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Promotion of Vegetative Growth in Force-ventilated *Protea cynaroides* L. Explants Cultured in Modified Temporary Immersion Culture Vessels

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Abstract. **Temporary immersion culture vessels were modified to culture *Protea cynaroides* L. microshoots on semisolid growth medium. The effects of different ventilation treatments and sucrose concentrations on the vegetative growth and physiological characteristics of *P. cynaroides* L. microshoots were investigated. Three ventilation treatments were used: microshoots were either ventilated naturally or forced ventilated for 2 minutes/2 hours or 2 minutes/4 hours. In addition, two sucrose concentrations were used in the growth medium: 30 and 10 g·L^{-1}. Significant interaction effects were found between ventilation and sucrose in the number of shoots formed. When cultured on growth medium with 10 g·L^{-1} sucrose, microshoots force-ventilated for 2 minutes/2 hours produced significantly higher number of shoots than those naturally ventilated or force-ventilated for 2 minutes/4 hours. In the 30 g·L^{-1} sucrose treatment, no significant differences in shoot numbers were observed among all ventilation treatments. The highest leaf areas were found in microshoots cultured in the 2 minutes/4 hours forced ventilation treatment, which were significantly higher than microshoots in the other ventilation treatments, irrespective of the sucrose concentration. Chlorophyll content was significantly higher in leaves of microshoots that were cultured in 30 g·L^{-1} sucrose compared with those grown in 10 g·L^{-1} sucrose in all ventilation treatments. Analysis of chlorophyll fluorescence of the leaves revealed that the F_v/F_m value of microshoots grown on 30 g·L^{-1} sucrose and force-ventilated for 2 minutes/4 hours was significantly higher than those naturally ventilated in the same sucrose treatment. Overall, the use of 30 g·L^{-1} sucrose in combination with 2 minutes/4 hours ventilation provided the best conditions for culturing *P. cynaroides* microshoots. This study demonstrated that these modified temporary immersion culture vessels can be used as a forced ventilation system to culture *P. cynaroides* microshoots and promote vegetative growth as well as improve their photosynthetic characteristics. The system described here introduces a simple and novel method of converting commercially available temporary immersion systems into force ventilation systems.**

The King Protea (*P. cynaroides* L.) is a semihardwood shrub that produces striking flowerheads consisting of hundreds of florets. It is an important cutflower in the floriculture industry. Because of its slow-growing nature and susceptibility to phenolic browning, it is known to be a difficult-to-propagate species in vitro. As a result, unlike other economically important plant species, in vitro propagation of *P. cynaroides* microshoots has yet to be achieved on a commercial scale. Previous studies investigating the effects of light-emitting diodes (Wu and Lin, 2012) and carbon dioxide enrichment (Wu and Lin, 2013) have provided a better understanding of the factors affecting their growth and development in vitro and resulted in improvements in their in vitro regeneration rate.

In conventional micropropagation, CO_2 concentration generally remains low in the culture vessel, which among other factors, is considered to be the cause of physiological stress in plantlets, and is known to directly affect their morphogenesis and growth (Xiao et al., 2011). Findings by Serret et al. (1996) showed that low availability of CO_2 may stimulate photoinhibition in plantlets cultured in vitro. Furthermore, Habash et al. (1995) demonstrated that by increasing the CO_2 concentration in the culture vessel, plantlets would be able to use more light energy with higher efficiency, which would reduce the occurrence of photoinhibition. The advantages of forced ventilation, which is often used to deliver enriched CO_2 into the culture vessel, are well documented. These include the following: improving the overall microenvironment of the culture vessel by lowering relative humidity, increasing CO_2 concentration, and removing buildup of ethylene gas, elimination of physiological and morphological disorders, and higher survival percentage during acclimatization. Forced ventilation of culture vessels has been shown to significantly promote in vitro growth and development of numerous plant species, these include, among others, *Cymbidium* sp. (Norikane et al., 2010), *Dendrobium candidum* (Xiao et al., 2007), *Momordica grosvenori* (Zhang et al., 2009), *Noppalea cochenilifera* (Houllou-Kido et al., 2009), and *Uniola paniculata* (Valero-Aracama et al., 2007). However, although the flow of CO_2 into the culture vessel in forced ventilation systems can be adjusted with an airflow controller to maximize photosynthetic rates and plantlet growth (Kozai and Kubota, 2001), it has been shown that long-term ventilation causes high water loss in the growth medium, which changes the concentration of additives in the medium (Goncalves et al., 2007). Consequently, plant growth may be adversely affected by excessive drying out of the growth medium. Results from our preliminary studies have showed that by culturing slow-growing plant species such as *P. cynaroides* in a forced ventilation setup similar to those described in literature, the detrimental effects of water loss on their growth over a prolonged period of time are particularly pronounced, which offset the benefits of forced ventilation. Moreover, a study by Gunderson and Wullschleger (1994) showed that plantlets exposed to elevated CO_2 on a long-term and continuous basis tend to suffer from downregulation of net photosynthesis. Reducing the ventilation frequency and duration to strike a balance between providing adequate ventilation and not causing excessive water loss from the growth medium may provide the explants with a more optimal environment, which would reduce physiological stress in particular, as well as enhance their growth.

Because of the low photosynthetic ability of explants in vitro, the addition of sucrose is required to provide the carbon and energy source for explants to grow (Gouk et al., 1999). Sucrose also plays a regulatory role in morphogenesis, growth, and development of plants in vitro. Several studies have showed that improvements in the photosynthetic characteristics of plantlets achieved by altering the sucrose concentration in combination with ventilation have significant effects on plantlet growth (Hdider and Desjardins, 1994). A reduction in sucrose concentration and an increase in CO_2 concentration lower the dependence of plantlets to exogenous carbohydrates, which promotes the gradual development of photoautotrophic characteristics (Kozai and Kubota, 2005).

According to Xiao et al. (2011), the most challenging task of ventilation is to regulate the ventilation rate of the culture vessel so that the in vitro environment, including the growth medium, is not altered in such a way that it is detrimental to plantlet growth. In light of the challenges described earlier, during in vitro propagation of *P. cynaroides*

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microshoots, a different approach to forced ventilation was investigated by modifying temporary immersion culture vessels so that microshoots could be cultured on semisolid medium. The system controller, which is normally used to regulate temporary immersion, was used to control ventilation frequency and duration. The aim of this study was to examine how *P. cynaroides* microshoots respond vegetatively and physiologically to different ventilation frequencies and durations when cultured on different sucrose concentrations and to determine the optimum ventilation and sucrose combination for their growth in vitro.

Materials and Methods

Plant material, growth medium, and growth conditions. *Protea cynaroides* microshoots, ≈10 mm in length with three nodes, were taken from in vitro–established stock plants that had been previously subcultured twice. The microshoots were cultured on full-strength woody plant medium (Lloyd and McCown, 1981) supplemented with 0.5 mg·L^{-1} IBA, 0.01 mg·L^{-1} kinetin, 100 mg·L^{-1} myo-inositol, 100 mg·L^{-1} silver nitrate, 200 mL·L^{-1} coconut water, and 9 g·L^{-1} agar. Two sucrose concentrations were used: 10 and 30 g·L^{-1}. The pH of the growth medium was adjusted to 5.5 before autoclaving. The growth medium was dispensed into Plantima® (A-Tech Bioscientific Co., Ltd., Taipei, Taiwan) culture vessels (250 mL/vessel) and autoclaved at 121 °C and 104 kPa for 30 min. Each culture vessel was placed on a PlanTower® shelf (A-Tech Bioscientific Co., Ltd.), which provided 50 µmol·m^{-2}·s^{-1} *PPF*. The PlanTower® with the cultures were placed in a CO_2-enriched growth chamber (≈1000 µmol·mol^{-1}) with the temperature and photoperiod adjusted to 26 °C ± 2 and 16 h, respectively.

Ventilation treatments. To set up a system that ensures efficient gaseous exchange between the inside and outside of the culture vessel, a novel approach was developed using modified Plantima® containers, which are normally used for culturing explants in temporary immersion systems. Plantima® culture vessels are usually separated into an upper section (where explants are placed) and a lower section (for the growth medium). In this study, the component that was used to partition each Plantima® container into the upper and lower sections was removed. The container was then left with a single growing space where *P. cynaroides* microshoots were grown on semisolid medium. The inlet and outlet valves that were originally on the culture vessel served as the entry and exit points for gas exchange to take place, either by natural ventilation or forced ventilation. Natural ventilation took place through the natural diffusion of air between the inside and outside of the culture vessel. Forced ventilation was regulated by the system's air-flow controller, whereby CO_2-enriched air was pumped at set intervals via silicon tubes first through a sterilant solution (Cu^{++}) and then through a 0.22 µm microfilter attached to the inlet valve and into the Plantima® container (Fig. 1). This process allowed the air in the headspace inside the culture container to be renewed, with the forced pressure escaping through the outlet valve, which also had a 0.22 µm microfilter attached to prevent microbes from entering the vessel. Three ventilation treatments were tested: natural ventilation (control), and air pumped every 2 h for 2 min or every 4 h for 2 min (forced ventilation).

Determination of chlorophyll content and chlorophyll fluorescence. An SPAD-502 Plus Chlorophyll Meter (Konica Minolta, Tokyo, Japan), which is a portable diagnostic device that takes nondestructive measurements of the greenness or relative chlorophyll concentration of leaves, was used to measure chlorophyll content. The mean of three readings was obtained from the uppermost unfolded leaf of each microshoot. Determination of chlorophyll fluorescence (Fluoropen FP100; Photon Systems Instruments, Drásov, Czech Republic) of *P. cynaroides* microshoots was carried out according to Molero and Lopes (2012). Briefly, microshoots were dark-adapted by placing them in a dark room for 24 h. The youngest unfolded leaf on the uppermost shoot was selected to take chlorophyll fluorescence readings. The leaf was placed into the sensor head, making sure that the leaf completely covers the aperture of the sensor. The same leaves that were used to take the dark-adapted readings were used to take the light-adapted readings. The mean of three readings was obtained for each microshoot. The F_v/F_m ratio obtained was used to interpret the photosynthetic efficiency of the microshoots.

Statistical analysis. One microshoot per culture vessel was used with seven replications per treatment. Data for the number of shoots, shoot length, leaf area (Version 1.6; Image J, Bethesda, MD), chlorophyll content, and F_v/F_m value were collected after 100 d in culture. Data were subjected to analysis of variation and mean comparison using SPSS version 17 for Windows.

Results and Discussion

Shoot and leaf growth. Significant interaction effects were found between ventilation and sucrose in the number of shoots formed, whereas no significant interaction effects were found in the other growth parameters (Table 1). When cultured on growth medium with 10 g·L^{-1} sucrose, microshoots force-ventilated for 2 min/2 h produced significantly higher number of shoots than those naturally ventilated or force-ventilated for 2 min/4 h (Table 2). However, in the same sucrose treatment, no significant differences in shoot numbers were found between the natural ventilation and 2 min/4 h treatments. In the 30 g·L^{-1} sucrose treatment, no significant differences in shoot numbers were observed among all ventilation treatments (Table 2). Thus, these findings indicated that when cultured on low sucrose concentration (10 g·L^{-1}), high frequency ventilation (2 min/2 h) is required to induce shoot formation, whereas in the 30 g·L^{-1} sucrose treatments where growth media have been supplemented with sufficient carbon and energy source, explants are able to develop similar number of shoots regardless of the type of ventilation treatment. According to Zobayed et al. (1999), high frequency ventilation will result in the relative humidity of the headspace in the culture vessel to be reduced to the point where the growth medium starts to lose water content, i.e., water loss from the growth medium caused by high ventilation rate (2 min/2 h) resulting in changes to its composition, most notably the sucrose concentration. As a result, it is probable that

Table 1. Interaction effects of ventilation and sucrose concentration on the vegetative growth, chlorophyll content, and F_v/F_m values of *Protea cynaroides* microshoots.

Treatment	*F* value	*P* value
No. of shoots		
Ventilation	6.72	0.00
Sucrose	0.98	0.33
Ventilation × sucrose	6.50	0.00
Shoot length (mm)		
Ventilation	4.42	0.02
Sucrose	0.03	0.86
Ventilation × sucrose	2.46	0.10
Leaf area (mm^2)		
Ventilation	34.03	0.00
Sucrose	3.01	0.09
Ventilation × sucrose	0.84	0.44
Chlorophyll content (SPAD)		
Ventilation	8.21	0.00
Sucrose	34.60	0.00
Ventilation × sucrose	1.76	0.19
F_v/F_m		
Ventilation	3.80	0.03
Sucrose	0.76	0.38
Ventilation × sucrose	0.96	0.39

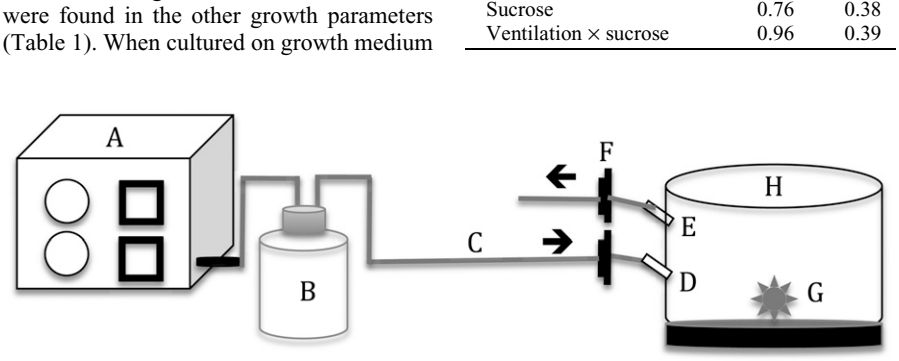

Fig. 1. Schematic drawing of the forced ventilation system using modified temporary immersion culture vessels. (**A**) Air-flow controller; (**B**) sterilant solution (1000 ppm Cu^{++}); (**C**) silicon tube; (**D**) inlet; (**E**) outlet; (**F**) air microfilter (0.22 µm); (**G**) *Protea cynaroides* microshoot; and (**H**) modified Plantima® culture vessel.

when *P. cynaroides* microshoots were force-ventilated for 2 min/2 h on medium containing 10 g·L^{-1} sucrose, the sucrose concentration increased to a level where it promoted the formation of shoots, leading to an increase in the number of shoots formed. By contrast, the low number of shoots that formed under natural ventilation and 2 min/4 h treatments could be due to insufficient CO_2 diffusion into the culture vessels combined with low sucrose content. Several studies have stated that in growth media with reduced sucrose levels, either the CO_2 concentration or the light intensity needs to be increased to ensure that a positive carbon balance is achieved (Hdider and Desjardins, 1994; Kozai, 1991). Under natural ventilation, a significantly higher number of shoots was produced on growth media containing 30 g·L^{-1} sucrose than those supplemented with 10 g·L^{-1} sucrose (Table 3). This demonstrated that when naturally ventilated, a higher sucrose concentration (30 g·L^{-1}) is required to induce the formation of shoots in *P. cynaroides* microshoots, which is to be expected because higher sucrose concentrations are generally needed in the absence of increased CO_2 levels. On the contrary, under 2 min/2 h ventilation, microshoots cultured with 10 g·L^{-1} sucrose produced significantly more shoots than those in the 30 g·L^{-1} sucrose treatment. As previously mentioned, this could be because of the reduced sucrose concentration (10 g·L^{-1}) increasing to more optimal levels as a result of water loss in the growth medium caused by high frequency ventilation, which at the same time, might have raised the 30 g·L^{-1} sucrose concentration to suboptimal levels, leading to lower shoot numbers. When the forced ventilation frequency was reduced, as in the 2 min/4 h treatment, the amount of sucrose in the growth medium did not significantly affect the number of shoots formed (Table 3). This suggests that at this ventilation frequency, conditions for the formation of shoots were similar, regardless of the sucrose concentration.

With regard to shoot length, different sucrose concentrations within the same ventilation treatment did not significantly affect the growth of microshoots (Table 4). These results are consistent with findings reported by Wojtania et al. (2015b). In their study, no significant differences in shoot length were found between *Pelargonium ×hortorum* explants grown in 15 and 30 g·L^{-1} sucrose. Similarly, no significant differences in the length of *Magnolia ×soulangiana* microshoots were observed between different sucrose concentrations in non-ventilated conditions (Wojtania et al., 2015a). By contrast, in the 10 g·L^{-1} sucrose treatment, *P. cynaroides* microshoots in the 2 min/4 h ventilation treatment produced significantly longer shoots than those in the other two ventilation treatments. Very few studies have examined the effects of different ventilation frequencies and durations on explant growth. Nevertheless, the results of our study are in agreement with the findings by Rahayu and Habibah (2015) where *Feronia limonia* explants cultured on reduced sucrose concentration led to significant increases in shoot growth. Similarly, Capellades et al. (1991) stated that culturing explants on reduced sucrose concentrations and increased gaseous exchange improved the growth rate of explants.

Microshoots force-ventilated for 2 min/4 h produced the largest leaves, irrespective of the sucrose concentration. This is shown by the significantly higher mean leaf area of microshoots grown in the 2 min/4 h force ventilation treatment in both the 10 and 30 g·L^{-1} sucrose treatments (Table 4). These results suggest that natural ventilation and 2 min/2 h forced ventilation represent the two extremes of the ventilation treatments, in which the ventilation conditions inside the culture vessel became suboptimal for leaf growth. It is likely that natural ventilation provided insufficient air exchanges, whereas the high ventilation frequency in the 2 min/2 h treatment caused excessive water loss in the growth medium. Goncalves et al. (2007) found that the higher water loss in ventilated culture vessels led to changes in media characteristics and concentration, which consequently affected plant growth. In particular, negative effects on vegetative biomass would become more pronounced with longer term culture (Mohamed and Alsadon, 2010). The results of the present study clearly indicated that this might have been the case because 100 d of frequent ventilation (2 min/2 h) most likely altered the media characteristics to the point where it adversely affected leaf growth, resulting in significantly lower leaf area. On the other hand, these findings illustrated that *P. cynaroides* microshoots force-ventilated for 2 min/4 h were optimal for leaf growth and development.

Chlorophyll content and F_v/F_m value. Within each ventilation treatment, the chlorophyll content of microshoots grown on 30 g·L^{-1} sucrose was significantly higher than those cultured on 10 g·L^{-1} sucrose (Table 4). These results are in agreement with those reported by Abbott and Belcher (1980) and Langford and Wainwright (1987). However, contradictory findings have also been reported with regard to the effect of sucrose on chlorophyll content. According to Grout and Donkin (1987), a high exogenous supply of sucrose is not required for the normal development of chlorophyll. In the in vitro culture of walnut explants, the chlorophyll

Table 2. Effects of different types of ventilation on the number of shoots formed in *Protea cynaroides* microshoots cultured under different sucrose concentrations.

Treatment	N/V	F/V, 2 min/2 h	F/V, 2 min/4 h	*F* value	*P* value
10 g·L^{-1} sucrose	2.5 ± 0.6 a	4.7 ± 0.5 b	2.8 ± 0.8 a	18.583	<0.001
30 g·L^{-1} sucrose	4.0 ± 0.6 a	3.7 ± 0.5 a	3.2 ± 1.5 a	1.273	0.307

Different letters in the same row indicate values differ significantly according to Duncan's multiple range test at $P \leq 0.05$.
N/V = natural ventilation; F/V = forced ventilation.

Table 3. Effects of different sucrose concentrations on the number of shoots formed in *Protea cynaroides* microshoots cultured under different ventilation treatments.

Treatment	10 g·L^{-1} sucrose	30 g·L^{-1} sucrose	*F* value	*P* value
N/V	2.5 ± 0.6	4.0 ± 0.6	17.182	0.003
F/V, 2 min/2 h	4.7 ± 0.5	3.7 ± 0.5	11.250	0.007
F/V, 2 min/4 h	2.8 ± 0.8	3.2 ± 1.5	0.244	0.632

N/V = natural ventilation; F/V = forced ventilation.

Table 4. Effects of sucrose concentration and ventilation treatment on the growth of *Protea cynaroides* microshoots after 100 d in culture.

Ventilation treatment	Shoot length (cm)	Avg leaf area (mm^2/microshoot)	Chlorophyll content[z]	F_v/F_m value[y]
N/V				
10 g·L^{-1} sucrose	5.6 ± 1.2 a[x]	44.3 ± 15.9 a	17.9 ± 2.7 ab	0.55 ± 0.10 ab
30 g·L^{-1} sucrose	6.0 ± 1.7 ab	44.6 ± 7.5 a	23.7 ± 6.0 c	0.53 ± 0.15 a
F/V (2 min/2 h)				
10 g·L^{-1} sucrose	4.5 ± 0.6 a	42.1 ± 2.8 a	12.6 ± 2.8 a	0.61 ± 0.07 ab
30 g·L^{-1} sucrose	5.4 ± 2.0 a	32.0 ± 7.0 a	25.3 ± 4.8 cd	0.63 ± 0.06 ab
F/V (2 min/4 h)				
10 g·L^{-1} sucrose	7.5 ± 0.8 b	72.4 ± 9.0 b	22.1 ± 2.0 bc	0.58 ± 0.05 ab
30 g·L^{-1} sucrose	5.9 ± 1.6 ab	65.0 ± 11.9 b	30.1 ± 5.0 d	0.66 ± 0.05 b

[z]SPAD value (Konica Minolta Chlorophyll Meter SPAD-502 Plus).
[y]Chlorophyll fluorescence (Fluoropen FP100, Photon Systems Instruments).
[x]Means in the same column with different letters are significantly different (Duncan's multiple range test at $P \leq 0.05$).
N/V = natural ventilation; F/V = forced ventilation.

content of explants cultured on low sucrose concentration (15 g·L^{-1}) were higher than those cultured on 30 g·L^{-1} sucrose in similar ventilation treatments (Hassankhah et al., 2014). Similarly, the chlorophyll content of *Billbergia zebrina* explants decreased as the sucrose concentration in the growth medium increased (Martins et al., 2015). These reports concluded that lower concentrations of sucrose in the growth medium stimulate chlorophyll production in explants cultured in vitro. By contrast, chlorophyll content in potato plantlets was not significantly affected by different sucrose concentrations; instead, significant effects were observed between different ventilation treatments (Mohamed and Alsadon, 2010). It is probable that the contradictory results of the relationship between sucrose concentration and chlorophyll content are associated with nitrogen levels, among other factors, in the growth medium. It has been suggested that because chlorophyll contains nitrogen in its structure, fluctuations in nitrogen levels in the growth medium would affect chlorophyll content in leaves (Richardson et al., 2002). Therefore, it can be concluded that in addition to sucrose, chlorophyll content of leaves is affected by interactions of several different factors.

F_v/F_m values of dark-adapted plant tissues are directly proportional to the quantum efficiency of PSII photochemistry (Butler, 1978), and are an excellent indicator for the PSII quantum yield, which provides a good indication of the photosynthesis efficiency of plants (Gouk et al., 1999). This in turn provides evidence as to whether a plant is stressed or not. A reduction in F_v/F_m values is associated with thermal damage of PSII reaction centers (Gouk et al., 1999). Plants that have F_v/F_m values less than 0.6 are considered stressed (Ritchie, 2006). In this study, *P. cynaroides* microshoots that had F_v/F_m values greater than 0.6 were cultured in 10 g·L^{-1} sucrose with 2 min/2 h forced ventilation, 30 g·L^{-1} with 2 min/2 h forced ventilation, and 30 g·L^{-1} with 2 min/4 h forced ventilation, whereas the lowest F_v/F_m value was observed in microshoots grown in the natural ventilation treatment containing 30 g·L^{-1} sucrose (Table 4). Although sucrose concentration in the growth medium has been shown to affect photosynthetic rate (Hdider and Desjardins, 1994; Mosaleeyanon et al., 2004), this was not observed in the present study. Instead, our findings showed significant differences in F_v/F_m values between forced ventilation (2 min/4 h) and natural ventilation in the 30 g·L^{-1} sucrose treatment. These results are consistent with several studies that demonstrated significantly high photosynthetic efficiency of explants cultured in forced ventilated treatments (Heo and Kozai, 1999; Xiao et al., 2005; Zobayed et al., 1999). The positive effects of forced ventilation are due to the presence of consistently high CO_2 levels in culture vessels, which improves the photosynthetic performance of plantlets (Zobayed et al., 1999). Our study shows that forced ventilation providing sufficient CO_2 is the overriding factor in determining in photosynthetic capacity of *P. cynaroides* microshoots.

In conclusion, sucrose and ventilation were found to have interaction effects in the number of shoots formed. Overall, compared with microshoots grown on 30 g·L^{-1} sucrose with natural ventilation, the use of 30 g·L^{-1} sucrose in combination with 2 min/4 h ventilation produced significantly higher leaf area, chlorophyll content, and F_v/F_m values. The modified temporary immersion culture vessels described in this study are suitable to be used as a forced ventilation system for culturing *P. cynaroides* microshoots. Further studies are needed to investigate the effects of these treatments on the rooting of *P. cynaroides* microshoots, as well as their acclimatization to ex vitro conditions.

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