pISSN 2302-1616, eISSN 2580-2909
Vol 10, No. 2, December 2022, pp. 215-224
Available online http://journal.uin-alauddin.ac.id/index.php/biogenesis
DOI https://doi.org/10.24252/bio.v10i2.31537

The effect of sucrose concentrations and different types of tube cap on *in vitro* growth of Dahlia (*Dahlia* sp.) using vermiculite as substrate

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ABSTRACT. *Dahlia* sp. is an ornamental plant belongs to Compositae. Plantlets produced through conventional *in vitro* culture have low growth ability during acclimatization. The use of ventilation on *in vitro* culture is expected to affect the condition of the plantlet micro environment. Reducing sucrose concentration will improve the physiological functions of the plant. This study aimed to investigate the effect of reduction of sucrose concentrations and the use of various culture tube caps on the growth of shoots cultures of *Dahlia* sp. The experiments used was factorial completely randomized design with two factors ie. concentrations of sucrose (10, 20, and 30 g/l) in combination with four different type of tube caps (Al foil, transparent plastic, filter 1 cm and 2 cm). The experiments used vermiculite as substrate and MS medium with 9 replicates. Parameters observed were plant height, number of leaves, number of nodes, and number of roots which observed every week up to 8 weeks of culture. At 8 weeks culture, fresh weight, and dry weight were also recorded. The results showed that reducing sucrose concentration in combination with tube cap significantly affected on the growth of shoot height, number of leaves, nodes and roots as well as length of root. The highest Dahlia shoot was found in the media containing 10 g/l sucrose in tubes with plastic cap and in 20 g/l sucrose with 2 cm filter cap. The highest number of leaves and nodes was found in media containing 30g/l of sucrose using Al foil and 1 cm filter caps, both were significantly different with others. The highest fresh weight was found in MS medium containing 30 g/l of sucrose with 1 cm filter caps. Highest number of roots were found in the 10 g/l of sucrose using 1cm filter cap and in 30 g/l sucrose with a plastic cap.

Keywords: *Dahlia* sp.; different tube caps; *in vitro*; filter cap; vermiculite sucrose reduction

Article History: Received 25 August 2022; Received in revised form 20 October 2022; Accepted 1 December 2022; Available online 30 December 2022. Ver: Pre-Press
How to Cite This Article: Rudiyanto R, Hapsari BW, Rantau DE, Ermayanti TM. 2022. The effect of sucrose concentrations and different types of tube cap on *in vitro* growth of Dahlia (*Dahlia* sp.) using vermiculite as substrate. *Biogenesis: Jurnal Ilmiah Biologi*. vol 10(2): 215–224. doi: https://doi.org/10.24252/bio.v10i2.31537.

INTRODUCTION

Dahlia spp. are originally from Mexico and Guatemala mountains. Numerous cultivars *Dahlia pinnata* with wide variations in flower color, size and shape have so far been produced by intra and interspecifc hybridizations (Otani *et al*., 2013). Dahlia is an erect plant, has some branches, and hairless (Yuliana, 2016). The decorative value of Dahlia is its inflorescences with appealing petals of various tones, sizes, shapes, and vivid leaves (Marcinek *et al.*, 2019). Dahlia is important for alternative food sources and medicinal plants. Its tubers contain carbohydrates, fiber and several bioactive compounds such as inulin (41.7-72.6%). Inulin is useful for maintaining the growth of bifidobacterium in the digesting system, stimulating the immune system, and reducing the risk of osteoporosis (Sandiya *et al.*, 2014; Yuliana *et al.*, 2014).

Dahlia can be propagated by seeds, tubers, and cuttings, so that it can be vulnerable to pathogens such as fungi, bacteria, and viruses (Nerway *et al*., 2020). Through plant tissue culture, Dahlia can be proliferated aseptically, unaffected by season, and generally more efficient and effective (Ibrahim and Daraj, 2015). Some reports in Dahlia micropropagation strategies have been published. Dahlia shoots cultured in MS medium containing Kinetin produced some lateral nodes. The addition of 2-iP did not stimulate shoot growth, while the addition of thidhiazuron causes vitrification on its shoots (Ermayanti & Al-Hafiizh, 2013). The development of Dahlia shoot culture was optimized in MS containing 0.5 mg/l BAP, while the best root development was in MS media containing 2.0 mg/l NAA (Wadankar and Malode, 2012). The highest multiplication rate from nodes was found with 0.25-0.5 mg/l BAP. The best quality shoots and the highest multiplication rate were also achieved in the media containing 2 mg/l BAP in combination with 5 mg/l GA_3 (Marcinek *et al.*, 2019). Some

studies were also done on regeneration from leaf and stem explants (Otani *et al.*, 2013), direct and indirect organogenesis (Ibrahim & Dara, 2015), and for viruses' elimination by meristem culture (Nerway *et al.*, 2020). Both the reduction of media compositions and modification on the in vitro environmental conditions have not been reported.

In plant tissue culture, sucrose is a source of energy for explant development and as an osmotic regulator (Rantau *et al*., 2017). On the other hand, plantlets growing under tissue culture condition do not fix enough CO_2 to sustain growth in the absence of sucrose which is mainly due to limited CO_2 inside the vessel (Gago *et al.*, 2014). Vented tube caps can increase airflow from and into the tube so that the rate of photosynthesis of plantlets will increase and produce robust plantlets (Mohamed & Alsadon, 2010), gave better growth in *Stevia rebaudiana* (Ermayanti *et al*., 2017; Rantau *et al.*, 2017). In walnut (*Juglans regia*) explants, by using 15 g/l sucrose in vented tubs gave healthier plantlets with higher total chlorophyll (Hassankhah *et al*., 2014). However, in *Tacca leontopetaloides* plantlets, there was no different growth between ventilated and non-ventilated culture (Wulandari *et al*., 2017).

The addition of vermiculite, a well aerated substrate, to the rooting medium enhanced root development in some hardwood species, including walnut, papaya, and pear (Allison *et al.*, 2016). By the use of ventilated tubes with filter, it is expected that the requirement for sugar can be reduced. Our previous experiments showed that ventilated polycarbonate tube in agar medium could increase growth of Dahlia, and sucrose concentration could also be reduced (Rudiyanto *et al.*, 2017). Therefore, aims of this study was to investigate the effect of the use of vermiculite as porous substrate, instead of agar, in culture tubes with different caps on shoots growth of *Dahlia* sp.

MATERIALS AND METHODS

The material used in this experiment was Dahlia shoot cultured on MS medium (Murashige and Skoog, 1962) for 8 weeks. The medium used was liquid MS medium containing 10, 20, and 30 g/l of sucrose. Twenty ml of liquid media was poured in 150 ml glass tubes filled with vermiculite as substrate at thickness of 2 cm. No plant growth regulator was added into the culture medium. Shoot tips was cut at 1.5 cm length with 2-3 leaves, then they were cultured on the treatment media. The pH of the medium was adjusted at 5.8, followed by sterilization using an autoclave at 121°C and 15 psi for 20 min. The culture was then incubated in a culture room at 24 ± 2°C, using 500-900 lux light intensity with continuous photoperiod.

The experiments used factorial complete randomized design, using two factors which were the concentrations of sucrose (10, 20, and 30 g/l) and four different types of tube caps on vermiculite substrate. The caps were aluminum foil (Al foil), transparent plastic without filter (no ventilation), plastic having ventilation filter with diameter of 1 and 2 cm. Pore size of filter was 0.22 micron. Sucrose at 30 g/l and Al foil cap were used as control treatment. Each treatment had 9 replicates. The variables observed were plant height, number of leaves, number of nodes, and number of roots observed every week up to 8 weeks of culture. At 8 weeks of culture, fresh and dry weights were also observed.

Data analysis. The data was performed using SPSS ver. 21 with one-way ANOVA at 95% of confidence level (α= 0.05%). The results then were proceed using the Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

In vitro plant cultures are not autotrophic. Therefore, they need sucrose as carbon, and as energy sources. However, its high concentrations may decrease net photosynthesis and causes explants abnormalities. It has been reported that the photosynthetic ability of tissues cultured *in vitro* with forced ventilation enhances culture performance and creates the possibility of decreasing or even eliminating sucrose supplementation, producing mixo-autotrophic cultures (Gago *et al.*, 2021). The

height of Dahlia shoot on vermiculite with different concentrations of sucrose and with different types of tube caps is presented in Fig. 1.

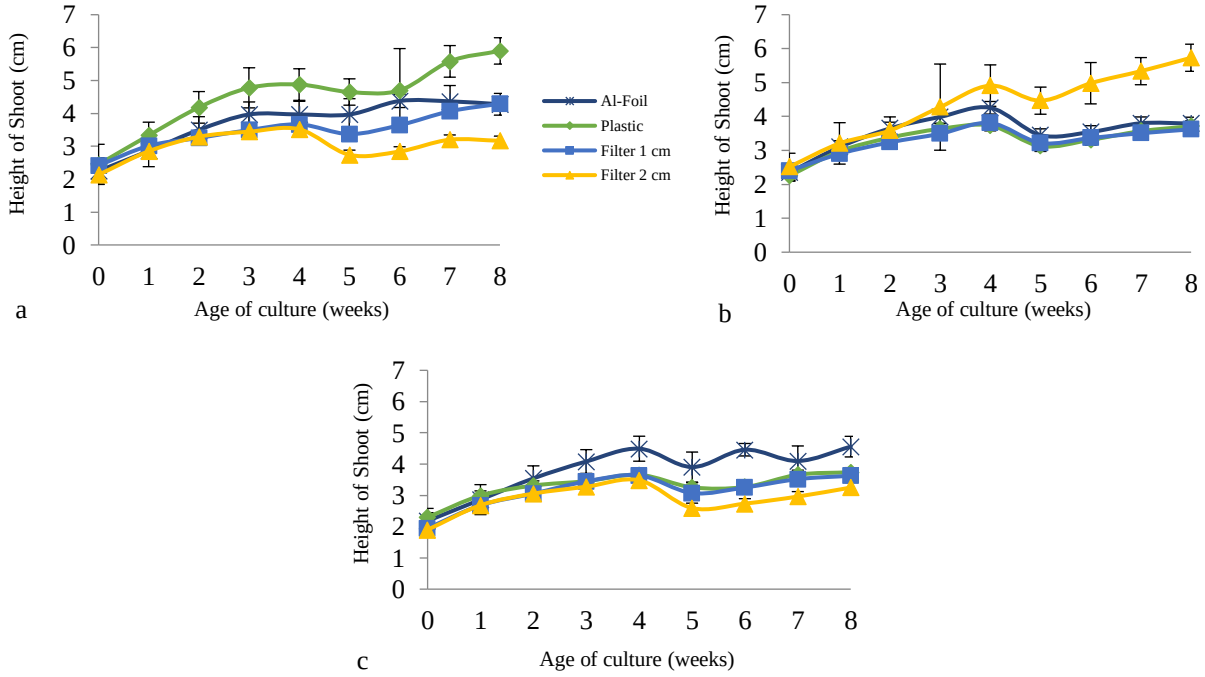

Fig. 1. Shoot height of *Dahlia* sp. 1-8 weeks of culture with tube caps Al foil, plastic, filter 1 cm, filter 2 cm grown on MS medium containing: a. 10 g/l; b. 20 g/l; c. 30 g/l sucrose with vermiculite substrate

At the medium containing 10 g/l of sucrose, height of Dahlia shoot started to increase one week after culture. In the plastic cap, shoot growth was the highest from 1 to 8 weeks of culture. The shoots growth with Al foil and 1 cm filter was similar at 8 weeks of culture. With 2 cm filter, Dahlia shoot growth was the slowest (Fig. 1a). In *Coleus amboinicus* the highest number of shoots was obtained by using 2 cm filter with 30 g/l sugar (Hapsari *et al*., 2018). Furthermore, in *Achras zapota*, node explants produced lateral shoots faster than apical shoots (Yuniastuti *et al*., 2016).

Our results indicated that on the medium containing 20 g/l sucrose, growth of Dahlia shoot height also started 1 week after culture. There was no significant different in shoot grown in tubes with Al foil, plastic, and 1 cm filter caps. High shoot growth was found in 2 cm filter cap with optimum growth occurring at 2-8 weeks of culture (Fig. 1b). In the medium containing 30 g/l sucrose, shoots growth began 1 week of culture. Shoot growth was not significantly different between plastic and 1 cm filter tube caps. The lowest shoot growth was found with 2 cm filter, and the highest was with the Al foil tube caps. Optimum shoot growth occurred 4 weeks after culture. There was no increase in shoot height from 4 to 8 weeks of culture (Fig. 1c). In shoot culture of Stevia and *C. amboinicus* grown on media containing 30g/l sugar using substrate of sorbarod and vermiculite gave the highest shoot height in Al foil caps compared to plastic caps with and without ventilation (Rantau *et al*., 2017; Hapsari *et al*., 2018). The use of Al foil as culture caps causes less optimal of light intensity compared to plastic caps, consequently the higher explant growth led to the etiolation process. Furthermore, Yudith *et al*. (2019) reported that reducing sucrose in the culture medium improved the morphophysiological, biochemical and anatomical development of the *Bambusa vulgaris* shoots. However, a high sucrose concentration decreased the total chlorophyll content and leaf area in shoots.

Number of Dahlia leaves at 0-8 weeks of culture on the medium containing different concentrations of sucrose with four different types tube caps cultured on the vermiculite substrate is shown in Fig. 2. In medium containing 10 g/l sucrose, high number of leaves was found with 1 cm filter, with optimum leaves numbers occurring at 2-8 weeks of culture. In the plastic tube caps and

with 1 cm filter, the leaf numbers were similar. In 2 cm filter, the growth of the number of leaves was low (Fig. 2a).

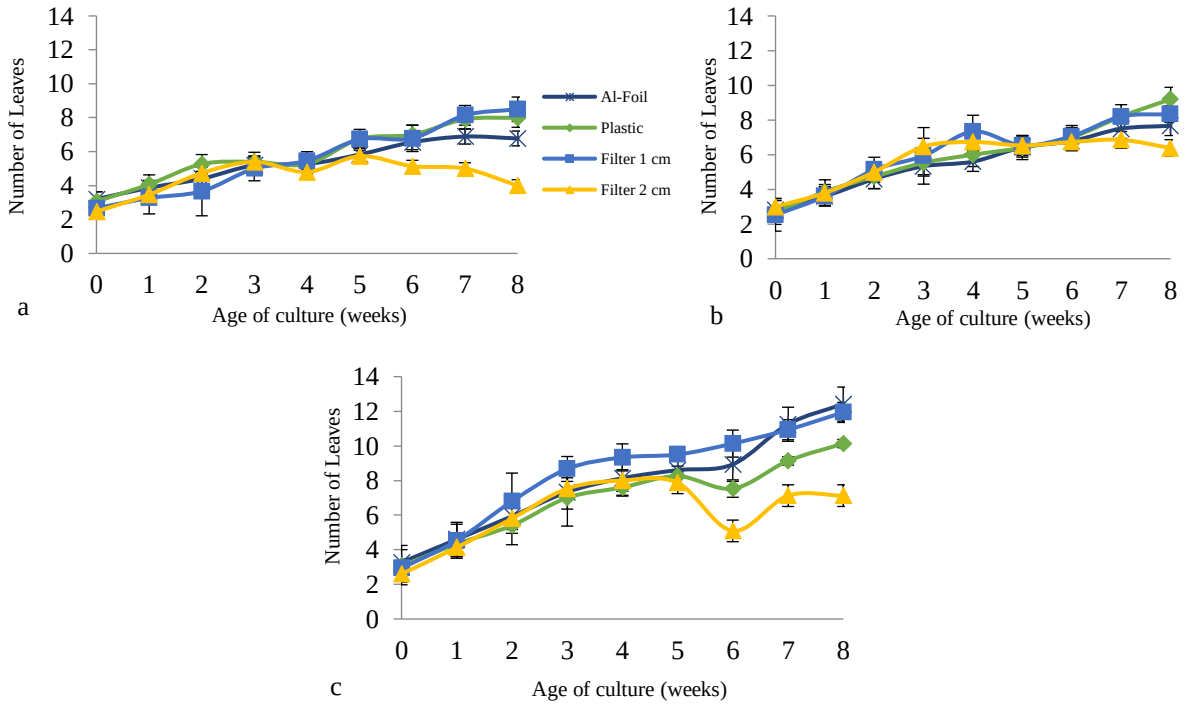

Fig. 2. Number of leaves of *Dahlia* sp. 1-8 weeks of culture with tube caps Al foil, plastic, filter 1 cm, filter 2 cm grown on MS medium containing: a. 10 g/l; b. 20 g/l; c. 30 g/l sucrose with vermiculite substrate

In MS medium containing 20 g/l of sucrose, there was no difference in the number of leaves in tubes with caps of Al foil, plastic, as well as with 1 and 2 cm filter on 0-3 weeks of culture. The increase in the number of leaves started after 1 week of culture. At 4 weeks, the highest number of leaves was produced in tube with 1 cm filter cap. Furthermore, at 8 weeks, the best development was found in the plastic cap tubes. In contrast, the lowest leaf numbers were found in shoot cultured in 2 cm filter caps (Fig. 2b). Sucrose at 30g/l with Al foil and 1 cm filter enhanced number of leaves. Optimum leaf growth was seen 2-8 weeks of culture. With plastic and 2 cm filter caps, the development of the number of leaves increased at 1-5 weeks of culture. However, at 6 weeks, the number of leaves went down. At 8 weeks of culture, the number of leaves in 2 cm filter cap was lower than that in Al foil, plastic, and 1 cm filter caps (Fig. 2c). In condition where the number of leaves decrease, the addition of liquid medium to the substrate needs to be done aseptically in order to develop further photomixotrophic process. In Dahlia, the addition liquid medium was done before 6 weeks of culture. Generally, sucrose enhanced the production of new *in vitro* leaves; whereas moderate and high light intensities inhibited their development during rooting and acclimation (Gago *et al.*, 2014).

The number of Dahlia nodes cultured in MS media with vermiculite substrate using different sucrose concentrations and with Al foil, plastic, 1 and cm filter caps is shown in Fig. 3. Our results showed that in the medium containing 10 g/l sucrose number of nodes increased after 1 week of culture. By using 2 cm filter cap the number of nodes increased at 1-3 weeks of culture, but remained steady afterwards, only a few nodes were produced. When shoots grown in tubes with 1 cm filter caps, the number of nodes increased at 1-8 of culture. The number of nodes in Al foil and plastic caps after 7 weeks had the best growth (Fig. 3a).

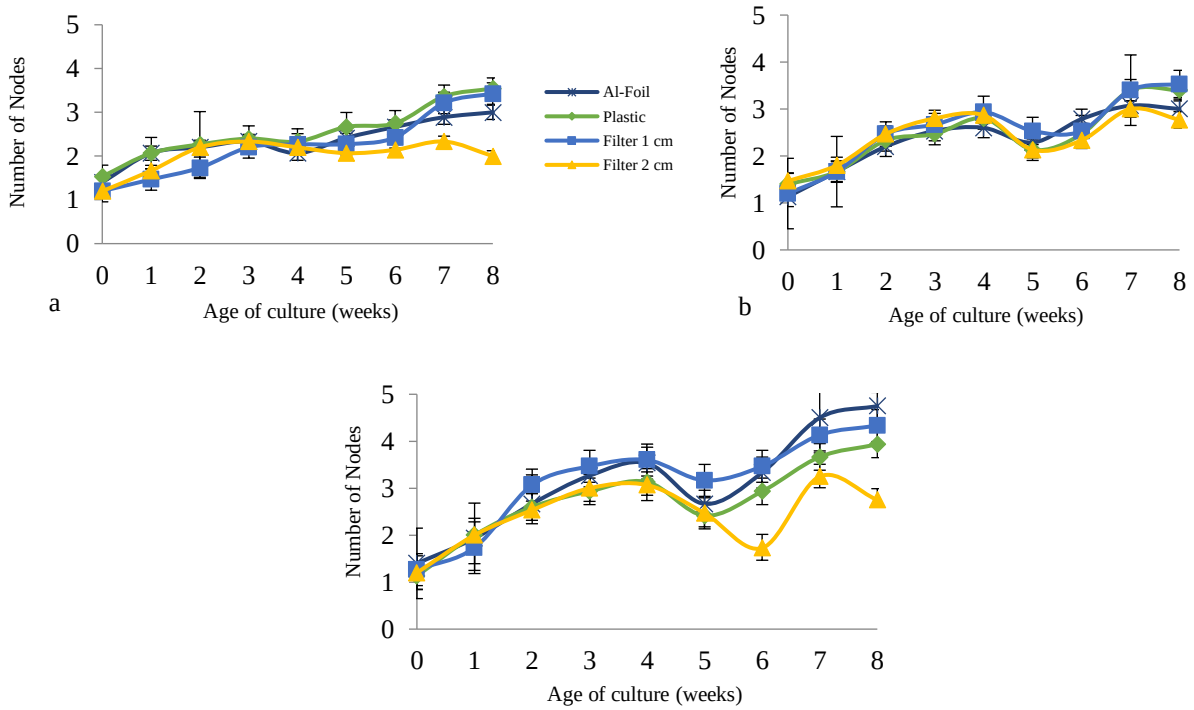

Fig. 3. Number of nodes of *Dahlia* sp. 1-8 weeks of culture with tube caps Al foil, plastic, filter 1 cm, filter 2 cm grown on MS medium containing: a. 10 g/l; b. 20 g/l; c. 30 g/l sucrose with vermiculite substrate

Our results indicated that shoot cultured on the medium containing 20 g/l of sucrose, the growth of number of nodes started at 1-4 weeks of culture. At 5-6 weeks of culture, the number of nodes on 2 cm filter cap reduced, but increased again at 7-8 weeks of culture. With Al foil, plastic, and 1 cm filter caps, the optimum number of nodes occurred at 5-8 weeks of culture. After 8 weeks of culture, the highest number of nodes was produced in Al- foil, and the lowest was found in 2 cm filter caps (Fig. 3b). On MS medium added with 30 g/l sucrose, the number of nodes in Al foil, plastic, 1 and 2 cm filter caps at 0-4 weeks were similar. The number of nodes began to increase at 1-4 weeks. The optimum number of nodes occurred at 6-8 weeks of culture. At 8 weeks of culture, the highest number of nodes was found in 1 cm filter cap, and the lowest was found in 2 cm filter cap (Fig. 3c).

Table 1. Shoot height, number of leaves, number of nodes, number of roots and root length of *Dahlia* sp. 8 weeks of culture on MS medium containing reduction of sugar concentration, with different tube caps in vermiculite substrate

Sucrose (g/l)	Tube cap	Shoot height	Number of leaves	Number of nodes	Number of roots	Roots length (cm)
10	Al foil	4.28 ab	6.78 cd	3.00 bc	0.22 b	0.22 cd
	Plastic	5.43 a	8.11 bc	3.56 ab	0.33 b	0.11 d
	Filter 1cm	4.36 ab	8.67 bc	3.44 ab	1.44 a	1.52 abcd
	Filter 2cm	3.17 b	4.00 d	2.00 c	0.44 ab	0.44 cd
20	Al foil	3.81 b	7.67 bc	3.00 bc	1.11 ab	1.15 abcd
	Plastic	3.68 b	9.22 bc	3.44 ab	1.33 ab	1.46 abcd
	Filter 1cm	3.67 b	8.33 bc	3.56 ab	1.11 ab	1.29 abcd
	Filter 2cm	5.73 a	6.33 cd	2.78 bc	0.89 ab	0.92 bcd
30	Al foil	4.59 ab	12.22 a	4.67 a	1.00 ab	1.27 abcd
	Plastic	3.70 b	10.22 ab	3.89 ab	1.44 a	1.77 abc
	Filter 1cm	3.63 b	11.89 a	4.33 a	1.33 ab	2.46 ab
	Filter 2cm	3.24 b	7.11 c	2.78 bc	1.00 ab	2.61 a

Notes: Numbers followed by the same letter in the same column were not significantly different according to Duncan's multiple distance test at α= 5%

In this study, CO_2 concentration was increased by ventilation by using plant tube caps with permeable filters. We expected to minimize any possible excess of sucrose present in the tissues which was cultured with 30 g/l of sucrose which used as control treatment. Increasing CO_2

concentration inside the tube enhanced the growth and rooting of several plants culture by increasing photosynthesis. High-level of CO_2 promoted the growth of shoots of *Eucalyptus tereticornis*, *Celastrus paniculatus* and *Vernonia condensate* supplemented with normal sucrose concentrations and allowed the growth of shoots cultured in low sucrose concentration (Gago *et al*., 2021). Vented tube caps also promoted growth of *Stevia rebaudiana* (Ermayanti *et al*., 2017; Rantau *et al*., 2017).

Table 1 represents shoot height, number of leaves, number of nodes, number of roots and length of roots of Dahlia after 8 weeks of culture on MS medium supplemented with 10-30 g/l sucrose with four types of tube caps. The results showed that the highest shoot height was found in 10 g/l sucrose using plastic cap, and in 20 g/l sucrose with 2 cm filter, significantly different from other treatments, except with 10 g/l sucrose with Al foil and 1 cm filter, as well as in 30 g/l sucrose with Al foil cap. The highest number of leaves was found in the 30g/l sucrose with Al foil and 1 cm filter caps, which was significantly different with others except with 30g/l sucrose using plastic cap (Table 1). In shoot culture of guava (*Psidium guajava*) the use of plastik cap without filter with 30g/l sugar and 0.5 mg/l BAP on agar media produced the highest number of nodes and shoots compared to ventilated caps with agar substrate (Rantau *et al.* 2019). Shin *et al*. (2012) reported that the heterotrophic cultures, where no filter and no gas exchange occurred between the inside and outside of the culture vessel, showed the poorest growth and leaf area decreased to approximately 56% as compared with plantlets grown in photomixotrophic conditions. Conventional micropropagation techniques are typically performed using small culture vessels under low light conditions without ventilation. The leaves were commonly smaller in no filter tube compared with the leaves from treatments with CO_2 enrichment. However, the application of ventilated cap may also lead to improve phyisiological and anatomical abnormalities such as; less ability of photosynthesis, low chlorophyl content, open stomatal with lack of cuticle layer in the leaf, abnormal leaf and xylem parenchyma (Hassankhah *et al*., 2013).

Our results showed that the number of Dahlia nodes after 8 weeks of culture varied. The highest number of nodes was found in the 30 g/l sucrose with Al foil and 1 cm filter caps, which was significantly different with 10 and 20 g/l sucrose with Al foil and 2 cm filter, as well as in 30 g/l sucrose using 2 cm filter cap (Table 1). Kozai (2012) reported that reduction of sugar concentrations more significantly affected shoot tips than nodal segment in terms of increasing shoot height. number of leaves and number of nodes. Types of closures gave variation effect on shoot growth. Furthermore. plantlet biomass (fresh and dry weights) also varied among treatments.

Table 2. Fresh and dry weights of *Dahlia* sp. 8 weeks of culture on MS medium containing reduction of sugar concentration, with different tube caps in vermiculite substrate

Sucrose (g/l)	Tube cap	Fresh weight (mg)	Dry weight (mg)
10	Al foil	63.98 b	3.57 b
	Plastic	104.44 b	7.71 b
	Filter 1cm	127.02 b	41.89 a
	Filter 2cm	48.78 b	5.32 b
20	Al foil	87.56 b	6.01 b
	Plastic	85.97 b	6.20 b
	Filter 1cm	51.49 b	6.36 b
	Filter 2cm	56.90 b	15.47 ab
30	Al foil	124.62 b	22.68 ab
	Plastic	109.57 b	8.64 b
	Filter 1cm	226.40 a	23.73 ab
	Filter 2cm	57.74 b	13.20 ab

Notes: Numbers followed by the same letter in the same column were not significantly different according to Duncan's multiple distance test at α= 5%

Number of Dahlia roots in the medium containing 10 g/l sucrose with 1 cm filter cap and 30 g/l sucrose with a plastic cap was the highest significantly different from that in the media containing10 g/l sucrose with Al foil and plastic caps. The highest root length was produced in 30 g/l sucrose with 2 cm filter, significantly different with 10 g/l sucrose using Al foil, plastic, and 2 cm filter, also with 20 g/l sucrose using 2 cm filter (Table 1). In papaya, by using a stationary liquid culture with

vermiculite and zeolite substrates supported more roots and increased the survival of shoots cultured with low sucrose concentration and exposed to high ventilation. Furthermore, in thyme, paulownia, potato, tobacco and wasabi, more roots were formed when less sugar was added to the culture medium (Gago *et al.*, 2021). In contrast, in strawberries, there was no increase in the root mass when sucrose was not added to the medium (Nguyen *et al.*, 2020). Rogayah *et al*. (2013) reported more and longer roots in photoautotrophic conditions Shoots culture of papaya on half-strength MS medium supplemented with vermiculite exhibited 92.5% rooting efficiency, and the survival rate of the vermiculite-grown transformed papaya plantlets *ex vitro* was 94%. Morpho-histological analyses revealed that the tap roots were more compact.

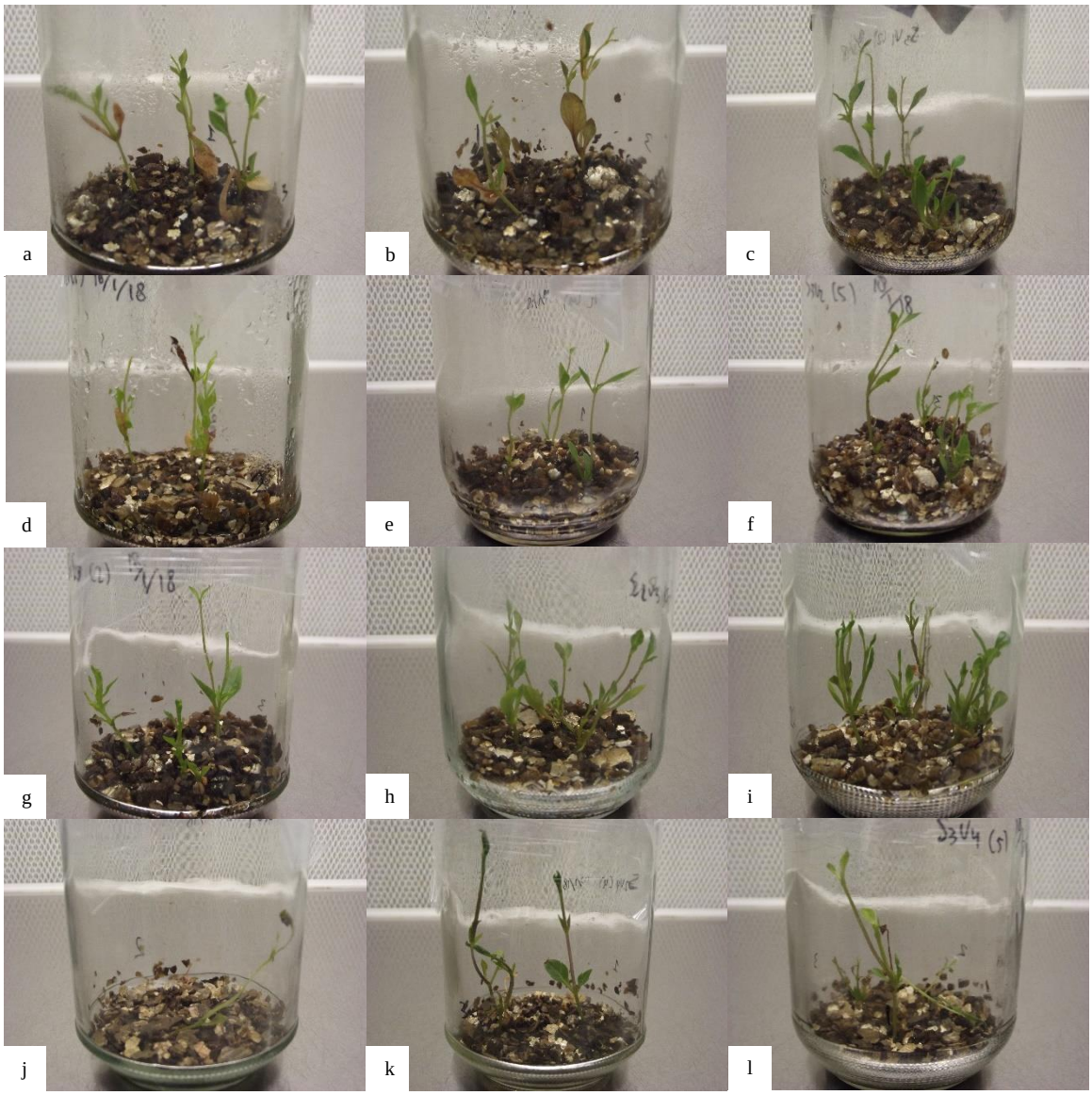

Fig. 4. Performance of *Dahlia* sp. cultured on MS medium containing reduction of sugar concentration, with different tube caps in vermiculite substrate: a-c: Al foil containing 10 g/l, 20 g/l, 30 g/l respectively; d-f: Plastic containing 10 g/l, 20 g/l, 30 g/l respectively; g-i: Filter 1 cm containing 10 g/l, 20 g/l, 30 g/l respectively; j-l: Filter 2 cm containing 10 g/l, 20 g/l, 30 g/l respectively

Fresh and dry weights of Dahlia shoots cultured in MS medium containing 10, 20, and 30 g/l sucrose with four different tube caps 8 weeks after culture is presented in Table 2. The highest fresh weight was found in the MS medium containing 30 g/l sucrose with 1 cm filter, significantly different from other culture condition. The high dry weight was found in 10 g/l sucrose with 1 cm filter,

significantly different with other treatments, except with 20 g/l sucrose using filter 2 cm and 30 g/l sucrose with Al foil, 1 and 2 cm filters (Table 2). Different sucrose concentrations may modify micro environmental conditions, thus affecting the photoautotrophic capacity and biomass accumulation of explants, ion absorption and their acclimation survival rate (Deng *et al*., 2012). Fortini *et al*. (2021) reported that sucrose concentrations and gas exchange gave significant interaction in plant biomass of *Vernonia condensata*. All growth variables were higher when plants were grown in an environment that used filters and allowed higher gas exchange rates.

Fig. 4 shows performance of Dahlia shoot culture after 8 weeks cultured on MS medium supplemented with 10-30 g/l sucrose with four types of tube caps. In the media containing 10 g/l sucrose using a plastic cap and filter 1 cm, shoots looked robust in contrast with that with Al foil cap, which had several leaves started to brown and shriveled. Furthermore, by using 2 cm filter, the most Dahlia leaves was withered. Shoot with 2 cm filter cap, at 10, 20, and 30 g/l sucrose did not grow well as others. Some shoots withered and brown. This was due to the lack of nutrients supplied to the explant, while the evapotranspiration in this treatment was high (Rudiyanto *et al*., 2017). The micropropagation rate was influenced by environment and substrate, increase in ventilation rate (using 2 cm filter of the vessel), increased the CO_2 concentration in the vessel during photoperiod, also decreased ethylene and increase the air movement in the vessel that affected the transpiration rate (Majada *et al*., 2020). Culture with ventilated vessel rather than without ventilation vessel faster loose water and consequently affect the growth rate (Vahdati & Hassankhah 2014). In contrast, using 1 cm filter, reduction of sucrose to 20 g/l did not significantly reduce Dahlia plant performance. The plantlets looked robust and green and produced many leaves and roots. In addition to have a carbon source supply from sucrose, explants also obtain a carbon source from mixotrophic condition by absorbing CO_2 through the filter and converting it to assimilation that can be absorbed by the Dahlia plantlet. Allison *et al.* (2016) reported that using a vermiculite substrate could potentially yield better root systems than a traditional semisolid medium. Increased aeration of the medium and a porous component of the vermiculite is thought to be responsible for improving root development.

CONCLUSION

Growth response of *Dahlia* sp. shoot culture was affected by sugar concentrations and caps of culture tubs. On vermiculite substrate, reduction of sucrose enhanced height of Dahlia shoots using plastic or ventilated tube caps, however, the highest number of leaves and nodes as well as the fresh weight was found in the 30 g/l of sucrose using Al foil and ventilated caps with 1 cm filter. Low sucrose concentration (less than 30 g/l) in combination with ventilated cap and normal (30 g/l) sucrose concentration using the plastic cap produced a high number of roots.

ACKNOWLEDGEMENTS

The authors would like to thank Evan Maulana and Lutvinda Ismanjani, from Plant Cell and Tissue Culture Laboratory at National Research and Innovation Agency-BRIN for their assistance during the experiment.

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