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Unsuccessful *in vitro* regeneration from *Phalaenopsis* (Orchidaceae) flowers

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Abstract: Regeneration *in vitro* has never been achieved from flower parts from members of the Orchidaceae and no report exists for *Phalaenopsis*. Studies that do exist all used immature (unopened) flower buds. In a bid to overcome this hurdle, *in vitro* organogenesis was attempted with various parts (dorsal sepal (base + center + tip); petal (base + center + tip); lateral sepal (base + center + tip); labellum (base + center + tip); pedicel (outer + inner tissue); column; anther cap; stigmatic surface) of immature and fully opened *Phalaenopsis* Gallant Beau 'George Vazquez' flowers. Despite the use of an extremely wide selection of plant growth regulators in Vacin and Went basal medium, and their concentrations, in both darkness and light conditions, no organogenesis could be achieved from any of the flower parts tested. Some callus was obtained on the column in response to 2 or 4 mg/l TDZ in the light, or to 1 mg/l 2,4-D and 1 mg/l dicamba in the dark, but the callus was hard and could not be further proliferated or induced to form organs. Although negative, these trials provide encouraging signs that organogenesis may be possible from flower parts with a little more insistence. To achieve such regeneration could allow for the generation of haploid or polyploidy plants, which may have practical use in orchid breeding and biotechnology if such plants (or plant parts) can be clonally propagated.

Keywords: anther cap; column; flower, labellum; Orchidaceae; pedicel; petal; sepal; stigmatic surface

Introduction

Phalaenopsis (Orchidaceae) species and hybrids are one of the most sought-after orchids on the global floriculture market, and for which well developed *in vitro* protocols exist [1,2]. Most of those protocols involve protocorm-like bodies (PLBs), *de facto* somatic embryos within the orchids [3], while a few others have employed thin cell layers (TCLs) [4]. PLBs and other regenerable parts of orchids *in vitro* can demonstrate different levels of (endo)polyploidy [5]. A recent study indicates how some parts of *Phalaenopsis* flowers contain some low levels of endopolyploidy [6]. Despite the extensive literature on the *in vitro* tissue culture of *Phalaenopsis*, no studies have been published on the *in vitro* regeneration from flowers although a few studies exist for some other members of the Orchidaceae, the largest family in the plant kingdom. In a bid to challenge this unbreakable record and to advance our basic knowledge on the *in vitro* regeneration of *Phalaenopsis*, this study aimed to attempt the regeneration from different parts of flowers of a well-studied *Phalaenopsis* hybrid, *P.* Gallant Beau 'George Vazquez', for which established tissue culture protocols exist (e.g., [7]). In general, media with low levels of micro- and macronutrients best support the growth and development of PLBs, including Vacin and Went (VW) medium [8], although nutrient-rich media also allow for PLB induction and proliferation, making the choice of basal medium difficult to make unless rigorous genotype-independent testing is done. Nonetheless, based on a survey of the wider *Phalaenopsis in vitro* literature, it was decided that the basal medium would be VW medium.

There are a few reports about regeneration *in vitro* from orchid flower tissues, including in *Oncidium* [9,10,11] and *Cymbidium sinense* [12,13]. Chen (1989) reported that PLBs could be induced from young floral buds of *Oncidium* Gower Ramsey on Murashige and Skoog (MS; [14]) medium supplemented with 3.0 mg/l 6-benzyladenine (BA) [9]. Santana and Chaparro (1999) reported that PLBs could be induced

from floral buds of immature inflorescences of *O*. Gower Ramsey on Knudson C [15] medium supplemented with 1 mg/l α-naphthaleneacetic acid (NAA), 120 ml/l pineapple juice, and 100 ml/l fruit pulp from green or ripe bananas [10]. PLBs could be multiplied and plantlets formed in liquid MS medium supplemented with 0.5 mg/l NAA and 5.0 mg/l BA and the growth rate of PLBs was 1.8 every 4 weeks. Abnormal PLBs formed in the presence of 1 mg/l NAA and 10 mg/l BA. Cui et al. (2004) reported that shoot buds could be induced in MS containing 4.0 mg/l BA, 0.5 mg/l NAA and 3% sucrose from primary flower buds of *O.* Kinse [11]. The best culture medium for shoot propagation was MS with 2.0-4.0 mg/l BA, 0.5 mg/l NAA and 3% sucrose. Zhang and Ou (1995) reported that PLBs could be induced from *C. sinense* flower primordia on modified 1/2 MS medium supplemented with 2 mg/l NAA, 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l BA, 2 g/l activated charcoal (AC) and 80 mg/l rutoside [12]. Zeng et al. (1998) reported the induction of PLBs from floral buds of immature inflorescences of *C. sinense* and its hybrid *C. sinense* X *C. eburneum* and *C. sinense* X *C. tracyanum* on MS medium supplemented with 0.5 mg/l NAA, 0.5 mg/l BA and 0.5 g/l AC [13]. The PLBs from flower primordia or from floral buds then slowly formed rhizomes and developed into plantlets when subcultured in the same medium. However, all of these studies used floral buds (immature, unopened flowers) as the starting material. To date, not a single study of the Orchidaceae has successfully reported on PLB induction from individual floral organ tissue.

This study aimed to induce regeneration and organogenesis *in vitro* of different parts of *Phalaenopsis* flowers, which have previously shown to be actively dividing cells, with some level of endopolyploidy [6]. This is important for two reasons. First**,** as a basal exploratory science, this has never been achieved, and thus needs to be explored. Second**,** if it were to be achieved, then it could be extremely useful for examination, using a flower-based protocol, flower development or *in vitro* flower induction. The benefits of having a flower-based regeneration protocol can already be seen for chrysanthemum, another popular ornamental plant [16]. Flow cytometric analyses using the protocol of Teixeira da Silva and Tanaka (2006) [5] and Teixeira da Silva et al. (2014) [6] indicated that polyploidy existed in some flower tissues (greenhouse) (for example 8C in the column; Fig. 1). In addition, the flowers of *P.* Gallant Beau 'George Vazquez' had higher levels of 4C cells than the flowers of other orchids tested; therefore, the existence of actively growing tissue indicated that such tissue would be an excellent potential target for possible regeneration *in vitro*.

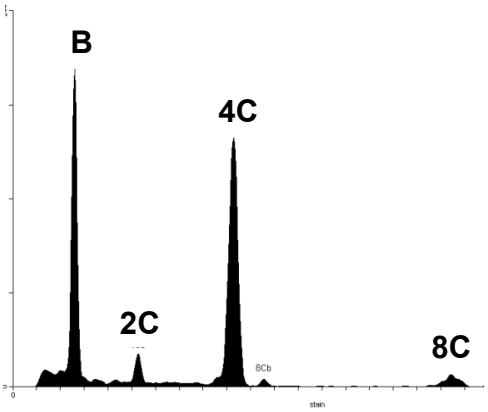

Figure 1. Ploidy levels in the *Phalaenopsis* Gallant Beau 'George Vazquez' flower column (I). B = barley (*Hordeum vulgare* L.) cv. 'Ryufu' (internal standard) Three samples were measured, and relative fluorescence intensity of the nuclei was analyzed when the coefficient of variation was < 3%. A minimum of 5000 nuclei were counted for any sample. Y-axis = number of nuclei.

Materials and Methods

Reagents, plant material and culture conditions

All chemicals and reagents, including PGRs, were of the highest analytical grade available and were purchased from Sigma-Aldrich (St. Louis, USA), Wako Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan). *P.* Gallant Beau 'George Vazquez' flowers were used. The first explant was immature (unopened) flower buds (1 cm long, 0.5 cm in diameter). In addition, various parts of fully-opened flowers were used: dorsal sepal (base + center + tip); petal (base + center + tip); lateral sepal (base + center + tip); labellum (base + center + tip); pedicel (outer + inner tissue); column; anther cap; stigmatic surface. In total, over 500 flowers from approximately 50 flower stalks (equivalent to 50 potted plants) were collected within a single flowering season. Not all flowers were used since some showed symptoms of senescence. All flowers on a flower stalk, representing younger flowers at the terminal base, ageing towards the pot-end of the flower stalk, were used and flower parts of all ages were pooled.

No established protocol was used although some conditions such as the choice of VW basal medium, the medium pH or the autoclaving conditions were based on widely employed parameters in the orchid tissue culture literature [1,2]. Basal medium throughout all trials was VW solidified with 8 g/l Bacto agar (Difco Labs., USA), 3% (w/v) sucrose, pH 5.3, which was adjusted with 1 N NaOH or HCL prior to autoclaving at 100 KPa for 17 min. All cultures were placed in the light (16-h photoperiod with a light intensity of 45 $\mu mol/m^2/s$ provided by 60 W plant growth fluorescent lamps; Homo Lux, Matsushita Electric Industrial Co., Japan) or in

the dark. Both light and dark cultures were placed at 25°C. All plant parts were arranged as 5 per Petri dish (Falcon, USA). Organogenesis and/or the response of cultures were monitored every 15 days until a maximum of 90 days.

Plant growth regulators tested

In vitro regeneration from flower tissue was attempted. At first, whole flowers (immature unopened buds or fully opened – 10 days after opening – flowers) were cut from pedicels with a sterilized (80% ethanol) blade and carried to the laboratory immediately and subjected to surface sterilization within 5 min to avoid oxidation of tissue. *P.* Gallant Beau 'George Vazquez' flowers only once a year (although a second bloom can be induced, although flowers are poor quality and irregular size). Flowers were washed in running tap water for 10 min. They were then washed in soapy water for 5 min. Whole flowers were then dipped in a solution of sodium hypochlorite (1% active chlorine) containing 2-3 drops of Tween-20 for 1 min then dipped directly into 80% ethanol for < 5 sec. Whole flowers were rinsed in three washes of sterile distilled water on a laminar flow bench and flower parts, as indicated above, were dissected for tissue culture. Tissues that showed browning, or symptoms of damage or senescence, were discarded. A range of common plant growth regulators (PGRs), applied singly in three concentrations each (1, 2, 4 mg/l) was used to assess the ability to induce organogenesis from different flower parts. Control treatments contained no PGRs.

Three broad groups were tested (at 0, 1, 2, and 4 mg/l each: auxins (3,6-dichloro-2-methoxybenzoic acid, dicamba (pesticide that mimics an auxin); 2,4-D (pesticide that mimics an auxin); indole-3-acetic acid, IAA; indole-3-butyric acid, IBA; NAA; cytokinins (adenine hemisulphate, Ads; N^6–[2-isopentenyl] adenine, 2iP; 6-benzyladenine, BA (see notes in [17]); *N*-(chloro-4-pyridyl)-*N*′-phenylurea, 4-CPPU; kinetin (Kn); 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid, picloram; thidiazuron (*N*-phenyl-*N*-1,2,3-thidiazol-5-yl urea; TDZ) (cytokinin-like); zeatin riboside, ZR), other PGR (gibberellic acid, GA_3). All stocks were made up using the appropriate solvents as recommended in the Sigma-Aldrich products catalogue, and were added to cooled medium after filter sterilizing in 0.22 µm Millipore filters even if some PGRs were autoclavable, to avoid error caused by the autoclave/filter response. All stocks were made fresh every 3-6 months and were stored at -4°C until needed.

Experiments spanned a total of 90 days, and explants in all treatments were checked every 15 days. Dark treatments were checked under dim light to minimize exposure to light.

Experimental design and data analysis

Experiments were organized according to a randomized complete block design. *In vitro* propagation from flower parts consisted of 10 replicates (5 explants × 2 Petri dishes) per treatment, each repeated three times within a single flowering season to eliminate human error. Since there was no organogenesis, no quantitative statistical analysis was conducted.

Results and discussion

The sterilization protocol used was devised by the authors. Petals and sepals tended to be the most sensitive to the surface sterilization process and tended to become opaque during the sterilization process, occasionally turning brown. Oxidized (brown) tissues were discarded. No contamination was observed in tissue cultures, even after 90 days *in vitro*. Sepals and tepals tended to turn brown in all media after 10-15 days. It is possible that the lack of contamination, which reflects an effective sterilization protocol, may have negatively influenced organogenesis.

Three groups of PGRs were tested: auxins, cytokinins, other PGRs. No organogenesis (*neo*-PLBs, roots, shoots, flowers) was observed from any PGR, at any concentration, from any flower part. However, the column swelled and formed callus in response to 2 or 4 mg/l TDZ in the light (Fig. 2A), or to 1 mg/l 2,4-D (Fig. 2B) and 1 mg/l dicamba in the dark. Swollen column explants cultured in the light in the same PGR treatments remained green and responsive even after 6 months *in vitro*. Since the cells in the column tissue tend to divide more actively than other flower tissues (Fig. 1; [6]), they hold the most promise for tissue culture. Column tissue also displayed endopolyploidy (Fig. 1), which appears to be common in this genus [18,19]. Evidently, despite the large scale of these PGR trials, more effort needs to be focused on tissue culture protocols from flower tissues. Some callus could be induced at the cut ends of peduncles in response to 1 or 2 mg/l 2,4-D, but the response was weak. Protocols exist for generation of PLBs from pedicel (flower stalk) tissues or flower primordia of *Oncidium* [9,10,11] and *Cymbidium sinense* [12,13], although pedicels were not tested in this study since protocols for such and other tissues already exist (e.g., [7]).

Based on the slightly higher levels of 4C in *Phalaenopsis* flowers, the hypothesis was that, if placed in an *in vitro* environment, under favorable conditions, that organogenesis might be possible. Using *Phalaenopsis* flowers, this large-scale experiment was conducted in which all flower parts were exposed to several levels of PGRs. Regrettably, no organogenesis occurred, but several explants swelled and in some cases, formed callus (swollen, hardened, but not friable tissue). Due to limited material, available only once in the

flowering season each year, ensuing trials were not pursued, although the preliminary findings provide hope for future experiments, which would allow for the *in vitro* regeneration of orchids from greenhouse-derived flower tissue.

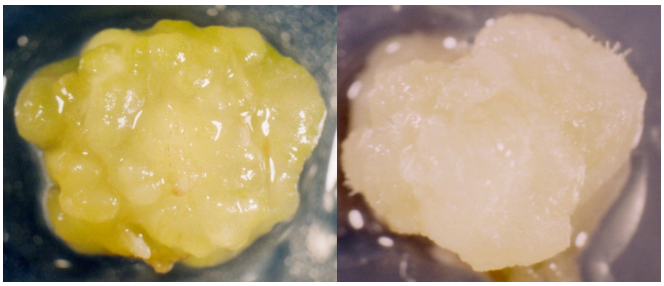

Figure 2. Callus formation on the column of mature *Phalaenopsis* Gallant Beau 'George Vazquez' flowers in response to 2 or 4 mg/l TDZ (left) in the light and 1 mg/l 2,4-D (right) in the dark.

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Conflicts of interest

The authors declare no financial or other conflicts of interest.

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