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In vitro flowering and shoot multiplication of *Gentiana triflora* in air-lift bioreactor cultures

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Abstract

Hormonal control of flower induction *in vitro* was investigated in Gentian. The effect of PBZ concentrations on flowering was studied in plantlets cultured in MS medium containing 30 g L^{-1}sucrose. Paclobutrazol (PBZ) concentration at 1.0 mg L^{-1} induced the highest flowering in terms of flowering percentage (91.5%), number of flowers, days to first flowering, flower length and flower diameter. PBZ did not trigger flowering but it rather stimulated flowering and its role seemed to be additive but not essential for flowering. Comparison between solid and bioreactor cultures (continuous immersion with a net) revealed that shoot multiplication and growth were more efficient using bioreactor culture. The highest shoot number per explant (29.9) was obtained in bioreactor culture. Regenerated shoots were cultured microponically for 6 weeks. Hundred percent of plants rooted and were acclimatized successfully in growing media containing perlite: vermiculite (1:1).

Key words: Bioreactor, *in vitro* flowering, Gentian, mass propagation, microponics

Introduction

Gentians (*Gentiana* spp.) are herbaceous perennial plants that are highly valued in the world market particularly in Japan as cut flowers. Flowering season of cut gentians lasts for only three months a year. *In vitro* flowers can, therefore, shorten the breeding period (Kumar *et al.*, 1995) and aid basic studies of the flowers throughout the year without the usual limitation of the season in the field. *In vitro* flowering of gentian has been reported and flowers were found to be similar to those of garden-grown plants in many aspects (Zhang and Leung 2000; 2002). Tissue culture techniques have important applications in flowering studies because a single parameter can be assayed *in vitro* to study its effect on flowering. Now, it is widely accepted that flowering results from subtle interactions of various promoters and inhibitors (Taiz and Zeiger, 2002). Growth retardants, among other plant hormones, are thought to play an essential role in the floral development of plants (Teixeira da Silva and Tan Nhut, 2003; Sudhakaran *et al.*, 2006; Taylor and van Staden, 2006). Exogenously applied paclobutrazol induce flowering in many plant species *e.g.*, *Euphorbia millii* (Dewir *et al.*, 2006a), Dendrobium (Wang *et al.*, 2006) and *Saposhnikovia divaricata* (Qiao *et al.*, 2008). It has been shown that flower-inducing or flower-inhibiting effect of paclobutrazol depends on its concentration (Dewir *et al.*, 2006a).

In vitro plant propagation methods have been developed for some *Gentiana* species (Ivana *et al.*, 1997; Lamproye *et al.*, 1987; Morgan *et al.*, 1997; Sharma *et al.*, 1993; Viola and Franz, 1989; Yamada *et al.*, 1991). Previous report on micropropagation of *Gentiana triflora* investigated the influences of several factors including the position of the explant, requirements for sucrose, cytokinin or GA_3, variations of pH and photosynthetic photon flux density (PPFD) on *in vitro* shoot development (Zhang and Leung, 2002). Bioreactor culture is a promising and efficient method for plant propagation. As compared to conventional tissue culture techniques using solid or semi-solid medium, bioreactors require fewer culture vessels, less labour, utilities and space. The aim of bioreactor application is to achieve either maximum yield, high quality of propagules, or to keep the production costs as low as possible by integrating automated facilities and simple low cost devices. The use of the bioreactor for micropropagation was first reported in 1981 for begonia propagation (Takayama and Misawa, 1981). Since then it has proved applicable to many species including shoot, bulbs, microtubers, corms and somatic embryos (Paek *et al.*, 2005).

In the present study, we investigated the effects of paclobutrazol (PBZ) as a growth retardant on flower induction of Gentian. We also applied the bioreactor culture system and microponics for the mass propagation of this important ornamental plant.

Materials and methods

Plant material: *In vitro* shoots of Gentian (*Gentiana triflora* Pall. var. axillariflora Akita Blue) were multiplied on MS (Murashige and Skoog, 1962) semisolid medium (30 g L^{-1}sucrose + 8.0 g L^{-1} agar supplemented with 1.0 mg L^{-1}BA) at 25 °C under a 16 h photoperiod (35 µmol m^{-2} s^{-1} PPFD). These cultures were sub-cultured and maintained on the same medium for one year. For the current experiments, shoots were individually separated and cultured on MS medium without growth regulators for 2 weeks and these shoots (3-4 cm in length at the 5-7 leaf-stage) were used as a plant material.

***In vitro* flowering in solid culture**: Young shoots of Gentian were cultured on MS medium supplemented with 3% (w/v) sucrose and different concentrations of PBZ at 0, 0.5, 1.0, 2.0, 4.0 and 6.0 mg L^{-1}. All media were solidified with 0.8% (w/v) Sigma agar-agar. The pH of the medium was adjusted to 5.7 (using Beckman 340 pH/Temp. Meter, USA) before autoclaving at 121°C (1.2 kg cm^{-2})

for 30 min. Each treatment contained five replicates and each replicate was represented by a cylindrical culture vessel (120 mL capacity) containing 25 mL MS medium with 4 shoots rendering a group of 20 shoots per treatment. MS medium supplemented with only 30 g L^{-1}sucrose was used as a control (non-flowering medium). The cultures were incubated under a 16 h photoperiod (35 µmol m^{-2} s^{-1} PPFD from cool-white fluorescent lamps) at 25 ± 2 °C. Observations on flowering, opened flowers, days to first flowering, number of flowers per explants, flower length and diameter, number of shoots per explants, shoot length and fresh and dry weights were recorded after 12 weeks of culture.

Shoot multiplication in solid/bioreactor culture: For shoot multiplication, double strength WPM medium (Lloyd and McCown, 1980) was supplemented with 0.5 mg L^{-1}BA and 30 g L^{-1}sucrose as optimal conditions (Zhang and Leung, 2002). For solid culture, Gentian shoots (4 per culture bottle) were inoculated to cylindrical culture bottle (500 mL capacity) containing 150 mL double strength WPM medium supplemented with 0.5 mg L^{-1}BA, 3% sucrose and solidified with 0.8% (w/v) Sigma agar-agar. The experiment contained fifteen replicates and each replicate was represented by a cylindrical culture bottle with 4 shoots rendering a group of 60 shoots. The pH of the medium was adjusted to 5.7 (using Beckman 340 pH/ Temp. Meter, USA) before autoclaving at 121 °C (1.2 kg cm^{-2}) for 30 min. The cultures were incubated under a 16 h photoperiod (40 µmol m^{-2} s^{-1} PPFD from cool-white fluorescent lamps) at 25 ± 2 °C. For bioreactor culture, a continuous immersion bioreactor culture system was used. A supporting net was used to hold the plant material in order to avoid the complete submersion of explants in the liquid medium. The volume of input air was adjusted to 0.1 vvm (volume per volume per min) using air-flow meter. Sixty shoots were transferred to 5 L balloon-type bubble bioreactor (BTBB) with 2 L of double strength WPM liquid medium supplemented with 0.5 mg L^{-1}BA and 3% sucrose. The layout of BTBB is shown in Fig. 1. The bioreactors were incubated under the same culture conditions as solid culture. After 6 weeks of culture, 20 explants were randomly selected and observation on the number of shoots, shoot height and fresh weight were recorded. Dry weight was measured after drying the shoots for 48 h at 60°C. The remaining shoot clumps from bioreactor culture were served as plant material for rooting experiments.

Microponics culture: Shoots (3-5 cm) at the 6-8 leaf stage, after being harvested from the bioreactor, were transplanted into plug trays {3.3 cm × 4.1 cm; 20 cells per tray (15.2 cm × 22.5 cm)} filled with a mixture of perlite and vermiculite (1:1, v/v). Cultured trays were placed in an individual container to supply the plants with nutrient solution. The nutrient solution contained half strength of MS (Murashige and Skoog, 1962) basal salts. The pH and the EC of the nutrient solution were adjusted to 5.8 and 1.2 dS m^{-1} using a pH and EC controller (HM-20E, and CM-20E, TOA, Tokyo, Japan). The nutrient solutions were drained off weekly and replaced with fresh solutions. Air was supplied through air pump to the nutrient solution at 0 and 0.1 vvm (volume per volume per min) using air-flow meter. Each individual container was covered with a clear plastic lid for the first 10 days thereafter the lid was removed. The environments in the growth chamber were adjusted to a 25 ± 2 °C air temperature, 40-50% relative humidity and a 100 µmol m^{-2} s^{-1} photosynthetic photon flux (PPF) with a 16 h photoperiod using cool-white fluorescent lamps. Observation on the rooting percentage, number of roots, shoot height and fresh weight were recorded after 4 weeks of culture and dry weight was measured after drying the shoots for 48 h at 60°C.

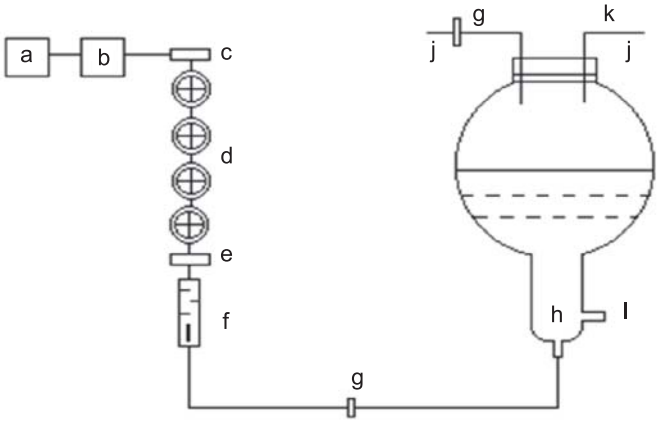

Fig. 1. Schematic diagram of an airlift bioreactor. a: air compressor, b: air reservoir, c: air cooling device, d: air filter system, e: air dryer, f: air flow meter, g: membrane filter, h: glass sparger, i: medium feeding port, j: vent, k: pre filter.

Statistical analysis: Each experiment was carried out under a completely randomized design with three replications and repeated twice. The data were subjected to ANOVA, LSD using SAS program (Version 6.12, SAS Institute Inc., Cary, USA) and Student's unpaired *t*-test, and the treatment mean values were compared at $P \leq 0.05 - 0.001$.

Results and discussion

Effects of PBZ on flowering and growth: PBZ had a florigenic effect on the apical meristems of Gentian plantlets in tissue culture (Fig. 2 A, B). Table 1 shows that PBZ concentration at 1.0 mg L^{-1}induced the highest flowering in terms of flowering percentage (91.5%), number of flowers, days to first flowering, flower length

Table 1. Effect of paclobutrazol on flower bud formation and vegetative growth of Gentian after 12 weeks in agar-solidified culture

PBZ (mg L^{-1})	Flowering (%)	Opened flowers %	No. of Flowers/ explant	Days to first flowering	Flower length (cm)	Flower diameter (cm^2)	No. of shoots/ explant	Shoot length (cm)	Fresh weight (mg)/ explant	Dry weight (mg)/ explant
0.0	58.3 ± 1.51	20.8 ± 1.85	1.0 ± 0.00	56 ± 0.34	1.7 ± 0.08	1.0 ± 0.02	3.6 ± 0.14	11.9 ± 0.46	1734 ± 123	284 ± 17
0.5	74.5 ± 2.52	26.5 ± 1.26	1.8 ± 0.24	37 ± 0.67	3.8 ± 0.22	1.1 ± 0.07	2.7 ± 0.18	7.5 ± 0.09	754 ± 44	128 ± 5
1.0	91.5 ± 1.49	91.5 ± 1.49	1.5 ± 0.14	28 ± 1.00	5.0 ± 0.20	2.0 ± 0.14	1.6 ± 0.08	6.7 ± 0.14	968 ± 14	155 ± 2
2.0	91.5 ± 1.49	33.2 ± 1.02	1.0 ± 0.00	35 ± 1.30	3.5 ± 0.20	1.2 ±0.03	1.4 ± 0.11	5.6 ± 0.19	746 ± 42	110 ± 5
4.0	33.0 ± 2.04	0.0 ± 0.00	1.0 ± 0.00	42 ± 0.67	1.7 ± 0.12	0.0 ± 0.00	1.0 ± 0.00	4.0 ± 0.12	446 ± 30	69 ± 2
6.0	8.3 ± 1.41	0.0 ± 0.00	0.3 ± 0.13	63 ± 2.00	0.6 ± 0.28	0.0 ± 0.00	1.0 ± 0.00	2.9 ± 0.09	282 ± 11	53 ± 3
LSD	6.83	5.99	0.60	4.79	0.97	0.33	0.89	1.12	336	46
Significance	**	**	**	***	***	***	***	***	***	***

Data presented are means ± SE. ** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively

Table 2. Effect of culture type on shoot multiplication and vegetative growth of Gentian after 6 weeks in culture

Shoot multiplication and growth characteristics	Culture type	
	Solid culture	Bioreactor culture
Number of shoot/ explant	5.1 ± 0.28	29.9 ± 1.79***
Length of main shoot (cm)/explant	3.3 ± 0.05	4.5 ± 0.09**
Fresh weight (g) / explant	0.523 ± 0.04	2.528 ± 0.12***
Dry weight (g) / explants	0.055 ± 0.01	0.282 ± 0.01***

Data presented are means ± SE. ** and *** = significantly different at $P \leq 0.01$ and $P \leq 0.001$, respectively, according to Student's unpaired *t*-test.

Table 3. Effect of air supply on rooting and vegetative growth of Gentian after 6 weeks in microponics culture

Rooting and growth characteristics	Air supply (vvm)	
	0.0	0.1
Rooting (%)	100 ± 0.00	88 ± 0.94**
Number of root/ plantlet	24.3 ± 0.93	9.3 ± 0.38 **
Length of main root (cm) / plantlet	1.25 ± 0.05	0.95 ± 0.03 NS
Length of main shoot (cm) / plantlet	4.9 ± 0.16	3.9 ± 0.17 NS
Fresh weight (g) / plantlet	1.523 ± 0.07	0.437 ± 0.06**
Dry weight (g) / plantlet	0.520 ± 0.04	0.103 ± 0.01**

Data presented are means ± SE. NS= non significant and ** = significantly different at $P \leq 0.01$ according to Student's unpaired *t*-test.

and flower diameter. The percentage of opened flowers and flower size were influenced by PBZ concentration. PBZ at 1.0 mg L^{-1} was optimal for flowering since the highest percentage of opened flowers (91.5%) was obtained at this concerntations. Higher concentrations of PBZ (>2.0 mg L^{-1}) reduced flowering and were associated with abnormal growth. The transition to flowering was earlier and faster in PBZ treated plantlets. However, there was a trend towards decreasing shoot length and fresh and dry weight with increasing PBZ concentration. Plantlets grown in PBZ free medium formed flower buds (58.3%) but large number of these flowers failed to open (Fig. 2 C). The above results indicate that Gentian plantlets were able to form flower buds without PBZ treatment. This indicates that PBZ does not trigger flowering but it rather stimulate flowering and its role seems to be additive but not essential for flowering. It is well established that during the change from the vegetative to the flowering state, the growth correlations within the apical meristem of a shoot are changed, which leads to loss of apical dominance (Pidkowich *et al.*, 1999). It is also reported that the mature state of the plantlets is usually a prerequisite for *in vitro* flowering and juvenility does not allow for flower production due to inability to produce a flowering factor or the inability of active meristems to respond to a flowering factor (Hackett, 1985). Very few studies have been conducted to investigate the effects of PBZ on *in vitro* flowering. PBZ induced *in vitro* flowering of *Euphorbia millii* and the response of flowering depended on the concentration of PBZ in the culture medium (Dewir *et al.*, 2006). PBZ also promoted *in vitro* flowering in *Dendrobium* (Wang *et al.*, 2006) and in *Cymbidium* (Zheng and Pang, 2006). In *Saposhnikovia divaricata*, low concentration of PBZ or ethephon promoted *in vitro* flowering of up to 26 or 21%, respectively (Qiao *et al.*, 2008). On the other hand, it has been reported that application of PBZ with N^6-benzyladenine (BA) totally abolished the promotive effect of cytokinin in flower induction of *Cymbidium niveo-marginatum* orchid *in vitro* (Kostenyuk *et al.*, 1999). PBZ, a widely used plant growth retardant in potted ornamental crops, provides height control in several floriculture crops (Prusakova *et al.*, 2004). *In vitro* studies showed that PBZ which prevents gibberellin biosynthesis, stimulate production of reproductive part at the expense of vegetative plant growth.

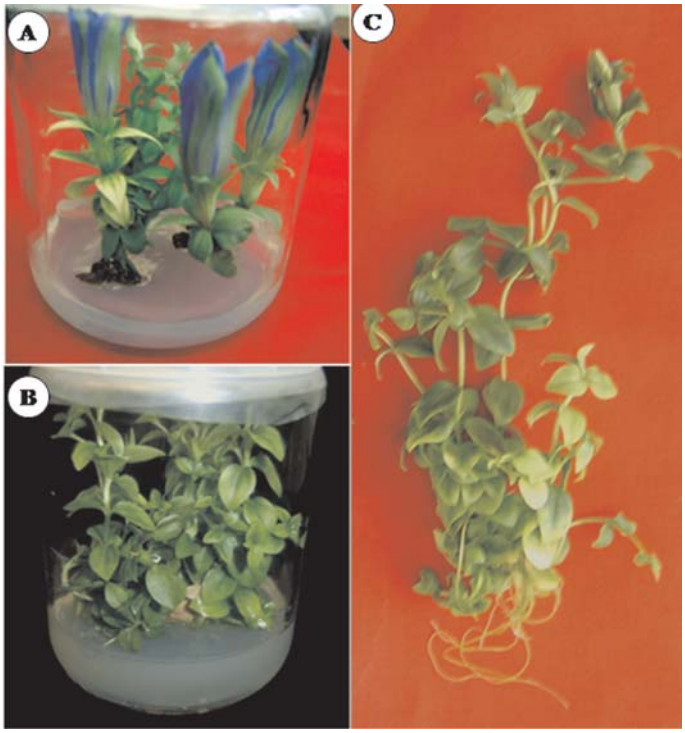

Fig. 2. *In vitro* flowering of Gentian plantlets. A and B) Flowering and non-flowering plantlets after 12 weeks' culture. C) Plantlets forming flower buds but failed to open after 12 week's culture.

Shoot multiplication and growth in solid/bioreactor culture: Comparative studies between solid and bioreactor culture (continuous immersion with net) revealed that shoot multiplication was more efficient in bioreactor culture (Table 2). Number of shoots, length of main shoot and fresh and dry weight significantly increased in bioreactor culture. The highest shoot

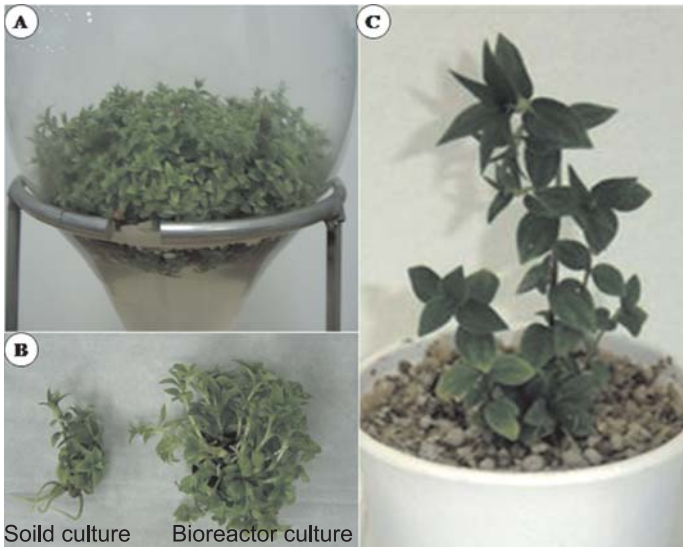

Fig. 3. Shoot multiplication and acclimatization of Gentian plantlets. A) Shoot multiplication after 6 weeks' culture in an air-lift bioreactor. B) Shoot clump developed in solid and bioreactor cultures. C) Acclimatized plantlet.

number per explant (29.9) was obtained in bioreactor indicating that bioreactor culture clearly stimulated shoot proliferation of Gentian (Fig. 3 A, B). In the case of immersion system with net, the basal area of shoots was continuously in contact with the medium, thus enabling a constant supply of nutrients as well as constant aeration to explants, leading to plantlet growth. Micropropagation by axillary or shoot meristem proliferation is typically labour-intensive methods of producing elite clones, but recently the adaptation of air-lift BTBB immersion bioreactors for propagation of shoots and bud-clusters has provided a workable technique for large-scale transplant production. Large-scale propagation using bioreactors has been applied to several horticultural and medicinal plants (Dewir *et al.*, 2005b, 2006b; Paek *et al.*, 2005; Jo *et al.*, 2008)

Rooting and acclimatization in microponics: After harvest from the bioreactor, shoots (3-5 cm) at the 6-8 leaf stage were microponically cultured for 30 d and 100% of plantlets rooted and acclimatized successfully (Fig. 3C). Plantlets grown in microponics without air supply had the highest rooting percentage, root number, and fresh and dry weights (Table 3). Air supplement in microponics usually improves rooting providing suitable aeration to the root system. However, in the present study, air supplement had a deleterious effect on Gentian rooting. The use of perlite and vermiculite as rooting substrate provided suitable aeration and more air supplement was unnecessary. Recently, because of the small dimensions of cultivated plants, a microponic culture system combining micropropagation with hydroponics has been applied to a number of horticultural plants (Chakrabarty *et al.*, 2003; Dewir *et al.*, 2005a, 2005b). The enhanced plantlet growth, rooting and *ex vitro* acclimatization rates prove that microponic culturing is a feasible system for efficient transition from *in vitro* to *ex vitro* acclimatization.

The immersion-type bioreactor (with net) system is a valuable option for Gentian micropropagation. Rapid and efficient multiplication rate, *ex vitro* rooting in microponics and successful transfer of plantlets to the greenhouse makes this protocol suitable for large-scale propagation of this important floricultural crop.

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