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Detection of Somaclonal Variation in Aquatic Plant Pearl Grass (*Hemianthus micranthemoides*)

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ABSTRACT

Hemianthus micranthemoides is an ornamental plan used for foreground plant in aquarium decorations. In Malaysia, the plant available on market is regenerated through tissue culture techniques which expose the risk for somaclonal variation. The objectives of this study are to identify suitable Random Amplified Polymorphic DNA (RAPD) markers for determining somaclonal variation rate in micropropagated plantlets of *H. micranthemoides* and to observe the effects of somaclonal variation on the plantlet growth (area of spreadability, shoot and root length). Out of 20 RAPD primers screened by polymerase chain reaction (PCR) using mother plant, one was selected as a suitable marker to detect somaclonal variation of *H. micranthemoides* which was P15. Somaclonal variation rate of the *H. micranthemoides* was determined by analyzing the RAPD-PCR bands. The results showed somaclonal variation was observed at the fifth subculture (S5) with the percentage of polymorphic bands of 50 % when compared to mother plant. Repeated subclutures has affected the growth of the regenerates negatively. It was shown that as the number of subculture increased, the growth of plantlet decreased in terms of decreasing shoot and root length as well as area of spreadability. This findings serves as a guide especially for tissue culturist to minimize subculture numbers when micropropagating *H. micranthemoides*.

Keywords: *Hemianthus micranthemoides*, somaclonal variation, RAPD, micropropagation

INTRODUCTION

Aquatic ecosystem is unique and diverse where all plants or animals live either in or on water. Aquatic plants are particularly grown because of their attractive appearance and ability to preserve the water quality. Besides, aquatic plants serve to provide food, shelter and breeding ground (Yapabandara & Ranasinghe, 2007). In fact,

the efficiency of any water body is controlled by the extent of aquatic plants because they constitute the primary and secondary producers of the aquatic ecosystem (Oyedeji & Abowei, 2012).

Hemianthus micranthemoides which originates from the family of Scrophulariaceae, is an ornamental aquatic plant natively found in North America. *H. micranthemoides* or also known as pearl grass is an elegant plant that is highly recommended for any planted tank with sufficient lighting. It has a characteristic trailing growth in intensive light, so it can be used as a foreground plant (Millar, 2011). A compact group of *H. micranthemoides* forms a light-green and beautiful cushion (Barpete et al., 2015). It is an ideal background plant in small tanks where the small leaves help to create an illusion of greater tank size. It is also a great selection for foreground highlights, filler and contrast in large tanks. This plant can grow both vertically and horizontally (Millar, 2011)

Although phytoremediation studies have not been conducted using *H. micranthemoides* but there is potential for it to be used as phytoremediator as shown in its close species, *Hemianthus callitrichoides*. *H. callitrichoides* has been demonstrated to have the capability to absorb heavy metals from polluted aquarium environment (Rai, 2009); (Singh et al., 2012). Zurayk et al. (2001) reported that *H. callitrichoides* is best used as bioindicator which monitors the pollution level in the aquarium. However, the use of aquatic plants for phytoremediation or as biomonitor in the aquarium are restricted by their limited availability.

H. micranthemoides is gaining popularity among aquascape hobbyist in Malaysia. It is mainly sold online and the price is relatively expensive. Due to climate differences, *H. micranthemoides* is difficult to regenerate when conventionally grown. Therefore, tissue culture techniques is indispensable. For commercial purpose, it requires micropropagation for it to be abundantly produced in a short period of time. Somaclonal variation or genetic variation as a result of *in vitro* culture of somatic cells is an unavoidable consequence of micropropagation. The methods to detect somaclonal variation may include phenotypic observation, cytological analysis and/or molecular approach (Saha et al., 2015). RAPD molecular markers is proved rapid, easy and less expensive in detecting somaclonal variation (Palombi & Damiano, 2002). However, there is no data available on micropropagation and somaclonal variation of *H. micranthemoides*. Without this information, it is difficult to fulfil market demand in the long-term as somaclonal variation will compromise the quality of regenerates.

The aim of his study was to determine somaclonal variation rate in in *H. micranthemoides* by PCR-RAPD. Suitable RAPD marker for detecting somaclonal variation was identified and the effects of somaclonal variation on the plantlet growth such as the shoot and the root length and as well as the area of spreadability were also observed.

MATERIALS AND METHODS

Subculture media

Healthy stem explants were cultured on 0.5 Murashige and Skoog (MS) media in jam jars. About 2.2 g of MS powder, 30 g/L sucrose and 3.2 g/L Gelrite™ were added in media. pH of the media was adjusted in the range of 5.7 to 5.8 before autoclaving for 15 minutes at 15 psi and 121 °C.

Micropropagation of *H. micranthemoides*

Mother plant *H. micranthemoides* was collected from Fisheries Research Institute (FRI), Glami Lemi, Negeri Sembilan. The healthy stem explants were selected and inoculated onto 0.5 MS medium. All cultures were incubated at 25 °C under light (16 hours) and dark (8 hours) conditions. Three replicates consisting of five explants were used. The explants were regenerated and multiplied by subculturing every six weeks. The length of shoot, the length of root and the area of spreadability of the plantlet of each subculture were recorded.

DNA Extraction

DNA was extracted from *H. micranthemoides* leaf by using PowerPlant® Pro DNA Isolation Kit (Mo Bio Laboratories, Inc) by following manufacturer's instruction.

RAPD-PCR Amplification

For RAPD-PCR amplification, twenty arbitrary, 10 mer RAPD primers (Kit A, Eurofin) were used by following the method of Williams et al. (1990).

Table 1. Primers sequences used for RAPD-PCR.

Name	Sequence (5'- 3')
Primer 1 (P1)	CAGGCCCTTC
Primer 2 (P2)	TGCCGAGCTG
Primer 3 (P3)	AGTCAGCCAC
Primer 4 (P4)	AATCGGGCTG
Primer 5 (P5)	AGGGGTCTTG
Primer 6 (P6)	GGTCCCTGAC
Primer 7 (P70	GAAACGGGTG
Primer 8 (P8)	GTGACGTAGG
Primer 9 (P9)	GGGTAACGCC
Primer 10 (P10)	GTGATCGCAG
Primer 11 (P11)	CAATCGCCGT
Primer 12 (P12)	TCGGCGATAG
Primer 13 (P13)	CAGCACCCAC
Primer 14 (P14)	TCTGTGCTGG
Primer 15 (P15)	TTCCGAACCC
Primer 16 (P16)	AGCCAGCGAA
Primer 17 (P17)	GACCGCTTGT
Primer 18 (P18)	AGGTGACCGT
Primer 19 (P19)	CAAACGTCGG
Primer 20 (P20)	GTTGCGATCC

PCR reaction was performed in the volume of 20 µl containing 100 ng of template DNA, 200 nM primer, 1X ThermoPol® reaction buffer, 0.1 mM dNTPs and 1 U *Taq* DNA polymerase. The PCR conditions for RAPD analyses consisted of an initial denaturation at 94 °C for 5 min followed by 45 cycles of 60 s at 94 °C, 30 s at 36 °C and 60 s at 72 °C and finally terminated with an extension of 5 min at 72 °C. Amplifications were carried out in Applied Biosystems™ Veriti™ 60-well Thermal Cycler. PCR products were separated in using in 1.4 % (w/v) agarose gel with 0.5X TBE buffer. The gels were visualized with Gel Documentation System (GelDoc 1000, v 1.5, BioRad). The PCR reaction was repeated at least twice to confirm reproducibility of the results.

Somaclonal variation rate

Somaclonal variation rate was determined by calculating percentage of polymorphic bands using the following equation:

(Number of polymorphic fragments/ Number of total fragments) x 100%.

Statistical analysis

The length of shoot, the length of root and the area of spreadability of all replicates were recorded after six weeks of each subculture. Data were analyzed by one-way ANOVA significant level of $p<0.05$ using Social Science Software (SPSS).

RESULTS AND DISCUSSION

Identification of RAPD marker for detecting somaclonal variation

RAPD primers as listed in Table 1 were screened using DNA extracted from leaf sample of mother plant of *H. micranthemoides*. The PCR products were electrophoresed on 1.4 % agarose gel.

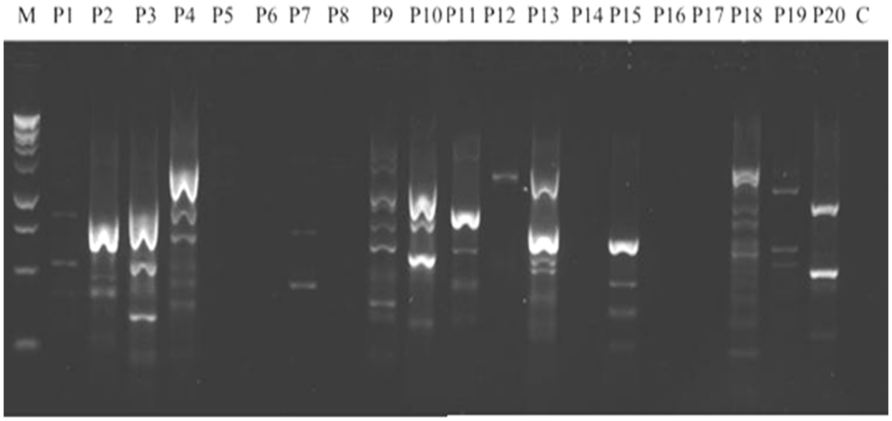

Fig.1. RAPD-PCR amplification products of from screening of P1-P20 with mother plant of *H. micranthemoides*. C - negative control; M - 1kb DNA marker.

Based on Fig. 1, 14 out of 20 primers tested showed PCR products. Those primers were P1, P2, P3, P4, P7, P9, P10, P11, P12, P13, P15, P18, P19 and P20. Meanwhile, the rest of the primers (P5, P6, P8, P14, P16 and P17) failed to show any amplification products, hence were immediately discarded from further investigation. Because of the sharper and clearer bands showed by primer P11, P15 and P20, they were chosen to be further examined.

With the PCR conditions as previously mentioned, P11, P15 and P20 were subjected to PCR using available DNA samples at that time which were the mother plant (MP) itself and DNA samples from subculture 1 to 4 (S1-S4). The PCR products are as shown in Fig. 2.

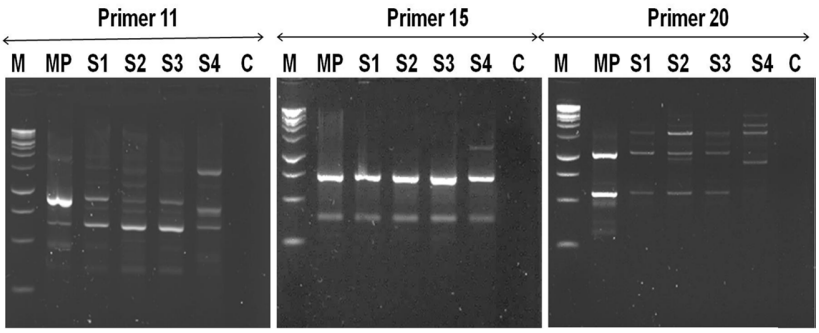

Fig. 2. RAPD-PCR amplification products of mother plant and subculture 1 to subculture 4 (S1-S4) with P rimer 11, 15 and 20. C- negative control, M- 1kb DNA marker, MP- mother plant

Based on the cross comparison between the amplification product banding patterns produced from the three primers tested, P15 was considered as the best primer as it showed the most defined fragments compared to P11 and P20. Hence, it was selected as the most appropriate RAPD marker for detecting somaclonal variation in *H. micranthemoides*.

Somaclonal variation rate in micropropagated plantlet of *H. micranthemoides*

DNAs were extracted from subculture 1 to subculture line 8 (S1-S8). All the DNAs were subjected to PCR-RAPD using primer P15 and the PCR products are shown in figure below.

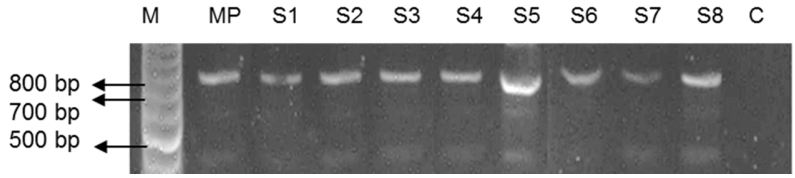

Fig. 3. Amplification banding patterns of mother plant (MP) and micropropagated plantlets (S1-S8) using primer 15. C- negative control, M- 100bp DNA marker.

Based on the fragments shown in Fig. 3, monomorphic bands consisted of two bands were observed in mother plant,S1, S2, S3, S4,S6 and S7. The size of the bands were 800 bp and 500 bp respectively. The presence of the monomorphic bands similar to the mother plant in those subcultures indicate that somaclonal variation had not occurred.

It was implied that somaclonal variation was only occur when the plant was subcultured or multiplied five times (S5) because polymorphic bands were observed at this stage. Polymorphism was also detected at S8. The percentage of polymorphism at S5 and S8 were 50 % and 60 % respectively. Since the percentage of polymorphism correlates with somaclonal variation, it can be said that the somaclonal variation in *H. micranthemoides* is rather arbitrary.

Somaclonal variation can occur at any stages of subculture. It had been long detected in commercial crop such as banana where micropropagation recognizable as a viable multiplication approach. According to Rodriguez et al. (1997), somaclonal variation in the Brazilian banana "Nanicao" appeared at fifth subculture but in this study phenotypic assessment was made rather than molecular one. It is generally accepted that increasing number of subcultures, increase the somaclonal variation, so the number of subcultures in micropropagation protocols should be kept to a minimum (Bhatia & Sharma, 2015).

The effects of somaclonal variation on the plantlet growth

Genotypic changes as a result of somaclonal variation might have effects on phenotypes of regenerates. In this study, phenotypic changes on the growth *of H. micranthemoides* were accessed by measuring shoot and root length as well as plantlet spreadability. Unfortified MS media were used to avert the effects of plant growth regulators. The measurements were recorded from the first up to eighth subculture.

Length of shoot

In this study, shoot length was measured six weeks after each subculture. Each subculture had five replicates.

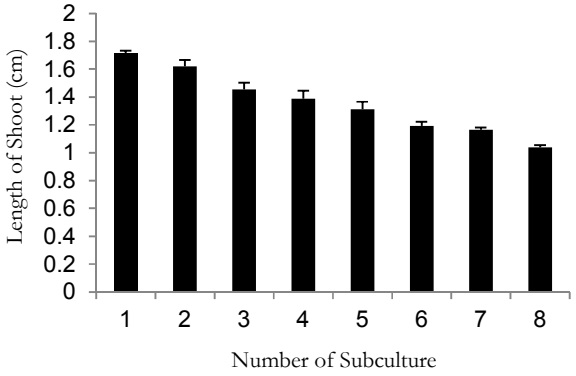

Fig. 4. The effects of number of subcultures on length of shoot of *H. micranthemoides*

Fig. 4 shows the length of shoot measured as the number of subculture progressed. It is clear that, as the number of subculture increased, the length of shoots produced steadily decreased. Statistical analysis shows there is a significant difference between the number of subculture and shoot length (p-value, 0.05). Decline in shoot length that occurred immediately after the first subculture showed the violent effects of subculturing in *H. micranthemoides*. Vujović et al. (2012) reported that the first significant decrease in shoot length was also observed after the first subculture in cherry Gisela 6 and plum Fereley Jaspi. However, there is no visible morphological variations or aberrations of shoots were found in successive subcultures although quality of shoots in terms of shoot length varied during subculturing.

Length of root

The root length was measured also after six weeks of subculture. Each subculture had five replicates. Based on Fig. 5, it obvious that the root length decreased as the number of subcultures increased, The root length at S8 was drastically decreased which was nearly half from the round of subculture. Statistical analysis shows that there is a significant difference between the number of subcultures and root length since the (p-value < 0.05). However, repeated subcultures can have opposite effects than observed in *H. micranthemoides*. For example, in the study of Chinese wild grape (*Vitis piasezkii* var. pagnucii) the rooting rate was increased significantly with subsequent subcultures (Zhang et al., 2006) and this might be due to addition of Indole Acetic Acid (IAA) in the growth medium.

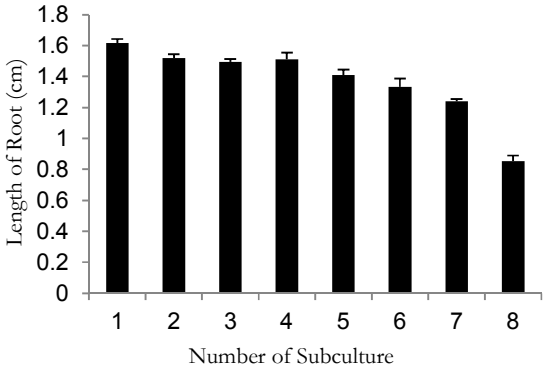

Fig. 5. The effect of subculture number on root length of *H. micranthemoides*

IAA is recognized to have a certain role in the rooting initiation (Štefančič et al., 2005) and apparently was influenced by number of subcultures.

Area of spreadability

As *H. micranthemoides* grows as a creep or carpeting-like plant it is wise to measure its area of spreadability. The area of spreadability was recorded after six weeks of subculturing using transparent grid paper.

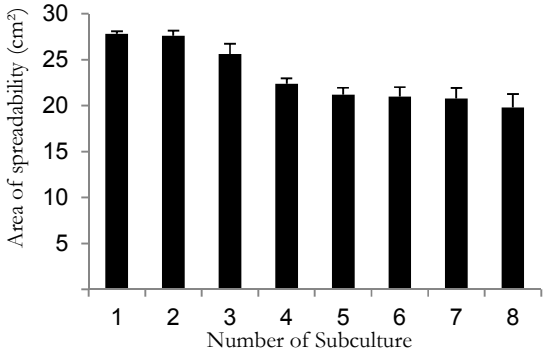

Fig. 6. The effect of number of subculture on area of spreadability of *H. micranthemoides*

Generally, as the subculture number increased, the area of spreadability of micropropagated plantlet *H. micranthemoides* decreased (Fig. 6). In the first two subcultures, there was no significant difference in the area of spreadability observed. The first significant decrease was observed only after the second subculture and it decreased further until the eighth subculture. This is expected because area of spreadability is closely related to root growth.

Continual multiplication is common in plant tissue cultures techniques because in theory it produces progenies of desired genetic as well as physical characteristics of the parental line quicker. However, the assessments of the phenotypes of the *H. micranthemoides* regenerates found that the plantlet growth was negatively affected as it was repeatedly multiplied. The plantlets produced were smaller than initially measured. The length of shoot and root as well as the area of spreadability decreased, reducing its quality. Parallel with genotypic changes as revealed by RAPD-PCR, number of subcultures should be kept minimum in order to preserve its original features.

CONCLUSION

At the end of this study, P15 was selected as a suitable marker to detect somaclonal variation in *H. micranthemoides* because it showed the most distinctive bands. Using P15, somaclonal variation was clearly observed at the fifth subculture due to the presence of polymorphic bands with percentage of polymorphism of 50 %. Therefore, it is suggested that the subculture process of *H. micranthemoides* should only be carried out until the fourth subculture or less. Moreover, the quality of the plantlet produced was significantly reduced as the plant was subcultured repeatedly.

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REFERENCES

Barpete, S., ÖZCAN, S. F., Aasim, M., & ÖZCAN, S. (2015). *In vitro* high frequency regeneration through apical shoot proliferation of Hemianthus callitrichoidesCuba'-a multipurpose ornamental aquatic plant. *Turkish Journal of Biology*, *39*(3), 493-500.

Bhatia S. & Sharma K., 2015. *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*, Academic Press.

Millar B., 2011. *Plant of the month Hemianthus micranthemoides. Tropical Fish Hobbyist Magazine.* Retrieved from http://www.tfhmagazine.com/details/plant-of-the-month/hemianthus-micranthemum-micranthemoides.htm in August 2019.

Oyedeji A.A. & Abowei J.F.N., 2012. The classification, distribution, control and economic importance of aquatic plants. *International Journal of Fisheries and Aquatic Sciences*, *1(2)*, 118-128.

Palombi M. & Damiano C., 2002. Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (Actinidiadeliciosa A. Chev). *Plant Cell Reports*, *20(11),* 1061-1066.

Rai P.K., 2009. Heavy metal phytoremediation from aquatic ecosystems with specialreference to macrophytes. Critical *Reviews in Environmental Science and Technology*, 39(9), 697-753.

Rodrigues P.H.V., Tulmann Neto A., Cassieri Neto P. & Mendes B.M.J., 1997. Influence of the number of subcultures on somaclonal variation in micropropagated nanicao (Musa spp., AAA Group). In II International Symposium on Banana: I International Symposium on Banana in the Subtropics 490, 469-474.

Saha, S., Adhikari, S., Dey, T., & Ghosh, P. (2015). RAPD and ISSR based evaluation of genetic stability of micropropagated plantlets of Morus alba L. variety S-1. *Meta gene*, *7*, 7–15.

Singh D., Tiwari A.& Gupta R., 2012. Phytoremediation of lead from wastewater using aquatic plants. *Journal of Agricultural Technology*, *8(1)*, 1-11.

Štefančič M., Štampar F. & Osterc G., 2005. Influence of IAA and IBA on root development and quality of Prunus' GiSelA 5'leafy cuttings. *Horticultural Science*, *40(7)*, 2052-2055.

Vujović T., Ružić D.J. & Cerović R., 2012. *In vitro* shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks. *Horticultural Science*, *39(3)*, 101-107.

Williams J.G., Kubelik A.R., Livak K.J., Rafalski J.A. & Tingey S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, *18(22)*, 6531-6535.

Yapabandara Y.M.H.B. & Ranasinghe P., 2007. Tissue culture for mass production of aquatic plant species. Retrieved from http://www.apctt.org/publication/pdf/tm_dec_tissue.pdf in August 2019.

Zhang J.L., Xu R., Cao Z.Y., Wang S.M. & Ren J.Z., 2006. Factors affecting in vitro propagation of a Chinese wild grape (Vitispiasezkii var. pagnucii): Shoot production and rhizogenesis. *New Zealand Journal of Crop and Horticultural Science, 34(3)*, 217-223.

Zurayk R., Sukkariyah B. & Baalbaki R., 2001. Common hydrophytes as bioindicators of nickel, chromium and cadmium pollution. *Water, Air, and Soil Pollution*, *127(1-4)*, 373-388.

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