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Effect of 2,4-Dinitrophenylhydrazine (2,4-D) and 6-Benzylaminopurine (BAP) on Callogenesis of Aquatic Plant Brazilian Microsword (*Lilaeopsis brasiliensis*)

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Lilaeopsis brasiliensis is one of popular ornamental aquatic plants and yet still being commercialized in Malaysia. In this study, the effect of 2,4-Dinitrophenylhydrazine (2,4-D) concentration on the survivability, callus induction and type of callus induced from the explants were determined. From the callus induction result, 0% of callusing was observed for leaf explant as there were no surviving explants. Treatment 2 which consisted of Murashige and Skoog (1962) media supplemented with 0.5 mg/L 2,4-D and 1 mg/L 6-Benzylaminopurine **(**BAP) shows a higher percentage of survivability (88.89 ± 19.24 %) and highest callus induction (66.67 ± 3.34 %) for internode explants after four weeks of culture compared to other protocols that were used. However, the duration of callus induction was observed at week 3 of culture observed in T4, media supplemented with 2.0 mg/L 2,4-D and 1 mg/L BAP. Callus induced from all treatments showed yellowish-white colour and friable.

Keywords: Aquatic plants, Callus induction, *Lilaeopsis brasiliensis*, 2,4-D, *Apiaceae*

INTRODUCTION

Lilaeopsis brasiliensis or its common name, Brazilian micro sword belongs to the family Apiaceae has sword-like narrow leaves appearances. It is a covering plant that spread out in thick carpet of grass at the base of the aquarium. In its native habitat in Brazil, it can be found growing along the shores of streams in both emerged and submerged states.

This plant is rarely found in Malaysia due to difficulty in growing and maintaining the plant (Nor Hasima et al. 2018). Due to the production of small quantity of plants, long cultivation period, diseases and large pace required for propagation, natural propagation of aquatic species become limited (Sulaiman, 2004). The plant can be propagated vegetatively using rhizome. However, propagation of matured rhizome to form a shoot is inefficient as it occur at very slow rate compared to other organs.Therefore, to meet the demands of aquarium industry, rapid and efficient *in vitro* protocol is suffiecient (Azmi et al. 2019).

L. brasiliensis or Brazilian micro sword are capable of inhibiting algae and phytoplankton growth by reducing nitrate content in water (Nor Hasima et al., 2018). There is no report on callus culture of the plant except some reports on *in vitro* micropropagation. *L. brasiliensis* has a high demand in the aquascape and popular among fish hobbist, but the supply is limited. The

conventional method to propagate this plant might be hard to fulfill the market demand. Moreover, these plants are foreign originated making it difficult to grow in actual environment in Asia. This micro sword plant is notable as an excellent foreground plant and are sold in the form of tissue culture production by aquaria and ornamental trade in Malaysia. However, there is no to limited report about *in vitro* micropropagation of *L. brasiliensis.* The plant are popular and has a high in demand in aquarium trade for their beautiful features. Nevertheless, the supply of these aquarium plants is inconsistent. (Nguang et al. 2019).

Different types of plant growth regulators such as NAA, BAP, indole-3-acetic acid (IAA),indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), zeatin, and kinetin could be used to compare the result of *L. brasilliensis in vitro* regeneration.(Jennielyn et al. 2019). *In vitro* callus can be induced by exogenous addition of auxin and cytokinin in a certain concentration. Callus has been proven to carry secondary metabolites from the plants (Satish et al. 2012). From callus tissue, the whole plant can be regenerated by changing the nutrients and hormone constituents in the culture medium. Through somatic embryogenesis, calli can be transformed into embryonal mass and the whole plant can be regenerated. Other than that, particular secondary metabolite can be obtained by extraction of that particular callus tissue (Pierik, 1987).

This study could provide information and reliable protocol for callogenesis of *Lilaeopsis brasiliensis*. This study could be used to produce callus with a significant success rate. Callus culture can be established which can promote somaclonal variations. Through this, it can be used for plant modification and improvement, and other potential uses.

MATERIALS AND METHODS

Explant sources of Lilaeopsis brasiliensis

Lilaeopsis brasiliensis plantlets original culture stocks were obtained from plant tissue culture laboratory at Universiti Sultan Zainal Abidin (UniSZA), Besut Campus, Terengganu. Healthy, sterile looking leaves and internodes were selected to culture.

Callus induction

Murashige and Skoog (1962) medium was used as the callus initiation medium containing 3 % (w/v) sucrose, 0.25 % (w/v) phytagel, plant growth regulators and pH between 5.7-5.8. Media pH was adjusted to 5.7 with KOH after adding growth regulators but before adding agar and before autoclaving. There were five (5) different concentrations of 2,4-dichlorophenoxyacetic acid with combination of 6-benzylaminopurine (2,4-D-BAP). The combination was labelled respectively as treatment 1 to 5 (T1 to T5, Table 1). MS medium without any supplementation of plant growth regulators was used as the control. Explants were placed on 15 ml culture medium contained in sterile petri dish and sealed with clear plastic polypropylene lids (Sigma, St. Louis, MO) and Parafiim (Fisher, Chicago, IL). All cultures were incubated in dark condition at 25 ± 2˚C.

Data on three parameters were observed which are explants survivability, percentage of explant that produce callus and characteristic of the callus produced.

Data analysis and measurement

All experiments were conducted in a Completely Randomized Design. There were five different treatments including control and 3 replicates for each treatment. After 4 weeks of culture, data was recorded. Observations were recorded on (i) percentage of explants survivability, (ii) percentage of callus induced and (iii) type of callus induced. Data collected were analysed using One-Way Analysis of Variance (ANOVA). The group means were considered significantly different at the level of $p<0.05$.

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RESULTS

Effect of 2,4-D on the survivability of explants

There was no significant difference for all the treatments ($p>0.05$) on survivability of both the internode and leaf explant. For internode explants, explants managed to survive throughout the four

weeks for all treatments although the survivability decreases by time. The highest percentage of survivability was observed in T_2 with 88.89%± 19.24. This followed by T_5 (77.78% ± 24.78), T_1 and T_3, (66.67% ± 33.34) respectively. While, the lowest percentage of survivability was observed in T_4 with 55.56% ± 19.24, (Table 1).

For all leaf explants, survivability only lasts for 1 week. Hence, further step of callus induction could not be done. This may be due to lack of nutrients and unproper culture techniques. Observation also show that there were also browning observed on some of the explants. Browning resulted in necrosis and death of explants.

Oxidative browning in the culture resulting in stunted growth of the explant, lower rates or even regeneration or recalcitrance failure and can ultimately lead to cell death (Jones & Saxena, 2013). Browning is mainly due to oxidation of polyphenols to quinones, which is catalyzed by browning enzymes such as polyphenol oxidase (PPO) (Luo et al. 1999). The browning of *L.brasiliensis* explants may be regulated to some degree by inhibiting PPO activity (Chuanjun et al. 2015). However, due to the pervasive existence of tissue browning and its severe consequences, many advances have been made towards reducing oxidative browning by modifying the environmental conditions used in tissue culture.

Table 1: Effect of 2,4-D concentrations on survivability, callus induction and type of callus induced of *Lilaeopsis brasiliensis* internode explants.

Treatment	Plant Growth Regulators (mg/L)		Percentage per explants (mean ± SD)		Type of callus induced
	2,4-D	BAP	Survivability	Callus induction	
T_1	0	0	66.67% ± 33.34	0	No callus induced
T_2	0.5	1.0	88.89% ± 19.24	66.67 ± 33.34	Friable yellowish-white in colour
T_3	1.0	1.0	66.67% ± 33.34	22.22 ± 19.24	Friable yellowish-white in colour
T_4	1.5	1.0	55.56% ± 19.24	11.11 ± 19.24	Friable yellowish-white in colour
T_5	2.0	1.0	77.78% ± 24.78	28.88 ± 30.52	Friable yellowish-white in colour

Each value is the mean of three replicates per explants.

Method that can be applied is by placing the culture in the dark, that can exhibit lower browning rates than those grown in the light (Lainé et al. 1994). Changing the basic media composition and plant growth regulator type or concentration may also decrease the degree of browning. Tissue browning can be minimized by pretreatments of explants or alteration of the culture media. The example of substances that can be added to the medium are antioxidants such as ascorbic acid, melatonin, or citric acid (Uchendu et al. 2011). Medium constituents such as medium strength, the concentration of sucrose, PGRs, and culture conditions play an important role in maintaining the survivability of explant. It is important to choose the correct constituents suitable for the type of explants.

Effect of 2,4-D on the callus induction of explants

There was no significant difference for all the treatments (p>0.05) except for T_2 in this study. The highest percentage of callus induction was observed in T_2 with 66.67 ± 33.34 % followed by T_5 (28.88 ± 30.52%) and T_3 (22.22 ± 19.24 %). While, the lowest percentage of callus induction was observed in T_4 with 11.11 ± 19.24 % (Table 1).

Callus initiation is usually initiated with the addition of optimal callus induction hormones. The medium that supports growth and sterile conditions are important in term of growth and development of callus. The supplementation of exogenous plant growth regulators affects the development of callus in the nutrient medium. Callus development hormone supplementation can be categorized into three which is auxin alone, cytokinin alone, auxin as well as cytokinin (Bhatia et al. 2015). Auxin and cytokinin combinations are believed to facilitate cell division and organogenesis (Lima et al. 2008). Plant growth regulator such as 2,4-D are known to be potentially efficient in the formation of callus, but the optimum concentration applied in the media varied from one plant to another (Fu et al., 2008). Long term cultures supplemented with 2,4-D or other potent auxins could cause the development

of somaclonal variation (Von Arnold et al. 2002). For the leaf explants, no callus production was recorded because leaf explants only managed to survive for 1 week only. Hence, callus production was halted and further step could not be done. For the internode explants, callus induction was observed after 3 weeks of culture. Not all explants produce callus, as there are some of the explants that died and could not proliferate callus. In terms of duration for callus induction, it was observed that the calli were induced within 3 weeks of culture in T_2 (Figure 1).

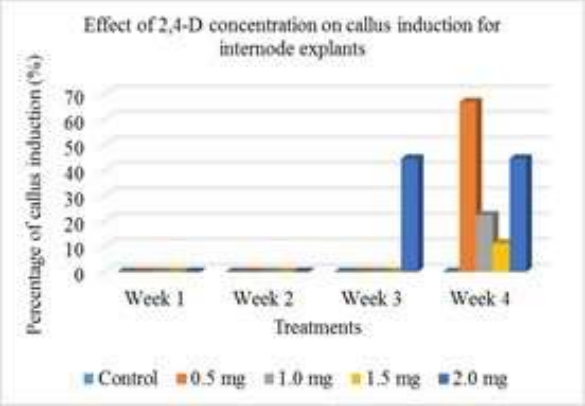

Figure1: The bar graph shows the effect of 2,4-D on callus inductiom for internode explants of *L. brasiliensis*.

These results is in paralel with Obembe et al., (2017) where they stated that callus can be induced from media supplemented with 2,4-D, but not from media that is free from 2,4-D when culturing *Cucurbita pepo* LCalli were induced at week 4 of culture for other treatments except for the T_1, control media because there were no PGRs added to the medium (Figure 1).

In *Solanum tuberosum*, Abdelaleem et al. (2009) found that BAP alone cannot induce callus efficiently for *in vitro* culture, but a combination of BAP and 2,4-D will induce callus efficiently. These results is in agreement with the findings of George, (2008) where almost all excised tissue that were cultured on media needs exogenous needs of one or more PGRs to initiate callus induction. The combination of 2,4-D with BAP does promote callus induction. Cytokinins plays the role in cellular division and cell expansion and are mostly used in combination with auxins to induce callus (Eckardt, 2003).

Effect of 2,4-D concentrations on type of callus induced by explants

All callus that were induced in the media appeared to be in the colouration of yellowish-white and friable (Figure 2). All induced calli have the same type of callus. Calli are numerous and can be divided into subgroups based on their macroscopic features (Ikeuchi et al. 2013). Calli with no regeneration of visible organs are typically referred to as friable or compact.

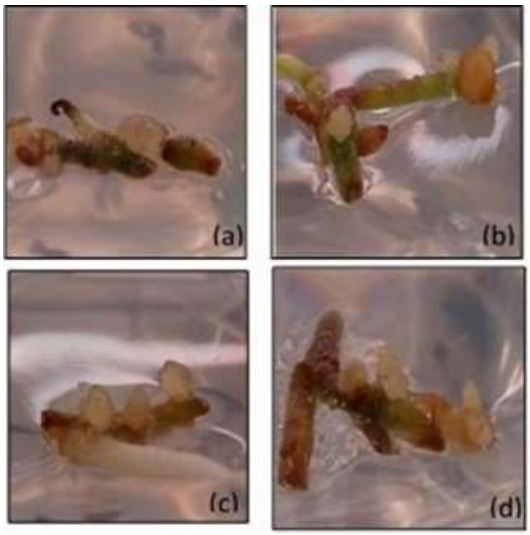

Figure2: Callus induced of *L.brasiliensis* internode explants in MS media supplemented with 2,4-D- BAP (a) 0.5; 1.0 mg/L, T_2 (b) 1.0; 1.0 mg/L, T_3(c) 1.5; 1.0 mg/L, T_4 (d) 2.0; 1.0 mg/L, T5

Depending on the organ they generate, calli showing certain degrees of organ regeneration are called rooty, shooty or embryogenic callus (Frank et al. 2000).

CONCLUSION

In this study, the effect of 2,4-D concentration on the survivability, callus induction and type of callus for the explants were determined. It can be concluded that callus of *L. brasiliensis* can be induced even at a very low concentration of 2,4-D and BAP. It is recommended that a combination of different types of plant growth regulators such as picloram, benzyladenine (BA), 1-naphthaleneacetic acid (NAA), kinetin, indole-3-actic acid (IAA), and indole-3-butyric acid (IBA) may be used to analyze the effects of callus induction for future analysis.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors wrote and approved the manuscript.

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