Malays. Appl. Biol. (2019) 48(5): 29–37

GENETIC FIDELITY ASSESSMENT OF TISSUE CULTURE-DERIVED *Neolamarckia cadamba* PLANTLETS USING DNA-BASED MARKERS

MOK PEI-KIENG and HO WEI-SENG*

Forest Genomics and Informatics Laboratory (fGiL), Resource Biotechnology Programme, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
**E-mail: wsho@unimas.my*

Accepted 25 September 2019, Published online 31 December 2019

ABSTRACT

Neolamarckia cadamba is one of the fast-growing tree species selected for industrial tree plantations (ITPs). As demand and supply of true-to-type planting material are increasing, micropropagation of elite *N. cadamba* planting material is required for the sustainable development of ITPs. However, somaclonal variation among tissue culture-derived plantlets is a bottleneck in micropropagation. The present study described the genetic fidelity of *in vitro* regenerated *N. cadamba* plantlets from first, second, third and sixth subcultures. These plantlets were obtained from axillary shoot multiplication and repeated subcultures of microshoots with three weeks interval period, which initially developed from nodal explants. By using five random amplified length polymorphism (RAPD) and two inter-simple sequence repeats (ISSR) primers, a total of 9,334 bands and 2,760 bands were amplified respectively, from a total number of 164 tested plantlets. The banding profiles for each primer was highly uniform, and the DNA bands were monomorphic across all tissue culture-derived plantlets from every four subcultures compared to the stock plants. A target gene-specific marker was also employed to detect single nucleotide polymorphism (SNP) within the targeted genomic sequence of *Susy* gene. There was no SNP detected from all the analysed plantlets. The current findings ascertained the efficiency and reliability of the *N. cadamba* micropropagation protocol at least up to six subculture cycles for mass production of true-to-type plantlets.

Key words: *Neolamarckia cadamba*, genetic fidelity, DNA marker, SNP, micropropagation

INTRODUCTION

Micropropagation of *Neolamarckia cadamba* (Roxb.) Bosser is vital for large scale commercial production of quality planting material for industrial tree plantations (ITPs) in Malaysia in order to fulfil the increasing demand for timber in future. This tree species is belonging to Rubiaceae family and widely distributed in South Asia and Southeast Asia, such as Malaysia, Indonesia, China, India, Thailand, Vietnam and Papua New Guinea (Krisnawati *et al*., 2011; Tchin *et al*., 2018). It is a light hardwood species that frequently harvested as raw materials for plywood, hardboard, paper, and furniture. It is also used for ornamental purposes, while its leaf and fruit extracts and dried bark are used for pharmaceutical purposes (Zaky *et al*., 2014; Dwevedi *et al*., 2015; Pandey & Negi, 2016).

Micropropagation is a well-established plant tissue culture technique that adopted to propagate commercially important plants by using part of the plants as explants. This technique is widely used for rapid clonal propagation in order to supply a large scale of planting material for afforestation and elite genotypes preservation (Kataria *et al*., 2013; Alizadeh *et al*., 2015). Hence, a high degree of genetic fidelity among the tissue culture-derived plantlets is very critical. The somaclonal variations could be detected in tissue culture-derived plantlets, which is the major bottleneck in the micro-propagation of plants. Level of plant growth regulators, types of explants, time in tissue culture conditions and subculture number are frequently reported as the main factors of this variation (Bairu, 2011; Krishna *et al*., 2016). It is reported that shoot proliferation from explants with pre-existing meristems poses a lower genetic instability risk (Mohanty *et al*., 2012; Behera *et al*., 2018). Besides,

* To whom correspondence should be addressed.

studies have shown that prolonged inorganized cell cultures are prone to somaclonal variations than shoot cultures (Khan *et al*., 2011; Devi *et al*., 2014). Somaclonal variations are often heritable and represent permanent genetic changes that occurred within the genetic makeup of propagated plantlets (Leva *et al*., 2012; Kasim *et al*., 2018). Such changes may lead to the loss of desired traits from the mother plants and produce variants that are not accepted by the end-users (Krishna *et al*., 2016).

DNA-based markers provide several benefits for genetic fidelity assessment of *in vitro* regenerated plants, such as high accuracy, environmental factors independent, high reliability and reproducibility (Kamle *et al*., 2014; Alizadeh *et al*., 2015). RAPD and ISSR are the common markers that widely applied for genetic uniformity assessment of plantlets in several genera derived from *in vitro* cultures (Kumar *et al*., 2011; Ahmed *et al*., 2017). They are easy to use and highly cost-effective, no requirement for radioactivity (Roostika *et al*., 2016). Although RAPD markers can produce a large number of loci, it also has a limited reproducibility (Costa *et al*., 2016). Besides RAPD and ISSR, the advances in DNA sequencing technology enable evaluation of genetic variation up to nucleotide level. SNP polymorphisms are identified through direct sequencing of DNA segments obtained by PCR amplification (Rafalski, 2002). This method allows reliable detection of SNPs; however, it requires enormous effort for the development of specific primers and a large number of samples needed to be amplified and sequenced (Ganal *et al*., 2009). A combination of different markers is efficient for genetic fidelity analysis as different regions of the whole DNA sequence are amplified (Martins *et al*., 2004).

The estimation of genetic diversity and relatedness of the selected *N. cadamba* trees from six natural forests and two planted forests in Sarawak had been reported using RAPD and ISSR markers (Tan *et al*., 2007; Tiong *et al*., 2014). However, there is no published report on the genetic fidelity assessment of tissue culture-derived plantlets of *N. cadamba* to date. Hence, the objective of this study was to assess the genetic fidelity of *N. cadamba* plantlets using DNA-based markers, namely: RAPD, ISSR and SNP markers. The plantlets of *N. cadamba* were propagated through direct shoot organogenesis by using nodal explants from *in vitro*-germinated seedlings of selected candidate plus tree. The subcultures of microshoots were done at three weeks interval period up to six times. Genetic fidelity assessment of tissue culture-derived plantlets at an early stage can assist in getting rid of genetically unstable seedlings, and thus reducing the maintenance cost in the field until maturity.

MATERIALS AND METHODS

Shoot multiplication and plantlet regeneration

Shoot culture was established through direct shoot organogenesis by using nodal explants of *N. cadamba* seedlings from selected candidate plus tree. Briefly, the explants were cultured in Gamborg B5 medium supplemented with 0.8 mg/L of Benzylaminopurine (BAP). Four *in vitro* seedlings were selected as stock plants based on their vigorous growth and then subcultured as nodal cultures. This was denoted as Subculture 1 (S1). The microshoots that less than 1 cm height after four weeks were excised and then subcultured into the new fresh medium with the same composition of nutrients and BAP (S2). Subcultures of microshoots were conducted every three weeks. The shoots were then rooted in Gamborg B5 medium supplemented with 0.1 mg/L of Paclobutrazol (PBZ) for four weeks.

DNA extraction and molecular analysis

The fresh young leaves from four stock plants and 160 randomly selected plantlets from four subcultures (S1, S2, S3 and S6) of the respective stock plants were collected and used as a source of DNA to assess genetic fidelity of the plantlets after repeated subculturing. The total genomic DNA was extracted from fresh leaf samples as prescribed by Murray and Thompson (1980).

RAPD-PCR amplification

Five RAPD primers from the previous study (Tan *et al*., 2007), namely OPA-05, OPA-08, OPA-10, OPA-15 and OPB-20, were used to evaluate the genetic fidelity of the plantlets. The 25 µL PCR reaction mixture containing 1 ng DNA, 2.5 mM $MgCl_2$, 10.0 pmol/µL of primer, 1.0 unit of *Taq* DNA polymerase, 0.2 mM dNTPs and 1× PCR buffer. The thermal cycling profile was programmed with an initial denaturation at 94°C for 3 minutes and then 45 s in 35 cycles, annealing at 40°C for 60 s, extension at 72°C for 1 min and final extension for 7 min at 72°C.

ISSR-PCR amplification

Two ISSR primers from the previous study (Tiong *et al*., 2014), which are $(GTG)_6$ and $(AC)_{10}$ were used to amplify the ISSR regions. The PCR amplifications were carried out in 25 µL reaction mixtures that containing 1 ng DNA, 2.5 mM $MgCl_2$, 10.0 pmol/µL of primer, 0.5 unit of *Taq* DNA polymerase, 0.2 mM dNTPs and 1× PCR buffer. The thermal cycling profile was programmed with initial denaturation at 95°C for 2 min and then 30 s in 40 cycles, the annealing of the primer $(GTG)_6$ and $(AC)_{10}$ at temperature of 60 and 57.8°C respectively for 30 s, extension at 72°C for 1 min and final extension for 10 min.

Target gene-specific PCR amplification and DNA sequencing

For further validation, the stock plants and their plantlets from each subculture (S1, S2, S3 and S6) were subjected for target gene-specific PCR amplification. PCR amplification of *Susy* gene was performed as prescribed by Tan *et al*. (2014). The PCR products were then sent for sequencing in both forward and reverse directions.

Data analysis

The amplified products from the RAPD and ISSR primers were resolved through 1.5% agarose gel electrophoresis. The patterns of bands were documented via Geliance 200 Imaging System (PerkinElmer, USA). The pattern of amplicons was compared between the stock plants and plantlets, which then manually scored. For sequence variation analysis, the raw data of DNA sequences were checked to remove low-quality base calling sequences by using Unipro UGENE 1.31 (UniPro, Russia). The partial *SuSy* genomic sequences from each stock plant and their plantlets were aligned to detect the presence of single nucleotide polymorphisms.

RESULTS

RAPD and ISSR analysis

All the five RAPD primers and two ISSR primers were found capable of generating reproducible, distinct and scorable bands (Table 1). A total of 9,334 bands and 2,760 bands (total number of scorable bands × total number of analysed plants) were generated by the RAPD markers and ISSR markers, respectively. All the bands produced were monomorphic across all the tissue culture-derived plantlets from all the four passages. The RAPD and ISSR banding profiles are shown in Figure 1(a, b) and Figure 1(c, d), respectively. The average number of scorable loci for each RAPD primer was varied from 9.8 to 13.0, with sizes ranged from 250 to 4,000 bp. Based on the fingerprinting profiles from the four stock plants, the five RAPD primers produced 56.8 scorable loci, with a mean of 11.4 loci per primer. Similarly, the average number of detectable loci from each ISSR primer was ranging from 8.0 to 8.5 within the size range of 600 to 2,500 bp. An average of 16.5 distinct and scorable bands was obtained from the two ISSR primers, with a mean of 8.3 loci per primer.

Target gene-specific PCR analysis

Sequence analysis was carried out to determine the nucleotide variation within the sequence of a selected gene in *N. cadamba* plantlets and the stock plants. The partial *SuSy* genomic sequence with a length of 708 bp was amplified and sequenced from four stock plants and 80 plantlets from four passages. The consensus sequences from the stock plants and plantlets were aligned to detect the presence of SNPs. No nucleotide polymorphism was detected among the analysed plantlets since these plantlets were derived from the respective stock plant. The alignment of partial *SuSy* genomic sequences from the four stock plants was also performed and found no variation among them. A part of the partial *SuSy* gene sequence alignment is shown in Figure 2.

DISCUSSION

For commercial utility, micropropagation is known to generate a mass number of clones from desired germplasms, which results in plantlets that are theoretically identical to the donor plant. However, the hindrance of micropropagation is frequently due to the *in vitro* stresses that might result in genetic instability of tissue culture-derived plantlets that commonly known as the somaclonal variation (Bello-Bello *et al*., 2014; Akdemir *et al*., 2016).

Table 1. Summary of RAPD and ISSR primers with their amplification products from four stock plants and 160 tissue culture-derived plantlets selected from four subcultures (S1, S2, S3 and S6)

Primer	Primer sequence (5'-3')	T_a (°C)	Mean no. of loci	Size range (bp)	Polymorphic band (%)			
					S1	S2	S3	S6
RAPD:								
OPA-05	*AGGGCTCTTG*	40.0	13.0	400-2000	0.0	0.0	0.0	0.0
OPA-08	*GTGACGTAGG*	40.0	9.8	250-2500	0.0	0.0	0.0	0.0
OPA-10	*GTGATCGCAG*	40.0	12.5	400-4000	0.0	0.0	0.0	0.0
OPA-15	*TTCCGAACCC*	40.0	11.3	600-3500	0.0	0.0	0.0	0.0
OPB-20	*GGACCCTTAC*	40.0	10.3	750-3500	0.0	0.0	0.0	0.0
ISSR:								
GTG_6	*GTGGTGGTGGTGGTGTG*	60.0	8.5	600-2500	0.0	0.0	0.0	0.0
AC_{10}	*ACACACACACACACACAC*	57.8	8.0	700-2500	0.0	0.0	0.0	0.0

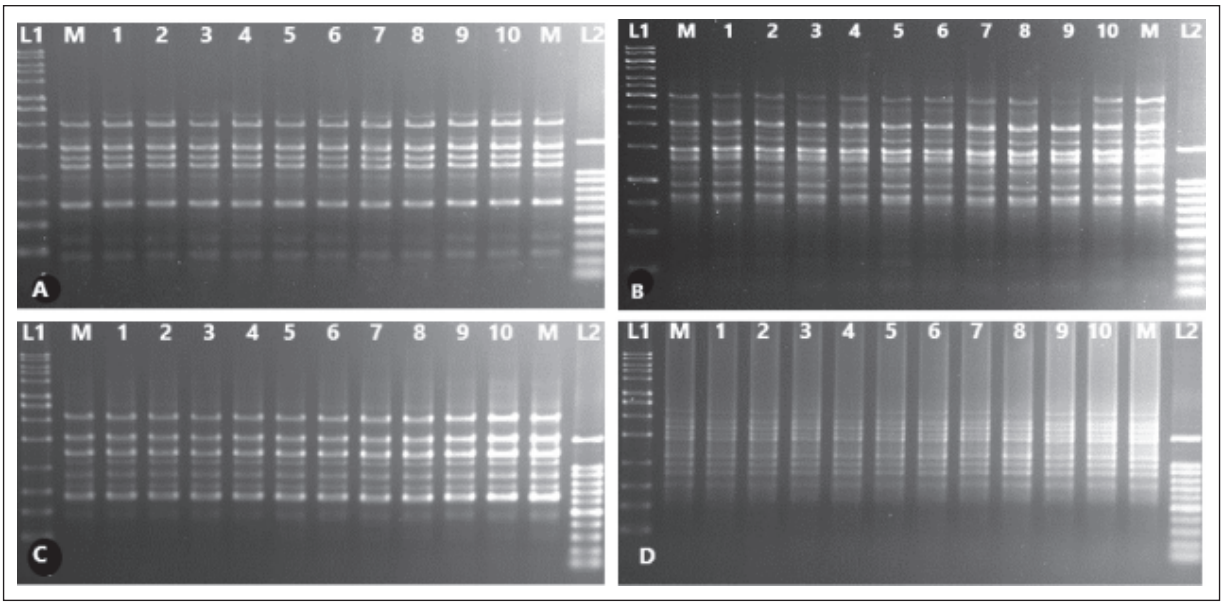

Fig. 1. PCR amplification profiles obtained by a) OPA-08, b) OPB-20, c) GTG_6, and d) AC_{10} for the stock plant, N5(14) and tissue culture-derived plantlets. Lane M, stock plant; Lanes 1-10, tissue culture-derived plantlets from axillary shoot multiplication. Lane L1, 1 kb ladder; Lane L2, 100 bp ladder.

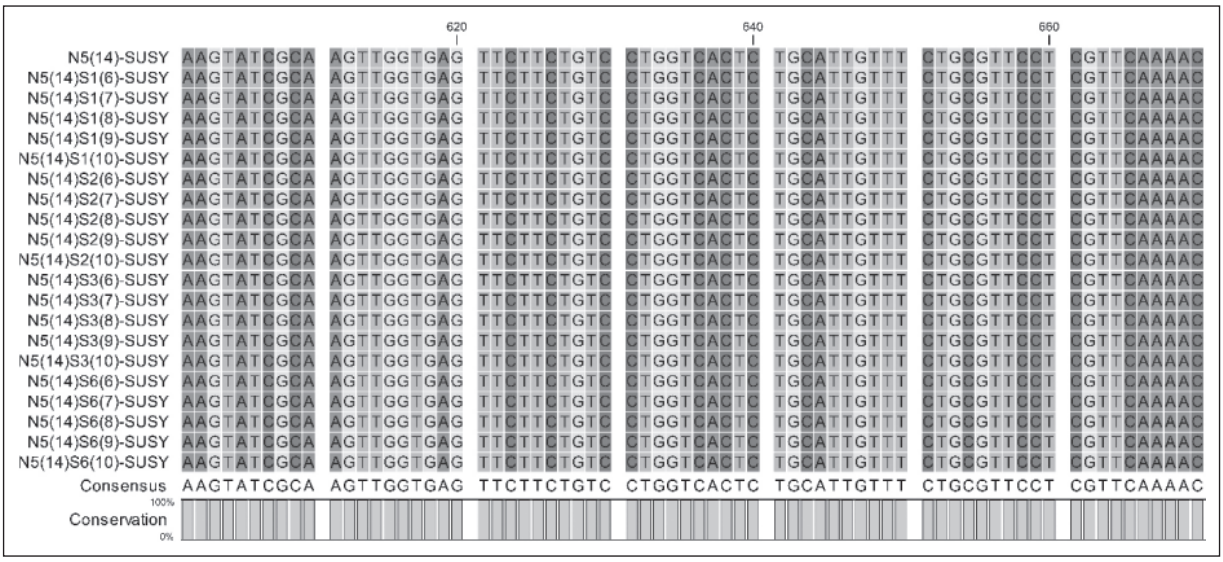

Fig. 2. A Part of the alignment result for the partial genomic sequences of *SuSy* gene from the stock plant, N5(14) and tissue culture-derived plantlets.

Somaclonal variations with frequency ranging from 60% to 98% had been documented in several reports on plantlets that regenerated from apical meristems and axillary buds (Phulwaria *et al*., 2014; Werner *et al*., 2015; Venkatachalam *et al*., 2018). Genetic variations may arise among the plantlets considering the factors that associated with tissue culture conditions, such as the type of explants and regeneration pathway (Linacero *et al*., 2000; Bramhanapalli *et al*., 2017). In the present investigation, propagation for *N. cadamba* was through direct axillary shoot organogenesis from nodal explants and no variation in all the DNA profiles generated. This regeneration pathway from organised meristematic cells without any intermediate callus formation is known to produce plantlets in mass amount within a short span of time with high genetic integrity and fidelity, and hence it is likely to be selected for mass production of quality planting materials with desired economic traits (Saha *et al*., 2014; Behera *et al*., 2018).

Several publications inferred that somaclonal variations result from interactions of three main factors which are the initial explants, genotype and *in vitro* culture conditions. For clonal propagation of *N. cadamba* with the absence of somaclonal variations, a low BAP concentration was used for axillary proliferation and subcultures of micro-shoots, while a low PBZ concentration was used for rooting in the present study. According to Farahani *et al*. (2011) and Khan *et al*. (2011), plant growth regulators are reported to be associated with somaclonal variations in tissue culture-derived plantlets. However, Venkatachalam *et al*. (2007) had shown that exposure to a high level of cytokinins up to 10 mg/L of banana cv. Nanjanagudu Rasabale (AAB) culture did not lead to genetic variation in all the plantlets screened. In contrast, a high concentration of BAP had resulted in chromosomal number variation in banana cultivar 'Williams' (Halim *et al*., 2018).

Findings from several reports had shown that the number of subcultures and the length of the culture period are associated with the occurrence of somaclonal variants, which the higher the number of subcultures and the longer the culture period, the higher the frequency of polymorphisms (Khan *et al*., 2011; Zoghlami *et al*., 2012; Devi *et al*., 2014). It is suggested that increase in culture duration is causing the cells or tissues being frequently exposed to *in vitro* environmental factors as well as various chemicals including plant growth regulators that lead to somaclonal variations (Turner *et al*., 2001; Peng *et al*., 2015). Conversely, some studies revealed that the level of subculture and culture duration did not affect the genetic integrity of tissue culture-derived plantlets, especially among regenerated plantlets obtained through shoot cultures and axillary branching (Gantait *et al*., 2010; Asthana *et al*., 2011; Chhajer & Kalia, 2016), which are in concordance with the present findings.

The genetic fidelity of the regenerated *N. cadamba* was confirmed by using five RAPD and two ISSR markers in the current study. Many studies on the molecular characterisation of micropropagated plantlets are conducted, especially in the assessment of clonal fidelity among the regenerated plants and donor plants by adopting the RAPD and ISSR techniques. Lattoo *et al*. (2006) had reported the 100% similarity among the *Chlorophytum arundinaceum* plantlets and the mother plant by using five RAPD marker. In a study by Kumar *et al*. (2013), the genetic uniformity of the micropropagated *Catharanthus roseus* plantlets was confirmed by five RAPD markers with no variation was detected. By using the cytogenetic and RAPD analysis from three primers, the genetic uniformity of regenerated *Iris sibirica* plantlets with identical profiles had been assessed in a study by Stanišiæ *et al*. (2015). Sharma *et al*. (2014) and Ramírez-Mosqueda *et al*. (2016) had shown a high genetic homology among the *Tylophora indica* and *Stevia rebaudiana* plantlets, respectively after the genetic fidelity evaluation via six ISSR primers. Besides that, the true-to-type of *Citrullus lanatus* plantlets was ascertained by six ISSR markers in the report by Vinoth and Ravindhran (2016).

Three different DNA marker systems were used for genetic fidelity assessment in the present study. It is suggested that a combination of different markers is efficient for genetic fidelity assessment as different regions of the genome are amplified and compared (Martins *et al*., 2004). The numbers of RAPD bands (11.4 loci per primer) and ISSR bands (8.3 loci per primer) were fairly high when compared with the reports on the other species. Therefore, the number of bands amplified in the current study seemed to be sufficiently satisfied with the evaluation of genetic fidelity among the *N. cadamba* plantlets. Bhatia *et al*. (2011) detected an average of 5.5 and 4.5 bands per primer from ten ISSR primers and twelve RAPD primers, respectively in the genetic stability assessment of *in vitro*-propagated gerbera. Saha *et al*. (2012) reported that an average of 4.11 fragments was obtained from nine RAPD primers. By using 31 RAPD and 11 ISSR primers, the average numbers of 2.96 and 4.81 bands per primer were generated in another study by Saha *et al*. (2014).

The PCR amplicons obtained in the present study were sequenced in both forward and reverse directions and aligned to distinguish the true single nucleotide polymorphisms (SNPs) from sequencing errors. SNPs are found in non-coding regions of the genome such as 3' untranslated regions (UTRs), introns and repetitive sequences; including in most of the coding sequences (Agarwal *et al*., 2008; Chagné *et al*., 2008). There was no SNP detected within the 708 bp of partial genomic sequence sucrose synthase (*SuSy*) gene among the analysed stock plants and plantlets. *SuSy* is a key enzyme that catalyses the reversible synthesis and degradation of sucrose (Carlson *et al*., 2002; Tan *et al.*, 2014). Coleman *et al*. (2009) implicated that overexpression of *SuSy* gene resulted in increased cellulose synthesis in the secondary cells, which demonstrated the tight association of *SuSy* gene with cellulose production and secondary cell wall formation. The SNP result further verifies the genetic integrity of regenerated *N. cadamba* plantlets from micropropagation technique. Due to the same clonal origin, it is expected that no sequence variation should be detected between the plantlets and their respective mother plant (Negi & Saxena, 2010).

Several studies had been reported that tissue culture conditions might cause stresses to the plantlets and results in the development of variants

that manifested from errors occurred in cellular control during plant regeneration process (Ruffoni & Savona, 2013; Khatun *et al*., 2018). The tissue culture-induced genetic changes including activation of transposon elements, chromosome rearrangements, duplication and point mutations such as base deletion, insertion, and substitution (Bairu *et al*., 2011; Krishna *et al*., 2016). The changes occurred within the genome, especially the gene coding regions may result in the generation of off-type plantlets.

CONCLUSION

To the best of our knowledge, this is the first report on the genetic fidelity assessment of tissue culture-derived plantlets of *N. cadamba* using DNA-based markers. The present study also successfully demonstrated the usefulness of molecular markers such as RAPD, ISSR and SNP in determining the trueness-to-type of tissue culture-derived plantlets of *N. cadamba*. The genetic integrity of tissue culture-derived plantlets could be maintained and stable up to at least the sixth subcultures. The findings proved that micropropagation of *N. cadamba* from axillary shoot buds is a rapid, safe and efficient method for large scale production of true-to-type plants, and thus aids to meet the demands for quality planting materials for industrial tree plantation development.

ACKNOWLEDGEMENTS

This study was supported by the funding from the Sarawak Timber Association (STA) to Universiti Malaysia Sarawak (Grant No. GL(F07)/06/2013/STA-UNIMAS(06) and GL/F07/STA01/2019). The authors would like to acknowledge Mr Peter Ling Kwong Hung from WTK for providing the plant materials for this research project.

REFERENCES

Agarwal, M., Shrivastava, N. & Padh, H. 2008. Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Reports*, **27(4):** 617-631.

Ahmed, M.R., Anis, M., Alatar, A.A. & Faisal, M. 2017. *In vitro* clonal propagation and evaluation of genetic fidelity using RAPD and ISSR marker in micropropagated plants of *Cassia alata* L.: a potential medicinal plant. *Agroforestry Systems*, **91(7):** 637-647.

Akdemir, H., Suzerer, V., Tilkat, E., Onay, A. & Çiftçi, Y.O. 2016. Detection of variation in long-term micropropagated mature pistachio via DNA-based molecular markers. *Applied Biochemistry and Biotechnology*, **180(7):** 1301-1312.

Alizadeh, M., Krishna, H., Eftekhari, M., Modareskia, M. & Modareskia, M. 2015. Assessment of clonal fidelity in micro-propagated horticultural plants. *Journal of Chemical and Pharmaceutical Research*, **7(12):** 977-990.

Asthana, P., Jaiswal, V.S. & Jaiswal, U. 2011. Micropropagation of *Sapindus trifoliatu*s L. and assessment of genetic fidelity of micro-propagated plants using RAPD analysis. *Acta Physiologiae Plantarum*, **33(5):** 1821-1829.

Bairu, M.W., Aremu, A.O. & Van Staden, J. 2011. Somaclonal variation in plants: Causes and detection methods. *Plant Growth Regulation*, **63(2):** 147-173.

Behera, B., Sinha, P., Gouda, S., Rath, S.K., Barik, D.P., Jena, P.K., Panda, P.C. & Naik, S.K. 2018. *In vitro* propagation by axillary shoot proliferation, assessment of antioxidant activity, and genetic fidelity of micropropagated *Paederia foetida* L. *Journal of Applied Biology and Biotechnology*, **6(2):** 41-49.

Bello-Bello, J.J., Iglesias-Andreu, L.G., Avilés-Vinas, S.A., Gómez-Uc, E., Canto-Flick, A. & Santana-Buzzy, N. 2014. Somaclonal variation in habanero pepper (*Capsicum chinense* Jacq.) as assessed ISSR molecular markers. *Hortscience*, **49(4):** 481-485.

Bhatia, R., Singh, K.P., Sharma, T.R. & Jhang, T. 2011. Evaluation of the genetic fidelity of *in vitro*-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers. *Plant Cell, Tissue and Organ Culture*, **104(1):** 131-135.

Bramhanapalli, M., Thogatabalija, L. & Gudipalli, P. 2017. Efficient *in vitro* plant regeneration from seedling-derived explants and genetic stability analysis of regenerated plants of *Simarouba glauca* DC. by RAPD and ISSR markers. *In Vitro Cellular and Developmental Biology-Plant*, **53(1):** 50-63.

Carlson, S.J., Chourey, P.S., Helentjaris, T. & Datta, R. 2002. Gene expression studies on developing kernels of maize sucrose synthase (SuSy) mutants show evidence for a third SuSy gene. *Plant Molecular Biology*, **49(1):** 15-29.

Chagné, D., Gasic, K., Crowhurst, R.N., Han, Y., Bassett, H.C., Bowatte, D.R., Lawrence, T.J., Rikkerink, E.H., Gardiner, S.E. & Korban, S.S. 2008. Development of a set of SNP markers present in expressed genes of the apple. *Genomics*, **92(5):** 353-358.

Chhajer, S. & Kalia, R.K. 2016. Evaluation of genetic homogeneity of *in vitro*-raised plants of *Tecomella undulata* (Sm.) Seem. *Tree Genetics and Genomes*, **12:** 100.

Coleman, H.D., Yan, J. & Mansfield, S.D. 2009. Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultrastructure. *Proceedings of the National Academy of Sciences*, **106(31):** 13118-13123.

Costa, R., Pereira, G., Garrido, I., Tavares-de-Sousa, M.M. & Espinosa, F. 2016. Comparison of RAPD, ISSR, and AFLP molecular markers to reveal and classify orchardgrass (*Dactylis glomerata* L.) germplasm variations. *PLoS One*, **11(4)**.

Devi, S.P., Kumaria, S., Rao, S.R. & Tandon, P. 2014. Single primer amplification reaction (SPAR) methods reveal subsequent increase in genetic variations in micropropagated plants of *Nepenthes khasiana* Hook. f. maintained for three consecutive regenerations. *Gene*, **538(1):** 23-29.

Dwevedi, A., Sharma, K. & Sharma, Y.K. 2015. Cadamba: A miraculous tree having enormous pharmacological implications. *Pharmacognosy Reviews*, **9(18):** 107-113.

Farahani, F., Yari, R. & Masoud, S. 2011. Somaclonal variation in Dezful cultivar of olive (*Olea europaea* subsp. *europaea*). *Gene Conserve*, **10(41):** 216-221.

Ganal, M.W., Altmann, T. & Röder, M.S. 2009. SNP identification in crop plants. *Current Opinion in Plant Biology*, **12(2):** 211-217.

Gantait, S., Mandal, N., Bhattacharyya, S. & Das, P.K. 2010. Determination of genetic integrity in long-term micropropagated plantlets of *Allium ampeloprasum* L. using ISSR markers. *Biotechnology*, **9(2):** 218-223.

Halim, N.A.A., Tan, B.C., Midin, M.R., Madon, M., Khalid, N. & Yaacob, J.S. 2018. Abscisic acid and salinity stress induced somaclonal variation and increased histone deacetylase (HDAC) activity in *Ananas comosus* var. MD2. *Plant Cell, Tissue and Organ Culture*, **133(1):** 123-135.

Kamle, M., Kumar, P., Bajpai, A., Kalim, S. & Chandra, R. 2014. Assessment of genetic fidelity of somatic embryogenesis regenerated guava (*Psidium guajava* L.) plants using DNA-based markers. *New Zealand Journal of Crop and Horticultural Science*, **42(1):** 1-9.

Kataria, N., Yadav, K., Kumari, S. & Singh, N. 2013. Micropropagation: An important tool for conserving forest trees. *Pertanika Journal Tropical Agricultural Science*, **36(1):** 1-26.

Khan, S., Saeed, B. & Kauser, N. 2011. Establishment of genetic fidelity of *in vitro* raised banana plantlets. *Pakistan Journal of Botany*, **43(11):** 233-242.

Khatun, M.M., Tanny, T., Yesmin, S., Salimullah, M. & Alam, I. 2018. Evaluation of genetic fidelity of *in vitro*-propagated *Aloe vera* plants using DNA-based markers. *Science Asia*, **44(2):** 87-91.

Krishna, H., Alizadeh, M., Singh, D., Singh, U., Chauhan, N., Eftekhari, M. & Sadh, R.K. 2016. Somaclonal variations and their applications in horticultural crops improvement. *3 Biotech*, **6(1):** 54.

Krisnawati, H., Kallio, M. & Kanninen, M. 2011. *Anthocephalus cadamba* Miq.: *Ecology, Silviculture and Productivity.* CIFOR, Indonesia. 11 pp.

Kumar, A., Prakash, K., Sinha, R.K. & Kumar, N. 2013. *In vitro* plant propagation of *Catharanthus roseus* and assessment of genetic fidelity of micropropagated plants by RAPD marker assay. *Applied Biochemistry and Biotechnology*, **169(3):** 894-900.

Lattoo, S.K., Bamotra, S., Dhar, R.S., Khan, S. & Dhar, A.K. 2006. Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker – An endangered medicinal herb. *Plant Cell Reports*, **25(6):** 499-506.

Leva, A.R., Petruccelli, R. & Rinaldi, L.M. 2012. Somaclonal variation in tissue culture: A case study with olive. In: *Recent Advances in Plant In Vitro Culture*. A. Leva and L.M.R. Rinaldi (Eds.). IntechOpen, Rijeka. pp. 123-150.

Linacero, R., Alves, E.F. & Vázquez, A.M. 2000. Hot spots of DNA instability revealed through the study of somaclonal variation in rye. *Theoretical and Applied Genetics*, **100(3-4):** 506-511.

Martins, M., Sarmento, D. & Oliveira, M.M. 2004. Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. *Plant Cell Reports*, **23(7):** 432-496.

Mohanty, S., Joshi, R.K., Subudhi, E., Sahoo, S. & Nayak, S. 2012. Genetic stability assessment of micropropagated Mango Ginger (*Curcuma amada* Roxb.) through RAPD and ISSR markers. *Research Journal of Medicinal Plant*, **6(7):** 529-536.

Kasim, N.F.M., Yahya, H.N., Kadzimin, S. & Awang, Y. 2018. Micropropagation and assessment of genetic variability of *Cyclanthus bipartitus*. *Asian Journal of Plant Sciences*, **17:** 19-26.

Murray, M.G. & Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, **8(19):** 4321-4326.

Negi, D. & Saxena, S. 2010. Ascertaining clonal fidelity of tissue culture raised plants of *Bambusa balcooa* Roxb. using inter simple sequence repeat markers. *New Forest*, **40(1):** 1-8.

Pandey, A. & Negi, P.S. 2016. Traditional uses, phytochemistry and pharmacological properties of *Neolamarckia cadamba*: A review. *Journal of Ethnopharmacology*, **181(2):** 118-135.

Peng, X., Zhang, T.T. & Zhang, J. 2015. Effect of subculture times on genetic fidelity, endogenous hormone level and pharmaceutical potential of *Tetrastigma hemsleyanum* callus. *Plant Cell, Tissue and Organ Culture*, **122(1):** 67-77.

Phulwaria, M., Patel, A.K., Rathore, J.S., Ram, K. & Shekhawat, N.S. 2014. An improved micropropagation and assessment of genetic stability of micropropagated *Salvadora oleoides* using RAPD and ISSR markers. *Acta Physiologiae Plantarum*, **36(5):** 1115-1122.

Rafalski, A. 2002. Applications of single nucleotide polymorphisms in crop genetics. *Current Opinion in Plant Biology*, **5(2):** 94-100.

Ramírez-Mosqueda, M.A., Iglesias-Andreu, L.G., Ramírez-Madero, G. & Hernández-Rincón, E.U. 2016. Micropropagation of *Stevia rebaudiana* Bert. in temporary immersion systems and evaluation of genetic fidelity. *South African Journal of Botany*, **106:** 238-243.

Roostika, I., Khumaida, N. & Ardie, S.W. 2016. RAPD analysis to detect somaclonal variation of pineapple *in vitro* cultures during micropropagation. *BIOTROPIA-The Southeast Asian Journal of Tropical Biology*, **22(2):** 109-119.

Ruffoni, B. & Savona, M. 2013. Physiological and biochemical analysis of growth abnormalities associated with plant tissue culture. *Horticulture, Environment, and Biotechnology*, **54(3):** 191-205.

Saha, S., Kader, A., Sengupta, C. & Ghosh, P. 2012. *In vitro* propagation of *Ocimum gratissimum* L. (Lamiaceae) and its evaluation of genetic fidelity using RAPD marker. *American Journal of Plant Sciences*, **3(1):** 64-74.

Saha, S., Sengupta, C. & Ghosh, P. 2014. Evaluation of the genetic fidelity of *in vitro* propagated *Ocimum basilicum* L. using RAPD and ISSR markers. *Journal of Crop Science and Biotechnology*, **17(4):** 281-287.

Sharma, M.M., Verma, R.N., Singh, A. & Batra, A. 2014. Assessment of clonal fidelity of *Tylophora indica* (Burm. f.) Merrill "*in vitro*" plantlets by ISSR molecular markers. *SpringerPlus*, **3(1):** 400.

Stanišić, M., Raspor, M., Ninković, S., Milošević, S., Ćalić, D., Bohanec, B., Trifunović, M., Petrić, M., Subotić, A. & Jevremović, S. 2015. Clonal fidelity of *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis in leaf-base culture – RAPD and flow cytometer analyses. *South African Journal of Botany*, **96:** 42-52.

Tan, C.J., Ho, W.S. & Pang, S.L. 2014. Resequencing and nucleotide variation of Sucrose Synthase (SuSy) gene in a tropical timber tree *Neolamarckia macrophylla*. *International Journal of Scientific and Technology Research*, **3(7):** 135-140.

Tan, S., Abdullah, J. & Ho, W. 2007. Genetic variation of kelampayan (*Neolarmarckia cadamba*) trees for planted forest development in Sarawak. In: *Proceedings of Applied Forest Science Research Seminar*. Sarawak.

Tchin, B.L., Ho, W.S. & Pang, S.L. 2018. Isolation and characterisation of *cinnamate 4-hydroxylase* (*C4H*) gene controlling the early stage of the phenylpropanoid biosynthetic pathway in developing xylem tissues of Kelampayan (*Neolamarckia cadamba*, Rubiaceae). *Asian Journal of Agriculture & Biology*, **6(2):** 278-286.

Tiong, S.Y., Chew, S.F., Ho, W.S. & Pang, S.L. 2014. Genetic diversity of *Neolamarckia cadamba* using dominant DNA markers based on inter-simple sequence repeats (ISSRs) in Sarawak. *Advances in Applied Science Research*, **5(3):** 458-463.

Turner, S., Krauss, S.L., Bunn, E., Senaratna, T., Dixon, K., Tan, B. & Touchell, D. 2001. Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. *Plant Science*, **161(6):** 1099-1106.

Venkatachalam, L., Sreedhar, R.V. & Bhagyalakshmi, N. 2007. Micropropagation in banana using high levels of cytokinins does not involve any genetic changes as revealed by RAPD and ISSR markers. *Plant Growth Regulation*, **51(3):** 193-205.

Venkatachalam, P., Jinu, U., Sangeetha, P., Geetha, N. & Sahi, S.V. 2018. High frequency plant regeneration from cotyledonary node explants of *Cucumis sativus* L. cultivar 'Green Long' via adventitious shoot organogenesis and assessment of genetic fidelity by RAPD-PCR technology. *3 Biotech*, **8(1):** 60.

Vinoth, A. & Ravindhran, R. 2016. Efficient plant regeneration of watermelon (*Citrullus lanatus* Thunb.) via somatic embryogenesis and assessment of genetic fidelity using ISSR markers. *In Vitro Cellular and Developmental Biology-Plant*, **52(1):** 107-115.

Werner, E.T., Soares, T.C.B., Gontijo, A.B.P.L., Neto, J.S. & Amaral, J.A.T. 2015. Genetic stability of micropropagated plants of *Crambe abyssinica* Hochst using ISSR markers. *Genetics and Molecular Research*, **14(4):** 16450-16460.

Zaky, M.Z., Ahmad, F.B., Ho, W.S. & Pang, S.L. 2014. GC-MS analysis of phytochemical constituents in leaf extracts of *Neolamarckia cadamba* (Rubiaceae) from Malaysia. *International Journal of Pharmacology and Pharmaceutical Science*, **6:** 123-127.

Zoghlami, N., Bouamama, B., Khammassi, M. & Ghorbel, A. 2012. Genetic stability of long-term micropropagated *Opuntia ficus-indica* (L.) Mill. plantlets as assessed by molecular tools: Perspectives for *in vitro* conservation. *Industrial Crops and Products*, **36(1):** 59-64.