南　華　大　學

自然生物科技學系自然療癒碩士班

碩 士 論 文

通風及蔗糖濃度對帝王花(*Protea cynaroides* L.)組培苗之器官發生的影響

The effects of ventilation and sucrose concentration on the organogenesis of *Protea cynaroides* L. microshoots *in vitro*

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Abstract

The aim of this study was to investigate the effects of different ventilation treatments and sucrose concentrations on the growth and development of *Protea cynaroides* L. microshoots *in vitro*. Three different ventilation treatments were used: microshoots were either ventilated naturally (control), forced ventilated for 2 min/2 h, or 2 min/4 h. In addition, two sucrose concentrations were used in the growth medium: 30 $g.L^{-1}$ and 10 $g.L^{-1}$. The following data were collected: number of buds, bud length, leaf area, chlorophyll content, chlorophyll fluorescence, fresh weight, total phenol content, and DPPH free radical scavenging activity.

Results showed that the highest number of buds were formed in microshoots cultured in 10 $g.L^{-1}$ sucrose that were forced ventilated for 2 min/2 h, which were significantly higher than those grown in 10 $g.L^{-1}$ sucrose in the natural ventilation treatment (control). Microshoots

cultured in 10 g.L^{-1} sucrose with 2 min/4 h forced ventilation produced the longest buds, whereas the shortest buds were found in the 2 min/2 h forced ventilation treatment cultured in the 10 g.L^{-1} sucrose. In addition, microshoots that formed the largest leaves were found in the 2 min/4 h forced ventilation treatment, irrespective of the sucrose concentration. These microshoots produced significantly higher leaf areas than microshoots in the other ventilation treatments. Furthermore, chlorophyll content were significantly higher in leaves of microshoots that were cultured in 30 g.L^{-1} sucrose compared to those grown in 10 g.L^{-1} sucrose, irrespective of the ventilation treatment.

Moreover, analysis of chlorophyll fluorescence of the leaves revealed that the highest Fv/Fm value was observed on microshoots that were forced ventilated for 2 min/4 h in the 30 g.L^{-1} sucrose treatment, which was significantly higher than microshoots cultured in 30 g.L^{-1} sucrose in the control treatment (natural ventilation). With regard to the fresh weight of the microshoots, the highest fresh weight was found in the 30 g.L^{-1}

sucrose treatment that was forced ventilated for 2 min/2 h, while microshoots cultured in 10 $g.L^{-1}$ sucrose in same forced ventilation treatment had the lowest fresh weight. The total phenol content of the microshoots was highest all the 30 $g.L^{-1}$ sucrose treatments, irrespective of the ventilation treatment, which indicated the strong effects of sucrose concentration on phenolic compounds production in *P. cynaroides* microshoots. Similarly, the highest DPPH radical scavenging activity were detected in the 30 $g.L^{-1}$ sucrose concentration treatments in all the ventilation treatments, particularly in those that were forced ventilated for 2 min/2 h and 2 min/4 h. These results suggest that sucrose concentration affected total phenol production and DPPH activities in *P. cynaroides* microshoots. In general, the ventilation and sucrose treatments used in this study clearly demonstrated their effects on the growth and development of *P. cynaroides* microshoots.

Keywords: forced ventilation; natural ventilation; sucrose; plant tissue culture

摘要

本研究目的為探討不同的通風及蔗糖濃度對帝王花（*Protea cynaroides* L.）組培苗的生長影響。本研究採用自然通風（對照組）及強制通風（2 min/2 h 及 2 min/4 h）三種不同的通風處理及 10 $g.L^{-1}$ 和 30 $g.L^{-1}$ 兩種不同的蔗糖濃度對帝王花組培苗的生長影響，以分析帝王花的芽數、芽長度、葉面積、葉綠素、葉綠素螢光、鮮重、總酚及總抗氧化能力之測定。研究結果顯示，芽數最高為 10 $g.L^{-1}$ 蔗糖的強制通風（2 min/2 h）處理，最低為 10 $g.L^{-1}$ 自然通風（對照組）處理，兩者間有顯著性差異。芽長度生長狀態最佳處理為 10 $g.L^{-1}$ 蔗糖的強制通風（2 min/4 h）處理，最低為 10 $g.L^{-1}$ 自然通風與強制通風（2 min/2 h）處理。研究也發現，不論是在 10 $g.L^{-1}$ 或 30 $g.L^{-1}$ 的蔗糖濃度，強制通風（2 min/4 h）的處理在葉面積，結果顯示其生長影響為最佳。接著，在自然通風（對照組）及強制通風(2 min/2 h 及 2 min/4 h)三種不同通風處理，30 $g.L^{-1}$ 蔗糖處理組的葉綠素含量都高於 10 $g.L^{-1}$ 蔗糖處理組，兩者之間有顯著性差異。研究證實葉綠素會因不同的通風時間及蔗糖濃度，而受到影響。另外，在葉綠素螢光(Fv/Fm

值)的研究結果顯示，Fv/Fm 值最高的處理為 30 $g.L^{-1}$ 蔗糖的強制通風 (2 min/4 h)，Fv/Fm 值最低的處理為 30 $g.L^{-1}$ 蔗糖的自然通風(對照組)，兩者間有顯著性差異。在鮮重的實驗結果顯示，最重的處理為 30 $g.L^{-1}$ 蔗糖的強制通風（2 min/2 h）處理，最輕的處理為 10 $g.L^{-1}$ 蔗糖的強制通風（2 min/2 h）處理。接著，在總酚的實驗結果顯示，30 $g.L^{-1}$ 蔗糖處理的多酚類都高於 10 $g.L^{-1}$ 蔗糖組，表示帝王花會因蔗糖濃度的高低而影響多酚類的含量。在總抗氧化能力的實驗分析研究結果顯示，在強制通風（2 min/2 h、2 min/4 h）的處理，30 $g.L^{-1}$ 蔗糖條件的清除 DPPH 自由基能力高於 10 $g.L^{-1}$ 蔗糖組，表示清除 DPPH 自由基能力高低會因不同的通風及蔗糖濃度而受到影響。本研究結果顯示不同通風處理與蔗糖濃度會影響帝王花組培苗之生長。

關鍵字：自然通風、強制通風、蔗糖、組織培養

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Chapter 1

Introduction

1.1 Research background

Currently, mass propagation of *Protea cynaroides* L. plants for commercial production has mainly relied on rooting stem cuttings in propagation beds. These rooted cuttings are then transplanted and established in the field to be used to produce cut flowers. Over the years, breeding new varieties, shortening the time from plant establishment to flower production, improvements in pre- and post-harvest qualities, and overcoming pest and disease problems have resulted in improvements in the commercial production of *P. cynaroides* cut flowers and enhancements in quality and vase life. However, attempts to replace traditional methods (stem cuttings) in the initial stages of the production cycle – plant propagation and regeneration, with micropropagated

plantlets have not been as successful.

1.2 Aim of this research study

Despite some recent successes being reported in the *in vitro* propagation of *P. cynaroides* explants with regard to light quality, phenolic compounds, carbon dioxide enrichment, and growth media composition, several challenges still remain. First and foremost, due to the slow-growing nature of the *P. cynaroides* plants, and the fact that they are inherently difficult to propagate *in vitro*, stimulating regeneration of microshoots and enhancing bud formation and proliferation have yet to achieve satisfactory results. Second, improvements in the success rate and reducing the length of time required for the *P. cynaroides* explants to grow and develop *in vitro* have remained challenging. An investigation into new *in vitro* propagation methods of *P. cynaroides* explants will provide a better understanding of the factors that affect their *in vitro* growth and development, and perhaps overcome the aforementioned

obstacles. Therefore, the objectives of this study were:

1. To study the effects of natural ventilation and forced ventilation on the growth and development of *P. cynaroides* microshoots.
2. To study the effects of sucrose concentration in the growth medium on the vegetative growth of *P. cynaroides* microshoots.
3. To analyze the effects of sucrose concentration and ventilation treatment on the total phenol content and DPPH radical scavenging activity of *P. cynaroides* microshoots.

Chapter 2

Literature Review

2.1 Plant tissue culture

Plant tissue culture, also called *in vitro* culture, is a term used to describe the propagation of plant cells, tissues, and organs. The parental cells are directed to dedifferentiate into meristematic or embryogenic cells, which then divide and redifferentiate into whole plants. Theoretically, every plant cell has the potential to produce a whole plant, known as totipotency. It is this fundamental principle that allows the mass production of genotypes that are identical to the parent cells, in a process known as micropropagation (Watt, 2012).

In practice, protocols for the *in vitro* culture of plants are determined on a case-by-case basis. The components for growth medium, such as

nutrients and plant growth regulators, and environmental conditions (light and temperature) for each stage of growth and morphogenesis require repeated testing to obtain the optimum concentration and conditions. In most cases, a gelling agent is mixed with the water, nutrients, growth regulators, and other additives to obtain a semi-solid substrate in which the cultures are placed (Watt, 2012). The entire process is carried out in aseptic conditions to ensure no microbial contamination occurs. *In vitro* culture is therefore a technique that is ideal for the clonal propagation of commercially-important crops, rare or threatened species, and plants that are difficult to propagate through other means.

2.2 *Protea cynaroides* L.

Protea cynaroides L., also known as the King Protea, belongs to the Proteaceae family. It is subdivided into 14 genera comprising an estimated 1700 species (Paterson-Jones, 2000). Of all the genera, *P. cynaroides* is the most popular species because of its magnificent

flowerhead (Fig. 1). The King Protea is a slow-growing, semi-hardwood shrub that can grow up to a height of two meters (Fig. 2). It has sparse branches, with hairless stems. The leaf stalks are reddish and can reach lengths of 50 to 100 mm. The plant's leaves are round, oval or narrowly elliptic, ranging from 50 to 120 mm in length and 50 to 75 mm in width. The flowerhead sizes range from 120 mm to 300 mm in diameter and the color of the bracts, which are either hairy or hairless, range from pink to creamy-white (Paterson-Jones, 2000).

Most plants belonging to the Proteaceae family are found in the southern hemisphere, with the richest concentration of species in eastern and western Australia and the western Cape region of South Africa (Paterson-Jones, 2000). The *Protea* genus has the widest distribution area of all the southern Africa Proteaceae, ranging from the predominantly winter or all-year round rainfall area of the Cape in South Africa to the subtropical and tropical areas of southern Africa (Paterson-Jones, 2007). They occupy a variety of habitats from sea level to up to 1,500 meters.

Due to their wide variability, flowers can be seen throughout the year, depending on the variety.

P. cynaroides is endemic to South Africa. The majority of the areas where the King Protea flourishes are characterized by hot, dry summers. However, *P. cynaroides* is one of the most adaptable species in the Proteaceae family, which is why it can also be grown in tropical areas such as Tropical Africa and Tropical South America, where humidity is high and the rainfall is spread more evenly throughout the year. Evidence of its adaptability is shown in the fact that cultivation of King Protea has been successfully introduced into Hawaii, which has become an important Protea-producing region, and tropical areas such as Peru and Ecuador.

In their natural habitat, King Proteas are found in nutrient-deficient soils. However, they are renowned as plants that are difficult to grow and fertilize (Littlejohn, van den Berg, & Matlhoahela, 2003). The major

factors that have been identified for successful cultivation are well-drained, sandy acidic soils with low phosphor content and pH ranging from 3.5 to 5.8 (Silber, Mitchnick, & Ben-Jaacov, 2001). Although higher pH levels can be tolerated, these plants have low mineral requirements and are therefore not tolerant to salt concentrations that would appear normal to other plants (Montarone, & Allemand, 1995).

The King Protea is a well-known cut flower in many parts of the world. Its striking flowerhead, each consisting of hundreds of individual florets, is among one of the most recognizable flowers in the world. In addition to its attractive flowerhead and unique floral features, its long vase life have made it a highly sought after cut flower. Current important production areas include: Australia, South Africa, California, Portugal, Israel, Zimbabwe, Hawaii, Chile, New Zealand, and Ecuador (Dorrington, 2009). The total area of Proteaceae planted worldwide is 5218 ha. Leading the way in the total area planted are Australia (2500 ha), South Africa (900 ha) and the United States (800 ha). Besides Hawaii, which is

becoming an important producer of Proteaceae, growers in China have started Protea plantations and is currently expanding rapidly in terms of size and species planted (Dorrington, 2009).

Fig. 1 *Protea cynaroides* flowerhead (Photograph: G. Bredenkamp)

Fig. 2 *Protea cynaroides* (Photograph: H.C. Wu)

2.3 *In vitro* culture of *P. cynaroides* explants

Some progress has been made in recent years with regard to understanding the factors affecting *in vitro* propagation of *P. cynaroides*, particularly root induction and vegetative growth. These include: *in vitro* establishment of *P. cynaroides* explants (Ben-Jaacov, & Jacobs, 1986) and reduction in phenolic browning of established axillary buds (Wu, & du Toit, 2004); the establishment of the relationship between stem blanching and starch accumulation during root formation of stem cuttings (Wu, & du Toit, 2006); the discovery of 3,4-dihydroxybenzoic acid to be an important regulator of root formation in *P. cynaroides* stem cuttings and *in vitro* explants (Wu et al., 2007c); the successful regeneration of *P. cynaroides* plantlets through micrografting (Wu, du Toit, & Reinhardt, 2007b); the establishment of a protocol for the direct formation of somatic embryos from *P. cynaroides* cotyledons (Wu, du Toit, & Reinhardt, 2007a). Results showed that somatic embryos were able to form directly on mature zygotic embryos and cotyledons, and germinate

into plantlets.

In addition, alternating temperatures was found to be a key factor in improving *in vitro* germination of excised *P. cynaroides* embryos (Wu, & du Toit, 2010). Furthermore, an investigation into the fluctuation of total phenolic compounds during the rooting of *P. cynaroides* stem cuttings demonstrated the regulating effects of endogenous total phenol concentrations on adventitious root formation (Wu, & du Toit, 2011). Furthermore, the study of the influence of phosphate concentration in the growth medium on explant growth led to the successful *in vitro* multiplication of *P. cynaroides* microshoots (Wu, & du Toit, 2012a).

More recently, red light-emitting diode (LEDs) irradiation was found to induce organogenesis in *P. cynaroides* shoot-buds *in vitro* (Wu, & du Toit, 2012b). Moreover, the use of LEDs as an alternative light source not only induced organogenesis, further investigations also showed correlations between light quality, root formation and endogenous

concentrations of phenolic compounds such as 3,4-dihydroxybenzoic acid, gallic acid, caffeic acid and ferulic acid (Wu, & Lin, 2012). In particular, results revealed that significant correlations exist between red LEDs, root formation and phenolic compounds. A relationship was also established between CO_2 enrichment and improvements in vegetative growth, net photosynthetic activity, and acclimatization of *P. cynaroides* microshoots (Wu, & Lin, 2013).

2.4 Photomixotrophic micropropagation

Conventional plant tissue culture is considered photomixotrophic as sugars, such as sucrose (20 – 30 $g.L^{-1}$), are usually added into the growth medium as the main source of carbon for *in vitro* plantlet growth (Mohamed, & Alsadon, 2010). In general, plantlets grown photomixotrophically tend to have low chlorophyll content, and possess low photosynthesizing enzyme activity (Hdider, & Desjardins, 1994). As a result, the photosynthetic ability of plantlets grown

photomixotrophically on growth medium supplemented with sucrose is relatively poor. In addition, under conventional photomixotrophic conditions, the presence of sugar in the growth medium and high relative humidity in the culture vessel often result in the growth of abnormal plantlets. The development of morphological, anatomical, and physiological abnormalities in plantlets subsequently reduces their survival rate upon transfer to *ex vitro* conditions (Zobayed, 2005). As a result, several studies have showed that by culturing plantlets on reduced sucrose concentrations and increased gaseous exchange improved photosynthetic ability and growth rate of plantlets (Capellades, Lemeur, & Debergh, 1991).

2.5 Ventilation of culture vessel

As mentioned previously, photomixotrophic conditions without adequate gaseous exchange tend to adversely affect the growth and development of *in vitro* plantlets. Often, leaves of these plantlets have

low numbers of functioning stomata, and poorly developed mesophyll (Hazarika, 2006). In combination with poor cuticle development, the malfunctioning stomata on the leaves cause excessive water loss, and low photosynthetic activity (Mohamed et al., 2010). Furthermore, Yokota et al. (2007) reported that *Arata eleta* plantlets grown *in vitro* possessed thinner leaves with undeveloped palisade layer and large intercellular mesophyll airspaces, compared to those grown in *ex vitro* conditions. Therefore, in order to improve the micro-environment of the culture vessels in which the plantlets are grown, ventilation of the culture vessels is needed. Optimal ventilation will lower the relative humidity, increase CO_2 concentration, and remove buildup of ethylene gas, which would result in improved growth and development of the plantlets. Common methods of ventilation are by natural ventilation and forced ventilation.

2.5.1 Natural ventilation

Natural ventilation is carried out by attaching a gas-permeable filter

on the lid or wall of culture vessels. The diffusion through the filter will allow the gaseous composition inside the vessels to gradually change toward the composition outside the vessel (Tichá, 1996). A number of studies have reported the benefits of the use of natural ventilation on plantlet growth and quality: *Gerbera jamesonii* (Liao, Wang, Zhang, Xu, & Lian, 2007), *Myrtus communis* (Lucchesini, Monteforti, Mensuali-Sodi, & Serra, 2006) and *Saccharum* spp. (Xiao, Lok, & Kozai, 2003).

2.5.2 Forced ventilation

Forced ventilation is achieved by directly supplying CO_2-enriched air into the culture vessel using an air pump (Solárová, & Pospíšilová, 1997). Xiao, and Kozai (2004) found that growth period of forced-ventilated *Zantedeschia elliottiana* plantlets was reduced by 50%, while the leaf area, shoot and root dry weight were, respectively, 5.2, 4.6 and 3.8 times higher than those cultured under a conventional micropropagation system. In addition, the net photosynthetic rate and chlorophyll concentration of

Gerbera jamesonii plantlets were, respectively, 9.2 and 2.2 times greater than those cultured photomixotrophically (Xiao, He, Liu, & Yang, 2005).

2.6 Chlorophyll fluorescence

When radiant energy strikes a leaf, the light energy that is absorbed by chlorophyll molecules in the photosynthetic system can undergo three processes: a) it can be used for photosynthesis (photochemistry), b) it can be dissipated as heat, or c) it can be re-emitted as light, which is known as chlorophyll fluorescence (Maxwell, & Johnson, 2000; Misra, Misra, & Singh, 2012). These three processes occur in competition. As a result, any changes in the efficiency of one of these processes will affect the yield of the other two. Therefore, the measurement of the yield of chlorophyll fluorescence will provide information about the efficiency of photochemistry and heat dissipation (Maxwell et al., 2000). The spectrum of the fluorescent light has a longer wavelength than the light that is absorbed. Hence, when a leaf is exposed to a light of a defined

wavelength, fluorescence yield can be quantified by measuring the amount of light re-emitted at longer wavelengths.

When light energy enters the leaf, it is absorbed by chlorophyll present in the thylakoid membranes of plants. The light enters the reaction centers called Photosystem I (PSI) and Photosystem II (PSII), which are situated on membranes in the chloroplasts (Ritchie, 2006). When PSII absorbs a photon of energy, one of its chlorophyll *a* electrons is raised to a higher energy state. While the electron is in this state, it is captured by an electron acceptor and funneled into PSI. While in PSI, NADPH is generated through a photochemical process, and used to provide energy to convert carbon dioxide into sugar (known as the Calvin Cycle).

In addition, in order to replenish the electrons that are lost from chlorophyll *a* in PSII, water molecules are split providing electrons for PSII, as well as releasing oxygen atoms into the atmosphere (Ritchie,

2006). When the chlorophyll *a* electrons at a higher energy state in PSII are not captured by the acceptor, they fall back to their ground state. Energy is lost during this process, and fluorescent light is released. Chlorophyll fluorescence is the measurement of the emission of the fluorescent light.

Kautsky and Kirsch first reported chlorophyll florescence in 1931 (Govindje, 1995). They placed plant cells in the dark for a few minutes, which removed all excited electrons and emptied the acceptor pools. They then illuminated the cells with a high intensity light, and measured the increase and decrease of fluorescent light emitted by the cells (Ritchie, 2006). By measuring the fluorescent emissions, Kautsky and Kirsch reported that the emissions first increase to a point (F_0), indicating that all reaction centers are open, which is then followed by a sharp increase to the point of maximum fluorescence (F_m). The increase from F_0 to F_m is known as variable fluorescence (F_v). Once fluorescent emissions have reached F_m, it is followed by a marked decrease to the steady state (F_t).

Genty, Briantais, and Baker (1989) observed that the ratio of F_v/F_m measures the optimal quantum efficiency of the plant. In other words, it is an indication of the efficiency of the light reaction in photosysnthesis. Generally, healthy and unstressed plants have an F_v/F_m ratio of between 0.70 to 0.83, while stressed plants, which have inefficient light reactions and/or damaged photosynthetic systems, tend to have values lower than 0.6 (Ritchie, 2006).

Chapter 3

Materials and Methods

3.1 Chemicals

Chemicals, growth medium, and plant growth regulators used in this study were purchased from the following companies: Woody Plant Medium (PhytoTechnology Laboratories, USA); indole-butyric acid (IBA) (Sigma, U.S.A); kinetin (Sigma, U.S.A); meso-inositol (Koch-light Laboratories Ltd, U.K.); sucrose (Choneye Pure Chemicals, Taiwan); Agar (BD Biosciences, U.S.A); silver nitrate (Shimakyu's Pure Chemicals, Japan); Folin-Ciocalteu reagent (ICN Biochemicals, Inc., U.S.A); gallic acid (Sigma, U.S.A)

3.2 Plant material, growth medium, and growth conditions

P. cynaroides plantlets that have been in culture for at least six months were used as explants. Microshoots, approximately 13 mm in length (Fig. 3) with three nodes, were excised and placed on growth medium. All microshoots were cultured on Woody Plant Medium (WPM) (Lloyd, & McCown, 1981) supplemented with 0.5 $mg.L^{-1}$ indole-3-butyric acid (IBA), 0.01 $mg.L^{-1}$ kinetin, 100 $mg.L^{-1}$ meso-inositol, 100 $mg.L^{-1}$ silver nitrate, 200 $ml.L^{-1}$ coconut water, and 9 $g.L^{-1}$ agar. 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 (Fig. 3). Each culture vessel contained 250 ml growth medium. The growth medium was autoclaved at 121°C and 104 kPa for 30 min.

Each culture vessel was placed on a Plantower® shelf (A-Tech Bioscientific Co., Ltd., Taipei, Taiwan) (Fig. 3). The Plantower® with the cultures were placed in a CO_2-enriched growth chamber (≈1000 μmol. mol^{-1}) (Table 1) with the temperature and photoperiod adjusted to 25°C ± 2 and 16 h, respectively. Photosynthetic photon flux was adjusted to 30 $\mu mol.m^{-2}.sec^{-1}$.

3.3 Sucrose and ventilation treatments

Two different sucrose concentrations were used to investigate their effects on the growth of *P. cynaroides* microshoots: 10 $g.L^{-1}$ and 30 $g.L^{-1}$. In addition, the effect of gas exchange on the vegetative growth of the microshoots was studied. In order 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. 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 solid medium (Fig. 3). The inlet and outlet valves 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 an air flow controller (Fig. 3), whereby CO_2-enriched air was pumped at set intervals via silicon tubes through a 0.22 μm microfilter attached to the inlet valve and into the Plantima® container. 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 (Fig. 4). 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) (Fig. 5).

3.4 Determination of chlorophyll fluorescence

Determination of chlorophyll fluorescence (Fluoropen FP100, Photon Systems Instruments, 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 upper-most bud 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. After all the leaves of microshoots in each treatment were measured, light was turned on in the room to take light-adapted readings. The same leaves were used to take the light-adapted readings as those used in the dark-adapted readings. The Fv/Fm ratio obtained was used to interpret the photosynthetic efficiency of the microshoots.

3.5 Determination of total phenol content

P. cynaroides microshoots were dried in an oven (DS60, DENG YNG Instruments Co., Ltd) at 38°C for 24 h, and ground into fine powder. The dried sample (0.8 g) was homogenized with a ceramic mortar and pestle in 70% alcohol for 24 h, and filtered. The extraction solution was then placed into a flask and dried under vacuum in a rotary evaporator and a water-bath (SB-1000, EYELA, Japan). A standard curve for gallic acid was prepared (25 - 125 ppm). The total phenol content was determined using a modification of the Folin-Ciocalteu method described by Singleton, and Rossi (1965).

A 125 ppm sample solution was prepared, from which an aliquot of 0.5 mL was taken and mixed with 1.5 mL Folin-Ciocalteu reagent (1: 10 v/v diluted with DDW). After ten minutes, 2 mL sodium carbonate (7.5% w/v) was added to the sample solution. The mixture, which was shaken intermittently, was allowed to stand for 90 minutes in the dark. The

absorbance was measured with a spectrophotometer (Microplate Spectrophotometer, Biotek Instruments, Inc., U.S.A) at 264 nm. The phenolic content was expressed as gallic acid equivalent (mg/g).

3.6 Determination of DPPH radical scavenging activity

The preparation of the *P. cynaroides* sample for the DPPH radical scavenging activity was the same as described for the total phenol content. DPPH activity was determined according to the method of Blois (1958) with slight modifications. The extract was dissolved in methanol in the following concentrations: 1, 2, 5, 10, and 25 $\mu g.mL^{-1}$. An aliquot of 100 μL and 200 μL of the sample solutions and DPPH (0.1 mM) solution, respectively, was added into a 96-well dish. The mixture was allowed to stand for thirty minutes in the dark at room temperature. Absorbance was measured at 517 nm using a spectrophotometer (Microplate Spectrophotometer, Biotek Instruments, Inc., U.S.A).

3.7 Statistical Analysis

One microshoot per culture vessel was used with seven replications per treatment. Data for number of buds, mean bud length, mean leaf area (Image J, Version 1.6, U.S.A), chlorophyll content (SPAD value, Konica Minolta Chlorophyll Meter SPAD-502 Plus, Japan), Fm/Fv value, fresh weight of microshoots were collected after 100 days in culture. A completely randomized design was used. Where appropriate, data were analyzed using Duncan's multiple range test, and least significant difference (LSD) to compare treatment means using SPSS version 17.

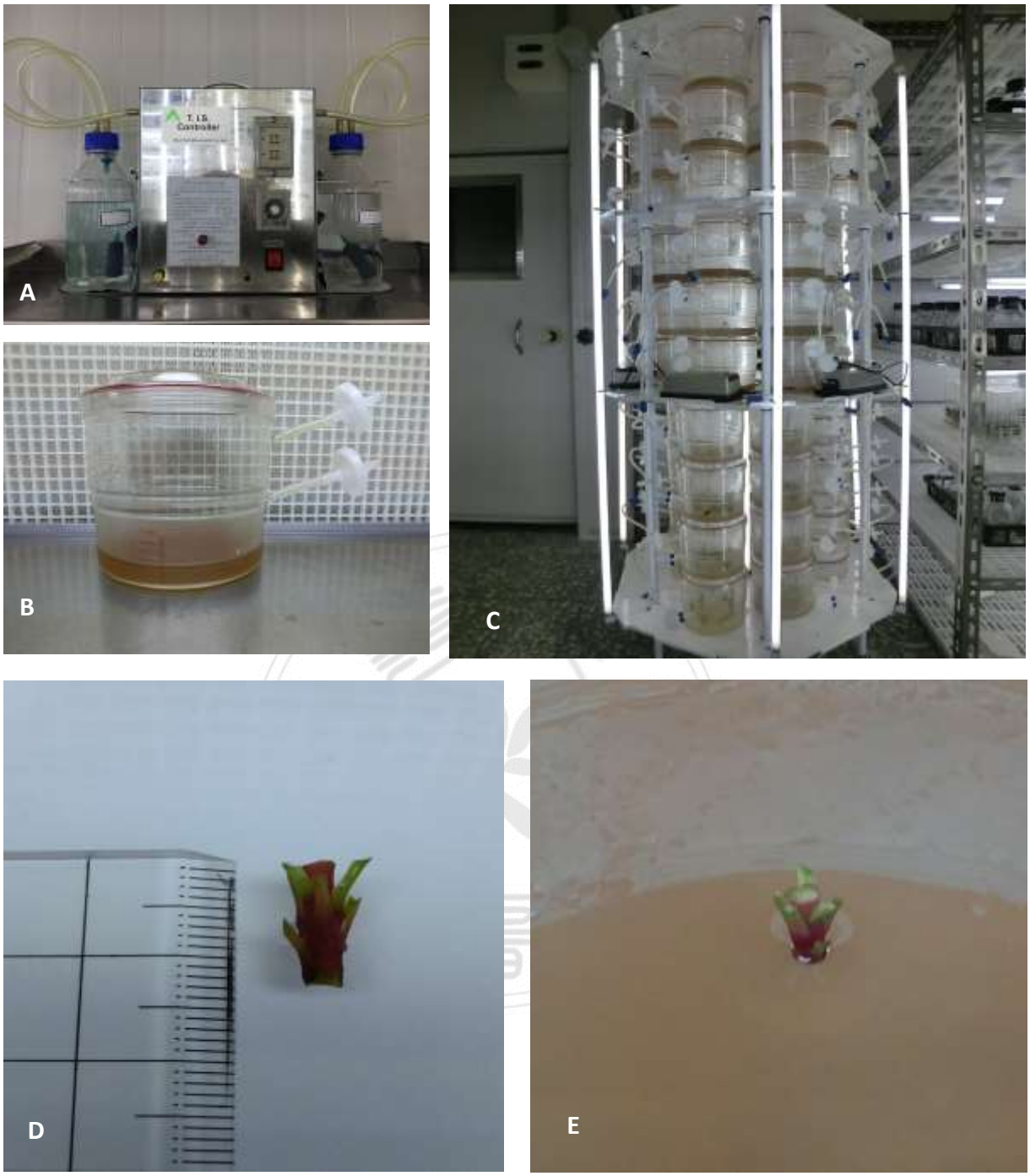

Fig. 3 (A) Air flow controller; (B) Plantima® culture vessel; (C) Plant tower; (D) Microshoot length (13 mm); (E) Microshoot in Plantima® culture vessel

Table 1 Carbon dioxide concentration in culture vessel of *P. cynaroides* microshoots under different ventilation treatments.

Treatment	CO_2 ($\mu mol.mol^{-1}$)
Natural ventilation	
10 $g.L^{-1}$ Sucrose	465
30 $g.L^{-1}$ Sucrose	480
Forced ventilation (2 min/ 2 h)	
10 $g.L^{-1}$ Sucrose	980
30 $g.L^{-1}$ Sucrose	1360
Forced ventilation (2 min/ 4 h)	
10 $g.L^{-1}$ Sucrose	1150
30 $g.L^{-1}$ Sucrose	990

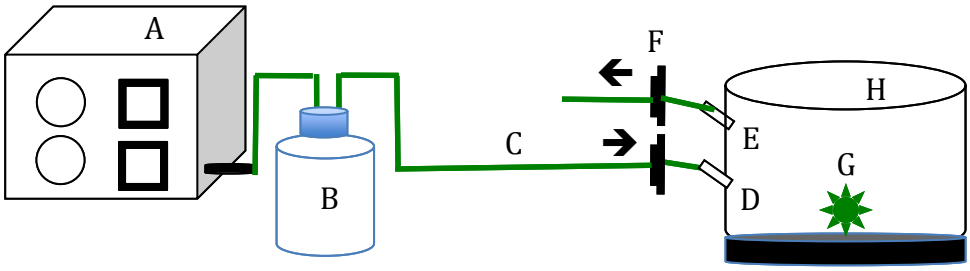

Fig. 4 Schematic drawing of the forced ventilation system using modified temporary immersion culture vessels. (A) Air flow controller; (B) Sterilant (1000 ppm Cu^{++}); (C) Silicon tube; (D) Inlet; (E) Outlet; (F) Air microfilter; (G) *P. cynaroides* microshoot; (H) Modified Plantima® culture vessel

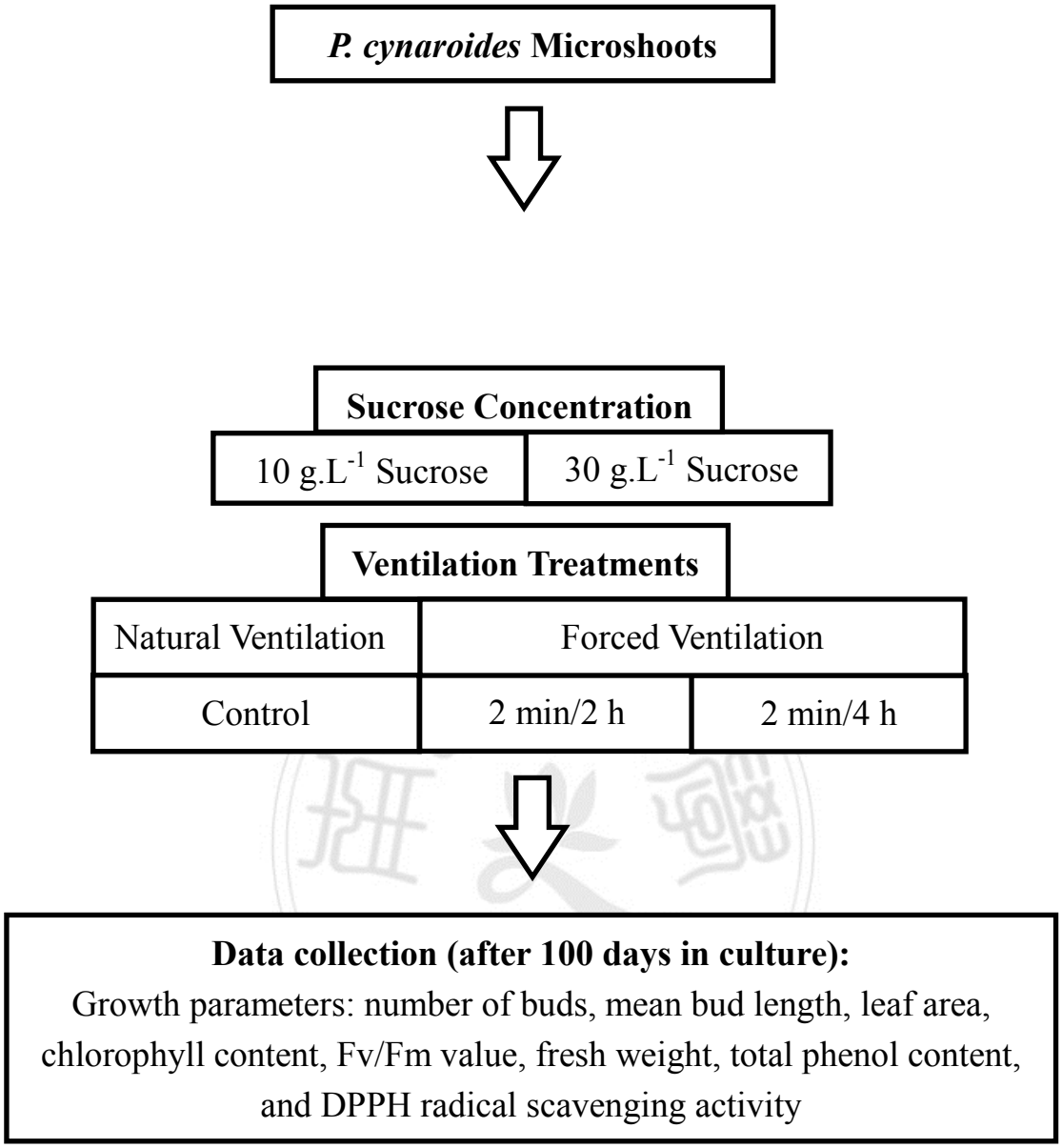

Fig. 5 Experimental flow chart

Chapter 4

Results

The vegetative growth of the microshoots varied between the different sucrose concentration and ventilation treatments. Through visual observation on day 15, it was found that all microshoots cultured on 30 $g.L^{-1}$ sucrose in the 2 min/4 h force ventilation treatment produced buds, compared to only one microshoot in the other treatments forming buds (Fig. 6). Overall, compared to the 10 $g.L^{-1}$ sucrose treatments, microshoots cultured on growth medium supplemented with 30 $g.L^{-1}$ sucrose produced the highest number of buds at day 15, irrespective of the ventilation treatment.

At day 55, microshoots grown on 30 $g.L^{-1}$ sucrose continued to produce the highest number of buds with the largest leaf area, compared to those cultured on 10 $g.L^{-1}$ sucrose (Fig. 7). However, microshoots that

were force-ventilated seemed to produce higher numbers of leaves than those that were naturally ventilated. In addition, leaf yellowing were evident in microshoots cultured on 10 $g.L^{-1}$ sucrose, particularly in the 2 min/2 h ventilation treatment (Fig. 7).

After 100 days when data were collected, results showed that microshoots that were force-ventilated for 2 min/2 h in the 10 $g.L^{-1}$ sucrose treatment produced the highest mean number of buds (4.7) (Fig. 8; Fig. 9). In contrast, the lowest mean number of buds (2.5) was produced by microshoots in the same sucrose concentration in the control treatment (natural ventilation). Moreover, except for the natural ventilation treatment with 30 $g.L^{-1}$ sucrose, the number of buds formed on microshoots grown in the 2 min/2 h force ventilation treatment was significantly higher ($P = 0.002$) than all the other treatments, irrespective of the sucrose concentration. In addition, no significant differences were observed in the number of buds formed between the different ventilation treatments in microshoots cultured on growth medium containing 30 $g.L^{-1}$

sucrose. Within each ventilation treatment, the number of buds formed were significantly different between those cultured on 10 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose, except for the 2 min/4 h forced ventilation treatment (Fig. 9).

With regard to bud length, within the same ventilation treatment sucrose concentration did not significantly affect their growth (Fig. 10). In contrast, significant differences in bud length were found between microshoots subjected to the different ventilation treatments in the 10 $g.L^{-1}$ sucrose treatment. In particular, microshoots in the 2 min/4 h ventilation treatment produced significantly longer buds than the other treatments. On the other hand, no significant differences were observed between the different ventilation treatments in the 30 $g.L^{-1}$ sucrose treatment (Fig. 10).

Microshoots force-ventilated for 2 min/4 h produced the largest leaves, irrespective of the sucrose concentration (Fig. 11). 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 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose treatments. In addition, similar to the elongation of buds described above, sucrose concentration did not affect the expansion of leaves formed on microshoots cultured in the same ventilation treatment. Microshoots force-ventilated for 2 min/2 h produced the smallest leaves, however, these were not significantly different to the control treatment (natural ventilation) (Fig. 11).

The chlorophyll content of microshoots was significantly affected by both sucrose concentration and ventilation treatment (Fig. 12). The highest chlorophyll content was found in the leaves of microshoots grown on 30 $g.L^{-1}$ sucrose and ventilated for 2 min/4 h, whereas the lowest chlorophyll content was detected on microshoots cultured on 10 $g.L^{-1}$ sucrose and ventilated for 2 min/2 h. Most importantly, in each type of ventilation treatment, microshoots grown on 30 $g.L^{-1}$ sucrose were significantly higher than those cultured on 10 $g.L^{-1}$ sucrose. Furthermore,

the leaves of microshoots cultured on growth medium supplemented with 10 $g.L^{-1}$ sucrose and forced ventilated for 2 min/4 h produced significantly higher chlorophyll content than those in the 2 min/2 h force ventilation treatment. Similar results were found in the 30 $g.L^{-1}$ sucrose treatments with significantly higher chlorophyll content detected in leaves of microshoots that were force-ventilated for 2 min/4 h than those ventilated naturally (control). However, no significant differences were observed between the two force ventilation treatments when cultured on 30 $g.L^{-1}$ sucrose (Fig. 12).

The Fv/Fm ratio of the microshoots are shown in Fig. 13. Within each ventilation treatment, no significant differences were found between the microshoots cultured on 10 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose. Similarly, on growth medium containing 10 $g.L^{-1}$ sucrose, no significant differences were observed between microshoots ventilated naturally, forced ventilated for 2 min/2 h, and forced ventilated for 2 min/4 h. On the contrary, in growth medium containing 30 $g.L^{-1}$ sucrose, the Fv/Fm ratio

of microshoots forced ventilated for 2 min/4 h was significantly higher than those in the control treatment (natural ventilation) (Fig. 13).

The fresh weight of microshoots was significantly affected by the sucrose concentration (Fig. 14). However, this was only evident in the 2 min/2 h ventilation treatment, where the fresh weight of microshoots cultured on 30 $g.L^{-1}$ sucrose were significantly higher than those grown on 10 $g.L^{-1}$ sucrose. The effect of sucrose concentration in the 2 min/2 h ventilation treatment was particularly pronounced since the highest and lowest fresh weights were observed in microshoots grown on 30 $g.L^{-1}$ and 10 $g.L^{-1}$ sucrose, respectively. In contrast, no significant differences were found between microshoots subjected to different sucrose concentrations in the other ventilation treatments. In addition, within the same sucrose treatment, no significant differences were found between the fresh weights of microshoots between each ventilation treatment (Fig. 14).

Results from the analyses of total phenol content of the microshoots

showed that microshoots cultured on medium supplemented with 30 $g.L^{-1}$ sucrose contained higher amounts of total phenols, irrespective of the type of ventilation treatment (Fig. 15). Overall, the highest amount of total phenols were found in the 2 min/4 h force-ventilated treatment (30 $g.L^{-1}$ sucrose), whereas the lowest phenol content was detected in microshoots cultured on 10 $g.L^{-1}$ sucrose in the 2 min/2 h force-ventilated treatment (Fig. 15).

In the analyses of DPPH radical scavenging activity, results found that microshoots cultured on 10 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose in the control treatment (natural ventilation) had similar scavenging activity (Fig. 16). However, in the 2 min/2 h and 2 min/4 h forced ventilation treatments (Fig. 17; Fig. 18), the DPPH analysis results showed that microshoots grown on 30 $g.L^{-1}$ sucrose had higher scavenging activities than those cultured on 10 $g.L^{-1}$ sucrose. Therefore, excluding the natural ventilation treatment, high sucrose concentration had a pronounced effect on free radical scavenging activities in *P. cynaroides* microshoots.

Interaction effects between ventilation and sucrose concentration on the vegetative growth, chlorophyll content, and Fv/Fm values of *P. cynaroides* microshoots are shown in Table 1. Statistical analyses revealed that no significant interactions between sucrose concentration and ventilation treatment were present in the bud length, leaf area, Fv/Fm values, chlorophyll content, and fresh weight parameters. However, significant interactions were found between sucrose concentration and ventilation treatment in the number of buds formed by the microshoots.

Further analysis (least significant difference, LSD) of the number of buds parameter was carried out (Table 2; Table 3). Results showed that the highest number of buds was found in microshoots cultured on 10 $g.L^{-1}$ sucrose that were force-ventilated for 2 min/2 h, while the lowest number of buds was produced by microshoots grown in the same sucrose concentration that were naturally ventilated (control treatment) (Table 2). In the 10 $g.L^{-1}$ sucrose treatment, significant differences ($P \leq 0.001$) were observed between the 2 min/2 h force ventilation treatment and the

natural ventilation treatment, as well as between the 2 min/2 h treatment and the 2 min/4 min force ventilation treatment. In the 30 $g.L^{-1}$ sucrose treatment, no significant differences were found between the three ventilation treatments (P = 0.307). Furthermore, in the natural ventilation treatment and the 2 min/2 h force ventilation treatment, significant differences were detected between the 10 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose treatments (P = 0.003, P = 0.007, respectively) (Table 3). On the other hand, no significant differences were found in the 2 min/ 4 h force ventilation treatment (P = 0.632).

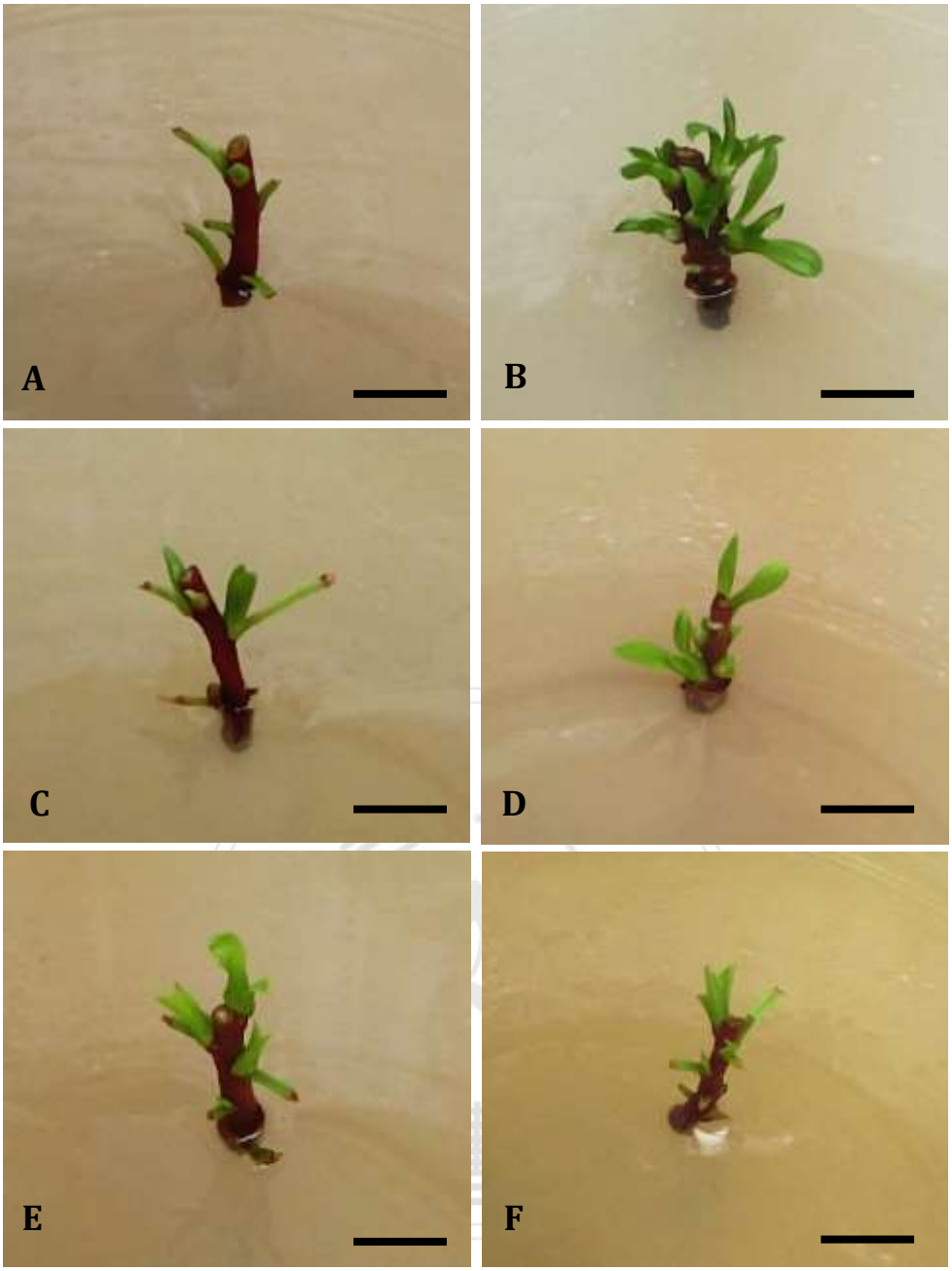

Fig. 6. *P. cynaroides* microshoots after 15 days in culture. (A) 10 g.L^{-1} sucrose in natural ventilation treatment (control); (B) 30 g.L^{-1} sucrose in natural ventilation treatment; (C) 10 g.L^{-1} sucrose in 2 min/2 h treatment; (D) 30 g.L^{-1} sucrose in 2 min/2 h treatment; (E) 10 g.L^{-1} sucrose in 2 min/4 h ventilation treatment; (F) 30 g.L^{-1} sucrose in 2 min/4 h ventilation treatment. Bar ≈ 1 cm

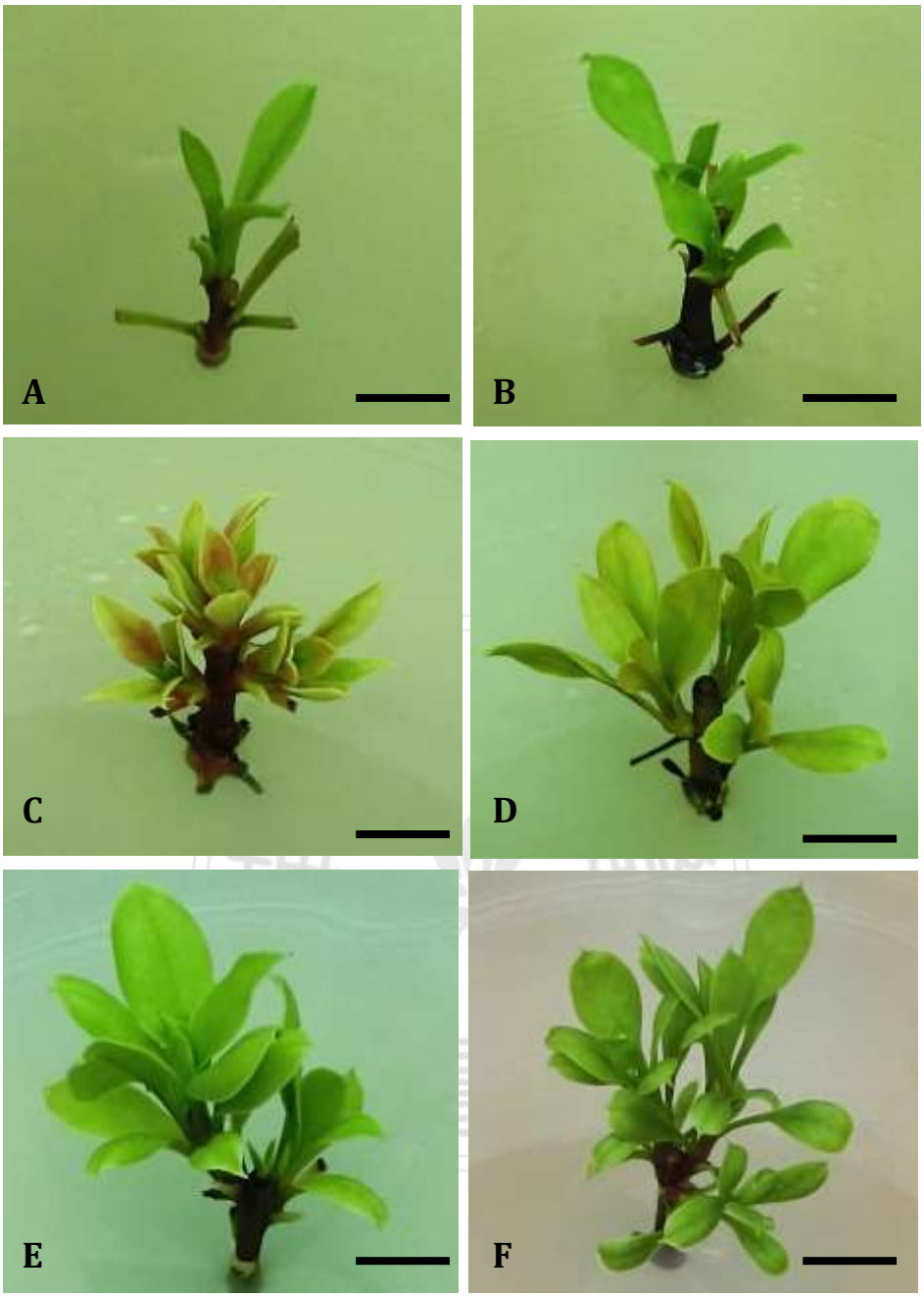

Fig. 7. *P. cynaroides* microshoots after 55 days in culture. (A) 10 g.L^{-1} sucrose in natural ventilation treatment (control); (B) 30 g.L^{-1} sucrose in natural ventilation treatment; (C) 10 g.L^{-1} sucrose in 2 min/2 h treatment; (D) 30 g.L^{-1} sucrose in 2 min/2 h treatment; (E) 10 g.L^{-1} sucrose in 2 min/4 h treatment; (F) 30 g.L^{-1} sucrose in 2 min/4 h treatment. Bar ≈ 1 cm

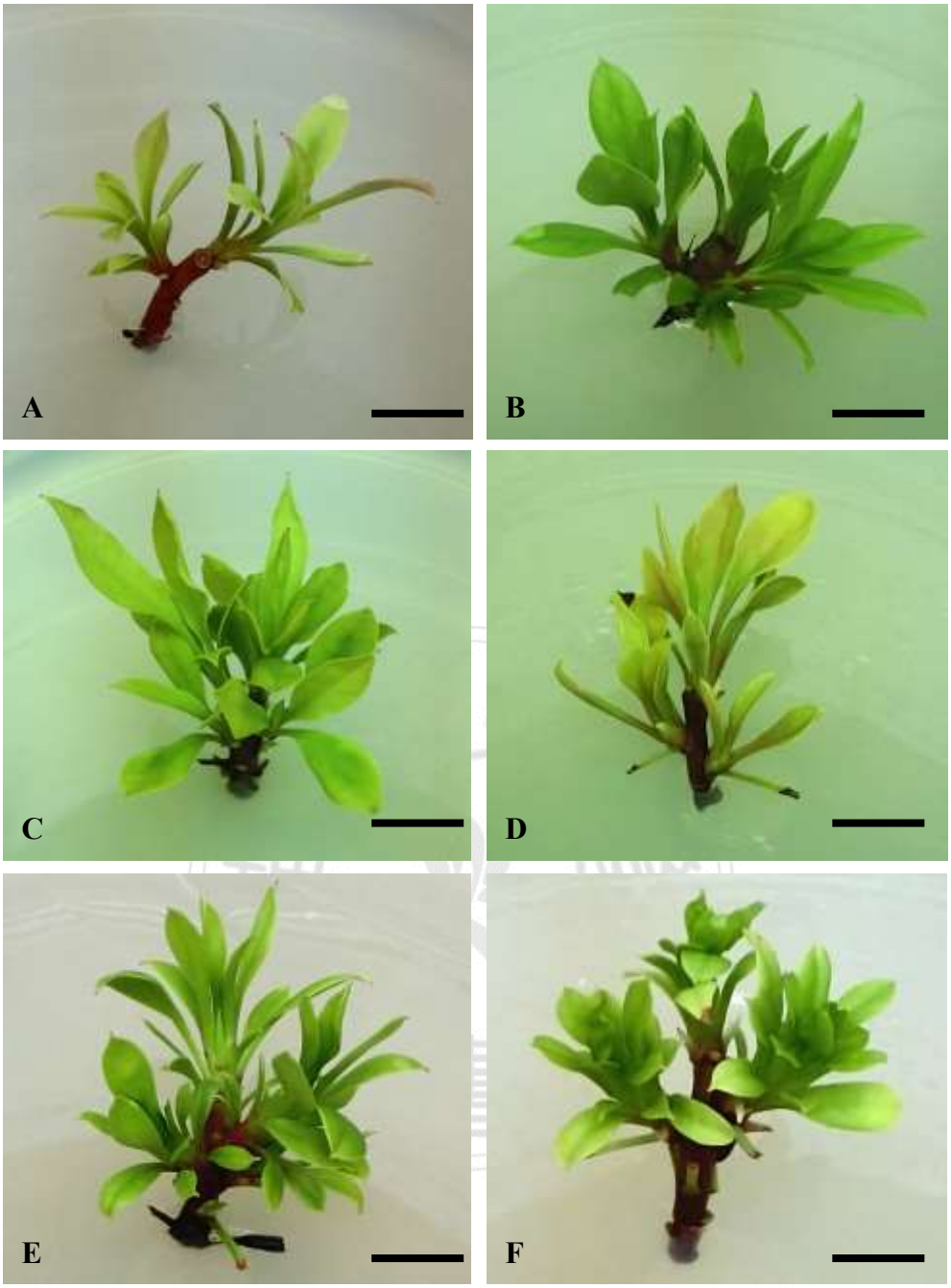

Fig. 8. *P. cynaroides* microshoots after 100 days in culture. (A) 10 g.L^{-1} sucrose in natural ventilation treatment; (B) 30 g.L^{-1} sucrose in natural ventilation treatment; (C) 10 g.L^{-1} sucrose in 2min/2h treatment; (D) 30 g.L^{-1} sucrose in 2min/2h treatment; (E) 10 g.L^{-1} sucrose in 2min/4h treatment; (F) 30 g.L^{-1} sucrose in 2min/4h treatment. Bar ≈ 1 cm

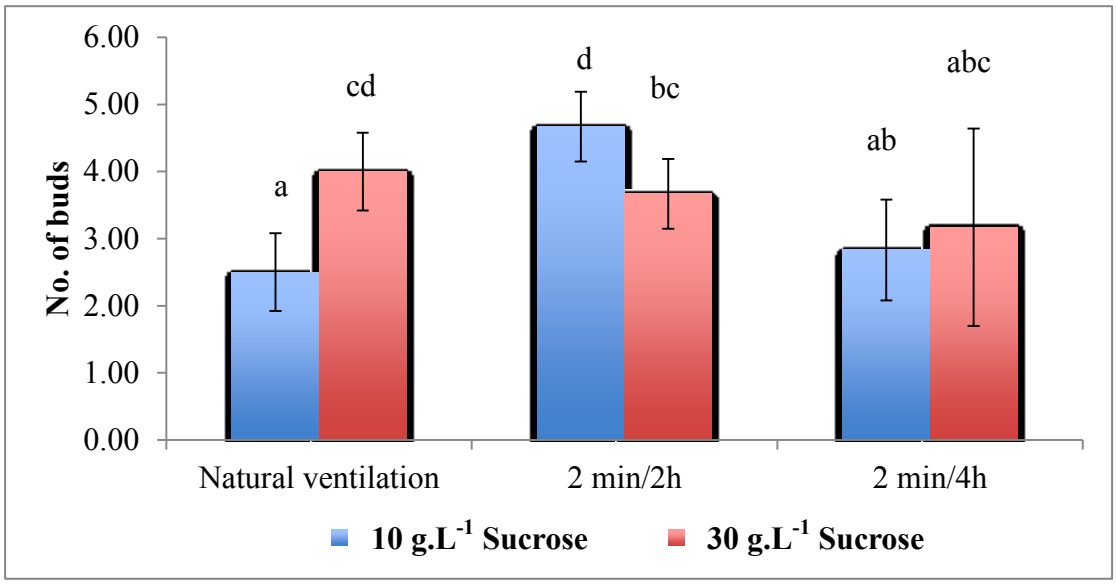

Fig. 9 Effects of sucrose concentration and ventilation treatment on formation of new buds after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

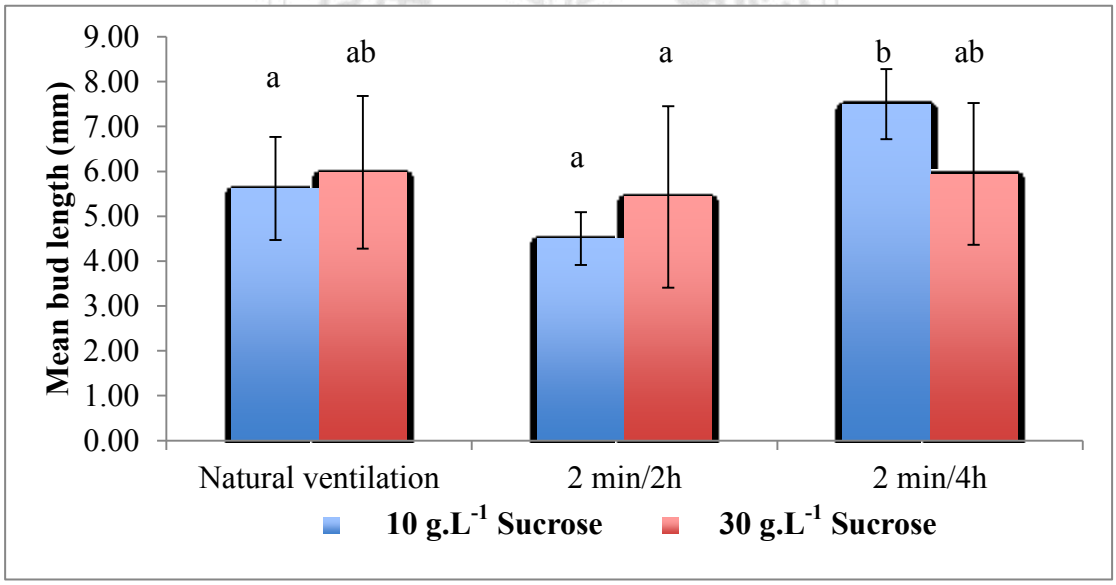

Fig. 10 Effects of sucrose concentration and ventilation treatment on elongation of buds after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

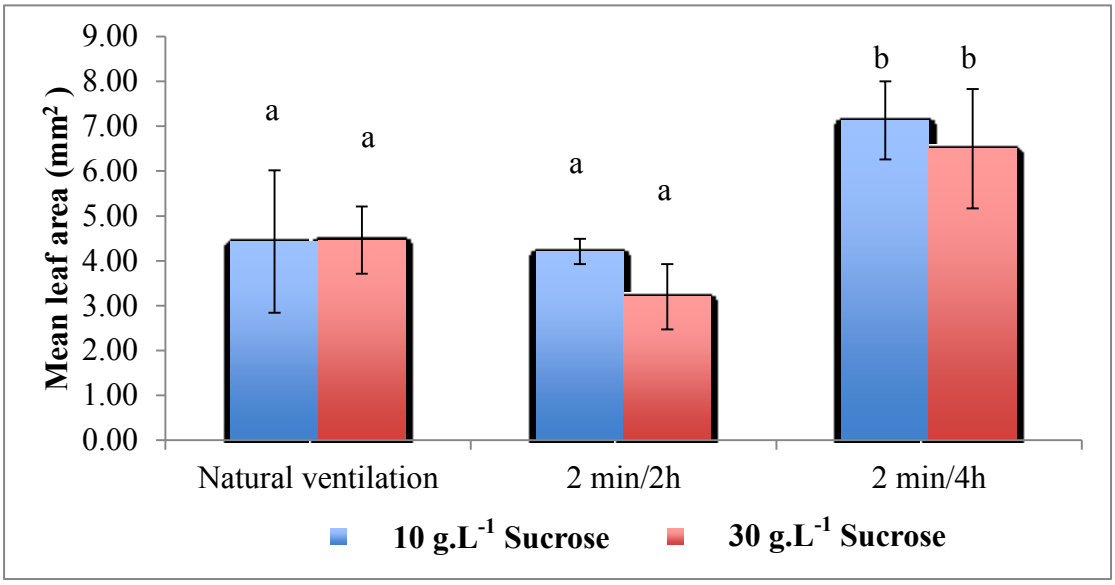

Fig. 11 Effects of sucrose concentration and ventilation treatment on leaf growth after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

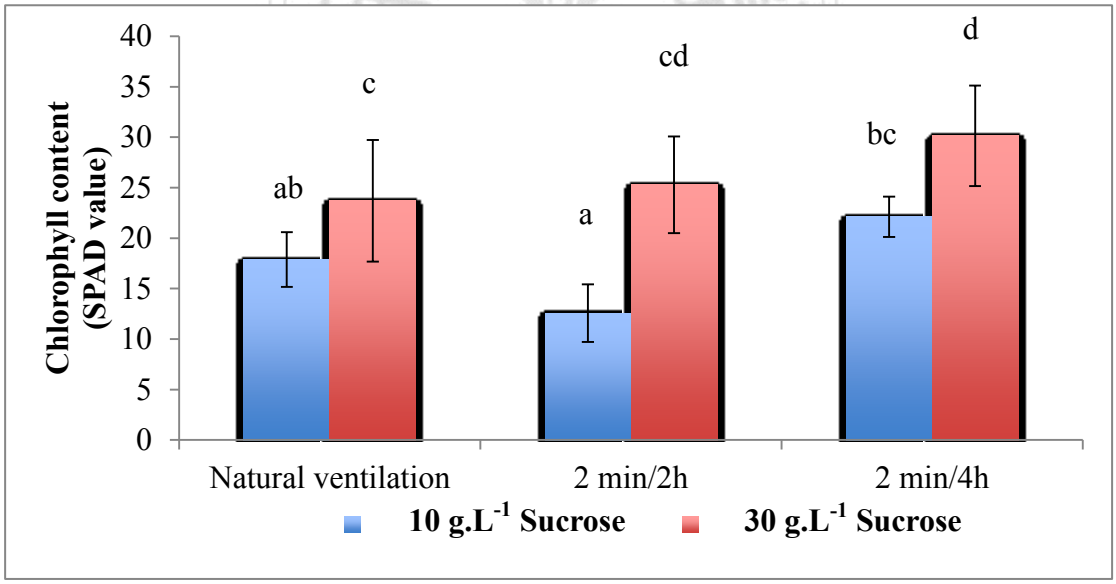

Fig. 12 Effects of sucrose concentration and ventilation treatment on chlorophyll content after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

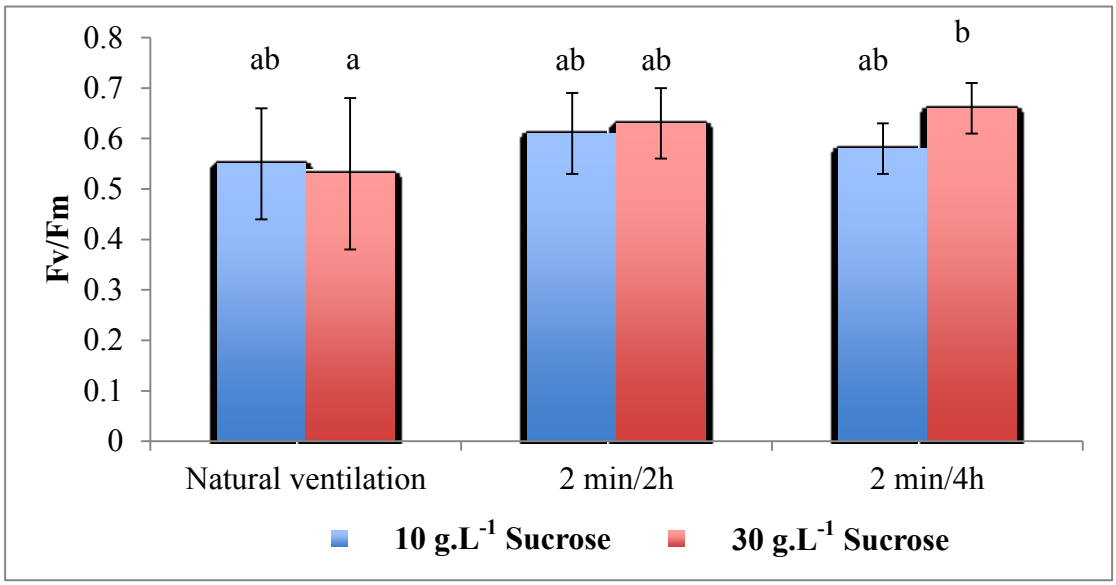

Fig. 13 Effects of sucrose concentration and ventilation treatment on Fv/Fm values after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

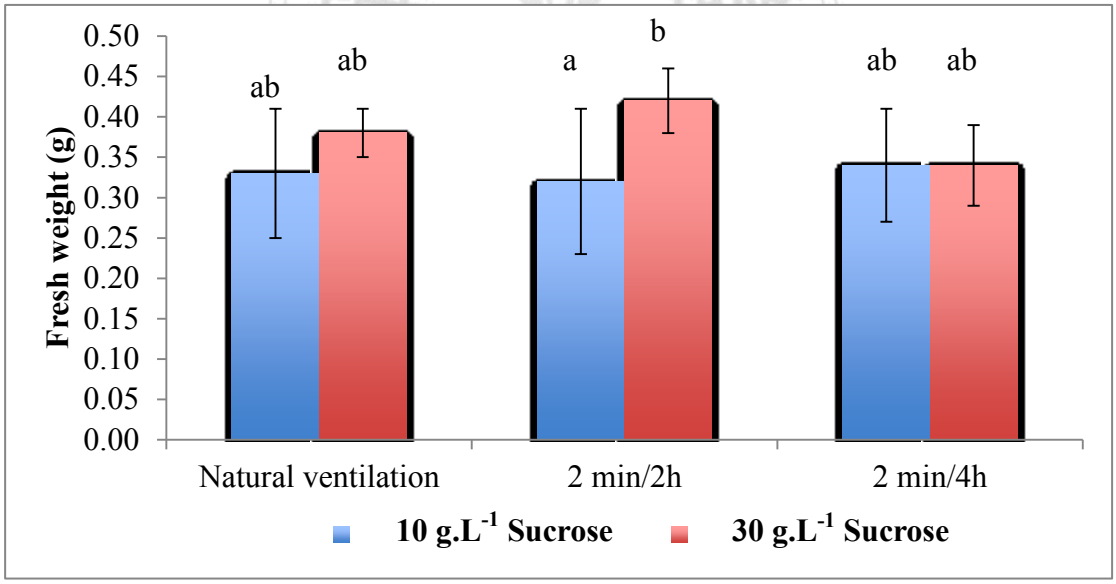

Fig. 14 Effects of sucrose concentration and ventilation treatment on fresh weight after 100 days in culture. Treatment means with different letters are significantly different based on Duncan's Multiple Range test ($P < 0.05$).

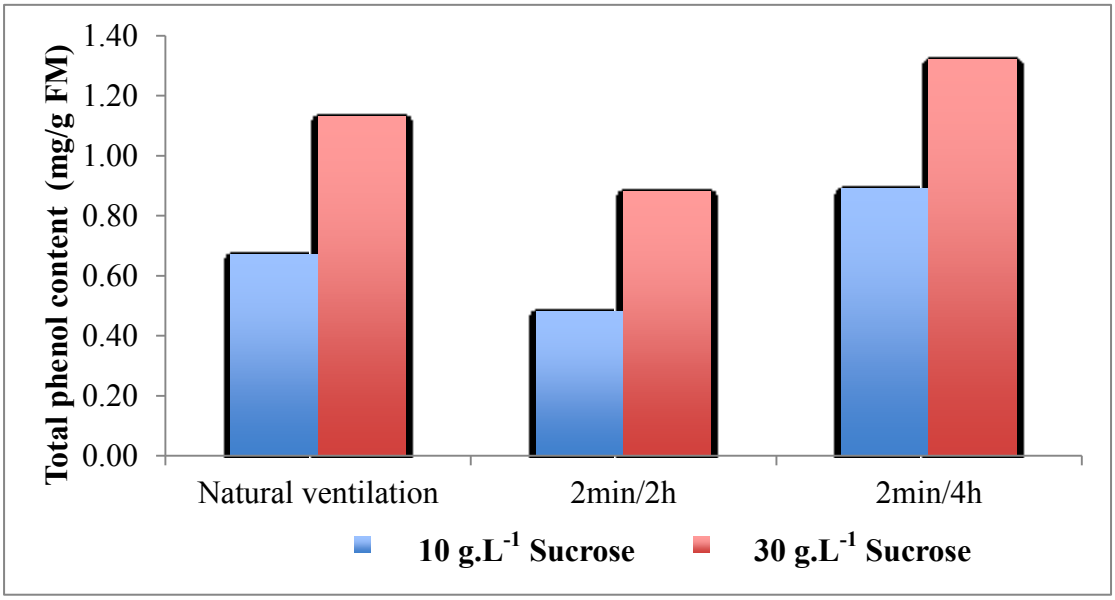

Fig. 15 Effects of sucrose concentration and different ventilation treatments on total phenol content of *P. cynaroides* microshoots after 100 days in culture.

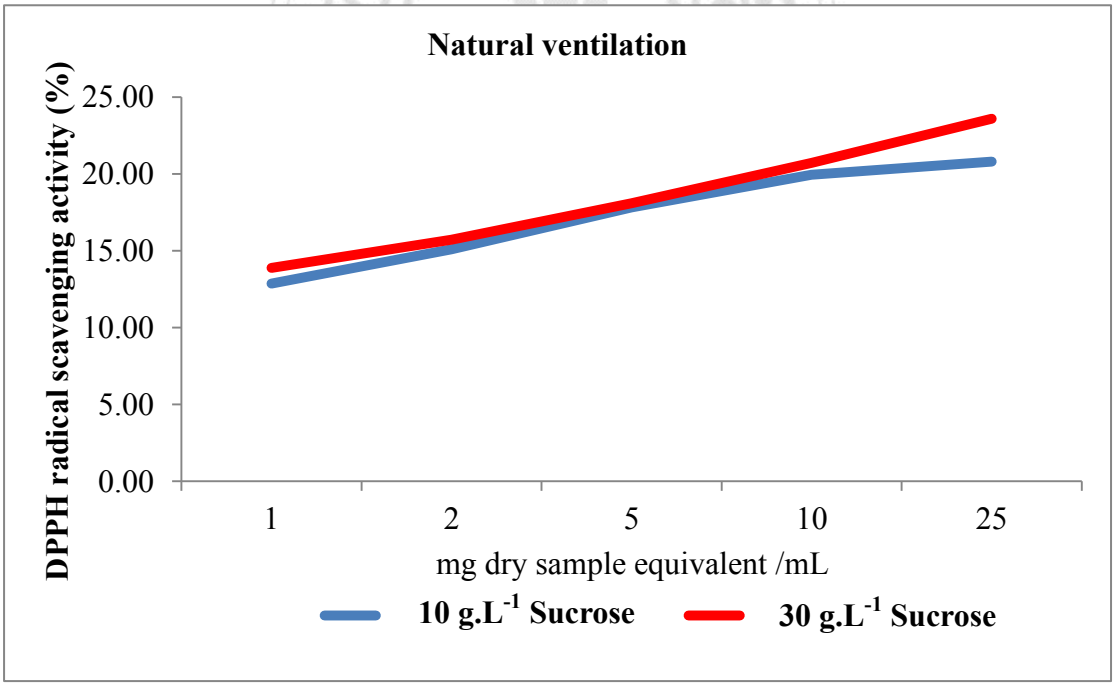

Fig. 16 Effects of sucrose concentration under natural ventilation on the scavenging effects (%) of *P. cynaroides* microshoots after 100 days in culture.

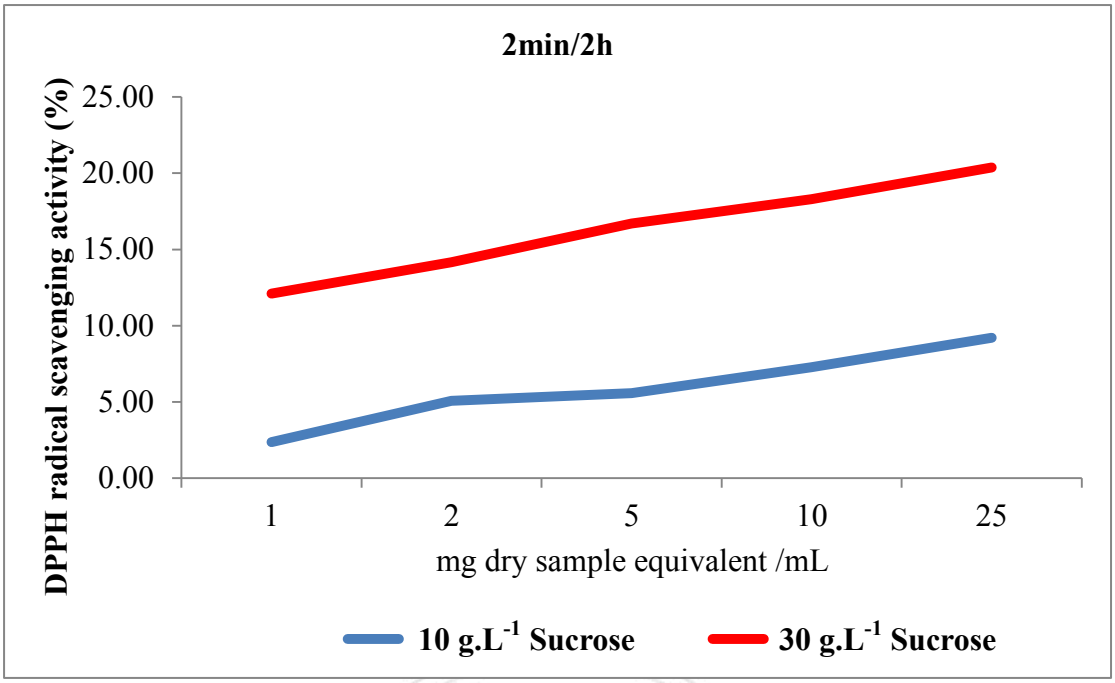

Fig. 17 Effects of sucrose concentration under 2 min/2 h force ventilation on the scavenging effects (%) of *P. cynaroides* microshoots after 100 days in culture.

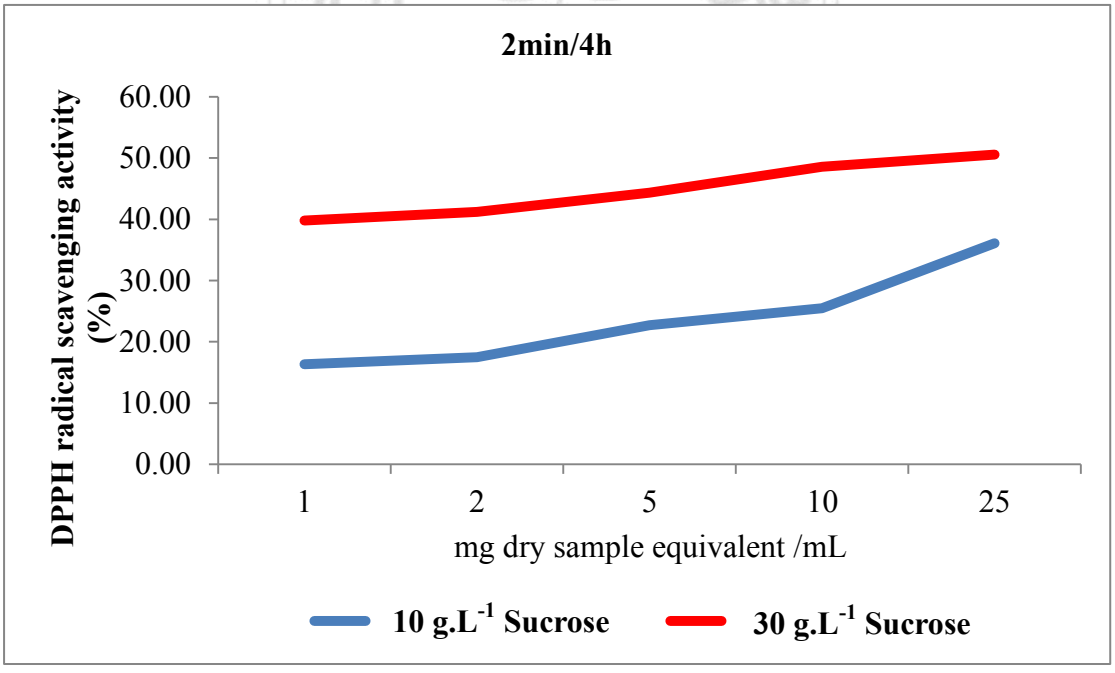

Fig. 18 Effects of sucrose concentration under 2 min/4 h force ventilation on the scavenging effects (%) of *P. cynaroides* microshoots after 100 days in culture.

Table 2 Interaction effects of ventilation and sucrose concentration on the vegetative growth, chlorophyll content, and Fv/Fm values of *P. cynaroides* microshoots.

Treatment		*F* value	*P* value
No. of buds			
	Ventilation	6.72	0.00
	Sucrose	0.98	0.33
	Ventilation*Sucrose	6.50	0.00
Bud 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
Fv/Fm			
	Ventilation	3.80	0.03
	Sucrose	0.76	0.38
	Ventilation*Sucrose	0.96	0.39
Fresh weight (g)			
	Ventilation	0.71	0.50
	Sucrose	4.28	0.05
	Ventilation*Sucrose	1.77	0.19

Table 3 The effects of different types of ventilation on the number of buds formed in *P. cynaroides* microshoots under different sucrose concentrations.

Treatment	Natural ventilation	2 min/2 h	2 min/4 h	*F* value	*P* value
10 g.L^{-1} Sucrose	2.50±0.58^{a}	4.67±0.52^{b}	2.83±0.75^{a}	18.583	<0.001
30 g.L^{-1} Sucrose	4.00±0.58^{a}	3.67±0.52^{a}	3.17±1.47^{a}	1.273	0.307

Different letter in the same row indicate values differ significantly according to least significant difference (LSD).

Table 4 The effects of different sucrose concentrations on the number of buds formed in *P. cynaroides* microshoots under different ventilation treatments.

Treatment	10 g.L^{-1} Sucrose	30 g.L^{-1} Sucrose	*F* value	*P* value
Natural ventilation	2.50±0.58	4.00±0.58	17.182	0.003
2 min/2 h	4.67±0.52	3.67±0.52	11.250	0.007
2 min/4 h	2.83±0.75	3.17±1.47	0.244	0.632

Chapter 5

Discussion

The number of buds formed by *P. cynaroides* microshoots in the control treatment (natural ventilation) were significantly affected by sucrose concentration, where microshoots cultured on 30 $g.L^{-1}$ produced significantly higher number of buds than those grown in 10 $g.L^{-1}$ sucrose (Fig. 9). Similar results were reported in *Vaccinium corymbosum* and *Eucomis autumnalis* cultures (Cao, Fordham, Douglass, & Hammerschlag, 2003; Taylor, & van Staden, 2001), where a high sucrose concentration increased axillary shoot formation. In addition, a study by Rahayu, and Habibah (2015) showed that significantly higher number of shoots were produced by *Feronia limonia* explants cultured on growth medium supplemented with 30 $g.L^{-1}$ sucrose than on 10 $g.L^{-1}$ sucrose in similar environmental conditions. It is known that a high concentration of sucrose, up to a point, is initially needed for morphogenesis of buds and

leaves (Solarova, Posphilsilova, Catsky, & Santrucek, 1989). On the other hand, excessive sucrose concentrations have been shown to inhibit growth and development of axillary buds (Gabryszewska, 2009).

In contrast to the control treatment (natural ventilation), significantly higher number of buds were formed in 10 $g.L^{-1}$ sucrose of the 2 min/2 h forced ventilation treatment compared to the 30 $g.L^{-1}$ sucrose treatment. According to Zobayed, Kubota, and Kozai (1999), high frequency ventilation will result in the relative humidity of the headspace in the culture vessel being reduced to the point where the growth medium starts to lose water content, i.e., a sizable amount of water loss from the growth medium caused by high ventilation (2 min/2 h), resulting in changes to its composition, most notably the sucrose concentration. Consequently, the 10 $g.L^{-1}$ sucrose concentration increased to a level where it was optimal for the growth of *P. cynaroides* microshoots, resulting in enhanced bud formation. Conversely, the 30 $g.L^{-1}$ sucrose concentration in the 2 min/2 h forced ventilation treatment

rose to excessive levels, adversely affecting bud formation.

Therefore, it seems that under natural ventilation, which relies on the natural diffusion of air between the inside and outside of the culture vessel to take place, 30 $g.L^{-1}$ sucrose was an optimal concentration for the formation of *P. cynaroides* buds, whereas 10 $g.L^{-1}$ sucrose was optimal concentration in the 2 min/2 h forced ventilation treatment (Fig. 9). The importance of ventilation frequency is further highlighted by the fact that the number of buds formed by *P. cynaroides* microshoots in the 2 min/4 h forced ventilation treatment, which were ventilated less frequently, did not differ significantly to those in the control treatment in their respective sucrose concentrations (Fig. 9).

With regard to bud elongation, results showed that sucrose concentration does not have a significant effect in *P.cynaroides* microshoots, irrespective of the ventilation treatment (Fig. 10). These results are consistent with findings reported by Wojtania,

Wegrzynowicz-Lesiak, Dziurka, and Waligorski (2015). In their study, no significant differences were found between *Pelargonium* x *Hortorum* explants grown in 15 $g.L^{-1}$ and 30 $g.L^{-1}$ sucrose. Similarly, no significant differences in the length of *Magnolia* x *soulangiana* microshoots were observed between different sucrose concentrations in non-ventilated conditions (Wojtania et al., 2015).

Several studies have demonstrated that growth and development of buds and shoots depend on sucrose, nitrogen, and cytokinin levels (Coruzzi, & Zhou, 2001; Zheng, 2009). Furthermore, recent findings have provided evidence that activity of shoot growth is affected by coordination between nutritional and hormonal signaling (Rubio et al., 2009). Moreover, Krouk et al. (2011) suggested that growth and development of plantlets are controlled by hormonal signaling pathways, which are regulated by fluctuations in nutrient levels. Although examining the effects of plant growth regulators and nutrient levels was not within the scope of this study, it is probable that further development,

including elongation, of the buds formed on *P. cynaroides* microshoots were directly affected by changes in plant growth regulator concentrations and nutrient levels caused by the effects of ventilation.

The type of ventilation significantly affected leaf growth of *P. cynaroides* microshoots, as indicated by their mean leaf area (Fig. 11). Significant differences were observed between the two forced ventilation treatments, with the leaf area in the 2 min/4 h treatment being significantly higher. This is likely due to the high ventilation frequency in the 2 min/2 h treatment causing excessive water loss in the growth medium than those in the 2 min/4 h and natural ventilation treatments. Goncalves, Santos, Nina, and Che-Vreuil (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, effects on vegetative biomass would become more pronounced with longer-term growth (Mohamed et al., 2010). The results of the present study clearly indicated that this might have been the case

since 100 days 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 (Fig. 11). These results suggest that natural ventilation and 2 min/2 h forced ventilation represent the two extremes of the ventilation treatments, in which the growth media composition became suboptimal for leaf growth in *P. cynaroides* microshoots. On the other hand, these findings illustrated that growth media characteristics in culture vessels that were force-ventilated for 2 min/4 h were optimal for the growth and development of leaves in *P. cynaroides* microshoots (Fig. 11).

In contrast to leaf growth, chlorophyll content were positively affected by sucrose concentration, irrespective of the ventilation treatment (Fig. 12). These results are in agreement with those reported by Langford, and Wainwright (1987) and Abbott, and Belcher (1980). However, contradictory findings have also been reported with regard to the effect of sucrose on chlorophyll content. According to Grout, and

Donkin (1987), exogenous supply of sucrose is not required for the normal development of chlorophyll. In the *in vitro* culture of walnut explants, the chlorophyll 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, Pasqual, Martins, & Francisca, 2015). These authors concluded that lower concentrations of sucrose in the growth medium stimulate chlorophyll production in plantlets cultured *in vitro*. In contrast, chlorophyll content in potato plantlets was not significantly affected by different sucrose concentrations, instead, significant effects were observed between different ventilation treatments (Mohamed et al., 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, Duigan, & Berlyn, 2002). These reports seem to indicate that, in addition to sucrose, chlorophyll content of leaves is affected by interactions of several different factors.

The F_v/F_m values are a good indicator for photosynthesis efficiency, which in turn, provides evidence as to whether a plant is stressed or not. Plants that have F_v/F_m values below 0.6 are considered stressed (Ritchie, 2006). In this study, *P. cynaroides* microshoots that had F_v/F_m values above 0.6 were cultured in 10 $g.L^{-1}$ sucrose with 2 min/2 h forced ventilation (0.61), 30 $g.L^{-1}$ with 2 min/2 h forced ventilation (0.63), and 30 $g.L^{-1}$ with 2 min/4 h forced ventilation (0.66) (Fig. 13). Interestingly, all *P. cynaroides* microshoots subjected to natural ventilation had F_v/F_m values lower than 0.6, and therefore are considered stressed with poor photosynthetic efficiency. Although sucrose concentration in the growth

medium has been shown to affect photosynthetic rate (Mosaleeyanon, Cha-um, & Kirdmanee, 2004; Hdider et al., 1994), this was not observed in the present study (Fig.13). Instead, our findings showed significant differences between forced ventilation (2 min/4 h) and natural ventilation in the 30 $g.L^{-1}$ sucrose treatment on photosynthetic performance (F_v/F_m) of *P. cynaroides* microshoots. These results are consistent with several studies that demonstrated significantly high photosynthetic rates of explants cultured in forced ventilated treatments (Heo, & Kozai, 1999; Xiao et al., 2005; Zobayed et al., 1999). The positive effects of forced ventilation with high frequency gaseous exchange 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).

Overall, the fresh weight of *P. cynaroides* microshoots were higher in growth medium containing 30 $g.L^{-1}$ sucrose than those grown on 10 $g.L^{-1}$ sucrose (Fig. 14). These results are similar to those reported in walnut explants (Hassankhah et al., 2014). The importance of sucrose as a

carbon source has been well documented (Hazarika, Parthasarathy, & Nagaraju, 2004). The fresh weight of plantlets cultured on high sucrose concentration is almost always higher than those in lower sucrose treatments (Kozai, Koyama, & Watanabe, 2002; Rahman, & Alsadon, 2007). However, with regard to ventilation, studies often report that the fresh weights of ventilated plantlets are lower than those without or with very little ventilation (Mohamed et al., 2010). This may be due to reduced relative humidity in the culture vessel caused by gaseous exchange, resulting in higher amounts of water loss from the plantlets. It is important to note that the frequency of ventilation has a direct effect on the amount of water that is lost from plantlets.

The availability of carbohydrate is a major factor affecting secondary metabolite production, including phenolic compounds (Bryant, Chapin, & Klein, 1983). Several studies have reported that increasing the sucrose concentration in the growth medium caused an increase in endogenous phenolic concentration in explants (Yildiz, Onde, & Ozgen,

2007; Cui, Murthy, & Paek, 2014). According to Cui et al. (2014), the high accumulation of phenolics may also be due to elevated levels of osmotic stress caused by high sucrose concentration in the growth medium. Findings by Julkunen-Tiito (1996) showed that by increasing the sucrose concentration in the growth medium, accumulation of phenolic content in willow explants also increased. Similar results were reported by Curtis, and Shetty (1996), where high endogenous phenolic levels were associated with an increase in sucrose concentration in oregano plants.

These results are consistent with the results of the present study, where the endogenous total phenol content of *P. cynaroides* microshoots cultured on growth medium supplemented with 30 $g.L^{-1}$ sucrose were higher than those in the 10 $g.L^{-1}$ sucrose treatments in all ventilation treatments. Yildiz et al. (2007) concluded that the accumulation of large amounts of phenolics in the leaves due to increases in sucrose concentration is the cause of necrosis and tissue death. Necrosis and

tissue death were not however, observed in the *P. cynaroides* microshoots throughout the duration of this study.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) activity is a good indicator for determining free radical scavenging activities of phenolic compounds. Furthermore, Cui et al. (2014) stated that elevated levels of phenolics in plantlets are related to the osmotic stress caused by high concentrations of sucrose, which results in increased DPPH activity. Therefore, DPPH scavenging activities can also be used to assess the osmotic stress effects of sucrose.

Results clearly showed higher DPPH activity in the 30 $g.L^{-1}$ sucrose concentrations compared to the 10 $g.L^{-1}$ sucrose treatment, particularly in the forced ventilation treatments (Fig. 17; Fig 18). This strongly suggests that the relatively high sucrose concentration (30 $g.L^{-1}$) caused osmotic stress in *P. cynaroides* microshoots, which led to an increase in phenolic production, resulting in higher DPPH activity. These results are in

agreement with those reported by Cui et al. (2014), who found that increases in sucrose concentration resulted in increased total phenol accumulation, which in turn, led to increases in DPPH activity in *H. perforatum* explants. Moreover, these findings further increases the possibility that the high frequency forced ventilation (2 min/2 h) altered the growth medium composition, most notably the 30 $g.L^{-1}$ sucrose concentration, causing osmotic stress on *P. cynaroides* microshoots, which negatively affected their growth. This was particularly pronounced in bud formation (Fig. 9; Fig. 11). Conversely, as a result of the changes in the growth medium characteristics, significantly higher number of buds was instead produced in 10 $g.L^{-1}$ sucrose by *P. cynaroides* microshoots, which were not subject to osmotic stress. The relationship between ventilation treatment and sucrose concentrations are further demonstrated by their significant interaction effects (Table 1; Table 2; Table 3).

Chapter 6

Conclusion

This study investigated the effects of different ventilation treatments and sucrose concentrations on the growth and development of *P. cynaroides* microshoots. Findings from this study showed significant effects of both ventilation and sucrose treatments on the growth of the microshoots. Overall, microshoots that were forced ventilated performed better than those that were naturally ventilated. In particular, the 2 min/4 h forced ventilation treatment seemed to be best suited for the elongation of buds and leaf growth, as well as for the production of chlorophyll. In addition, the addition of 30 $g.L^{-1}$ sucrose in the growth medium produced better results in general, including total phenol content and DPPH scavenging activity.

Research Limitations

Although valuable results were obtained from this study, however, the size of the microshoots used in each treatment was a bit small. This resulted in the inability to obtain the dry weight for each microshoot after drying. Consequently, the combined dry material of all microshoots used in each treatment had to be used for the analyses of total phenol content and DPPH activity. Furthermore, due to the limited amount of plantlets available, a low number of replications was used in each treatment. A higher number of replications would provide an even better insight into the growth of the *P. cynaroides* microshoots as affected by the ventilation and sucrose treatments.

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