Effective Acclimatization of *Epidendrum in vitro*  
Using a Novel Micropropagation Vessel

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**Abstract:** To overcome various disadvantages of conventional culture vessels and to employ photoautotrophic micropropagation techniques and in order to reduce the cost of film culture vessels, a multiple-layered and very inexpensive OTP® film made of TPX (4-methyl-1-pentane polymer) and CPP (a polypropylene) with characteristics similar to the PFA (tetrafluoroethylene perfluoroalkyl vinyl ether copolymer) film, has been used to make a novel disposable film culture vessel, termed the Vitron. In this study, *Epidendrum* (E. Rouge Magic x E. Joseph Lii Mother’s Day Koto) shoots at the three leaf stage were cultured under high CO₂ enrichment at a low photosynthetic photon flux density in three film culture systems: Miracle Pack (MP) using PFA film, MP using OTP film and Vitron with sugar-free liquid modified Vacin and Went medium and rockwool multiblock™ as a substrate. The *in vitro* and *ex vitro* growth of *Epidendrum* plantlets cultured in the three film culture systems were significantly similar, producing normal and vigorous plantlets. The net photosynthetic rate of *in vitro* *Epidendrum* plantlets cultured in the three film culture systems were also significantly similar, suggesting that the novel Vitron film culture system can replace conventional culture vessels for *Epidendrum* photoautotrophic micropropagation. This study further emphasizes that *Epidendrum* plantlets can be well-rooted and acclimatized *in vitro* and transfer to a greenhouse does not require any additional acclimatization step, which is usually an additional economic and time-consuming step.

**Key words:** CO₂ enrichment, orchid, photoautotrophic, PPFD

**INTRODUCTION**

The culture vessel used for micropropagation may be thought of as a miniature greenhouse or growth chamber with *in vitro* culturing of the miniature vegetative cutting explants[1]. However, the differences in the *in vitro* physical environment of conventional tissue culture systems from that found in greenhouses often results in undesirable physiological and pathological problems[2].

The conventional vessel environment has been characterized as having high humidity, constant temperature, low Photosynthetic Photon Flux Density (PPFD), large diurnal fluctuations in CO₂ concentration, the presence of high concentrations of sugar, salt and plant growth substances and the accumulation of ethylene[3]. These conditions often affect the uptake of water, nutrients and CO₂ transpiration, dark respiration and development of photosynthetic machinery resulting in poor plantlet growth.

Photoautotrophic micropropagation has been shown to produce plantlets with anatomical and physiological characteristics better suited for acclimatization than conventional heterotrophic and photomixotrophic micropropagation techniques[4]. Photoautotrophic micropropagation holds several advantages over conventional methods in achieving enhanced growth of plantlets with lower contamination levels, reduced dependence on exogenous growth regulators and more rapid and vigorous plant growth and development during the acclimatization state[5].

In order to facilitate the application of photoautotrophic micropropagation, a film culture vessel, the Culture Pack (CP) made of fluorocarbon polymer film was developed[6] with the trade name of Neoflon® PFA (tetrafluoroethylene perfluoroalkyl vinyl ether copolymer) and supported by a stainless frame. This film possesses superior thermal stability, high light transmittance, low water vapor transmittance, chemical inertness and most notably, high gas permeability[7]. Superior gas permeability is the unique characteristic of this vessel; that is, gases can diffuse across the vessel wall to compensate differences in gas concentrations internal and external.

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to the vessel. Suitability of the CP system under CO2-enrichment with low Photosynthetic Photon Flux Density (PPFD), sugar-free medium and rockwool multiblock (RW) substrate was demonstrated for the following plant species: Gerbera, Limonium, Fragaria, Chrysanthemum, Vitis, Spathiphyllum, Cymbidium, Phalaenanopsis, Eucalyptus, Azadirachta excelsa and banana.

Later a practical application of the CP, the Miracle Pack (MP), was developed. The MP-PFA system is constructed of PFA film supported by a clear polycarbonate frame with RW substrate. Use of this system in CO2-enrichment resulted in enhanced in vitro growth of many plant species when compared with those cultured in a conventional culture vessel. Examples include: Anthurium, Syngonium, Spathiphyllum, Agapanthus, Hascup, Cymbidium and Eucalyptus. However the MP-PFA vessel is very expensive due to the high price of the PFA film and the polycarbonate frame, making it ill-suited for widespread application in commercial plant tissue culture laboratories.

The present study assesses an apparatus, the Vitron, which is of a similar size and shape to the MP-PFA but which can be produced at a fraction of the cost by using a much cheaper film and frame material. The novel OTP film (a multi-layer film, made of TPX (4-methyl-1-pentene polymer) and CPP (a polypropylene), has characteristics similar to PFA but is available at lower cost. The frame of the Vitron apparatus is made of polypropylene, which also greatly reduces its cost.

The Vitron system has already been shown to be satisfactory for the micropropagation of Eucalyptus, sweet potato and Spathiphyllum (unpublished data) and in this research the focus was on comparing the in vitro and ex vitro growth of Epidendrum plantlets cultured in different film culture vessels: MP-PFA; MP-OTP (MP with OTP film) and the Vitron. This is the first study employing the photoautotrophic micropropagation of Epidendrum species (Ochidaceae). Therefore, this research examines whether in vitro Epidendrum plantlets photoautotrophically cultured in the novel Vitron vessel are able to develop and produce healthy ex vitro plantlets when transferred to the greenhouse.

**MATERIALS AND METHODS**

**Culture vessels:** Two types of film were used to make vessels: PFA and OTP. PFA (Neoflon®) is a fluorocarbon polymer film (Daikin Industries, Ltd) made of tetrafluoroethylene perfluoroalkyl vinyl ether copolymer (Table 1). OTP® (Otsuka Technology Production, Ltd) is a multi-layer film (Fig. 1) consisting of three layers. The

<table>
<thead>
<tr>
<th>Layer</th>
<th>Thickness</th>
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<tbody>
<tr>
<td>Outer layer TPX</td>
<td>10 or 15 μm</td>
</tr>
<tr>
<td>Adhesive layer</td>
<td>10 μm</td>
</tr>
<tr>
<td>Inner layer CPP</td>
<td>10 μm</td>
</tr>
</tbody>
</table>

Fig. 1: Structure of OTP film
1: 4 methyl-1-pentene polymer; 2: polylephin resins; 3: polypropylene

Fig. 2. Diagram of Miracle Pack® film culture system
1, lid (polycarbonate); 2, clamp (polycarbonate); 3, frame for lining film to the lid (polycarbonate); 4, silicon foam band; 5, main frame (108x108x130 mm; polycarbonate); 6, Neoflon® PFA film (25 μm) or OTP® film (30 μm) sheet; 7, Neoflon® PFA film bag (25 μm) or OTP® film bag (30 μm); 8, Rockwool (Grodan, Denmark) multiblock

outer layer of TPX (4-methyl-1-pentene polymer) and the inner layer of CPP (a polypropylene) are bonded together by the middle layer of polylephin resins. The MP-PFA and MP-OTP vessels (Fig. 2) are set-ups, consisting of a frame made of clear polycarbonate and a PFA or OTP film which is in the shape of a bag when the set-up is initially introduced, respectively. The film bags were set properly inside the frame as shown in Fig. 2. The polycarbonate lid of these vessels was lined with a sheet of PFA or OTP film using a silicon foam band. The lid was secured to the frame of the main vessel with two clamps at the sides. The Vitron (Fig. 3) consists of a 3-dimensional injection-molded polypropylene frame, covered by an OTP film sheet heat-sealed on all sides except the top. A top seal film (OTP) was affixed to the top of the vessel after removing the paper backing to expose the adhesive. The
Table 1: Characteristics of films used for the Miracle Pack and the Vitron

<table>
<thead>
<tr>
<th>Kind of film</th>
<th>Thickness of film (μm)</th>
<th>Oxygen permeability (cm³/m² d Pa)</th>
<th>CO₂ permeability (cm³/m² d Pa)</th>
<th>Water vapor permeability (g m⁻² d Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTP**</td>
<td>30-35</td>
<td>10,900</td>
<td>30,100</td>
<td>38.0</td>
</tr>
<tr>
<td>PFA**</td>
<td>25</td>
<td>15,000</td>
<td>24,200</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*OTP* film
**Neoflon* PFA film

Table 2: Description of film culture vessel systems

<table>
<thead>
<tr>
<th>Film culture systems</th>
<th>Kind of film</th>
<th>Thickness of film (μm)</th>
<th>Kind of frame</th>
<th>Amount of medium (mL)</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-PFA</td>
<td>PFA</td>
<td>25</td>
<td>Polycarbonate</td>
<td>180</td>
<td>RW</td>
</tr>
<tr>
<td>MP-OTP</td>
<td>OTP</td>
<td>30</td>
<td>Polycarbonate</td>
<td>180</td>
<td>RW</td>
</tr>
<tr>
<td>Vitron</td>
<td>OTP</td>
<td>35</td>
<td>Polypropylene</td>
<td>180</td>
<td>RW</td>
</tr>
</tbody>
</table>

Fig. 3: Diagram of Vitron vessel
1: main frame; 2: top seal film; 3: adhesive area

film was secured on the flange of the vessel and the edges of the film were folded to the underside of the flange to achieve a hermetic seal.

Preparation of culture systems: The characteristics of the three culture systems are shown in Table 2. RW (25 joined-blocks, 5x5, Grodan A/S, Denmark) was used as substrate. Substrate was sterilized on a dry sterilizer at 150°C for 1 h prior to being placed in the sterilized vessels. Sterile liquid medium was then poured onto the RW.

Plant materials: The explants (*Epidendrum* Rouge Magic x *E. Joseph Lii Mother’s Day Koto*) used in this study were three-leaf shoots without roots, obtained from a mass of shoots cultured in *vitro*. The *Epidendrum* shoots were inserted in a hole (3 mm in diameter and 10 mm in depth) made in each block of the RW. Twenty-five explants were cultured in each culture vessel. Three vessels were used for each treatment. For acclimatization, 25 plantlets were transferred to pine bark in plastic pots (5x5 cm) and placed in a greenhouse for three months.

Culture medium: A sugar and hormone-free, liquid, modified Vacin and Went (1957) culture medium supplemented with 1 mg L⁻¹ Nitsch microelements (1971) was used. The pH of the medium was adjusted to 5.7 before autoclaving at 121 kPa for 17 min.

Culture conditions: The culture conditions were: Temperature (25±1°C); photoperiod (16 h/day); light intensity (45 μmol m⁻² s⁻¹); Homo-Lux. National Electric Co., Ltd. Tokyo, Japan; CO₂ enrichment (3000 ppm/24 h/day).

Measurement of growth: Plantlet growth was quantified by the number of leaves, plant height, fresh and dry weight of shoots, number of roots, root length and fresh and dry weight of roots. Chlorophyll content in the third leaf (counting downward from the top) of the plantlets three months after the start of culturing was measured as by the SPAD value by a chlorophyll meter (SPAD-502, Minolta Co., Ltd., Japan).

Measurement of photosynthetic rate: Photosynthetic rate of the leaves were measured using a LI-COR portable gas exchange system (LI-6400, LICOR, Lincoln, USA). Measurements were performed at 25°C and the vapor pressure deficit at the leaf surface was maintained between 2.3 and 3.1 kPa. The CO₂ concentration in the sample chamber was set at 400 μL L⁻¹. Measurement of CO₂ uptake between the range of 0 μmol m⁻² s⁻¹ and 300 μmol m⁻² s⁻¹ was conducted using a built-in red Light Emitting Diode (LED) lamp.

Data analysis: Data analysis was carried out using the IRRISTAT, version 3.0. Duncan’s Multiple Range Tests at p = 0.05 was used for statistical comparisons.

RESULTS AND DISCUSSION

In vitro growth of *Epidendrum* plantlets cultured in different film culture systems: All plantlets were normal and vigorous (Fig. 4 and Table 3) and the plant height, number of leaves, number of roots, root length, top and root fresh weight and root dry weight were equivalent in all treatments. Top and total dry weights were highest in
Table 3: In vitro growth of *Epidendrum* plantlets cultured in different film culture systems

<table>
<thead>
<tr>
<th>Film culture systems</th>
<th>Plant height (cm)</th>
<th>No. of leaves</th>
<th>No. of roots (cm)</th>
<th>Root length (cm)</th>
<th>SPAD value of leaves*</th>
<th>Fresh weight (mg)</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-PFA†</td>
<td>6.2a</td>
<td>5.7a</td>
<td>20.6a</td>
<td>40.5b</td>
<td>994.6a</td>
<td>108.4a</td>
<td>549.2a</td>
</tr>
<tr>
<td>MP-OTP†</td>
<td>6.5a</td>
<td>5.7a</td>
<td>18.6a</td>
<td>45.1a</td>
<td>965.5a</td>
<td>123.7a</td>
<td>552.4b</td>
</tr>
<tr>
<td>Vitron†</td>
<td>6.4a</td>
<td>7.0a</td>
<td>34.4a</td>
<td>20.1a</td>
<td>1058.1a</td>
<td>106.1a</td>
<td>618.6b</td>
</tr>
</tbody>
</table>

*Different letters within a column indicate significant differences at p = 0.05 by Duncan’s Multiple Range Test.

Table 4: Subsequent growth of in vitro *Epidendrum* plantlets cultured in the different film culture systems three months after transferring to greenhouse

<table>
<thead>
<tr>
<th>Film culture systems</th>
<th>Plant height (cm)</th>
<th>No. of leaves</th>
<th>No. of roots (cm)</th>
<th>Root length (cm)</th>
<th>SPAD value of leaves*</th>
<th>Fresh weight (mg)</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-PFA†</td>
<td>10.1a</td>
<td>11.0a</td>
<td>10.2a</td>
<td>11.1a</td>
<td>47.1a</td>
<td>38.7a</td>
<td>50.1a</td>
</tr>
<tr>
<td>MP-OTP†</td>
<td>10.8a</td>
<td>11.7a</td>
<td>12.8a</td>
<td>11.2a</td>
<td>37.1a</td>
<td>39.0a</td>
<td>58.6b</td>
</tr>
<tr>
<td>Vitron†</td>
<td>9.7a</td>
<td>11.7a</td>
<td>10.3a</td>
<td>10.7a</td>
<td>41.2a</td>
<td>39.0a</td>
<td>60.4a</td>
</tr>
</tbody>
</table>

*Different letters within a column indicate significant differences at p = 0.05 by Duncan’s Multiple Range Test.

Fig. 4: In vitro growth of *Epidendrum* plantlets cultured in different film culture systems, 90 days after culturing (left: MP-PFA, middle: MP-OTP, right: Vitron)

The Vitron system, while in the two other treatments these values were equivalent, SPAD values of leaves of those from the MP-PFA and Vitron systems were similar, but lower than those from the MP-OTP system.

Many researchers achieved enhanced gas exchange between the culture vessel atmosphere and growth chamber environment by using a ventilated culture vessel. These findings stimulated the development of culture containers and container enclosures that facilitate control of the vessel atmosphere. Among the many different proposed solutions, the simplest incorporates gas permeable membranes into the container enclosure or uses a film that is permeable to air for the closure (natural ventilation); the more complicated ones either employ a large culture vessel with CO₂ supply system, or forced ventilation system or use a diffusive and humidity-induced convective throughflow ventilation system.

By increasing CO₂ concentration inside the vessel through the use of Neoflon® PFA film, the growth and development in the rooting stage of the plantlets of some horticultural plants was remarkably improved by using a novel culture system, which consists of a box-shaped film culture vessel, the Culture Pack and is made of PFA film. Plantlets grown under these culture conditions were more vigorous compared to those cultured in conventional Elrendenravels covered with aluminum foil or in aerated glass bottles with polycarbonate screw caps with a 3 mm diameter hole fitted with a circular self-adhesive gas permeable membrane (Millisip™, pore size 0.5 µm). These results could be due to better gas exchange in the Culture Pack than in the flask since PFA film has a high gas permeability. Better growth of Anthurium, Syngonium, Spadixiphium, Agapanthus, haccrupee, Cymbidium and Eucalyptus were obtained with the MP-PFA system compared to CP and conventional vessel systems.

The present study was a continuation of the above-mentioned studies and therefore, the conventional vessel was not used for a control treatment in this study. As the
in vitro growth of *Epidendrum* plantlets in MP-OTP and Vitron were equivalent to those cultured in the MP-PFA system (Table 3), it is suggested that the culture systems using OTP film (used in MP-OTP and Vitron), which has high gas exchange capability as the PFA film, that supports enhanced *in vitro* growth of *Epidendrum* plantlets compared to conventional culture systems.

*Epidendrum* plantlets grew vigorously in the culture systems using all three film culture systems with sugar-free liquid medium under CO₂ enriched condition[30]; sucrose, an essential exogenous carbon source in conventional vessel system, can be replaced by culture atmosphere with elevated CO₂ for micropropagation of *Cymbidium*. This is important as bacterial and fungal growth can be prevented if sugars are excluded from the culture medium.

**Photosynthetic capacity of in vitro Epidendrum plantlets cultured in different film culture systems:** Net photosynthetic rate of *Epidendrum* plantlets (Fig. 5 and Table 3) cultured in the Vitron system was almost equal to that of plantlets cultured in MP-PFA and MP-OTP systems regardless of differences in PPFD over a range of 0 to 300 μmol m⁻² s⁻¹, confirming that the enhanced growth of *in vitro* Epidendrum plantlets is comparable in the three treatments and is in accordance with the similar photosynthetic capacity observed in the treatments.

Plantlets growing under heterotrophic conditions *in vitro* have low photosynthesis rates due to low light intensity, low CO₂ concentration[31] and inhibition of photosynthesis by high sugar concentrations in the medium[21,31,32].

Poor photosynthetic activity of plantlets cultured *in vitro* is considered to be one of the major factors inhibiting the improvement of micropropagation efficiency and acclimatization success. In this study, the CO₂-enrichment, gas permeable film culture vessel and the absence of exogenous sugars in the medium, regardless of the low PPFD, might have a profound impact on the photosynthetic capacity of *in vitro* Epidendrum plantlets.

**Subsequent growth of Epidendrum plantlets from different film culture systems:** Survival was 100% for plantlets three months after being transferred to a greenhouse. All plantlets were normal and vigorous. The plantlets cultured in the Vitron system were similar to those from MP-PFA and MP-OTP systems for all growth parameters (Table 4).

The plantlets were grown photoautotrophically in the film culture systems; therefore, they experienced fewer changes in physical, physiological and nutritional environments upon transfer from *in vitro* to *ex vitro* conditions. Thus, higher growth and survival rates are expected following transfer compared to conventional culture methods. The positive *ex vitro* growth of plantlets in all treatments obtained in this experiment is in agreement with other results[30], which indicated that *ex vitro* growth can be achieved more easily with photoautotrophic micropropagation. In addition, the film vessels have a large opening, which makes it easy to avoid damaging the plantlets when they were removed for transfer.

In the present study, the survival rates of the plantlets cultured in the film vessels were 100% without any specialized *ex vitro* acclimatization treatment. This is an important factor, as one of the most serious problems of micropropagation is the poor survival of plantlets after transfer to soil[31].

The growth of plantlets cultured photoautotrophically in all three film culture systems was equally enhanced (Table 4). The *in vitro* plantlets had high photosynthetic capacity and were vigorous and became high quality/healthy *ex vitro* plantlets. However, the newly developed Vitron vessel has many advantages over the other two culture systems: cost (10 times cheaper than MP-PFA systems and 6 times cheaper than MP-OTP system), weight (only 25 grams per Vitron compared to 144 grams per MP) and the most important, disposability. In addition, *Epidendrum* plantlets were able to grow photoautotrophically in Vitron system, under low PPFD. This is an important consideration, since high PPFD results in electricity cost for lighting as well as increased temperature in the culture room, which in turn increases the additional cost for cooling systems. These attractive features of the Vitron vessel help to reduce both production and labor costs and overcome many difficulties encountered with using other existing conventional vessels, thereby promoting the effectiveness of micropropagation. In conclusion, the
Vitron vessel is recommended for photoautotrophic *Epidendrum* micropropagation. The Vitron will be marketed in the near future at a low price (approximately 1 US$ per unit). This price supposedly will be reduced by large-scale production, especially of high-value crops such as many orchid species.

REFERENCES


