Research Article

Effects of culture medium components and container type on growth and development of blackberry micropropagation

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Abstract

Blackberry is an imported and challenging crop to grow in Thailand. This study aimed to investigate the effects of medium formulations, sucrose concentration and containers on plant growth of blackberry micropropagation. The salt medium formulations, concentrations of sucrose (0, 1, 2 and 3%) and two types of containers (glass bottles with normal lids and vented lids) were tested. The highest overall quality as 2.58 and 2.91 scores were found in shoots grown on MS medium + 3% sucrose using vented lids, and 0.5× MS + 3% sucrose using vented lids, respectively. The shoots grown on a medium without sucrose had poor growth, while shoots grown on a medium with low sucrose concentrations using vented lids had normal growth and similar quality as shoots grown on MS. Selected conditions of the culture medium were conducted and evaluated for antioxidant properties. Shoots grown on containers using vented lids had a higher fresh weight. There were no significant differences in total phenolic contents and DPPH.

Keywords: Micropropagation, Mineral nutrition, Sucrose, Containers, Blackberry

Introduction

Plant tissue culture medium consists of 13 minerals, growth regulators, carbon sources, vitamins, and gelling substances. Mineral nutrition from those 13 minerals plays a vital role in plant growth and development [1, 2]. Although the Murashige and Skoog (MS) [2] formulation is commonly used for *in vitro* culture of various plants, it was designed to grow tobacco callus, and is not suitable for all plants. Several studies indicated that some plants cannot grow or be propagated well on MS. Shoot or organ cultures showing non-optimal growth on MS were observed in many plant species including red raspberries (*Rubus idaeus* L.) [3, 4, 5, 6, 7, 8]. A recent study of micropropagation of red raspberry and blackberry of the *Rubus* genus showed that MS mineral nutrients were unsuitable for growth, multiplication, and development of the five *Rubus* cultivars tested [7, 8]. Investigating the effects of *in vitro* mineral nutrients is very challenging because these minerals are very important components for *in vitro* plant growth. Plant species or cultivars may have diverse requirements for nutrition [5, 6, 7, 8]. The optimal level of sucrose, a typical carbon and energy source in *in vitro*

culture systems, also varies with the plant species. Still, there are few studies reporting its effect on plant growth and development of *Rubus* [9, 10].

Another critical factor is the environmental condition within containers or vessels. For example, low gas exchange, high moisture or ethylene accumulation may cause poor growth or abnormalities [11]. To overcome this limitation, gas-permeable membranes on lids (vented lids) have been used on species such as eggplants, passion fruit and neem [12, 13, 14]. A photoautotrophic or semi-photoautotrophic condition was defined as the culture technique (using sugar-free medium). It provides environmental condition that allow micropropagated plants to photosynthesize all or most of their carbon [15, 16].

Traditional methods of blackberry propagation including division of the crown or suckers have been used for a long time [17]. However, these methods are ineffective for producing large amount of plant stock in commercial cultivation. Moreover, creating rooted plants or suckers from different fields may spread pathogens such as nematodes, fungi, bacteria, or viruses. Given these limitations of traditional methods, micropropagation of blackberries was first attempted in 1977 and successfully used in 1978 [18]. Currently, micropropagation quickly produces several uniformly healthy stocks of many plant species, including blackberries.

In Thailand, blackberry crop production is mostly found in the northern highlands (e.g., Chiang Mai, Chiang Rai Phayao), where temperature is cool enough for flowering. However, production is still mostly from rooted plants propagated by cutting or digging suckers. The goal of this study was to delineate micropropagation methods, suitably container types and levels of MS mineral salts and sucrose, that could replace these traditional propagation methods and thereby improve the quantity and quality of domestic blackberry production.

Material and Method

Plant materials and establishment

Blackberry mother plants were obtained from Thai royal project (Doi Pui Research Station) and stocked in a temporary greenhouse. Shoot tips were taken from the mother plants as explants and washed with tap water for 30 min. After surface sterilization with 10% Clorox for 10 min and rinsing with sterilized deionized water for three times (5 mins each), meristems about 0.2-0.5 mm were cut under a stereo microscope. The meristems were placed on MS medium with free plant growth regulators (PGR). After a few weeks, shoot elongation occurred and these shoots were transferred to MS medium with 1.0 mg/L N6-benzylaminopurine (BAP), 30 g/L sucrose, and 8 g/L agar at pH 5.7 before autoclaved. Shoots were grown in glass bottles (8 oz.) with 30 mL of medium per container at 25±1^{o}C with a 16 hr photoperiod using cool and warm fluorescent bulbs (PPFD = 35 µmol m^{-2} s^{-1}). Those shoots were re-subculture every 4 weeks until sufficient shoots were obtained for subsequent experiments (Fig. 1).

Figure. 1 Blackberry shoots grown on MS basal medium for re-subculture in every 4 weeks.

The effect of semi-photoautotrophic condition on blackberry growth and development

Plantlets (about 1 cm long shoots) were transferred onto media of changing MS concentration (0.5, 1.0, 1.5×) and sucrose concentration (0, 1, 2, 3%), and changing container types (16-ounce glass bottles with normal lids and vented lids). The experiment was conducted in Completely Randomized Design (CRD). There were 24 treatments (Table 1), four replicates (bottles) per treatment, and four plants per bottle. Shoots were cultured for 6 weeks at 25±1°C with a 16 hr photoperiod using cool and warm fluorescent bulbs (PPFD = 35 μmol m^{-2} s^{-1}). Overall quality was defined. Blackberry shoots showing poor growth were scored as 1, intermediate growth with pale leaves were scored as 2, ideal growth with green leaves were scored as 3.

The effect of semi-photoautotrophic condition on phytochemical properties

The five conditions shown in Table 2 were selected and repeated to evaluate after 6 weeks of culturing. *In vitro* growth, phytochemical content, and antioxidant activity (2,2-Diphenyl-1-picrylhydrazyl or DPPH) were assayed, using the methods described in Poothong et al. [19].

Table 1 The 24 treatments testing sucrose and MS basal medium concentrations and container types

Treatment (Tr.)	Factors		
	Sucrose (%)	Medium (× MS)	Lid
1	0	0.5	Normal
2	1	0.5	Normal
3	2	0.5	Normal
4	3	0.5	Normal
5	0	0.5	Vented
6	1	0.5	Vented
7	2	0.5	Vented
8	3	0.5	Vented
9	0	1.0	Normal
10	1	1.0	Normal
11	2	1.0	Normal
12	3	1.0	Normal
13	0	1.0	Vented
14	1	1.0	Vented
15	2	1.0	Vented
16	3	1.0	Vented
17	0	1.5	Normal
18	1	1.5	Normal
19	2	1.5	Normal
20	3	1.5	Normal
21	0	1.5	Vented
22	1	1.5	Vented
23	2	1.5	Vented
24	3	1.5	Vented

Results

The effect of semi-photoautotrophic condition on blackberry growth and development

Different MS strengths and sucrose concentrations had a significant effect on plant growth. Blackberry shoots were grown on 0.5×MS strength supplemented with 1% sucrose in vented containers. They had statistically (p<0.05) higher overall quality scores, shoot length, leaf number and leaf area than shoots grown on control medium (Fig. 2A, 1C, 1D and 2A). Shoots grown on increased MS strength (1.5×) supplemented with 3% sucrose in vented containers had the highest shoot number (Fig. 2B). However, in this experiment, the result showed that blackberry had a low shoot number representing common multiplication.

Shoots grown with the highest sucrose concentration (3%) had increased leaf colour regardless of MS strength or container type (Fig. 3B). Shoots grown on reduced MS strength (0.5×) in vented containers had high rooting scores and the longest roots (Fig. 3C and 3D). Moreover, growing shoots on 0.5×MS without sucrose in vented lid containers did not affect rooting score. The result also showed that growing this blackberry in vented containers had a higher rooting score and longer roots (Fig. 4).

The effect of semi-photoautotrophic condition on phytochemical properties

To investigate the effect of all the above conditions on plant growth and phytochemical properties, the second experiment was conducted. In this study, blackberry shoots were grown on 0.5× MS strengths supplemented with either 1% or 3% sucrose in containers with vented lids. They had improved leaf color, leaf area, rooting and root length (Table 2). Using any MS strengths supplemented with either 1% or 3% sucrose in containers with vented lids, shoots had increased fresh weight and dry weight (Table 3). Although the total phenolic contents found in each treatment had no statistically significant differences, shoots grown on reduced MS strengths (0.5×MS) supplemented with 3% sucrose in containers with vented lids had the highest total flavonoid contents (Table 3). However, those shoots had the lowest antioxidant activity (Table 3).

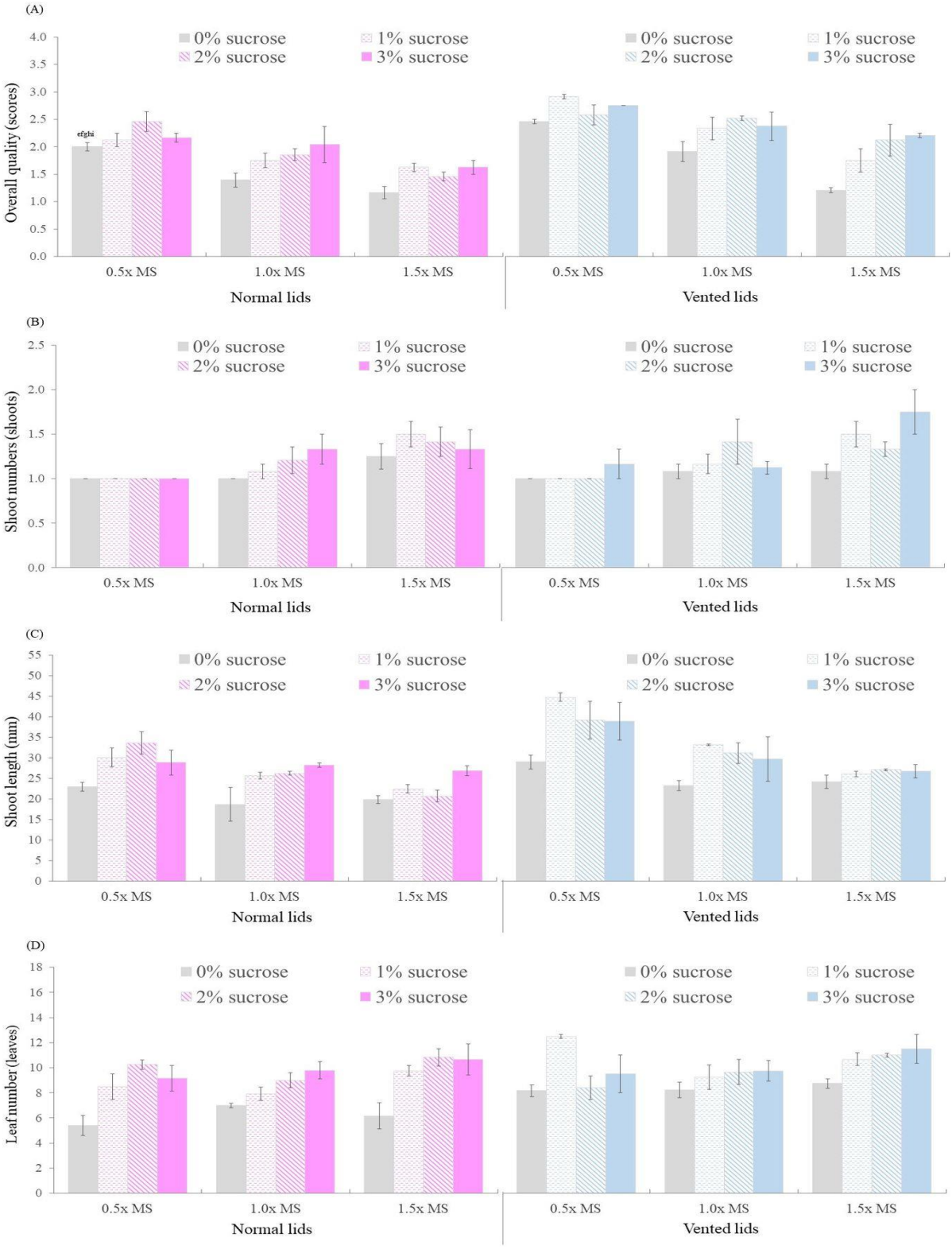

Fig. 2 Blackberry shoot growth when cultured on MS basal medium of various strength supplemented with various sucrose concentration in containers of two lid types. (A) overall quality scores, (B) shoot numbers, (C) shoot length and (D) leaf numbers. Data represented as mean ± SE (n=3)

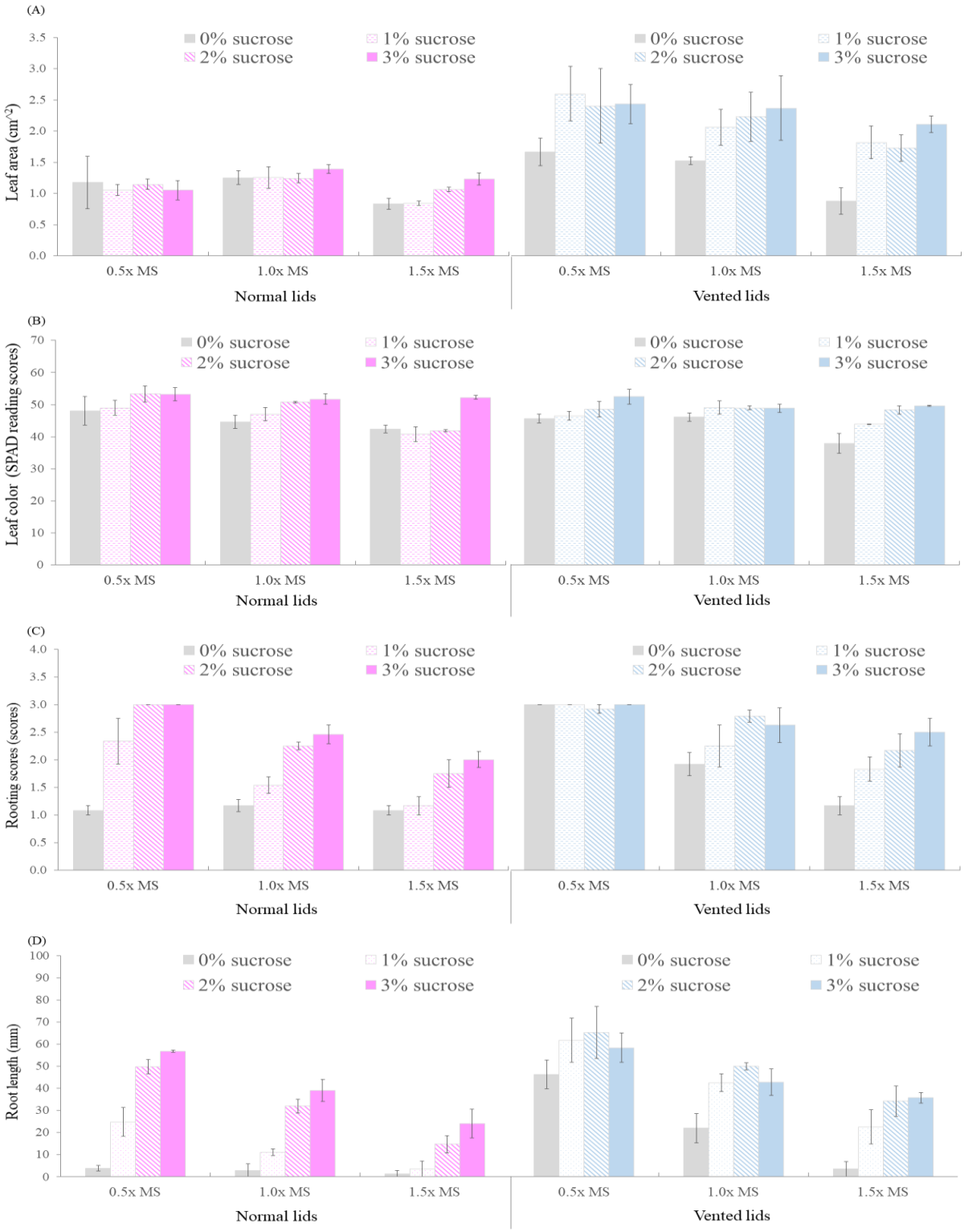

Fig. 3 Blackberry shoot growth when cultured on MS basal medium of various strength supplemented with various sucrose concentration in containers of two lid types. (A) leaf area, (B) leaf color, (C) rooting scores and (D) root length. Data represented as mean ± SE (n=3)

Fig. 4 Blackberry shoot appearance after 6 weeks of culturing on MS basal medium of various strength supplemented with various sucrose concentration in containers of two lid types ● = normal lids and ○ = vented lids (bar **I** = 1 cm).

Table 2 The effect of semi-photoautotrophic condition on blackberry growth and development. Data represented as mean ± SE (n=24).

Basal medium	Sucrose concentration (%)	Types of lids	Shoot length (mm)	Shoot number (shoots)	SPAD (scores)	Leaf area (cm^2)	Rooting (scores)	Root length (mm)
1×MS	3	Normal	54.2±2.2	1.4±0.1	48.4±0.8^{b}	1.7±0.1^{c}	2.9±0.1^{a}	43.3±2.3
0.5×MS	1	Vented	50.9±2.7	1.2±0.1	46.1±1.0^{c}	3.2±0.3^{a}	3.0±0.0^{a}	50.8±1.9
0.5×MS	3	Vented	40.4±2.1	1.3±0.1	49.0±1.0^{b}	2.8±0.2^{ab}	3.0±0.0^{a}	48.2±1.8
1.5×MS	1	Vented	55.5±1.4	1.3±0.1	49.6±0.9^{ab}	2.4±0.1^{b}	2.6±0.1^{b}	44.2±3.3
1.5×MS	3	Vented	59.6±2.0	1.3±0.1	51.8±0.6^{a}	2.7±0.2^{ab}	2.9±0.1^{a}	49.8±2.2
			NS	NS	Sig	Sig	Sig	NS

Means in the same column that are followed by different letters are significantly different ($p \leq 0.05$) using Duncan's Multiple Range Test. Sig means significantly different and NS means not significantly different.

Table 3 The effect of semi-photoautotrophic condition on blackberry weights and phytochemical properties. Data represented as mean ± SE (n=6).

Basal medium (×MS)	Sucrose concentration (%)	Types of lids	Fresh weight (g)	Dry weight (g)	Total phenolic contents (mg GAE/g dry weight)	Total flavonoid contents (mg CE/g dry weight)	Antioxidant activity (DPPH assay)
1×	3	Normal	0.58±0.05^{b}	0.14±0.01^{c}	21.56±3.66	4.66±0.68^{ab}	236.75±43.37
0.5×	1	Vented	1.12±0.10^{a}	0.19±0.01^{ab}	20.82±1.16	3.33±0.66^{b}	226.86±27.30
0.5×	3	Vented	1.15±0.10^{a}	0.21±0.01^{a}	25.06±1.31	5.74±0.46^{a}	186.07±9.25
1.5×	1	Vented	0.80±0.05^{b}	0.17±0.01^{bc}	21.78±4.59	1.67±0.20^{c}	275.64±37.60
1.5×	3	Vented	1.02±0.04^{a}	0.21±0.01^{a}	23.37±0.51	4.67±0.22^{ab}	293.23±19.77
			Sig	**Sig**	NS	**Sig**	NS

Means in the same column that are followed by different letters are significantly different ($p \leq 0.05$) using Duncan's Multiple Range Test**.** Sig means significantly different and NS means not significantly different.
GAE = gallic acid equivalent, CE = catechin equivalent

Discussion

Adjustment of the mineral nutrient concentrations was one of effective protocols for micropropagation of *Rubus* [20]. Until recently, Murashige and Skoog (MS) medium firstly developed for tobacco callus culture has been used for micropropagation of many plants [2]. At the initial stage, most studies focused on use and optimization of basal medium for those plants. Different plant species or cultivars might require different amount of mineral nutrients. In the other words, not all plants can grow well on MS medium. Therefore, modification of MS basal medium has been used for improving or increasing growth of several types of plants such as woody or herbaceous plants [21]. According to our result, it showed that modified MS strength improved shoot and root length (Fig. 1C and 2D). Besides plant hormones or growth regulators, *in vitro* mineral nutrients and sugars also play a critical role in plant growth and development [22, 23]. Optimization of the *in vitro* mineral component resulted in the reduction of the plant growth regulators required in the culture medium [24, 25]. Furthermore, sucrose provides an energy source and affects the water

use of *in vitro* plants based on the osmotic potential [10, 23]. Mineral and water uptake are governed by osmolarity of culture medium. Water availability is determined by altering nutrients, carbon sources or agar. It importantly affected growth and response in the anther culture or cell suspension culture [26].

Type of container was a significant factor when cultivating *in vitro* shoots under semi-photoautotrophic condition. Our data indicated that the use of ventilated lids improved overall quality scores, shoot length, leaf number and leaf area. When growing shoots in containers with vented lids, blackberry shoots cultured on medium supplemented with low mineral nutrients and sucrose had average growth compared to shoots cultured on medium supplemented with 3% sucrose. According to the results, growing blackberry shoots under semi-photoautotrophic condition also need sucrose because shoots cultured on a sugar-free medium had poor growth compared to shoots cultured on a medium supplemented with 3% sucrose. There were several studies on the advantages of air exchange by using porous or ventilated membranes. The advantages of ventilated membranes generally dealt with improving leaf morphogenesis such as greenness or leaf color including chlorosis. However, in this study, growing shoots in vented lids increased leaf area but not leaf color (Fig. 2A and 2B). Rodrigules et al. (2012) reported that growth of micropropagated neem (*Azadirachta indica* A. Jues.) was improved by using flasks sealed with membranes (polytetrafluoro-ethylene: PTFE) [14]. When blackberry shoots were cultured using vented lids, elevated carbon dioxide or ethylene may escape by diffusion or convection. The alternative microporous tape and PTFE membranes were used on a nodal culture of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen] [12]. The result showed that gas-permeable membranes resulted in improvement of shoot height, and there was no contamination observed in any treatments [12].

In conclusion of this study, semi-photoautotrophic condition with reduced MS minerals and sucrose (1%) promoted blackberry growth and rooting. This finding can be helpful for further research on the effect of pre-culture on transplanting for low-cost mass micropropagation.

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