JOURNAL OF AGROBIOTECHNOLOGY 2019, VOL 10(1S):88-93
e-ISSN: 2180-1983
https://journal.unisza.edu.my/agrobiotechnology/index.php/agrobiotechnology/index

In Vitro Micropropagation of Aquarium Plants Pearl Grass *Hemianthus micranthemoides* (Nuttall) and Micro Sword Grass *Lilaeopsis brasiliensis* (Glaziou) Affolter (Apiaceae)

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Received: 05/08/2019, Accepted: 06/10/2019, Available online: 31/10/2019

ABSTRACT

Hemianthus micranthemoides and *Lileaopsis brasiliensis* are nowadays 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 both aquarium plants. In this study, an experimental procedure for micropropagation of both plants was documented. The propagation were done in Murashige and Skoog (MS) medium with eight combination of different concentration of 6-Benzylaminopurine with Naphteneacetic acid (BAP-NAA) and 1-phenyl-3-1,2,3-thiadizol-5-yl urea with Naphteneacetic acid (TDZ-NAA) following the rooting technique for 42 days. All treatments for 1 L MS medium were regulated to pH 5.7 to 5.8 and added with sucrose (30 g/L) and phytagel (2.5 g/L). All combination treatments had induced rooting. There was a significance different in the shoot regeneration of *H. micranthemoides* in all treatment (95% confidence level, F = 13.907, n = 240, p = 0.000), on the contrary there was no different were observed in shoot regeneration of *L. brasiliensis* treatment. Overall, all treatment of TDZ-NAA were significantly higher in the shoot regeneration than those in BAP-NAA treatment. Particularly the T7 (TDZ-NAA, 0.075: 0.1 mg/L) given highest result in diameter (1.626±0.213 cm) and area (1.724±0.308 cm^2) of shoot clumps in *H. micranthemoides*, and formation of shoots (51.5±4.95), nodes (20.0±2.83) and roots (45.5±13.4) in *L. brasiliensis*. Hardening of in vitro cultured plantlets was done in aquatic laboratory. All of the plants survived and transferred into aquarium. This study has provided beneficial information in further micropropagation protocol for the mass production of both aquarium plants.

Keywords: Aquarium plants, *Hemianthus micranthemoides*, *Lilaeopsis brasiliensis*, Micropropagation

INTRODUCTION

Aquatic plants are used to make the freshwater aquarium into a natural water environment, which is important to sustain the dissolve oxygen levels and organic matter, feeding and propagation of other aquatic organisms (Micheli et al., 2006; Barpete et al., 2015). The number of aquatic plants in any water body could be used as a parameter to

determine the efficiency since they are the primary and secondary producers in natural ecosystem (Oyedeji and Abowei, 2012). Nowadays the popularity of aquatic plants in aquaria and water gardens are rising and various types of aquatic plants have been across the geographic barrier for aquaria and ornamental trade purposes (Petruzella et al., 2018).

The targeted aquarium plants in this study are *Hemianthus micranthemoides* and *Lilaeopsis brasiliensis*. *H. micranthemoides* belonging to the family of Scrophulariaceae, is an ornamental aquatic plant from the Central America known as the pearl grass. The plant is best used as decoration in aquarium as carpeting plant since it helps to create an illusion of greater tank size with its delicate, cushion forming, and small leaves features (Barpete et al., 2015). It is generally slow growing in an aquarium as it requires plenty lighting, large nutrient levels, fine substrate material and optimal carbon dioxide levels (Othman et. al, 2014).

L. brasiliensis commonly known as micro sword grass or copragrass as the sword-like narrow leaves appearance. It is originated from the South America and belongs to the family Apiaceae. This aquarium plant is an attractive carpet plant with long green grass-like leaves. This micro sword is notable as an excellent foreground plant and great spawning medium which is the good choice for beginners.

Both aquarium plants are nowadays 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 *H. micranthemoides* and *L. brasiliensis*. They are popular and high in demand in aquarium trade for their beautiful features. Nevertheless, the supply of these aquarium plants is inconsistent. It is expensive for fish hobbyist to obtain and grow a variety of aquarium plants. Moreover, these plants are foreign originated making it difficult to grow in actual environment in Asia. Besides, it takes a longer period to propagate it conventionally. Thus, it is suggested that a method of propagation should be applied to *H. micranthemoides* and *L. brasiliensis* in order to shorten the propagation period, such as in vitro micropropagation. Therefore, it is important to develop a reliable in vitro regeneration protocol for these aquarium plants species.

The objective of this study were to compare the effects of different concentration of 6-Benzylaminopurine with Naphteneacetic acid (BAP-NAA) and 1-phenyl-3-1,2,3-thiadizol-5-yl urea with Naphteneacetic acid (TDZ-NAA) for micropropagation of *H. micranthemoides* and *L. brasiliensis* by concentrating on the proliferation activity. This study is also conducted to increase higher quality and quantity of in vitro both aquarium plants by hormone manipulations within a time frame. This is to spread their use as aquarium plants by setting up a consistent and repeatable in vitro regeneration protocol. This study analyses the importance of plant growth regulators (PGRs) and the effects of different PGRs concentration for the shoot regeneration and rooting development in regenerated shoots of the targeted plants.

MATERIAL AND METHODS

Preparation of materials

H. micranthemoides and *L. brasiliensis* were obtained from Fisheries Research Institute Glami Lemi, Negeri Sembilan. Both aquarium plants were then incubated in the incubation room (25±2ºC) with adequate light for the plants adjustment in new environment. Only disease free, young and healthy stems with node were selected for culture. Both plants were washed under running tap water for 30 minutes to wash off the contaminants with minimal damage to plant cells for surface sterilization. The apical shoots, internodes and shoots stem were cut in size of 1 cm^2 each and cultured in the shoot regeneration medium in vented vessels for 42 days.

Experiments for the optimal concentration of three constituents

The shoots regeneration medium contained the Murashige and Skoog (MS) mineral salts and vitamin), sucrose (30 g/L) and pytagel (2.5 g/L) were added, and combine with eight different concentration of 6 benzylminopurine with α-naphtaleneacetic acid (BAP-NAA), and 1-phenyl-3-1,2,3-thiadizol-5-yl urea with α- naphtaleneacetic acid (TDZ-NAA) (Table 1). All the combination then was labeled respectively as treatment 1 to 8 (T1 to T8).

Culture conditions and measurements

The pH of the medium was regulated between 5.7 to 5.8 with 1N NaOH or 1N HCl, then autoclaving at 15 psi and 121ºC for 15 minutes. All cultures were incubated at 25±2ºC under light conditions for 42 days. Each treatment has five replicates containing six explants.
The regenerated shoots were then used for in vitro rooting by using MS basal medium without PGRs. After 42 days of rooting, the plantlets were washed carefully under running tap water to eliminate agar-solidified medium. For acclimatization process, the plantlets were transferred into an aquarium containing sand and gravel with tap water as a foreground surface. After a week, the acclimatized plants were kept in the aquariums with fish under daylight conditions. The data on each treatment was subjected to a one way analysis of variance (ANOVA) and test the significance tested using Tukey's b test at the $p < 0.05$ level of significance.

RESULTS AND DISCUSSION

The results showed the utilization of all PGRs were interacted significantly to the growth of *H. micranthemoides* (95% confidence level, F = 13.907, n = 240, $p = 0.00$). There were significantly difference in the diameter and area of the shoot clumps that ranged from 1.06 to1.63 cm, and 0.73 to 1.89 cm^2, respectively (Table 1). The BAP-NAA and TDZ-NAA had promoted the induction of shoot clumps in *H. micranthemoides*. The shoot clump refers to the high amount of shoots per explant, which was produced particularly from the stem axis. Therefore, the counting of shoots per explant became not practicable regardless the growth variation. Similar observation were attained in the microprogation of grass pea (*Lathyrus sativus L*) (Barpete et al., 2015) and some other aquatic plants of *Cryptocoryne becketti*, *C. lutea* and *Rotala rotundifolia* (Micheli et al., 2006). In this study, the data were recorded based on the diameter and area of the shoot clumps during the 42 days of culture (Fig. 1).

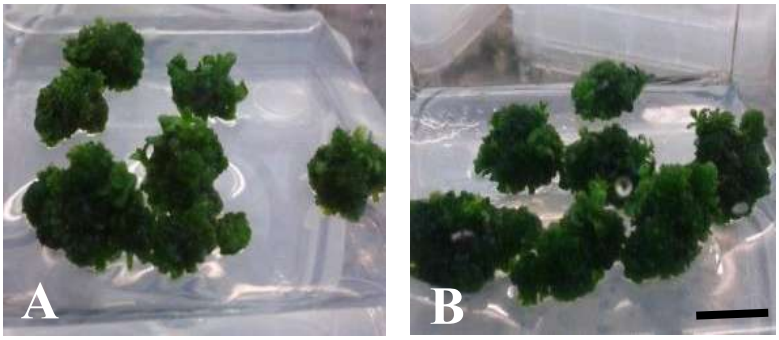

Fig. 1. Proliferated clumps of *H. moicranthemoides* in MS medium supplemented with (A) BAP-NAA (1.0; 0.1 mg/L, T2); and (B) TDZ-NAA (0.05; 0.1 mg/L, T6) after 42 days of culture (scale, 1.0 cm).

Table 1. Shoot regeneration of *H. micranthemoides* explant after 42 days culture in MS medium addition with different concentrations of BAP-NAA and TDZ-NAA.

Treatment	Plant growth regulator, PGRs (mg/L)			Clump diameter (cm)*	Clump area (cm^2)*
	NAA	BAP	TDZ		
T1	0.1	0.5	0.000	1.598± 0.630 c	1.892±1.382 z
T2	0.1	1.0	0.000	1.296± 0.277 b	1.122± 0.375 y
T3	0.1	2.0	0.000	1.062± 0.130 a	0.730± 0.157 x
T4	0.1	3.0	0.000	1.088± 0.207 a	0.740± 0.313 x
T5	0.1	0.0	0.025	1.528± 0.234 bc	1.728± 0.366 yz
T6	0.1	0.0	0.050	1.430± 0.233 bc	1.508± 0.655 yz
T7	0.1	0.0	0.075	1.626± 0.213 c	1.724± 0.308 z
T8	0.1	0.0	0.100	1.410± 0.363 bc	1.388± 0.642 yz

*Each value is the mean of five replicates for each explant. The values in column followed by different letters are significantly different at the 0.05 level of significance using the Tukey's b test.

This is the preliminary micropropagation study of *H. micranthemoides*. In this study, the PRGs appliance was strategy based on the related micropropagation studies and similar PRGs usage in aquatic plants, and the features of the targeted species (Öztürk et al., 2004; Micheli et al., 2006; Aasim et al., 2013; Cinar et al., 2013; Barpete et al., 2015). The results revealed that TDZ-NAA combinations were more effective for shoot initiation in all explants compare to those in BAP-NAA combinations. Contrarily, the Barpete et al. (2015) study reported that MS medium with BAP were most suitable and effective for shoot growth and proliferation compare to TDZ, 2iP and GA3. Whereas the Öztürk et al. (2004) study stated that the shoot proliferation was more effective in extremely low concentrations of TDZ-NAA (0.05: 0.1 mg/dm) that even the slightest increase resulting in shoot proliferation reductions.

In the present study, the shoots regenerated in the medium supplemented with PGRs had significantly reduced number of roots compared to the control treatment. Similar result was obtained in the other aquarium plant *Ludwigia repens* (Öztürk et al., 2004). The control treatment of *H. micranthemoides* explant cultured in MS medium without PGR and T1 (BPA-NAA, 0.5: 0.1mg/L) were produced roots. However, the roots formed in MS medium without PGRs were longer and thinner compare to the roots formed in T1 (BAP-NAA, 0.5: 0.1mg/L), which have shorter and thicker roots. Conversely, previous study showed that the presence of neither BAP nor TDZ in the regeneration medium did not restrain the frequency of rooting in aquatic plant like *H. callitrichoides* (Barpete et al., 2015).

The negative effect on the elongation of shoots was lessen and minimized by culturing in the MS medium without PRG that also act as the rooting medium after 42 days of culture. This has given positive results in which shoot elongation accelerated and formation of roots increase tremendously. Similar results were also obtained in *L. repens* (Öztürk et al., 2004). Thus, this indicates that with the presence of BAP and TDZ in low concentration of NAA may facilitate the proliferation of shoots but is not necessary for the shoots growth and elongation.

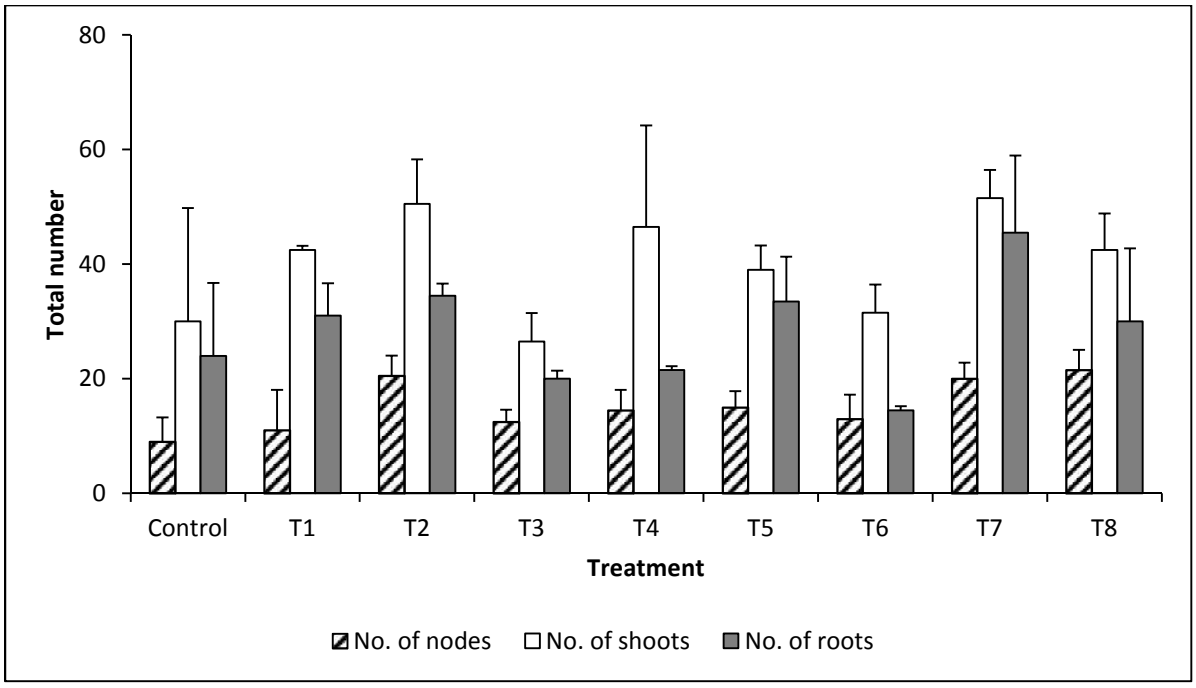

Fig. 2. Shoot regeneration of *L. brasiliensis* explant after 42 days culture in MS medium addition with different concentrations of BAP-NAA and TDZ-NAA.

There were no significant difference in the proliferation of *L. brasiliensis* and PGRs interaction with respect to average number of shoots, nodes and roots (95% confidence level, n = 30, p>0.05). Nevertheless, there is difference in the formation of shoots, nodes and roots of *L. brasiliensis* after 42 days of culture (Fig. 2 and 3). For the production of nodes, the T8 produced the highest number of nodes (21.5±3.54) whereas the lowest number of nodes was those grown in MS medium lacking in no PGR (9.0±4.24). The T7 gives the highest number of shoots (51.5±4.95) followed by T2 (50.5±7.78). On the other hand, the lowest number of nodes is recorded in T3 (26.5±4.95). Results showed that BAP-NAA and TDZ-NAA exerted variable effects in root formations. The T7 produced the highest number of roots (45.5±13.44) whereas the lowest number of roots is those grown in T6

(14.5±0.71). Shoot regeneration was inconsistent on medium supplemented with BAP-NAA (Orturk et al., 2004). The differences of shoot regeneration in each treatment might due to the hormone (endogenous factor) presented in the aquatic tissue plants (Saad et al., 2012). In this study, the possible explanation for the results would be the balance between the exogenous growth regulators (NAA, BAP and TDZ) and the endogenous hormone in the explants.

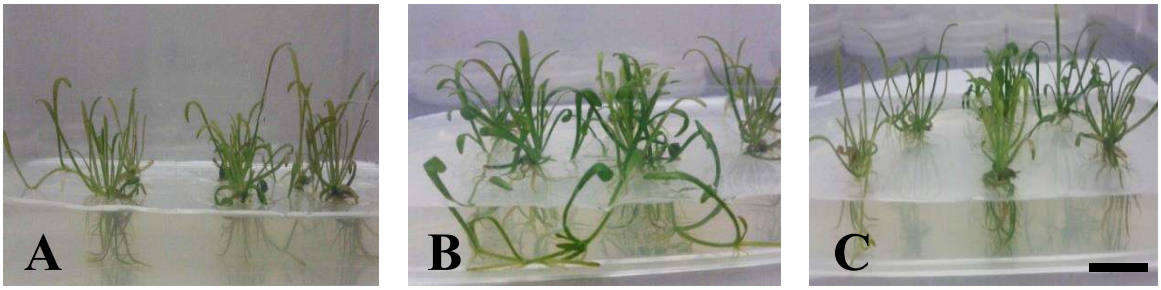

Fig. 3. Shoot regeneration of *L. brasiliensis* in MS medium supplemented with (A) no PGRs; (B) BAP-NAA (0.5: 0.1 mg/L, T1); and (C) TDZ-NAA (0.075; 0.1 mg/L, T7) after 42 days of culture (scale, 1.0 cm).

Overall, the presence of BAP, TDZ and NAA stimulated the shoot regeneration of both aquarium plants. Even at very low concentrations of PGRs have been induced the production of shoot clumps in *H. micranthemoides*. Based on this study, it is suggested that the regenerated mass of *H. micranthemoides* (shoot clumps) could be used as one of the parameter. However, the risk of contamination might become higher due to the high amount shoot regenerated per explant. Thus, the explant should be undertaken using sterile apparatus throughout the micropropagation process. Since the shoot regeneration could occur even at very low concentrations of PGRs. It is also suggested to investigate the minimal concentration of BAP-NAA and TDZ-NAA in the further experiment. Besides, different types and lower costs of PGRs such as indole-3-acetic acid (IAA), indole-3-butric acid (IBA), 2,4-dichlorophenoxy-acetic acid (2,4-D), zeatin, kinetin and benzyladenine (BA) could be used to compare the shoot regeneration by BAP-NAA and TDZ-NAA.

CONCLUSION

Overall treatment TDZ-NAA gives higher number in shoot proliferation of both *H. micranthemoides* and *L. brasiliensis*. However, other than to maintain the quality and quantity of in vitro both aquarium plants, further research could be done for optimal and minimal application of cheaper PGRs.

ACKNOWLEDGEMENTS

This study was partially supported by grants University (UniSZA/2016/DPU/04 and UniSZA/2017/DPU/09). The authors wish to express high gratitude to the laboratory staff at Faculty Bioresources and Food Industry for their invaluable assistance and hospitability throughout the aquarium plant tissue experiment.

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How to cite this paper:
Nguang, S.I., Anis, A.K., Norhaniza, S., Rokiah, Z., Nor Hasima, M., Tajul, A.A. & Ha, H.C. (2019). *In vitro* micropropagation of aquarium plants pearl grass *hemianthus micranthemoides* (Nuttal) and micro sword grass *lilaepsis brasiliensis* (Glaziou) affolter (Apiaceae). Journal of Agrobiotechnology, *10*(1S), 88-93