OF EMBRYOGENIC CELLS OF *DORITAENOPSIS* NEW TOYOHASHI (DTP643) AFTER 4 MO. OF CULTURE ON GELLAN GUM MEDIA WITHOUT SUBCULTURE

| Carbohydrate (58.4 m*M*) | Dried yeast (g $l^{-1}$) | BA ($\mu M$) | Fresh weight[a] (mg) | No. of PLBs | Fresh weight of PLBs (mg) |
|---|---|---|---|---|---|
| Glucose | − | − | 704.6 ± 90.7 | 3620.0 ± 295.7 | 1452.2 ± 194.0 |
| Fructose | − | − | 346.9 ± 112.5 | 2.6 ± 1.3 | 6.6 ± 3.5 |
| Maltose | − | − | 69.0 ± 11.2 | 286.8 ± 48.3 | 52.8 ± 11.9 |
| Lactose | − | − | 4.4 ± 2.6 | 2.4 ± 1.5 | 1.3 ± 0.8 |
| Sorbitol | − | − | 77.8 ± 12.1 | 103.4 ± 36.1 | 21.8 ± 8.7 |
| Sucrose | − | − | 657.4 ± 56.1 | 1.6 ± 1.0 | 0.4 ± 0.4 |
| Sucrose | 0.1 | − | 1366.0 ± 136.6 | 3.8 ± 1.5 | 2.0 ± 0.8 |
| Sucrose | 1.0 | − | 1293.4 ± 164.2 | 26.4 ± 8.9 | 211.4 ± 75.4 |
| Sucrose | 10.0 | − | 50.5 ± 2.2 | 0 | 0 |
| Sucrose | − | 0.4 | 1018.3 ± 96.8 | 0 | 0 |
| Sucrose | − | 4.4 | 190.0 ± 43.5 | 0 | 0 |

Each value (± SE) represents the average of the data obtained from five replicates.
[a] Initial fresh weight of cells was 100 mg. Weight of non-necrotic cells, excluding PLBs, was measured.

Parade (PP1776) and *P.* Wedding Promenade (PP1572), examined (Figs. 3 and 4). Fresh weight of cells excluding that of PLBs increased with an increase in the concentrations of sucrose and glucose in both genotypes, except that growth of the cells of *P.* Wedding Promenade (PP1572) in Fig. 4 was inhibited at the highest concentration (87.6 m*M*) of sucrose tested. For *P.* Snow Parade the carbohydrate sucrose gave the greatest fresh weight (Fig. 3), while for *P.* Wedding Promenade glucose was the best. On the other hand, optimum concentrations of sucrose and glucose for PLB formation were different between these two genotypes. The number of PLBs produced was the highest at 58.4 m*M* for glucose and 29.2 m*M* for sucrose, respectively, in *P.* Snow Parade (PP1776) (Fig. 4), whereas the highest number of PLBs (more than 1500) was obtained at 14.6–29.2 m*M* glucose and 14.6 m*M* sucrose, in *P.* Wedding Promenade (PP1572). These results suggest that sucrose may have the ability to induce embryogenesis at about half the level of glucose. A similar result was also obtained in shoot regeneration from leaf pieces of *Solanum melongena* (Mukherjee et al., 1991). In contrast, high concentrations of sucrose (58.4 m*M*) and glucose (87.6 m*M*) resulted in callus proliferation. The data obtained in this study indicate that the morphogenetic response of the cells could be greatly modified with the concentrations as well as the kind of carbon source. When sucrose is supplied to the medium, it is broken down into monosaccharides (glucose and fructose) by the release of extracellular enzyme during the *in vitro* culture (George, 1993), which, in turn, increases the osmotic pressure in the medium. Therefore, it is necessary to note that differences in the effects of glucose and sucrose on PLB formation and callus proliferation observed in our study might be caused not only by the difference in the sugar composition during the culture, but also by changes in the osmotic pressure by the breakdown of sucrose into monosaccharides. Consequently, the nature of breakdown products and osmolarity change need to be considered when disaccharides are supplied in the medium.

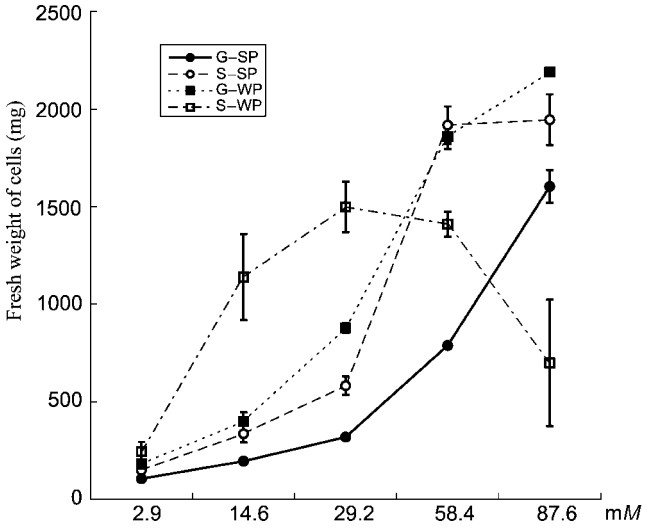


FIG. 3. Effects of glucose and sucrose concentrations on proliferation of embryogenic cells in *Phalaenopsis* Snow Parade (SP, PP1776) and *P.* Wedding Promenade (WP, PP1572). G, glucose; S, sucrose. *Bars* = standard error.

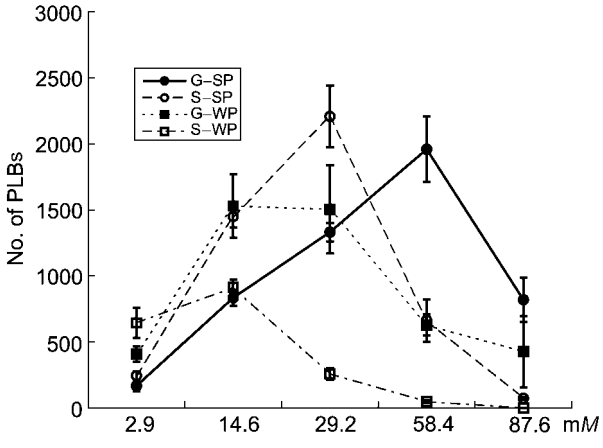


FIG. 4. Effects of glucose and sucrose concentrations on PLB formation derived from suspension cells of *Phalaenopsis* Snow Parade (SP, PP1776) and *P.* Wedding Promenade (WP, PP1572). G, glucose; S, sucrose. *Bars* = standard error.

Our present study shows that development of PLBs from embryogenic cell suspension culture of *Phalaenopsis* can be controlled by simply changing the concentration of glucose or sucrose. PLBs thus obtained further developed into plantlets without any trouble. Therefore, these simple methods would enable us to obtain numerous plantlets from cultured fine cells (Tokuhara and Mii, 2001), which are efficiently utilized for micropropagation and *Agrobacterium*-mediated transformation.

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