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# ANALYSIS OF GENETIC STABILITY OF MICROPROPAGATED SUGARCANE IN DIFFERENT SUBCULTURE FREQUENCIES USING SSR MARKER

## *Analisis Kestabilan Genetik Tebu Hasil Mikropropagasi pada Beberapa Frekuensi Subkultur Menggunakan Marka SSR*

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ABSTRACT

*In vitro* technique is an effective method to produce high quality and uniform sugarcane seedlings. This study was aimed to determine genetic stability based on SSR marker analysis of six varieties of sugarcane subcultured in regeneration media. It was conducted at the ICABIOGRAD Molecular Biology Laboratory, Bogor, from May 2015 to October 2016. Six sugarcane varieties (PS 862, PS 865, PS 881, PSJK 922, TK 386, and GMP 3) derived from apical shoot explants were subcultured on MS regeneration media enriched with 0.3 mg/l BAP; 0.5 mg/l IBA; and 100 mg/l PVP, for 3, 6 and 9 times. Sugarcane DNA was extracted using the CTAB method; then, the genetic stability was analyzed using 20 pairs of SSR primers. Data were analyzed in groups using the UPGMA method in the SAHN subprogram available on NTSYS software. The results showed that five sugarcane varieties (PS 865, PS 881, PSJK 922, TK 386, and GMP 3) subcultured up to nine times on the regeneration media remained genetically stable with similarity coefficient to their mother plants value more than 0.94. However, PS 862 variety had genetically unstable after the sixth and the ninth subcultures, the similarity coefficient value to its mother plant was only 0.64, indicated that it experienced somaclonal variations. The study concluded that the *in vitro* shoots of the other varieties were more genetically stable during subcultures compared to PS 862 sugarcane variety based on SSR marker analysis. Further study is needed to find out the cause of genetic changes in PS 862.

**Keywords**: *Saccharum officinarum, apical shoots, in vitro propagation.*

ABSTRAK

Perbanyakan tebu secara *in vitro* merupakan metode yang efektif untuk menghasilkan benih berkualitas yang seragam. Penelitian ini bertujuan untuk mengetahui kestabilan genetik berdasarkan analisis marka SSR terhadap enam varietas tebu yang disubkultur pada media regenerasi. Penelitian dilaksanakan di Laboratorium Biologi Molekuler, BB Biogen, Bogor mulai Mei 2015 hingga Oktober 2016. Varietas tebu yang digunakan adalah PS 862, PS 865, PS 881, PSJK 922, TK 386, dan GMP 3 hasil perbanyakan eksplan menggunakan tunas apikal. Eksplan tebu disubkultur pada media regenerasi MS yang diperkaya dengan 0,3 mg/l BAP; 0,5 mg/l IBA; dan 100 mg/l PVP, selama 3, 6, dan 9 kali. DNA tebu diekstraksi menggunakan metode CTAB kemudian kestabilan genetiknya dianalisis menggunakan 20 pasang primer SSR. Data dianalisis secara gerombol menggunakan perangkat lunak NTSYS dengan metode UPGMA dalam subprogram SAHN. Hasil analisis gerombol menunjukkan bahwa tunas *in vitro* lima varietas tebu (PS 865, PS 881, PSJK 922, TK 386, dan GMP 3) yang disubkultur sampai sembilan kali pada media regenerasi tetap stabil secara genetik dengan nilai koefisien kemiripan dengan tetuanya sebesar >0,94. Namun demikian, varietas PS 862 secara genetik tidak stabil setelah disubkultur enam dan sembilan kali dengan nilai koefisien kemiripan dengan tetuanya hanya 0,64. Hal ini mengindikasikan hanya varietas PS 862 yang mengalami variasi somaklonal setelah disubkultur lebih dari enam kali. Hasil penelitian menyimpulkan bahwa tunas *in vitro* dari lima varietas tebu lainnya lebih stabil secara genetik selama subkutur dibandingkan dengan varetas PS 862. Perlu penelitian lebih lanjut untuk mengetahui penyebab terjadinya perubahan genetik pada varietas PS 862 selama subkultur.

**Kata kunci**: *Saccharum officinarum*, tunas apikal, perbanyakan *in vitro*.

## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the essential crops because it is the main sugar-producing plant. Besides, it can be used as an alternative to renewable fuel. Sugarcane has a chromosome number 2n = 8x = 80 (Yu et al. 2018), so it is challenging to reproduce generatively. Vegetatively, sugarcane is propagated using stem cuttings. Farmers usually use 2-3 nodes of sugarcane stems as new plant materials. However, these methods have shortcomings, such as long propagation time, need for mother plants are high, pathogenic attacks that are difficult to avoid, and dependence on the growing season.

Propagation of plants, including sugarcane through *in vitro* culture, can produce seedlings in large quantities, in a relatively shorter time, uniform, pathogen-free, and the production of seedlings that do

not depend on the season (Hamza and Alebjo 2017). One of the conditions in large-scale seedlings production is that the seedlings produced have the same characteristics as the mother plant or true-to-type. Off-type seedlings can be caused by genetic changes (mutations) during the process of mass propagation through tissue culture. The research of Azizi et al. (2017) showed that the frequency of sugarcane *in vitro* culture, which subcultured in multiplication media could reach nine times the subculture. It is feared that the influence of subculture frequency can affect the genetic stability of sugarcane seedlings.

According to Minarsih et al. (2016), the increase in sugarcane callus abnormalities in subculture frequency five times reached 6%. The same response was shown in citrus callus subcultured every 4-6 weeks for four years, causing morphological diversity in the resulting plantlets (Wulansari et al. 2015). The genetic variation is thought to occur during the subculture process due to the regeneration system used, explant sources, media components, culture duration, number of culture cycles, and genotype influences (Dubrovina and Kiselev 2016; Leva et al. 2012).

This study was aimed to determine the genetic stability of six varieties of sugarcane subcultured in regeneration media based on Simple Sequence Repeat (SSR) marker analysis.

## MATERIALS AND METHODS

This research was carried out from May 2015 to October 2016 at the Cell and Tissue Biology Laboratory and the Molecular Biology Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Bogor, Indonesian Agency for Agricultural Research and Development (IAARD).

### Sugarcane Varieties

Plant genetic materials used in this study consisted of six varieties of sugarcane (PS 865, PS 881, PS 862, PSJK 922 TK 386, and GMP 3) propagated using *in vitro* shoots. PS 865 variety was originated from the Cibinong Experimental Station collection (Bogor), whereas PS 881, PS 862, and PSJK 922 came from the Muktiharjo Experimental Station (Pati). TK 386 variety came from the Jati Tujuh Sugar Factory (Majalengka), GMP 3 from the Gunung Madu Plantation (Lampung), and the mother plant of the six varieties of sugarcane, as a comparison.

### Propagation of Plants

Apical shoots from the sugarcane plants were isolated, sterilized, and planted on *in vitro* regeneration media (MS plus 0.3 mg/l BAP, 0.5 mg/l IBA, and 100 mg/l PVP). This regeneration media caused explants to regenerate directly without callus formation (Azizi et al., 2017). After the *in vitro* shoots were grown and multiplied, the shoots of six varieties of sugarcane were subcultured nine times on regeneration media. Next, the green leaf samples from sugarcane varieties propagated by *in vitro* at frequencies of the third, sixth, and ninth subcultures were taken to be analyzed for their genetic stability. Leaf samples of the mother plant varieties grown in glass-house were used as controls.

### Total Genomic DNA Extraction

The genomic DNA of sugarcane plants was extracted in the Cetyl Trimethyl Ammonium Bromide (CTAB) (Hoisington et al., 1994). Sugarcane leaf samples (0.5 g) were crushed in a mortar added with liquid nitrogen. Then, the extract was put in a 2 ml microtube containing 500 µl extraction buffer (Tris-HCl 100 mM (pH 8.0), NaCl 1.4 M, EDTA 20 mM (pH 8.0), cetyl trimethyl ammonium bromide (CTAB) 2% (w/v), and 0.5% (w/v) sodium disulfite. The mixture was incubated at 65 °C for 45 minutes and homogenized by flipping through the microtubes every 15 minutes. Furthermore, the addition of chloroform: isoamyl alcohol solution with a ratio of 24:1 (v/v) of 500 µl into a microtube and homogeneous using vortex. The mixture was then centrifugated for 15 minutes at 12000 rpm. The supernatant formed was transferred into a new microtube (1.5 ml size), followed by the deposition of DNA by adding 2X the volume of absolute ethanol solution and 1 M sodium acetate (pH 5.2) by 1/10 the volume of the solution. The DNA precipitate formed was separated from the ethanol solution by centrifuging the mixture at a speed of 12000 rpm for 10 minutes. The absolute ethanol solution was removed, and the DNA pellets formed were washed with 1000 µl 70% ethanol. Next, centrifugation was carried out at 12000 rpm for 5 minutes. Washing solution (ethanol 70%) was discarded, and the pellets formed were dried overnight. The DNA pellets were resuspended by adding 50 µl TE 1X buffer (Tris 10 mM pH 8, EDTA 1 M). The contaminated RNA in the DNA preparation was removed by adding 2 µl of RNAse 10 mg/ ml enzyme and incubated for one hour at 37 °C. The concentration and quality of DNA produced were determined spectrophotometrically at a wavelength of 260 nm

using NanoDrop MD-100 (NanoDrop Technologies, Inc., Wilmington, DE USA). The measurement of DNA purity was carried out based on the absorbance ratio of 260/280. Furthermore, the concentration of each DNA sample was uniformed to 20 ng/µL before the PCR analysis was carried out.

**Primer Polymorphism Survey**

A total of 20 SSR primer pairs (Abdullah et al. 2013; Sharma et al. 2014) were used to analyze the genetic stability of sugarcane varieties (Table 1). Polymorphic Information Content (PIC) values of each SSR marker were analyzed using PowerMarker 3.25 software following the method described by Liu and Muse (2005).

*Table 1. Twenty pairs SSR primers used in the genetic stability analysis of six in vitro propagation of sugarcane varieties*

Tabel 1. Dua puluh pasang primer SSR yang digunakan dalam menganalisis kestabilan genetik enam varietas tebu hasil perbanyakan *in vitro*

| No/ *No* | SSR Primer/ *Primer SSR* | Ta (°C)/ *Ta (°C)* | Sequence (Forward/ Reverse)/ *Sekuens (Forward/ Reverse)* |
|---|---|---|---|
| 1 | SKM 01 | 54 | TATGGAGAGAGCAACCTATCA/ GACGGAAGATTGGGATTC |
| 2 | SKM 02 | 55 | GGCCTTCGATTAACCGAT/ ACAGGACGCTGCTTCTTG |
| 3 | SKM 03 | 52 | CCTATCGAATTGTGCTACTC/ GCATGTGTATTGTGTTAGAGAA |
| 4 | SKM 04 | 54 | TTATTTGTCCAACCTGCTTCTG/ CATGGATGCTTTTGCGTTAG |
| 5 | SKM 05 | 54 | ACCACCACCACTTTGTCTT/ GGATTGCTAAAGCATTGGT |
| 6 | SKM 06 | 54 | ACCACCACCACTTTGTCTT/ CGTGAGAAGGTAGGGAAACA |
| 7 | SKM 07 | 54 | CCAAACCACATTGTAGCAG/ CTTCTTGTCATCATCACTTGAG |
| 8 | SKM 08 | 54 | TTATCCCTTTCGTTCAGTAGAG/ ATTTTGCGTAGGGTCTGAG |
| 9 | SKM 09 | 54 | GGTGGCTAACAGACAGGG/ TTGCTGCCGAGAGTCATA |
| 10 | SKM 10 | 53 | GCGCCTATTTAATACCAGA/ CTTTCCCTATACCCATGATAG |
| 11 | SMs 03 | 52 | CATCTGCTCCCTCTTCCT/ CTCTGGCGGCTTGGTCCTG |
| 12 | SMs 08 | 55 | CTGACTAAGGAGGAAGTGGAG/ GACGACGATAGATGAAACA |
| 13 | SMs 10 | 55 | CTCTCTTCTCGTCTCCTCATT/ GTCCTTCTTCTTCTCGTGGT |
| 14 | SMs 12 | 55 | AAATGTCTTCGCACTAACC/ AAGGAGATGCTGATGGAGA |
| 15 | SMs 16 | 50 | CCCAGAGGACAAGGAACT/ GTAATGGAAGGAAGCAACTGA |
| 16 | SMs 45 | 55 | CTTCCCTCCCTCTCCTCT/ AGCCTTCTACTAAACTATCTGCT |
| 17 | SMs 46 | 50 | GTGAGTGAGACCAGACCAG/ CCGTGCTGTAGTTGTTGTAG |
| 18 | SMs 47 | 50 | ATACGCTACTCTGAATCCCAC/ CAATCACTATGTAAGGCAACA |
| 19 | SMs 48 | 53 | ACTCCTCTTCCTCTTCCTCTT/ GTTGTTCCCGTTCCCGCC |
| 20 | SMs 49 | 55 | ACTCGGTCATCTCATCACTC/ GTTCTTCGGGTCATCTGG |

Note: Ta = Annealing temperature of primer.
*Keterangan: Ta = Suhu penempelan primer.*

**Polymerase Chain Reaction (PCR) Analysis**

The PCR reaction was carried out in a total volume of 20 µL containing a 20 ng DNA template, 1X PCR reaction buffer, dNTPs of 0.2 mM each, primers (forward and reverse) of 20 µM each and 1 unit of DNA polymerase. The process of DNA amplification was carried out following the PCR profile described by Sharma et al. (2014). PCR amplification was performed on a PCR machine (Biorad T100TM Thermal Cycler). The reaction mixture was denatured at 94 °C for 5 minutes, followed by 35 cycles of denaturation stages at 94 °C for 40 seconds, priming the primer at 52-56 °C (depending upon the annealing temperature of each primer) for 40 seconds, and elongation stage at 72 °C for 30 seconds. In the final step, the last elongation cycle was carried out at 72 °C for 7 minutes. The amplified DNA was electrophoresed in an 8% non-denatured polyacrylamide gel (MGV Model, CBG Scientific Co.) with 1X TBE buffer, and run for 2 hours at 90 volts. The polyacrylamide gel was then stained with 0.5 µg/ml ethidium bromide, and the resulting DNA banding patterns were visualized under UV light using a digital system contained in the Chemidoc Gel Documentation (Biorad, USA). The PCR product sizes were estimated by comparing it with a DNA ladder of known size (100 bp DNA ladder).

**Data analysis**

DNA bands derived from PCR amplification of each sample at a marker locus were considered as one allele. DNA bands that have the same migration rate were assumed to be homologous loci of the sample. The SSR bands were scored as binary data in which the presence and absence of bands in a given size class were converted to 1 and 0, respectively. The binary data were then used in clustering analysis to determine genetic stability for each sample of sugarcane varieties that had been treated with several subcultures. Cluster analysis was performed using the UPGMA (Unweighted Pair Group Method Arithmetic with Mean) method in the SAHN (Sequential Agglomerative Non-overlapping) subprogram available on NTSYS-PC software version 2.1. (Rohlf 2000). The genetic relationships of sugarcane samples were evaluated based on similarity coefficients as implemented in the NTSYS-PC software.

## RESULTS AND DISCUSSION

**Total Alleles and Polymorphic Values**

Twenty pairs of SSR primers used to genotype 24 samples (6 varieties x 4 subcultural populations) resulted in a total of 60 alleles. Of these, 47 out of 60 alleles produced by 12 SSR primers showed polymorphism. SSR primer SKM 10 generated the highest polymorphic information content (PIC) with a value of 0.82, while SKM 01 produced the lowest PIC with a value of 0.26 (Table 2). The higher the value of the PIC given by a primer, the more informative the primer in detecting the genetic diversity of the population being tested. Molecular markers with a PIC value greater than 0.5 were considered useful in discriminating genotypes (DeWoody et al. 1995). In the present study, the use of such primer is not only informative in detecting diversity among sugarcane varieties but also informative in identifying heterogeneity among sugarcane culture populations at the frequency of subcultures three, six, and nine times in the same sugarcane variety.

**Genetic Stability of Sugarcane Varieties during Subcultures**

Clustering analysis of the six sugarcane varieties genotyped by SSR markers resulted in two main groups at 53% coefficient of similarity. The first cluster contained 12 subcultures plants derived from four sugarcane varieties and mother plants of each subculture plant, which consisted of two sub-clusters. The second cluster contained six subculture plants and each mother plant of each subculture plant. The second cluster consisted of two sub-clusters (Figure 1). Based on the dendrogram performed from the SSR data, each subculture's sugarcane variety (3, 6, and 9 times) tended to group at the same cluster along with the respective mother plant.

In comparison to their mother plant, GMP 3 sugarcane varieties differentiated into two clusters, which showed the highest coefficient of similarity (0.98) among other sugarcane varieties. Similarly, four sugarcane varieties, such as TK 386, PS 881, PSJK 922, and PS 865, also consisted of two clusters with the similarity coefficient value of 0.94, 0.95, 0.96, and 0.97, respectively. This result indicated that the *in vitro* propagation of such sugarcane varieties with 3-9 times of subcultures frequency showed to have genetic material identical to the mother plant. However, in the case of PS 862 sugarcane variety, the effect of subculture frequency was observed. In the three times

subculture frequency, the subcultures plants were grouped in the same cluster along with the mother plant. In comparison, after six and nine subcultures times, subcultures plants of the PS 862 variety were separated from their mother plant at a similarity coefficient value of 0.64 (Figure 1).

These findings revealed that subcultures frequency of three times showed the genetic stability of the subcultures plants. In other words, there are no genetic changes that occurred in the six sugarcane varieties tested in the present study. The same results were also found in bamboo commodities. Genetic variation did not occur in bamboo propagation results *in vitro* with a subculture three times (Goyal et al. 2015). The use of a direct organogenesis method in the present study was able to minimalize the risk of generating somaclonal variation. Also, the technique produced genetic similarity to its mother plant (Pandey et al. 2012; Roostika et al. 2015).

The results of the cluster analysis showed that the mother plant samples (control) and the effects of *in vitro* propagation in one variety had a high level of genetic similarity, which was more than 0.94 except for the PS 862 genotype (Figure 1). The similarity coefficient found in the current study was 0.94 higher than that previously reported by Thorat et al. (2017), which analyzed two varieties of sugarcane that produced a genetic similarity coefficient of 0.90 and 0.87, respectively. Also higher than the study of Tiwari et al. (2011), which detected a genetic similarity coefficient of more than 85% based on molecular tests using SSR markers. It means that the subculturing of five sugarcane varieties (PS 881, PS 865, GMP 3, PSJK 922, and TK 386) on the regeneration media was sufficient to produce genetically similar seedlings. *In vitro*, sugarcane regeneration media used in this study can be recommended in the production of seedlings and sugarcane conservation materials

Table 2. The total number of alleles and PIC values from 20 SSR primers for genetic stability analysis of sugarcane *in vitro* propagation

*Tabel 2. Jumlah alel total dan nilai PIC dari 20 primer SSR untuk analisis kestabilan tebu hasil perbanyakan in vitro*

| No/ *No.* | SSR Primer/ *Primer SSR* | TNA/ *JAT* | PIC value/ *Nilai PIC* |
|---|---|---|---|
| 1 | SKM 01 | 4 | 0.26 |
| 2 | SKM 02 | 3 | 0.31 |
| 3 | SKM 03 | 5 | 0.68 |
| 4 | SKM 04 | 8 | 0.78 |
| 5 | SKM 05 | 5 | 0.77 |
| 6 | SKM 06 | 2 | 0.37 |
| 7 | SKM 07 | 3 | Monomorphic |
| 8 | SKM 08 | 4 | 0.65 |
| 9 | SKM 09 | 3 | 0.57 |
| 10 | SKM 10 | 6 | 0.82 |
| 11 | SMs 03 | 1 | Monomorphic |
| 12 | SMs 08 | 1 | Monomorphic |
| 13 | SMs 10 | 1 | Monomorphic |
| 14 | SMs 12 | 2 | 0.36 |
| 15 | SMs 16 | 1 | Monomorphic |
| 16 | SMs 45 | 2 | Monomorphic |
| 17 | SMs 46 | 2 | Monomorphic |
| 18 | SMs 47 | 2 | 0.27 |
| 19 | SMs 48 | 3 | 0.58 |
| 20 | SMs 49 | 2 | Monomorphic |
| Total | | 60 | |

Note: TNA = Total number of alleles, PIC = Polymorphic Information Content

*Keterangan: JAT = Jumlah Alel Total, PIC = Konten Informasi Polimorfik.*

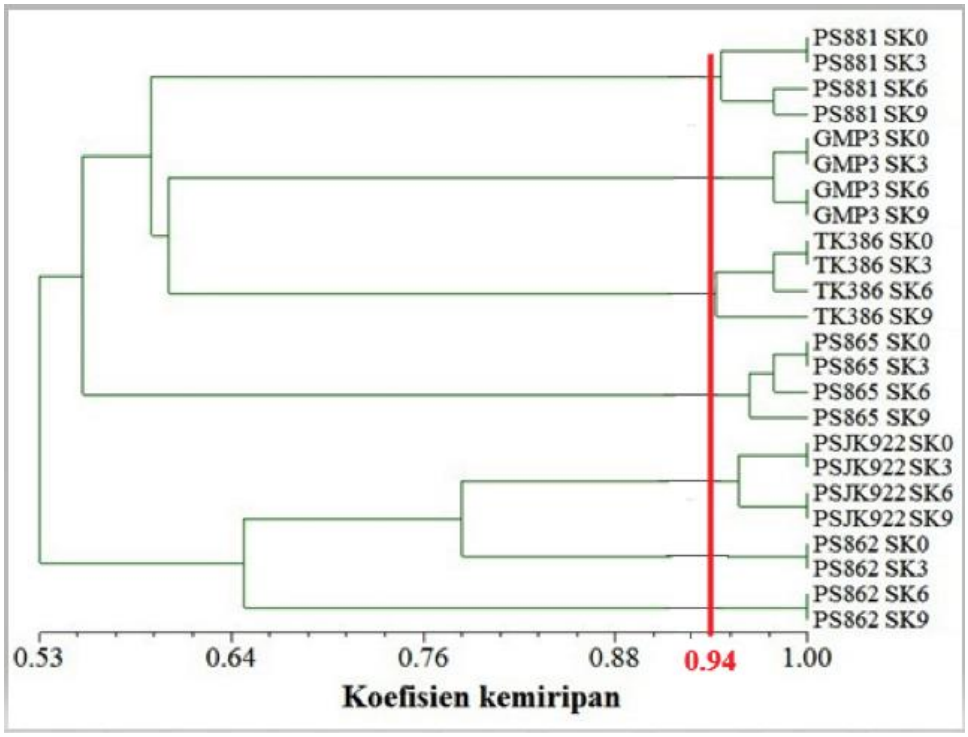


Figure 1. Similarity coefficient of 24 samples sugarcane culture derived from *in vitro* propagation at three (SK3), six (SK6), nine (SK9) times of subcultures frequency, and mother plant (SK0) based on 12 SSR primers.

*Gambar 1. Koefisien kemiripan 24 sampel tebu hasil perbanyakan in vitro pada frekuensi subkultur tiga (SK3), enam (SK6), sembilan (SK9), dan tanaman induk (SK0) berdasarkan 12 primer SSR.*

The genetic changes in the PS 862 variety might occur during subculture more than six times. Based on shoot multiplication experiments, PS 862 reached the highest multiplication level at the frequency of subcultures six times. High chance mutations occur when cells are increasingly very actively dividing. In tissue culture, the use of growth regulators can increase cell division activity, and the chance of variations will be higher. Propagation of shoots *in vitro* PS 862 both for mass seedlings production and cryopreservation materials can be applied by lowering BAP concentrations lower than 0.3 mg/l and IBA lower than 0.5 mg/l to avoid genetic changes. It is believed that the plant growth regulator type and concentration, culture period, genotype used, and culture age can cause somaclonal variations in the culture of tissue culture results (Aydin et al. 2015; Bradai, Romero and Martin 2019; Chin et al. 2019).

Genetic changes that occur are thought to be mutations at the DNA level, but variations in the PS 862 variety were not detected morphologically in the culture. The presence or absence of morphological changes in PS 862 can be known after acclimatization and the planting of sugarcane seedlings in the field. With the help of molecular analysis, genetic variations in culture can be detected early in the *in vitro* stage. The SSR primers used in this study proved to be able to identify genetic diversity in sugarcane cultures.

Subcultures repeatedly affected the growth of *in vitro* cherry, plum and pear buds (Vujović, Ružíc and Cerović 2012), and *Ficus carica* (Mustafa and Taha 2012). Also, the number of subculture frequencies could reduce the multiplication level of *in vitro* shoots as in *Simmondsia chinensis* after the rate of subcultures four times (Hegazi et al. 2014), as well as affected the secondary metabolite content of *Hypericum perforatum* (Figueiró et al. 2010). Based on this study, the frequency of subculture six times changed the genetic stability of sugarcane variety PS 862 according to molecular tests using 12 SSR primers. However, the sugarcane plantlets did not show morphological changes. Therefore, molecular testing is beneficial for

detecting genetic changes in sugarcane culture more quickly so that it is efficient in the process of *in vitro* propagation to produce seedlings that are uniform and identical to the mother plant.

Genetic stability is essential in plant propagation. The *in vitro* propagation technique of direct organogenesis is the most popular method of reproduction because it can produce large amounts of seedlings that are identical to the mother plant. The results of the study were in line with the previous reports that the *in vitro* propagation sugarcane varieties GT54-C9 (Alla et al. 2017), *Albizia procera* (Mohammad et al. 2016), *Gloriosa superba* L. (Yadav et al. 2013), Mulberry (Saha et al. 2016), and Coconut (Bandupriya et al. 2017).

The results of this study indicated that five of the six varieties of sugarcane (PS 865, PS 881, PSJK 922, TK 386, and GMP 3) are more genetically stable during subculture nine times on regeneration media, so it is safe to be used in propagating sugarcane seedlings. However, the PS 862 variety can only be reproduced maximally six times because it is genetically unstable. Similar results were shown in sorghum seedlings and sugarcane, which were propagated *in vitro*, experienced the phenomenon of genotype-dependent (Flinn et al. 2020; Ijaz et al. 2015; Jamil et al. 2017). Therefore, further studies are needed to find out the factors that cause the instability of PS 862 varieties during subcultures in regeneration media

## CONCLUSION

The seedlings of five sugarcane varieties (PS 865, PS 881, PSJK 922, TK 386, and GMP 3) showed genetic stability for nine subcultures in regeneration media based on the analysis using twelve pairs of SSR primers with similar coefficients >0.94 to their mother plants. However, the genetic stability of PS 862 variety could only be maintained until three times of subcultures in regeneration media because in the sixth subculture, the similarity coefficient value to its mother plant was only 0.64. Further research is needed to determine the cause of genetic changes in PS 862 variety during subcultures.

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## DECLARATIONS

Alfia Annur Aini Azizi, Ika Roostika, Reflinur, and Darda Efendi contributed equaly as the main contributor of this paper.

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